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The Roles of Inflammation, Oxidative Stress, and Neurotransmitters in an Animal Model of Post-Traumatic Stress Disorder

Carl Brad Wilson
Louisiana State University and Agricultural and Mechanical College, brad.wilson21@gmail.com

THE ROLES OF INFLAMMATION, OXIDATIVE STRESS, AND
NEUROTRANSMITTERS IN AN ANIMAL MODEL OF POST-TRAUMATIC
STRESS DISORDER

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Carl Brad Wilson
B.A., University of West Florida, 2001
M.F.S., George Washington University, 2008
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"Back off man, I'm a scientist!"

- Dr. Peter Venkman (Bill Murray); Ghostbusters

I like to tell people that one of my most memorable moments on this highly unexpected journey occurred late one night in December 2010. I was halfway through a 6-month deployment in Baghdad, Iraq, and my soon-to-be major professor, Dr. Joseph Francis, sent an email asking me to call him and discuss the possibility of joining his laboratory. Sometime after midnight, I climbed on the roof of our building with my notebook, flashlight, and satellite phone and made the call that would change my future. While we spoke there were helicopters passing overhead, random explosions in the distance, and the ever-present sound of gunfire downtown (you never really knew if they were actually shooting at you or celebrating after a soccer game). After I had made my best pitch that a Ph.D. could in fact be done in an accelerated time frame and failure was not an option, Dr. Francis decided to take a chance and be my major professor. As I was climbing off the roof, I remember thinking, "Huh…wonder how many other Ph.D.'s started this way?"

With that being said, I would first and foremost like to thank Dr. Francis for giving me the opportunity to live up to my promise. His constant leadership, guidance, and professionalism have been the backbone of this entire endeavor. Most importantly, though, was his patience as I transitioned from an untested, unproven, and often hardheaded student into someone I hope he is proud to call an academic. I will be forever grateful. I would also like to thank my committee members for their constant advice and direction. Dr. David Baker has been a mentor and guiding hand from day one. He was always willing to stop what he was doing and discuss whatever I was facing at the time. Dr. Ryoichi Teruyama opened his lab to me as if I was one of his

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HIAA   5-hydroxyindoleacetic acid

5-HT   5-hydroxytryptamine

ACTH   adrenocorticotropic hormone

AIC   anti-inflammatory cytokine

ANOVA   analysis of variance

ANS   autonomic nervous system

AVP   arginine vasopressin

BBB   blood brain barrier

BDNF   brain-derived neurotrophic factor

CA   cornu ammonis

cDNA   complementary DNA

CHF   congestive heart failure

CIITA   class II major histocompatibility complex transactivator

CMH   1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine

CNS   central nervous system

CO_2 carbon dioxide

CPH   1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine

CRH   corticotropin-releasing hormone

CSF   cerebrospinal fluid

DA   dopamine

DNA   deoxyribonucleic acid

DOPAC   3,4-dihydroxyphenylacetic acid

DSM-III	Diagnostic and Statistical Manual of Mental Disorders III
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders IV-Text Revision
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5
EDTA	ethylenediaminetetraacetic acid
EPM	elevated plus-maze
EPR	electron paramagnetic resonance
FDA	U.S. Food and Drug Administration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G.B.W.	grams body weight
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HET-E	hydroxyeicosateraenoic acid
HF	heart failure
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
IACUC	Institutional Animal Care and Use Committee
IDO	indoleamine-2,3-dioxygenase
IL	interleukin
IML	intermediolateral nucleus
LC	locus coeruleus
LRR	leucine-rich repeat

LTP	long-term potentiation
MetS	metabolic syndrome
mRNA	messenger RNA
NACHT	NAIP, CIITA, HET-E, TP-1
NAIP	neuronal apoptosis inhibitory protein
NALP3	NACHT, LRR, PYD domains containing protein 3
NE	norepinephrine
NF-κB	nuclear factor-*kappa*B
NIH	National Institutes of Health
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide oligomerization domain
$O_2 \cdot -$	superoxide
OONO−	peroxynitrite
PCR	polymerase chain reaction
PEG-SOD	polyethylene glycol superoxide dismutase
PFC	prefrontal cortex
PIC	pro-inflammatory cytokines
PRR	pattern-recognition receptors
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
PYD	pyrin domain
RNA	ribonucleic acid

ROS   reactive oxygen species

RT-PCR   reverse-transcriptase PCR

RVLM   rostral ventrolateral medulla

SAM   sympathetic adrenomedullary system

SEM   standard error of the mean

SSRE   selective-serotonin reuptake enhancer

SSRI   selective-serotonin reuptake inhibitor

TH   tyrosine hydroxylase

Th1/Th2   T helper 1/T helper 2

TLR   toll-like receptor

TP-1   transition protein 1

TPH   tryptophan hydroxylase

VA   valproic acid

VTA   ventral tegmental area

WB   western blot

ABSTRACT

Post-traumatic stress disorder (PTSD), a trauma- and stressor-related disorder, is a condition that

can develop in response to life-threatening situations. According to the Diagnostic and

Statistical Manual of Mental Disorders 5 (DSM-5), a diagnosis of PTSD necessitates exposure to

a life-threatening event, intrusive recollections, avoidance of associated stimuli, hyperarousal,

and a significant social impairment. All of these symptoms must persist for at least 30 days and

not be due to illness, medication, or substance abuse. To date, no definitive diagnostic

biomarkers have been identified for PTSD. Recent research, however, points toward

physiological abnormalities in the brain, hypothalamic-pituitary-adrenal (HPA) axis, and

immune system that may be partially responsible. Many chronic conditions such as

hypertension, heart failure, and metabolic syndrome perpetuate in a state of increased

inflammation and oxidative stress, exacerbating their pathophysiology. In many psychiatric

conditions such as depression and anxiety disorders, neurotransmitter modulation may play a

critical role in their pathogenesis. Based upon the literature and work from our laboratory, we

hypothesized that similar pathophysiological mechanisms may play a role in PTSD development.

We tested our theory by creating a PTSD-like syndrome in rats with the use of a predator

exposure/psychosocial stress animal model. We then conducted a series of *in vivo* and *ex vivo*

experiments in an attempt to discover the roles of inflammation, oxidative stress, and

neurotransmitter modulation in PTSD development. First, we evaluated inflammation and

oxidative stress in the brain, adrenal glands, and blood in response to the predator exposure

model. We then analyzed neurotransmitter modulation in the hippocampus and prefrontal

cortex. Next, we investigated the anti-inflammatory and neuromodulating effects of the histone

deacetylase inhibitor (HDACi) valproic acid (VA) on inflammation/oxidative stress and

neurotransmitters. Finally, we employed the selective-serotonin reuptake inhibitor (SSRI) sertraline to ascertain why SSRIs have historically been ineffective in treating PTSD. Taken together, our findings indicate inflammation, oxidative stress, and aberrant neurotransmitter profiles may play a significant role in PTSD development and progression. In addition, VA may prove to be a legitimate pharmacologic alternative in PTSD treatment, as SSRIs may increase the noradrenergic response and actually exacerbate anxiety in a clinical setting.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND AND EPIDEMIOLOGY OF PTSD

The term post-traumatic stress disorder (PTSD) was first introduced as a diagnosis in the Diagnostic and Statistical Manual of Mental Disorders III (DSM-III) in 1980 (American Psychiatric Association, 1980). Trauma- and stress-related disorders, however, have probably existed since the beginning of recorded history. Trimble believed Shakespeare's Henry IV, written over 400 years ago, displayed many of the symptoms currently associated with PTSD (Trimble, 1985). Ezra even describes trauma-related stress reactions dating back to 2100 BC, when King Urnamma was killed in battle in the ancient city of Ur and citizens of the city subsequently experienced sleep disturbances (Ezra, 2001). Since then, what is now known as PTSD has been described by many different names. Soldier's heart, shell shock, stress-response syndrome, and battered woman syndrome are among the many, but they all seem to possess similar symptoms. It seems evident that some type of stress response to extremely traumatic situations has long been a part of the human condition.

Lifetime prevalence rates for PTSD in the United States are estimated to be between 8-14%, with current rates close to 15%. For combat veterans, estimates range as high as 31% (Magruder et al., 2004). The average age of onset for PTSD is 23, but the highest incidence is reported in the 45-59 year-old age group (Kessler et al., 2005). In post-conflict countries, the rates are considerably higher. In a study by de Jong et al. of four countries with a history of conflict, they reported PTSD rates of 37% in Algeria, 28% in Cambodia, 18% in Gaza, and 16% in Ethiopia (de Jong et al., 2001). There also appear to be gender differences in prevalence rates. According to a recent report, women are twice as likely as men to develop PTSD, but the reasons for this disparity are unknown (Cramer, 2013). In one study, researchers found that females

diagnosed with PTSD responded considerably stronger to traumatic visual cues than male PTSD patients (Inslicht et al., 2013). The rationale behind these findings could be biological, psychological, social, or a combination of these factors and others. In various animal models of PTSD, the results are the opposite. Male animals tend to be more susceptible to stress, whereas females are considerably more resilient (Cohen & Yehuda, 2011). Studies have also shown that susceptibility to stress in females varies with their menstrual cycle. During lower estrogen levels (follicular phase), females tend to have impaired fear inhibition, but during higher estrogen levels (luteal phase), estrogen may be protective (E. M. Glover et al., 2013). Men, on the other hand, tend to underreport psychiatric symptoms, further blurring the lines between gender differences (Peters, Issakidis, Slade, & Andrews, 2006). It is clear that further research into gender susceptibility and response is necessary.

One of the unique features of PTSD is that it rarely occurs as an isolated diagnosis, and comorbidity with other disorders is more the rule than the exception. Data from the National Comorbidity Survey indicate that 88.3 percent of men and 79.0 percent of women diagnosed with PTSD meet the criteria for at least one other psychiatric disorder, with many of those meeting the criteria for three or more (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). The most common comorbid disorders with PTSD are depressive disorders, substance abuse disorders, and other anxiety disorders. According to Brady et al., females with PTSD are 4.1 times as likely to develop depressive disorders and 4.5 times as likely to develop mania as women without PTSD. Men who have PTSD are 6.9 times as likely to develop depression and 10.4 times as likely to develop mania as men without PTSD (Brady, Killeen, Brewerton, & Lucerini, 2000). In addition, a large portion of both males and females with PTSD experience alcohol and other substance abuse disorders (Kessler et al., 1995). Suicide attempts are also a

2

common occurrence among PTSD sufferers, and it is estimated that approximately 20% have attempted suicide at least once (Davidson, Hughes, Blazer, & George, 1991).

Despite the increase in PTSD research in the last 10-15 years, it is difficult to determine if rates are increasing or remaining stable. According to Brunet, global PTSD diagnoses remained relatively unchanged from 1997-2007. The article states that while PTSD criteria have become stricter over the years, mental health professionals have become better at correctly assessing and identifying PTSD, leading to diagnoses that may have previously been missed (Brunet, Akerib, & Birmes, 2007). The true number of those suffering from PTSD, however, may never be known. In certain subgroups such as battered women, inmates, and the homeless, they may not have the resources or ability to seek help. There also exists a prevailing social stigma that might cause those in certain professions to downplay their symptoms for fear of being ostracized. Current diagnostic approaches for PTSD are self-assessment questionnaires and mental health provider interviews, which although effective rely exclusively on the candidness of the patient and are thus inevitably subjective. This subjectivity can result in missed diagnoses, underreported symptoms, or even malingering, which necessitates better diagnostic tools. To this end, exploring the pathophysiology occurring in the brain and body during progression of the disorder may lead to the discovery of PSTD biomarkers and greatly improve the accuracy and speed of PTSD diagnoses and treatment.

1.2 PATHOPHYSIOLOGY OF PTSD

PTSD, an anxiety disorder recently reclassified as a trauma- and stressor-related disorder, can develop in response to real or perceived life-threatening situations. According to the DSM-5, a diagnosis of PTSD necessitates exposure to a life-threatening event, intrusive recollections of the event, avoidance of associated stimuli and numbing of general responsiveness, negative

cognitions/mood, hyperarousal not present before the trauma, and a significant social

impairment. All of these symptoms must persist for at least 30 days and not be due to illness,

medication, or substance abuse (American, 2013). To date, no definitive diagnostic biomarkers

have been identified for PTSD. Recent research, however, points toward physiological

abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary

system, immune system, and brain neurotransmitters that may be implicated in the disorder

(Liberzon, Abelson, Flagel, Raz, & Young, 1999; Oosthuizen, Wegener, & Harvey, 2005;

Sondergaard, Hansson, & Theorell, 2004; Wilson et al., 2013, 2014).

1.2.1 Inflammation and Oxidative Stress

Exposure to psychologically traumatic events, such as those experienced during combat

or other situations posing a legitimate threat to safety and survival, place individuals at

significant risk for developing PTSD. A growing body of evidence suggests that exposure to

traumatic stressors and subsequent psychological trauma may result in increased morbidity and

premature demise of patients. Much of the data available suggest traumatic exposure and

subsequent PTSD may lead to increased incidence of cardiovascular disease, diabetes, chronic

fatigue syndrome, and other conditions (Dansie et al., 2012; Edmondson & Cohen, 2013; Gupta,

2013; Lukaschek et al., 2013). Most of these diseases have detrimental inflammatory

components that may exacerbate their progression. Inflammation is a critical component of the

immune response, but acute and chronic inflammation can damage cellular mechanisms.

Stressful events affect the immune system by reducing the cellular response to mitogen

stimulation, decreasing production of natural killer cell activity and altering levels of cytokines.

Cytotoxic T lymphocytes, which regulate the balance between Th1 and Th2 cells, are altered by

stress and may lead to a Th2 dominant response. This response can result in an unrestrained

production of pro-inflammatory cytokines (PICs). These PICs, especially the interleukins, have been shown to play an important role in modulating disease processes. An important and detrimental consequence of increased cytokine production is the induction of nitric oxide (NO) and reactive oxygen species (ROS) (Hu, Peterson, & Chao, 1998; Mokuno et al., 1994).

Elevated levels of PICs and ROS can cause cell death and tissue damage, although the cellular mechanisms responsible for initiating these processes during the stress response have remained poorly understood. In addition to leukocytic responses, PIC upregulation may also be due to the activation of inflammasomes (Salminen, Ojala, Kaarniranta, & Kauppinen, 2012). Inflammasomes are multiprotein complexes that cooperate with pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). When the inflammasome complex is activated, it cleaves pro-caspase-1 into its active form, which results in the production of PICs and initiates the inflammatory response (Figure 1.1). When proliferation of PICs exceeds the ability of local cellular receptors to utilize them in autocrine or paracrine functions, they become blood-borne. These cytokines can then be transported across the blood-brain barrier (Banks, 2005), where they activate microglial cells and induce the production of more cytokines. The process results in a positive feedback loop, which can become self-sustaining and cause systemic organ dysfunction (Figure 1.2). Research from our lab has demonstrated the damaging effects of PICs when quantities reach uncontrolled levels. We have also shown that blocking certain downstream transcription factors and gene modifiers of these cytokines reduces oxidative stress, inflammation, and associated damage in hypertension, heart failure (HF), and metabolic syndrome (MetS) (Elks & Francis, 2010; Elks et al., 2009; Guggilam et al., 2011).

5
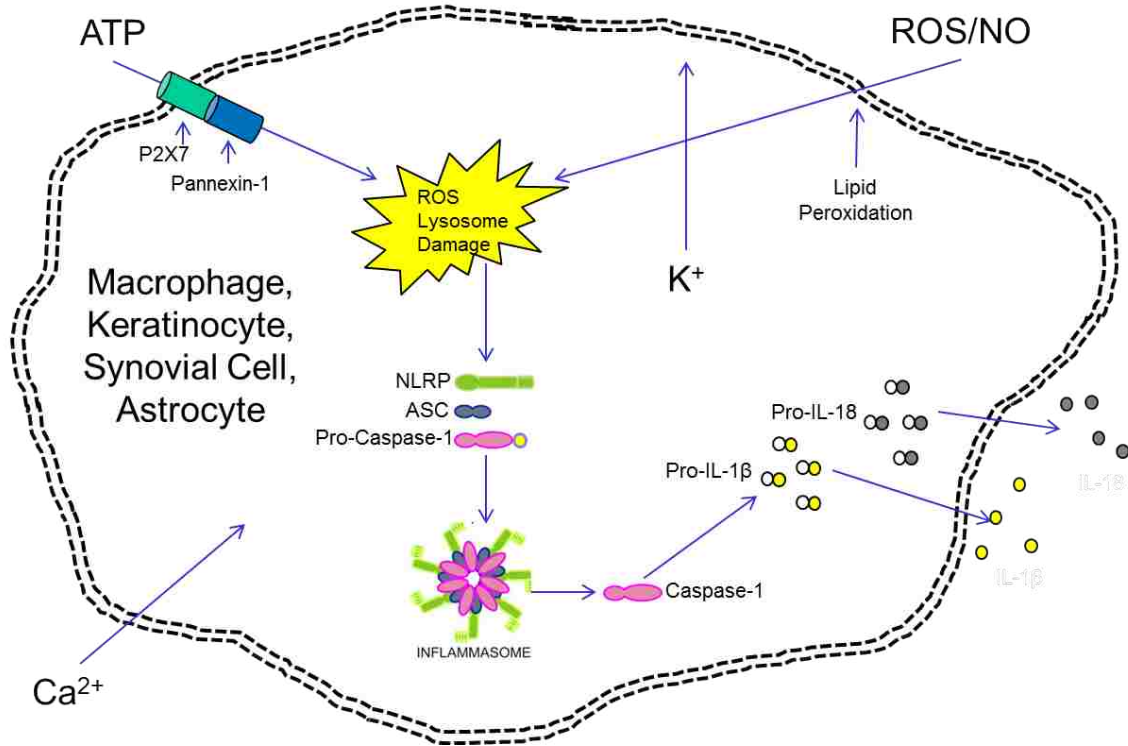
Figure 1.1: The inflammasome complex, once activated, cleaves pro-caspase-1 into active caspase-1, which then cleaves pro-IL-1β and pro-IL-18 into active IL-1β and IL-18. The active interleukins are then released and perpetuate the inflammatory response in neighboring cells.
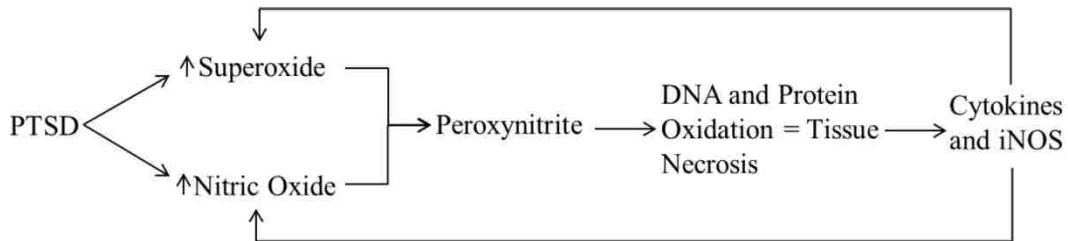
Figure 1.2: The upregulation of reactive oxygen species can cause tissue damage, which leads to increased cytokines and further upregulation of reactive oxygen. The process results in a detrimental positive feedback loop.

1.2.2 Histone Deacetylases

Inflammation is a critical component of the immune response, but chronic inflammation can damage cellular mechanisms. There are a host of triggers initiating the inflammatory response, many of which are initiated via TLR4 and subsequent nuclear factor (NF)-κB activation. Transcription by NF-κB requires DNA and chromatin remodeling, which enables access to the pertinent genomic sequences. Gene expression is regulated via highly controlled acetylation/deacetylation of histone N-terminal tails, which either increases or decreases gene availability (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003). Acetylation/deacetylation is accomplished by histone acetyltransferases (HAT) and histone deacetylases (HDAC), which enable and restrict genome access, respectively. When oxidative stress and inflammation are increased, upregulated PICs can correspond with heightened HDAC activity and NF-κB transcription, resulting in perpetual PIC production (Keslacy, Tliba, Baidouri, & Amrani, 2007). Although HDACs restrict access for transcription and should depress PIC production, the exact opposite may be the case. The effects of HDAC inhibitors (HDACi) extend to non-histone proteins that are reversibly acetylated, which markedly affects their function (Nair, Boersma, Schiltz, Chaudhry, & Muschel, 2001; Tong, Yin, & Giardina, 2004). This functional shift means HDACs may actually enhance the inflammatory response. In addition to histone modification, HDACs may also modulate neurotransmitters by modifying levels of pertinent rate-limiting enzymes (Sharma, Grayson, & Gavin, 2008). For example, tyrosine hydroxylase, the rate-limiting enzyme for dopamine (DA) and norepinephrine (NE) synthesis, is depressed by HDACi (Akiba et al., 2010). Studies have also shown antidepressant effects of HDACi (Covington et al., 2009), possibly be due to epigenetic modifications of tryptophan hydroxylase, the rate-limiting enzyme for serotonin (5-HT) synthesis.

1.2.3 The HPA Axis

The HPA axis is a critical component of the central nervous system (CNS) stress response. During a stressful event, afferents from the thalamus and sensory cortex signal the amygdala of possible danger. The amygdala has efferents that project to the hypothalamus, where the parvocellular neurons of the paraventricular nucleus (PVN) release corticotropin-releasing hormone (CRH) at the median eminence and it is carried to the anterior pituitary gland via the hypophyseal portal system. In response, the anterior pituitary releases adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH causes the release of cortisol (corticosterone in rodents and non-humans) from the adrenal cortex, which serves a variety of purposes in the stress response. Primarily, cortisol functions to increase blood sugar, depress immune function, and acts as a negative feedback signal at the pituitary and PVN. In PTSD, however, the system may not function in the same manner. Current research has shown that the interaction between cortisol and the immune system may not be as simplistic as previously believed. Cytokines are upregulated in the brain and systemic circulation during the stress response (Wilson et al., 2013), which contradicts the belief that cortisol is always immunosuppressive. Researchers have also demonstrated that CRH and glucocorticoids could influence the immune system in both directions, indicating a stress response could increase PICs and inflammation and alter normal HPA axis function (Chrousos, 1995; Chrousos & Gold, 1992).

Numerous studies have shown PTSD patients to have lower cortisol levels than the general population (Yehuda, 2009), but there have also been studies reporting no difference (Baker et al., 1999) or elevated levels (Liberzon et al., 1999). Cortisol functions effectively in a narrow therapeutic range, and hypercortisolism (Cushing's syndrome) or hypocortisolism

(Addison's disease) both have deleterious effects. The difference in study results underscores the rapid nature of glucocorticoid changes in response to even the slightest stressor, and may provide a rationale for often inconsistent results reported in PTSD. Despite these differences, glucocorticoid abnormalities seem to play a legitimate role in PTSD progression, but to what extent remains unanswered.

1.2.4 The Sympathoadrenal Medullary (SAM) System

The SAM system works in concert with the HPA axis during the stress response. The system is very complex and most pathways between nuclei are bi-directional with multiple connections. As mentioned previously, the amygdala receives inputs from sensory areas of the brain and transmits signals to the appropriate regions for response. The amygdala and the PVN have efferents to the rostral ventrolateral medulla (RVLM), which projects to the spinal cord intermediolateral nucleus (IML) (Fisher & Paton, 2012). The amygdala and PVN also project to the locus coeruleus (LC), the brainstem nucleus responsible for most NE synthesis in the CNS, which contains sympathetic projections to the IML. The IML sends preganglionic efferents to the adrenal medulla from the thoracic spinal cord, and the adrenal medulla acts as a specialized sympathetic ganglion (Sapru, 2007). In response to stimulation, chromaffin cells in the adrenal medulla synthesize and release catecholamines into the systemic circulation. Catecholamine release during stress influences neuroplasticity, memory, emotions, behavior, and other actions in the CNS (Benarroch, 2009). In the systemic circulation, epinephrine and NE increase heart rate, trigger glucose release, and prepare the body for a "fight-or-flight" response.

The persistent sympathetic drive and noradrenergic responsiveness by the interaction of the LC and amygdala have received attention as possible contributors to stress-induced disorders such as PTSD. One postmortem study of combat-related PTSD patients discovered an aberrant

number of neurons in the LC, indicating the LC may play a more important role in PTSD development than previously believed (Bracha, 2005). These data support our findings that NE is significantly increased in the hippocampus and PFC in response to the predator exposure model, and that constant noradrenergic stimulation may be a primary contributor to PTSD (Wilson et al., 2014).

1.2.5 Neurotransmitters

Neurotransmitters are small, endogenous chemicals that relay information from one neuron to the next at a synaptic cleft. Most neurotransmitters are packaged in synaptic vesicles and released in response to an action potential. At the post-synaptic cell they act on either excitatory or inhibitory receptors, which determines the receiving cell's response. Although the primary drugs for treating PTSD are the selective-serotonin reuptake inhibitors (SSRI), the actions of neurotransmitters in PTSD are poorly understood. Serotonin (5-HT), for example, is a neurotransmitter responsible for many functions in the CNS and peripheral organs. 5-HT influences aggression, arousal, sleep, anxiety, appetite, fear, learning, and other actions (Dubovsky, 1994). 5-HT is also the principle regulator of mood. A study by Peirson et al. (Peirson & Heuchert, 2000) found lower platelet $5\text{-}HT_2$ receptor function was associated with depressed mood, while Williams et al. (Williams et al., 2006) demonstrated higher blood 5-HT levels were correlated with better mood. An increased mood and overall sense of well-being has been shown, in both psychiatric and physical disorders, as protective and positively correlated with resiliency behavior (Delamothe, 2005). Research has demonstrated that 5-HT uptake sites in platelets were lower in PTSD patients vs. controls (Arora, Fichtner, O'Connor, & Crayton, 1993). Lower 5-HT has also been implicated in diminished physical health. Muldoon et al.

showed that a low prolactin response to fenfluramine, a drug that increases 5-HT levels, was associated with metabolic syndrome (Muldoon et al., 2004).

Norepinephrine (NE) is a neurotransmitter that is also involved in the regulation of psychiatric and physical mechanisms. Under normal conditions, NE is a principle component of the stress response, directly increasing heart rate and blood flow to skeletal muscles and triggering the release of glucose, all in preparation for the 'fight-or-flight' response. Persistent noradrenergic activity, however, has been linked with negative outcomes in patients with congestive heart failure (CHF) (Francis et al., 1993) and diabetes (Ganguly, Dhalla, Innes, Beamish, & Dhalla, 1986). Studies have also shown that individuals with PTSD have elevated cerebrospinal fluid (CSF) levels of NE (Geracioti et al., 2001) and noradrenergic hyperresponsiveness to various stimuli (Liberzon et al., 1999). Dysregulation of noradrenergic neurons has also been associated with hyperarousal and intrusive recollections attributable to PTSD (Southwick et al., 1999).

Dopamine (DA) is a neurotransmitter that plays a major role in emotion and the reward system of the brain. It optimally functions within a narrow range and dopaminergic hypo- or hyperactivity is implicated in both physical and psychiatric illnesses. Parkinson's disease is characterized by a loss of dopaminergic neurons, and evidence suggests schizophrenia and psychosis are linked to elevated levels of DA (Paterlini et al., 2005). DA may also have a role in PTSD, and studies have shown dopaminergic hyperactivity in male combat veterans (Yehuda, Southwick, Giller, Ma, & Mason, 1992), traumatized adult females (D. A. Glover et al., 2003), and abused children (De Bellis et al., 1999) with PTSD. The dopamine metabolite homovanillic acid (HVA), often used as a diagnostic test for catecholamine-producing tumors of the adrenal glands, has also demonstrated aberrant levels in PTSD patients. Geracioti et al. found HVA was

significantly reduced in the CSF of combat-related PTSD patients immediately after viewing

traumatic imagery (Geracioti et al., 2013). Based on the previous research, it is clear that

elucidating the complex interactions of the numerous neurotransmitter systems may be a critical

link in understanding PTSD progression.

1.3 THE HIPPOCAMPUS

The hippocampus lies deep in the temporal lobe (Figure 1.3) and is part of the limbic

system. It exerts important influences on the endocrine and autonomic systems, and it also

affects motivation and mood. It plays an important role in the consolidation of both long term

and short term memories, and it is chiefly responsible for mediation of stress responses via the

HPA axis. The hippocampus has been implicated in fear conditioning (Fendt, Fanselow, & Koch,

2005), spatial memory (Kessels, de Haan, Kappelle, & Postma, 2001), depression (Malberg,

2004), epilepsy and seizure susceptibility (McEwen & Magarinos, 2001), CNS-mediated

glucoregulation through cholinergic epinephrine secretion (Uemura et al., 1989), cognitive

disorders such as Alzheimer's Disease (Garzon, Yu, & Fahnestock, 2002) and PTSD (Wilson et

al., 2013, 2014)

The hippocampal formation is comprised of six distinct regions, linked by primarily

unidirectional projections (Hasselmo, 1995). These regions include the dentate gyrus, the

hippocampus proper (Cornu Ammonis (CA) 1, 2, 3), subicular cortex (comprised of the

subiculum, the presubiculum, the parasubiculum), and the entorhinal cortex (composed of two or

more subdivisions) (Hasselmo, 1995). Although the neurophysiological roles for each of these

areas are now being elucidated and reported, distinct functions remain incompletely understood

and are based mainly on computational models derived from hippocampal connectivity studies

(Guzowski, Knierim, & Moser, 2004; Strange & Dolan, 1999).

What is well known and widely reported, however, is that the hippocampus exhibits a large

degree of functional and structural plasticity and throughout adulthood generates large numbers

of new neurons (Kozorovitskiy & Gould, 2004; van Praag, Christie, Sejnowski, & Gage, 1999;

van Praag et al., 2002).

Figure 1.3: Computer generated model of the rat hippocampus illustrating its shape and position
deep within the temporal lobe of the brain (Laboratory of Neuro Imaging, 2005).

1.4 THE PREFRONTAL CORTEX

The prefrontal cortex (PFC) is the anterior portion of the frontal lobe of the brain (Figure

1.4), and it is considered the executive center for higher order functions such as complex

behavior and decision-making, personality, and social "control" (Yang & Raine, 2009). Other

executive actions of the PFC include determining good vs. bad, actions and consequences, and

working toward a goal (DeYoung et al., 2010). The exact definition of the PFC differs among

researchers, and there are varying descriptions. It has been characterized by the presence of a

granular layer IV (only in primates) (Uylings, Groenewegen, & Kolb, 2003), as being the

projection zone of the mediodorsal nucleus of the thalamus (primates and nonprimates) (Preuss

& Goldman-Rakic, 1991), and as the area of the frontal cortex whose electrical stimulation does

not lead to observable movements (contains granular and non-granular areas) (Preuss, 1995).

During anxiety and fear responses, the PFC is responsible for the extinction of fear

conditioning and the retention of extinction (Milad & Quirk, 2002). This extinction does not

occur when the PFC is damaged (Morgan, Romanski, & LeDoux, 1993; Quirk, Russo, Barron, &

Lebron, 2000), and research has demonstrated inflammation and oxidative stress are increased in

the PFC during PTSD progression (Wilson et al., 2013). Damage caused by inflammation and

oxidative stress, coupled with the fact that individuals with PTSD exhibit abnormal fear

responses and attenuated fear extinction (Orr et al., 2000; Rothbaum, Kozak, Foa, & Whitaker,

2001), supports the hypothesis that the PFC may be impaired in PTSD.

Figure 1.4: Diagram showing the location of the PFC and its afferent projections from the locus
coeruleus (LC), dorsal raphe nuclei (DRN), and the ventral tegmental area (VTA). Efferents (not
shown) include projections to the hippocampus (HIP), amygdala (AMG), thalamus (TH), and
paraventricular nucleus (PVN), among others (Ash, Zanatta, Williams, Lawrence, & Djouma,
2011).

1.5 PHARMACOTHERAPY

Since PTSD has a high comorbidity with other psychiatric conditions, there are many drugs available that mental health providers prescribe to manage symptoms. Treatment for PTSD directly, however, is comprised of only two drugs that are Food and Drug Administration (FDA) approved: the SSRIs sertraline and paroxetine (Roman, 2010; Sullivan & Neria, 2009). Although there have been studies that demonstrated some improvement in PSTD symptoms with the use of SSRIs, they are generally regarded as only moderately effective as a first-line therapy and even less so in combat-related PTSD (Tawa & Murphy, 2013). Recent research found that efficacy rates of SSRIs in PTSD therapy rarely reached 60%, and less than 20-30% achieved full remission (Berger et al., 2009).

Based on their mechanism of action, SSRIs should increase 5-HT levels and attenuate many of the detrimental effects of lowered 5-HT. This concept has proven effective in the treatment of depression (Doogan & Caillard, 1992; Miller et al., 1998). In PTSD, however, results are less promising. In a popular study by Davidson et al. which was a part of the FDA approval process for sertraline use in PTSD, they demonstrated decreased severity of symptoms and an overall increase in functioning in the PTSD patients vs. controls (Davidson, Rothbaum, van der Kolk, Sikes, & Farfel, 2001). The results were achieved with multiple investigator- and self-rated assessments. This study, however, had uneven gender distribution (84% female), racial distribution (83% white), and traumatic event distribution (64% physical or sexual assault). The effectiveness of sertraline, therefore, may be limited to a certain demographic and type of incident. The data showed a 45% increase in symptom improvement in the treatment group, but also a 36% increase in symptom improvement in the placebo group. Taken together, the numbers indicate that the majority of the noted improvement may be due to a placebo effect. In

addition, there were no physiological measures in the study that could analyze actual

neurotransmitter modulation during treatment. This information could be critical in determining

the true efficacy of SSRIs as neurotransmitter changes are not mutually exclusive events.

1.6 ANIMAL MODELS OF PTSD

Most of the current PTSD research is focused on human patients, which has its

advantages and disadvantages. The advantages are self-evident, but disadvantages include the

variable backgrounds among patients, the type of stressful event (e.g., combat, rape, kidnapping,

etc.), reduced experimental control in treatment studies, and the inability to determine baseline

physiological data before PTSD developed. An animal model mitigates those variables, but it

also has drawbacks. True PTSD is a human disorder that is diagnosed primarily through the

verbal and written responses of the individual, so there is a certain amount of extrapolation

necessary with animal models. It is impossible to ask an animal how they are feeling or if they

have persistent memories of a traumatic event. Devices such as the elevated plus-maze, fear-

conditioning system, or Morris water maze are thus employed to ascertain anxiety changes vs.

control animals. Animal models provide excellent experimental control and negate many of the

physiological and ethical barriers encountered in human PTSD research. There are multiple

animal models used in anxiety disorder research with varied approaches and methods. Current

models include inescapable foot shock (Rudy, Huff, & Matus-Amat, 2004), restraint stress

(Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002), social defeat (Huhman, 2006), and

maternal separation (Diehl et al., 2012), among others. Many of these models have been shown

to be effective for establishing depression and/or fear conditioning, but not necessarily PTSD.

Although a single traumatic event can cause PTSD in humans, the persistent stress of normal

everyday life may significantly contribute to the progression of the disorder. Animal models that

employ a single stressful event may simply be causing a temporary fear response that subsides after pertinent anxiety testing. When certain methods such as social instability and additional stressors are combined, however, the results tend to bear more of a resemblance to human PTSD (Saavedra-Rodriguez & Feig, 2013). For our experiments, it was important to select an animal model of PTSD that matched, as closely as possible, the behavioral, psychological, and physiological elements of PTSD in humans. The predator exposure/psychosocial stress model by Zoladz et al., (Figure 1.5) possesses both predictive and construct validity, meaning the model is sensitive to clinically effective pharmacologic agents and the rationale underlying the model displays similarities to human PTSD (Bourin, Petit-Demouliere, Dhonnchadha, & Hascoet, 2007). The model demonstrates three hallmark features of PTSD: hormonal abnormalities, a long-lasting traumatic memory, and persistent anxiety (Zoladz, Conrad, Fleshner, & Diamond, 2008; Zoladz, Fleshner, & Diamond, 2012).

In the predator exposure/psychosocial stress model, PTSD rats were individually isolated in cylindrical, Plexiglas containers and canned cat food was smeared on the outside of the cylinders. The cylinders prevented direct contact with the cats, and the cat food induced movement in the cats. Rats were then placed in a stainless steel cage consisting of a solid metal floor with a hinged, metal rod door, with a cat for one hour. The first cat exposure was conducted during the light cycle (0700-1900). Ten days later, a second cat exposure was conducted during the dark cycle (1900-0700). In addition, the rats were subjected to psychosocial stress by changing their cage cohort daily. The predator exposure/psychosocial stress regimen continued for 31 days (Figure 1.6).

Figure 1.5: Pictures showing the rat enclosed in the Plexiglas cylinder (top) and inside the cage with the cat during the predator exposure (bottom). Canned cat food was smeared on the outside of the cylinders to invoke predatory movement in the cats.
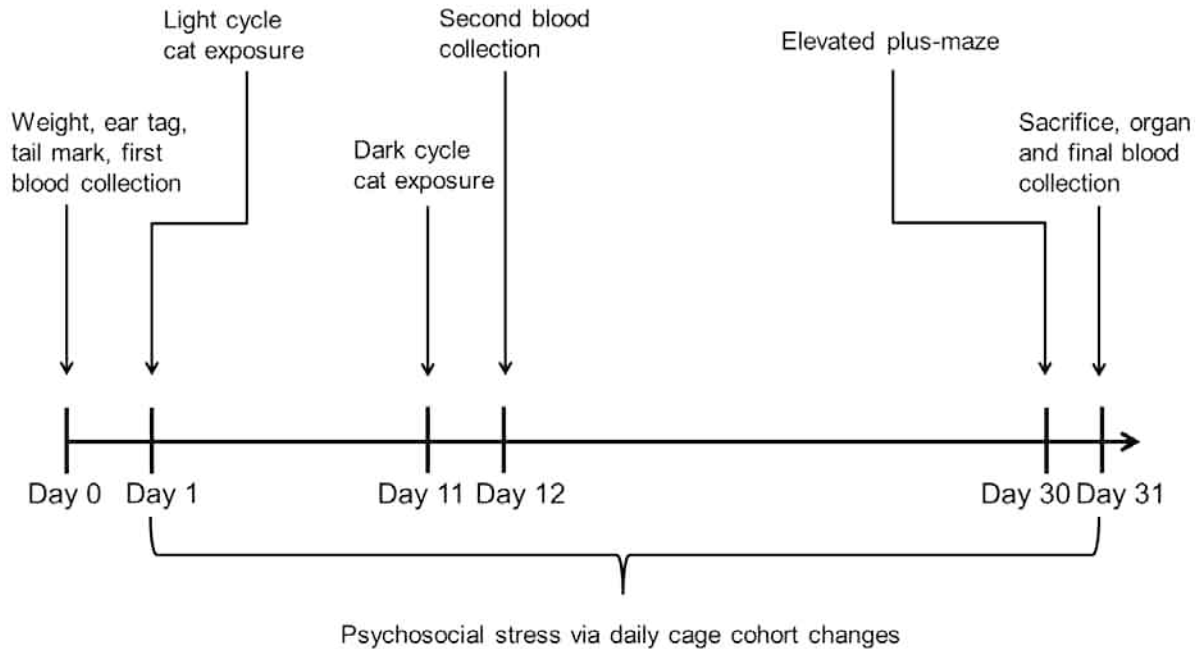
Figure 1.6: Diagram showing the 31-day predator exposure/psychosocial stress regimen. This model was also modified beyond 31 days including additional cat exposures and continued cage cohort changes during treatment phases. The extended model was necessary to ensure any noted improvement was due to treatment and not natural subsiding of anxiety.

1.7 ANXIETY TESTING

One of the diagnostic criteria for PTSD is hyperarousal, which includes an exaggerated startle response and heightened anxiety (American, 2013). To measure anxiety levels, we used the elevated plus-maze (EPM). Rodents have a natural tendency to explore novel environments, but open areas or alleys (without protective walls) invoke a greater fear and avoidance response (Montgomery, 1955). The EPM is widely used as a measure to test fear or anxiety and has been extensively validated for use in rats (Korte & De Boer, 2003; Pellow, Chopin, File, & Briley, 1985). The EPM is a four-arm maze, shaped like a "plus" sign, that consists of two open arms and two closed arms (Figure 1.7). The rats were placed in the middle of the maze and allowed to roam freely for 5 minutes. The premise behind the EPM is based on rodents' natural aversion of

19

open spaces versus their desire to explore novel environments. Entry into the open areas is associated with increased freezing behavior as well as increased plasma corticosterone levels, indicating heightened anxiety (Pellow et al., 1985). Anxiogenic compounds or procedures can increase avoidance of the fear-provoking open arms, whereas anxiolytic compounds or procedures can increase open arm exploration (Pellow et al., 1985). The primary criteria correlated with anxiety levels are total arm entries (overall ambulations) and the percent time spent in the open vs. closed arms.

Figure 1.7: Photograph of the elevated plus-maze showing the two enclosed and two open arms. The overhead camera mounted on the tripod captured the animals' movements onto each different arm and measured distance traveled, total arm entries, and the amount of time spent in the open vs. closed arms.

1.8 STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

PTSD is a serious condition that affects millions of people, and with the Middle Eastern conflicts drawing down and troops returning home, the numbers are likely to rise. Although PTSD is fairly well characterized from a behavioral standpoint, we still know very little about the molecular mechanisms involved with its development. There has been research exploring the HPA axis and glucocorticoids, neurotransmitters, the immune system, brain peptides, the SAM system, and many others. Most of this research, however, has been conducted on human PTSD patients. A major hurdle to that approach is making valid comparisons to control subjects. Since PTSD is a psychiatric diagnosis involving both physiological and environmental elements, it is reasonable to state that there is simply too much variability between individual experiences to make legitimate comparisons. An animal model of PTSD, however, enables us to control the process and make comparisons between PTSD and control groups that would otherwise be impossible in humans. Another problem facing PTSD understanding is the lack of pharmacotherapies available. Sertraline and paroxetine are the only drugs that are FDA approved for treating PTSD, but their effectiveness has been questionable. These drugs work well for depression, but they do not seem to manage the anxiety element of PTSD.

Recent evidence demonstrated certain inflammatory and oxidative stress components might play a role in PTSD development, but to what extent is unknown. In addition, neurotransmitter modulation might also be implicated, but the mechanisms are poorly understood. We hypothesized that inflammation, oxidative stress, and increased sympathetic drive due to heightened catecholamines might be key elements to PTSD progression. We tested our hypothesis through a series of *in vivo* experiments using the predator exposure/psychosocial stress model of PTSD on rats. In our first experiment, we sought to determine whether inflammatory and reactive oxygen components were up-regulated in the PTSD model. Second,

we examined how neurotransmitters were modulated in response to the PTSD model. Once we

obtained this information, we conducted additional experiments to analyze the effects of certain

drugs on inflammation, oxidative stress, and neurotransmitters. In our third experiment, we

administered VA and measured its effects on our target molecules. Our final experiment

involved the SSRI sertraline, and we attempted to elucidate a possible mechanism explaining

why its efficacy in PTSD is so poor. The specific aims of this project were as follows:

Aim 1: Determine which inflammatory and reactive oxygen components, if any, were up-

regulated in an animal model of PTSD.

Aim 2: Analyze neurotransmitter modulation in an animal model of PTSD and describe

possible mechanisms responsible.

Aim 3: Evaluate the effects of VA, a non-traditional PTSD therapy, on inflammation,

oxidative stress, and neurotransmitters.

Aim 4: Investigate potential factors responsible for sertraline's minimal effectiveness in

treating PTSD.

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CHAPTER 2
INFLAMMATION AND OXIDATIVE STRESS ARE ELEVATED IN THE BRAIN, BLOOD, AND ADRENAL GLANDS DURING THE PROGRESSION OF POST-TRAUMATIC STRESS DISORDER IN A PREDATOR EXPOSURE ANIMAL MODEL

2.1 INTRODUCTION

Post-Traumatic Stress Disorder (PTSD) is an anxiety disorder that can develop in response to real or perceived life-threatening situations. According to the Diagnostic and Statistical Manual of Mental Disorders IV-Text Revision (DSM-IV-TR), a diagnosis of PTSD necessitates exposure to a life-threatening event, intrusive recollections of the event, avoidance of associated stimuli and numbing of general responsiveness, hyperarousal not present before the trauma, and a significant social impairment. All of these symptoms must also persist for at least 30 days (American Psychiatric Association, 2013). To date, no definitive diagnostic biomarkers have been identified for PTSD. Recent research, however, points toward physiological abnormalities in the brain, hypothalamic-pituitary-adrenal (HPA) axis, and immune system that may be responsible for the psychological manifestations of the disorder (Liberzon, Abelson, Flagel, Raz, & Young, 1999; Oosthuizen, Wegener, & Harvey, 2005; Sondergaard, Hansson, & Theorell, 2004). Many chronic conditions such as hypertension, heart failure, and metabolic syndrome perpetuate in a state of increased inflammation and oxidative stress, exacerbating their pathophysiology (Cardinale et al., 2010; Elks & Francis, 2010; Guggilam et al., 2011). We hypothesize that similar physiological mechanisms may play a role in PTSD development.

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Exposure to psychologically traumatic events, such as those experienced during combat or other situations posing a legitimate threat to safety and survival, place individuals at significant risk for developing PTSD. A growing body of evidence suggests that exposure to traumatic stressors and subsequent psychological trauma may result in increased morbidity and premature demise of patients. Much of the data available suggest traumatic exposure and subsequent PTSD may lead to increased incidence of cardiovascular disease, diabetes, chronic fatigue syndrome, and other conditions (Dansie et al., 2012; Edmondson & Cohen, 2013; Gupta, 2013; Lukaschek et al., 2013). Most of these diseases have detrimental inflammatory components that may exacerbate their progression. Inflammation is a critical component of the immune response, but acute and chronic inflammation can damage cellular mechanisms. Stressful events affect the immune system by reducing the cellular response to mitogen stimulation, decreasing production of natural killer cell activity and altering levels of cytokines. Cytotoxic T lymphocytes, which regulate the balance between Th1 and Th2 cells, are altered by stress leading to a Th2 dominant response, resulting in an unrestrained production of pro-inflammatory cytokines (PICs). These PICs, especially the interleukins, have been shown to play an important role in modulating disease processes. An important and detrimental consequence of increased cytokine production is the induction of nitric oxide (NO) and reactive oxygen species (ROS) (Hu, Peterson, & Chao, 1998; Mokuno et al., 1994).

Elevated levels of PICs and ROS can cause cell death and tissue damage, although the cellular mechanisms responsible for initiating these processes during the stress response have remained poorly understood. In addition to leukocytic responses, PIC upregulation may also be due to the activation of inflammasomes (Salminen, Ojala, Kaarniranta, & Kauppinen, 2012). Inflammasomes are multiprotein complexes that cooperate with pattern-recognition receptors

(PRRs) such as Toll-like receptors (TLRs) and NOD (nucleotide oligomerization domain)-like receptors (NLRs). When the inflammasome complex is activated, it cleaves pro-caspase-1 into its active form, which results in, among other things, the production of PICs and initiates the inflammatory response. When proliferation of PICs exceeds the ability of local cellular receptors to utilize them in autocrine or paracrine functions, they become blood-borne. These cytokines can then be transported across the blood-brain barrier (Banks, 2005), where they activate microglial cells and induce the production of more cytokines. The process results in a positive feedback loop, which can become self-sustaining and cause systemic organ dysfunction. Research from our lab has demonstrated the damaging effects of PICs when quantities reach uncontrolled levels. We have also shown that blocking certain downstream transcription factors and gene modifiers of these cytokines reduces oxidative stress, inflammation, and associated damage in hypertension, heart failure (HF), and metabolic syndrome (MetS) (Elks & Francis, 2010; Elks et al., 2009; Guggilam et al., 2011). In light of this information, this study investigates whether oxidative stress and inflammation increase in the brain, adrenal glands, and systemic circulation during the progression of PTSD using a predator exposure/psychosocial stress animal model.

2.2 MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Institute for Laboratory Animal Research's 2011 *Guide for the Care and Use of Laboratory Animals*, under the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee (Protocol Number: 12-067).

Animals

Naïve adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in all experiments. The rats were the same age (12 weeks) and approximately the same weight (±15g) upon delivery. Rats were pair-housed in standard plastic microisolator cages and had access to food and water *ad libitum*. The cages were maintained in ventilated racks (racks hold eight cages vertically and five horizontally) and each cage was randomly assigned to a specific rack location to ensure groups were evenly distributed. The vivarium room was kept on a 12-hour light/dark cycle (0700-1900), room temperature was maintained at 20±1ºC, and humidity ranged from 23-42%. After a one-week acclimation period, the mean weight of all rats was 347.9g ± 4.5. Two cats, one male and one female (Harlan Laboratories, Indianapolis, IN (male), and Tulane University, New Orleans, LA (female)) were used for all predator exposures. Cats were seven and ten years old, respectively. They were housed in an open room (15' x 15') in the vivarium with access to food, water, and enrichment devices *ad libitum*. The cat room was on the same light/dark cycle and maintained at similar temperature and humidity as the rat room.

Stress Induction

Following the acclimation period, rats were brought to the laboratory and under isoflurane anesthesia were weighed, ear-tagged, tail-marked (ear tag number written on tail for easy identification), and 250-500µL of blood was drawn from either the tail or lateral saphenous vein. The rats were then randomly assigned to the "PTSD" or "control" group and returned to the vivarium for 24 hours. The following day, PTSD rats were started on a predator exposure/psychosocial stress regimen, published and validated by Zoladz, et al., designed to produce a pre-clinical PTSD that closely mimics signs and symptoms seen in human patients (Zoladz, Fleshner, & Diamond, 2012) (Figure 2.1). Briefly, PTSD rats were individually

isolated in cylindrical, Plexiglas containers (IITC Life Science, Inc., Woodland Hills, CA; tail

cuff restraint containers for 400-600g rats and Kent Scientific, Torrington, CT; tail cuff restraint

containers for 300-500g rats) and canned cat food (Friskies, Purina, St. Louis, MO) was smeared

on the outside of the cylinders. The cylinders prevented direct contact with the cats, and the cat

food induced predatory movement in the cats. Studies show a moving cat invokes a greater fear

response than a sedentary cat (Blanchard, Mast, & Blanchard, 1975). Rats were then placed in a

stainless steel holding cage (76cm x 76cm x 60cm) consisting of a solid metal floor with a

hinged, metal rod door, with a cat for one hour. The first cat exposure was conducted during the

light cycle (0700-1900). Ten days later, a second cat exposure was conducted during the dark

cycle (1900-0700). In addition to the cat exposures, starting on day one the rats were subjected

to psychosocial stress by changing their cage cohort daily. The cage cohort rotation was

established prior to the start of the experiment, whereby each rat was never housed with the same

rat on consecutive days and never housed with the same rat more than four times in a month.

The predator exposure/psychosocial stress regimen was continued for 31 days. After 31 days,

PTSD and control group rats were euthanized via CO_2 inhalation, perfused with a phosphate

buffered vascular rinse solution, and the brains were removed. The hippocampus, amygdala, and

pre-frontal cortex (PFC) were dissected and flash-frozen in liquid nitrogen. The thymus and

adrenals glands were also removed, trimmed, weighed, and flash-frozen.

Blood Collection

Approximately 250-500μL blood per animal was drawn from either the tail or lateral

saphenous veins of rats in both groups, allowing a minimum of 24 hours between any

consecutive blood draws to prevent anemia. Anesthesia was induced in an isoflurane anesthesia

chamber and maintained on low-flow isoflurane via a nose cone throughout the procedure.
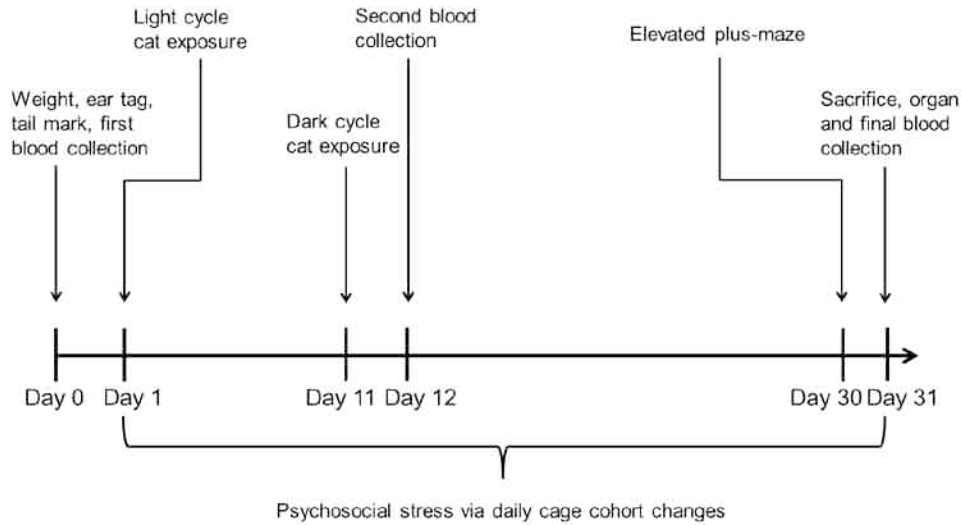
Figure 2.1: The predator exposure/psychosocial stress model includes two cat exposures over a 31-day period, combined with daily cage cohort changes. In addition, blood was collected at three different time points and ROS levels were measured to determine oxidative stress within groups and between groups. Anxiety was measured at the end of the stress regimen via EPM.

The hind legs were shaved to allow access to the lateral saphenous veins. Blood was collected using a heparinized 22g needle and microcentrifuge tube with 50µL of heparin. Petroleum jelly (Vaseline, Unilever, Englewood Cliffs, NJ) was applied at the puncture site when necessary to reduce clotting. Alternatively, a lateral tail vein was used to collect blood after warming the tail in water and using the same gauge heparinized needle and microcentrifuge tube with 50µL of heparin.

Elevated Plus-Maze

Rats were placed in the center of the elevated plus-maze (EPM) (EB-Instruments (Bioseb), Tampa Bay, FL) facing an open arm and allowed to roam freely for five minutes. Movement was monitored via an overhead camera and captured with a specifically designed software program (BioEPM3C, EB-Instruments, Tampa Bay, FL). The primary measurements were the number of entries into each arm (total ambulations) and the total time spent in the open vs. closed arms. An arm entry was defined as all four feet crossing into a different arm. The

stand for the EPM was approximately 36" above the floor, and each arm measured 11 cm x 50 cm.

Growth Rates and Organ Weights

At the beginning of the experiment and after the 31-day stress induction period, rats were weighed to determine average growth rate/day of PTSD and control animals. Following euthanasia, the adrenal glands and thymus were removed and weighed. Those organs were chosen, as their structure and function have been shown to be adversely affected by stress (Gruver & Sempowski, 2008; Ulrich-Lai et al., 2006). The adrenal weights were combined as one weight for each animal, and weight was expressed as mg/100g body weight. Thymus weight was also expressed as mg/100g body weight.

Corticosterone Analysis

Plasma corticosterone was measured using the DetectX Corticosterone ELISA (K-014-H1, Arbor Assays, Ann Arbor, MI). The samples were diluted and prepared as per the protocol, optical density was analyzed at 450nm with a plate reader (VersaMax, Molecular Devices, Hayward, CA), and a standard curve was created based on the concentrations in each sample.

Electron Paramagnetic Resonance Spectroscopy

Total ROS, superoxide, and peroxynitrite were measured in whole blood (baseline, day 12, and day 31), brain tissue (hippocampus and PFC), and the adrenal glands via electron paramagnetic resonance (EPR) as previously described (Mariappan, Elks, Fink, & Francis, 2009; Mariappan et al., 2010). Blood was drawn from all rats at the beginning of the experiment (baseline), one day after the second cat exposure (day 12), and at the end of the predator exposure/psychosocial stress regimen (day 31). Superoxide, peroxynitrite, and total ROS levels in the blood were compared as repeated measures within the control and PTSD groups, and also

37

between groups (control vs. PTSD), to analyze oxidative stress during PTSD progression. Analysis of oxidative stress between groups was also compared with tissue collected following euthanasia. Two different spin probes were used for EPR studies. 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine(CMH) was used to measure tissue ROS and superoxide $O2^{\bullet}-$, and 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine(CPH) was used for measurement of tissue peroxynitrite ($OONO-$). Briefly, pieces of tissue were incubated at 37°C with CMH (200 µM) for 30 min for ROS measurement; PEG-SOD (50 U/µL) for 30 min, then CMH (200 µM) for an additional 30 min for $O2^{\bullet}-$ measurement; or CPH (500 µM) for 30 min for $OONO-$ measurement. Aliquots of incubated probe media were then taken in 50-µl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of ROS, $O2^{\bullet}-$, or $OONO-$ production. All EPR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor microwave cavity (Bruker Company, Germany).

Real-Time PCR Analysis

Semi-quantitative real-time RT-PCR (n = 6/group) was used to determine the mRNA levels of IL-1β and the NALP3 inflammasome in the hippocampus. The primer sequences used for real-time PCR are given (Table 2.1). In brief, the rats were euthanized using CO_2 inhalation, perfused with a phosphate buffered solution directly into the left ventricle, and the brains were quickly removed, dissected, and immediately flash-frozen in liquid nitrogen. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal, Welsch, Keller, & Francis, 2011). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data is presented as fold change of the gene of interest relative to that of control animals.

Table 2.1: Rat primers used for real-time RT-PCR.

Gene	Sense	Antisense
GAPDH	agacagccgcatcttcttgt	cttgccgtgggtagagtcat
IL-1β	cagaccactttggcagacttcact	ggattcgttggctgttcggtcg
NALP3	cagaaggcatgtgagaagca	tgggtgtagcgtctgttgag

IL, (Interleukin); NALP3, (NACHT, LRR, PYD domains containing protein 3); GAPDH, (Glyceraldehyde 3-phosphate dehydrogenase).

Western Blot Analysis

Tissue homogenates from the hippocampus and PFC were subjected to Western Blot (WB) analysis (n =10/group) for the determination of protein levels of IL-1β, the NALP3 inflammasome, and GAPDH. The extraction of protein and WB was performed as previously described (Agarwal et al., 2011). The specific antibodies used included: IL-1β, NALP3, and GAPDH. Primary antibodies were commercially obtained: IL-1β and GAPDH, 1:1000 dilution (SC-7884 and SC-20358 respectively, Santa Cruz Biotechnology, Santa Cruz, CA); and NALP3, 1:1000 dilution (Biorbyt, San Francisco, CA). Secondary antibodies were commercially obtained: anti-mouse, 1:500 dilution and anti-rabbit, 1:500 dilution (SC-2314 and SC-2004 respectively, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using ImageJ imaging software (NIH), and were normalized with GAPDH.

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was done by one-way ANOVA with a Tukey's post hoc test for multiple comparisons, and unpaired Student's T-tests were used for two-column analyses. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using Prism (GraphPad Software, Inc, La Jolla, CA; version 5.0).

2.3 RESULTS

Growth Rates and Organ Weights

The PTSD group displayed a significantly diminished growth rate over the 31-day stress period, $t(18) = 2.78$, $p < 0.05$. The same group also showed an increase in adrenal gland weight, $t(18) = 5.66$, $p < 0.0001$, and a decrease in thymus weight, $t(18) = 4.81$, $p < 0.0001$ relative to the control group (Table 2.2).

Table 2.2: Growth rate and organ weights.

Group	Growth Rate (g/day)	Adrenal Wt. (mg/100 g.b.w.)	Thymus Wt. (mg/100 g.b.w.)
Control (n=10)	1.72 (0.14)	10.73 (0.49)	80.24 (2.11)
PTSD (n=10)	1.11* (0.17)	14.03* (0.32)	61.51* (3.28)

*$p < 0.05$ relative to the control group.

Plasma Corticosterone

The PTSD group displayed higher plasma corticosterone levels, $t(14) = 2.24$, $p < 0.05$ relative to the control group (Figure 2.2).

Elevated Plus-Maze Performance

The comparison of anxiety levels revealed the PTSD group spent considerably less time in the open vs. closed arms, $t(18) = 3.88$, $p < 0.05$. Overall ambulations, however, were not affected as both groups were still relatively active inside the maze, $t(18) = 0.34$, $p > 0.05$ (Figure 2.3).

Oxidative Stress Analysis

To investigate the influence of the predator exposure/psychosocial stress regimen on oxidative stress/redox balance, we examined levels of superoxide, peroxynitrite, and total ROS in the hippocampus, PFC, and adrenal glands. In the hippocampus, PFC, and adrenal glands, analysis of the EPR data revealed superoxide, peroxynitrite, and total ROS were elevated in all
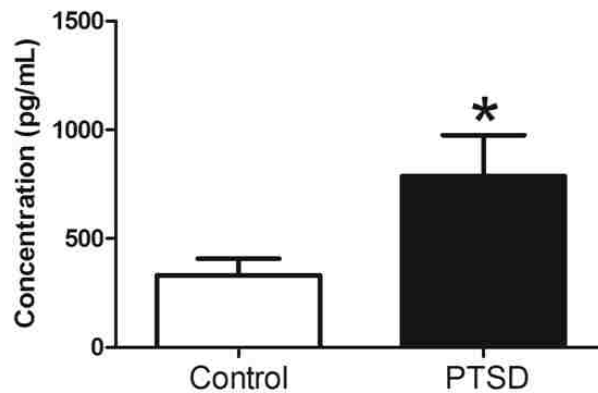
Figure 2.2: After 31 days of the predator exposure/psychosocial stress regimen, plasma corticosterone levels were higher in the PTSD group. Corticosterone was measured in plasma collected at the time of sacrifice and frozen prior to testing. Data are presented as ± SEM. *p < 0.05 relative to the control group.

three regions (p < 0.05). Peroxynitrite in the PFC, however, did not reach significance (p > 0.05) (Figure 2.4). In whole blood drawn at three different time points, superoxide levels were nearly identical at baseline, elevated at 12 days, and further elevated at 31 days relative to the control group (p < 0.05) (Figure 2.5). Within-group comparison via repeated measures with the same animals of the PTSD group demonstrated superoxide levels were elevated (day 12 vs. baseline, p < 0.05) and further elevated (day 31 vs. day 12, p < 0.05). Repeated measures of total ROS levels yielded similar results (Figure 2.5). Repeated measures in the control group revealed superoxide and total ROS levels remained relatively unchanged (p > 0.05).

Brain Inflammatory Markers

To investigate the influence of the predator exposure/psychosocial stress regimen on inflammation, we examined mRNA (Figure 2.6) and protein (Figure 2.7) levels of IL-1β and NALP3 in the hippocampus, PFC, and amygdala. The PTSD group demonstrated significantly

elevated mRNA levels of IL-1β and NALP3 in all three regions. In the hippocampus and PFC, protein for IL-1β and NALP3 was significantly higher in the PTSD group relative to controls.
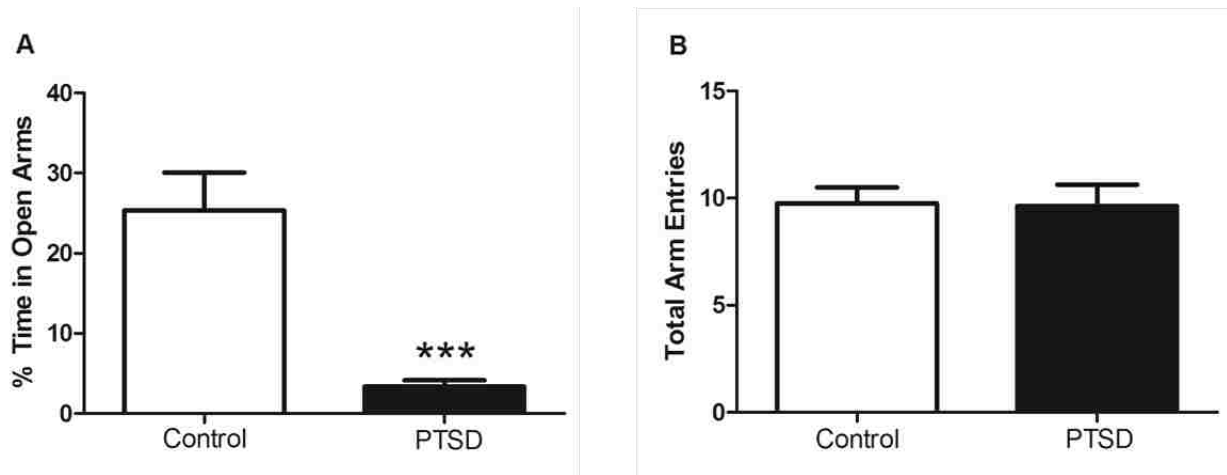
Figure 2.3: The PTSD group displayed significantly higher anxiety than the control group, as evidenced by their reluctance to spend time in the open arms of the EPM (A). Total ambulations, however, were essentially equal between the two groups (B). Anxiety on the EPM was tested within 24 hours of the final day of the 31-day stress regimen. Data are presented as mean ± SEM. ***$p < 0.0001$ relative to the control group.

2.4 DISCUSSION

The present study sought to analyze specific pathophysiological mechanisms involved in the progression of PTSD by employing a predator exposure/psychosocial stress regimen. Few animal models of PTSD exist, but the model by Zoladz et al. has been shown to cause heightened anxiety, exaggerated startle response, impaired cognition, and increased cardiovascular reactivity (Zoladz et al., 2012), all of which are common symptoms reported in humans with PTSD (Brewin, Andrews, & Valentine, 2000; Nemeroff et al., 2006). Although animal models have their limitations, a major component missing from human PTSD research is the ability to ascertain physiological data prior to PTSD development and while the disorder is progressing.
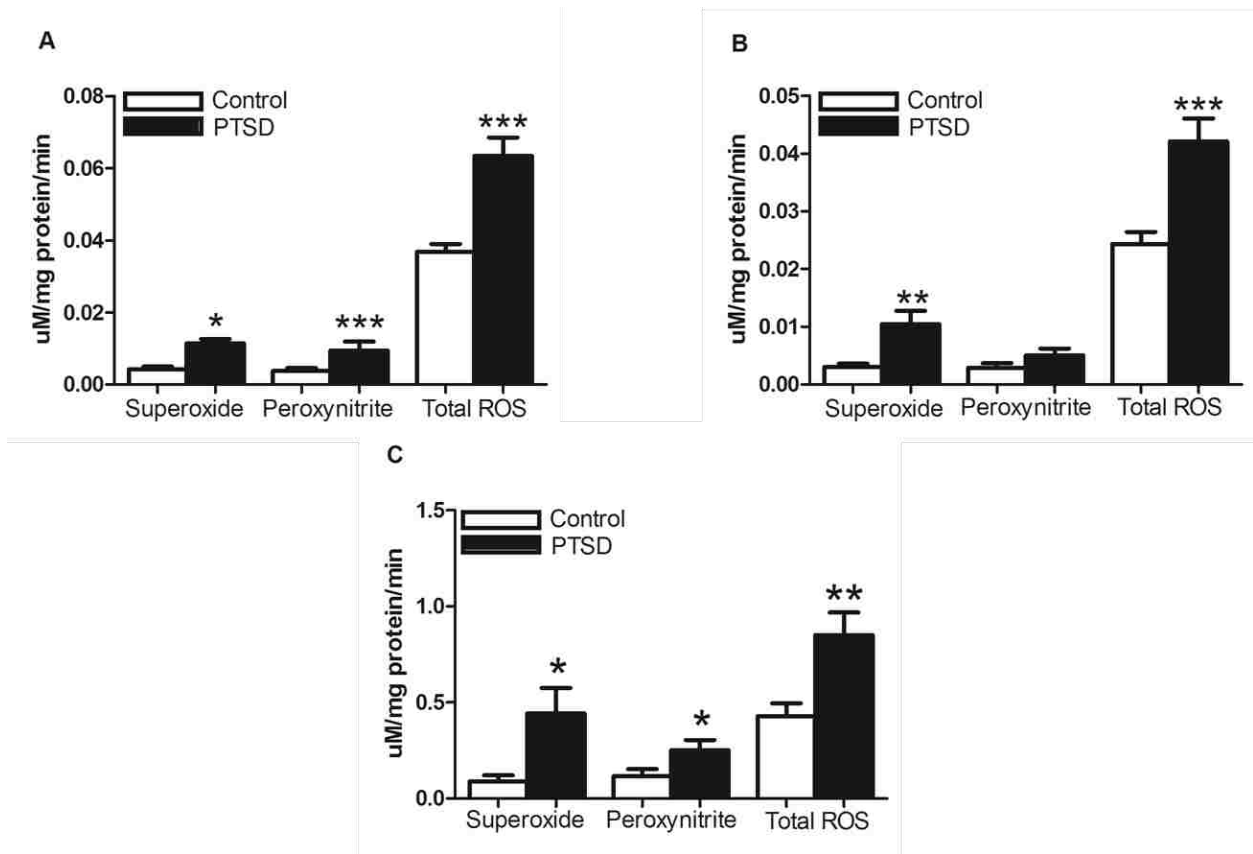
Figure 2.4: Superoxide, peroxynitrite, and total ROS were all significantly elevated in the hippocampus (A) and adrenal glands (C) in the PTSD group. Superoxide and total ROS were also elevated in the pre-frontal cortex (B) in the PTSD group, but peroxynitrite did not reach significance. All data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ relative to the control group.
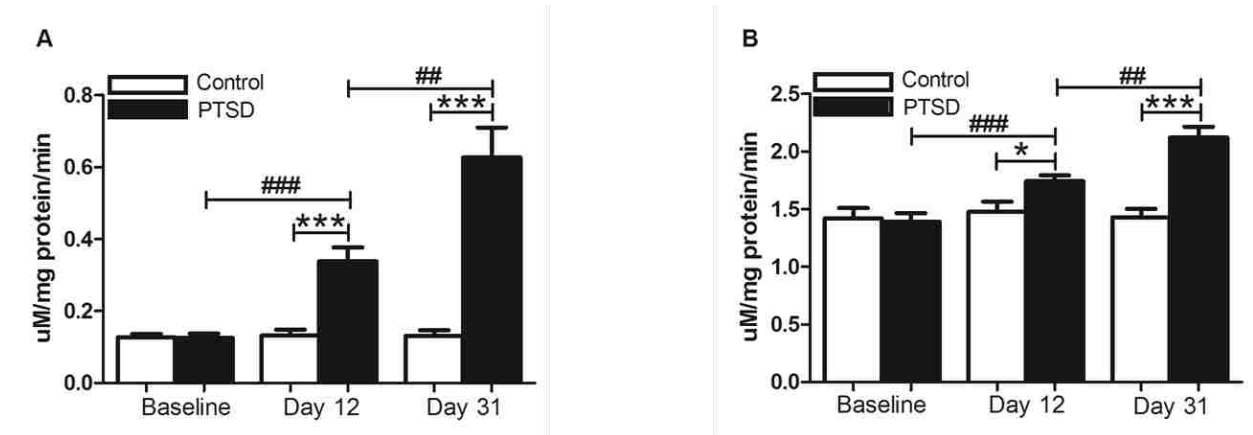
Figure 2.5: Superoxide (A) and total ROS (B) in the blood were measured between groups and within groups at three time points during the predator exposure/psychosocial stress regimen. Superoxide and total ROS were at approximately the same level for the PTSD and control groups at the beginning of the experiment, but progressively rose during stress. All data are presented as

mean ± SEM. *$p < 0.05$, ***$p < 0.0001$ relative to the control group. ##$p < 0.001$, ###$p < 0.0001$ relative to the previous measurement of the same group.
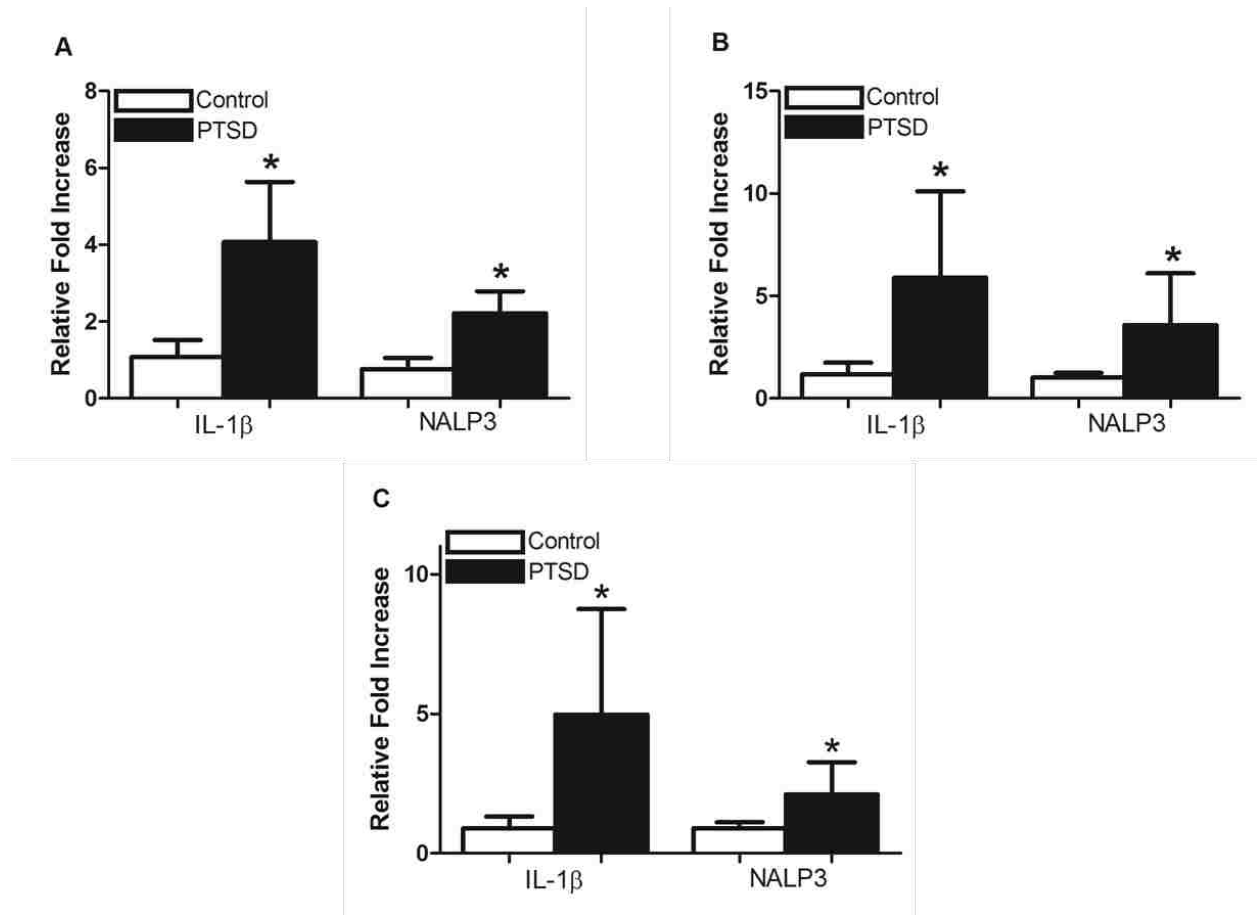
Figure 2.6: RT-PCR revealed IL-1β and NALP3 mRNA were significantly elevated in the hippocampus (A), PFC (B), and amygdala (C) in the PTSD group. All data presented as mean ± SEM. *$p < 0.05$ relative to the control group.

We have successfully obtained such data with this model, and to our knowledge, we are the first

to report the time-dependent progression of oxidative stress in the blood in PTSD animals. In

addition, we discovered damaging ROS in the hippocampus, PFC, and adrenal glands were also

upregulated in response to the predator exposure/psychosocial stress regimen. In the brain,

mRNA and protein for cytokines and cytokine-producing mechanisms were significantly

elevated, demonstrating a neuroinflammatory component in PTSD. Three novel and important

findings emerged from this study. First, oxidative stress and inflammation are upregulated in the
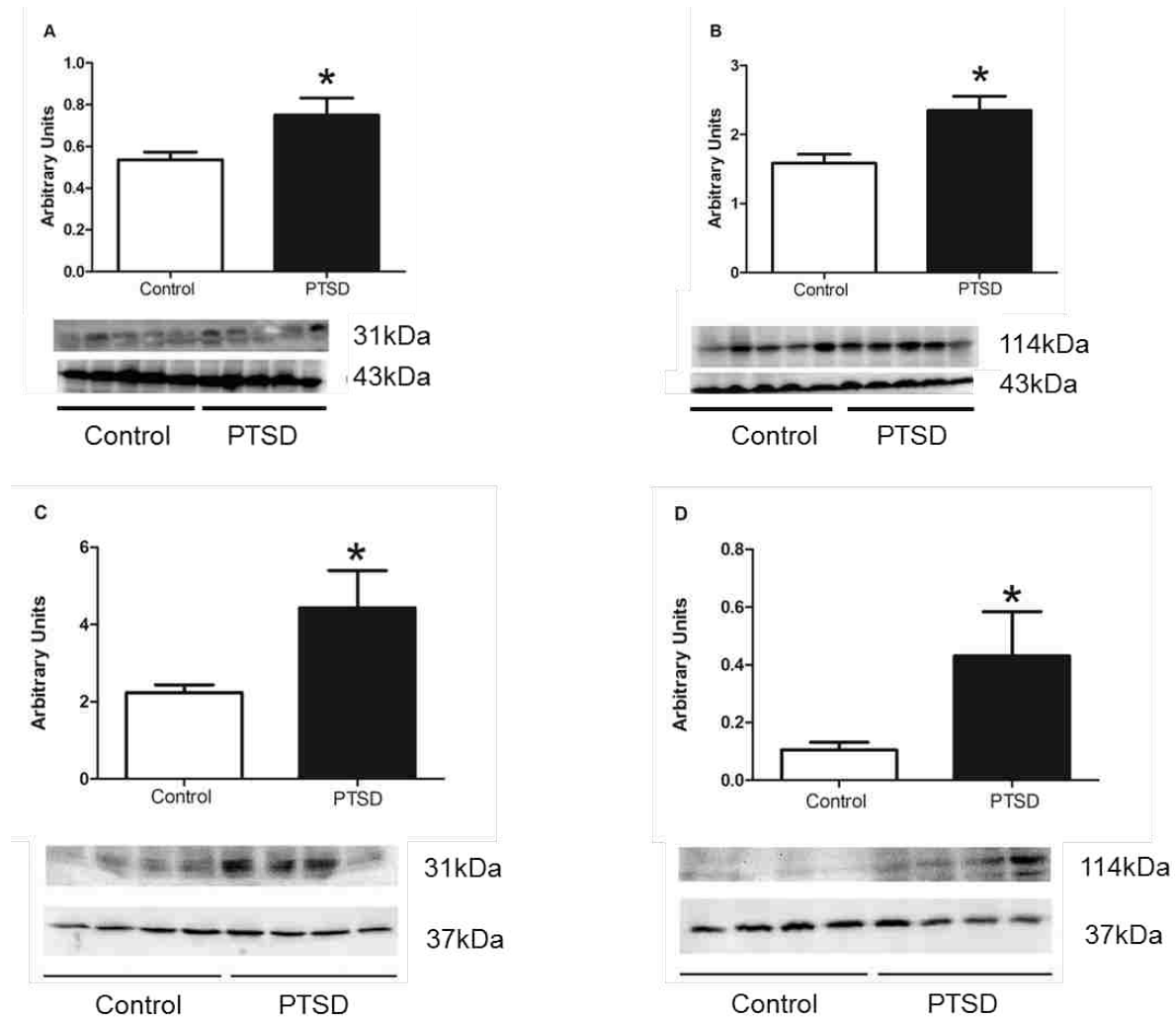
Figure 2.7: Western Blot showed IL-1β and NALP3 protein in the hippocampus (A & B respectively) and PFC (C & D respectively) were significantly elevated in the PTSD group. All data presented as mean ± SEM. *$p < 0.05$ relative to the control group.

brain, namely the hippocampus, PFC, and amygdala, in response to psychological stress.

Second, oxidative stress increases not only in the brain but also in the blood and adrenal glands,

indicating PTSD may progress as a systemic condition involving multiple organ systems. Last,

and possibly most important, oxidative stress increases in the blood in a time-dependent manner

within the same group of animals.

Most of the current PTSD research is focused on human patients, which has its advantages and disadvantages. The advantages are self-evident, but disadvantages include the variable backgrounds among patients, the type of stressful event (e.g., combat, rape, kidnapping, etc.), reduced experimental control in treatment studies, and the inability to determine baseline physiological data before PTSD developed. An animal model mitigates those variables, but as there are multiple animal models used in anxiety disorder research with varied approaches and methods, careful selection was necessary. For our experiments, it was important to select an animal model of PTSD that matched, as closely as possible, the behavioral, psychological, and physiological elements of PTSD in humans. The predator exposure/psychosocial stress model by Zoladz et al., possesses both predictive and construct validity, meaning the model is sensitive to clinically effective pharmacologic agents and the rationale underlying the model displays similarities to human PTSD (Bourin, Petit-Demouliere, Dhonnchadha, & Hascoet, 2007). The model demonstrates three hallmark features of PTSD: hormonal abnormalities, a long-lasting traumatic memory, and persistent anxiety (Zoladz et al., 2012).

The roles of oxidative stress and inflammation in other pathological conditions including cardiovascular disease, diabetes mellitus, metabolic syndrome, and neurological diseases are well established (Agarwal et al., 2011; Alexopoulos et al., 2012; Elks & Francis, 2010; Pall & Satterlee, 2001). Reactive oxygen species, or free radicals, have unpaired valence shell electrons and cause damage by oxidizing proteins, lipids, nucleic acids, and other cellular components. They are produced naturally via mitochondrial leakage, xanthine oxidase, and other pathways, and they are important in cell signaling, homeostasis, and host defense. Under normal conditions, the body's antioxidant mechanisms (e.g., superoxide dismutase, glutathione peroxidase, uric acid) scavenge ROS and convert them to inert compounds. Oxidative stress, by

46

contrast, occurs when there is an imbalance between naturally occurring ROS and the body's ability to convert them via antioxidants. As ROS levels increase, they can cause DNA and protein oxidation, leading to tissue necrosis and upregulation of PICs (Pall & Satterlee, 2001). An important function of cytokines is to transmit information concerning inflammatory responses to the CNS (Chrousos, 2000; Dunn, 2000; McCann et al., 2000; Turnbull, Lee, & Rivier, 1998). The CNS then participates in negative feedback regulation of the peripheral immune response by releasing pituitary hormones (adrenocorticotropic hormone [ACTH] and arginine vasopressin [AVP]) and increasing sympathetic drive. Cortisol, AVP, and sympathetic nerve activity all act to suppress further peripheral production of cytokines, and cortisol also acts centrally to inhibit further production of corticotropin-releasing hormone (CRH). When cytokine upregulation exceeds cellular usage, however, they may then be transported across the blood-brain barrier (Banks, 2005), activate microglial cells, and induce the production of more cytokines. The process becomes a positive feedback loop, which can become self-sustaining and result in severe organ dysfunction. A proposed pathway by which this pathophysiology may occur is presented in Figure 2.8.

Recent research has sought to determine the pathophysiology of PTSD development and to identify diagnostic biomarkers, but progress has been slow on both endeavors. Glucocorticoid derangements have been found to be variable (Baker et al., 1999; Liberzon et al., 1999; Yehuda, 2009), hippocampus structural changes may or may not occur (Gilbertson et al., 2002; Oosthuizen et al., 2005), and studies have not been able to consistently delineate the specific roles of neurotransmitters (Sutherland & Davidson, 1994) in the disorder. These conflicting reports indicate a need to explore other mechanisms possibly involved in PTSD pathophysiology. As mentioned previously, oxidative stress and inflammation are implicated in

many disease processes. The involvement of oxidative stress and inflammation in PTSD, however, has only recently garnered attention. Oosthuizen et al., reported that PTSD was
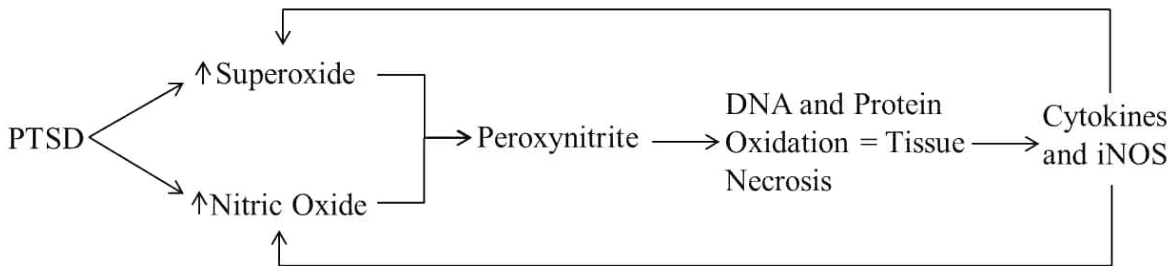
Figure 2.8: Cytokine production in PTSD leads to upregulation of superoxide and nitric oxide, which bind to form the very potent oxidizer peroxynitrite. The resulting tissue damage sustains the positive feedback loop causing further detrimental effects.

exacerbated by increased levels of NO and other ROS, causing cellular damage in the hippocampus (Oosthuizen et al., 2005). Diehl et al., discovered DNA breakage, a sign of oxidative stress, in the hippocampus of rats subjected to stress via a maternal separation model (Diehl et al., 2012). Our results demonstrate ROS and PICs are significantly upregulated during the progression of PTSD, indicating an increase in oxidative stress and inflammation in the brain, adrenals, and systemic circulation. Whether or not the structural and functional damage these mechanisms may cause is contributory or a by-product of PTSD progression is yet to be determined.

Previous research regarding plasma glucocorticoid (cortisol in humans, corticosterone in rodents) levels in PTSD has been met with varied results. Numerous studies have shown PTSD patients to have lower cortisol levels than the general population (Yehuda, 2009), but there have also been studies reporting no difference (Baker et al., 1999) or elevated levels (Liberzon et al., 1999). Cortisol is the primary hormone involved in the stress response, and its primary roles include gluconeogenesis and suppression of the immune system. Cortisol functions effectively

in a narrow therapeutic range, and hypercortisolism (Cushing's syndrome) or hypocortisolism (Addison's disease) both have deleterious effects. In this experiment we found plasma corticosterone was elevated in the PTSD group vs. controls, which contrasted with the results of Zoladz et al., in this model (Zoladz et al., 2012). In their experiment, lower baseline corticosterone levels were obtained in the undisturbed (non-dexamethasone/vehicle injected) group, whereas we measured corticosterone levels from plasma collected during euthanasia procedures immediately after the predator exposure/psychosocial stress regimen. The difference in results underscores the rapid nature of glucocorticoid changes in response to even the slightest stressor, and may provide a rationale for often inconsistent results reported in animal models of PTSD. Despite these differences, glucocorticoid abnormalities seem to play a legitimate role in PTSD progression, but to what extent still remains unanswered.

Chronic stress affects normal growth patterns, possibly due to increased HPA axis stimulation or other mechanisms acting in concert with upregulated glucocorticoids (Krahn, Gosnell, & Majchrzak, 1990). The decrease noted in body weight growth rates in our rats may be attributable to endocrine abnormalities similar to those seen in human PTSD patients. During traumatic or stressful events, there is a profound release of cortisol from the adrenal cortex (Bremner, 1999). The hypothalamic-pituitary-adrenal (HPA) axis is activated by the hypothalamus via corticotropin releasing hormone (CRH) at the median eminence, which stimulates the release of ACTH from the anterior pituitary gland. In turn, ACTH causes the release of cortisol from the adrenal cortex. In non-PTSD individuals, cortisol exerts negative feedback control at the hypothalamus and pituitary, but recent research indicates PTSD patients may display aberrant endocrine profiles (Krystal & Neumeister, 2009; Vidovic et al., 2011). One such abnormality is enhanced negative feedback inhibition of the HPA axis, resulting in

increased levels of CRH (Rohleder, Joksimovic, Wolf, & Kirschbaum, 2004; Stein, Yehuda,

Koverola, & Hanna, 1997; Yehuda, Boisoneau, Lowy, & Giller, 1995). Higher levels of CRH

can inhibit feeding behavior, even in food-deprived animals (Krahn et al., 1990; Mazjoub, 2006).

Increased adrenal gland weight may also be due to excessive glucocorticoid production without

proper negative feedback from the hypothalamus, resulting in adrenal hypertrophy and

hyperplasia (Ulrich-Lai et al., 2006). The substantive decrease seen in thymus weight may be a

result of increased oxidative stress or cortisol toxicity causing thymocyte apoptosis (Salgo &

Pryor, 1996).

One of the diagnostic criteria for PTSD is hyperarousal, which includes an exaggerated

startle response and heightened anxiety (American Psychiatric Association, 2013). To measure

anxiety levels, we used the elevated plus-maze (EPM). Rodents have a natural tendency to

explore novel environments, but open areas or alleys (without protective walls) invoke a greater

fear and avoidance response (Montgomery, 1955). The EPM is widely used as a measure to test

fear or anxiety and has been extensively validated for use in rats (Korte & De Boer, 2003;

Pellow, Chopin, File, & Briley, 1985). The EPM is a four-arm maze, shaped like a "plus" sign,

that consists of two open arms and two closed arms. The premise behind the EPM is based on

rodents' natural aversion of open spaces versus their desire to explore novel environments.

Entry into the open areas is associated with increased freezing behavior as well as increased

plasma corticosterone levels, indicating heightened anxiety (Pellow et al., 1985). Anxiogenic

compounds or procedures can increase avoidance of the fear-provoking open arms, whereas

anxiolytic compounds or procedures can increase open arm exploration (Pellow et al., 1985).

The primary criteria correlated with anxiety levels are total arm entries (overall ambulations) and

the percent time spent in the open vs. closed arms. We found that the predator

50

exposure/psychosocial stress regimen had a significant anxiogenic effect regarding time spent in the open vs. closed arms. These findings demonstrate the model induced a marked increase in anxiety in the PTSD group vs. controls. The lack of difference in overall ambulatory activity between the groups suggests activity level and anxiety may be independent measures in animal models of PTSD.

In summary, this study sought to determine if, as in many other disease processes, oxidative stress and inflammation increased during PTSD progression. To answer this question, we used a predator exposure/psychosocial stress animal model of PTSD. We validated stress induction via an EPM and also found plasma corticosterone levels to be elevated in the PTSD group. In addition, growth rate and thymus weights were lower in the PTSD group, while adrenal gland weight was higher vs. controls. Our findings indicate ROS and PICs are upregulated in the adrenal glands, circulating blood, and the areas of the brain associated with PTSD, indicating an increase in oxidative stress and inflammation as the disorder progresses.

2.5 CONCLUSIONS

We utilized a predator exposure/psychosocial stress animal model of PTSD to demonstrate how oxidative stress and inflammation may play a key role in PTSD development. We found ROS and PICs were elevated in all three regions of the brain commonly associated with PTSD, indicating increased oxidative stress and inflammation. In addition, oxidative stress and inflammation were elevated systemically, as evidenced by increased ROS and PICs in the adrenal glands and circulating blood. We also noted a time-dependent nature of oxidative stress by analyzing whole blood drawn at different time points. Levels of superoxide, peroxynitrite, and total ROS in the blood rose in an exponential nature throughout the stress period, while corresponding ROS levels in the control animals remained relatively constant. Our use of the

51

model established by Zoladz et al., (Zoladz et al., 2012) produced similar behavioral and physiological results, including a diminished growth rate, larger adrenal glands, a smaller thymus, and decreased time spent in the open arms of the EPM, confirming their results. In contrast to their findings, we obtained higher post-stress corticosterone levels. Their results, however, were obtained following a dexamethasone suppression test, whereas we tested plasma corticosterone on frozen blood collected immediately following euthanasia. Overall, our results demonstrate the progressive nature of oxidative stress and inflammation during PTSD development and may provide new targets for pharmacologic and non-pharmacologic treatments. Future studies by our lab will seek to elucidate neurotransmitter modulation in response to the predator exposure/psychosocial stress regimen and the subsequent response to various treatment modalities.

ACKNOWLEDGEMENTS:

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CHAPTER 3
PREDATOR EXPOSURE/PSYCHOSOCIAL STRESS ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER MODULATES NEUROTRANSMITTERS IN THE RAT HIPPOCAMPUS AND PREFRONTAL CORTEX

3.1 INTRODUCTION

Post-Traumatic Stress Disorder (PTSD), recently reclassified as a trauma- and stressor-related disorder, can develop in response to real or perceived life-threatening situations. According to the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), a diagnosis of PTSD necessitates exposure to a life-threatening event, intrusive recollections of the event, avoidance of associated stimuli and numbing of general responsiveness, negative cognitions/mood, hyperarousal not present before the trauma, and a significant social impairment. All of these symptoms must persist for at least 30 days and not be due to illness, medication, or substance abuse (American Psychiatric Association, 2013). To date, no definitive diagnostic biomarkers have been identified for PTSD. Recent research, however, points toward physiological abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary system, and immune system that may be implicated in the disorder (Liberzon et al., 1999; Oosthuizen et al., 2005; Sondergaard et al., 2004; Wilson et al., 2013). In the brain, neurotransmitter modulation may also play a critical role in PTSD development, and they continue to be the primary target for pharmacologic interventions. It still remains unclear, however, exactly which neurotransmitters are up- or down-regulated during PTSD progression.

A growing body of evidence suggests that exposure to traumatic stressors and psychological trauma may result in increased morbidity and mortality. Much of the data available suggest traumatic exposure and subsequent PTSD may lead to increased incidence of cardiovascular disease, diabetes, chronic fatigue syndrome, and other conditions (Dansie et al., 2012; Edmondson & Cohen, 2013; Gupta, 2013; Lukaschek et al., 2013), but the involvement of neurotransmitters has yet to be clearly delineated. Serotonin (5-HT), for example, is a neurotransmitter responsible for many functions in the central nervous system (CNS) and peripheral organs. 5-HT influences aggression, arousal, sleep, anxiety, appetite, fear, learning, and other actions (Dubovsky, 1994). 5-HT is also the principle regulator of mood. A study by Peirson et al. (Peirson & Heuchert, 2000) found lower platelet 5-HT_2 receptor function was associated with depressed mood, while Williams et al. (Williams et al., 2006) demonstrated higher blood 5-HT levels were correlated with better mood. An increased mood and overall sense of well-being has been shown, in both psychiatric and physical disorders, as protective and positively correlated with resiliency behavior (Delamothe, 2005). PTSD research has demonstrated that 5-HT-uptake sites in platelets were lower in PTSD patients vs. controls (Arora, Fichtner, O'Connor, & Crayton, 1993). Lower 5-HT has also been implicated in diminished physical health. Muldoon et al. showed that a low prolactin response to fenfluramine, a drug that increases 5-HT levels, was associated with metabolic syndrome (Muldoon et al., 2004).

Norepinephrine (NE), a neurotransmitter principally affecting excitatory receptors, is also involved in the regulation of psychiatric and physical mechanisms. Under normal conditions, NE is a principle component of the stress response, directly increasing heart rate and blood flow to skeletal muscles and triggering the release of glucose, all in preparation for the 'fight-or-

flight' response. Persistent noradrenergic activity, however, has been linked with negative outcomes in patients with congestive heart failure (CHF) (Francis et al., 1993) and diabetes (Ganguly, Dhalla, Innes, Beamish, & Dhalla, 1986). Studies have also shown that individuals with PTSD have elevated cerebrospinal fluid (CSF) levels of NE (Geracioti et al., 2001) and noradrenergic hyperresponsiveness to various stimuli (Liberzon et al., 1999). Dysregulation of noradrenergic neurons has also been associated with hyperarousal and intrusive recollections attributable to PTSD (Southwick et al., 1999).

Dopamine (DA), a neurotransmitter with primarily inhibitory effects, plays a major role in emotion and the reward system of the brain. It optimally functions within a narrow range and dopaminergic hypo- or hyperactivity is implicated in both physical and psychiatric illnesses. Parkinson's disease is characterized by a loss of dopaminergic neurons, and evidence suggests schizophrenia and psychosis are linked to elevated levels of DA (Paterlini et al., 2005). DA may also have a role in PTSD, and studies have shown dopaminergic hyperactivity in male combat veterans (Yehuda, Southwick, Giller, Ma, & Mason, 1992), traumatized adult females (Glover et al., 2003), and abused children (De Bellis et al., 1999) with PTSD. The dopamine metabolite homovanillic acid (HVA), often used as a diagnostic test for catecholamine-producing tumors of the adrenal glands, has also demonstrated aberrant levels in PTSD patients. Geracioti et al. found HVA was significantly reduced in the CSF of combat-related PTSD patients immediately after viewing traumatic imagery (Geracioti et al., 2013). Based on the previous research, the primary focus of this study was to determine how neurotransmitters were modulated in response to a predator exposure/psychosocial stress rodent model of pre-clinical PTSD.

3.2 MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Institute for Laboratory Animal Research's 2011 *Guide for the Care and Use of Laboratory Animals*, under the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee (Protocol Number: 12-067). Upon completion of all PTSD-related experiments and in adherence with the approved protocol, the cats will be adopted out to approved families in the local area.

Animals

Naïve adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in all experiments. The rats were the same age (12 weeks) and approximately the same weight ($\pm$15g) upon delivery. Rats were pair-housed in standard plastic microisolator cages and had access to food and water *ad libitum*. The cages were maintained in ventilated racks (racks hold eight cages vertically and five horizontally) and each cage was randomly assigned to a specific rack location to ensure groups were evenly distributed. The vivarium room was kept on a 12-hour light/dark cycle (0700-1900), room temperature was maintained at 20 ± 1ºC, and humidity ranged from 23-42%. After a one-week acclimation period, the mean weight of all rats was 347.9g $\pm$ 4.5. Two cats, one male and one female (Harlan Laboratories, Indianapolis, IN (male), and Tulane University, New Orleans, LA (female)) were used for all predator exposures. Cats were seven and ten years old, respectively. They were housed in an open room (15' x 15') in the vivarium with access to food, water, and enrichment devices *ad libitum*. The cat room was on the same light/dark cycle and maintained at similar temperature and humidity as the rat room.

Stress Induction

Following the acclimation period, rats were brought to the laboratory and under isoflurane anesthesia were weighed, ear-tagged, tail-marked (ear tag number written on tail for easy identification), and 250-500µL of blood was drawn from either the tail or lateral saphenous vein. The rats were then randomly assigned to the PTSD (n=10) or control (n=10) group and returned to the vivarium for 24 hours. The following day, PTSD rats were started on a predator exposure/psychosocial stress regimen, published and validated by Zoladz et al., designed to produce a pre-clinical PTSD that closely mimics signs and symptoms seen in human patients (Zoladz, Conrad, Fleshner, & Diamond, 2008; Zoladz et al., 2012). Briefly, PTSD rats were individually isolated in cylindrical, Plexiglas containers (IITC Life Science, Inc., Woodland Hills, CA; tail cuff restraint containers for 400-600g rats and Kent Scientific, Torrington, CT; tail cuff restraint containers for 300-500g rats) and canned cat food (Friskies, Purina, St. Louis, MO) was smeared on the outside of the cylinders. The cylinders prevented direct contact with the cats, and the cat food induced predatory movement in the cats. Studies show a moving cat invokes a greater fear response than a sedentary cat (Blanchard et al., 1975). Rats were then placed in a stainless steel holding cage (76cm x 76cm x 60cm) consisting of a solid metal floor with a hinged, metal rod door, with a cat for one hour. The first cat exposure was conducted during the light cycle (0700-1900). Ten days later, a second cat exposure was conducted during the dark cycle (1900-0700). In addition to the cat exposures, starting on day one the rats were subjected to psychosocial stress by changing their cage cohort daily. The cage cohort rotation was established prior to the start of the experiment, whereby each rat was never housed with the same rat on consecutive days and never housed with the same rat more than four times in a month. The predator exposure/psychosocial stress regimen was continued for 31 days. After 31

days, PTSD and control group rats were euthanized via decapitation and the brains were immediately removed. The hippocampus, amygdala, and prefrontal cortex (PFC) were dissected and flash-frozen in liquid nitrogen.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Preparation of Standard Solution

Neurotransmitter concentrations were detected using an Eicom HTEC 500 high performance liquid chromatography system. The standard solutions of norepinephrine (NE; MW 337.3), 3, 4- dihydroxyphenylacetic acid (DOPAC; MW 168.15), dopamine (DA; MW 158.17), 5-hydroxyindoleacetic acid (5-HIAA; MW 218.68), homovanillic acid (HVA; MW 182.18), 5-hydroxytryptamine (5-HT; MW 212.68) and isoproterenol (internal standard; MW 247.7), each 1 ng/µL concentration, were prepared by serial dilution. 5-HT and 5-HIAA were dissolved in 0.1M acetic acid including 1mg/mL EDTA and other salts were prepared in 0.1M hydrochloric acid including 1mg/mL EDTA. These solutions were prepared and filtered through a 0.45µm centrifuge tube filter before injection into the HPLC system. Different concentrations were injected by maintaining the volume of injection at 10µL in order to quantify sample values after authenticating the retention time of individual neurotransmitters.

Preparation of Samples

Sample preparations from the experimental animals were carried out according to the procedure of Deyama et al. (Deyama et al., 2008). Hippocampus and PFC tissue were weighed and dissected before homogenizing at 4º C with 0.2 M perchloric acid including 100µM EDTA-2Na in a Teflon/glass homogenizer. The homogenate was centrifuged at 4º C for 15 min at 20,000 x g. The supernatant was collected and filtered through a 0.45µm centrifuge tube filter before injection into the HPLC system.

HPLC–EC Detection of Neurotransmitters

The following working conditions were maintained in the HPLC system: isocratic elution; mobile phase (citrate buffer in methanol with EDTA and sodium octane sulfonate); Eicompak SC-3ODS (ID 3.0 X 100mm) column; flow rate 340 µL/min; graphite working electrode WE-3G (Gasket GS-25), (+750mV versus Ag/AgCl electrode); temperature 25°C. The levels of neurotransmitters are expressed as pg/µg of wet tissue.

Mobile phase

Citric acid monohydrate (8.84 g; mol wt. 210.14), and 3.10g of sodium acetate (mol. wt. 82.03) in 800 ml of MilliQ Ultrapure fresh water (>18.2MΩ/cm) and 200ml of HPLC grade methanol were added and shaken well (magnetic stirrer not used). EDTA (Dojindo Laboratories, USA, mol. wt. 372.24; 0.005g) and sodium octane sulfonate (Dojindo Laboratories, USA, and 0.220 g) were added and shaken well.

Western Blot

Tissue homogenates from the hippocampus and PFC were subjected to Western Blot (WB) analysis (n =10/group) for the determination of protein levels of the norepinephrine and dopamine rate-limiting enzyme tyrosine hydroxylase (TH), the 5-HT rate-limiting enzyme tryptophan hydroxylase (TPH), and GAPDH. The extraction of protein and WB was performed as previously described (Agarwal et al., 2011). The specific antibodies used included: TH, TPH, and GAPDH. Primary antibodies were commercially obtained: TH, 1:1000 dilution (AB-112, Abcam, Cambridge, MA.); TPH, 1:1000 dilution (AB-1541, Millipore, Billerica, MA.); GAPDH, 1:1000 dilution (SC-25778, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were commercially obtained: anti-rabbit, 1:5000 dilution, anti-sheep, 1:5000 dilution (SC-2004 and SC-2701 respectively, Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using ImageJ imaging software (NIH), and they were normalized with GAPDH.

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was done by Student's T-Test or one-way ANOVA with a Tukey's post hoc test. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using Prism (GraphPad Software, Inc, La Jolla, CA; version 5.0).

3.3 RESULTS

Neurotransmitters and their metabolites were modulated in the hippocampus and PFC

Table 3.1: Changes in the levels of biogenic amines and metabolites in the PFC and hippocampus after the 31-day predator exposure/psychosocial stress regimen. Average concentration in pg/µg of wet tissue (±SEM) in the hippocampus (n=10 for both groups).

Parameters	Prefrontal Cortex	Hippocampus
5-HT		
Control	3250.2 ± 503.3	349.8 ± 34.0
PTSD	2067.9 ± 148.2*	165.3 ± 14.9***
NE		
Control	421.5 ± 32.8	533.0 ± 64.6
PTSD	671.6 ± 55.3**	1277 ± 172.3***
DA		
Control	1637.4 ± 226.7	545.3 ± 95.6
PTSD	5640.1 ± 383.4***	646.4 ± 74.5
HVA		
Control	1419.6 ± 202.5	224.6 ± 19.3
PTSD	1386.2 ± 119.9	128.7 ± 18.3**
DOPAC		
Control	2446.8 ± 152.0	853.9 ± 114.4
PTSD	4135.7 ± 371.6***	1391.0 ± 27.9***
5-HIAA		
Control	1907.6 ± 253.4	976.1 ± 152.7
PTSD	1525.2 ± 175.4	854.1 ± 63.6

5-HT: 5-hydroxytrypamine, HVA: homovanillic acid, NE: norepinephrine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DA: dopamine, 5-HIAA: 5-Hydroxyindoleacetic acid. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$ relative to the control group.

To investigate the influence of the predator exposure/psychosocial stress regimen on

neurotransmitter modulation, we examined endogenous levels of biogenic amines and their

metabolites in the hippocampus and PFC of control and PTSD animals using HPLC (Table 3.1).

In the hippocampus, the levels of the tryptamine 5-HT and the DA metabolite HVA (Figure

3.1A) were significantly lower in the PTSD group vs. controls, $t(18) = 4.96$, $p < 0.0001$ and $t(18)$

$= 3.61$, $p < 0.05$, respectively. Conversely, the levels of the catecholamine NE and the DA

metabolite DOPAC (Figure 3.1B) were significantly higher in the PTSD group, $t(18) = 4.05$, $p <$

Figure 3.1: 5-HT and HVA were both significantly down-regulated (A), while NE and DOPAC
were both significantly elevated (B), in the hippocampus in response to the predator
exposure/psychosocial stress model. All data are presented as mean ± SEM. **$p < 0.001$, ***p
< 0.0001 relative to the control group.

0.001, and $t(18) = 4.56$, $p < 0.001$, respectively. There were no significant changes noted in DA

or 5-HIAA. In the PFC, the tryptamine 5-HT was significantly lower, while the catecholamine

NE was higher (Figure 3.2A), in the PTSD group vs. controls, $t(18) = 2.25$, $p < 0.05$, and $t(18) =$

3.89, $p < 0.001$. In addition, the levels of the catecholamine DA and the DA metabolite DOPAC

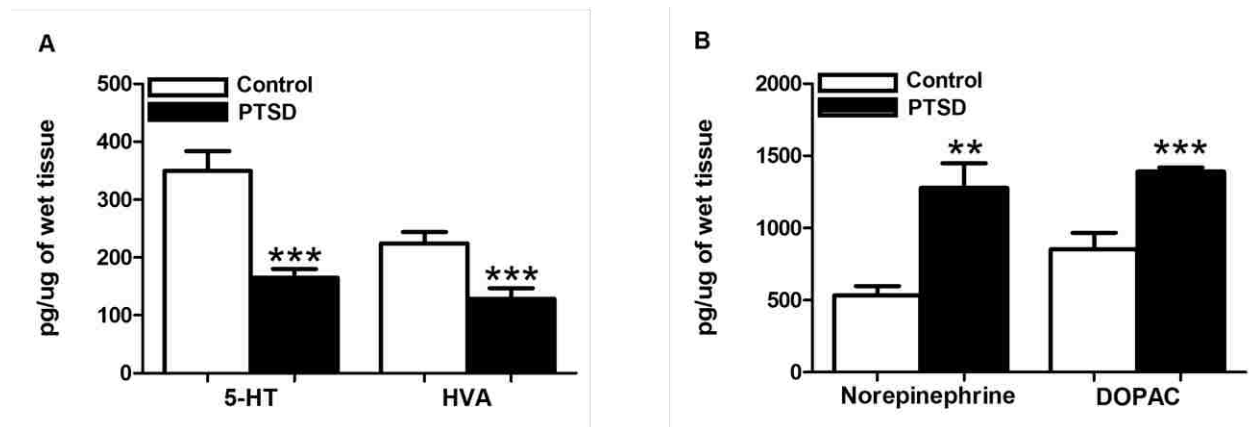(Figure 3.2B) were significantly higher in the PTSD group, $t(18) = 8.99$, $p < 0.0001$, and $t(18) =$

4.21, $p < 0.001$ respectively. There were no significant changes noted in HVA or 5-HIAA.

Rate-limiting enzyme fluctuations confirmed neurotransmitter modulation

To verify the observed changes in neurotransmitter levels in the hippocampus and PFC, we performed Western Blots of the rate-limiting enzymes for dopamine and norepinephrine (tyrosine hydroxylase (TH)) and 5-HT (tryptophan hydroxylase (TPH)). In both the hippocampus $t(6) = 5.00$, $p < 0.01$ and PFC $t(6) = 4.75$, $p < 0.01$, TH was elevated in the PTSD group vs. controls (Figures 3.3A and 3.3C). Conversely, TPH was significantly downregulated in the hippocampus $t(6) = 2.14$, $p < 0.05$ and PFC $t(6) = 5.20$, $p < 0.01$ in the PTSD group vs. controls (Figures 3.3B and 3.3D).

3.4 DISCUSSION

The present study sought to analyze neurotransmitter modulation in the hippocampus and PFC of rats subjected to pre-clinical PTSD via a predator exposure/psychosocial stress regimen. A myriad of animal models of PTSD exist, but the model by Zoladz et al. has been shown to cause heightened anxiety, exaggerated startle response, impaired cognition, and increased cardiovascular reactivity (Zoladz et al., 2008; Zoladz et al., 2012), all of which are common symptoms reported in humans with PTSD (Brewin et al., 2000; Nemeroff et al., 2006). Although animal models have their limitations, a major component missing from human PTSD research is the ability to ascertain physiological data directly from specific brain regions immediately after a stressful event. The majority of the human physiological data gathered *in vivo* are derived from CSF, blood, or urine, which may not accurately reflect neurotransmitter modulation in the brain and certainly cannot distinguish between changes in specific brain regions. We have successfully obtained such data with this model, and to our knowledge, we are the first to report the modulation of biogenic amines in the brains of PTSD animals. Two novel
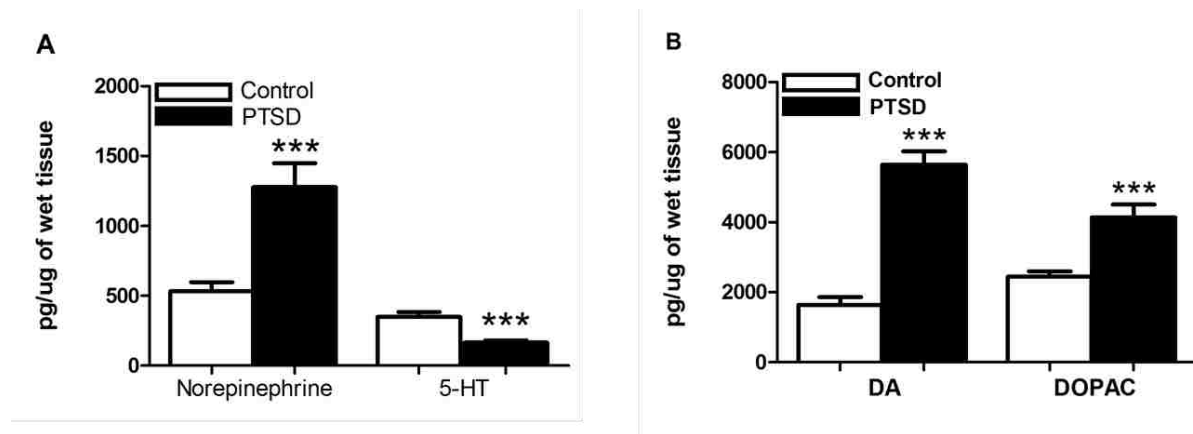
Figure 3.2: NE was elevated and 5-HT was down-regulated in the PFC in response to the predator exposure/psychosocial stress model (A). In addition, DA, and DOPAC were also significantly elevated (B). All data are presented as mean ± SEM. *p < 0.05, **p < 0.001, ***p < 0.0001 relative to the control group.
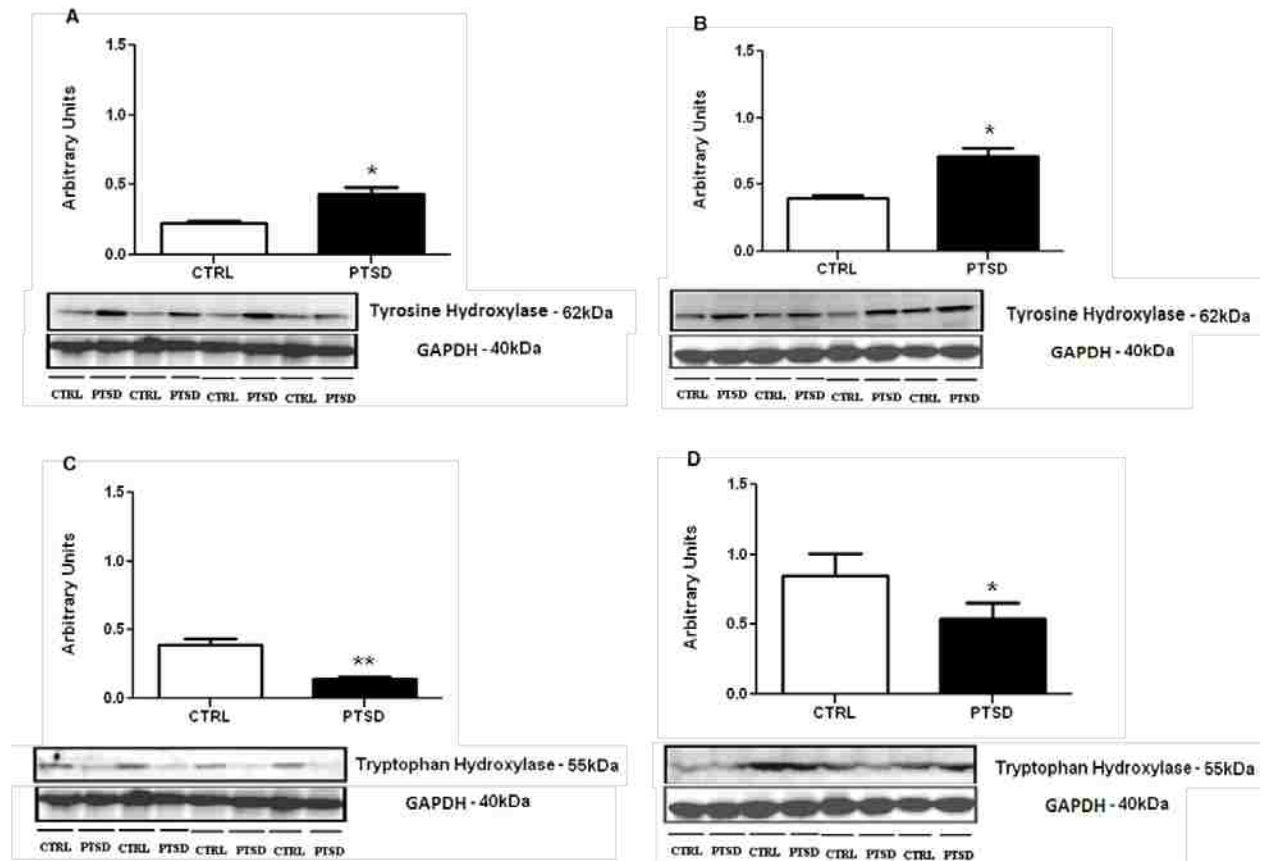
Figure 3.3: Tyrosine hydroxylase elevation in the PFC (A) and hippocampus (B) substantiated the findings of elevated levels of NE and DA, while down-regulated tryptophan hydroxylase in the PFC (C) and hippocampus (D) confirmed decreased levels of 5-HT. All data are presented as mean ± SEM. *p < 0.05, **p < 0.001.

and important findings emerged from this study. First, the predator exposure/psychosocial stress regimen of pre-clinical PTSD produced measureable changes in neurotransmitters in the rat brain. Second, and possibly most important, 5-HT decreased and NE increased in both the hippocampus and PFC, providing evidence that the neurotransmitters previously implicated in PTSD pathophysiology are in fact modulated in response to persistent stressors.

Human PTSD research has made significant advances, but certain undefined variables still exist. Inconsistencies in patient backgrounds, types of stressful events (e.g., combat, rape, natural disasters, etc.), innumerable epigenetic differences, and the inability to obtain physiological data before PTSD development all present challenges. An animal model mitigates these variables, but as there are multiple animal models used in PTSD research with varied approaches and methods, careful selection is necessary. For our experiments, it was important to select an animal model of PTSD that matched, as closely as possible, the behavioral, psychological, and physiological elements of PTSD in humans. The predator exposure/psychosocial stress model by Zoladz et al. possesses both predictive and construct validity, making the model sensitive to clinically effective pharmacologic agents and the rationale underlying the model displays similarities to human PTSD (Bourin et al., 2007). This model demonstrates three hallmark features of PTSD: hormonal abnormalities, a long-lasting traumatic memory, and persistent anxiety (Zoladz et al., 2008; Zoladz et al., 2012).

The modulation of various neurotransmitters observed with the predator exposure/psychosocial stress model is in concert with many of the neurotransmitter changes seen in human PTSD patients (Arora et al., 1993; Geracioti et al., 2001; Geracioti et al., 2013; Yehuda et al., 1992). Previous research has shown that stress blocks hippocampal long-term potentiation (LTP) and impairs its function (Diamond, Campbell, Park, Halonen, & Zoladz, 2007). The

68

hippocampus, the primary region for spatial and long-term memory storage, expresses all of the 5-HT receptor families and reflects the overall serotonergic functions relating to cognition and mood in this region (Berumen, Rodriguez, Miledi, & Garcia-Alcocer, 2012). During stress, glucocorticoid production can reduce the excitability of hippocampal neurons, and 5-HT may have a protective effect against such damage by activating 5-HT_{1A} receptors (Joca, Ferreira, & Guimaraes, 2007). Persistent activation of the HPA axis and excessive production of glucocorticoids, however, may directly reduce hippocampal 5-HT levels and adversely affect normal serotonergic transmission, thus contributing to heightened fear, depressed mood, and reduced resilience. The hippocampus also contains multiple NE receptors, which, when activated during stress, may contribute to the reinforcement of long-term memories (Jurgens et al., 2005). In a study by Geracioti et al. involving male combat veterans with PTSD, CSF concentrations of NE were significantly higher vs. controls (Geracioti et al., 2001). This finding could possibly explain why memories formed during extremely stressful events persist over time. Other evidence of catecholamine dysregulation in PTSD includes elevated urine catecholamine excretion, exaggerated biochemical responses to yohimbe, and clinical efficacy of adrenergic blockers (Southwick et al., 1999). Noradrenergic modulation was also noted with previous experiments utilizing the predator exposure/psychosocial stress animal model and treatments with selective serotonin reuptake enhancers (SSRE), α_2 agonists, and tricyclic antidepressants (Zoladz, Fleshner, & Diamond, 2013). Although we found no significant difference in DA levels in the hippocampus, reduction of HVA level was consistent with current human PTSD research (Geracioti et al., 2013). HVA is a downstream product of DA metabolism, and traumatic stress may impede CNS release of DA from the substantia nigra and ventral tegmental area (VTA) (Cabib & Puglisi-Allegra, 2012), the primary CNS regions of dopaminergic neurons, thus

reducing metabolite concentration. Another explanation may be that the majority of DA is directly converted to NE and not HVA, which also would further explain the marked increase in NE.

The PFC, responsible for executive functions such as consequences, drive, and social "control", is highly innervated by serotonergic neurons from the raphe nuclei and also expresses an abundance of 5-HT receptors. The serotonergic neurons and 5-HT receptors, specifically the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, are a key modulator of the PFC-amygdala corticolimbic circuit involved in threat and emotional responses (Fisher et al., 2011). PTSD-related aberrancies in this serotonergic system may cause inappropriate or incomplete extinction of conditioned fear. The PFC also contains NE receptors and receives input from NE neurons from the locus coeruleus, which are activated during the stress response (Finlay, Zigmond, & Abercrombie, 1995). Pathogical or stress-related elevations of NE in the PFC, however, may inhibit working memory and performance (Zhang, Cordeiro Matos, Jego, Adamantidis, & Seguela, 2013). Current neuroimaging research indicates the PFC is hyporesponsive during symptomatic PTSD states and responsiveness is inversely proportional to symptom severity (Shin, Rauch, & Pitman, 2006). Whether marked elevations in NE directly or indirectly diminish PFC responsiveness and subsequent performance on cognitive emotional tasks remains unclear. In contrast to unchanged DA levels in the hippocampus, DA levels were significantly increased in the PFC, which was similar to the CSF and urine DA elevations seen in humans with PTSD (De Bellis et al., 1999; Glover et al., 2003; Yehuda et al., 1992). In a similar manner to NE, stress-related elevations of DA may also impair working memory and performance. The PFC is densely innervated by dopaminergic neurons from the VTA, and dopamine release can be achieved via VTA or local stimulation. A recent study by Butts et al. demonstrated that stress-induced glucocorticoid

70

stimulation of DA neurons caused a local release of DA in the PFC (Butts, Weinberg, Young, & Phillips, 2011). These data support the theory that overstimulation of the HPA axis and the resulting elevation in glucocorticoid activity can directly modulate DA and possibly other neurotransmitters.

3.5 CONCLUSIONS

We utilized a predator exposure/psychosocial stress animal model of PTSD to determine if neurotransmitters were modulated in the rat hippocampus and PFC. We found that various neurotransmitters implicated in PTSD pathophysiology were altered in similar manners to those previously described in human PTSD research. In addition, we determined that other neurotransmitters were differentially expressed in the hippocampus and PFC. It would be an oversimplification, nonetheless, to presume that neurotransmitter modulation is the sole causal or resultant factor in PTSD development, as HPA axis, sympathoadrenal medullary pathway, and immune system alterations may also play an integral role in the pathophysiology of this complex disorder. Our use of the model established by Zoladz et al., (Zoladz et al., 2008; Zoladz et al., 2012) produced results consistent with current neurotransmitter levels obtained via CSF and urine analysis in humans, further validating the efficacy of the model. Overall, our results demonstrate that there are CNS-specific modifications in neurotransmitter activity and expression in response to predator exposure/psychosocial stress. Future studies by our lab will investigate neurotransmitter modulation in the hippocampus and PFC in response to FDA-approved pharmacologic interventions and possibly provide insight into to why selective serotonin reuptake inhibitors are only moderately effective in many PTSD patients.

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CHAPTER 4
VALPROIC ACID EFFECTS IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX IN AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER

4.1 INTRODUCTION

Post-Traumatic Stress Disorder (PTSD), an anxiety disorder recently reclassified as a trauma- and stressor-related disorder, can develop in response to real or perceived life-threatening situations and results in a prolonged stress response (Yehuda, 2002). According to the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), a diagnosis of PTSD necessitates exposure to a traumatic event, intrusive recollections, avoidance of associated stimuli, negative cognitions/mood, hyperarousal, and a significant social impairment. These symptoms must persist for at least 30 days and not be due to illness, medication, or substance abuse (American Psychiatric Association, 2013). To date, no definitive diagnostic biomarkers have been identified for PTSD. Current research, however, points toward physiological abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary system, immune system, and brain neurotransmitters that may be responsible for the progression of the disorder (Liberzon, Abelson, Flagel, Raz, & Young, 1999; Oosthuizen, Wegener, & Harvey, 2005; Sondergaard, Hansson, & Theorell, 2004; Wilson et al., 2014). Many chronic conditions such as hypertension, heart failure, and metabolic syndrome perpetuate in a state of inflammation and oxidative stress (Cardinale et al., 2010; Elks & Francis, 2010; Guggilam et al., 2011). In PTSD, inflammation and oxidative stress also persist.

Our lab recently demonstrated ROS and PICs were elevated in the brain and systemic circulation during PTSD progression (Wilson et al., 2013).

Inflammation is a critical component of the immune response, but chronic inflammation can damage cellular mechanisms. There are a host of triggers initiating the inflammatory response, many of which are initiated via Toll-like receptor-4 (TLR4) and subsequent nuclear factor (NF)-κB activation. Transcription by NF-κB requires DNA and chromatin remodeling, which enables access to the pertinent genomic sequences. Gene expression is regulated via highly controlled acetylation/deacetylation of histone N-terminal tails, which either increases or decreases gene availability (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003). Acetylation/deacetylation is accomplished by histone acetyltransferases (HAT) and histone deacetylases (HDAC), which enable and restrict genome access, respectively. When oxidative stress and inflammation are increased, upregulated PICs can correspond with heightened HDAC activity and NF-κB transcription, resulting in perpetual PIC production (Keslacy, Tliba, Baidouri, & Amrani, 2007). Although HDACs restrict access for transcription and should depress PIC production, the exact opposite may be the case. The effects of HDAC inhibitors (HDACi) extend to non-histone proteins that are reversibly acetylated, which markedly affects their function (Nair, Boersma, Schiltz, Chaudhry, & Muschel, 2001; Tong, Yin, & Giardina, 2004). This functional shift means HDACs may actually enhance the inflammatory response. In addition to histone modification, HDACs may also modulate neurotransmitters by modifying levels of pertinent rate-limiting enzymes (Sharma, Grayson, & Gavin, 2008). For example, tyrosine hydroxylase, the rate-limiting enzyme for dopamine (DA) and norepinephrine (NE) synthesis, is depressed by HDACi (Akiba et al., 2010). Studies have also shown antidepressant

effects of HDACi (Covington et al., 2009), possibly be due to epigenetic modifications of tryptophan hydroxylase, the rate-limiting enzyme for serotonin (5-HT) synthesis.

Currently, HDACi have proven beneficial as anticonvulsants and mood-stabilizers and have received attention as treatment for some forms of cancer (Alvarez-Breckenridge et al., 2012). They have also been shown to modify receptors and other neuronal cellular mechanisms that have a role in synaptic plasticity and learned behavior (Morris, Karra, & Monteggia, 2010). Research has demonstrated that administration of HDACi enhanced synaptic plasticity, dendritic growth, and the extinction of learned behavior in a drug-induced animal model (Malvaez, Sanchis-Segura, Vo, Lattal, & Wood, 2010). A recent study revealed that the HDACi valproate enhances fear extinction (Heinrichs, Leite-Morris, Rasmusson, & Kaplan, 2013). Since fear extinction is an important component of PTSD therapy, it follows that HDACi administration in PTSD may prove beneficial in diminishing negative learned behavior and enhancing resiliency. Our lab recently demonstrated the efficacy of valproic acid (VA) in diminishing ROS and PIC escalation in hypertension (Cardinale et al., 2010). Oxidative stress and inflammation can damage neurons and negatively influence synaptic plasticity, so we hypothesized that attenuating this effect may diminish PTSD symptoms and improve overall resiliency behavior. Based on this information, this study investigated whether chronic HDACi administration could modulate behavior, redox imbalances, inflammation, and neurotransmitters during the progression of PTSD, using a predator exposure/psychosocial stress animal model.

4.2 MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Institute for Laboratory Animal Research's 2011 *Guide for the Care and Use of Laboratory Animals*, under

the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee.

Animals

Naïve adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in all experiments. The rats were the same age (12 weeks) and approximately the same weight (±15g) upon delivery. Rats were pair-housed in standard plastic microisolator cages with access to food and water *ad libitum*. The cages were maintained in ventilated racks and randomly assigned to a rack location to ensure groups were evenly distributed. The vivarium room was kept on a 12-hour light/dark cycle (0700-1900), temperature was maintained at 20±1ºC, and humidity ranged from 23-42%. After a 1 week acclimation period, the mean weight of all rats was 347.9g ± 4.5. Two cats, a male and a female (Harlan Laboratories, Indianapolis, IN) were used for all predator exposures. They were housed in an open room (15' x 15') in the vivarium with access to food, water, and enrichment devices *ad libitum*. The cat room was on the same light/dark cycle and maintained at similar temperature and humidity.

Stress Induction

The predator exposure/psychosocial stress regimen is designed to induce a PTSD-like syndrome as true PTSD is clinically defined as a human disorder. Following the acclimation period, rats were weighed, ear-tagged, tail-marked, and 250-500μL of blood was drawn from the tail vein. The rats were then randomly assigned to the PTSD or control group and returned to the vivarium for 24 hours. The following day, PTSD rats were started on a predator exposure/psychosocial stress regimen. Briefly, PTSD rats were individually isolated in cylindrical, Plexiglas containers (IITC Life Science, Inc., Woodland Hills, CA.) and canned cat food was smeared on the outside of the cylinders. The cylinders prevented direct contact with

the cats, and the cat food induced movement in the cats.   Rats were then placed in a stainless

steel cage (76cm x 76cm x 60cm) consisting of a solid metal floor with a hinged, metal rod door,

with a cat for one hour. The first cat exposure was conducted during the light cycle (0700-1900).

Ten days later, a second cat exposure was conducted during the dark cycle (1900-0700). The

third exposure was during the light cycle. In addition, the rats were subjected to psychosocial

stress by changing their cage cohort daily. The predator exposure/psychosocial stress regimen

continued for 40 days, after which certain PTSD and control group rats were administered

valproic acid (VA) for 30 days. All groups were then euthanized, perfused with a phosphate

buffered solution, and the brains were removed. The hippocampus and PFC were dissected and

flash-frozen in liquid nitrogen.

Elevated Plus-Maze

Rats were placed in the center of the elevated plus-maze (EPM) (EB-Instruments

(Bioseb), Tampa Bay, FL) facing an open arm and allowed to roam freely for five minutes.

Movement was monitored via an overhead camera and captured with a software program

(BioEPM3C, EB-Instruments, Tampa Bay, FL). The primary measurements were the total

number of arm entries and the total time spent in the open vs. closed arms.

Valproic Acid

Rats were pair-housed and administered VA in their water bottles. Vehicle (water) or

valproic acid (VA, 0.71% wt/vol, Sigma) dissolved in water were prepared and provided daily

for 30 days as previously described (Cardinale et al., 2010).

Electron Paramagnetic Resonance Spectroscopy

Superoxide and total ROS (n = 6/group) were measured in whole blood via electron

paramagnetic resonance (EPR) as previously described (Mariappan et al., 2010). Superoxide and

total ROS levels were compared between all four groups. Briefly, blood was incubated at 37°C

with CMH (200 µM) for 30 min for ROS measurement, PEG-SOD (50 U/µl) for 30 min, then

CMH (200 µM) for an additional 30 min for $O_2\bullet-$ measurement. Aliquots of incubated probe

media were then taken in 50-µl disposable glass capillary tubes for determination of $O_2\bullet-$ or

total ROS production.

Real-Time PCR Analysis

Semi-quantitative real-time RT-PCR (n = 6/group) was used to determine the mRNA

levels of TLR4, NALP3, IL-1β, and IL-18 in the PFC and hippocampus. The primer sequences

used for real-time PCR are given (Table 4.1). Total RNA isolation, cDNA synthesis and RT-

PCR were performed as previously described (Agarwal, Welsch, Keller, & Francis, 2011).

Table 4.1: Rat primers used for real-time RT-PCR.

Gene	Sense	Antisense
GAPDH	agacagccgcatcttcttgt	cttgccgtgggtagagtcat
TLR4	attgttcctttcctgcctga	tccagccactgaagttgtga
NALP3	cagaaggcatgtgagaagca	tgggtgtagcgtctgttgag
IL-1β	cagaccactttggcagacttcact	ggattcgttggctgttcggtcg
IL-18	gaccactttggcagacttca	tagggtcacagccagtcctc

TLR4, (Toll-like receptor 4); NALP3, (NACHT, LRR, PYD domains containing protein 3); IL,
(Interleukin); GAPDH, (Glyceraldehyde 3-phosphate dehydrogenase).

Western Blot Analysis

Tissue homogenates from the PFC and hippocampus were subjected to Western Blot

(WB) analysis (n = 6/group) for the determination of protein levels of TLR4, NALP3, IL-1β, IL-

18, TH, TPH, and β-Actin. The extraction of protein and WB was performed as previously

described (Agarwal et al., 2011). Primary antibodies were commercially obtained: IL-1β, IL-18,

and β-Actin, 1:1000 dilution (SC-7884, SC-7954, and SC-1616R respectively, Santa Cruz

Biotechnology, Santa Cruz, CA); NALP3, 1:1000 dilution (ORB-101128, Biorbyt, San

Francisco, CA); TLR4, 1:1000 dilution, TH, 1:200 dilution (ab13556 and ab112 respectively,

ABCAM, Cambridge, MA); TPH, 1:1000 dilution (AB1541, Millipore, Billerica, MA).

Secondary antibodies were commercially obtained: anti-rabbit and anti-mouse, 1:5000 dilution

(SC-2004 and SC-2314 Santa Cruz Biotechnology, Santa Cruz, CA).

High-Performance Liquid Chromatography (HPLC)

Neurotransmitter concentrations were detected using an Eicom HTEC 500 HPLC system.

The standard solutions of NE (MW 337.3), DA (MW 158.17), 5-hydroxytryptamine (5-HT; MW

212.68) and isoproterenol (internal standard; MW 247.7) were each 1 ng/µL concentrations.

Sample preparations were carried out as previously described (Deyama et al., 2008).

HPLC Detection of Neurotransmitters

HPLC system working conditions: isocratic elution; mobile phase (citrate buffer in

methanol with EDTA and sodium octane sulfonate); Eicompak SC-3ODS (ID 3.0 X 100mm)

column; flow rate 340 µl/min; graphite working electrode WE-3G (Gasket GS-25), (+750mV

versus Ag/AgCl electrode); temperature 25°C.

HPLC Mobile Phase

Citric acid monohydrate (8.84 g; mol wt. 210.14), and 3.10g of sodium acetate (mol. wt.

82.03) in 800 ml of MilliQ Ultrapure fresh water (>18.2MΩ/cm) and 200ml of HPLC grade

methanol were added. EDTA (mol. wt. 372.24; 0.005g) and sodium octane sulfonate (0.220 g),

both from Dojindo Laboratories, Rockville, MD, were added.

HDAC Activity Analysis

Nuclear extracts of PFC and hippocampus tissue were obtained with a Nuclear Extraction

Kit (K266-100, BioVision, Milpitas, CA). Nuclear extracts were then analyzed for HDAC

activity with a colorimetric HDAC Activity Assay Kit (K331-100, BioVision), both according to

the manufacturer's instructions.

Assessment of NF-kB Activity

An ELISA kit (ab133112, Abcam, Cambridge, MA) was used to assess the activity of NF-kB in the nuclear extracts of the PFC and hippocampus according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was conducted by one-way ANOVA with a Bonferroni post hoc test for multiple comparisons, unpaired Student's T-tests for two-column analyses, and four-parameter logistic regression for curve fit. P-values less than 0.05 were considered significant. Statistical analyses were performed using Prism (GraphPad Software, Inc, La Jolla, CA; version 5.0).

4.3 RESULTS

Elevated Plus-Maze Performance

Immediately following the stress regimen, the PTSD group spent considerably less time in the open vs. closed arms, $t(10) = 3.99$, $p < 0.001$. Overall ambulations, however, were not affected, $t(10) = 0.88$, $p > 0.05$. After the 30-day VA treatment, the PTSD+VA group spent significantly more time in the open arms vs. the PTSD+Veh group, $F(3, 22) = 41.93$, $p < 0.01$. The control group showed no difference between the control+VA and control+Veh groups, $F(3, 22) = 5.17$, $p > 0.05$, (Figure 4.1A). No differences were found in overall ambulations between or within groups, $F(3, 22) = 1.52$, $p > 0.05$, (Figure 4.1B). Repeated measures were also conducted to analyze anxiety changes within the same groups over time. No changes were found in the control group between pre-treatment, control+Veh, and control+VA, $F(2, 21) = 3.07$, $p > 0.05$, (Figure 4.1C). In the PTSD group, however, significant differences were noted between pre-treatment, PTSD+Veh, and PTSD+VA, $F(2, 27) = 14.88$, $p < 0.0001$, (Figure 4.1D).
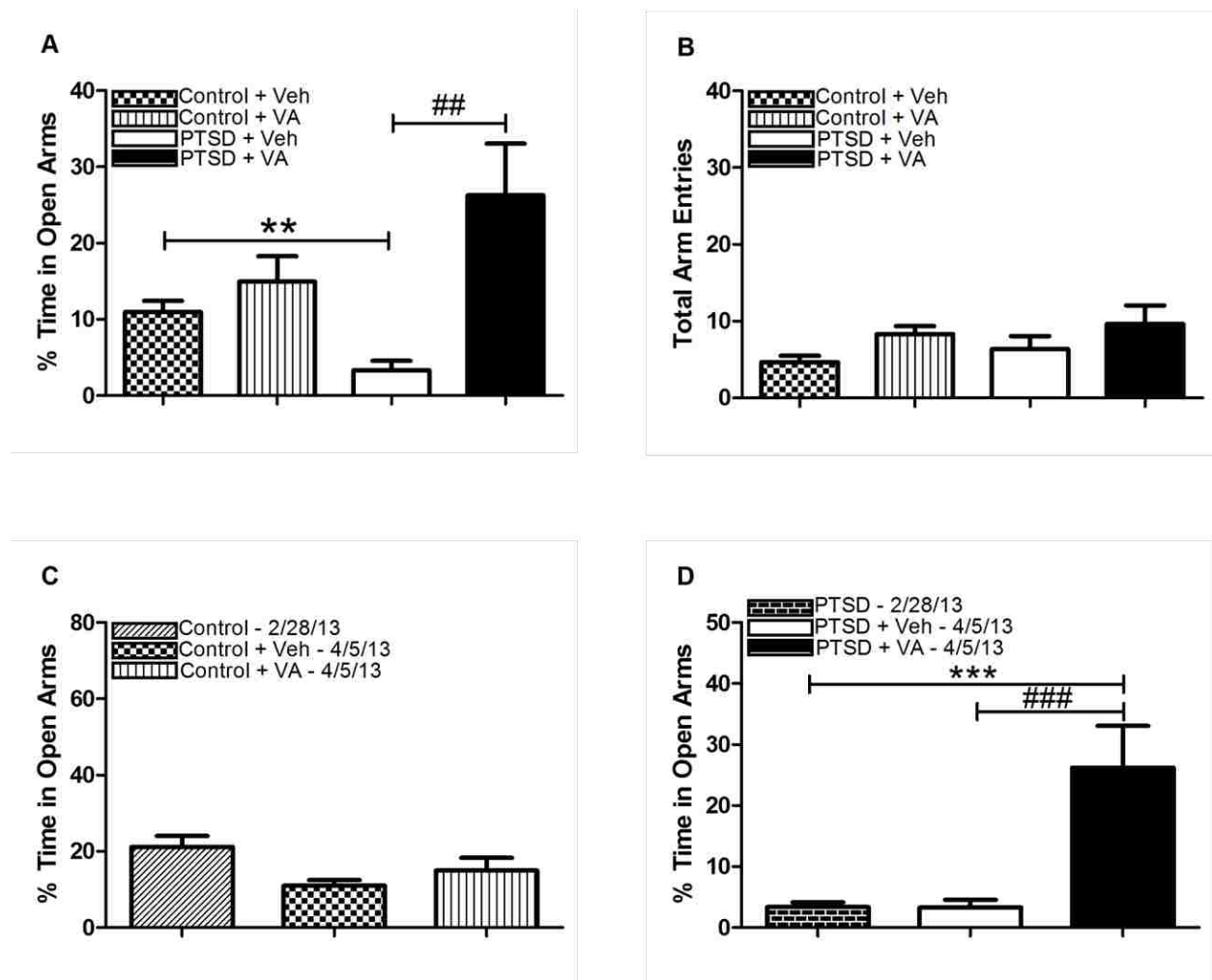
Figure 4.1: The PTSD group spent considerably less time in the open vs. closed arms. After VA treatment, the PTSD+VA group spent significantly more time in the open arms vs. the PTSD+Veh group. The control group showed no difference between the control+VA and control+Veh groups (A). No differences were found in overall ambulations between or within groups (B). Repeated measures found no difference in the control group between pre-treatment, control+Veh, and control+VA (C). In the PTSD group significant differences were noted between pre-treatment, PTSD+Veh, and PTSD+VA (D). All data are presented as mean ± SEM. **$p < 0.001$, ***$p < 0.0001$ relative to the control group. ##$p < 0.001$, ###$p < 0.0001$ relative to the treatment group.

Oxidative Stress Analysis

Analysis of the EPR data revealed superoxide was significantly elevated in whole blood in the PTSD group vs. controls, and it diminished with VA, $F(3,18) = 2.54$, $p < 0.05$. No difference was noted in the control+VA vs. the control+Veh groups, $t(8) = 0.07$, $p > 0.05$ (Figure 4.2A). Total ROS was also significantly elevated in the PTSD group vs. controls, and it

84

was also decreased with VA, $F_{(3,18)} = 3.13$, $p < 0.05$. No difference was noted in the

control+VA vs. the control+Veh groups, $t_{(8)} = 0.64$, $p > 0.05$ (Figure 4.2B).

Brain Inflammatory Markers

The PTSD group demonstrated elevated mRNA levels of TLR4, $F_{(3,12)} = 6.37$ and 7.14,

$p < 0.05$ (Figures 4.3A&B), NALP3, $F_{(3,12)} = 5.69$ and 17.10, $p < 0.05$ (Figures 4.3C&D), IL-

1β, $F_{(3,12)} = 6.08$ and 2.85, $p < 0.05$ and $p > 0.05$ (Figures 4.3E&F) and IL-18, $F_{(3,12)} = 44.84$

and 5.45, $p < 0.05$ (Figures 4.3G&H). Valproic acid administration normalized the upregulated

mRNA to levels similar to the untreated controls. In the PFC (Figure 4.3I) and hippocampus

(Figure 4.3J), both regions also demonstrated elevated protein levels of TLR4, $F_{(3,4)} = 30.52$

and 454.70, $p < 0.05$, NALP3, $F_{(3,4)} = 9.42$ and 27.67, $p < 0.05$, IL-1β, $F_{(3,4)} = 12.03$ and

12.83, $p < 0.05$, and IL-18, $F_{(3,4)} = 130.0$ and 50.18, $p < 0.05$. Valproic acid administration also

attenuated the upregulated protein to levels similar to the untreated controls.
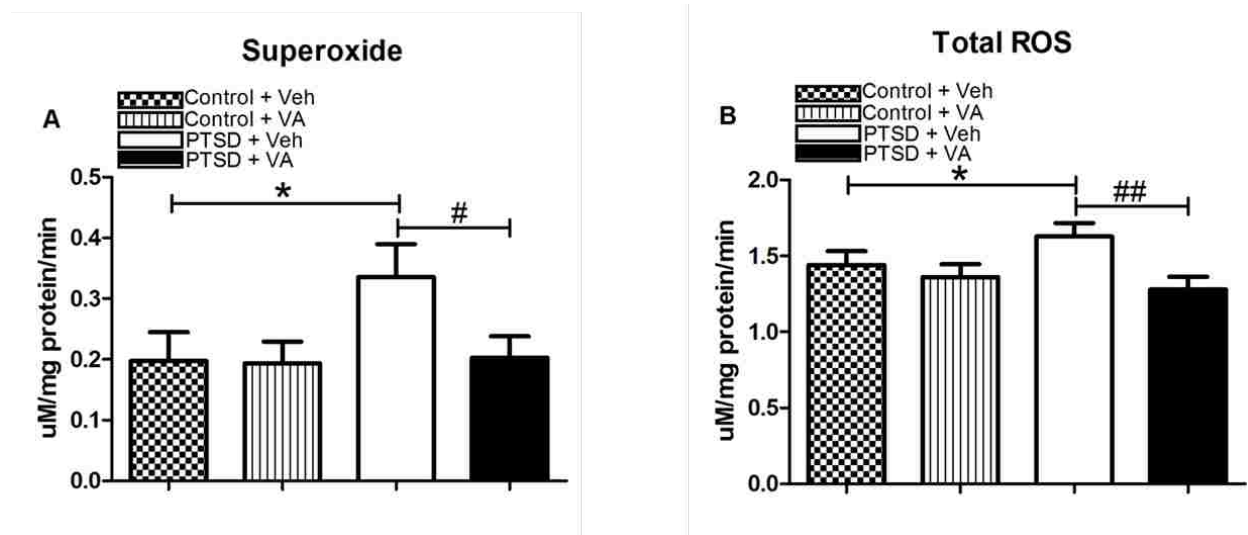
Figure 4.2: Superoxide (A) and total ROS (B) were significantly elevated in the circulating blood
in the PTSD group. The elevation was normalized to control group levels with VA. All data are
presented as mean ± SEM. *$p < 0.05$ relative to the control group. #$p < 0.05$, ##$p < 0.001$
relative to the treatment group.
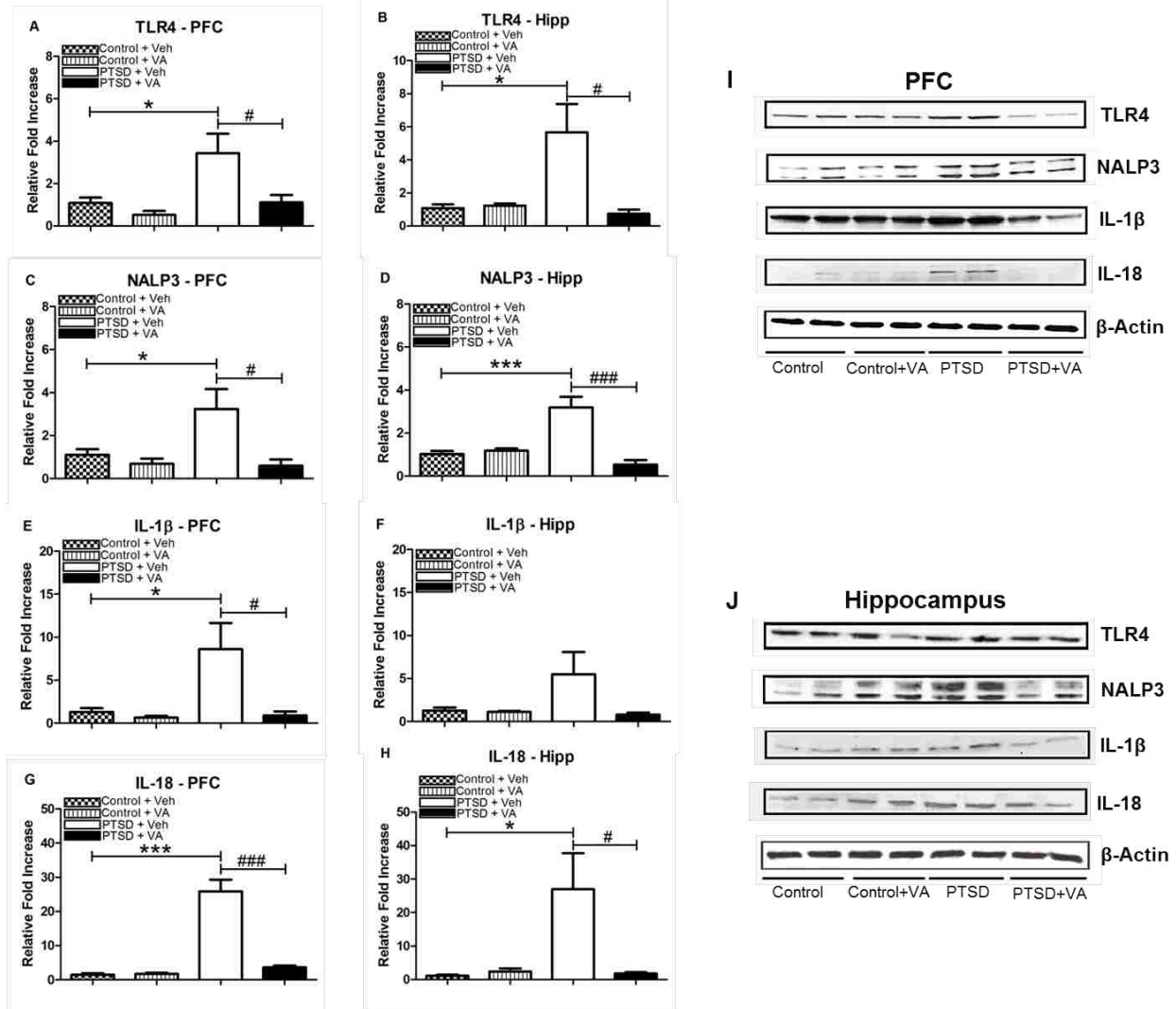
Figure 4.3: In the PFC, mRNA levels of TLR4, NALP3, IL-1β, and IL-18 were elevated in the untreated PTSD vs. control group, which was normalized with VA (A, C, E, G). In the hippocampus, mRNA levels of TLR4, NALP3, and IL-18 were elevated in the untreated PTSD vs. control group, which was normalized with VA (B, D, H). IL-1β was elevated vs. the control+Veh group and PTSD+VA group, but did not reach significance (F). All data presented as mean ± SEM. $*p < 0.05$, $***p < 0.0001$ relative to the control group. $\#p < 0.05$, $\#\#\#p < 0.0001$ relative to the treatment group.

HDAC Activity

Analysis of HDAC activity in the PFC revealed increased HDAC in the untreated PTSD group vs. controls, which was subsequently attenuated with VA (Figure 4.4A), $F(3,17) = 3.282$, $p < 0.05$. HDAC activity in the hippocampus was elevated in the untreated PTSD vs. control

group (did not reach significance), but it was significantly downregulated in the PTSD+VA

group (Figure 4.4B), $F(3,17) = 2.006$, $p < 0.05$.

NF-κB Transcription

The transcription factor NF-κB was significantly elevated in the PFC in the untreated

PTSD group vs. controls, which was normalized with VA (Figure 4.4C), $F(3,17) = 13.86$, $p < $

0.0001. In the hippocampus, NF-κB was also upregulated in the untreated PTSD group vs.

controls, which was subsequently downregulated with VA (Figure 4.4D), $F(3,17) = 16.66$, $p < $

0.0001.

Neurotransmitter Modulation

In the PFC, the catecholamines NE (Figure 4.5A) and DA (Figure 4.5C) were

significantly higher in the PTSD group vs. controls, $F(3,17) = 8.66$ and $F(3,17) = 36.32$, $p < $

0.05, respectively. Conversely, 5-HT (Figure 4.5E) was significantly lower in the PTSD group

vs. controls, $F(3,17) = 7.41$, $p < 0.05$. Valproic acid administration lowered catecholamine levels

and increased 5-HT to levels similar to the untreated controls. In the hippocampus, NE (Figure

4.5B) was significantly higher in the PTSD group vs. controls, $F(3,17) = 58.57$, but no

significant difference was found in DA levels, $F(3,17) = 1.56$, $p > 0.05$. The level of 5-HT

(Figure 4.5E) was lower in the PTSD group vs. controls, $F(3,17) = 30.65$, $p < 0.05$. Valproic

acid administration lowered NE levels and increased 5-HT to levels similar to the untreated

controls.

4.4 DISCUSSION

The present study sought to analyze the effects of HDACi administration on the behavior

and physiology associated with PTSD by employing a predator exposure/psychosocial stress

regimen. Multiple animal models designed to create PTSD-like effects are reported, but a
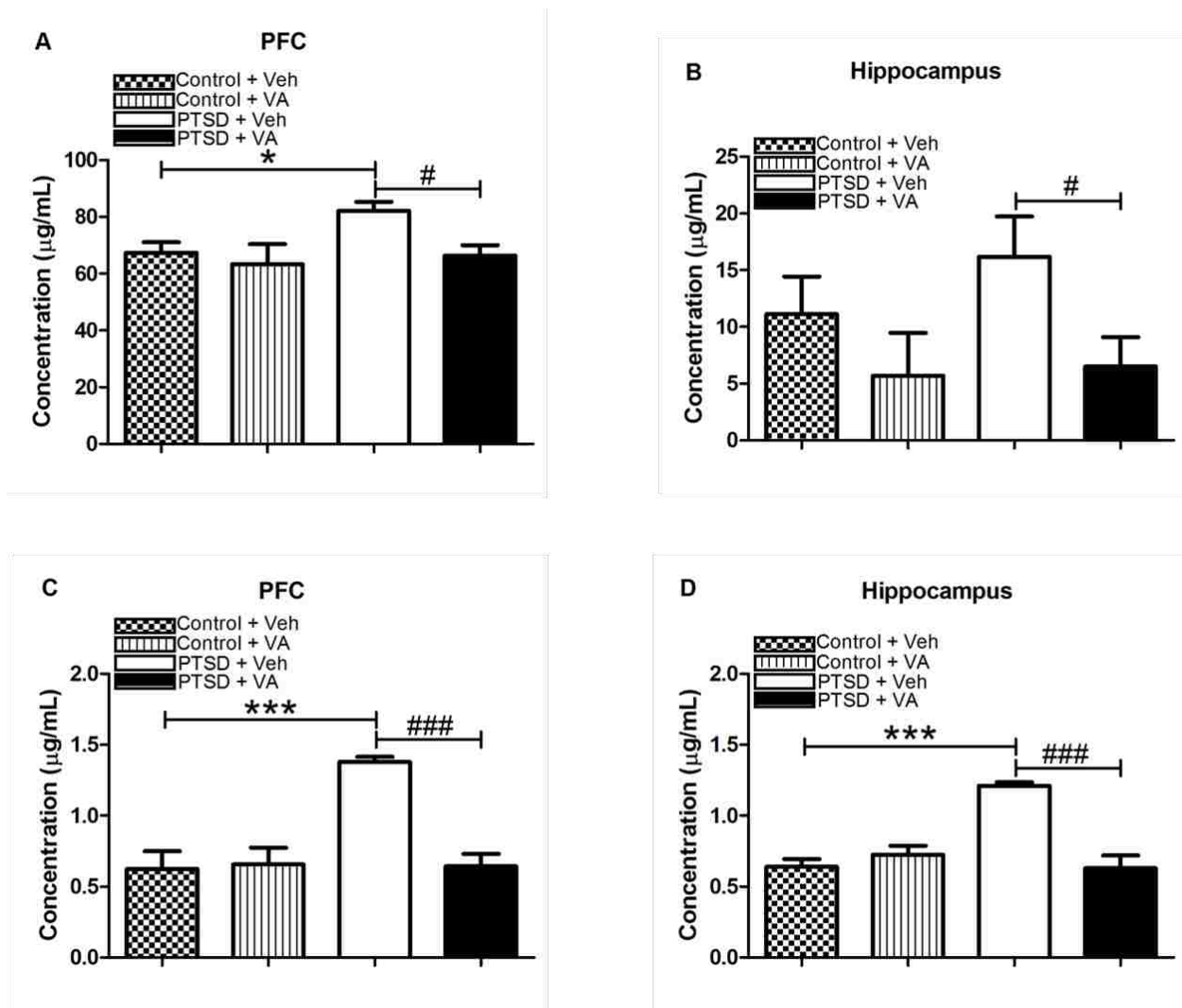
Figure 4.4: In the PFC, HDAC was increased in the untreated PTSD vs. control group, which was subsequently attenuated with VA (A). HDAC activity in the hippocampus was elevated in the untreated PTSD vs. control group (did not reach significance), but it was significantly downregulated in the PTSD+VA group (B). NF-κB was also elevated in the PFC (C) and hippocampus (D) in the untreated PTSD vs. untreated control group, which was normalized with VA. All data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.0001$ relative to the control group. #$p < 0.05$, ###$p < 0.0001$ relative to the treatment group.

particular model that seems to meet face, predictive, and construct validity is the predator

exposure/psychosocial stress regimen (Daskalakis, Yehuda, & Diamond, 2013). The model by

Zoladz et al. has been shown to cause heightened anxiety, exaggerated startle response, impaired

cognition, and increased cardiovascular reactivity (Zoladz et al., 2008; Zoladz et al., 2012), all of
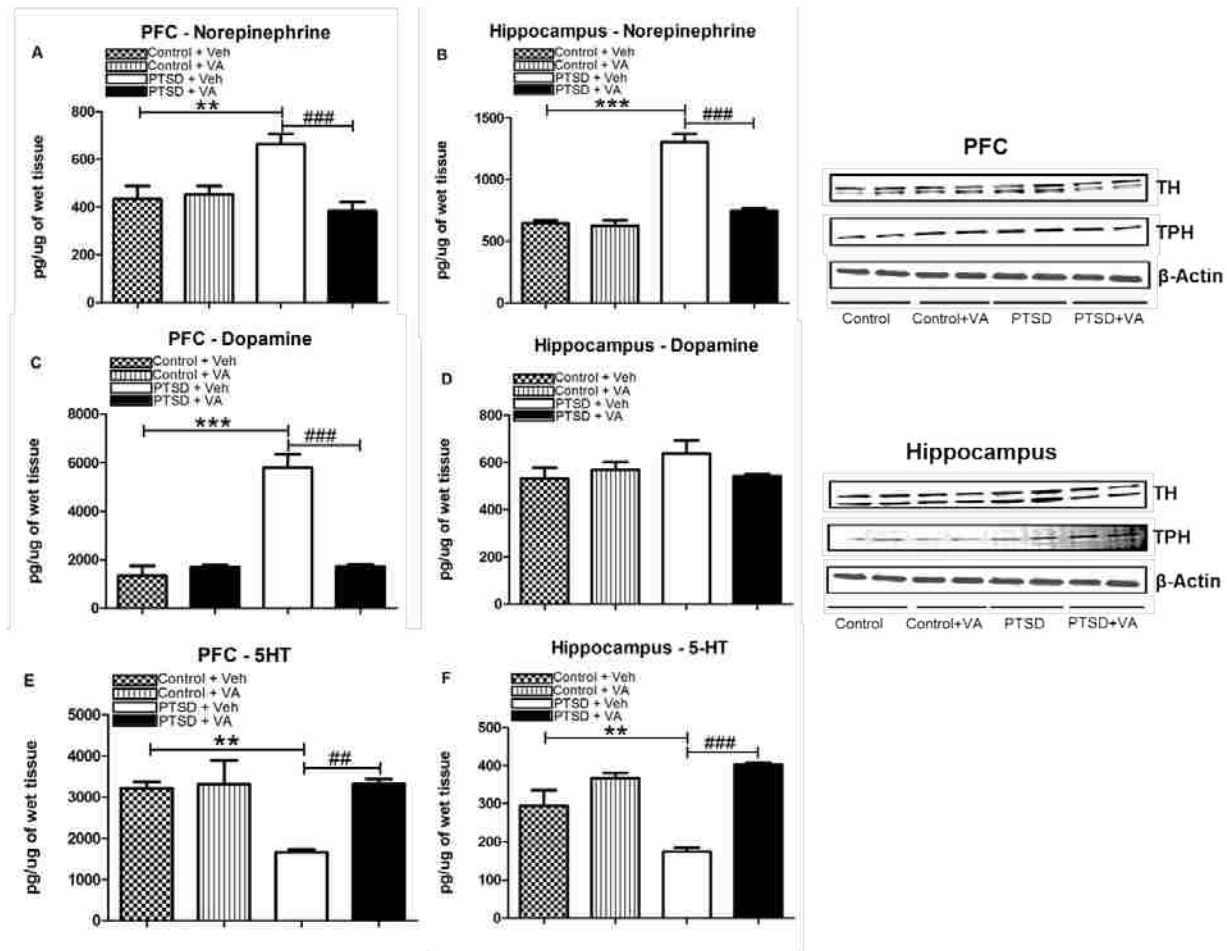
Figure 4.5: In the PFC, the catecholamines NE (A) and DA (C) were significantly higher in the PTSD group vs. controls, $F_{(3,17)} = 8.66$ and $F_{(3,17)} = 36.32$, $p < 0.05$, respectively. Conversely, the monoamine 5-HT (E) was significantly lower in the PTSD group vs. controls, $F_{(3,17)} = 7.41$, $p < 0.05$. VA administration lowered catecholamine levels and increased 5-HT to levels similar as the untreated controls. In the hippocampus, NE (B) was significantly higher in the PTSD group vs. controls, $F_{(3,17)} = 58.57$, but no significant difference was found in DA (D) levels, $F_{(3,17)} = 1.56$, $p > 0.05$. The level of 5-HT (F) was lower in the PTSD group vs. controls, $F_{(3,17)} = 30.65$, $p < 0.05$. Valproic acid administration lowered NE levels and increased 5-HT to levels similar as the untreated controls. **$p < 0.001$, ***$p < 0.0001$ relative to the control group. ##$p < 0.001$, ###$p < 0.0001$ relative to the treatment group.

which are common symptoms reported in humans with PTSD (Brewin, Andrews, & Valentine, 2000; Nemeroff et al., 2006). In addition, our lab recently demonstrated that the predator exposure model produced neurotransmitter modulation and altered catecholamine and 5-HT rate-limiting enzymes (Wilson et al., 2014). Although HDACi are currently prescribed for certain

neurologic disorders and show efficacy as anti-inflammatory agents, their utility in PTSD

treatment has been largely unexplored. We have successfully obtained data with this model

indicating HDACi (VA) may be a promising pharmacologic therapy for PTSD patients. Three

novel and important findings emerged from this study. First, increased oxidative stress and

inflammation in the brain and blood in response to psychological stress are attenuated with VA.

Second, the aberrant neurotransmitter profile of increased catecholamines and decreased 5-HT

was normalized with VA. Lastly, VA seems to enhance resiliency behavior as indicated by

reduced anxiety levels in the PTSD+VA group.

Histone deacetylases (HDAC), along with histone acetyltransferases (HAT), modulate

gene expression by decreasing or increasing gene availability, respectively. HDACs and HATs

act in concert with one another, maintaining homeostasis during normal cellular functions (de

Ruijter et al., 2003). During abnormal states, however, this imbalance is tilted in favor of HDAC

activity, resulting in increased oxidative stress and inflammation (Aung et al., 2006; Halili,

Andrews, Sweet, & Fairlie, 2009; H. J. Kim et al., 2007). It is an oversimplification to state that

acetylation increases and deacetylation decreases gene expression, as research has shown that

non-histone proteins can be reversibly acetylated, which drastically affects their function (M. S.

Kim et al., 2001). Current research has demonstrated that elevation of PIC expression can

increase HDAC activity, which is directly proportional to heighted NF-κB transcription and

further production of PICs (Keslacy et al., 2007). As a result, HDAC inhibition may be relevant

to treatment of neuroinflammatory disorders (Kazantsev & Thompson, 2008). Our findings that

a PTSD-like syndrome increases NF-κB expression and HDAC activity in the stressed animals

positively correlate with the increased ROS and PIC levels. In addition, the attenuation of

inflammatory mediators in the PTSD+VA group confirms previous findings that HDACi have anti-inflammatory properties.

The roles of oxidative stress and inflammation in pathological conditions such as cardiovascular disease, diabetes mellitus, metabolic syndrome, and neurological diseases are well established (Agarwal et al., 2011; Alexopoulos et al., 2012; Elks & Francis, 2010; Pall & Satterlee, 2001). We recently demonstrated increased oxidative stress and inflammation in the brain and systemic circulation of rats subjected to the predator exposure model (Wilson et al., 2013). Reactive oxygen and nitrogen radicals have unpaired electrons that can cause damage by oxidizing proteins, lipids, nucleic acids, and other cellular components. The damage caused by ROS can activate transcription factors such as NF-κB, leading to increased production of cytokines and chronic inflammation (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Immunomodulating cytokines function to transmit information concerning inflammatory responses to the CNS (Chrousos, 2000a; Dunn, 2000; McCann et al., 2000; Turnbull, Lee, & Rivier, 1998). The CNS then participates in negative feedback regulation of the peripheral immune response by releasing pituitary hormones and norepinephrine (NE) which increase sympathetic drive. In chronic stress-related conditions such as PTSD, however, a sustained sympathoexcitatory state can alter the Th1/Th2 cell balance and actually increase PIC production (Chrousos, 2000b).

A primary initiator of the inflammatory response is the inflammasome, a multi-protein complex that upregulates PICs. In our previous work, we demonstrated that the NALP3 inflammasome was upregulated in the predator exposure/psychosocial stress PTSD model (Wilson et al., 2013). NALP3, possibly in a two-stage process with TLR4, utilizes caspase-1 to cleave pro-IL-1β and pro-IL-18 to their active forms, leading to upregulation of other PICs and

inflammatory components. A few of these components have been implicated in PTSD

pathophysiology. Studies show that PTSD was exacerbated by increased levels of NO and other

ROS, causing cellular damage in the hippocampus (Oosthuizen et al., 2005). Other research

discovered DNA breakage in the hippocampus of rats subjected to stress (Diehl et al., 2012).

Inflammasomes are primarily found in resident macrophages and granulocytes, but they are also

key inflammatory mediators in non-myeloid cells (Yazdi, Drexler, & Tschopp, 2010), which

may explain the increase in PICs in the blood and periphery during PTSD progression.

One of the diagnostic criteria for PTSD is hyperarousal, which includes an exaggerated startle

response and heightened anxiety (American, 2013). Previous research with the predator

exposure model also demonstrated memory-related impairments of the hippocampus during

anxiety testing (Diamond, Park, Heman, & Rose, 1999). To measure anxiety levels, we used the

elevated plus-maze (EPM). Rodents have a natural tendency to explore novel environments, but

open areas or alleys invoke a greater fear and avoidance response (Montgomery, 1955). The

EPM is widely used as a measure to test anxiety and has been extensively validated for use in

rats (Korte & De Boer, 2003; Pellow, Chopin, File, & Briley, 1985). Entry into the open areas is

associated with increased freezing behavior as well as increased plasma corticosterone levels,

indicating heightened anxiety (Pellow et al., 1985). Anxiogenic compounds or procedures can

increase avoidance of the fear-provoking open arms, whereas anxiolytic compounds or

procedures can increase open arm exploration (Pellow et al., 1985). The primary criteria

correlated with anxiety levels are total arm entries and the percent time spent in the open vs.

closed arms. We found that VA had an anxiolytic effect, as rats in the PTSD+VA group

displayed an increase in open arm exploration vs. the PTSD+Veh group.

The modulation of various neurotransmitters observed with the predator exposure/psychosocial stress model is in concert with many of the neurotransmitter changes seen in human PTSD patients (Arora, Fichtner, O'Connor, & Crayton, 1993; Geracioti et al., 2001; Geracioti et al., 2013; Yehuda, Southwick, Giller, Ma, & Mason, 1992). Previous research has shown that stress blocks hippocampal long-term potentiation (LTP) and impairs its function (Diamond, Campbell, Park, Halonen, & Zoladz, 2007). The hippocampus is the primary region for spatial and long-term memory storage and expresses all of the 5-HT receptor families, which reflects the overall serotonergic functions relating to cognition and mood in this region (Berumen, Rodriguez, Miledi, & Garcia-Alcocer, 2012). In addition, the hippocampus has the highest density of glucocorticoid receptors compared to other brain regions (McEwen, Weiss, & Schwartz, 1969). During stress, glucocorticoid production can reduce the excitability of hippocampal neurons, and 5-HT may have a protective effect against such damage by activating 5-HT1A receptors (Joca, Ferreira, & Guimaraes, 2007). Persistent activation of the HPA axis and excessive production of glucocorticoids, however, may directly reduce hippocampal 5-HT levels and adversely affect normal serotonergic transmission, thus contributing to heightened fear, depressed mood, and reduced resilience. The hippocampus also contains multiple NE receptors which, when activated during stress, may contribute to the reinforcement of long-term memories (Jurgens et al., 2005). In a study by Geracioti et al. involving male combat veterans with PTSD, CSF concentrations of NE were significantly higher vs. controls (Geracioti et al., 2001). This finding could possibly explain why memories formed during extremely stressful events persist over time. Other evidence of catecholamine dysregulation in PTSD includes elevated urine catecholamine excretion, exaggerated biochemical responses to yohimbe, and clinical efficacy of adrenergic blockers (Southwick et al., 1999). Noradrenergic modulation was

also noted with previous experiments utilizing the predator exposure/psychosocial stress animal model and treatments with selective serotonin reuptake enhancers (SSRE), α2 agonists, and tricyclic antidepressants (Zoladz, Fleshner, & Diamond, 2013). Although we found no significant difference in DA levels in the hippocampus, we noted a reduction in homovanillic acid (HVA) levels (not shown), which was consistent with current human PTSD research (Geracioti et al., 2013). HVA is a downstream product of DA metabolism, and traumatic stress may impede CNS release of DA from the substantia nigra and ventral tegmental area (VTA) (Cabib & Puglisi-Allegra, 2012), the primary CNS regions of dopaminergic neurons, thus reducing metabolite concentration.

The PFC, responsible for executive functions such as consequences, drive, and social "control", is highly innervated by serotonergic neurons from the raphe nuclei and also expresses an abundance of 5-HT receptors. The 5-HT1A and 5-HT2A receptors are a key modulator of the PFC-amygdala corticolimbic circuit involved in threat and emotional responses (Fisher et al., 2011). PTSD-related aberrancies in this serotonergic system may cause inappropriate or incomplete extinction of conditioned fear. The PFC also contains NE receptors and receives input from NE neurons from the locus coeruleus, which are activated during the stress response (Finlay, Zigmond, & Abercrombie, 1995). Pathogical or stress-related elevations of NE in the PFC, however, may inhibit working memory and performance (Zhang, Cordeiro Matos, Jego, Adamantidis, & Seguela, 2013). Current neuroimaging research indicates the PFC is hyporesponsive during symptomatic PTSD states and responsiveness is inversely proportional to symptom severity (Shin, Rauch, & Pitman, 2006). In contrast to unchanged DA levels in the hippocampus, DA levels were significantly increased in the PFC, which was similar to the CSF and urine DA elevations seen in humans with PTSD (De Bellis et al., 1999; Glover et al., 2003;

Yehuda et al., 1992). In a similar manner to NE, stress-related elevations of DA may also impair

working memory and performance. The PFC is densely innervated by dopaminergic neurons

from the VTA, and dopamine release can be achieved via VTA or local stimulation. A recent

study by Butts et al. demonstrated that stress-induced glucocorticoid stimulation of DA neurons

caused a local release of DA in the PFC (Butts, Weinberg, Young, & Phillips, 2011). These data

support the theory that overstimulation of the HPA axis and the resulting elevation in

glucocorticoid activity can directly modulate DA and other neurotransmitters. Taken together,

our findings that VA was able to increase 5-HT and decrease catecholamine levels in the brain,

apparently by modulating levels of the rate-limiting enzymes for the respective

neurotransmitters, represents a completely novel finding in an animal model of PTSD and

demonstrates a valid justification for VA as an alternative to currently accepted

pharmacotherapy.

4.5 CONCLUSIONS

This study was conducted to test alternative pharmacologic therapies for PTSD, as the

currently approved selective-serotonin reuptake inhibitors (SSRI) have proven nominally

effective (Tawa & Murphy, 2013). Based on their mechanism of action, SSRIs should increase

5-HT levels and attenuate many of the detrimental effects of lowered 5-HT. This concept has

proven effective in the treatment of depression (Doogan & Caillard, 1992; Miller et al., 1998).

In PTSD, however, SSRI efficacy can be classified as questionable at best. In a study by

Davidson et al., they demonstrated decreased severity of symptoms and an overall increase in

functioning in the PTSD patients vs. controls (Davidson, Rothbaum, van der Kolk, Sikes, &

Farfel, 2001). This study, however, had uneven gender, racial, and traumatic event distribution.

The data showed a 45% increase in symptom improvement in the treatment group, but also a

36% increase in the placebo group. Taken together, the numbers indicate a significant portion of the noted improvement may be due to a placebo effect. The study did not perform physiological testing and therefore could not anticipate modulation of other neurotransmitters in response to serotonin reuptake. We have achieved such data and have successfully demonstrated that VA produces both physiological and behavioral changes in animals with a PTSD-like syndrome.

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4.6 REFERENCES

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CHAPTER 5
DIFFERENTIAL EFFECTS OF SERTRALINE IN A PREDATOR EXPOSURE
ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER

5.1 INTRODUCTION

Post-traumatic stress disorder (PTSD), an anxiety disorder recently reclassified as a trauma- and stressor-related disorder, can develop in response to real or perceived life-threatening situations. According to the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), a diagnosis of PTSD necessitates exposure to a traumatic event, intrusive recollections, avoidance of associated stimuli, negative cognitions/mood, hyperarousal, and a significant social impairment. All of these symptoms must persist for at least 30 days and not be due to illness, medication, or substance abuse (American Psychiatric Association, 2013). To date, no definitive diagnostic biomarkers have been identified for PTSD. Recent research, however, points toward physiological abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary system, immune system, and neurotransmitters that may be implicated in the disorder (Liberzon, Abelson, Flagel, Raz, & Young, 1999; Oosthuizen, Wegener, & Harvey, 2005; Sondergaard, Hansson, & Theorell, 2004; Wilson, Ebenezer, McLaughlin, & Francis, 2014; Wilson et al., 2013). Although neurotransmitters are modulated in PTSD development, it remains unclear whether 5-HT is the only neurotransmitter affected by selective-serotonin reuptake inhibitors (SSRI). Changes in levels of other neurotransmitters might explain why SSRIs have met with such mixed results in PTSD therapy (Davidson, Rothbaum, van der Kolk, Sikes, & Farfel, 2001; Watts et al., 2013). To evaluate the effects of sertraline on neurotransmitter modulation, we employed a well-documented predator exposure/psychosocial stress animal model of PTSD demonstrating three hallmark features of the disorder: hormonal abnormalities, a long-lasting traumatic memory, and persistent anxiety

(Zoladz, Conrad, Fleshner, & Diamond, 2008; Zoladz, Fleshner, & Diamond, 2012). This model also possesses both predictive and construct validity, making it sensitive to clinically effective pharmacologic agents while displaying similarities to human PTSD (Bourin, Petit-Demouliere, Dhonnchadha, & Hascoet, 2007).

Serotonin (5-HT) is a neurotransmitter responsible for many functions in the CNS and periphery. Serotonergic cell bodies originate primarily in the raphe nuclei, but every area of the central nervous system (CNS) receives 5-HT innervation (McGeer, 1987). It influences aggression, arousal, sleep, anxiety, appetite, fear, learning, and other processes (Dubovsky, 1994). 5-HT is also the principle regulator of mood. A study by Peirson et al. (Peirson & Heuchert, 2000) found lower platelet $5-HT_2$ receptor function was associated with depressed mood, while Williams et al. (Williams et al., 2006) demonstrated higher blood 5-HT levels were correlated with better mood. An increased mood and overall sense of well-being has been shown, in both psychiatric and physical disorders, as protective and positively correlated with resiliency behavior (Delamothe, 2005). Research has also demonstrated that 5-HT-uptake sites in platelets were lower in PTSD patients vs. controls (Arora, Fichtner, O'Connor, & Crayton, 1993). Lower 5-HT has also been implicated in diminished physical health. Muldoon et al. showed that a low prolactin response to fenfluramine, a drug that increases 5-HT levels, was associated with metabolic syndrome (Muldoon et al., 2004). Based on 5-HT's action, it is reasonable to surmise SSRIs should be effective in PTSD. The SSRIs sertraline and paroxetine are the only Food and Drug Administration (FDA) approved drugs for PTSD, but the modulation of other neurotransmitters in response to 5-HT reuptake has yet to be clearly delineated.

Norepinephrine (NE) is a neurotransmitter involved in the regulation of psychiatric and physical processes. In the brain, the locus coeruleus (LC) synthesizes and releases NE, which

modulates multiple functions such as neuroplasticity, attention and memory, emotions, and psychological stress (Benarroch, 2009). The LC also has projections to the spinal cord where NE is released from postganglionic neurons in the sympathetic nervous system to initiate the 'fight-or-flight' response. In addition, chromaffin cells in the adrenal medulla release NE and epinephrine into the bloodstream, increasing heart rate and blood flow to skeletal muscles and triggering the release of glucose. Persistent noradrenergic activity, however, has been linked with negative outcomes in patients with congestive heart failure (CHF) (Francis et al., 1993) and diabetes (Ganguly, Dhalla, Innes, Beamish, & Dhalla, 1986). Studies have also shown that individuals with PTSD have elevated cerebrospinal fluid (CSF) levels of NE (Geracioti et al., 2001) and noradrenergic hyperresponsiveness to various stimuli (Liberzon et al., 1999). Moreover, dysregulation of noradrenergic neurons has been associated with hyperarousal and intrusive recollections attributable to PTSD (Southwick et al., 1999). A study by Bracha et al. noted irregularities in the number of cells of the LC in postmortem examinations of combat veterans diagnosed with PTSD (Bracha, 2005). Thus, neurotransmitter modulation resulting in elevated NE levels might increase sympathetic drive and elevate anxiety.

Since the late 1980s, SSRIs have proven effective in the treatment of depression (Doogan & Caillard, 1992; I. W. Miller et al., 1998). In PTSD, however, SSRI efficacy can be classified as inconsistent at best (Friedman, Marmar, Baker, Sikes, & Farfel, 2007). A study by Davidson et al. which was a part of the FDA approval process for sertraline use in PTSD, demonstrated decreased severity of symptoms and an overall increase in functioning in the PTSD patients vs. controls (Davidson et al., 2001). The results were achieved with multiple investigator- and self-rated assessments. This study, however, had uneven gender distribution (84% female), racial distribution (83% white), and traumatic event distribution (64% physical or sexual assault). The

efficacy of sertraline in PTSD, therefore, may be variable due to gender, demographics, and/or type of incident. The data showed a 45% increase in symptom improvement in the treatment group, but also a 36% increase in symptom improvement in the placebo group. Taken together, these numbers indicate that the majority of the noted improvement may be due to a placebo effect. In addition, there were no physiological measures conducted to analyze actual neurotransmitter modulation during treatment. This information could be critical in determining the true efficacy of SSRIs, as neurotransmitter changes are not mutually exclusive events. With this in mind, this study sought to analyze the modulation of neurotransmitters and inflammatory components in the hippocampus, prefrontal cortex (PFC), CSF, and plasma after a 7-day sertraline treatment regimen.

5.2 MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Institute for Laboratory Animal Research's 2011 *Guide for the Care and Use of Laboratory Animals*, under the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee.

Animals

Naïve adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in all experiments. The rats were the same age (12 weeks) and approximately the same weight (±15g) upon delivery. Rats were pair-housed in standard plastic microisolator cages with access to food and water *ad libitum*. The cages were maintained in ventilated racks and randomly assigned to a rack location to ensure groups were evenly distributed. The vivarium room was kept on a 12-hour light/dark cycle (0700-1900), temperature was maintained at 20±1℃, and

humidity ranged from 23-42%. After a 1-week acclimation period, the mean weight of all rats was 308.5g ± 2.5. Two cats, a male and a female (Harlan Laboratories, Indianapolis, IN) were used for all predator exposures. They were housed in an open room (15' x 15') in the vivarium with access to food, water, and enrichment devices *ad libitum*. The cat room was on the same light/dark cycle and maintained at a similar temperature and humidity.

Stress Induction

The predator exposure/psychosocial stress regimen is designed to induce a PTSD-like syndrome as true PTSD is clinically defined as a human disorder. Following the acclimation period, rats were weighed, ear-tagged, tail-marked, and 250-500μL of blood was drawn from the tail vein. The rats were then randomly assigned to the PTSD or control group and returned to the vivarium for 24 hours. The following day, PTSD rats were started on a predator exposure/psychosocial stress regimen. Briefly, PTSD rats were individually isolated in cylindrical, Plexiglas containers (IITC Life Science, Inc., Woodland Hills, CA.) and canned cat food was smeared on the outside of the cylinders. The cylinders prevented direct contact with the cats, and the cat food induced movement in the cats. Rats were then placed in a stainless steel cage (76cm x 76cm x 60cm) consisting of a solid metal floor with a hinged, metal rod door, with a cat for one hour. The first cat exposure was conducted during the light cycle (0700-1900). Ten days later, a second cat exposure was conducted during the dark cycle (1900-0700). In addition, the rats were subjected to psychosocial stress by changing their cage cohort daily. The predator exposure/psychosocial stress regimen continued for 31 days, after which certain PTSD and control group rats were administered sertraline intraperitoneally (i.p.) for 7 days. All groups were then euthanized via CO_2 inhalation, blood was collected by intracardiac puncture,

exsanguination via perfusion was conducted with a phosphate buffered solution, and the brains were removed. The hippocampus and PFC were dissected and flash-frozen in liquid nitrogen.

Elevated Plus-Maze (EPM)

Rats were randomly selected for either the control or the PTSD group and were administered a baseline EPM prior to the predator exposure. The EPM was also administered after the 31-day stress regimen and again after the 7-day sertraline treatment. Rats were placed in the center of the elevated plus-maze (EPM) (EB-Instruments (Bioseb), Tampa Bay, FL) facing an open arm and allowed to roam freely for five minutes. Movement was monitored via an overhead camera and captured with a software program (BioEPM3C, EB-Instruments, Tampa Bay, FL). The primary measurements were the total number of arm entries and the total time spent in the open vs. closed arms.

Sertraline

Rats were pair-housed and treatment group animals were injected i.p. with sertraline HCl dissolved in 50% dimethyl sulfoxide (DMSO) and dH2O at 10mg/kg for 7 consecutive days (Maj & Rogoz, 1999). Control rats were injected i.p. with vehicle.

Real-Time PCR Analysis

Semi-quantitative real-time RT-PCR (n = 6/group) was used to determine the mRNA levels of interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-10 (IL-10), and Toll-like receptor-4 (TLR4) in the PFC and hippocampus. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal, Welsch, Keller, & Francis, 2011). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data is presented as fold change of the gene of interest relative to that of control animals.

GAPDH, (Glyceraldehyde 3-phosphate dehydrogenase); IL, (Interleukin); TLR4, (Toll-like receptor 4).

Western Blot Analysis

Tissue homogenates from the PFC and hippocampus were subjected to Western Blot (WB) analysis (n = 6/group) for the determination of protein levels of IL-1β, IL-4, IL-10, TLR4, and β-Actin. The extraction of protein and WB was performed as previously described (Agarwal et al., 2011). Primary antibodies were commercially obtained: IL-1β, and β-Actin, 1:1000 dilution (SC-7884 and SC-1616R respectively, Santa Cruz Biotechnology, Santa Cruz, CA), TLR4, IL-4, and IL-10, 1:1000 dilution (ab13556, ab9811, and ab9969 respectively, Abcam, Cambridge, MA). Secondary antibodies were commercially obtained: anti-rabbit, 1:5000 dilution (SC-2004, Santa Cruz Biotechnology, Santa Cruz, CA.).

High-Performance Liquid Chromatography (HPLC)

Neurotransmitter concentrations were detected using an Eicom HTEC 500 HPLC system. The standard solutions of NE (MW 337.3), 5-HT (MW 212.68) and isoproterenol (internal standard; MW 247.7) were each 1 ng/µL concentrations. Sample preparations were carried out as previously described (Deyama et al., 2008; Wilson et al., 2013, 2014).

HPLC Detection of Neurotransmitters

HPLC system working conditions: isocratic elution; mobile phase (citrate buffer in methanol with EDTA and sodium octane sulfonate); Eicompak SC-3ODS (ID 3.0 X 100mm) column; flow rate 340 µl/min; graphite working electrode WE-3G (Gasket GS-25), (+750mV versus Ag/AgCl electrode); temperature 25°C.

HPLC Mobile Phase

Citric acid monohydrate (8.84 g; mol wt. 210.14), and 3.10g of sodium acetate (mol. wt. 82.03) in 800 ml of MilliQ Ultrapure fresh water (>18.2MΩ/cm) and 200ml of HPLC grade methanol were added. EDTA (mol. wt. 372.24; 0.005g) and sodium octane sulfonate (0.220 g), both from Dojindo Laboratories, Rockville, MD, were added.

ELISA Analysis

An ELISA kit was used to measure NE (MBS881383, MyBioSource, San Diego, CA.) levels in the CSF and plasma according to manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was conducted by one-way ANOVA with a Bonferroni post hoc test for multiple comparisons, unpaired Student's T-tests for two-column analyses, and four-parameter logistic regression for curve fit. P-values less than 0.05 were considered significant. Statistical analyses were performed using Prism (GraphPad Software, Inc, La Jolla, CA; version 5.0).

5.3 RESULTS

Elevated Plus-Maze Performance

Prior to the start of the stress regimen, there were no differences noted in baseline open arm exploration (Figure 5.1A) or total arm entries (Figure 5.1B). After the 31-day stress regimen, the PTSD group spent considerably less time in the open vs. closed arms as compared to the control group, $t(22) = 5.10$, $p < 0.05$, and as compared to baseline, $t(22) = 3.86$, $p < 0.05$ (Figure 5.1A). Overall ambulations, however, were not affected, $F(3,44) = 0.974$, $p > 0.05$ (Figure 5.1B). After the 7-day sertraline treatment, the PTSD+Sert group displayed no increased open arm exploration vs. the PTSD+Veh group, $t(10) = 0.49$, $p > 0.05$, or the control+Sert group,

t(10) = 4.59, p < 0.05. The control group also showed no difference between the control+Sert

and control+Veh groups, t(10) = 0.43, p > 0.05, (Figure 5.2A). No differences were found in

overall ambulations between or within groups after the sertraline treatment, F(3, 20) = 0.55, p >

0.05, (Figure 5.2B).

CSF and Plasma NE Analysis

In the CSF, NE was elevated in the PTSD+Veh vs. the control+Veh group, t(8) = 4.22, p

< 0.05. Sertraline raised NE levels in the control+Sert vs. the control+Veh group, t(8) = 4.96, p

< 0.05, and NE was further elevated in the PTSD+Sert vs. the PTSD+Veh group, t(8) = 3.72, p <

0.05 (Figure 5.3A). In the plasma, NE was higher in the treatment groups, but it did not reach

significance in any comparisons (Figure 5.3B).

Inflammatory Markers

In the PFC and hippocampus, the PTSD group demonstrated elevated mRNA levels of

IL-1β, F(3,18) = 3.56, p < 0.05 and F(3,20) = 3.53, p < 0.05   (Figures 5.4A&B) and TLR4,

F(3,20) = 4.11, p < 0.05 and F(3,20) = 1.44, p > 0.05 (Figures 5.4C&D). Conversely, there were

diminished levels of IL-4, F(3,20) = 0.99, p > 0.05 and F(3,20) = 6.65,p < 0.05 (Figures 5.4E&F)

and IL-10, F(3,20) = 9.57, p < 0.05 and F(3,20) = 7.34, p < 0.05 (Figures 5.4G&H) in the same

regions. Sertraline administration normalized the elevated PIC mRNA and up-regulated anti-

inflammatory cytokine (AIC) to levels similar to or higher than the control+Veh group. The

PTSD group also displayed elevated protein levels in the PFC and hippocampus of IL-1β, F(3,4)

= 53.04, p < 0.05 and F(3,4) = 22.15, p < 0.05 (Figures 5.5A&B) and TLR4, F(3,4) = 25.78, p <

0.05 and F(3,4) = 43.14, p < 0.05 (Figures 5.5C&D). The levels of AIC protein were lower for

IL-4, F(3,4) = 25.59, p < 0.05 and F(3,4) = 27.01, p < 0.05 (Figures 5.5E&F) and IL-10, F(3,4) =

13.16, p < 0.05 and F(3,4) = 134.10, p < 0.05 (Figures 5.5G&H). Sertraline administration also

normalized the aberrant protein to levels similar to the control+Veh group.
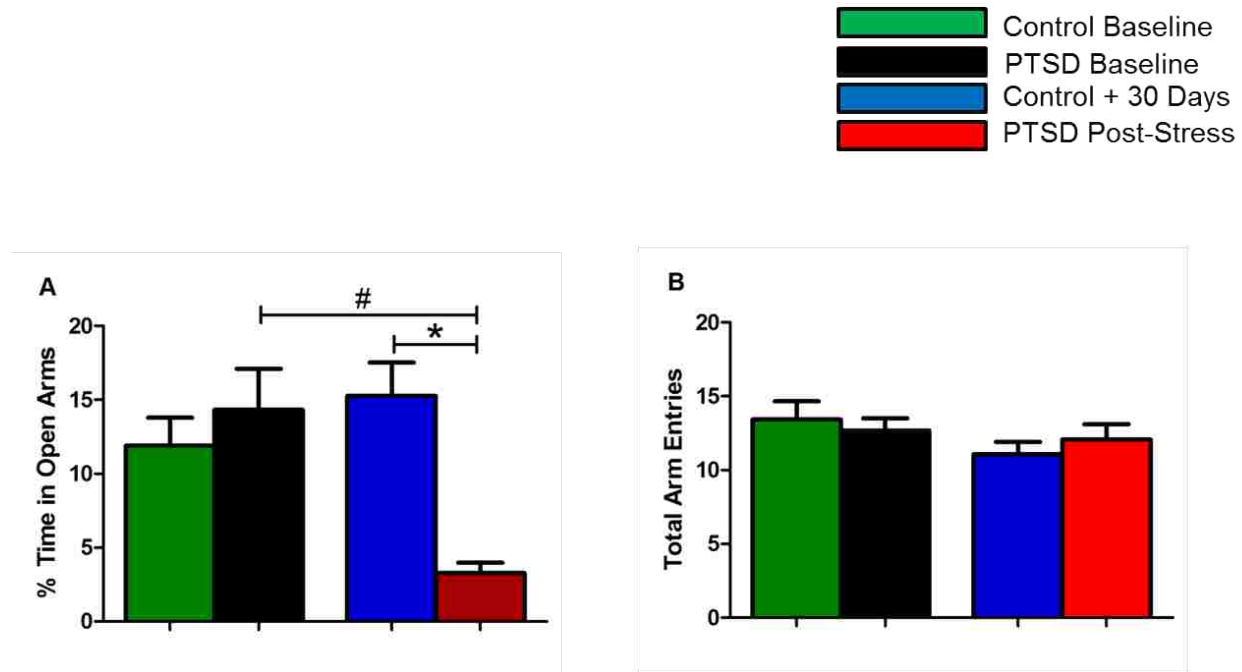

Figure 5.1: The PTSD group spent considerably less time in the open vs. closed arms compared
to the control group and to baseline (A). There were no differences noted in overall ambulations
between or within groups (B).   All data are presented as mean ± SEM. *p < 0.05 relative to the
control group. #p < 0.05 relative to within group measurements (baseline).

Neurotransmitter Modulation

To investigate the influence of sertraline on neurotransmitter modulation, we examined

endogenous levels of biogenic amines in the hippocampus and PFC of control and PTSD animals

using HPLC. In the PFC, the level of the tryptamine 5-HT (Figure 5.6A) was significantly lower

in the PTSD+Veh vs. the control+Veh group, t(10) = 6.64, p < 0.05. Conversely, the level of the

catecholamine NE (Figure 5.6B) was significantly higher in the PTSD+Veh group, t(10) = 8.04,

p < 0.05. Sertraline expectedly raised 5-HT levels in the PTSD+Sert and control+Sert groups to

levels higher than the control+Veh group F(3,20) = 32.62, p < 0.05 (Figure 5.6A), but it also
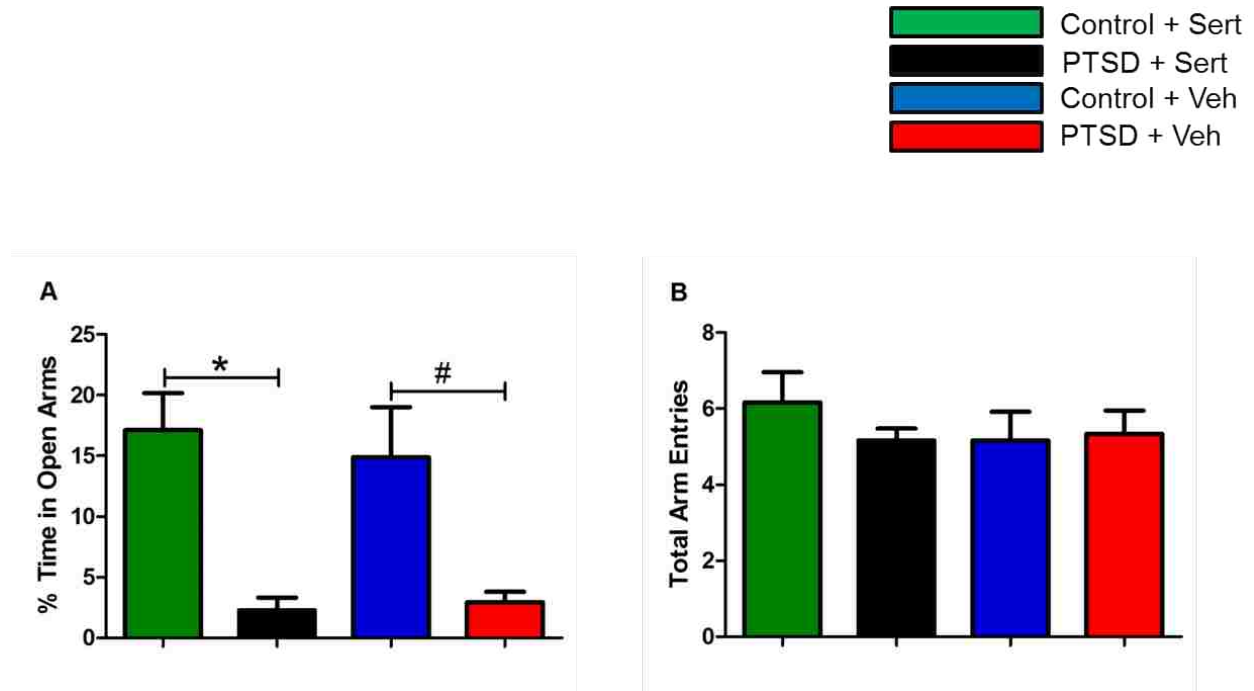
Figure 5.2: After sertraline treatment, the PTSD+Sert group demonstrated no measureable improvement vs. the control+Sert group, and the PTSD+Veh group displayed persistent anxiety vs. the control+Veh group (A). There were no differences in overall ambulations in any of the four groups after the treatment period (B). All data are presented as mean ± SEM. *$p < 0.05$ between the treatment groups. #$p < 0.05$ between the vehicle groups.

elevated NE in the PTSD+Sert and control+Sert groups to levels higher than the control+Veh group $F(3,20) = 26.59$, $p < 0.05$ (Figure 5.6B). In the hippocampus, 5-HT (Figure 5.6C) was significantly lower in the PTSD+Veh vs. the control+Veh group, $t(10) = 6.03$, $p < 0.05$, while NE (Figure 5.6D) was higher, $t(10) = 8.94$, $p < 0.05$. Similar to results in the PFC, sertraline normalized 5-HT to pre-stress levels, $F(3,20) = 18.35$, $p < 0.05$ (Figure 5.6C), but it doubled NE in the control+Sert group and more than tripled NE in the PTSD+Sert group compared to the control+Veh group, $F(3,20) = 124.10$, $p < 0.05$ (Figure 5.6D).
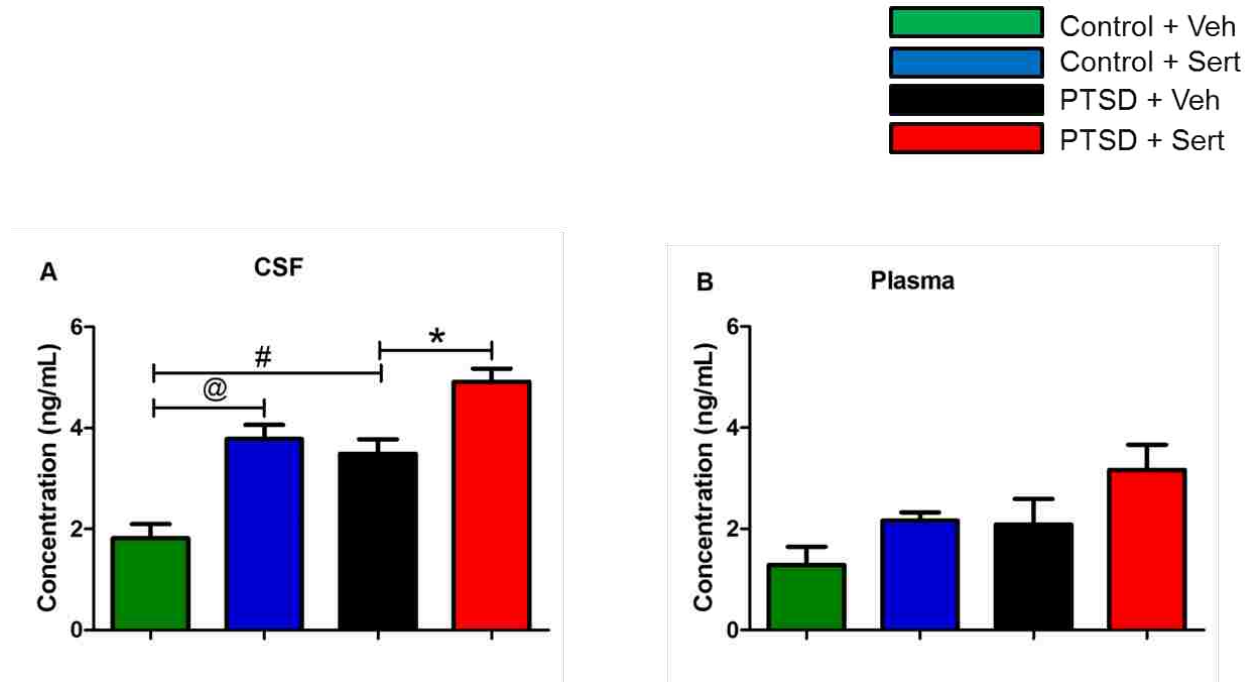
Figure 5.3: In the CSF, NE was elevated in the PTSD+Veh vs. the control+Veh group and in the control+Sert vs. the control+Veh group. NE was further elevated in the PTSD+Sert vs. the PTSD+Veh group (A). In the plasma, NE was higher in the treatment groups, but it did not reach significance in any comparisons (B). All data are presented as mean ± SEM. $*p < 0.05$ between the PTSD groups. @$p < 0.05$ between the control groups. #$p < 0.05$ between the vehicle groups.

5.4 DISCUSSION

The present study sought to analyze neurotransmitter modulation and pro- and anti-inflammatory cytokines in the PFC, hippocampus, CSF, and plasma of rats subjected to a PTSD model and subsequently treated with sertraline. A myriad of animal models designed to create PTSD-like effects are reported, but the model by Zoladz et al. has been shown to cause common symptoms reported in humans with PTSD (Brewin, Andrews, & Valentine, 2000; Nemeroff et al., 2006) such as heightened anxiety, exaggerated startle response, impaired cognition, and increased cardiovascular reactivity (Zoladz et al., 2008; Zoladz et al., 2012).
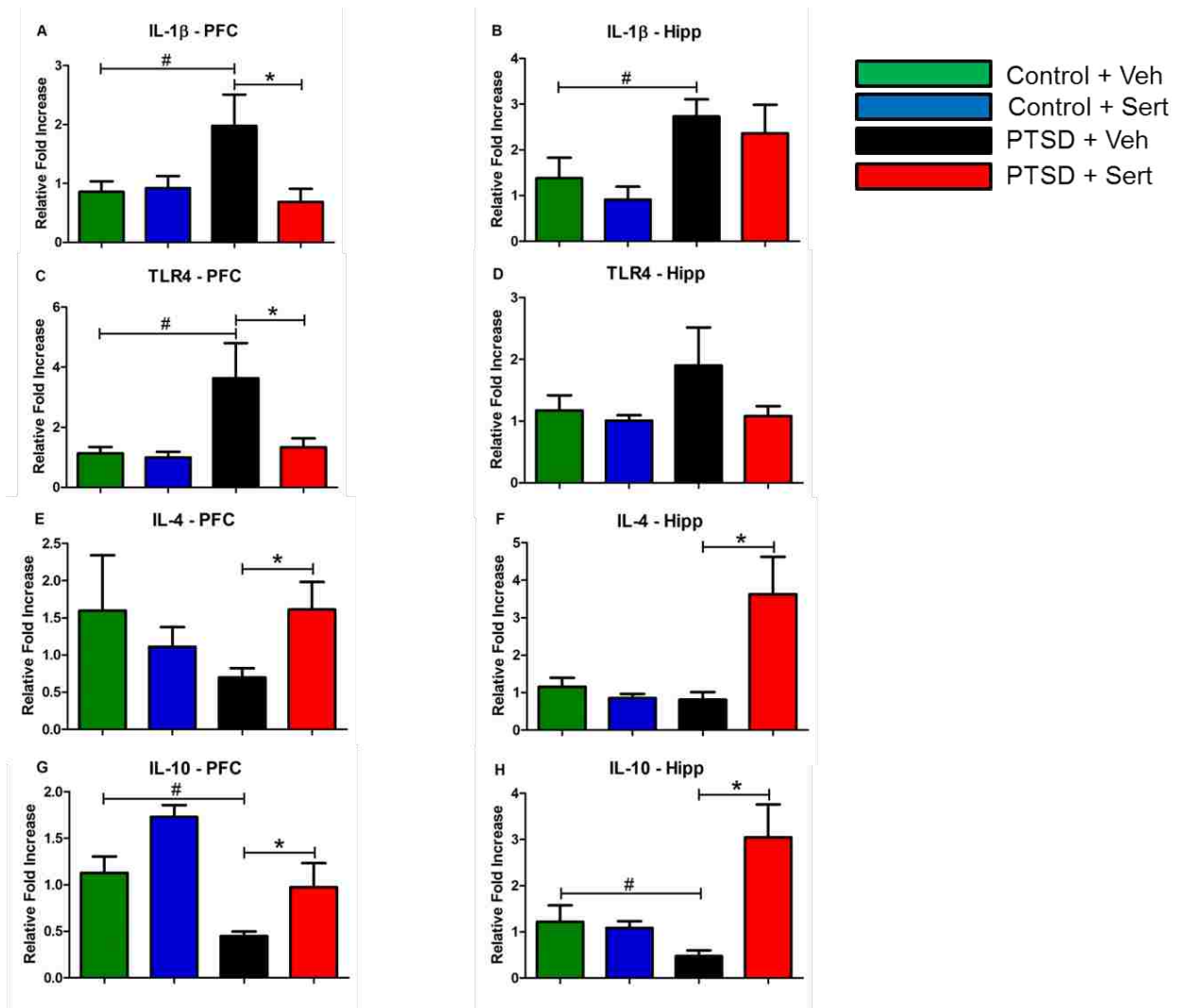
114

Figure 5.4: In the PFC and hippocampus, the PTSD group demonstrated elevated mRNA levels of IL-1β (A&B) and TLR4 (C&D). Conversely, there were diminished levels of IL-4 (E&F) and IL-10 (G&H) in the same regions. Sertraline administration normalized the elevated PIC mRNA and up-regulated anti-inflammatory cytokine (AIC) to levels similar to or higher than the control+Veh group. All data are presented as mean ± SEM. *p < 0.05 between the PTSD groups. #p < 0.05 between the vehicle groups.
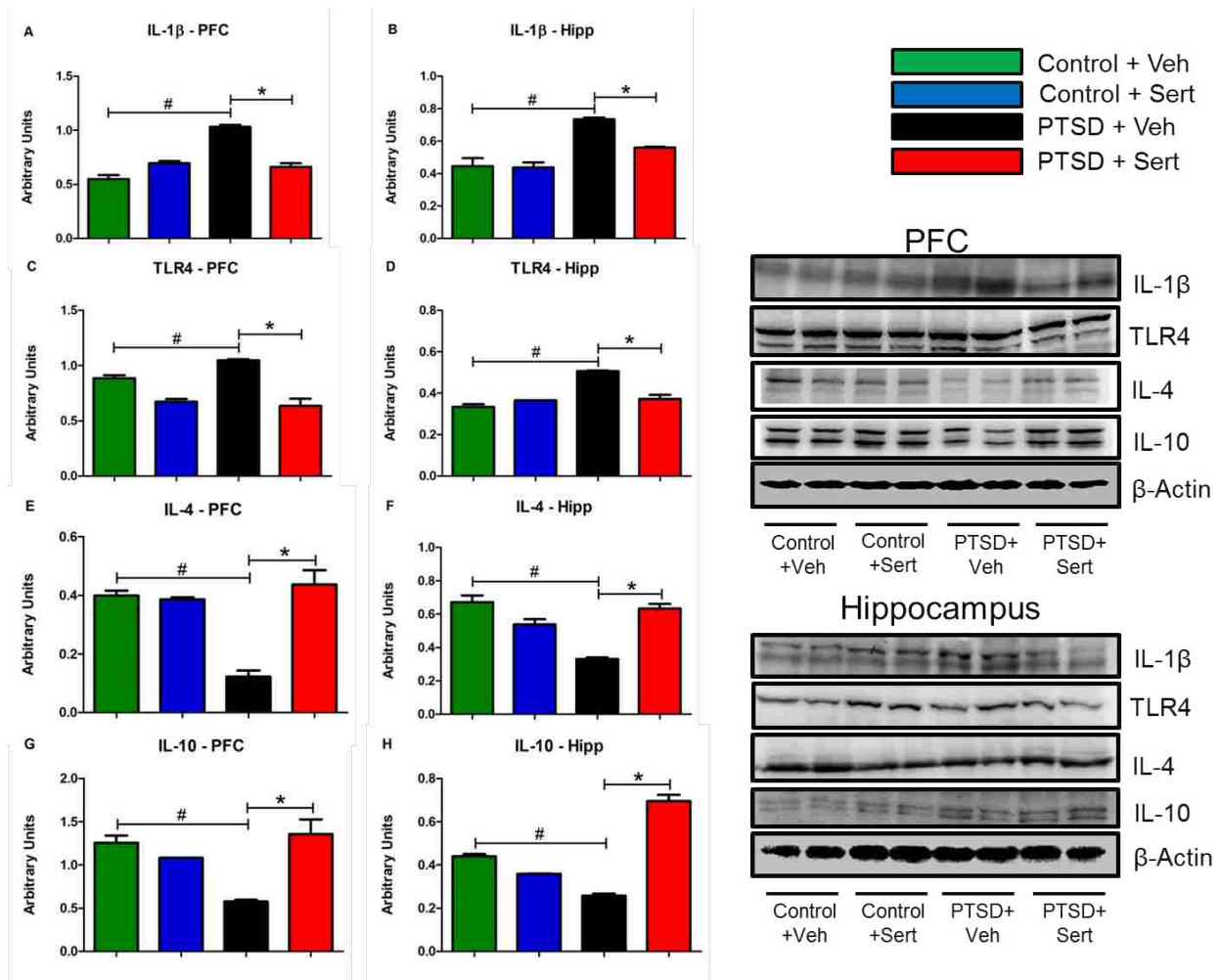
Figure 5.5: The PFC and hippocampus demonstrated elevated protein levels of IL-1β (A&B) and TLR4 (C&D) in the PTSD group. Conversely, the levels of AIC protein in these regions were lower for IL-4 (E&F) and IL-10 (G&H). Sertraline administration also normalized the aberrant protein to levels similar as the control+Veh group. All data are presented as mean ± SEM. *$p < 0.05$ between the PTSD groups. #$p < 0.05$ between the vehicle groups.

Although animal models have certain well-understood limitations, a major component missing from human PTSD research is the ability to ascertain physiological data directly from specific brain regions immediately after a stressful event. The majority of the human physiological data gathered *in vivo* is derived from saliva, blood and urine, which may not accurately reflect neurotransmitter modulation in the brain and certainly cannot distinguish between changes in specific brain regions. We have successfully obtained such data with this Sprague-Dawley rat
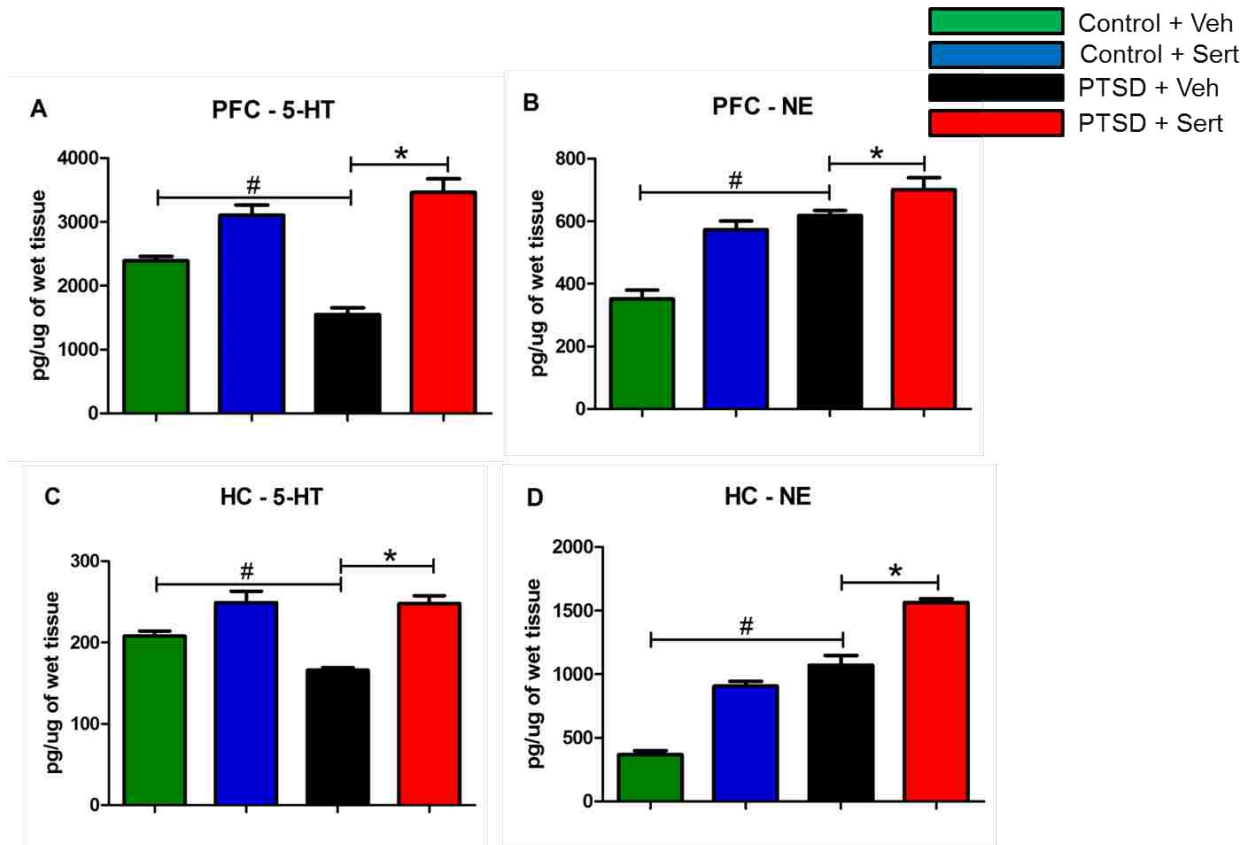
Figure 5.6: In the PFC, the level of 5-HT (A) was significantly lower in the PTSD+Veh vs. the control+Veh group. Conversely, the level of NE (B) was significantly higher in the PTSD+Veh group. Sertraline expectedly raised 5-HT levels in the PTSD+Sert and control+Sert groups to levels higher than the control+Veh group (A), but it also elevated NE in the PTSD+Sert and control+Sert groups to levels higher than the control+Veh group (B). In the hippocampus, 5-HT (C) was significantly lower in the PTSD+Veh vs. the control+Veh group, while NE (D) was higher. Similar to results in the PFC, sertraline normalized 5-HT to pre-stress levels, but it doubled NE in the control+Sert group and more than tripled NE in the PTSD+Sert group compared to the control+Veh group (D). All data are presented as mean ± SEM. *$p < 0.05$ between the PTSD groups. #$p < 0.05$ between the vehicle groups.

model, and to our knowledge, we are the first to report the modulation of biogenic amines and inflammatory components in response to sertraline administration in the brains of PTSD animals. Three novel and important findings emerged from this study. First, 5-HT suppression in the brain regions examined was normalized with sertraline, but NE levels also significantly increased in response to the treatment. Second, sertraline produced anti-inflammatory effects as

117

evidenced by decreased PICs and elevated AICs. Lastly, despite attenuating inflammatory markers, sertraline provided no positive benefit in relation to anxiety or behavior.

The modulation of various neurotransmitters observed with the predator exposure/psychosocial stress model is in concert with many of the neurotransmitter changes seen in human PTSD patients (Arora et al., 1993; Geracioti et al., 2001; Geracioti et al., 2013; Yehuda, Southwick, Giller, Ma, & Mason, 1992). Previous research has shown that stress blocks long-term potentiation (LTP) in the hippocampus as well as impairs hippocampal function (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Kim & Diamond, 2002). The hippocampus, the primary region for spatial and long-term memory storage, expresses all of the 5-HT receptor families and reflects overall serotonergic functions relating to cognition and mood in this region (Berumen, Rodriguez, Miledi, & Garcia-Alcocer, 2012). During stress, glucocorticoid production can reduce the excitability of hippocampal neurons, and 5-HT may have a protective effect against such damage by activating 5-HT$_{1A}$ receptors (Joca, Ferreira, & Guimaraes, 2007). Persistent activation of the HPA axis and excessive production of glucocorticoids, however, may directly reduce hippocampal 5-HT levels and adversely affect normal serotonergic transmission, thus contributing to heightened fear, depressed mood, and reduced resilience. The hippocampus also contains multiple NE receptors which, when activated during stress, may contribute to the reinforcement of long-term memories (Jurgens et al., 2005). In a study by Geracioti et al. involving male combat veterans with PTSD, CSF concentrations of NE were significantly higher vs. controls (Geracioti et al., 2001). This finding could possibly explain why memories formed during extremely stressful events persist over time. Other evidence of catecholamine dysregulation in PTSD includes elevated urine catecholamine

excretion, exaggerated biochemical responses to yohimbe, and clinical efficacy of adrenergic blockers (Southwick et al., 1999).

The PFC is responsible for executive functions such as consequences, drive, and social "control." It is highly innervated by serotonergic neurons from the raphe nuclei, and it expresses an abundance of 5-HT receptors. The serotonergic neurons and 5-HT receptors, specifically the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, are key modulators of the PFC-amygdala-corticolimbic circuit involved in threat and emotional responses (Fisher et al., 2011). PTSD-related aberrancies in this serotonergic system may cause inappropriate or incomplete extinction of conditioned fear. The PFC also contains NE receptors and receives input from NE neurons from the LC, which are activated during the stress response (Finlay, Zigmond, & Abercrombie, 1995). Pathological or stress-related elevations of NE in the PFC, however, may inhibit working memory and performance (Zhang, Cordeiro Matos, Jego, Adamantidis, & Seguela, 2013). Current neuroimaging research indicates that the PFC is hyporesponsive during symptomatic PTSD states and that this responsiveness is inversely proportional to symptom severity (Shin, Rauch, & Pitman, 2006). Whether marked elevations in NE directly or indirectly diminish PFC responsiveness and subsequent performance on cognitive emotional tasks remains unclear.

The role of inflammation in pathological conditions such as cardiovascular disease, diabetes mellitus, metabolic syndrome, and neurological disease is well established (Agarwal et al., 2011; Alexopoulos et al., 2012; Elks & Francis, 2010; Pall & Satterlee, 2001). We recently demonstrated oxidative stress and inflammation were up-regulated in the brain and systemic circulation of rats subjected to the predator exposure model (Wilson et al., 2013). In chronic stress-related conditions such as PTSD, a sustained sympathoexcitatory state can alter the T$_H$1/T$_H$2 cell balance and increase PIC production (Chrousos, 2000). Research presents strong

evidence that cytokines and inflammation may be directly linked to psychiatric disorders, but whether they are causal or nonspecific immunological side-effects remains unresolved (Schiepers, Wichers, & Maes, 2005). Inflammatory cytokine levels have been shown to be inversely proportional to 5-HT levels, and it is hypothesized that PICs can diminish tryptophan by activating the tryptophan-metabolizing enzyme indoleamine-2,3-dioxygenase (IDO) (Heyes et al., 1992). There is also evidence that PICs counteract the negative feedback of glucocorticoids on the HPA axis, altering its function (A. H. Miller, Pariante, & Pearce, 1999). In our previous work, we found that valproic acid (VA) attenuated inflammation, but it differed from sertraline in that it did not result in noradrenergic hyperresponsiveness and actually modulated NE to levels similar to untreated controls. In addition, VA lowered anxiety and resulted in vast improvements on the EPM (Wilson, McLaughlin, Ebenezer, Nair, & Francis, 2014). The fact that both of these compounds decreased inflammatory components but did not equally improve EPM performance indicates inflammation and oxidative stress may be contributors, but not the sole causal factors in terms of PTSD pathophysiology.

We have demonstrated that sertraline increases 5-HT and NE, and that it attenuates inflammation in the hippocampus, PFC, and CSF. Based on these mechanisms, its administration should result in decreased anxiety and improved resilience. In our experiments, however, we observed no improvement on the EPM that indicated reduced anxiety in the rats. The EPM is widely used as a measure to test fear or anxiety and has been extensively validated for use in rats (Korte & De Boer, 2003; Pellow, Chopin, File, & Briley, 1985). Anxiogenic compounds or procedures can increase avoidance of the fear-provoking open arms, whereas anxiolytic compounds or procedures can increase open arm exploration (Pellow et al., 1985). It should be noted, however, that not all anxiolytic compounds modify behavior in PTSD. Zoladz

et al. demonstrated the ineffectiveness of clonidine and amitriptyline, but showed a marked improvement in behavior with tianeptine (Zoladz, Fleshner, & Diamond, 2013). The anxiolytic effects of increased 5-HT and attenuated inflammation should have resulted in increased open arm exploration compared to the PTSD+Veh group. The fact that no significant changes were noted between the treated and untreated PTSD groups suggests other mechanisms might be acting as endogenous anxiogenic agents. One such mechanism might be elevated CNS NE and increased sympathetic tone. Noradrenergic hyperresponsiveness has previously been shown to contribute to PTSD pathophysiology (Southwick et al., 1993). It has also been suggested that the LC neurons, responsible for CNS NE production, constitute the first or second step of the PTSD circuit (Bracha, 2005). Our findings of elevated NE in the hippocampus, PFC, and CSF despite sertraline administration provide sound evidence that exaggerated sympathoexcitation may be a primary reason underlying the modest efficacy of SSRIs in PTSD.

5.5 CONCLUSIONS

We utilized a predator exposure/psychosocial stress animal model of PTSD to analyze the effects of sertraline on neurotransmitter modulation and inflammation in the rat hippocampus, PFC, CSF, and plasma. We found that sertraline increased 5-HT and NE levels in the hippocampus, PFC, and CSF. We also discovered that sertraline attenuated inflammation by lowering PICs and elevating AICs. Despite these seemingly beneficial changes, however, anxiety did not diminish in the PTSD+Sert vs. the PTSD+Veh groups. We propose that noradrenergic hyperresponsiveness, evident by exaggerated levels of NE present in the hippocampus, PFC, and CSF, might be a primary factor in persistent anxiety and a major reason that SSRIs have demonstrated poor efficacy in PTSD. To our knowledge, this is the first study to provide a molecular rationale for this unsatisfactory record of accomplishment. This insight

might allow for more targeted pharmacologic therapies with an emphasis on inflammatory suppression and control of sympathetic drive. It would be an oversimplification, nonetheless, to presume that a persistent noradrenergic tone is the sole causal factor in PTSD development. The autonomic nervous system (ANS), endocrine system, and immune system are indelibly linked with CNS disorders and identifying one system as the cause of PTSD might be impossible. Overall, our results demonstrate that there are CNS-specific modifications in neurotransmitters, immunomodulators, and ANS activity in response to sertraline in the predator exposure/psychosocial stress model. Further research is critical to delineate, if possible, which of these systems actually contributes to PTSD pathophysiology and which are producing nonspecific responses common to multiple psychiatric etiologies.   In addition, direct comparisons with SSRIs and other compounds demonstrating effectiveness in PTSD such as VA and tianeptine are warranted.

6. Acknowledgements

7. Funding and Disclosures

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CHAPTER 6
SUMMARY AND CONCLUSIONS

6.1 OVERALL FINDINGS

Post-Traumatic Stress Disorder (PTSD) is a disorder that does not discriminate based on age, race, sex, occupation, or nationality. According to the National Center for PTSD, it is estimated that approximately 60% of men and 50% of women in the U.S. will face some type of serious traumatic event in their lives. As a result, approximately 5.2 million people (2%) in the U.S. have PTSD in a given year, and 20-25 million (7-8%) will experience PTSD during their lifetime. For combat veterans, estimates range from 11-20% (Veterans Affairs, 2014). PTSD, an anxiety disorder recently reclassified as a trauma- and stressor-related disorder, can develop in response to real or perceived life-threatening situations. According to the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), a diagnosis of PTSD necessitates exposure to a life-threatening event, intrusive recollections of the event, avoidance of associated stimuli and numbing of general responsiveness, negative cognitions/mood, hyperarousal not present before the trauma, and a significant social impairment. All of these symptoms must persist for at least 30 days and not be due to illness, medication, or substance abuse (American Psychiatric Association, 2013). To date, no definitive diagnostic biomarkers have been identified for PTSD. Recent research, however, points toward physiological abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary system, immune system, and brain neurotransmitters that may be implicated in the disorder (Liberzon, Abelson, Flagel, Raz, & Young, 1999; Oosthuizen, Wegener, & Harvey, 2005; Sondergaard, Hansson, & Theorell, 2004; Wilson et al., 2013, 2014). Current therapies include drugs (SSRIs) and cognitive behavioral approaches, but due to varied backgrounds, experiences, epigenetic differences, and high comorbidity with other psychiatric disorders, many patients have shown limited improvement.

As such, a greater understanding of the molecular, behavioral, and environmental relationship inherent in the disorder is paramount for future prevention and treatment strategies.

Exposure to psychologically traumatic events, such as those experienced during combat or other situations posing a legitimate threat to safety and survival, place individuals at significant risk for developing PTSD. A growing body of evidence suggests that exposure to traumatic stressors and subsequent psychological trauma may result in increased morbidity and mortality. Much of the data available suggest traumatic exposure and subsequent PTSD may lead to increased incidence of cardiovascular disease, diabetes, chronic fatigue syndrome, and other conditions (Dansie et al., 2012; Edmondson & Cohen, 2013; Gupta, 2013; Lukaschek et al., 2013). Most of these diseases have detrimental inflammatory components that may exacerbate their progression. Inflammation is a critical component of the immune response, but acute and chronic inflammation can damage cellular mechanisms. Stressful events affect the immune system by reducing the cellular response to mitogen stimulation, decreasing production of natural killer cell activity and altering levels of cytokines. Cytotoxic T lymphocytes, which regulate the balance between Th1 and Th2 cells, are altered by stress leading to a Th2 dominant response, resulting in an unrestrained production of pro-inflammatory cytokines (PICs). These PICs, especially the interleukins, have been shown to play an important role in modulating disease processes. An important and detrimental consequence of increased cytokine production is the induction of nitric oxide (NO) and reactive oxygen species (ROS) (Hu, Peterson, & Chao, 1998; Mokuno et al., 1994).

Chapter two was our initial experiment with the predator exposure/psychosocial stress animal model, and the primary focus was to analyze inflammatory components and reactive oxygen, in both the brain and systemic circulation, in response to PTSD. The roles of oxidative

129

stress and inflammation in other pathological conditions such as cardiovascular disease, diabetes mellitus, metabolic syndrome, and neurological diseases are well established (Agarwal, Welsch, Keller, & Francis, 2011; Alexopoulos et al., 2012; Elks & Francis, 2010; Pall & Satterlee, 2001). We hypothesized similar inflammatory mechanisms may also be implicated in PTSD. First, we validated stress induction via an EPM and determined plasma corticosterone levels were elevated in the PTSD group. In addition, growth rate and thymus weights were lower in the PTSD group, while adrenal gland weight was higher vs. controls. The abnormal growth rates confirmed HPA axis hyperactivity. Higher levels of CRH can inhibit feeding behavior, even in food-deprived animals (Krahn, Gosnell, & Majchrzak, 1990; Mazjoub, 2006). Increased adrenal gland weight may be due to excessive glucocorticoid production without proper negative feedback from the hypothalamus and pituitary, resulting in adrenal hypertrophy and hyperplasia (Ulrich-Lai et al., 2006). The substantive decrease seen in thymus weight may be a result of increased oxidative stress or cortisol toxicity causing thymocyte apoptosis (Salgo & Pryor, 1996). We discovered that damaging ROS in the hippocampus, PFC, and adrenal glands were upregulated in response to the predator exposure/psychosocial stress regimen. We also found that oxidative stress in the blood increased in a time-dependent manner in the PTSD animals. In the brain, mRNA and protein for cytokines and cytokine-producing mechanisms were significantly elevated, demonstrating a neuroinflammatory component in PTSD. Taken together, these data demonstrated aberrancies in both the HPA axis and immune system during PTSD progression and confirmed the fact that inflammation and oxidative stress, whether causal or resultant, may play a key role in the development and exacerbation of the disorder.

Chapter three discusses the interaction between various neurotransmitters in the primary brain regions associated with PTSD in response to the predator exposure/psychosocial stress

model. In the brain, neurotransmitter modulation may play a critical role in PTSD development, and they are currently the primary target for pharmacologic interventions. It remains unclear, however, exactly which neurotransmitters are up- or down-regulated during PTSD progression. 5-HT is the principle regulator of mood. A study by Peirson et al. (Peirson & Heuchert, 2000) found lower platelet $5\text{-}HT_2$ receptor function was associated with depressed mood, while Williams et al. (Williams et al., 2006) demonstrated higher blood 5-HT levels were correlated with better mood. In addition, elevated levels of NE and DA have been associated with hyperarousal, intrusive recollections, and psychosis (Paterlini et al., 2005; Southwick et al., 1999). We found that 5-HT decreased and NE increased in both the hippocampus and PFC, providing evidence that the neurotransmitters previously implicated in PTSD pathophysiology were in fact modulated in response to persistent stressors. In addition, we determined that neurotransmitters were differentially expressed in the hippocampus and PFC. In the hippocampus, 5-HT and the DA metabolite HVA were lower in the PTSD group vs. controls. Conversely, the levels of NE and the DA metabolite DOPAC were significantly higher in the PTSD group. In the PFC, 5-HT was significantly lower, while NE was higher in the PTSD group vs. controls. The levels of DA and the DA metabolite DOPAC were significantly higher in the PTSD group. The neurotransmitter data were supported by the down-regulation of tryptophan hydroxylase, the rate-limiting enzyme for 5-HT, and the up-regulation of tyrosine hydroxylase, the rate-limiting enzyme for NE and DA. It would be an oversimplification, nonetheless, to presume that neurotransmitter modulation is the sole causal or resultant factor in PTSD development, as HPA axis, sympathoadrenal medullary pathway, and immune system alterations may also play an integral role in the pathophysiology of this complex disorder.

In chapter four we demonstrated that HDAC inhibition could decrease inflammation and oxidative stress and modulate neurotransmitters, resulting in decreased anxiety and improved performance in the EPM. Gene expression is regulated via highly controlled acetylation/deacetylation of histone N-terminal tails, which either increases or decreases gene availability (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003). Acetylation/deacetylation is accomplished by histone acetyltransferases (HAT) and histone deacetylases (HDAC), which enable and restrict genome access, respectively. When oxidative stress and inflammation are increased, upregulated PICs can correspond with heightened HDAC activity and NF-κB transcription, resulting in perpetual PIC production (Keslacy, Tliba, Baidouri, & Amrani, 2007). This study was conducted to test alternative pharmacologic therapies for PTSD, as the currently approved selective-serotonin reuptake inhibitors (SSRI) have proven nominally effective (Tawa & Murphy, 2013). Previous data from our lab demonstrated that HDACi were effective at reducing inflammation and oxidative stress (Cardinale et al., 2010), and we hypothesized it may have a similar effect in PTSD. We found that increased oxidative stress and inflammation in the brain and blood in response to psychological stress are attenuated with VA, possibly due to attenuated NF-κB transcription. In addition, the aberrant neurotransmitter profile of increased catecholamines and decreased 5-HT was normalized with VA. The mechanism responsible for the neurotransmitter modulation may be HDACi interaction with the rate-limiting enzymes (Akiba et al., 2010; Covington et al., 2009). VA also seems to enhance resiliency behavior as indicated by reduced anxiety levels in the PTSD+VA group. These findings indicate VA may be a promising therapy with broad-spectrum effects in PTSD patients.

In chapter five, we determined that a possible mechanism responsible for the negligible effects of SSRIs in some PTSD patients might be an overactive sympathetic drive. We utilized the predator exposure/psychosocial stress model of PTSD to analyze the effects of sertraline on neurotransmitter modulation and inflammation in the rat hippocampus, PFC, CSF, and plasma. We found that sertraline increased 5-HT and NE levels in the hippocampus, PFC, and CSF. We also discovered that sertraline attenuated inflammation by lowering PICs and elevating anti-inflammatory cytokines (AIC). Despite these seemingly beneficial changes, however, anxiety did not diminish in the PTSD+Sert vs. the PTSD+Veh groups. We propose that noradrenergic hyperresponsiveness, evident by exaggerated levels of NE present in the hippocampus, PFC, and CSF, might be a primary factor in persistent anxiety and a major reason that SSRIs have demonstrated poor efficacy in PTSD. This insight might allow for more targeted pharmacologic therapies with an emphasis on inflammatory suppression and control of sympathetic drive. It would be an oversimplification, nonetheless, to presume that a persistent noradrenergic tone is the sole causal factor in PTSD development. The autonomic nervous system (ANS), endocrine system, and immune system are indelibly linked with CNS disorders and identifying one system as the cause of PTSD might be impossible. Overall, our results demonstrate that there are CNS-specific modifications in neurotransmitters, immunomodulators, and ANS activity in response to sertraline in the predator exposure/psychosocial stress model. Further research is critical to delineate, if possible, which of these systems actually contributes to PTSD pathophysiology and which are producing nonspecific responses common to multiple psychiatric etiologies.

6.2 SIGNIFICANCE OF RESEARCH AND FUTURE DIRECTIONS

Current treatment regimens for PTSD are focused primarily on counseling-based therapies that help break the association between the traumatic event and the lingering anxiety, hyperarousal, intrusive thoughts, and other symptoms. These approches have proven effective to a degree, but they do not take into account the aberrant pathophysiological profile of PTSD. The drugs that are currently available can suppress some of the comorbid symptoms such as depression, but they fail to correct the underlying molecular mechanisms in PTSD. This work was significant because we used an animal model to elucidate some of the pathophysiological mechanisms involved in PTSD progression, discovered a rationale as to why SSRIs might be ineffective in treating PTSD, and demonstrated the efficacy of an alternative drug in attenuating inflammatory cytokines, reactive oxygen, and neurotransmitter fluctuations. Our findings, however, merely scratch the surface of this complex disorder and continued research is paramount.

One of our original goals was to locate a specific biomarker that could be diagnostic for PTSD. One project that we have on the immediate horizon is the total RNA sequencing and gene comparison between PTSD animals and controls. Based on the pathophysiological components we have previously described, we hypothesize that there might be gene expression changes occuring as the disorder progresses. We aim to analyze the potential changes, isolate certain genes, and attempt to identify a small number of genes that are characteristic of PTSD. We will also evaluate whether differentially expressed genes might be correlated with behavior. Our long-term goal concerning gene expression analysis is to block or overexpress certain genes in an attempt to attenuate the pathophysiologic changes.

The potential to diagnose PTSD both psychologically and physiologically could drastically broaden treatment options and hopefully improve recovery. PTSD, however, is a very complex disorder with multiple system aberrancies occuring simutaneously. The truth is there might not be a single physiologic biomarker that is indicative of and exclusively diagnostic for PTSD. Taken together, the data presented herein will help add to the collective knowledge base concerning PTSD and assist in the constant pursuit of understanding and treating this confounding disorder.

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APPENDIX
LETTER OF PERMISSION

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VITA

Carl Brad Wilson was born in 1969 in Ft. Wayne, IN. He was adopted in early July, 1969 by Carl Douglas and Dolores Alene Wilson of Lexington, KY. As the son of an active duty Air Force member, Brad grew up at March AFB, CA, Scott AFB, IL, Torrejon AB, Spain, and finally Eglin AFB, FL. In 1984, his dad retired and they moved 30 miles north to the small town of Crestview, FL, where he completed high school at Crestview High in 1987. Brad enlisted in the Air Force in 1988 and spent his first two years at Spangdahlem AB, Germany. In 1990, he was stationed at Hurlburt Field, FL, where he remained until his separation in 1995. Brad completed EMT and paramedic school in 1998 and was a full-time paramedic with Okaloosa County EMS until 2002.

During his last few years as a paramedic, Brad completed his B.A. degree in psychology at the University of West Florida and returned to active duty as an Air Force officer in 2002. In 2004, he became a special agent with the Air Force Office of Special Investigations. After a 12-month tour in Iraq, Brad completed his M.F.S. in forensic sciences at The George Washington University in 2008.

In 2010, Brad was offered the opportunity to become a professor at the U. S. Air Force Academy, contingent upon his successful completion of a Ph.D. Taking full advantage of his circumstances, he secured a position as a graduate student in the laboratory of Joseph Francis, B.V.Sc., M.V.Sc., Ph.D., Department of Comparative Biomedical Sciences, Louisiana State University School of Veterinary Medicine. He is a candidate for the Doctor of Philosophy degree in Veterinary Medical Sciences and will return to Colorado Springs, CO, in Aug 2014. Brad's research is focused on the molecular aspects of PTSD and he plans to continue searching for specific pathophysiological mechanisms associated with the disorder.