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DISSERTATION APPROVAL
FOR THE DOCTORAL DISSERTATION
IN THE NEUROSCIENCE
GRADUATE PROGRAM

Title of Dissertation: "Effects of Estradiol on Post-Traumatic Stress Disorder Symptoms"

Name of Candidate: LTC Danette F. Cruthirds
Doctor of Philosophy Degree
March 25, 2010

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAR 2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Effects Of Estradiol On Post-Traumatic Stress Disorder (PTSD) Symptoms	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences,F. Edward Herbert School Of Medicine,4301 Jones Bridge Rd,Bethesda,MD,20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Post-traumatic stress disorder (PTSD) is a complex anxiety-related disorder that is caused by a traumatic or life-threatening event. This disorder is defined by a triad of clustered symptomatology including: re-experiencing through intrusive thoughts and dreams; persistent avoidance of stimuli associated with the trauma along with numbing of general responsiveness; and hyperarousal not present before the event. Current treatments are beneficial to some patients but many continue to suffer from this debilitating disease and no preventative treatments are available.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**208**	

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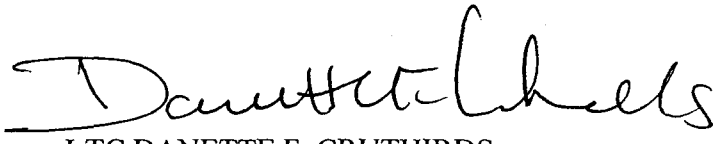

LTC DANETTE F. CRUTHIRDS
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ABSTRACT

"EFFECTS OF ESTRADIOL ON POST-TRAUMATIC STRESS DISORDER (PTSD) SYMPTOMS"

LTC Danette F. Cruthirds

Thesis Directed By: T. John Wu, Ph.D. Associate Professor, Department of Obstetrics and Gynecology

Post-traumatic stress disorder (PTSD) is a complex anxiety-related disorder that is caused by a traumatic or life-threatening event. This disorder is defined by a triad of clustered symptomatology including: re-experiencing through intrusive thoughts and dreams; persistent avoidance of stimuli associated with the trauma along with numbing of general responsiveness; and hyperarousal not present before the event. Current treatments are beneficial to some patients but many continue to suffer from this debilitating disease and no preventative treatments are available. The underlying biological mechanism leading to PTSD is still elusive; thus, the present research evaluates a potentially useful chronic stress paradigm in a rat model to mimic PTSD symptomatology. Additionally, the efficacy of estrogen receptor (ER) agonists as a preventative treatment for PTSD symptomatology was examined. Adult ovariectomized Sprague-Dawley rats were treated with vehicle (VEH), 17β-estradiol (E2), propylpyrazoletriol (PPT:ERα agonist), or diarylpropionitrile (DPN:ERβ agonist). Half the animals from each treatment were immobilized 60 min/day for 22 days. Rats that were chronically stressed had reduced body weight gain without affecting food intake. These rats also had attenuated corticosterone levels, suggesting that the hypothalamic-pituitary-adrenal axis (HPA) response to stress was altered. Behavioral measures show stressed animals had

exaggerated startle responses and an increase in tolerance to thermal pain. In response to hormone treatment, rats administered E2, PPT and DPN had attenuated exaggerated startle response. Furthermore, E2 and PPT were anxiolytic while E2, PPT, and DPN treatment improved the animal's latency to escape from an anxious environment. In summary, the results of this study show that chronic immobilization stress disrupts the HPA axis and exaggerates certain behaviors reminiscent of PTSD in humans. Treatment with E2 or its agonists counteract some of the negative effects of chronic immobilization stress in the female rat.

“EFFECTS OF ESTRADIOL ON POST-TRAUMATIC STRESS DISORDER (PTSD) SYMPTOMS”

By

Lieutenant Colonel Danette F. Cruthirds

US Army Nurse Corps

Dissertation submitted to the Faculty of the
Neuroscience Graduate Program
Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, 2010

DEDICATION

To the United States Military Personnel, for your service and sacrifice.

ACKNOWLEDGEMENTS

It has been an honor and a privilege to be a part of Uniformed Services University Graduate Program. I have had the opportunity to meet so many dedicated, intelligent, and kind people in my studies here. This journey, completing a doctoral degree in Neuroscience, was a success because of those around me and their can do spirit, encouragement, mentorship and sacrifice.

First and foremost I want to thank my family. To my dad and mom, for instilling in me the importance of hard work, the belief I could do anything and the importance of generosity. To my brothers, for always taking care of your baby sister. To my in-laws, for your love and support. My deepest gratitude goes to my husband and two children for their sacrifice, patience, support and compromise. Nicole and Bradley you are the joy and light in my life. Eddie, without you this would have been an impossible task. You deserve as much credit, if not more, for my completion of this milestone. Our accomplishments together pale in comparison to anything we've done alone.

I am most grateful to my mentor, Dr. T. John Wu. You have inspired and challenged me to achieve beyond the boundaries of my expectations and given me a deep understanding of scientific research. Your steadfast support and guidance to the advancement of my education has been unyielding and compassionate.

I offer a heartfelt "thank you" to Dr Joseph McCabe, my committee chair who always took the time to give me his full attention. Your insight, wisdom, and positive reinforcement kept me focused to complete the rigorous requirements of this program.

To my committee members, Dr Regina Day, Dr Thomas Cote, Dr Mary Lou Cutler and Dr Bob Handa who demonstrated a true interest in the focus of this project and put forth tireless efforts in editing the written document ensuring a scholarly manuscript.

A dept of gratitude goes to to my numerous coworker and friends in the Wu Lab including Omar Larco, Soha Ashrafi, Kevin Oakley, Jamie Grimes, Madelaine Clark, Florencia Vasta, Elysia Pagano and Meg Keil for all you contributions to my research.
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To the US Army Nurse Corps, for allowing me to embrace my past experiences, engage in the present challenges and envision future endeavors. Also appreciated is the TriService Nursing Research Program (TSNRP) for recognizing the importance of this research and providing the means necessary to accomplish the work.

I would like to extend my sincere thanks to those that made completing this major research project a success: Dr. Regina Armstrong (Previous Director, Neuroscience Program); Dr Sharon Juliano (Present Director, Neuroscience Program) (Dr. Eleanor Metcalf (Dean, Graduate Education) and the graduate education office personnel.

There are others that have encouraged me to reach beyond my potential in research and science. Deserving recognition are Drs. Bruce Schoneboom, and Patricia Nishimoto without their contributions, I would not be here today

A deep appreciation to my extended military family: LTC (ret) Judith Rozelle, COL (ret) Patricia Cordts, LTC Wendall Holladay, COL Patti Lederer, LTC (ret) Sandra Bruner, Lt Col Susan Perry, LTC Joseph O'Sullivan and MAJ Micheal Bently just to name a few. Over the years your leadership qualities, professionalism, sense of duty, moral compass and lasting friendship have inspired and propelled me to where I am today.

Lastly, I want to thank my best friends, Joy, Shelly, Liza and Carol for their undeniable friendship in good times and bad.

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LIST OF ABBREVIATIONS

5-HTTLPR	serotonin transporter gene
ACTH	adrenocorticotropin hormone
AIS	acute immobilization stress
ANOVA	analysis of variance
ARC	arcuate nucleus
ASR	acoustic startle reflex
BM	Barnes maze
BMI	body mass index
CIS	chronic immobilization stress
CORT	corticosterone
CRH	corticotropin releasing hormone
DEX	dexamethasone
DHEA	dehydroepiandrosterone
E2	estradiol
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
ER	estrogen receptor
DEX-CRH	dexamethasone-corticotrophin releasing hormone test
DPN	diarylpropionitrile
GC	glucocorticoid
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal

Hsp	heat shock proteins
ICV	intracerebroventricular
LC	locus ceruleus
MBH	medial basal hypothalamus
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
MDD	major depressive disorder
Ob-Rb	leptin receptor
OVX	ovariectomized
pAKT	phospho-protein kinase B
PFC	prefrontal cortex
POMC	proopiomelanocortin
PPI	prepulse inhibition
PPT	propylpyrazoletriol
PTSD	post-traumatic stress disorder
PVDF	polyvinylidene difluoride membrane
PVN	hypothalamic paraventricular nucleus
rACC	rostral anterior cingulated cortex
SEM	standard error of the mean
SERMs	selective estrogen receptor modulators
SOCS3	cytokine signaling 3
SSRIs	selective serotonin reuptake inhibitors
STAT3	transcription 3

TBI	traumatic brain injury
US	United States
USUHS	Uniformed Services University of the Health Sciences
WHR	waist/hip circumference ratio

"Effects of estradiol on post-traumatic stress disorder symptoms"

CHAPTER 1

GENERAL INTRODUCTION

The endocrinologist Hans Selye (1907-1982), is largely credited for introducing the term "stress" in the 1930's. A pioneer in stress research, he defined stress as a nonspecific response of the body to any demand and stressor as an agent that produced stress at any time [1-2]. The stressors, which may be physical, chemical or psychological, cause a wide range of responses from mild reactions to severe dysfunction [3-4]. These early concepts of stress and stressors were built upon the concept of homeostasis defined by physiologists Claude Bernard (1813-1878) and Walter B. Cannon (1871-1945). Homeostasis relies on the proper coordination of physiological and biochemical processes the body uses to maintain a stable "milieu interieur"[5-7]. This concept has led to the current view of stress as "a state of threatened homeostasis (physical or perceived threat to homeostasis)" [8 9]. The mechanism of response was coined the "General Adaptation Syndrome" and has three stages: the alarm reaction stage, the resistance stage, and the exhaustion stage [3]. The alarm reaction stage, occurring after the nocuous stimulus, involves activation of the hypothalamic-pituitary-adrenal (HPA) axis and the peripheral adrenomedullary hormonal system. The resistance stage, evolving after the alarm reaction stage, triggers adaptive responses in the form of glucocorticoids to replenish energy stores. Disease and illness occur when chronic stimulation of these adaptive responses compromise energy stores resulting in development of the exhaustion stage [10]. Dr Selye's early experiments further found an additive effect of exposure to multiple damaging stressors that result in a more rapid depletion of the adaptive response

[11-12]. Activation of the HPA axis is critical to coordinating the restoration of a stable internal milieu while its dysfunction may lead to disease [6].

Alterations in the function of the HPA axis from chronic stress are thought to be a risk factor in psychological disorders such as anxiety and depression. Anxiety disorders are the most common psychiatric disorders, affecting nearly one-fifth of the adult population in any given year [13]. On the forefront of anxiety disorders is post-traumatic stress disorder (PTSD) underscored by recent events that include 9/11, hurricane Katrina, and ongoing military operations [14]. This disorder is defined by a triad of clustered symptomatology, including re-experiencing the traumatic event through intrusive thoughts and dreams, persistent avoidance of stimuli associated with the trauma often accompanied by a "numbing" of general responsiveness, and a state of hyperarousal that was not present before the event. Additionally cognitive deficits and alterations in pain sensations can occur. While current treatments help some, many continue to suffer and little has been done to develop preventative pharmacotherapy targeting the development of this disorder [15].

A valid animal model for PTSD has yet to be established even while there are a multitude of stress paradigms. Animal models are used to reflect the manner and variability of induction along with the time course for development of core symptoms associated with PTSD. Rats exposed to unpredictable or uncontrollable stress have shown similar symptoms exhibited by individuals with PTSD. These symptoms include, exaggerated startle responses, irritability or aggression, social avoidance or inhibition, and memory dysfunction [16]. Techniques used thus far in an attempt to recapitulate human PTSD in an animal model include; (1) brief sessions of electrical shock (electric

tail shock or inescapable foot shock), (2) aversive signals (a predator, predator odor, or social stress), (3) single prolonged stress (2hr restraint stress, 20 minutes of swimming, and ether exposure until loss of consciousness), and (4) restraint/immobilizations stress [17].

Of the animals models put forward chronic immobilizations stress appears to be most promising. This stressor is associated with PTSD symptoms (startle and anxiety) and is believed to simulate the human perception of lack of control [18]. In addition, repeated restraint or immobilization causes structural remodeling in areas of the brain responsible for emotional memories and regulation of the stress response (amygdala, hippocampus and prefrontal cortex) [19-21]. Acute immobilization stress also produces reliable elevations in hormones associated with a stress response including the hormones, adrenocorticotropin (ACTH) and corticosterone (CORT) [22].

These neuroendocrine, physiological and behavioral findings establish face validity of the immobilization model and strengthen the developing convergent validity. What is missing is the long-term persistence and possible delayed-onset actions of PTSD which is often overlooked in acute short-term models. One such long-term persistence action that may also develop as a potential neuroendocrine marker of PTSD is enhanced inhibition of the HPA axis [23-24] or a decrease sensitivity level to pituitary and adrenal stimulation [25]. Multiple studies have demonstrated lower cortisol outputs when compared to controls [25-31]. Further, PTSD patients subjected to the dexamethasone suppression test demonstrate significant suppression of plasma cortisol levels [32-34].

The goal of the research presented here is to further develop an operationally useful animal model to mimic PTSD symptomatology. We began by establishing face

validity, characterizing biochemical and behavioral symptoms. Biochemical measurements focused on the stress hormone, glucocorticoid, that are altered in PTSD patients [26-28]. Multiple behavioral symptoms characteristic of PTSD were measured including startle reflex, locomotor activity, anxiety and declarative memory. In addition long-lasting changes subsequent to a brief stressor and corresponding progression of PTSD-like symptoms that grow over time should ideally be shown in a relevant animal model [33]. The experiments presented here used an animal model consisting of a 1 hour daily immobilization stress paradigm using a finger-like Centrap cage over a 22 day period fit these mentioned criteria. In addition to the development of the animal model, a secondary goal of this study is to determine whether estrogen may ameliorate the PTSD-like symptoms in this animal model. Estrogens, which are steroid hormones, have been shown to have positive effects on both mood and cognition including these PTSD-like symptoms [35-41]. A well established ovariectomized rat model with implanted osmotic pumps delivering reliable levels of hormone treatments prior to the stressor was used to determine our overarching hypothesis that estrogen treatment can prevent symptomatology of PTSD.

This dissertation follows the manuscript–based format as outlined by the USUHS Neuroscience Program. Following this brief introduction is a review of the literature, three first-authored manuscripts and finally a discussion chapter unifying the two manuscripts.

CHAPTER 2

REVIEW OF THE LITERATURE

The regulation of stress evolved as a biological means to protect an organism. When confronted with a real or perceived threat physiological systems necessary to allow the body to cope are activated. Alterations of normal state through the activation of the various physiological systems including neural, endocrine, immune, and digestive systems, allows for either fight or flight thereby maintaining self preservation[42]. Psychological disorders, such as post-traumatic stress disorder (PTSD), have chronic alterations from the normal state brought forth by 2 criterions. The traumatic event, as defined by two Criterion A1 of the DSM-IV-TR, is "an event a person experiences, witnesses, or confronts that involves actual or threatened death or serious injury, or a threat to the physical integrity of self or others". The emotional response as defined by Criterion A2 "the involved person's response of intense fear, helplessness, and horror" [43]. Physiologic systems having the greatest impact into this disorder are the neural and endocrine systems.

Post-Traumatic Stress Disorder

PTSD is defined as a psychological disorder that results from a traumatic event (or events) that involves actual or threatened death, serious injury, or threat to the physical integrity of oneself or others with a person's response that involves intense fear, helplessness, or horror [43-44]. This definition has evolved from early observations first described in the 19th century in which a pattern of responses (pallor, nervousness, anxiety, tearful, thready pulse) was associated with traumatic life-events [45]. The

diagnostic term used to describe the multiple signs and symptoms has also undergone many changes.

The first medical articles in 1867, by English surgeon John Erichsen, credited the associated psychological sequelae to injury of the spinal cord following railroad accidents, the most advanced technology at this time in history [46-47]. This led to an early descriptive term of the developing syndrome, railroad spine syndrome [48]. Even though Erichsen believed the damaged spinal cord caused the symptoms others during this time believed the symptoms to be psychological in nature. Subsequently the syndrome was replaced by a new diagnostic term “traumatic neurosis” [46] where "trauma" was used to include the psychological reaction to a traumatic experience [48]. At this same time the American Civil War saw common symptoms among the soldiers from both sides that were attributed to the heart. Symptoms included chest pain, palpitations of the heart, shortness of breath, and increased heart rate [49]. This condition was formally described in a paper entitled "On Irritable Heart" by Jacob DaCosta, an American, in 1871. Terms such as “cardiac weakness”, “irritable heart”, and “soldier’s heart” were used to describe this condition [45, 47].

Over the next 100 plus years, war continued to influence the evolution of PTSD. The term “Shell Shock” became the new phrase during WWI when describing the reactions of some soldiers to ear splitting cacophony induced by exploding shells and firearms [50]. Reactions included tremors, increased startle, mutism, irritability, restlessness, and nightmares [51]. This term quickly fell out of favor once the war ended because the vast majority of the cases were found to be psychogenic [47]. Subsequent to WWII and during the Korean War the term “gross stress reaction” was entered into the

Diagnostic and Statistical Manual of Mental Disorder (DSM)-I. Although this term was not operationally defined, thereby limiting its reliability of diagnoses, it was justified for individuals experiencing extreme physical or psychological demands. In the 1960's, during the writing of the DSM-II, the United States (US) was no longer at war and the term "gross stress reaction" was dropped from the new edition. It was replaced by the term “transient situational disturbance” which still lacked an operational definition but now included psychotic reactions following overwhelming stress and all kinds of acute reactions[48]. It was not until 1980, after the devastating effects of the Vietnam War and long term studies of Holocaust survivors that the operationally defined term “post-traumatic stress disorder” occurred.

Formally introduced into the third edition of the DSM (DSM-III) in 1980, PTSD is a unique debilitating anxiety-related disorder that has a presumed cause—exposure to a traumatic event. Currently the revised fourth edition (2000) of the DSM (DSM-IV-TR) defines PTSD as a traumatic event (or events) that involves actual or threatened death, serious injury, or threat to the physical integrity of oneself or others with a person's response that involves intense fear, helplessness, or horror [43-44]. Three clusters of diagnostic criteria include re-experiencing through intrusive thoughts and dreams, persistent avoidance of stimuli associated with the trauma along with numbing of general responsiveness, and hyperarousal not present before the event [43] (Figure 1). Victims also are likely to have disturbed sleep, increased irritability, concentration problems, and an exaggerated startle response. Feelings of guilt, anger, and shame often emerge [52].

Individuals suffering symptoms from the three diagnostic criteria (re-experiences, numbing and avoidance, and hyperarousal) within 1 month of the traumatic event are diagnosed with acute stress disorder (ASD) not PTSD according to DSM-IV-TR. However, if there is no improvement of symptoms after this period of time, acute PTSD is diagnosed. If these symptoms persist longer than three months, then the diagnosis is changed to chronic PTSD. Another diagnostic variation of PTSD is delayed onset PTSD, which involves symptoms first appearing more than six months after the traumatic event [43](Figure 2).

Today as the war on terrorism continues in Afghanistan and Iraq the diagnostic criteria of PTSD is once again being revisited. Unique to this war are the perilous conditions of urban warfare, to include door to door close combat, suicide bombers, improvised explosive devices and the difficulty in differentiating civilians from enemies. Recent studies show deployed personnel exposed to combat have a threefold increase in new onset self reported symptoms of PTSD when compared to non-deployed personnel [53]. The "signature injury" of the today's wars is traumatic brain injury (TBI) due to greater survival rates from improved body armor and medical intervention [54]. While the vast majority of TBI's are mild, a recent study showed soldiers with loss of consciousness or altered mental status also met criteria for PTSD in 43.9% and 27.3% of the cases respectively [54]. What is learned from modern wars of today will once again likely influence future definitions of stress and PTSD.

Epidemiology and Prevalence

The rate of overall lifetime exposure to trauma varies depending on research methodology and ranges from 39% to 89% among civilian and military adults [55]. It has

been estimated that the lifetime prevalence rate of PTSD in the general civilian population is up to 14% [13, 55-56]. Studies of some military populations, Gulf War veterans and veterans of Iraq and Afghanistan, showed comparable risks for PTSD [57-58]. In the National Comorbidity Survey, men were at higher risk for exposure to a traumatic event then women (60% vs 51%) but women were more likely to develop PTSD (10.4% vs 5%)[13, 59].

Among military service members, the potential for exposure to trauma and development of PTSD increases markedly during times of war with a prevalence rate of 10.9-58% [60-63]. Presently, ongoing operations for the war on terrorism, Afghanistan and Iraq, have an estimated risk for PTSD of 11% and 18% respectively [64].

PTSD affects not only the afflicted individual but family and society as well. The consequences of this debilitating disorder are more far-reaching; epidemiological data indicate that 1 out of every 12 adults are affected by PTSD [65]. The social and personal costs to PTSD patients and their families are incalculable.

Comorbidities.

Maxmen & Ward (1995) reported that half of PTSD cases occur with an additional psychiatric diagnosis [66]. PTSD is commonly associated with major depression, anxiety disorders, and substance abuse [56]. In fact, patients with PTSD also show depressive symptoms in 30%-50% of cases, with more women than men to having comorbid depression [66-67]. On the other hand, men are more likely to have comorbid substance abuse leading to a more severe clinical profile such as lower functioning and poorer well being than those with either disorder alone[68].

Many patients have symptoms of other anxiety disorders, with 53% to 90% of victims experiencing a panic attack during an extreme stressor [69]. Memory problems are also associated with PTSD; individuals suffering from PTSD have deficits in hippocampal-dependent tests of immediate recall [70]. Further, Magnetic Resonance Imaging (MRI) studies have revealed that PTSD patients have decreased hippocampal volume, presumably secondary to cortisol-related hippocampal atrophy [70].

Neuroanatomy

Traumatic stress is a psychobiological response to a life-threatening event [71]. The response to the traumatic event is complex; thus an interest in the functional neuroanatomy of traumatic stress to understand the clinical presentation has exploded in the past two decades. Prior to this explosion, early neuroanatomist, began mapping neuroanatomic and neurophysiologic evidence associated with stress and emotion. A universal brain system of emotion culminated in the 1950's with the development of the limbic system concept [72]. This term, coined by Paul MacLean, considered subcortical regions of the brain (hypothalamus, hippocampus, thalamus, medial temporal cortex, and cingulate) described by earlier anatomists as the "rhinencephalon". These areas together with other brain areas including the amygdala, septum, and prefrontal cortex (PFC) function as a system within the brain that responds to stress and emotion [73-74].

In the last ten years neuroimaging studies have just begun to piece together the possible neural circuitry involved in PTSD. Findings from neuroimaging studies resulted from a variety of stress paradigms: symptom provocation, cognitive activation, and functional connectivity studies [75]. The thalamus (a gateway for sensory input), the

medial prefrontal cortex (mPFC) including the anterior cingulate and orbitofrontal, the amygdala, and the hippocampus, have been implicated in mediating symptom formation in PTSD [73, 75-77].

Adaptation to a stressful experience requires integration of cognition, behavior, and complex neuroendocrine responses. The prefrontal cortex is vital for this to occur through the regulation of the hypothalamic paraventricular nucleus (PVN), which controls hypothalamic-pituitary-adrenocortical (HPA) and sympathoadrenal responses. More specifically, emotional processing falls under the mPFC as amygdala activity is suppressed through their intricate connections [78-80]. Many believe the underlying neural mechanism of PTSD symptomatology is decreased activity of the ventral mPFC, particularly the rostral anterior cingulated cortex (rACC). This is important for mediating deficits in extinction. The rACC also suppresses attention/response to trauma-related stimuli and hyperactivation of the amygdala subserving exaggerated acquisition of fear associations and expression of fear responses [81-83]. The dorsal mPFC and locus ceruleus (LC), the principal noradrenergic cell group in brain, have also been shown to modulate emotional stress-induced HPA activation. Radley and colleagues, when selectively ablating noradrenergic inputs into the mPFC, found Fos and corticotropin-releasing hormone (CRH) mRNA expression attenuated during acute stress [80].

The interaction between memory function and traumatic stress play a crucial role in PTSD as well. In magnetic resonance imaging (MRI) studies of patients with post-traumatic stress disorder, without comorbid conditions, have a smaller hippocampus, which is a major player in short term memory, to be decreased in size.[83-85]. The susceptibility of neurons of the hippocampus to stress is most likely due to the fact they

display a high percentage of mineralcorticoid and glucocorticoid receptors [86-87]. Dendritic branching and growth in CA3 pyramidal cells of the hippocampus are decreased when exposed to corticosteroids over a 21 day period [88]. In addition, apical dendrites of the CA3 pyramidal neurons showed atrophy after repeated daily restraint stress for 21 days [20].

The amygdala, which is responsible for fear learning, fear processing, and fear memory, also experiences changes [89]. Subnuclei of the amygdala receive input about the stressful experience from the thalamus and hippocampus. An emotional valance is created and signals are sent via projection to the hypothalamus, triggering neuroendocrine and autonomic responses, the periaqueductal gray area located in the midbrain, triggering lifesaving response such as flight or fight, and to subcortical structures of the brain stem, eliciting reflexes and motor responses. Input is also received from the PFC providing guidance to the event of such responses. Functional neuroimaging studies of the amygdala reveal exaggerated responses during stressful situations in PTSD patients [82, 90]. In contrast to the decreased hippocampal dendritic growth, using a chronic restraint stress paradigm, the amygdala displayed increased branching and length growth [21]. Understanding what is happening in the various regions of the brain during stress and chronic stress states remains a major research challenge but can lead to improved preventive techniques as well as targeted treatments.

Neuroendocrine Regulation of Stress: the HPA axis, Allostasis and Allostatic Load

Physiological and behavioral coping responses to daily events and major stressors are under the control of the hippocampus, amygdala, and prefrontal cortex [91]. The

hippocampus is an important brain region for declarative, explicit memory about facts, and spatial, orientation to one's environment, learning and memory. Enriched with glucocorticoid (GC) receptors, the hippocampus is vital in processing information about stressful events to allow for adaptation [86, 92]. The amygdala, which is important in regulating anxiety and affective responses, [93] also contains CRH-expressing neurons which express GC receptors [94]. In addition, studies show a properly functioning basolateral amygdala (BLA) with GC receptors is needed to facilitate memory consolidation. This has been demonstrated by lesions to the basolateral amygdala or pharmacological inactivation with GC antagonists to the BLA impairing memory consolidation [95-96]. Direct effects of GC on BLA neurons include increasing their intrinsic excitability and decreasing the impact of $GABA_A$ inhibition (an inhibitory neurotransmitter) [97]. Furthermore, neurons of the amygdala show a growth response when exposed to repeated stress as evidenced by dendritic growth in this region of the brain in rats exposed to chronic restraint stress [21]. The prefrontal cortex (PFC), which is important for executive function and working memory, also contains GC receptors but their precise role when modulated remains to be elucidated [98-100].

The HPA axis regulates the stress response, a short term process allowing the body to adapt, accommodate or escape from an actual or potential threat (Figure 3). First, during stress corticotrophin releasing hormone (CRH) is released by the paraventricular nucleus (PVN) of the hypothalamus. Neuroendocrine neurons of the PVN project to the median eminence releasing CRH via the nerve terminals. Second, CRH travels through the portal vascular system to stimulate the anterior pituitary to release adrenocorticotropin hormone (ACTH). Third, ACTH is transported through the blood

system stimulating the adrenal cortex to produce and release the stress hormone cortisol secretion in humans and corticosterone release in rats [101]. Lastly, stress hormones promote the conversion of protein and lipids to carbohydrates replenishing energy reserves in the body. Excess glucocoricoids then exert a feedback effect onto the hypothalamus causing it to stop producing CRH. This replenishment returns the body back to balance also known as allostasis.

If functionally impaired, from damage, atrophy, or psychological stress, a prolonged HPA response can occur after because the hippocampus is crucial for shutting off the HPA stress response [102-103]. One study reported blocking CRH neurons in the medial PFC with a non-selective receptor antagonist thereby significantly attenuating HPA responses to either acute or chronic restraint stress [104]. All three regions discussed, not only are targets of stress hormones but also appear to be involved in PTSD.

Allostasis and allostatic load are two terms that respectively encompass both the protective and potentially damaging effects of stress mediators on both the brain and body [100]. Allostasis is protective responses by the body to maintain homeostasis during acute stress via the release of hormones and autonomic regulators (such as cortisol and adrenalin) [105]. In contrast, allostatic load, the long term effect of failed allostasis or adaptation, leads to undue “wear and tear” on the body and can have deleterious effects on psychological and physiological function [100]. This a risk factor for the development of PTSD [100]. Types of maladaptions to allostatic load include "(1) frequent activation of allostatic systems, (2) failure to shut off allostatic activity after stress, (3) inadequate response of allostatic systems leading to elevated activity of other normally counter-regulated allostatic systems after stress" (Figure 4) [6].

Numerous neuroendocrine studies on PTSD patients show alterations at all regulatory steps of the HPA axis to suggest allostasis is disrupted. Levels of CRH in the cerebral spinal fluid (CSF) are shown to be elevated in patients with PTSD when compared to healthy subjects [106-107]. A recent study using the dexamethasone-corticotrophin releasing hormone (DEX-CRH) test, an HPA-axis function test, showed PTSD patients with co-morbid major depressive disorder (MDD) had an attenuated ACTH response [108]. Furthermore circulated cortisol levels have been found to be decreased in urine [26-27] saliva[108-109] and plasma[107, 110] Decreased cortisol levels has not been found in all studies. Wheler and colleagues found no differences in mean level or circadian pattern of cortisol secretion when comparing PTSD patients to controls [111]. PTSD has also been characterized by increased sensitivity for the HPA negative feedback inhibition [112]. This increased sensitivity results in a below normal stress hormone response. These studies reinforce the concept that allostatic load created by the traumatic experience exceeds the body's coping capacity.

Treatments for PTSD

There are two main approaches to the clinical management of PTSD: psychotherapy and pharmacotherapy. Psychotherapy treatment options include: exposure therapy, which aims to desensitize the patient to the traumatic experience; cognitive behavior therapy, which aims to change the patient's perception to the traumatic experience; anxiety management training, which aims to provides methods to manage anxiety levels; and eye movement desensitization and reprocessing therapy, the newest treatment option allowing patient's to analyze the traumatic event from a detached

perspective[59, 113]. These various psychotherapy treatment options interlace with potential PTSD models. For example, exposure therapy facilitates extinction learning which is believed to be impaired in PTSD sufferers [114-115]. Fear extinction, when the fear response is attenuated in the absence of the fear-induced stimuli [116], is mediated by inhibitory control of the vmPFC over the amygdala. During exposure therapy, an MRI study shows an increase in rACC activity with a corresponding decrease in amygdala activity [115, 117-118]. Furthermore, a multideminsional meta-analysis shows 67% of patients no longer meet criteria for PTSD when these psychotherapy approaches were fully completed [114]. While psychotherapy treatment options look promising, not all patients are able to fully complete therapy and greater that 30 percent of patients continue to suffer.

First-line pharmacologic treatment for PTSD, aimed at reducing severity and improving function, is selective serotonin reuptake inhibitors (SSRIs) [119-120]. Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter, has been implicated in a variety of disorders most notably mood, anxiety, and depression as well as in normal functions like sleep, sexual activity, and appetite[121]. Although typically prescribed to treat depression, many of the SSRIs have significant anxiety-relieving effects. In fact two SSRI's, sertraline and paroxetine, are FDA approved in the treatment of PTSD. The efficacy of SSRIs is believed to result from desensitization of the serotonin autoreceptors that occurs with long-term treatment, which then increases the amount of serotonin available in the synapse [122-123]. Asnis and coworkers provide an excellent review of the open-label and placebo designed SSRIs studies [124]. While many significant improvements in all three clusters of symptoms occurred during these trials, significant and even incapacitating residual symptoms remained in over 50% of patients. Disadvantages of SSRI's in the

treatment of PTSD include limited remission rates, lack of response to treatment, and inability to tolerate medications [125-128]. In addition, military veterans who are at high risk to combat-related trauma often do not improve with sertraline treatment. Two studies involving military veterans treated with sertraline showed no improvement or no statistical difference in the Clinician-Administered PTSD Scale when compared to placebo [129-130] suggesting that SSRI's are not efficacious in all patient populations. The difference in baseline severity and chronicity of symptoms compared to civilians is one possible explanation for these results.

The community one lives in can also assist in treatment of individuals and groups following a traumatic event creating a psychosocial-intervention continuum. Community-based psychological trauma interventions allow members of the community (ie religious leaders, political and school officials. coaches), who may also be affected by the traumatic event, to play a central role in the resolution and adaptation of the traumatic losses [131].

Psychotherapy and pharmacotherapy offer effective treatment options for individuals suffering from PTSD. However, these options often produce only significant clinical responses not full remission and relapses can often occur. In addition, certain patient populations are refractory to current treatments. These gaps highlight the need to develop more effective treatment therapies targeting PTSD symptomatology as well as preventing the onset of this debilitating disease.

Gender Differences.

In the general population, women are about twice as likely as men to suffer from PTSD [132]. Multiple factors including type of trauma exposure, definition of the trauma, and biological response appear to be involved in this gender difference. Many studies attribute the increase of PTSD in women to the type of trauma suffered which is a higher frequency of sexual (child and adult) and domestic physical abuse [133-134] which is the most common cause of PTSD among women [44]. Evidence also supports women experiencing a sensitizing effect to a nonassaultive trauma if it was preceeded by an assaultive trauma (ie rape) thereby increasing the risk for PTSD [53, 135].

Criteria used to define the traumatic event are another possible reason PTSD is diagnosed more frequently in women than men. The definition of the event went from features of the event itself, in DSM-III and DSM-III-R, to what the cognitive and emotional responses are to the event, in DSM-IV-TR. Criterion A1, which defines the person's direct involvement and criterion A2, which accounts for the personal response to the event, compose the event definition in DSM-IV-TR [43]. Tolin and Foa suggest that the cognitive and emotional responses women experience when exposed to traumatic events make a diagnosis of PTSD more likely. Men do not experience the same emotional responses even though men may experience more traumas [133].

In addition, there is growing evidence in both human and animal studies that genetic variation in the serotonin transporter gene (5-HTTLPR) might influence anxiety and depressive behaviors when exposed to traumatic events [136-138]. A study by Barr and colleagues on rhesus macaques showed that 5-HTTLPR polymorphism (short allele versus long allele) in females not males with a history of adversity had an associated

increase in adrenocorticotropin hormone (ACTH) responses to stress. In addition, peer-reared females also exhibited lower cortisol responses to stress [139] a problem also noted in PTSD. Many factors appear to be involved in the gender differences associated with PTSD and its complexity requires further study.

Estrogen and Estrogen Production

Estrogens, a class of steroids best known for their role in reproductive behaviors, has established actions in the brain for modulating anxiety, mood, cognition, pain, homeostasis and neuroprotection [140-141]. 17β–estradiol (E2) is the most abundant and potent endogenously produced estrogen and is the focus of this paper [142]. Estrogens with a lower binding affinity to the estrogen receptors, to include estriol and estrone, are mostly formed in the liver from estradiol. Estrogens are lipid based steroids derived from cholesterol. In short, the production of E2 begins with the cleavage of cholesterol's side chain by cytochrome P450 enzymes located in the inner membrane of the mitochondria. Following a series of hydroxylations, production ends with the aromatization of testosterone by p450 aromatase monooxygenase enzyme complexes in the smooth endoplasmic reticulum (Figure 5) [143-144].

In premenopausal women, E2 is mainly produced in the ovaries and is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis will be described in more detail later. E2 released from the ovaries circulates in the blood stream to target tissues either as free molecules or protein bound. Free molecules of E2 (1.5%) are considered biologically active. The two states of bound E2 include loose binding to albumin (37%), which can dissociate for tissue uptake, and tight binding to sex-hormone

binding globulin (61%), which is thought to be active only in the liver [145-146]. Extragonadal biosynthesis of estrogens includes the liver, mesenchymal cells of the adipose tissue, skin, and bone, cells of the aortic smooth muscle, and various regions of the brain. Because the ovaries shut off in postmenopausal women, they must rely on extragonadal estrogen production. The principal site for this biosynthesis appears to be adipose tissue [147]. Evan Simpson conceptualized that the adrenal glands produce androgens (ie testosterone) and their precursors [dehydroepiandrosterone (DHEA) and (DHEA sulfate)], which then circulate to specific extragonadal sites for conversion by aromatization to estrogens [148]. Estrogens produced in this fashion act locally in a paracrine or intracrine manner; in other words acting within the tissues or cells that produced it [149]. Interestingly, a study using ovariectomized rats suggests E2 production increases over time as aromatase protein and mRNA expressions in subcutaneous abdominal adipose tissue and blood (from leakage into the circulation) increase [150].

Men also require estrogen for a variety of processes. Testes produce some estrogen but the majority comes from extragonadal aromatization of circulating androgen [151]. The critical importance of estrogen action in males stems from aromatase knockout mice models and human aromatase deficient case reports which will be discussed shortly. The distinction between males and postmenopausal women is the availability of greater volumes and uninterrupted supplies of precursor steroids (testosterone). This means that local production of E2 is always supported thereby offering protection against estrogen deficient diseases [151]. However advancement in age does cause decreases in both sexes.

The Role of Estrogen in Stress and Anxiety

Estrogen plays an important role in behavioral and hormonal responses to stress. Many have shown that estrogen works synergistically with corticosterone to alter behavior [152-156]. In intact animals, stress increases estrogen levels [157-160], suggesting that increased estrogen is a compensatory response to stress. Estrogen also modulates activity of neurotransmitters implicated in PTSD (i.e., norepinephrine; NE) as well as in depression (i.e., 5HT, NE, dopamine; DA) [157-162].

Important to the proposed work, estrogen alleviates anxiety and depression in humans [37, 163-164]. Yet studies in mice and rats have shown mixed results, with some studies indicating that estrogen increases anxiety and others showing that estrogen decreases anxiety [165-166]. These inconsistencies may arise due to differences in methodology including the form of estrogen used and the behavioral task employed. However, manipulation of estradiol alters the behaviors to be studied in the proposed work, including the acoustic startle reflex and open-field activity[157-162, 167-168]. Taken together, human and animal data provide a clear conceptual framework for further testing the efficacy of estrogen and estrogen like compounds to prevent PTSD. Furthermore, since estrogen is heavily involved in 5HT system modulation, administration of estrogen or estrogen-like compounds might be a method of protecting individuals likely to be exposed to trauma and/or treating individuals previously exposed to trauma**.**

Risks and Benefits of Estrogen Administration.

Estrogen replacement therapy has a long history, offering many benefits but there have also been some associated risks. Benefits of estrogen replacement include

prevention of osteoporosis and reduction in risk and onset of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [169]. The importance of preventing osteoporosis is highlighted by the fact that mortality and morbidity rates from hip fractures alone are as high as 16% within the first 3 months after injury [170]. While the risks of estrogen replacement therapy are still being evaluated, it was discovered during the 2002 Women's Health Initiative (WHI), that a trial of combined estrogen and progesterone in postmenopausal women with a uterus showed and increased risk in coronary heart disease, stroke, pulmonary emboli, and breast cancer as compared to the placebo group. This was the case even though estrogen is known to improve cholesterol levels [171].

The development of selective estrogen receptor modulators (SERMs) such as Tamoxifin and Raloxifene provide the benefit of selectively activating or inhibiting different target tissues. Although SERMs are non-hormonal they are able to act like estrogen in some parts of the body for instance, increasing bone density while at the same time blocking estrogen effects on the breast. SERMs are not completely risk free. Work continues to develop SERMs that exhibit beneficial effects without harmful side-effects.

Hypothalamic-Pituitary-Gonadal Axis

Regulation of the major source of sex steroid production requires a functional endocrine axis composed of the hypothalamus, pituitary and gonads (Figure 6). In females the ovaries are the major gonadal source of E2 production. While development of the ovary is not part of this study it is important to know that significant E2 release

from the ovaries begins when they are first stimulated by gonadotropins at the onset of puberty [172].

A functional HPG axis is under the control of feedback loops, both positive and negative. It is hypothesized that prior to puberty, the HPG axis is mainly under the control of the negative feedback mechanism causing the hypothalamus to be very sensitive to very low levels of E2. This response is attenuated during late puberty and the positive feedback loop is activated. Normal menstruation begins with episodic secretion of gonadotrophic releasing hormone (GnRH) from the hypothalamus which acts on the anterior pituitary to release lutenizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation. LH and FSH are released in a pulsatile manner to stimulate the theca and granulosa cells of the ovaries to produce progesterone and estrogens, respectively. Released into the blood stream, E2 in unbound and bound form travel to and act on target tissues. Excess E2 cycles back to the level of the hypothalamus and pituitary thereby decreasing the release of GnRH [173-174]. The relationship between the ovaries and HPG axis now depends on the delicate balance between the two feedback loops.

Estrogens play significant roles in reproduction and cognitive processes in both sexes, even though serum levels of estrogen are higher in females than males. As already mentioned E2 is also generated in a number of extragonadal sites. In these areas estrogen acts locally in a paracrine and intracrine fashion and by doing so it does not significantly affect circulating levels of estrogen [175-176]. Indeed, estrogen is synthesized endogenously from androgens via the aromatase enzyme. Functional aromatase is necessary for conversion of androgens to estrogens.

Males with aromatase deficiency have a mutation located within exons V and IX in the CYP19 gene [177]. The Jones et al. study establishes many common phenotypical features shared by aromatase deficient men. Features include undetectable estrogen levels with high levels of testosterone and gonadotrophins, tall stature with associated delayed bone maturation and osteoporosis, hyperinsulinemia, impaired lipid metabolism and impaired reproductive function [177]. Moreover, the development of aromatase deficient mice has allowed for further characterization of the roles or aromatase and subsequent estrogen production in males. Some associated phenotypical characteristics associated with these mice include impaired spatial reference memory, significant decrease in structures of the medial amygdala, and increased aggression toward estrus females [177]. One study utilizing the aromatase knockout mouse linked the anxiety disorder Obsessive-Compulsive Disorder, with estrogen deficiency [175]. Once these mice were treated with 17β estradiol over a three week time frame excessive anxious activities were significantly reduced. From these studies and others it is apparent that estrogen plays an important role in the overall health and normal mating and cognitive behaviors in males.

Molecular Mechanisms of Estrogen Action

Estrogen exerts most of its biological effects via estrogen receptors (ER), members of the nuclear hormone receptor superfamily. These receptors are ligand-dependent transcription factors that increase gene transcription through direct binding of the receptor to specific DNA target sequences called estrogen response elements (EREs).

Onset of action is delayed requiring hours or days for effects to occur. This is known as a classical or genomic pathway.

Unbound intranuclear or cytoplasmic ER monomers have attached inhibitory complex composed of receptor-associated proteins most notably heat shock ptoteins (Hsp) 90 [178]. Easily crossing the plasma membrane, the lipophilic E2 binds to the ER releasing the receptor from its inhibitory complex. Once bound to ligand ERs dimerize forming either a homodimer or heterodimer. The receptor-ligand complex diffuses into the cell nucleus to bind to the specific ERE of the target gene stimulating transcription [144, 179]. Co-regulatory Proteins are essential to either enhance or repress estrogen action following this association [180-183]. For example, recent studies show that the ER in cerebral cortex explant may form a multimeric complex in the cytoplasm with hsp90, *src*, and β-Raf which can activate the MAP kinase signaling cascade [184]. The balance of receptors with coactivators, and corepressors is critical for specific responses in different tissues.

Estrogen Receptor Subtypes

Recognition of estrogen binding proteins that regulate gene expression culminated in the early 1960's with the discovery of the "classical" estrogen receptor [185]. Now referred to as ERα, this receptor was considered for over 30 years to be the only one if its kind and indispensable for maintenance of life [186]. However in the 1990's several important discoveries occurred permanently changing this view. This started with the case presentation of a male lacking functional ERs (α) [187]followed by the development of

the ERα knockout mouse [188] and then culminating with the first cDNA clone of second estrogen receptor (ERβ) [186].

The ER protein structure consists of two activating domains. They are the AF-1 region at the N-terminus and the AF-2 region in the ligand-binding region [189]. In addition, there is the DNA binding domain that is involved in receptor binding and dimerization. The ER subtypes share 97% homology in their DNA binding domain but only 56% homology in the ligand binding domain [161]. It is now known that two proteins serve as receptors for 17β-estradiol, the estrogen receptors α and β.

It is now well accepted that estrogens regulate many physiological processes and their targets include reproductive tissues, the cardiovascular system, bone and the most of the brain [140, 190-191]. The different tissue distributions of the two ER subtypes and their different responses suggest that each subtype will have different functions [152, 154-155].

Additional Estrogen Site of Action May be the Plasma Membrane.

The classical view of estrogen action requires E2 binding to a nuclear receptor, which, in turn binds to specific estrogen response elements within the DNA. This mechanism does not explain the diverse actions of estrogen in the central nervous system [192] in which E2 can regulate genes that do not contain any of the estrogen regulatory elements [193-194]. Furthermore, E2 has a rapid effect on cellular function (seconds to minutes) that cannot be explained with a transcriptional mechanism [195-197]. Recent work in the hippocampus indicates that E2 rapidly increases kainate receptor-induced currents by altering the G protein-coupled, cAMP-dependent phosphorylation of potassium channels [198-199]. On a similar time course and at similar concentrations, E2 can

hyperpolarize GnRH neurons in hypothalamic explants of ovariectomized guinea pigs [200]. Many of the effects of E2 in the hippocampus and other neural tissue can be replicated with E2 conjugated to hydrophilic molecules such as albumin (E2-BSA) [198-199, 201-207]. Because E2-BSA is not likely to enter the cell, these studies suggest that one alternative site of E2 action may occur at the plasma membrane [208]. Collectively, the studies underscore the complexities of estrogen regulation and suggest that its effect is not strictly restricted to reproductive function.

Estrogen Enhancement of SSRIs.

In humans, the efficacy of fluoxetine, a SSRI, is significantly enhanced when co-administered with estrogen [209-211]. There is solid neurobiological evidence that enhanced SSRI efficacy in humans in the presence of estrogen is directly related to estrogen's modulation of brain serotonin systems. For example, estrogen facilitates the down-regulation of the serotonin receptor, 5-HT1A [212-214]. Estrogen may also be involved in altering the pattern of serotonin transporter binding sites to enhance the effects of paroxetine, an SSRI [215]. Estrogen may potentiate increases in the density of 5-hydroxytryptamine-2a (5-HT2a) binding sites in the brain [216]. Hence, estrogen may have both pre-synaptic and post-synaptic activities to enhance the effectiveness of SSRIs. Further investigation into estrogen's role in the brain and interaction with mood and emotion is necessary for continued advances in this field.

Animal Models of Stress.

Animal models of stress are all characterized by exposure to unpredictable or uncontrollable stress. These models include exposure to tail shock, foot shock, forced cold water swimming, fear conditioning, and immobilization. Exposure to uncontrollable or unpredictable stress produces symptoms in rats similar to those exhibited by individuals with PTSD. Signs and symptoms include neuroendocrine, physiological and behavioral responses. Table 1 compares the human condition of PTSD to results of rodent studies utilizing acute immobilization stress and chronic immobilization stress. Behavioral symptoms include, exaggerated startle responses, anxiety, irritability or aggression, social avoidance or inhibition, and memory dysfunction. These symptoms also include behaviors associated with a depressive state.

Our preliminary data indicate that animals exposed to repeated immobilization stress exhibit exaggerated startle responses and depressive behaviors (in females), indicating that our model is reliable and exhibits good convergent validity with other models of stress-induced behavioral changes [22, 217]. This immobilization procedure produces reliable elevations in hormones associated with a stress response, including adrenocorticotropin (ACTH) and corticosterone (CORT). Immobilization models are believed to simulate the human perception of lack of control that is associated with greater PTSD symptom severity following traumatic experience [18]. Although immobilization stress appears to be a minor stress for rats, it has been found to induce fear conditioning [218] and potentiate anxiety [21, 219]. This animal model will include hormone replacement (VEH, E2, Propylpyrazoletriol (PPT), an ERα agonist and Diarylpropionitrile (DPN), an ERβ agonist in gonadectomized animals prior to

behavioral testing. This is a well established paradigm that has been used in over a thousand behavioral neuroendocrine outcome studies. Examples include molecular effects of stress response [220], anxiolytic properties of estrogen [41, 221] and neuroprotective effects of estrogen [222].

The present research begins by evaluating a potentially useful chronic stress rat model to mimic PTSD symptomatology in addition to examining the effects of estrogen receptor agonists as a preventative treatment of such symptoms. The research addressed four specific aims: (1) to determine the neuroendocrine (plasma glucocorticoid levels), physiological (body weight, locomotor activity) and behavioral (ASR, EPM, Barnes maze) outcomes induced by repeated immobilization stress (2) to determine the efficacy of estradiol to prevent PTSD behavioral symptoms in an animal model (3) to determine the extent to which chronic stress and estrogen receptor agonists affect acute thermal pain tolerance (4) to determine the effect chronic stress has on hormone regulation on feeding and body weight.

Tables and Figures

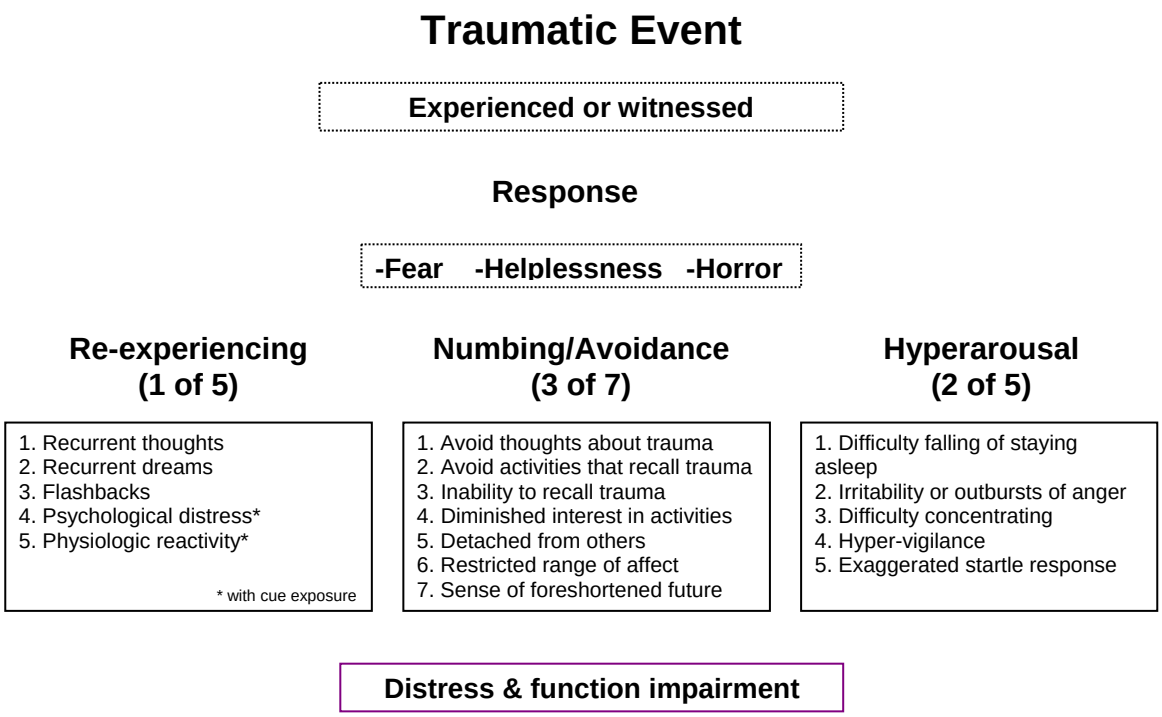

Figure 1. Diagnostic criteria for Post-Traumatic Stress Disorder as defined in DSM-IV [43].

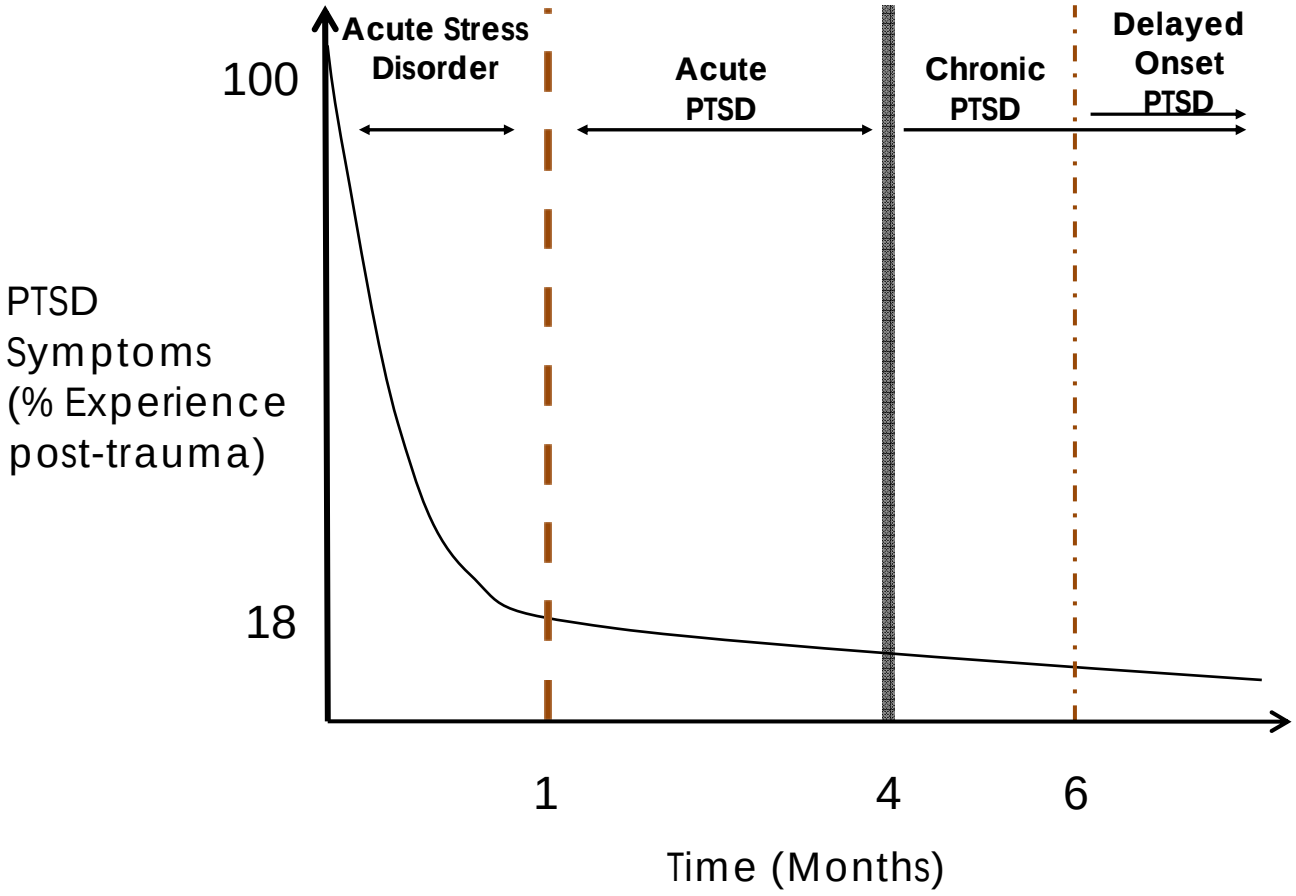

Figure 2. Schematic representation: percent of patients with symptoms in relation to acute stress disorder (ASD) and post-traumatic stress disorder (PTSD). Symptoms from the three diagnostic criteria (re-experiences, numbing and avoidance, and hyperarousal) within 1 month of the traumatic event are diagnosed with acute stress disorder (ASD) if no improvement of symptoms classified as acute PTSD (1 to 4 months), if symptoms persist > 3 months after acute PTSD is diagnosed then classified as chronic PTSD. Delayed PTSD is diagnosed when symptoms first appear >6 months after the traumatic event [223].

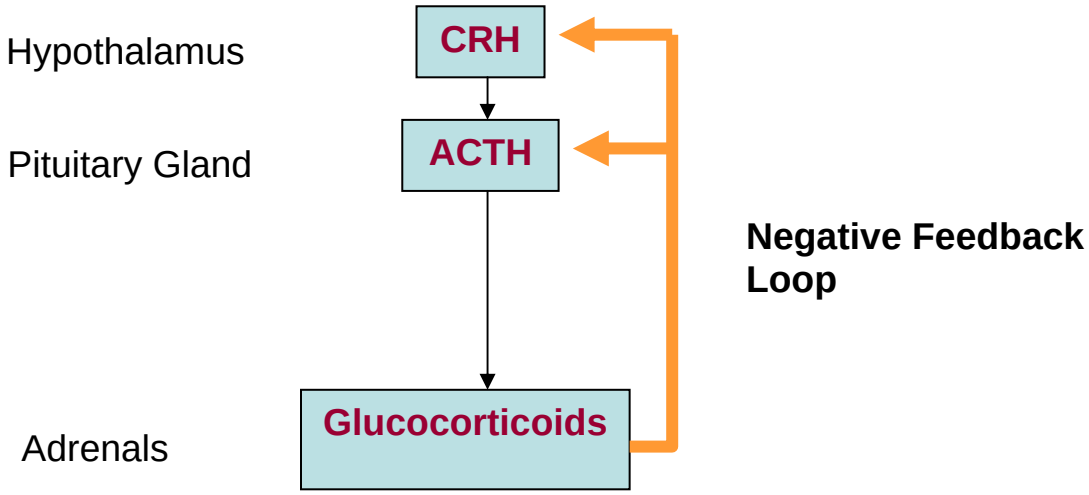

Figure 3. HPA axis and hormonal cascade of the stress response. The hypothalamus produces and secretes corticotropin releasing hormone (CRF) into the blood to stimulate the pituitary gland to produce and secrete adrenocorticotropin hormone (ACTH) which enters the general circulation and triggers adrenal production of glucorticoids. The proper regulation of the HPA axis depends on glucocorticoid negative feedback regulation on CRH secretion from the hypothalamus.
HPA= hypothalamic-pituitary-adrenocortical axis
CRH=Corticotropin releasing hormone
ACTH= adrenocorticotropin hormone

Types of Allostatic Load

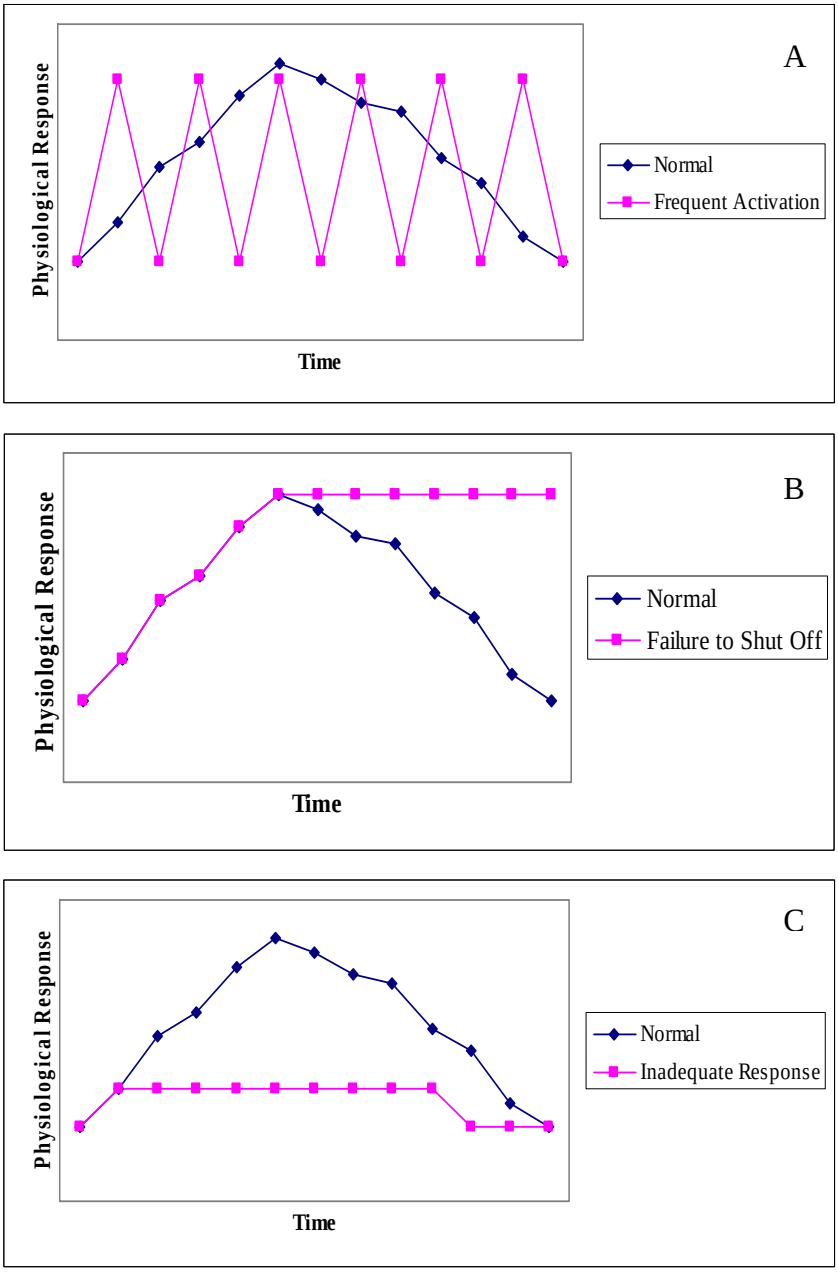

Figure 4. Types of allostatic load in regards to mediators of the stress response. (A) Frequent activation of mediators (B) Failure of mediators to shut off (C) Inadequate mediator response [6]

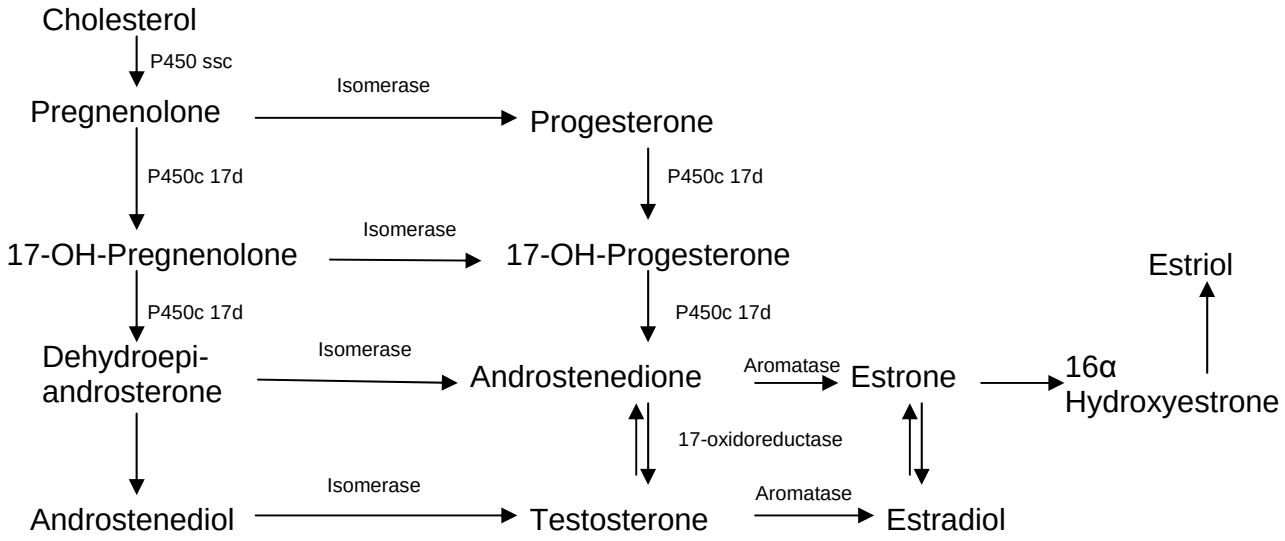

Figure 5. Steroid Metabolism Pathways

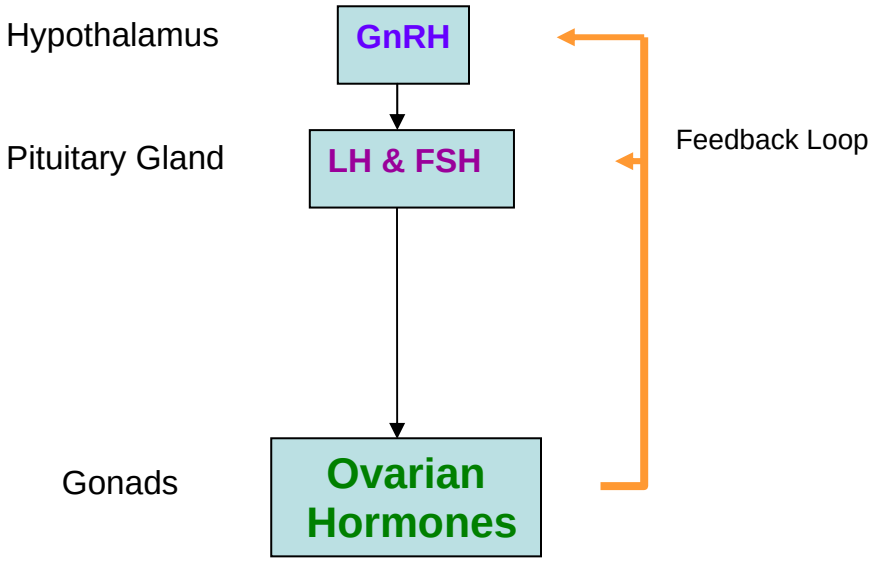

Figure 6. HPG axis. The hypothalamus produces and secretes Gonadotropin-releasing hormone (GnRH) into the blood to stimulate the pituitary gland to produce and secrete two hormones, leutenizing hormone (LH) and follicle stimulating hormone (FSH) which enter the general circulation and trigger ovarian production of hormones estrogen and progesterone. HPG = hypothalamic-pituitary-gonadal axis

	Human PTSD	Rodent Immobilization (Acute)	Rodent Immobilization (Chronic)
Body Weight	Weight loss [224-226]	Decreased weight gain and decreased feeding[227]	Decreased weight gain and decreased feeding [228-229]
Heart Rate	Elevated basal heart rate [230-231]	Elevated heart rate & mean arterial pressure post stressor [232]	Unknown
Glucocorticoid	Decreased/no change in cortisol levels [109, 233]	Elevations in corticosterone levels [41]	Unknown
Norepinephrine	Elevations of urine and CNS norepinephrine levels [233-235]	Elevated microdiasylate norepinephrine levels in the medial amygdala[236]	Elevated plasma norepinephrine [237]
Hippocampus	Decrease in size of Hippocampus [83-85, 238]	No change in apical dendrites of the CA3 pyramidal neurons [239]	Atrophy of apical dendrites of the CA3 pyramidal neurons [20-21]
Amygdala	Increased activation of the amygdala after symptom provocation [238]	Reduced dendritic arborization posterodorsal medial region [240]	Enhanced dendritic arborization in basolateral region[21]
Anxiety	Elevated anxiety levels [241]	Elevated anxiety levels [242]	Elevated anxiety levels [242]
Memory	Logical memory deficits [112, 243] Enhanced declarative memory [244]	Impaired spatial memory [245-246]	Unknown
Startle	Exaggerated Startle Response [32, 247]	Exaggerated Startle Response [22]	Exaggerated Startle Response (males only)[22]
Pain	Increased pain thresholds to thermal pain [248]	Tail flick hypoanalgesic [249] Thermal hot plate hypoanalgesic [250-253]	TMJ/Tail Flick-hyperalgesia [242, 249] Thermal Unknown

Table 1: Comparison of PTSD Symptoms to Acute and Chronic immobilization stress induced dysfunction in rodents

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Title: Effects of Stress and Estradiol in Ovariectomized Sprague-Dawley Rats

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Sources of Financial Support:
Department of Defense (Tri-Service Nursing Program Grant #600-085-00000-00-104622 and USAMRMC 04182001)

Disclaimer: The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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Key Words: Anxiety, estradiol, elevated plus maze, restraint stress, and estrogen receptor

Abstract

Chronic immobilization stress is known to disrupt the hypothalamic-pituitary-adrenal (HPA) axis while 17β-estradiol (E2) has been shown to produce anxiolytic effects. In this study, we sought to examine the effects of chronic immobilization stress and E2 on the neuroendocrine axis. Ovariectomized (OVX) Sprague-Dawley rats were assigned to one of 8 treatment groups based on a 2x4 design that comprise of stress (1 h immobilization a day for up to 22 days vs no stress) x hormone treatment (comprising of vehicle (VEH), E2, propylpyrazoletriol (PPT; a selective ERα agonist) or diarylpropionitrile (DPN; a selective ERβ agonist). Stress treatment was divided into 3 periods: acute stress (1-10 days of immobilization stress), mid-period stress (11-16 days of immobilization stress) and late period stress (greater than 16 days of stress). During the stress phase, rats were tested for their startle response, activity, and anxiety using behavioral tasks Acoustic Startle Reflex (ASR), Locomotor, and Elevated Plus Maze (EPM) respectively. Acute immobilization stress in rats increased corticosterone (CORT) levels while chronic immobilization stress significantly reduced CORT levels relative to nonstressed rats Stressed rats administered with VEH had exaggerated startle responses in all stress phases while treatment with E2, PPT, and DPN attenuated this effect. Prepulse inhibition (PPI) was also enhanced with E2 and PPT in the late stress phase. Interestingly, only E2 treatment consistently reduced horizontal locomotor activity in nonstressed and stressed rats. Acute and chronic stressed rats were more anxious than nonstressed rats although E2 and PPT treatment exerted anxiolytic effects in all rat conditions. Our results suggest chronic immobilization stress alters the negative feedback regulation of glucocorticoid secretion. Furthermore, the anxiolytic effect of E2 is likely to be mediated by ERα and its

effects on locomotor activity seem to be regulated independent of the classical nuclear receptors.

Introduction

Stress activates the hypothalamic-pituitary-adrenocortical (HPA) axis, which evokes the release of glucocorticoids and autonomic regulators to maintain homeostasis [1]. HPA activation protects by allowing the body to return to homeostasis via a glucocorticoid mediated negative feedback mechanism. However, this negative feedback can be maladaptive with sustained activation of HPA axis leading to a diseased-state [2]. Allostatic load, the cumulative negative effects the body suffers for being forced to adapt to various challenges and adverse environments, occurs when mediators of the stress response overwhelm the system through hyperactivity, lack of adaptation to repeated stress, or inadequacy during the initial response period [3 4 5].

Multiple studies have demonstrated that in contrast to the effects of an acute stressor, certain patient populations who have experienced chronic or severe trauma, have lower basal glucocorticoids compared to corresponding non-patient populations [6 7-8]. Such patient populations include those suffering from post-traumatic stress disorder (PTSD) [9 10-11 12]. Furthermore, patients with PTSD subjected to the dexamethasone suppression test demonstrate significantly enhanced suppression of plasma glucocorticoid levels when compared to those withour PTSD [13 14 15]. These findings suggest that PTSD patients have altered pituitary and adrenal sensitivity to glucocorticoids [10]. Several researchers have proposed that diminished glucocorticoid levels in response to DEX treatment may serve as a neuroendocrine marker of PTSD [16 17].

Exposure to unpredictable or uncontrollable stress is characteristic techniques used in the development of animal models for PTSD. The wide rage of paradigms include exposure to tail shock [18 19], foot shock [20 21], under-water trauma [22], predator

exposure [23] and immobilization [24]. These current models vary dramatically in duration, combination, and intensity. Immobilization models of stress are believed to simulate the human perception of lack of control that is associated with greater PTSD symptom severity following traumatic experience. Although restraint stress appears to be a minor stress for rats, it has been found to induce fear conditioning [25] and potentiate anxiety [26-27]. In addition, restraint stress causes structural remodeling in areas of the brain responsible for emotional memories and regulation of the stress response (amygdala, hippocampus and prefrontal cortex) [28 29]. This model also reliably produces elevations in the stress hormones, adrenocorticotropin (ACTH) and corticosterone (CORT)[30-31]. While the results of acute mild immobilization stress (less than 10 days) are well-characterized, the chronic effect (greater than 14 days) of mild immobilization stress has not been determined.

Estradiol-17β (E2), the most abundant and potent estrogen, has both anxiolytic and antidepressant effects[32-34]. Working synergistically with corticosterone to alter behavior, E2 is an important steroidal hormone in behavioral and hormonal responses to stress [35 36]. It is known that two proteins serve as receptors for E2, the estrogen receptors (ER) α and β [37]. In intact animals, stress increases estrogen levels[38-41] suggesting that increased E2 is a compensatory response to stress. E2 also modulates activity of neurotransmitters implicated in PTSD (i.e., norepinephrine; NE) as well as in depression (i.e., 5HT, NE, dopamine; DA) [42-43]. Studies in mice and rats show that E2 decreases anxiety [44-45]. Taken together, human and animal data provide a rationale for examining the possibly interactions between E2 and stress.

Based on the literature, the chronic repeated daily immobilization stress paradigm may be a suitable animal model to study the underlying mechanism of PTSD pathology. In this study, we examined the effects of long-term repeated daily immobilization stress on neuroendocrine and behavioral markers in the ovariectomized rat to determine whether this mode of stress recapitulates CORT effects and behavioral modifications observed in humans with PTSD. We also wanted to characterize the effects of treatment with selective estrogen receptor modulators (SERMs) to determine the role of the estrogen receptors (ER), ERα and ERβ, on the HPA axis in our chronic stress paradigm.

Material and Methods

Animals

Adult female Sprague-Dawley rats (200-225 g) were purchased (Harlan, Indianapolis, IN). Upon arrival, the rats were randomly housed in pairs in standard polycarbonate shoebox cages (42 x 20.5 x 20 cm) with hardwood chip bedding (Pine-Dry; need to cite company that this is purchased from) and changed 2-3 times a week. The housing room was maintained at 21-24° C at 50% humidity. Rats were maintained on a 12-h reversed light/dark cycle with *ad libitum* access to food and water.

Daily body weights were recorded over the entire length of the study. The rats were assigned to treatment groups based on a design counterbalanced for body weight after 7-9 days of acclimation to the new housing facility and to human contact. Upon completion of the experimental phase, the rats were euthanized by carbon dioxide overdose with rapid decapitation. Brains were quickly removed from each animal and stored at -80° C for further analysis. All procedures conducted were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Health Research Extension Act, 1985).

Chemicals

Estradiol 17-β (E2), purchased from Sigma Chemical Co (St Louis, MO), binds with nearly equal affinity to both estrogen receptors [46]. In order to study the respective biological roles of ERα and ERβ, specific estrogen receptor agonists, PPT and DPN, were purchased from Tocris Cookson, Inc (Ellisville, MO). PPT binds to ERα with high

affinity, 410-fold grater relative binding affinity than to ERβ [47]. DPN, while it acts as an agonist at both ER subtypes, it has a 70-fold higher relative binding affinity to ERβ than to ERα [48]. Hydroxypropyl-β-cyclodextrin served as vehicle (Sigma Chemical Co., St. Louis, MO)

Experimental Design

Two experiments were conducted here. The first experiment served as the preliminary experiment to determine the effect of restraint stress on peripheral blood CORT levels after 7, 14 and 21 days of daily 60-min restraint. Thirty-six ovariectomized rats were assigned to one of 2 treatment groups (daily restraint stress or no stress) counter-balanced for body weight (n=6 per treatment group per time point). At the end of each time points, randomly assigned rats within each treatment groups were euthanized and trunk blood collected for hormone analysis by radioimmunoassay (see below).

In the second experiment, the rats were assigned to one of 8 treatment groups. The experiment was conducted in a full 2 x 4 factorial design comparing the effect(s) of stress (no stress x daily 60-min immobilization stress) and hormone treatment (vehicle (VEH) x E2 x Propylpyrazoletriol (PPT), an ERα agonist x Diarylpropionitrile (DPN), an ERβ agonist),. Briefly, after the acclimation period, the rats were ovariectomized and implanted with hormonal treatments delivered by an Alzet osmotic pump (see below). Daily immobilization stress was initiated 3 days after the surgery and lasted for 22 days (see below for details). Within the study design, the animals were randomly assigned to the treatment groups subsequent to the ovariectomy and onset of the hormonal treatment (n = 12 per treatment group). Stress periods and behavioral testing started one hour after

the start of the dark phase and stressed animals were tested within 20 minutes after removal from restraints.

Immobilization Stress

During the experimental phase, animals (beginning post-OVX/implant day 4) assigned to stress groups underwent immobilization using finger-like restraining devices (Centrap Cages, Fisher Scientific) for 60 min/day. The Centrap cage and the restraining 'fingers' were tightened until the rats are immobilized (unable to turn/barrel-roll) but not compressed, pinched, or in pain. This restraint procedure reliably produces elevations in hormones associated with an acute stress response including adrenocorticotropin (ACTH) and corticosterone (CORT)[49 30] (Shaham, 1992 #165}. Control animals were transported along with the stress groups but were not placed in the restraining devices. Behavioral testing occurred within 15 minutes of removal from the restrainers on stated dates.

Ovariectomy and Alzet osmotic pump implant

After acclimations, all rats underwent bilateral ovariectomy and Alzet mini-osmotic pump implantation under isoflurane anesthesia. Each rat was implanted with two 14-day Alzet mini-osmotic pumps (Model 2002 0.5ML, Durect Co., Cupertino, CA, USA) in order to deliver the vehicle or steroids for the duration of the experiment. Each pump was subcutaneously implanted between the shoulder blades. Pumps delivered VEH, 0.25 mg/kg body weight (bw)/d of E2, 1 mg/kg bw/d of PPT (ERα agonist) or 1 mg/kg bw/d of DPN. (ER β agonists). Mini-osmotic pumps release their contents at a slow continuous infusion rate of 0.5 µl/h. This dose represents physiological levels of circulating estradiol between the proestrous period [50]. The dose for ER agonists, PPT and DPN, represent previously established doses for these agonists [38].

Tissue Collection

All rats were euthanized by carbon dioxide overdose with rapid decapitation 1 hour after the end of the final immobilization. Trunk blood was collected into ice-cold tubes containing 20 µL 5 M EDTA, mixed, and centrifuged at 4000 x g for 20 minutes. Plasma was then collected and stored at -80 C until further use. Brains were rapidly removed from each animal over ice, snapped frozen in dry-ice and subsequently stored at -80 C until further analysis.

Behavioral Measures

Acoustic Startle Reflex (ASR). ASR responses have been used extensively to understand the neurobiological basis of PTSD characterized by exaggerated or hyperreactive startle responses [51-52]. ASR amplitudes was measured in an Acoustic Response Test System (MEDASR-310; Med Associates, Georgia, VT) consisting of weight-sensitive platforms inside individual sound-attenuated chambers with speakers, an audio generator, a high-speed serial microcontroller, and a high-speed analog to digital converter. Responses were measured periodically throughout all experiments. Procedure followed previously studied protocol [53]. In short, each rat was placed in a ventilated holding cage then the cage was placed on a weight-sensitive platform. A fan built into the chamber provided background noise of 48 dB. Following placement of animals in the chambers, a 3-minute adaptation period occurred in which no stimuli was presented. Startle stimuli consisted of 110 and 120 dB noise bursts of 20 msec duration interleaved with prepulse non-startling stimuli of 68 and 82 dB. Each of the three trial types was presented eight times, for a total of 24 trials, in random order to prevent habituation. The intertrial interval was random, ranging from 30 to 60 sec. All testing was conducted within the first 4 hours of

the dark cycle. Animals were immediately returned to their home cages at end of testing period. Higher values during ASR indicated an exaggerated response whereas lower PPI values reflect deficits in sensorimotor gating.

ASR startle results were expressed as percent change from baseline ± SEM [(peakvalue ASR-baseline ASR)/basline ASR]. PPI values were computed using the formula [{peakvalue ASR- prepulse ASR)/peakvalue ASR].

Elevated Plus Maze (EPM). The EPM is a widely used test to examine effects of drugs and other manipulations on anxiety {Benwell, 1994 #5;Brioni, 1993 #13;Bhattacharya, 1995 #170}. Rats were placed individually on the central platform facing a closed arm, and allowed to explore the maze for 5 minutes. Animal activities were recorded and analyzed using a video-tracking system (ANYMAZE, Stoelting, Wood Dale, IL). Behaviors scored included: percent time spent in the open arms [(time spent in open arms/total time) ×100], percent time spent in the closed arms [(time spent in closed arms/total time) ×100], and percentage of open arms entries (open arm entries/total arm entries) × 100. An entry into an arm was defined as the animal having two paws on the arm and an exit was defined as two paws leaving the arm. Time spent in the open arms index anxiety because this behavior repeatedly correlates with anxiety. Testing occurred once per experimental group (post-drug day 12-mid period & 19-late period) and animals were tested only once. The maze, floor and walls, were cleaned with 35% ethanol after each animal test period was complete.

Locomotor Activity. Locomotor responses refer to an animal's activity level when placed in a non-home cage arena. Locomotor activity was measured using an Omnitech Electronics Digiscan infrared photocell system [Test box model RXYZCM (16 TAO);

Omnitech Electronics, Columbus, OH] located in a dedicated room. Animals were placed singly in a 40 X 40 X 30 cm clear Plexiglas arena with ventilated lid. A photocell array measured activity using 16 pairs of infrared photocells located every 2.5 cm from side-to-side and 16 pairs of infrared photocells located front-to-back in a plane 2 cm above the floor of the arena. Data was automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. The apparatus monitors animal activity continuously with data recorded as cumulative activity every 5 minutes for a total testing period of 60 minutes. All testing was conducted within the first 4 hours of the dark cycle. The Plexiglas arenas were cleaned with 35% ethanol after each animal test period was complete.

Radioimmunoassay

Plasma levels of corticosterone were measured as described previously [54]. Samples were diluted 1:25 in 0.01M PBS and corticosterone binding proteins were heat denatured at 65°C for 1 h. The assay was conducted in 13 x 100 mm glass tubes (Fisher Scientific). Standard curves were constructed from dilutions of corticosterone (4-pregnen-11β, 21-diol-3, 20-dione; 5–500 ng/ml; Steraloids) dissolved in 95% ethanol. Rabbit anti-corticosterone serum (ICN Biomedicals, Costa Mesa, CA) was used at a final dilution of 1:2000, according to manufacturer's protocol. H^3-Corticosterone was purchased from Amersham (Waltham, MA). The limit of detection for the assay was 5.4 ng/ml and the intraassay coefficient of variation was 5.26%.

Data Analysis

Data analysis was based on approaches commonly used in the literature. An analysis of variance (ANOVAs) was performed to detect a difference in CORT over time

in the respective non-stress and stress groups. A t-test was performed to compare the stress and non-stress effects on CORT levels at 7, 14 and 21 days of stress. Results from the behavioral analyses are expressed as mean values ± SEM. Multiple two-way analysis of variance (ANOVAs), with random block design, were used to determine stress (stress and non-stress) and treatment (E2, PPT, DPN) effects on ASR,EPM and locomotor behavioral tasks. Two-way analysis of variance (ANOVAs), with random block design, was also performed to determine stress (stress and non-stress) and treatment (E2, PPT, DPN) effects on corticosterone levels. After and overall significant treatment effect was determined, Fisher's least Significant difference post-hoc test was conducted to determine differences among groups. Prior to statistical analyses, the behavioral results were also subject to Hartley's Test of Homogeneity. When appropriate, the data was transformed. Significance was set at $p<0.05$.

Results

Effects of chronic restraint stress and hormone treatment on CORT levels

Plasma CORT levels were different between rats that were stressed for varying periods of time (Figure 1). During the early period (7 days of stress), CORT levels were elevated in the stressed group (683.905± 70.531ng/ml) compared to the non-stressed group (410.067±68.472ng/ml, $p<0.05$). After 14 days of stress, plasma CORT levels in stressed rats (388.413± 133.978ng/ml) were not different from non-stressed rats (450.717±132.994ng/ml). Interestingly, in the late period (22 days), plasma CORT levels trended lower in stress rats (357.638± 43.054ng/ml) compared to non-stressed rats (515.738±61.472ng/ml, $p<0.10$).

A main effect of chronic stress and hormone treatment was next examined ($F(1, 83) = 15.336$, $p<0.05$) (Figure 2). There was no hormone treatment effect in non-stressed rats with E2, PPT, or DPN compared to rats treated with VEH. However, stressed rats treated with DPN, the selective ERβ agonist, showed greater CORT compared to stressed rats treated with VEH (362.458± 57.021ng/ml vs 226.953± 30.230ng/ml, respectively, $p<0.05$). As with Figure 1, chronically stressed VEH-treated rats had lower CORT compared to non-stressed animals VEH-treated rats ($p<0.05$).

Effects of chronic restraint stress and hormone treatment on startle response

In order to determine the effect of chronic stress on the response to the acoustic startle, we examined the startle response in the non-stressed and stressed rats 3 times during 3 stress periods – early period (day 4), mid-period (day 11) and late stress period (day 21) (Figure 3). Following acoustic startle, the total of the startle amplitude was greater in stressed rats compared to non-stressed rats (early and late periods, $p<0.05$).

Our data indicated that both non-stressed and stressed rats treated with E2 and PPT consistently had lower startle amplitude compared to VEH-treated animals ($p<0.05$) (Figure 4). Those treated with DPN had lower startle amplitude when they were acutely stressed ($p<0.05$). There was no effect of treatment on pre-pulse inhibition until the late period in the stressed animals (Figure 5). Chronically stressed rats (late period) that were treated with VEH exhibited lower pre-pulse inhibition compared to other VEH-treated stressed rats (early and mid period) and non-stressed rats (late period) ($p<0.05$). Late period stressed rats treated with E2 and PPT showed a trend of greater pre-pulse inhibition when compared to their respective VEH-treated group ($p<0.10$).

Effects of chronic restraint stress and hormone treatment on locomotor activity

A comparison of horizontal activity in nonstressed and stressed rats showed that there was no difference in activity until the late stress period (days15-22). Stressed animals in this time period had decreased activity when compared to non-stressed animals ($p<0.05$) (Figure 6).

To determine the possible interaction between stress and hormone treatment, we compared these effects on the total horizontal activity in non-stressed (Figure 7a) and stressed rats (Figure 7b). All groups treated with E2 regardless of time point and stress status were lower ($p<0.05$) when compared to control animals. Two-way ANOVA showed significant main effects of treatment in all time periods and of stress compared with controls (stressed: early period $F(3,88)=10.364$, $p<0.05$; mid period $F(3,88)=9.261$, $p<0.05$; late period $F(3,88)=7.567$, $p<0.05$: controls: mid period $F(1,88)=7.307$, $p<0.05$; late period $F(1,88)=7.964$, $p<0.05$).

Effects of chronic restraint stress and hormone treatment on non-locomotor activity

Treatment with E2 also decreased stereotypic count, a non-locomotor activity associated with non-purposeful behavior, in both non-stressed and stressed groups at all timepoints tested (Figure 5). Two-way ANOVA showed significant main effects of treatment in all time periods and of stress compared with controls (stressed: early period $F(3,88)=17.983$, $p<0.05$; mid period $F(3,88)=13.170$, $p<0.05$; late period $F(3,88)=18.696$, $p<0.05$; controls: mid period $F(1,88)=5.770$, $p<0.05$; late period $F(1,88)=6.451$, $p<0.05$).

Effects of chronic restraint stress and hormone treatment on anxiety.

To test for relative levels of anxiety, rats were tested after 19 days of immobilization stress. Open arm entry ratio showed that stressed animals exposed to 1hr daily restraint stress for 19 days (late period) entered the open arms less than non-stressed animals (39.7%±2.9% vs47.1%±2.8%, respectively, $p<0.05$).(data not shown). Analysis of the ratio of time in the open arm by two-way ANOVA showed a significant main effect of hormone treatment ($F(3,80)=7.2$, $p<0.05$) and stress ($F1,80)=4.2$, $p<0.05$) (Figure 9). Both non-stressed and stressed animals treated with E2 spent more time ($P<0.05$) in the open arm compared to the VEH-treated animals. Likewise, stressed animals treated with PPT also spent more time ($p<0.05$) in the open arms whereas there is a trend ($p<0.10$) for PPT treated non-stressed animals to spend more time in the open arms.

Discussion

In the present study, we have examined an animal model of chronic immobilization for the development if markers that resemble human PTSD. We chose immobilization stress for our study since it is a mild stressor and has been shown to effectively increase circulating stress hormones such as ACTH and CORT after acute stress [55]. Our data indicate that mild but chronic daily immobilization stress (1 h/d) attenuated peripheral glucocorticoid levels in OVX rats subjected to 1 hour immobilization stress for 22 days when compared to control animals. To our knowledge, this is the first report correlating this developmental HPA response with the period of immobilization stress in the rodent**.** The result of the present study supports clinical observations that sustained stress leading to PTSD symptomology is correlated with low glucocorticoid levels in patients [56]. For example, basal or stimulated cortisol levels are attenuated in combat veterans [6 57] and rape victims [58] who are diagnosed with PTSD. These results suggest sustained activation of the HPA axis alters the system's response to negative feedback inhibition. Together, these findings indicate that chronic immobilization may lead to significant alterations in the HPA axis in a manner similar to that of human PTSD patients.

Our data also show that acutely stressed rats showed differences in glucocorticoid regulation compared to chronically stressed animals. In contrast, glucocorticoid levels of rats subjected to the same immobilization stress for only 7 days were significantly increased. This early stress effect on glucocorticoid levels is consistent with other studies examining the effects of acute stress [55 29, 59]. Interestingly, rats stressed for 14-days had glucocorticoid levels that were not different from the non-stressed rats suggesting a

developmental transition from HPA responsiveness during the early stress period to the mid-stress period into the later stress period. The underlying mechanism for this phenomenon is unclear; it is possible that glucocorticoid receptor expression is increased in the PVN or pituitary, thereby enhancing glucocorticoid negative feedback. Moreover, it is possible that chronic immobilization stress desensitizes the adrenals to the effects of ACTH where further secretion of CORT is inhibited. Apart from the actions of stress hormones, it is likely other modulators of the stress axis are altered leading to diminished CORT. One study examining stress responses in serotonin transporter null mice reported these mice had consistently reduced CORT levels whether they were exposed to stress or not (Jiang 2008).

Our results show that the rats chronically stressed in the immobilization model demonstrated both exaggerated startle response and an elevated anxiety level during the late stress period when glucocorticoid levels are attenuated. This is similar to the key clinical features of PTSD of an exaggerated startle response and elevated anxiety levels [60]. In this study, stressed rats demonstrated exaggerated startle at all time points (early, mid and late stress periods). This is important as the animals were not habituated to the immobilization. A number of studies have shown that acute stress causes structural remodeling in the amygdala, an area important for the regulation of anxiety and affective responses [28], [29, 61]. The amygdala contains CRH containing neurons which express glucocorticoid receptors [62]. It is thought that direct effects of glucocorticoids on amygdala neurons can increase their intrinsic excitability and decreasing the impact of $GABA_A$ inhibition (an inhibitory neurotransmitter) [63] Furthermore neurons of the amygdala show a growth response when exposed to repeated stress as evidence by

dendritic growth in rats exposed to chronic restraint stress [26]. Growth and increase in excitability of the amygdala may enhance the retrieval of traumatic events, induce fear conditioning, and induce the release of noradrenalin thereby increasing the startle response[64-65] Interestingly, the enhanced startle response in chronically stressed rats was maintained even when CORT levels diminished in the late stress phase. We speculate chronic immobilization stress is not only altering negative feedback inhibition of the HPA axis but is sensitizing components of the CNS that regulate the startle response such as the central nucleus of the amygdala. This region is receptive to dopaminergic input, which has been shown in rats to facilitate CRH-induced increase in startle response (Meloni 2006). However, since we did not assess CRH levels or dopamine input in the amygdala, it is still not clear how these factors are altered in our chronic stress paradigm.

Certain psychiatric disorders, such as schizophrenia and PTSD, have been linked with disrupted PPI responses (Braff et al., Arch Gen Psychiatry 49:206-215, 1992; [66]; Adamcio et al, Behavioral Brain Research 204:246-249, 2009). PPI is a form of reflex modification by providing a weak prestimulus prior to the startling stimulus and is thought to be a measure of sensorimotor gating and pre-attentive information processing (Braff and Light, Psychopharmacology 174:75-84, 2004). The results here show that chronic immobilization stress does influence PPI, even though hormone treatment (estradiol and the selective ERα agonist) alleviate the chronic stress effects.

The EPM is a behavioral test examining the conflict rodents are confronted with between the drive to explore a new environment and the fear of bright, open, elevated spaces [67-69]. This test is frequently used in rats showcasing anxiety like behavior which is at the core of PTSD represented in numbing and avoidance activity [70]. The results here

showed that 1 h daily immobilization stress after 19 days was sufficient enough to change animal emotionality. Our results suggest that stressed rats were more anxious when compared to the non-stressed rats which are in agreement with other studies that have used repeat immobilization stress showing increased anxiety levels[25 26]. In addition, our finding further supports the clinical literature for our animal model. It should be noted that in the present study, EPM was only conducted once in each rat as it has been previously shown that learning may occur [71].

Locomotor activity reflects the general activity level of an animal. Our results showed reduced activity in stressed animals treated with VEH only during the late period (d15-22). Reduced activity levels following a stressor can indicate a depressive state [72-73]. A frequent comorbid disorder associated with PTSD [74] in depression which often is associated with a decrease in physical activity. In addition, a study specifically addressing physical activity in patients with PTSD found a significant reduction in physical activity following the onset of the disease [75].

The results of the present study suggest that there is a hormone treatment effect. In animal models, estrogen has been shown to exert effects on behaviors that are symptomatic of PTSD, such as anxiety, startle reflex and cognitive behavior [35, 76-79]). This is likely due to its ability to influence or regulate major pathways that are involved in these behaviors. These include the serotonergic [35, 80-84], dopaminergic [85-87] and cholinerestase [88-89] pathways. Interestingly, E2 decreased locomotor and non-locomotor (stereotypy) activity across all timepoints similar to results shown by Palmero-Neto and collogue. While not fully understood it is postulated that E2 may have an inhibiting effect on dopamine receptors which are centrally located in motor function regions

(mesostriatal and mesolimbic systems) of the brain [90]. In the present study, E2 effects in conditions of no stress is consistent with the literature [91-94]. Further, under conditions of chronic stress, E2 improved startle reflex, PPI, anxiety and latency to target in the Barnes maze. These are all conditions that would suggest E2 may be an important pharmacological therapy for these symptoms under conditions of stress. It would appear that the underlying mechanism of action remains to be elucidated as the interaction between stress and the ERs remain unclear.

The results of the present study show a developmental HPA response to chronic daily immobilization stress. This appears to support the clinical literature for PTSD in that glucocorticoid levels are attenuated under conditions of stress [6-7, 12]. This is in contrast to the proper regulation of the HPA axis in which adrenal hormones may lead to a negative feedback of CRF release from the hypothalamus as well as the downregulation of central glucocorticoid receptors [95]. The behavioral data from our studies largely support the clinical observations though some refinement may be needed to further characterize this model. The results of these studies have identified significant transitions during chronic stress that suggest a developmental change within the neural sites that lead to alterations in the HPA axis. The results further suggest that targets of E2 may be suitable for improving PTSD symptoms. The precise mechanisms, particularly the interaction of E2 pathways and chronic stress, remain to be examined. Thus in future studies it would be interesting to assess the effects of chronic immobilization stress on the serotonergic system to modulate glucocorticoid-mediated negative feedback inhibition.

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Figures

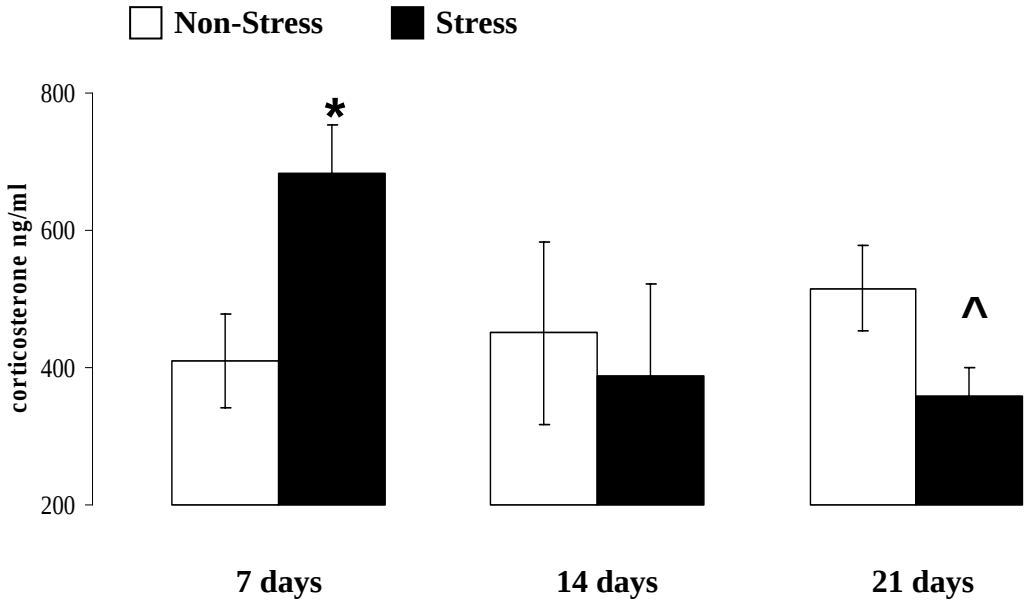

Figure 1. Effect of 7-, 14- and 22-days of immobilization stress (1 h/d) on plasma CORT levels (mean±SEM ng/ml) in OVX Sprague-Dawley rats. *p<0.05 vs VEH non-stress, ^p<0.10 vs VEH non-stress.

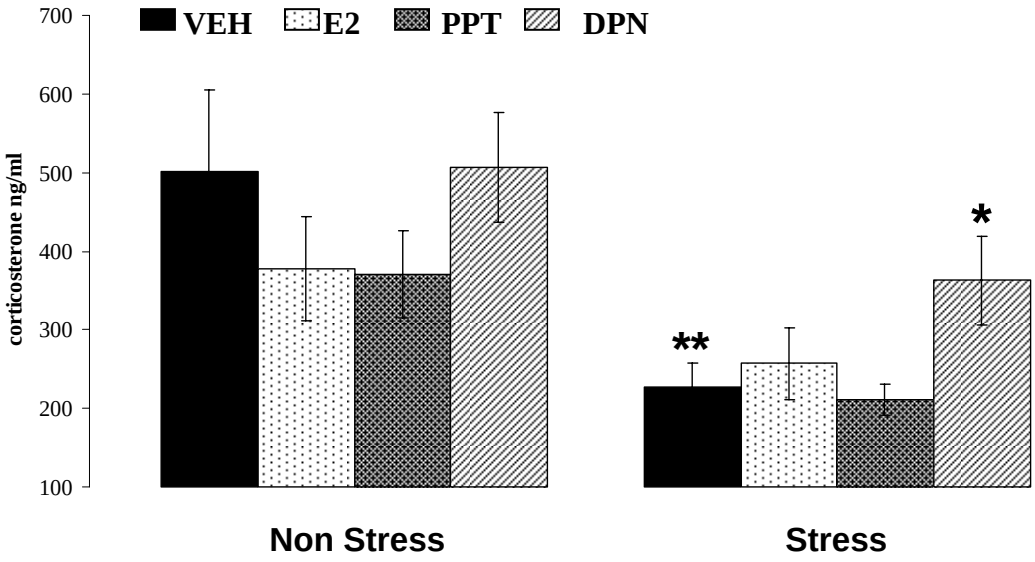

Figure 2. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in OVX Sprague-Dawley rats that were also immobilization stressed for 22-days (1 h/d) on plasma CORT levels (mean±SEM ng/ml). *p<0.05 vs respective VEH group, ** p<0.05 VEH Stressed vs VEH Non-stressed group

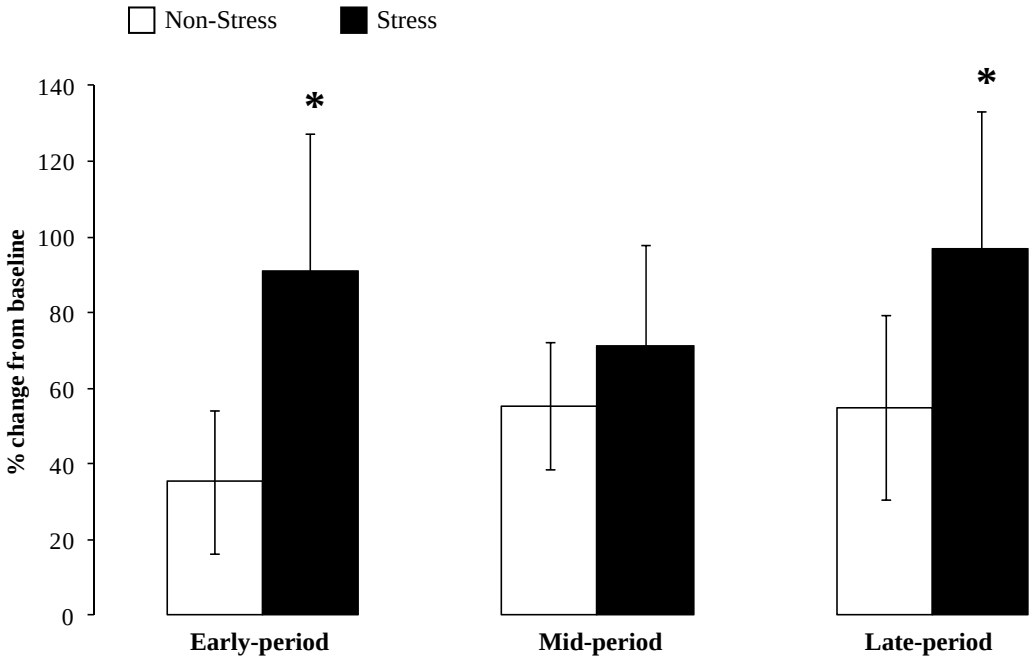

Figure 3. Effect of early stress period (d. 4), mid-stress period (d.11) and late stress period (17- 22-days) (immobilization stress at 1 h/d) on total startle response (mean±SEM relative units) in OVX Sprague-Dawley rats. This represents total acoustic startle response to 110- and 120-db during a 1-h test period in non-stressed and stressed OVX female Sprague-Dawley rats treated with VEH. *$p<0.05$ vs respective non-stressed group.

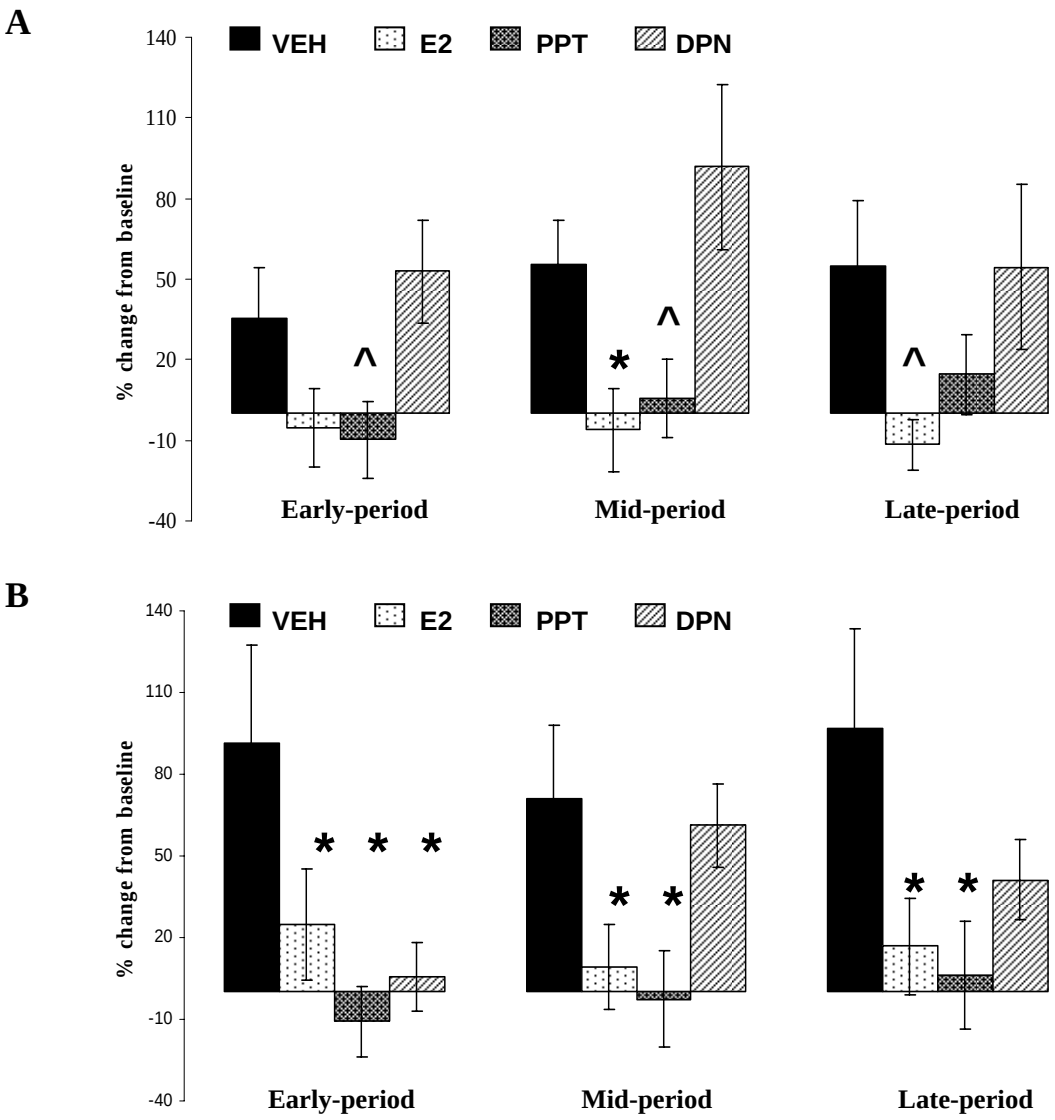

Figure 4. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in OVX Sprague-Dawley rats that were also immobilization stressed for 22-days (1 h/d) on startle response (mean±SEM ng/ml). *p<0.05 vs respective VEH group, ^ p<0.10 vs VEH

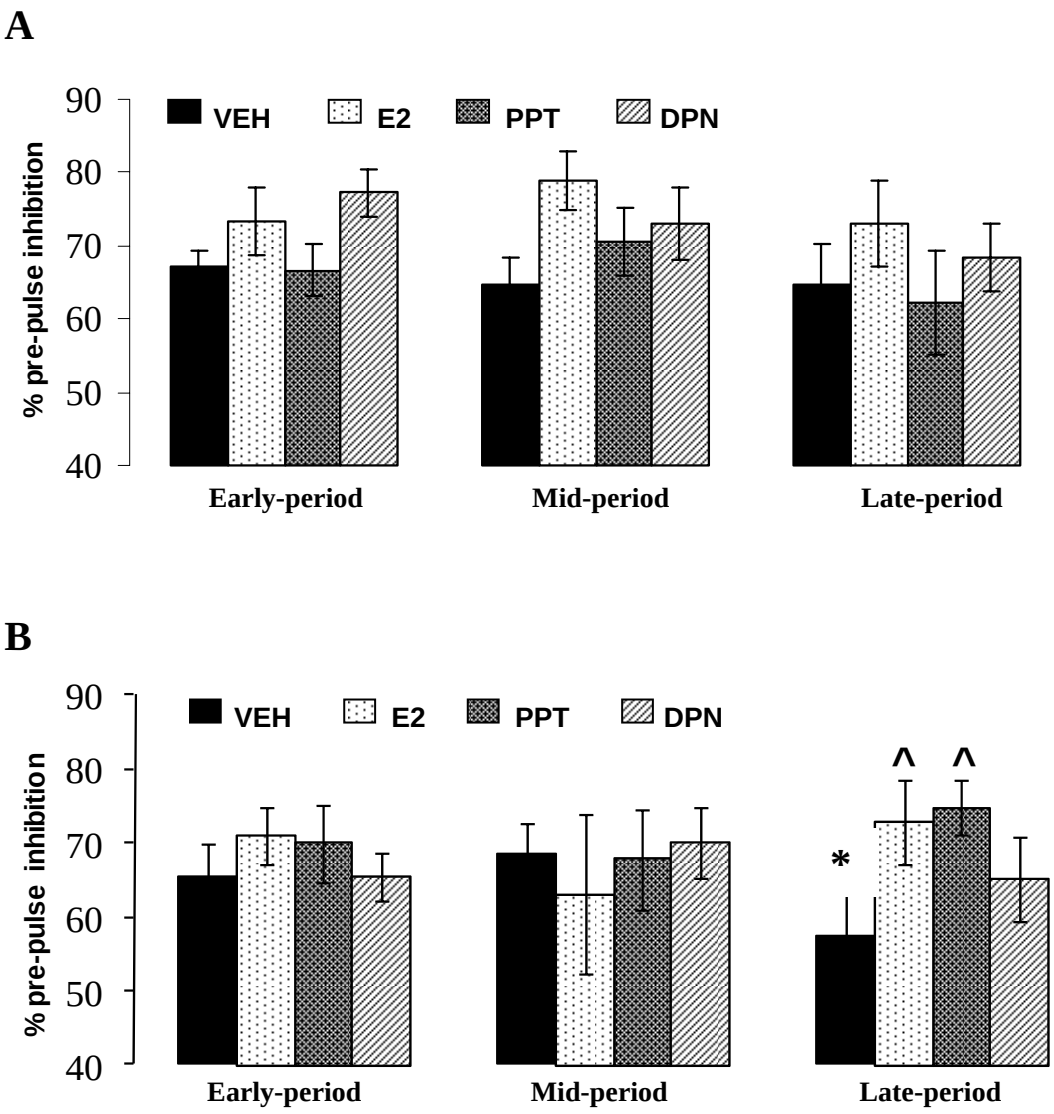

Figure 5. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in OVX Sprague-Dawley rats that were also immobilization stressed for 22-days (1 h/d) on pre-pulse inhibition (mean±SEM ng/ml). (A) non-stressed (B) stressed. * $p<0.05$ vs non-stressed VEH rat in late period, and vs VEH group of stressed rats in early and mid period, ^ $p<0.10$ vs VEH.

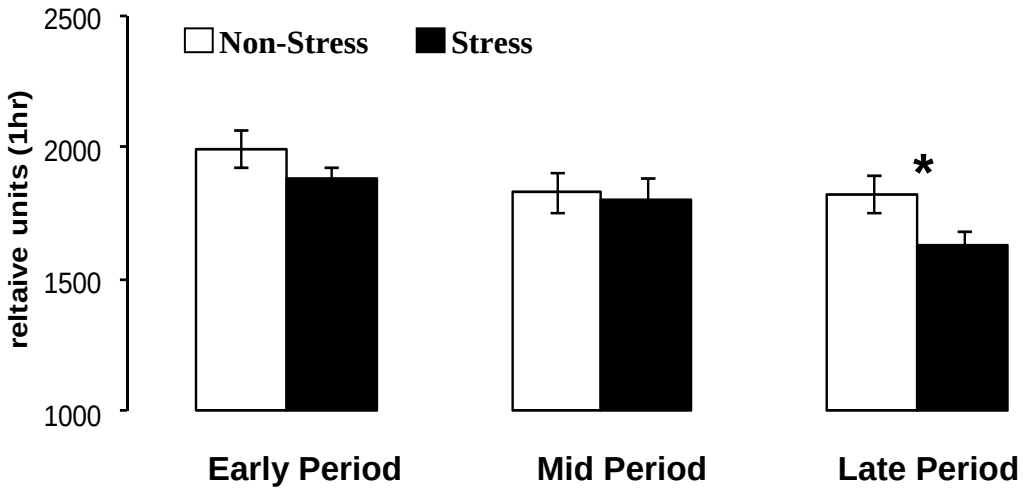

Figure 6. Effect of acute (4-7 days), mid-length (10-14 days) and chronic (17- 22-days) immobilization stress (1 h/d) on horizontal locomotor activity (mean±SEM relative units) in OVX Sprague-Dawley rats. Horizontal activity represented by beambreaks averaged over a 1 hour period of non-stressed and stressed OVX female Sprague-Dawley rats treated with VEH during early (1-7 days), mid (8-14 days), and late (15-22 days) periods of the experimental phase. *$p<0.05$ comparing non-stressed vs stressed groups.

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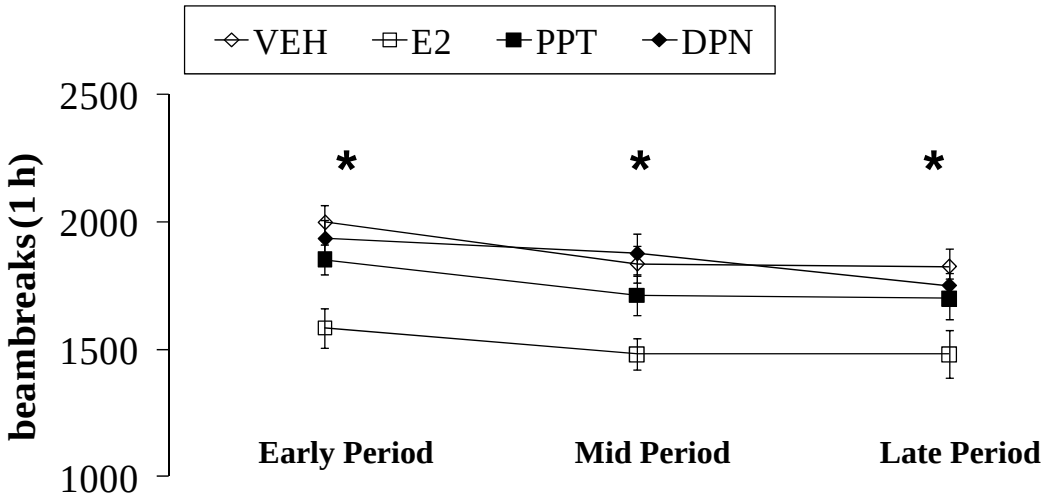

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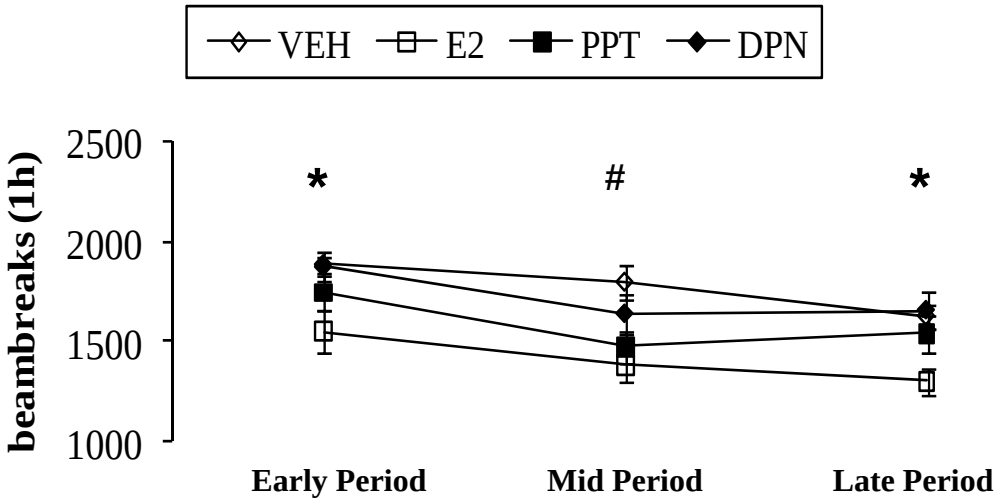
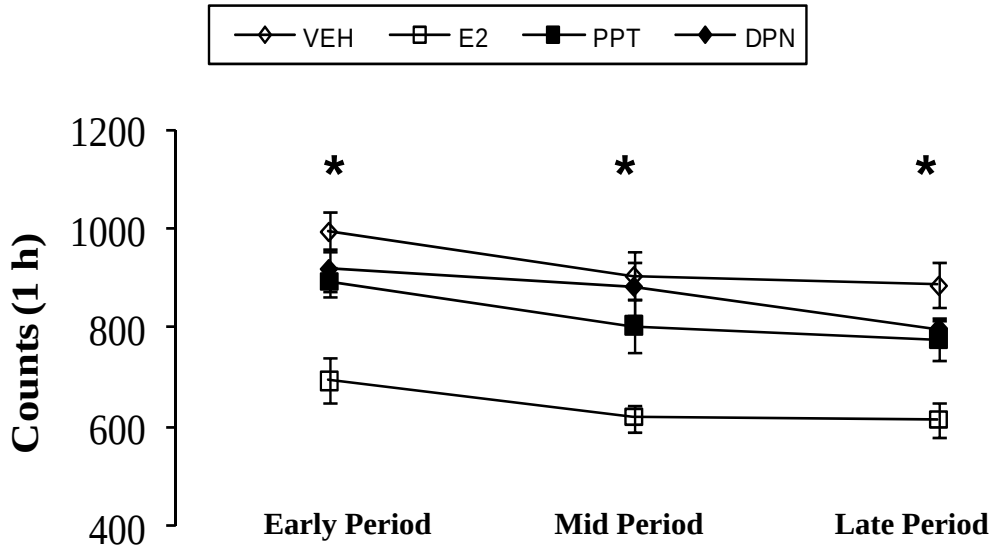


Figure 7. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in OVX Sprague-Dawley rats that were also immobilization stressed for 7 days (Early Period), 14 days (Mid Period) and 22-days (Late Period) for 1 h/d on horizontal activity (beam breaks mean±SEM). (A) nonstressed rats and (B) stressed rats. *$p<0.05$ E2 vs VEH; #$p<0.05$ PPT vs VEH.

A



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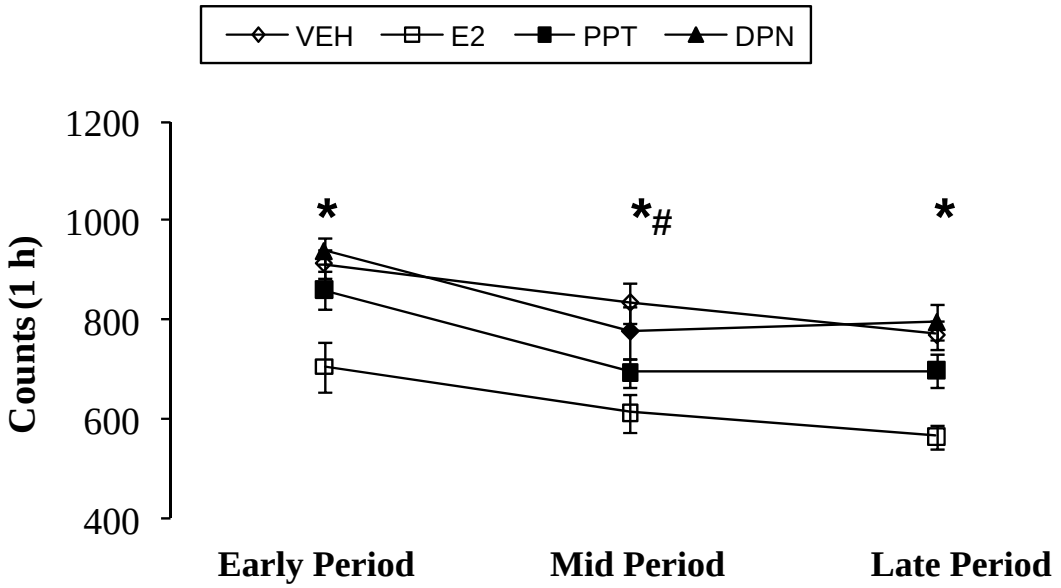

Figure 8. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in OVX Sprague-Dawley rats that were also immobilization stressed for 7 days (Early Period), 14 days (Mid Period) and 22-days (Late Period) for 1 h/d on stereotypy (mean±SEM). (A) nonstressed rats and (B) stressed rats. * $p<0.05$ E2 vs VEH; # $p<0.05$ PPT vs VEH.

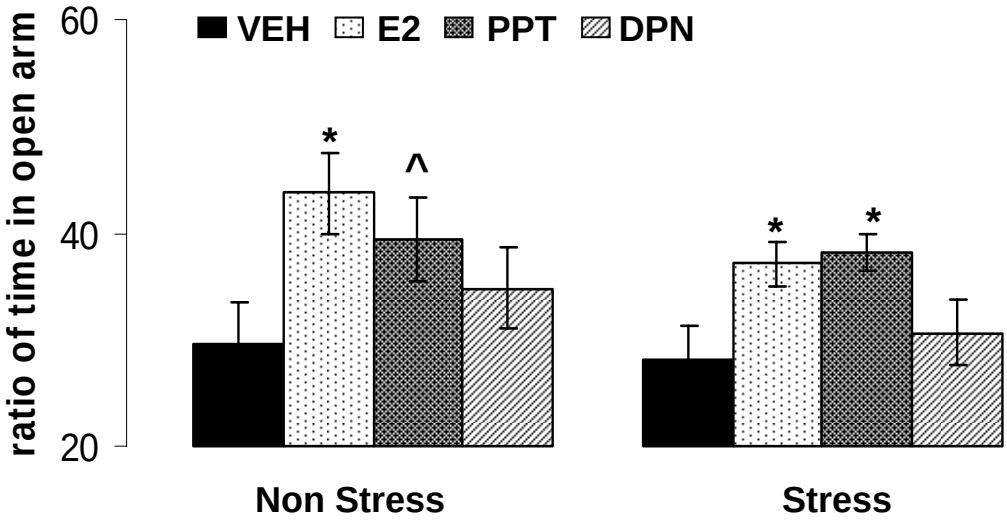

Figure 9. Effect of estradiol (0.25 mg/kg bw), selective ERα agonist (PPT; 1 mg/kg bw) and selective ERβ agonist (DPN; 1 mg/kg bw) treatment in rats that were also immobilization stressed for 22-days (1 h/d) on open arm time in the elevated plus maze (mean±SEM ng/ml) in OVX Sprague-Dawley rats. *$p<0.05$ vs respective VEH group, ^$p<0.10$ vs VEH.

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Title: Effects of Immobilization Stress and Hormonal Treatment on Nociception

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Sources of Financial Support:
Department of Defense (Tri-Service Nursing Program (TSNRP Grant #600-085-00000-00-104622; and USAMRMC 04182001)
John F. Garde Grant, American Association of Nurse Anesthetists Foundation

Disclaimer: The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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ABSTRACT

The purpose of this study was to evaluate the effects of stress and estradiol on pain tolerance. Ovariectomized rats were assigned to treatment groups based on a 2x4 factorial design comprising stress (nonstress x stress) and hormone treatment (vehicle x estradiol (E2; 0.25mg/kg/d) x ERα selective agonist, Propylpyrazoletriol (PPT; 1mg/kg/d) x ERβ selective agonist, Diarylpropionitrile (DPN; 1mg/kg/d)). Stressed animals underwent daily 60-min immobilization for 22 days. Pain tolerance was assessed with the hotplate test, an acute thermal pain test. In this study, stressed rats showed increased ($p<0.05$) pain tolerance compared to nonstressed rats (25.0+/-1.92 s vs 20.4+/-1.02 s, respectively). Increased ($p<0.05$) pain threshold was observed in rats treated with estradiol and the ERα agonist in both nonstressed and stressed rats when compared to vehicle-treated rats. Interestingly, the ERβ agonist only increased ($p<0.10$) pain thresholds in stressed rats. Stressed rats exhibited greater ($p<0.05$) β-endorphin levels compared with non-stressed rats in all hormone-treatment groups. There was no hormone effect on β-endorphin levels except for stressed rats treated with the ERβ agonist. These studies suggest that the E2's effect on pain thresholds may be mediated via the ERα, while the interaction between chronic stress and ERβ may also enhance pain threshold.

Key Words: pain, stress, hot plate test, restraint stress, estrogen receptor agonists

INTRODUCTION

Pain is a complex subjective phenomenon that the body uses to signal tissue injury and danger. It can involve both sensory and emotional components that can be influenced by physical and psychological factors [1,2]. Pain is classified by time course (acute to chronic) and severity (mild to complete agony) [3]. As one of the most common reasons to seek medical attention [4], pain can lead to clinical and psychological changes that increase an individual's morbidity and mortality if not treated properly [5]. Furthermore, delays and inadequate relief of acute pain can lead to changes in the central nervous system (CNS) contributing to the development of chronic and neuropathic pain [6].

The body's response to any actual or potential threat is regulated by the hypothalamic-pituitary-adrenal (HPA) axis through hormonal feedback. The HPA axis involves the release of corticotrophin-releasing hormone from the hypothalamus, which regulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary, that then regulates the secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal glands. The glucocorticoids, in turn, provide negative feedback to regulate central pathways [7,8]. In the context of pain, glucocorticoids have been shown to reduce pain responses and increase pain threshold through the activation of glucocorticoid receptors [9-10] and through the secretion of β-endorphin from the pituitary [11].

In addition, steroid hormones can have an important role in behavioral and hormonal responses to stress and in regulation of pain. Increased pain perception has been correlated with periods of low estradiol (E2) during the menstrual cycle [12]. Abundant evidence now exists from both human and animal studies showing that E2

treatment may elicit anti-nociceptive effects [12-13]. Interestingly, E2 works synergistically with corticosterone to alter behavior including mood, cognition and pain [13-15].

Behavioral, neuroendocrine and autonomic components are involved in maintenance of homeostasis [8]. Behavioral tests and serum assays of neuroendocrine markers thus represent reliable methods to quantify stress and pain in rat models [16]. The hotplate test, an acute thermal pain test, is a method to study responses to a noxious stimulus without causing nerve injury. A supraspinal thermal pain test, the hotplate test, requires integration at the brain stem level of the CNS. Pain and temperature systems involve specific regions of the CNS including axons of the dorsal root ganglion, dorsal horn, spinal cord, and thalamic nuclei located in the diencephalon just superior to the hypothalamus. First-order neurons from the free nerve endings of the periphery synapse on second-order neurons located in the substantial gelatinosa of the spinal cord. These second-order neurons traverse the midline of the spinal cord and ascend to nuclei located in the thalamus. A number of centers located in the thalamus, brainstem, and cortex are responsible for the emotional component of pain. There are also descending pathways from higher centers that network with ascending pathways to modulate the transmission of pain signals to the cortex [17]. Nociceptive afferent pathways that operate and relay signals through the limbic area of the brain are of primary importance in communicating the aversive emotional affect of pain; particularly, the locus coeruleus, HPA axis, and the dorsal/ventral noradrenergic bundles [18]. Studies show the transmission of pain is modulated by β-endorphin, a neurohormone [19-21].

The purpose of this study was to determine whether E2, under chronic stress conditions (immobilization for 1 h/day for 22 days), modulate responsiveness to an acute

painful stimulus. To that end, we hypothesize that chronic immobilization stress and E2 would alter pain tolerance which in turn is mediated by β-endorphin.

MATERIALS AND METHODS

Subjects.

Ninety-six female Sprague-Dawley rats (200-225 g) were purchased from an approved vendor (Harlan, Indianapolis, IN). Upon arrival, rats were randomly housed in pairs in standard shoebox cages (42 x 20.5 x 20 cm) with hardwood chip bedding (Sani-Chip, Laboratory Grade, Harlan Teklad, Madison, WI) and changed 2-3 times a week. Rats were housed on a 12-h reversed light/dark cycle (lights out at 1200 h) with *ad libitum* access to food and water. The animal facility was maintained at consistent temperatures (21-24° C) and humidity (40-70%). All rats were acclimated to the facility for 9 days prior to experimentation. The rats were handled daily during the acclimation period and throughout the entire experiment to become familiar with the investigators. Daily body weights, as well as food and water consumption, were recorded over the entire length of the study. Rats were weighed individually, while food and water consumption was recorded for each pair. All procedures conducted were approved by the Institutional Animal Care and Use Committee and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Experimental Design.

The experiment was based on a 2 x 4 factorial design comparing the effect(s) of stress (nonstress x daily 60-min immobilization stress) and hormone treatment (VEH, E2, Propylpyrazoletriol (PPT), an ERα agonist and Diarylpropionitrile (DPN), an ERβ agonist). Rats were assigned to one of the 8 treatment groups by counter-balancing based on body weights taken after acclimation. In order to accommodate for consistent procedural times and behavioral testing the study was designed to run 16 or 24 rats at a

time where each run included representatives for all treatment groups. Briefly, after acclimation, the rats were ovariectomized (OVX) and implanted with hormonal treatments delivered by an Alzet osmotic pump (see below). Rats assigned to the stressed groups underwent daily immobilization stress during the experimental phase which was initiated 3 days after surgery and lasted for a total of 22 days (see below). All rats were tested for pain tolerance using the hotplate test, in week 3 of the experimental phase. At the end of treatment, the rats were euthanized by carbon dioxide overdose (via gaseous delivery to home cage) with rapid decapitation. Trunk blood of each animal was obtained immediately following decapitation into individual 50 ml conical flasks containing 150 u heparin, centrifuged (1000xg) at 4°C for 20 minutes. Plasma was harvested and stored at -80° C until further analysis.

Drugs.

Estradiol 17-β (Sigma Chemical Co, St Louis, MO), binds with nearly equal affinity to both estrogen receptors [22]. In order to study the respective biological roles of ERα and ERβ, specific estrogen receptor agonists, PPT and DPN, were also purchased (Tocris Cookson, Inc., Ellisville, MO). Hydroxypropyl beta cyclodextrin (27%) (Sigma) served as VEH.

Surgery.

After acclimation, all rats underwent bilateral ovariectomy and Alzet osmotic pump implantation under isoflurane anesthesia. Animals were subcutaneously implanted with osmotic pumps between the shoulder blades (Alzet Model 2002; Durect Co., Cupertino, CA). Pumps contained VEH, E2, PPT or DPN. The estradiol, PPT and DPN were dissolved in 27% hydroxypropyl beta cyclodextrin (Sigma Chemical). Due to pump

reservoir capacity, new pumps were rapidly replaced (less than 2 min after anesthesia) under sterile conditions and isoflurane anesthesia at 13 days after the initial pump insertion. The pump insertion site was monitored daily for any unusual bleeding, drainage, or swelling of which no animals displayed at any point during the study**.** Pumps delivered 0.25 mg/kg body weight (bw) of E2 (subcutaneous [s.c.]), 1 mg/kg bw of PPT (s.c.) and 1 mg/kg bw of DPN (s.c.). This dose represents physiological levels of circulating estradiol between the proestrous period [23]. The dose for ER agonists, PPT and DPN, represent previously established doses for these agonists [24]. Control animals in both the stress and non-stress groups received VEH (hydroxypropyl beta cyclodextrin) only.

Immobilization Stress.

Animals assigned to stress groups underwent immobilization (3 days after OVX) using finger-like restraining devices (Centrap Cages, Fisher Scientific, St. Louis, MO) for 60 min/day. Rats were placed in the Centrap cage and the restraining 'fingers' were tightened until the animals were immobilized but not compressed, pinched, or screaming /screeching. Animals were unable to turn or even barrel-roll in the Centrap cage. Studies show that repeated immobilization stress (most significantly at 21 days) causes structural remodeling in areas of the brain responsible for emotional memories and regulation of the stress response (amygdala, hippocampus and prefrontal cortex) [25-26].

Hot Plate Test.

The Hot Plate Test is an acute thermal pain test used to measure the latency to hindpaw lick, which is considered indicative of an antinociceptive response. The testing apparatus, consisting of a clear plastic box with a flat metal surface, was heated to 51° C.

Each rat was sequentially tested three times at 10-minute intervals. All animals were returned to their cages after each test session. Basal latency was determined as the average of the three measurements. The exposure to the thermal stimulus lasted no more than 60 seconds. During the baseline phase, animals were acclimated to the hot plate test using the same protocol. Testing occurred in the late part (week 3) of the experimental phase. All non-stressed animals were tested first, followed by stressed animals. Testing started 1 hour after the start of the dark phase and stressed animals were tested within 20 minutes after removal from restraints. The hot plate platform and box were cleaned with 35% ethanol solution between animals to minimize olfactory cues.

ELISA. Serum concentrations of beta endorphin were measured using a standard ELISA (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

*Data Analysis***.**

Hot plate results on latency to hindpaw lick was expressed as mean latency ± standard error of the mean (SEM) and converted to percent maximal possible effect (%MPE) according to the formula: (TL-BL)/(60-BL) x 100, where TL = test latency and BL = basal latency. Statistical significance of differences for pain sensitivity was determined by either t-test or two-way analysis of variance (ANOVA) with a random block design. After an overall significant treatment effect was determined with the ANOVA, a Fishers LSD post-hoc test was conducted to determine differences between groups.

Serum data was collected by performing beta-endorphin immunoassay tests and from readings based on a V-Max kinetic microplate reader. The V-Max kinetic reader provides the capability to monitor and automatically analyze enzyme kinetics in a microplate. Upon completion of microplate readings, raw data was mathematically

converted to active serum levels of beta-endorphin per the manufacturer's protocol (Phoenix Pharmaceuticals). Statistical analyses were conducted using a two-way ANOVA with treatment groups of stress or hormone treatment (VEH, E2, PPT, and DPN) as independent variables followed by Tukey's post-hoc test.

RESULTS

Effects of stress and hormone treatment on nociception.

Following daily 1-h immobilization, VEH-treated stressed rats showed increased ($t(21) = -2.09$, $p<0.05$) latency to hindpaw lick during the hotplate test compared to the VEH-treated non-stressed rats (25.0+/-1.9s vs 20.4+/-1.0s, respectively) to suggest a decreased sensitivity to pain (Figure 1). When comparing the effect of stress and hormone treatment, a significant main effect of hormone treatment ($F(3, 84) = 7.85$, $p<0.05$) and of stress ($F(1,84)=6.47$, $p<0.05$) was observed. Both non-stressed and stressed groups treated with E2 and PPT (ERα agonist) showed an increase ($p<0.05$) in latency to hindpaw lick compared to their respective VEH-treated controls (Figure 2). In contrast, only stressed rats treated with DPN, the ERβ selective agonist, showed an increase ($p=0.08$) in latency to hindpaw lick compared to their respective controls whereas there was no ERβ agonist effect ($p>0.10$) in the non-stressed group (Figure 2).

Effects of Stress and Hormone treatment on plasma β-endorphin levels.

Serum level of β-endorphin was analyzed using an ELISA. There was a significant main effect of stress ($F(1,77)=82.18$, $p<0.05$), treatment ($F(3,77)=17.72$, $p<0.05$) and stress treatment interaction ($F(3,77)=14.95$, $p<0.05$). Stressed rats showed an increase ($p<0.05$) in β-endorphin levels compared to non-stressed rats in all hormone treatment groups (Figure 3). In nonstressed animals, β-endorphin levels were not altered by hormone treatments ($p>0.10$). Among the stressed rats, E2- and PPT-treatment did not affect ($p>0.10$) circulating β-endorphin levels, compared to VEH-treatment, whereas DPN (ERβ)-treated rats had greater ($p<0.05$) circulating β-endorphin levels compared to all other groups.

DISCUSSION

In the present study, chronic daily immobilization stress enhanced pain tolerance in OVX rats by increasing the latency to hind-paw lick during hot plate testing. Furthermore, 17β-estradiol (E2) and PPT, the ERα agonist, enhanced pain tolerance compared to the corresponding control group in both the non-stressed and stressed animals. Interestingly, treatment with the ERβ selective agonist, DPN, appears to enhance tolerance to pain only when the rat is chronically stressed. Lastly, stressed rats have increased peripheral levels of β-endorphin to suggest its involvement in the increase in pain threshold during acute thermal pain.

Pain threshold is modulated by both acute and chronic stress. Paradigms of acute stress show anti-nociceptive effects immediately following the stressor [27], known as stress-induced analgesia. More controversial are the nociceptive effects during chronic stress conditions which show mixed anti-nociceptive effects [27-29]. Additionally, the interactive effect of chronic stress and estradiol on response to pain remains unclear. The predominant use of male animals as subjects and acute stress as the paradigm of choice in the current literature explains the inadequacies. For example, chronic immobilization in male rats is hyperalgesic when tested by the temporomandibular joint (TMJ) formalin test [30] or tail flick response [27]. In the present study, our results showed that chronically stressed OVX rats showed an increase in pain threshold when exposed to an acute thermal pain assay. These findings are suggestive that chronic stress may alter pain perception in a gender-specific manner.

Our present results are consistent with other reports that show that the administration of E2 or a selective ERα agonist enhanced pain tolerance in both

nonstressed and stressed animals [13, 31]. A neuropathic pain study by Tsao and coworkers showed OVX rats receiving daily injection of E2 had significantly decreased autonomy (self-mutilation) scores following sciatic nerve ligation compared to rats not treated with E2 [13]. Moreover, OVX rats treated with E2 had decreased TMJ nociception. This effect is possibly mediated independent of opioid receptors through a nitric oxide/cyclic guanosine mono-phosphate (cGMP) signaling pathway [32]. Similarly, ERα is expressed and activated by pain in the medullary dorsal horn, a region critical in nociceptive transmission, providing support for our finding that ERα stimulation may directly regulate pain transmission at specific levels of the spinal cord [33]. Interestingly, there does not appear to be an additive effect of the ERα agonist and stress on pain tolerance. It is possible that may be a convergence of effects downstream from the stress and the activation of ERα.

In contrast, our study showed that treatment with the ERβ selective agonist, DPN, appears to enhance tolerance to pain only when the rat is chronically stressed. Previous studies have shown ERβ mRNA in the PVN of the hypothalamus is up-regulated by stress and is CORT-dependent [34-35]. In turn, this suggests chronically stressed animals are more sensitive to DPN administration. Moreover, we saw a pronounced increase in beta-endorphin levels with DPN and stress exposure.

Previous studies have shown that under conditions of stress, estradiol may disinhibit the negative feedback of corticotrophin releasing hormone secretion by glucocorticoids [36], though it would appear to occur via ERα [34, 37]. However, our present results suggest that the selective activation of ERβ may enhance the effect of stress on pain perception and may do so hormonally via the secretion of β-endorphin. A possible

explanation for this quandary may lie in the form of stress. The studies suggesting the interaction between ERα and the glucocorticoid pathway were conducted under conditions of acute or short-term stress [33-34, 36-37], whereas the current study was conducted under conditions of chronic stress.

In the present study, the reduced pain sensitivity in the animals that were restrained-stressed is consistent with clinical observations. For example, combat veterans suffering from post-traumatic stress disorder (PTSD) reported reduced pain sensitivity during fixed temperature (43°C) thermal pain testing when compared to those who are not diagnosed with PTSD [2, 38]. Other similar responses exhibited by animals exposed to repeated immobilization in the manner described include exaggerated startle and depressive behaviors while reliably producing elevations in the stress hormones, ACTH and corticosterone [39]. Collectively, our studies along with these others strongly suggest that the rodent immobilization model simulates the human perception of a lack of control that is associated with greater severity of symptoms following traumatic experience [40]. Although immobilization stress appears to be a mild stressor, when applied to rats it has been found to induce fear conditioning [41] and potentiate anxiety [42]. Interestingly, inflammatory mediators are released in response to stressful stimuli. In turn, the CNS signals the activation of the HPA axis and the sympatho-adrenal axis [43]. Stress produces an increase in the adrenergic activity of the hypothalamus [19]. In response to the increase in adrenergic activity, proopiomelanocortin (POMC) is released and cleaved to produce β-endorphin [44].

In conclusion, chronic restraint stress decreases pain sensitivity to acute thermal pain and increases serum plasma levels of β-endorphin. This finding suggests stress-

induced analgesia may be a result of the μ-opioid receptor stimulation. Furthermore, E2 administration enhanced pain tolerance during hot plate testing and under nonstressed conditions is mediated through ERα. Interestingly, ERβ activation reduced pain sensitivity only in stressed rats, which in part could be acting through the up-regulation of β-endorphin to depress pain afferents. Additional studies are needed to determine the interactions with neuropeptides or neurotransmitters, such as, dopamine, histamine, serotonin, and/or excitatory amino acids, which can alter nociceptive responses. Whether E2 administration regulates the expression of these receptors in response to pain and/or stress will be of importance in determining the underlying mechanism of actions.

FIGURES

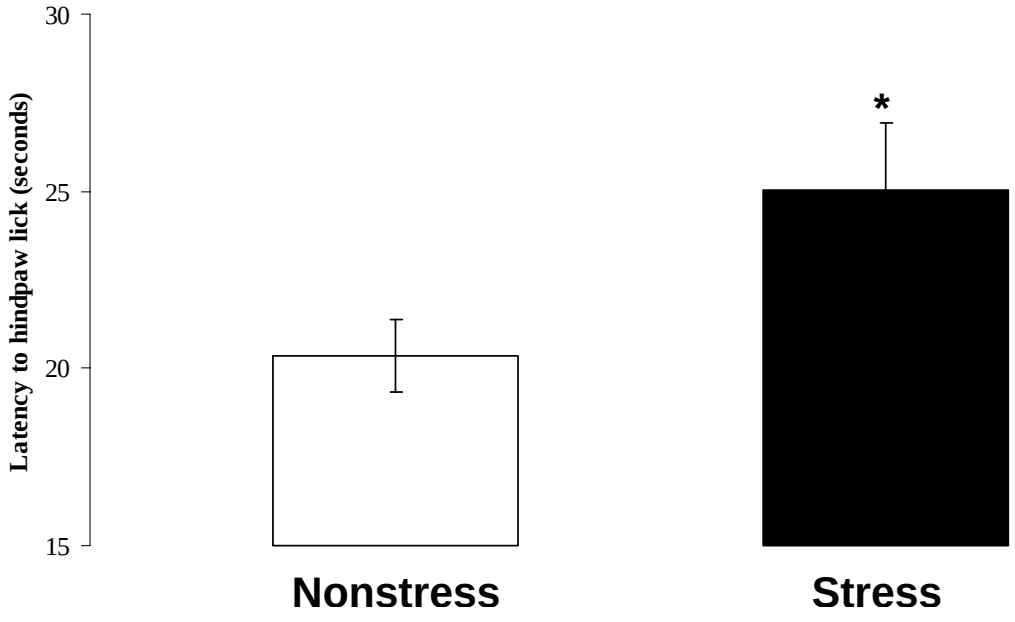

Figure Legends

Figure 1: Effect of immobilization stress on basal nociception in the hotplate test (51°C). Two groups of ovariectomized rats were tested and compared – these were rats that were not stressed and those that were stressed (immobilized for 1 h/day for 19 days prior to testing). Each bar represents the latency to hindpaw lick (determined in seconds) during hotplate testing (means ±SEM) of non-stressed and stressed OVX rats (*$p<0.05$ vs non-stress group).

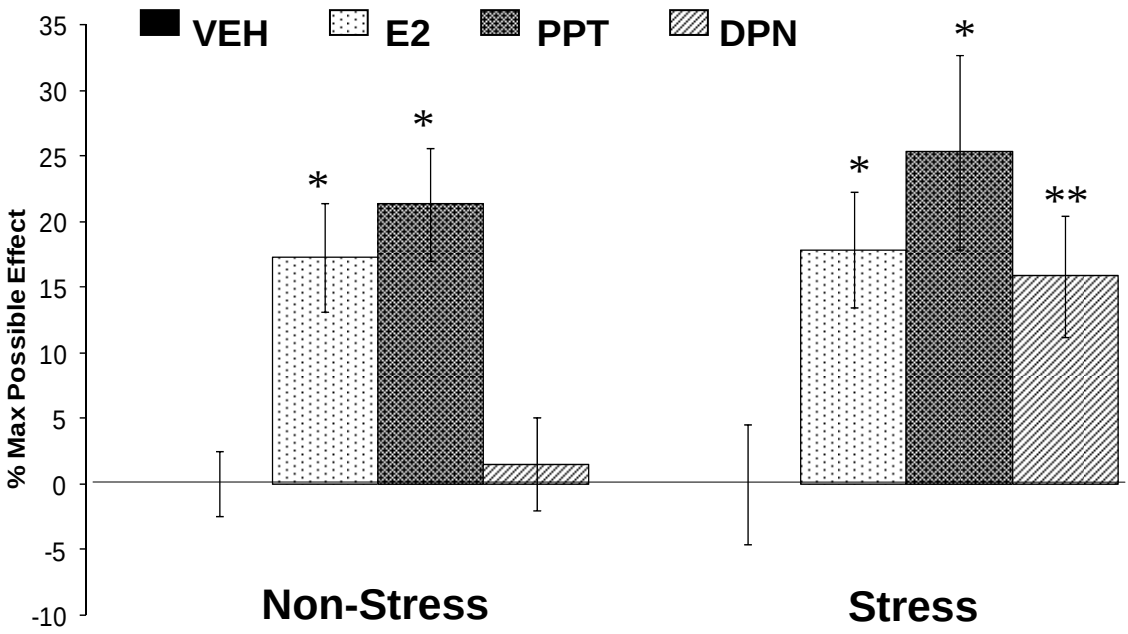

Figure 2: Effects of immobilization stress and hormone treatment (VEH, E2, PPT and DPN) on basal nociception in the hotplate test (51°C) expressed as a percent of the respective VEH-treated rats. The rats were randomly assigned to one of eight treatment groups in a 2x4 factorial design of stress (non-stress vs stress groups) and hormone treatments (VEH, E2, PPT and DPN). Each bar represents the mean percentage (+/- SEM) latency to hindpaw lick in the hotplate test relative to the respective VEH group (*$p<0.05$ vs VEH group).

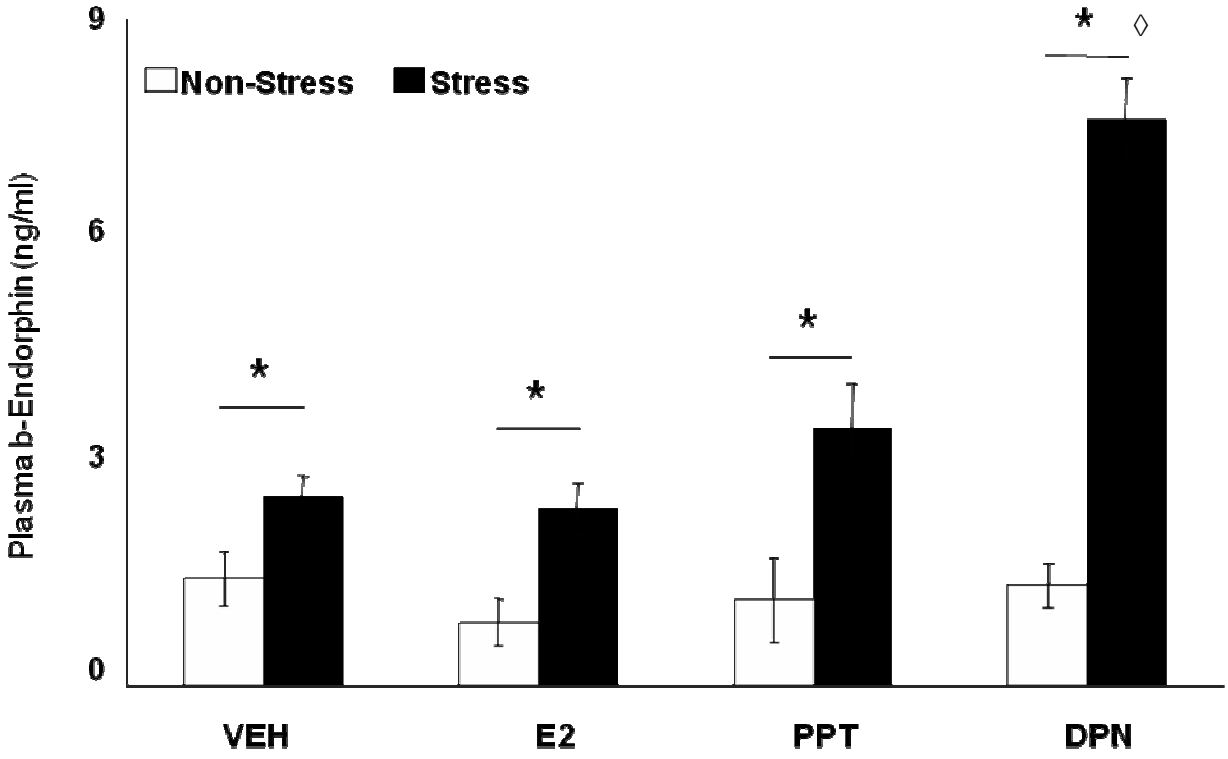

Figure 3. Effects of immobilization stress and hormone treatment (VEH, E2, PPT and DPN) on plasma concentrations of β-Endorphin concentrations. Each bar represents the mean (+/- SEM) concentration for the hormone (* $p<0.05$ vs the respective non-stress groups; ◊ $p<0.05$ vs all other stress groups).

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Title: The interaction of 17β-estradiol and chronic stress to regulate body weight in the ovariectomized rat

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Sources of Financial Support:

Tri-Service Nursing Program, Department of Defense Grant #600-085-00000-00-104622, and Department of Defense Grant CO85DP.

Disclaimer: The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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Key Words: body weight, stress, leptin, estrogen, restraint stress, estrogen receptors

Abstract

17β-estradiol (E2) treatment attenuates post-ovariectomy weight gain in the female rat, suggesting E2 may overlap in mechanism with the adipokine leptin to regulate body weight. In addition, the influence of chronic stress on the anorectic action of E2 remains unclear. This study examined the contribution of each estrogen receptor (ER) and the effects of chronic stress on body weight. Adult OVX Sprague-Dawley rats were infused with either vehicle (VEH), E2, propylpyrazoletriol (PPT:ERα agonist), or diarylpropionitrile (DPN:ERβ agonist). Half the animals from each treatment were immobilized 60 min/day for 22 days. E2 and PPT treatment inhibited weight gain and food intake in OVX rats. In contrast, chronic stress reduced body weight in VEH, PPT, and DPN treated animals but did not affect food intake. E2 and PPT treatment also reduced leptin levels and increased insulin, an effect that was absent in animals subjected to immobilization. Chronic stress also diminished the diurnal rise in corticosterone (CORT) during the early dark phase compared to nonstressed rats. Western blot analysis indicated that E2 treatment increased leptin receptor (Ob-Rb) expression in the MBH; however, this treatment also increased SOCS3, which is an inhibitor of leptin signaling. Unusually, PPT and DPN did not overlap in E2 effects to regulate leptin associated proteins within the MBH, suggesting E2 is partially regulating leptin independent of the classical ERs. Furthermore, chronic immobilization stress blunted the E2-induced increase in Ob-Rb and SOCS3 expression. These results suggest that leptin signaling is differentially regulated by E2 and chronic immobilization stress.

Introduction

Menopausal women have reduced circulating estrogen levels increasing their risk for developing central obesity and associated pathologies such as diabetes and cardiovascular disease (1-3). The ovariectomized rat has been used as a model for studying the complex interaction between estrogen and body weight. Surgical removal of both ovaries in the female rat induces a hypoestrogenic state resulting in excess accumulation of abdominal fat whereas estrogen replacement restores normal fat distribution. In non-pregnant female rats the predominant estrogen is 17β-estradiol (E2), and its actions are mediated through the activation of two estrogen receptor (ER) isoforms, ERαand ERβ (4-6). Transgenic mice deficient in ERα but not ERβ become hyperphagic and obese compared to corresponding wild-type mice (7). Our lab and others have shown that selective activation of ERα attenuates post-ovariectomy weight gain in ovariectomized and ovariectomized/adrenalectomized rats implicating ERαmediated regulation of body weight (8, 9).

Regulation of feeding and body weight is partly a consequence of circulating levels of leptin, a hormone of adipose origin. Intracerebroventricular (ICV) injections of leptin in mice act to reduce food intake and increase energy expenditure (10). Furthermore, knockout mice deficient only in the long form of the leptin receptor (Ob-Rb) develop severe obesity and are unresponsive to leptin treatment, implicating this receptor as the signal-transducing element for leptin's effects on body weight (11). Ligand-activated Ob-Rb activates the signal transducer and activator of transcription 3 (STAT3) (12) to regulate the expression of proopiomelanocortin (POMC) and suppressor of cytokine signaling 3 (SOCS3), which, in turn, modulates energy homeostasis (13-15).

In this study, we explored the interaction between E2 and leptin to regulate feeding and body weight. The hypothalamic arcuate nucleus (ARC) is a central integrative loci for both E2 and leptin effects (16). Furthermore, long-term E2 has been shown to up-regulate Ob-Rb expression in the hypothalamus and white adipose tissue (17). However, other studies indicated that treatment with E2 in leptin and leptin receptor deficient mice significantly reduced food intake and body weight suggesting that E2 can elicit its effects regardless of leptin status (18). These investigators further reported that E2 activated the STAT3 pathway, thereby mimicking leptin signaling, and that brain-specific STAT3 knockout mice were unresponsive to E2 administration suggesting E2's mode of action is STAT3-dependent (18). Consequently, E2 seems to act downstream of Ob-Rb to activate STAT3 and facilitate transcription of target genes including POMC to regulate food intake (18, 19).

In animal models the effects of stress on body weight have produced variable results and largely depend on the type of stressor applied (20). For the most part, physical stressors such as immobilization stress reduce food intake and body weight in rats (20, 22). Yet, the biological mechanism behind chronic stress effects on body weight is for the most part unclear especially in female rats since most studies have focused on males. Thus, in this study, we chose to examine female rats to model a chronic stress state using daily sessions of a physical stressor, immobilization. This stressor activates the hypothalamic-pituitary-adrenal (HPA) axis to release adrenocorticotropic hormone (ACTH) and corticosterone (CORT) (23, 24). However, body weight sensitivity in response to immobilization stress has been shown to depend on gender and strain in rats (25). Male rats are reportedly more sensitive to stress-induced changes in feeding and

body weight than females, suggesting that gonadal hormones may play an important role in mediating stress effects on metabolism (25). Furthermore, insight from another chronic stress model where male rats were treated with dexamethasone (DEX), a CORT analog, resulted in elevated plasma leptin, reduced feeding, and lower body weight compared to untreated controls (26). Such results indicate a potential interaction between the stress response and leptin; yet, whether this is the case for the female rat is still not clear. Therefore, further investigation into the effects of immobilization stress on body weight in female rats is necessary to address these gaps.

The experiments presented here examined the effects of chronic stress and the differential role of ERβ and ERα on leptin and insulin signaling in post-ovariectomy body weight regulation. A chronic stress state was induced by exposing hormone treated female Sprague-Dawley rats to daily immobilization stress. To assess the effects of hormone treatment and chronic stress on body weight, markers of metabolism (serum insulin, leptin and CORT) were measured. In addition, the relative levels of downstream effectors including phospho-protein kinase B (pAKT), Ob-Rb, pSTAT3, and SOCS3 expression was measured across groups within the medial basal hypothalamus.

Material and Methods

Subjects

Ninety-six 200-225g female Sprague-Dawley rats (Harlan, Indianapolis, IN) were pair-housed in standard polycarbonate shoebox cages (42 x 20.5 x 20 cm) containing hardwood chip bedding (Sani-Chip, Laboratory Grade, Harlan Teklad, Madison, WI). All animals had *ad libitum* access to soy-free rodent chow (Dyets, Inc. Bethlehem, PA) (27) and water. The housing room was maintained at 22-25° C at 50 % humidity on a 12-hour reverse light/dark cycle. Upon arrival, all animals were individually handled daily and acclimated to facilities and equipment prior to commencement of experiments.

Body weight, food intake, and water consumption were measured daily over the entire length of the study. Subjects were weighed individually, while food and water consumption were recorded for each pair. All procedures conducted were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Experimental Design

The experiment was conducted as a 2 (no stress or 60 min immobilization/day) x 4 (VEH, E2, PPT or DPN) factorial design with 12 animals per treatment group for a total of 96 animals. Groups were counter-balanced for body weight prior to any experimental manipulation.

Drugs

17β-Estradiol (E2 (Sigma Chemical Co., St Louis, MO), binds with nearly equal affinity to both estrogen receptors (28). In order to study the respective contributions of ERα and ERβ to estradiol signaling, the selective estrogen receptor agonists, PPT and DPN (Tocris Cookson, Inc., Ellisville, MO) were used in the study. PPT binds to ERα with a 410-fold grater relative binding affinity than to ERβ (29). DPN has a 70-fold higher relative binding affinity to ERβ than to ERα (30).

Ovariectomy and osmotic pump implant

Nine days after arrival, all animals underwent bilateral ovariectomy and Alzet mini-osmotic pump implantion under isoflurane anesthesia. Animals were implanted with mini-osmotic pumps (Alzet Model 2002 0.5ML, Durect Co., Cupertino, CA, USA), subcutaneously between the shoulder blades. Pumps contained either vehicle, E2, PPT (selective ERα agonist) or DPN (selective ERβ agonist). E2, PPT and DPN were dissolved in vehicle (VEH; 27% hydroxypropyl-β-cyclodextrin in sterile water, Acros Organics, Fair Lawn, NJ) (27). On post-implant day 13 pumps were replaced under isoflurane anesthesia. Pumps delivered 0.25 mg/kg body weight (bw)/d of E2, 1 mg/kg bw/d of PPT, and 1 mg/kg bw/d of DPN. Mini-osmotic pumps release their contents at a slow continuous infusion rate of 0.5 µl/h. The dose for ER agonists, PPT and DPN, represent previously established doses for these agonists (31). Control animals in both the stress and non-stress groups received VEH only.

Immobilization Stress

Beginning on post-ovariectomy day 4, assigned stress groups were immobilized for 60 min/day for 22 days using finger-like restraining devices (Centrap Cages, Fisher Scientific). Rats were placed in the Centrap cage and the restraining 'fingers' were tightened until the animals were immobilized but not compressed, pinched, or in pain. This device allows for very little movement. Animals were unable to turn or barrel-roll in the Centrap cage. This restraint procedure, acute or chronic, reliably produces elevations in hormones associated with a stress response including ACTH and CORT (24). Stress exposure was conducted 1 hours after lights out during the dark phase of the daily light cycle to prevent any light-induced effects on the stress response (32).

Locomotor

In order to determine whether the effect of these drugs on body weight were due to changes in activity, we measured locomotor activity using an Omnitech Electronics Digiscan infrared photocell system [Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, OH] located in a dedicated room. Animals were placed singly in a 40 X 40 X 30 cm clear Plexiglas arena with a ventilated lid. A photocell array measured activity using 16 pairs of infrared photocells located every 2.5 cm from side-to-side and 16 pairs of infrared photocells located front-to-back in a plane 2 cm above the floor of the arena. Data was automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer (33). The apparatus monitors animal activity continuously with data recorded as cumulative activity every 5 minutes for a total testing

period of 60 minutes. All testing was conducted within the first 4 hours of the dark cycle. The Plexiglass arenas were cleaned with 35% ethanol between each animal.

Collection of Serum and Brain Tissue

Upon completion of the experimental phase, all rats were killed by carbon dioxide overdose with rapid decapitation within 1 h after the end of the final immobilization. Brains were quickly removed, snap-frozen on dry ice, and stored at -80° C prior to protein analysis. Trunk blood was collected into ice-cold tubes followed by centrifugation at 4000g for 20 minutes. Blood serum was extracted and stored at -80° C prior to further analysis.

Western Blot

The medial basal hypothalamus was dissected on ice as previously described (34, 35). Brain tissue was either homogenized in RIPA buffer (50mM Tris-HCl pH 7.5; 150mM NaCl; 1% NP-40; 1% NaDOC; 0.1% SDS; Roche Complete Protease Inhibitor, Indianapolis, IN) for measuring Ob-Rb expression or O'dell's buffer (50mM Tris-HCl pH 7.5; 50mM NaCl; 10mM EGTA; 10mM EDTA; 80uM Na_2MoO_4; 5mM $NaPO_4$; 1mM Na_3VO_4; 1mM PMSF; 4mM pNPP; 1% Triton; Sigma Cocktail I and II; Roche Complete Protease Inhibitor, Indianapolis, IN) for phosphorylated proteins. Lysates were centrifuged at 20,000g at 4 C for 30 minutes and subjected to SDS-PAGE according to a previously established method (36) under reducing conditions using 4-15% Tris-HCl gels (Bio-Rad, Hercules, CA). The resolved proteins were electroblotted onto a polyvinyldifluoride (PVDF) membrane (Bio-Rad, Hercules, CA) in a Tris/glycine buffer (25 mM Tris, 0.192 M glycine, 0.01% SDS, pH 8.3) and blocked overnight with 5%

(w/v) non-fat dry milk in Tris buffered saline (TBS: 25 mM Tris-HCl, 125 mM NaCl, pH 7.4) prior to probing overnight at 4° C with a specific antibody (anti-mouse monoclonal Ob-Rb , 1:500, Santa Cruz Technologies, Santa Cruz, CA; monoclonal anti-rabbit pSTAT3 1:1000, STAT3 1:1000, pAKT 1:1000, and AKT 1:1000, Cell Signaling, Danvers, MA). Blots were subsequently washed 3 times with 0.1% tween TBS and subjected to 1 hour incubation at room temperature with the appropriate secondary antibody conjugated to a peroxidase enzyme. A SuperSignal West Femto chemiluminescence kit (Rockford, IL) was used to develop immunoblots with subsequent exposure to Fujifilm LAS-3000 western blot imager (Stamford, CT). Densitometric analyses of digitized western blot images were quantitated using Fujifilm Image Gauge (Valhalla, NY). Correct loading amounts were determined by Coomassie staining (Bio-Rad, Hercules, CA). All values are expressed relative to non-stressed VEH-treated animals.

Enzyme-linked immunosorbent assays

A competitive enzyme-linked immunosorbent assay (ELISA) kit was used to determine the serum concentrations of insulin (SPI Bio, Montigny Le Bretonneux, France) and corticosterone (Cayman Chemical, Ann Arbor, MI); intra-assay coefficient of variance was 8% and 5%, respectively. A sandwich ELISA kit was used to determine leptin (SPI Bio, Montigny Le Bretonneux, France) and resistin (B-Bridge International, Inc., Mountain View, CA) serum concentrations; intra-assay coefficients of variance were 7% and 8%, respectively.

Statistical Analysis

Using the statistical software SPSS 16.0 (Chicago, Illinois), hormone and stress interactions were examined using a 2-way multivariate analysis of variance followed by Fischer's least significant difference post-hoc test. A value of $p<0.05$ was considered significant and a trend was noted at $p<0.10$.

Results

Body Weight and Food Consumption

We examined the relative contributions of ERα and ERβ on feeding and body weight in addition to the effects of chronic immobilization. Body weights on the last day of the stress phase experiment (Post-OVX Day 26) revealed a hormone ($F(3,88)=110.438$, $P<0.05$) and stress ($F(1,88)=8.235$, $P<0.05$) effect but not an interaction ($F(3,88)=1.158$, $P>0.10$). Both nonstress and stress female OVX rats receiving constant infusion of E2 or PPT for 22 days had decreased body weight ($p<0.05$) compared to rats treated with VEH or DPN (Figure 1a). DPN-treated stressed rats weighed less than VEH-treated stressed rats. Comparison of weights from nonstress and stress groups receiving the same hormone treatment show chronic stress exposure decreased body weight in VEH ($p<0.1$), PPT ($p<0.1$) and DPN ($p<0.05$) treated rats but not with E2 treatment (Figure 1c). Further, stressed rats receiving PPT had decreased body weight ($p<0.05$) on SD3 relative to VEH-stressed rats while PPT-non-stressed rats took longer to lower body weight reaching significance on SD8 (Figure 1b).

To determine whether hormone treatment or chronic stress effects on body weight were due to changes in food consumption, we measured daily food intake from all groups. Daily values were averaged to calculate average food consumption per day in the respective week. Analysis of weekly food intake revealed an effect of hormone treatment ($F(3,40)=33.633$, $p<0.05$) , but not of stress ($F(1,88)=0.004$, $p>0.1$) or a hormone treatment x stress interaction ($F(3,40)=0.580$, $p<0.631$). Post hoc tests showed food consumption decreased in rats administered E2 or PPT independent of stress exposure in

the early ($p<0.05$), middle ($p<0.05$), and late ($p<0.05$) stress phase of the experimental phase. During the third week (late stress phase), E2 and PPT treatment decreased ($p<0.05$) food consumption per day relative to VEH, yet, this effect appeared to be diminishing in comparison to earlier weeks. Analogous to body weight effects, treatment with DPN produced no changes in food consumption compared to VEH. However, there was a significant reduction in food intake during the middle stress phase of DPN-treated stressed rats ($p<0.05$) relative to VEH-treated stressed rats. Other than this there were no significant effects of stress on food consumption (Figure 2a, b). In order to determine whether the effect of these drugs on body weight were due to changes in activity, all rats were exposed to a locomotor chamber four times throughout the stress phase for 1 hour. Testing occurred after the stress session in the case of stressed animals and total distance traveled was measured as an indicator of activity. Hormone and stress had no observable effect on total distance traveled relative to VEH treatment (Figure 3).

Serum Analysis

Since our results demonstrated an effect of hormones and stress on weight gain, we assessed the effect of chronic stress and hormone treatment on markers of metabolism by measuring serum insulin, leptin, resistin, and CORT on the final day of the stress phase in all animals. Stressed rats were immobilized for 60 minutes and blood was collected within 1 hour following the cessation of immobilization.

Analysis of leptin levels revealed a hormone treatment effect [$F(3,88)=13.420$, $p<0.05$]. Furthermore, there was a trend for both the effect of stress [$F(1,88)=8.528$, $p<0.1$] and the hormone x stress interaction [$F(3,88)=7.348$, $p<0.1$]. Rats treated with E2 or PPT had

lower serum leptin ($p<0.05$) compared to rats receiving only VEH. There was no effect of DPN on leptin relative to VEH. Interestingly, the reduction in serum leptin was prevented by chronic stress with both E2 and PPT treatment (Figure 4a).

There was an effect of hormone treatment [$F(3,88)=26.406$, $p<0.05$] in addition to a significant hormone treatment x stress interaction [$F(3,88)=2.956$, $p<0.05$] on resistin levels and no effect of stress alone [$F(1,88)=0.071$, $p>0.1$]. Nonstressed rats treated with E2 or PPT had reduced resistin levels ($p<0.05$) compared to rats receiving VEH or DPN treatment. Stressed rats treated with PPT had significantly higher resistin ($p<0.05$) levels than nonstressed PPT-treated rats (Figure 4c).

There was a significant effect of chronic stress $F(1,88)=8.956$, $p<0.05$] on serum insulin levels whereas there was no effect of hormone treatment [$F(3,88)=2.304$, $p>0.1$]. There was a trend for the hormone x stress interaction [$F(3,88)=2.655$, $p<0.1$]. Insulin was higher in rats treated with E2 ($p<0.05$) and slightly elevated in rats treated with PPT ($p<0.10$) while their corresponding stressed-group had levels comparable to VEH and DPN treatment (Figure 4b).

Stress significantly decreased CORT levels compared to nonstressed animals regardless of hormone treatment [$F(1,88)=52.589$, $p<0.05$]. Additionally, there was a hormone treatment effect on CORT [$F(3,88)=4.041$, $p<0.05$]. Stressed E2 and DPN treated groups had significantly higher ($p<0.05$) CORT levels relative to stressed VEH treated animals, although these levels did not reach nonstressed CORT levels. Analysis revealed no hormone treatment x stress interaction [$F(3,88)=0.358$, $p>0.1$] (Figure 5).

Chronic stress alters hormone-induced changes in leptin signaling

There is evidence suggesting E2 up-regulates leptin receptor (Ob-Rb) expression in the hypothalamus (Meli 2004). Analysis of Ob-Rb expression in the MBH revealed a hormone treatment [$F(3,22)=5.470$, $p<0.05$], stress effect [$F(1,22)=6.344$, $p<0.05$], and a hormone treatment x stress interaction [$F(3,22)=3.587$, $p<0.05$]. Chronic E2 treatment significantly increases ($p<0.05$) expression in the MBH relative to control animals. Ob-Rb expression was not altered with PPT or DPN treatment. Interestingly, chronic stress exposure reduced Ob-Rb levels in animals treated with E2 ($p<0.1$), PPT ($p<0.05$), and DPN ($p<0.05$) (Figure 6a).

To further characterize downstream effectors of leptin signaling, we also measured pSTAT3 and SOCS3 levels in the MBH. There was no effect of hormone treatment [$F(3,20)=1.289$, $p>0.1$] or stress [$F(1,20)=2.337$, $p>0.1$] on pSTAT3 levels; however, there was a trend for a hormone treatment x stress interaction [$F(3,20)=2.489$, $p<0.1$]. Stress down-regulated pSTAT3 with VEH ($p<0.05$) and E2 ($p<0.05$) treatment while PPT and DPN treatment inhibited this effect (Figure 6b). Analysis of SOCS3 revealed an effect of hormone treatment [$F(3,21)=3.804$, $p<0.05$] but not stress [$F(1,21)=0.624$, $p>0.1$] . There was a significant hormone treatment x stress interaction [$F(3,21)=7.460$, $p<0.05$]. E2 ($p<0.05$) and to a lesser extent DPN ($p<0.1$) treatment increased SOCS3 expression. Stress moderately reduced SOCS3 only with E2 treatment ($p<0.1$). PPT and DPN treatment in stressed rats significantly increased SOCS3 expression ($p<0.05$) relative to stressed VEH-treated animals (Figure 6c).

We also measured pAKT, as an estimate of insulin activity, since we noticed hormone and stress had differential effects on serum insulin (Figure 4a). Hormone treatment [$F(3,21)=6.312$, $p<0.05$] had a an effect on pAKT but not stress [$F(1,21)=0.468$ $p>0.1$] or the hormone treatment x stress interaction [$F(3,21)=0.408$, $p>0.1$]. PPT and DPN treatment, regardless of stress exposure, significantly up-regulated pAKT ($p<0.05$) in the MBH (Figure 6d).

Discussion

Consistent with previous findings (8, 9), our results implicate that ERα mediates E2 regulation of feeding and body weight. Treatment of ovariectomized (OVX) female rats with E2 or PPT (ERα agonist) attenuated post-ovariectomy weight gain and transiently decreased food consumption. Conversely, animals treated with the ERβ agonist, DPN, increased in body weight paralleling the weight gain observed in control rats receiving no hormone treatment. We also observed resistin levels were reduced in E2 and PPT treated animals, which is consistent with previous findings showing that estrogen down-regulates resistin mRNA expression in adipose tissue (37). Furthermore, E2 or PPT treatment increased insulin, which has been shown to negatively regulate resistin (38); and suggests that this regulation is mediated by ERα. Similarly, peripheral leptin levels were reduced in E2 and PPT-treated rats indicative of decreased adiposity in these animals. These results suggest E2 and PPT treatment prevents resistin and leptin resistance, which is commonly associated with obesity (39).

Previously, we reported ERα regulation of body weight may be HPA axis independent since adrenalectomy did not alter E2 or PPT effects on body weight (8). ERα modulation of body weight perhaps is regulating leptin sensitivity in critical control centers of metabolism and food consumption within the hypothalamus. Therefore, we examined the MBH for Ob-Rb, STAT3 and SOCS3 expression levels as measures of leptin signaling (12-15, 18). Additionally, we examined the effect of hormone treatment on pAKT levels as a measure of insulin activity. We show E2 administration up-regulated Ob-Rb levels within the MBH, suggesting E2 can regulate central leptin sensitivity by modulating the expression of Ob-Rb. This finding is supported by previous research implicating an E2

and Ob-Rb interaction (17). Furthermore, OVX rats administered ICV injections of leptin were resistant to its anorectic effects suggesting E2 deficiency reduces Ob-Rb expression (40). Interestingly, we did not observe an effect with any of the hormone treatments on the phosphorylation status of STAT3 (Y705), which is necessary for dimerization and subsequent translocation to the nucleus for gene transcription. Gao and colleagues reported hypothalamic pSTAT3 increased after 30 minutes of an intraperitoneal injection of E2 (18). Therefore, it is possible that chronic E2 treatment eventually activates a negative feedback mechanism to modulate STAT3 responsiveness. Indeed, we saw an E2-induced increase in SOCS3 expression, which inhibits Ob-Rb activation of the STAT3 pathway (41). It has been shown SOCS3, in addition to regulating STAT3 responsiveness, is also a negative regulator of insulin signaling (42). E2-treated animals did not have altered pAKT (Thr308) levels while PPT and DPN treatment significantly up-regulated pAKT. Therefore, our results suggest chronic E2 treatment potentially mediates a negative feedback mechanism involving SOCS3 to regulate STAT3 and AKT activation.

In this study we also examined the roles played by ERα and ERβ to mediate E2 effects on central leptin and insulin signaling. As already mentioned, PPT-treated animals had significantly reduced body weight compared to those treated with VEH and DPN, thus, we expected PPT treatment to mimic the expression profile we obtained from the MBH of E2-treated animals. However, PPT and E2 treatment diverged in efficacy to regulate Ob-Rb, SOCS3, and pAKT. A study conducted by Thammacharoen and colleagues reported *sc* injections of E2 and PPT differentially induced cFos expression on the PVN of the hypothalamus, suggesting E2 and PPT may not completely overlap in mechanism

to regulate body weight (43). As for the effects seen in response to DPN, there was a slight increase in SOCS3, which may suggest stimulation of ERβ in part can negatively regulate STAT3, however, this remains to be determined. Other evidence showing ERβ can modulate ERα activity comes from an in vitro study conducted by Liu and colleagues who reported E2 activation of ERβ repressed ERα induced cell proliferation by regulating cyclinD1 levels (44). Furthermore, we cannot rule out the involvement of other receptors, which might be preferentially stimulated by E2 alone in contrast to that of the agonists. A membrane ER (mER) or a G protein-coupled receptor (GPR) may contribute to the regulation of feeding and energy independent of the classical ERs (45). In an in vitro study using a breast cancer cell line, Filardo and colleagues showed E2 binds GPR30 (46), which served to counteract E2-mediated activation of the Erk1/2 pathway (47)

In this study we were also interested in the effect of chronic immobilization stress on body weight. Studies report a gender-specific response to stress (25, 48), thus, it is important to consider the putative interaction between E2 and glucocorticoids to modulate feeding and energy balance across genders. Our results show that daily immobilization stress for 22 days decreased body weight in all hormone treatment groups, except with E2 treatment, without altering locomotor activity or food consumption. The stress and feeding interaction is consistent with another study using repeated immobilization stress (48); however, the investigators reported no significant effects on body weight in intact female rats. This difference is most likely a consequence of the varying E2 levels in these rats throughout the estrous cycle. Furthermore, we extended the duration of the stress period to 22 days while in the previous study it was for 13 days, which could mark a transition point between acute and chronic stress effects on

physiology. Another study using 60 minute daily immobilization stress for 40 days found female rats gained less weight than nonstressed rats (49), which is consistent with our findings. Collectively, hormone status and varying periods of stress exposure may have different effects on feeding and body weight regulation.

Acute activation of the HPA axis leads to the secretion of glucocorticoids followed by a reduction in feeding. Short-term exposure to the synthetic glucocorticoid, dexamethasone, increased leptin, reduced feeding and body weight in male rats (26). This observation suggests an acute interaction between glucocorticoids and leptin to reduce food intake. Consistent with these finding, we show chronically stressed rats treated with E2 and PPT had elevated leptin levels. The rise in serum leptin may potentially explain the reduced body weight seen in stressed rats treated with PPT; yet, this stress-induced reduction in body weight was not seen with E2 treatment even though stress also increased leptin levels. Such a different response suggests that E2-treated animals may have reached a point where further loss of fat and weight would have been deleterious. It is possible that by decreasing the E2 concentration in our delivery system we would have seen a similar stress-induced reduction in body weight analogous to PPT treatment. On the other hand, leptin levels in stressed VEH and DPN treated rats did not increase relative to nonstressed VEH-treated animals even though there was stressed-induced decrease in body weight. This could be due to increased adiposity seen with VEH and DPN treatment leading to elevated leptin levels in both nonstressed and stressed groups as circulating leptin has been shown to correlate with body fat distribution (50, 51). Any stress-induced differences in leptin would have likely diminished due to increasing fat stores while this was not the case for E2 and PPT treated animals.

To assess the HPA axis response to chronic immobilization stress, we measured CORT levels across all groups. Our results show chronically stressed rats regardless of hormone treatment had diminished CORT. This finding is rather interesting since a previous study using the same immobilization protocol reported females exposed to daily immobilization for 13 days had increased ACTH and CORT levels, which were measured around the same time of the dark cycle we used in this study (48). However, with the current results, it is difficult to interpret whether chronic immobilization stress actually decreased the stress-induced secretion of CORT or it inhibited the diurnal rise in CORT since these values were measured in the early dark phase. In efforts to understand this difference, we measured CORT levels in OVX rats exposed to 1 week, 2 weeks, or 3 weeks of daily immobilization stress. Animals stressed for 1 week indeed had increased CORT; yet by 2 weeks of immobilization stress, CORT levels began to decrease; and finally by 3 weeks of chronic stress, circulating CORT was significantly reduced compared to nonstressed controls (unpublished data, Cruthirds DF, Larco DO, Wu TJ). Repeated immobilization stress has been shown to increase the expression of glucocorticoid receptors (GC) in the PVN (52). Thus, in our study, chronically stressed animals may have increased GC expression in brain regions that regulate the negative feedback response of the HPA axis to blunt further the diurnal release of CORT from the adrenals. Additionally, although not to the extent of nonstressed rats, E2 and DPN-treated stressed rats had higher CORT levels than stressed rats treated with VEH. This suggests that if the diurnal rise in CORT was inhibited by chronic immobilization stress, then ERβ may serve to counteract this effect. Whether this is the case in our stress paradigm remains to be directly addressed.

In the MBH, chronic stress altered leptin signaling. Stressed rats treated with E2 had reduced Ob-Rb and pSTAT3 levels. As a result, we expected SOCS3 levels to be elevated, however, chronic stress reduced SOCS3 in E2-treated rats in contrast to the increased SOCS3 levels in nonstressed E2-treated rats. It has been proposed SOCS3 is a negative regulator of CRH expression (53), thus, a stress-induced decrease in SOCS3 may enhance CRH action to activate the HPA axis under chronic stress conditions. However, this diminution in SOCS3 levels was not observed in stressed PPT or DPN-treated rats. This lends the possibility E2 may regulate SOCS3 expression independent of the classical ERs under chronic stress conditions. Furthermore, we observed divergent effects of E2 and the agonists on Ob-Rb and pSTAT3 expression in the MBH. Chronically stressed rats treated with PPT or DPN had reduced Ob-Rb expression similar to E2 treatment, yet, pSTAT3 levels were not altered. Others have reported, even though *sc* injections of E2 or PPT reduced food intake, that PPT treatment preferentially induced cFos expression in the PVN of the hypothalamus (43). They attributed this difference in cFos induction to the idea PPT is associating with a mER, which could activate cFos expression more rapidly than a classical ER (43). This is consistent with another study where female rats had increased cFos after 4 hours of E2 treatment in the ARC (54), in contrast, to the PPT-induced cFos that occurred as early as 90 minutes in the previous study (43). Collectively, these observations suggest PPT and E2 may not completely overlap in their mechanism of action to regulate body weight.

Other players of the HPA axis could also be contributing to stress effects on body weight. As already mentioned, we measured serum CORT and we observed that stressed rats had a significantly lower diurnal rise in CORT than nonstressed rats; however, we did not

measure CRH, ACTH, or vasopressin, which are involved in the initial steps of HPA activation. Mice over-expressing CRH have been shown to feed less and weigh less than their wild-type littermates (55) suggesting CRH could be acting on important energy control centers in the brain to regulate food consumption and body weight homeostasis. Additionally, ICV injection of CRH reduces food intake and is likely mediated by modulation of POMC transcription and processing (56-58). Further, central CRH can activate the sympathetic nervous system thereby increasing peripheral metabolism (59). In our chronic stress model, we did not see differences in feeding between nonstressed and stressed female rats even though stress decreased body weight with PPT and DPN treatment. Therefore**,** we speculate the stress induced increase in leptin as seen with PPT-treated stressed rats could be driving another system to increase energy expenditure and reduce body weight independent of feeding and possibly independent of STAT3 since we saw no significant effects of stress on pSTAT3 with PPT treatment. Interestingly, Morimoto and colleagues showed central injections of leptin increases CRH mRNA expression in the PVN (60) while treatment with a CRH antagonist blunts leptin's effects on metabolism. These observations suggest that CRH and leptin mediate their actions through a common pathway and may depend on each other to elicit their functions. Further investigation into the CRF and leptin interaction in our chronic stress paradigm is certainly warranted.

In summary, OVX female rats treated with E2 or PPT had reduced body weight and consumed less food than VEH and DPN treated animals. Furthermore, E2 treatment may enhance leptin action in the MBH to inhibit food intake and increase expenditure by up-regulating the Ob-Rb. However, chronic E2 treatment seems to activate a negative

feedback mechanism to modulate the leptin and insulin pathway by increasing SOCS3 levels. As for PPT and DPN, the ER agonists diverged in function to regulate the central markers measured. We speculate other ER complexes such as a mER is contributing to E2 effects in the MBH. Chronic immobilization stress, on the other hand, decreased body weight without any apparent effects on food consumption. We saw a stress-induced increase in leptin, suggesting this adipokine could be contributing to the reduction in body weight. Yet, in the MBH, chronic immobilization stress reduced Ob-Rb expression indicating other mediators are involved. Finally, chronically stressed rats had altered HPA axis activity since these rats had diminished levels of CORT at the end of the stress phase. The results of the present study suggest that the estrogen and stress interaction on body weight is complex. Future studies should consider the differential effects of E2 to PPT and DPN treatment in the brain; and how glucorticoids under chronic stress feeds into this pathway.

Figure Legends

Figure 1. Effect of hormone treatment and stress on body weight. (A) Constant infusion of E2 or PPT (ERα agonist) for 26 days prevents post-ovariectomy weight gain in female rats compared to VEH and DPN (ERβ agonist) treatment. (B) Similarly, stressed rats treated with either E2 or PPT had lower body weight compared to stressed rats treated with VEH or DPN treatment; however, the combination of stress and DPN treatment decreased body weight relative to VEH. (C) Daily immobilization stress decreased body weight in VEH, PPT and DPN treated rats relative to respective hormone counterparts. Data points represent daily mean body weight values ± SEM of n=12 animals per group. * $p<0.05$ or # $p<0.1$

Figure 2. Effect of hormone treatment and stress on food consumption. (A,B) E2 and PPT (ERα agonist) treatment decreased food intake in all weeks of the stress phase compared to VEH treated rats. B) DPN treatment reduced food intake only during the middle week of the stress phase. No other effects of stress reached significance. Data points represent daily mean weight values ± SEM for food consumption during each week of the experimental phase of n=12 animals per group. * $p<0.05$ vs. VEH

Figure 3. Effect of hormone treatment: VEH, E2, PPT (ERα agonist), or DPN (ERβ agonist); and stress (no stress, open bars vs. 1 hour immobilization stress per day, dark bars) on activity of rats measured by total distance traveled during a 1 hour period in a locomotor chamber summed over four separate time points throughout the stress phase. Hormone and stress had no effect on total distance traveled relative to control. Bars represent mean values ± SEM of n=12 animals per group.

Figure 4. Effect of hormone and stress on serum leptin, insulin, and resistin. Blood serum was extracted from all subjects on the final day of stress (Post-OVX day 26) and analyzed for leptin, insulin, and resistin. (A) Leptin levels in animals treated with E2 and PPT (ERα agonist) were significantly lower compared to VEH and DPN (ERβ agonist) groups but not in stressed animals. (B) E2 significantly elevated insulin levels, yet is reversed by stress. Similarly, PPT treated animals, which were not stressed, trended ($p<0.1$) towards having higher circulating insulin compared to control but stress reversed this increase. (C) Conversely, E2 and PPT significantly reduced resistin levels compared to VEH and DPN treated animals. Bars represent mean values ± SEM of n=12 animals per group. * $p<0.05$ or # $p<0.1$

Figure 5. Effect of hormone and stress on serum corticosterone. Blood serum was measured for CORT on Post-OVX day 26. Daily exposure to immobilization for 22 days significantly decreased CORT levels compared to non-stressed animals. Interestingly, E2 and DPN (ERβ agonist) treated groups that were stressed had elevated CORT levels relative to stressed VEH treated animals. Bars represent mean values ± SEM of n=12 animals per group. * $p<0.05$ or # $p<0.1$

Figure 6. Effect of hormone treatment and stress on leptin signaling in the medial basal hypothalamus. The medial basal hypothalamus (MBH) was dissected on ice and expression of Ob-Rb, pSTAT3, SOCS3, and pAKT was assessed by western blot analysis. (A) E2 treatment significantly increased ($p<0.05$) Ob-Rb expression in the MBH relative to control animals. Chronic stress reduced expression in animals treated with E2 ($p<0.10$), PPT ($p<0.05$), and DPN ($p<0.10$) compared nonstressed counterparts. (B) Stress decreased pSTAT3 in VEH ($p<0.05$) and E2 ($p<0.05$) treated animals while

PPT and DPN treatment attenuated this effect. (C) E2 ($p<0.05$) and to a lesser extent DPN ($p<0.10$) treatment increased SOCS3 expression. Stress moderately reduced SOCS3 only with E2 treatment ($p<0.10$) while PPT and DPN treatment significantly increased expression ($p<0.05$) relative to stressed VEH-treated animals. (D) PPT and DPN treatment regardless of stress exposure was significantly up-regulated ($p<0.05$) in the MBH. Bars represent mean values ± SEM of n=3-4 animals per group. * $p<0.05$ or # $p<0.1$

Figure 1

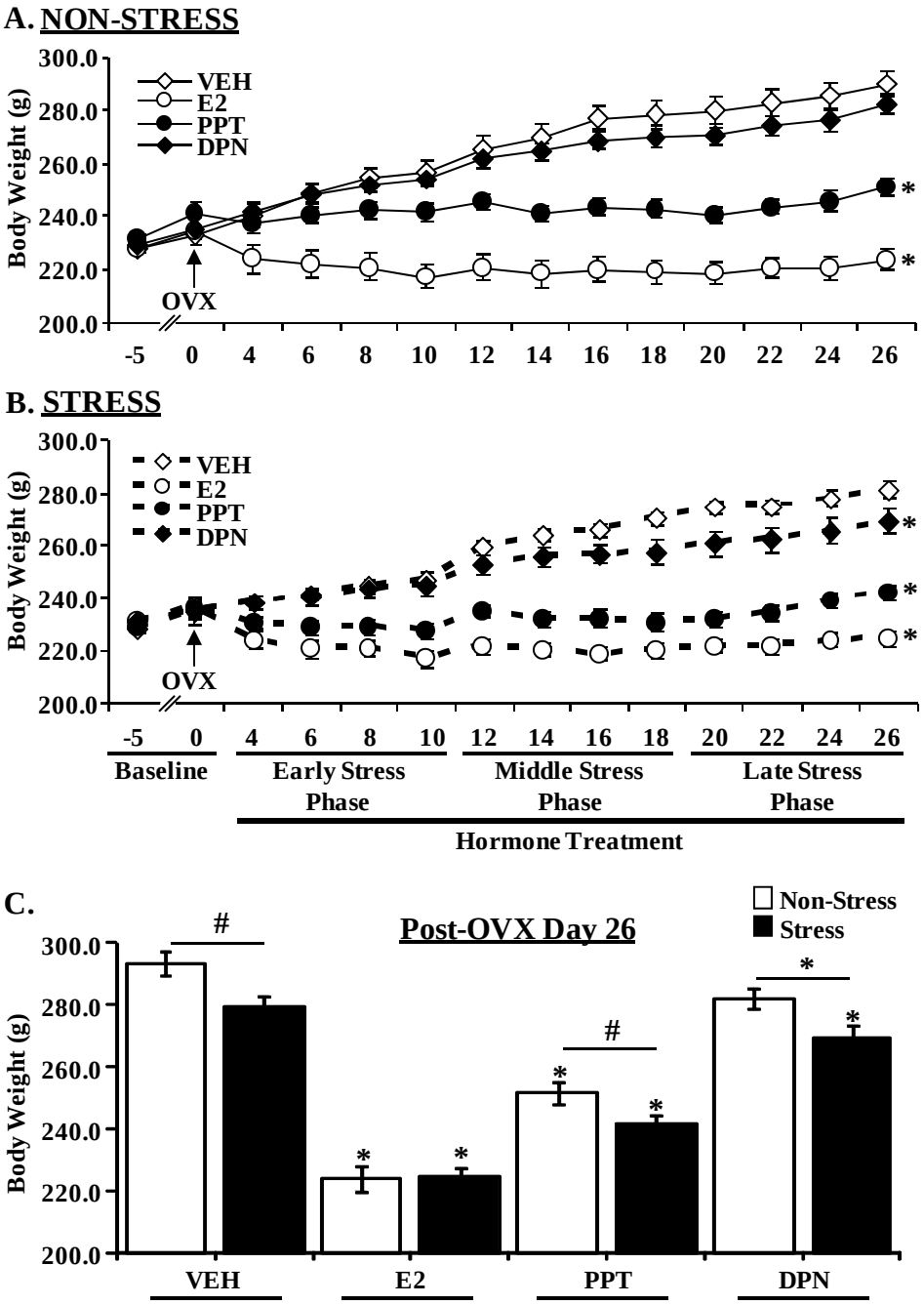

A. NON-STRESS
Body Weight (g)
300.0
280.0
260.0
240.0
220.0
200.0
VEH
E2
PPT
DPN
OVX
-5 0 4 6 8 10 12 14 16 18 20 22 24 26
B. STRESS
Body Weight (g)
OVX
Baseline
Early Stress Phase
Middle Stress Phase
Late Stress Phase
Hormone Treatment
C.
Post-OVX Day 26
Non-Stress
Stress
Body Weight (g)
VEH
E2
PPT
DPN

Figure 2

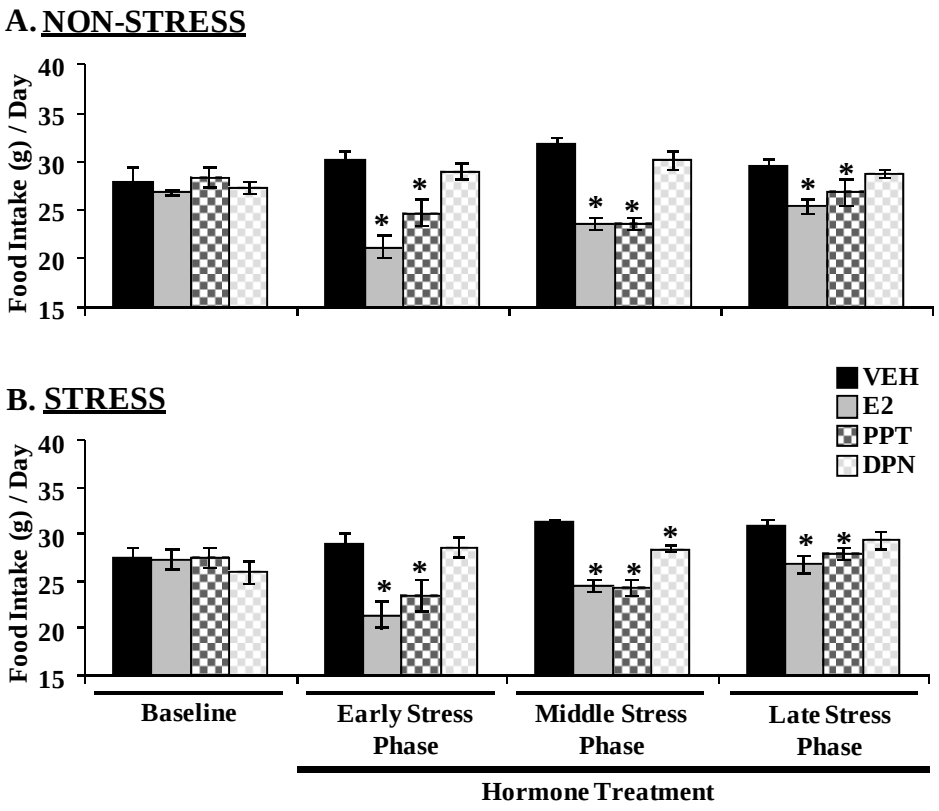

A. NON-STRESS
Food Intake (g) / Day
40
35
30
25
20
15
B. STRESS
VEH
E2
PPT
DPN
Baseline
Early Stress Phase
Middle Stress Phase
Late Stress Phase
Hormone Treatment

Figure 3

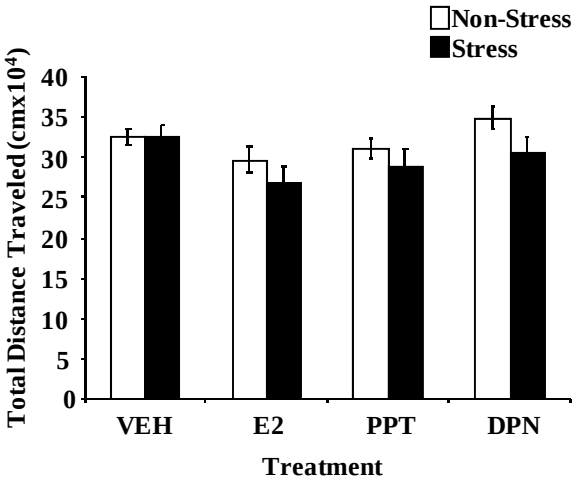

Non-Stress
Stress
Total Distance Traveled (cmx10⁴)
40
35
30
25
20
15
10
5
0
VEH
E2
PPT
DPN
Treatment

Figure 4

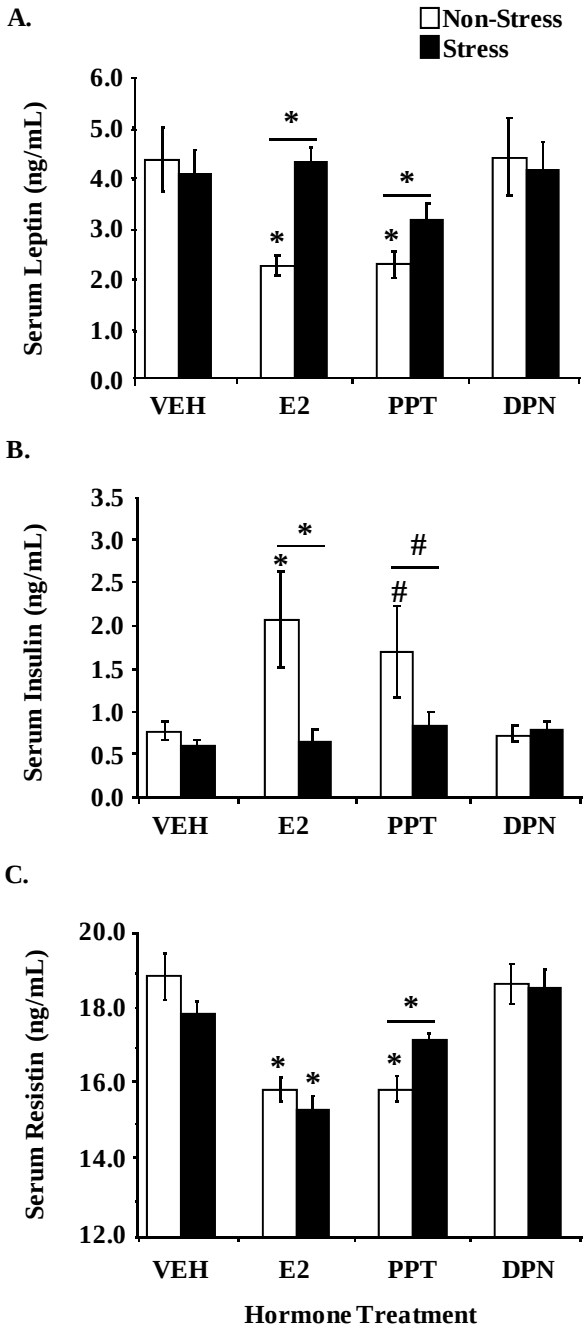

A.
Non-Stress
Stress
Serum Leptin (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VEH
E2
PPT
DPN
B.
Serum Insulin (ng/mL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
VEH
E2
PPT
DPN
C.
Serum Resistin (ng/mL)
20.0
18.0
16.0
14.0
12.0
VEH
E2
PPT
DPN
Hormone Treatment

Figure 5

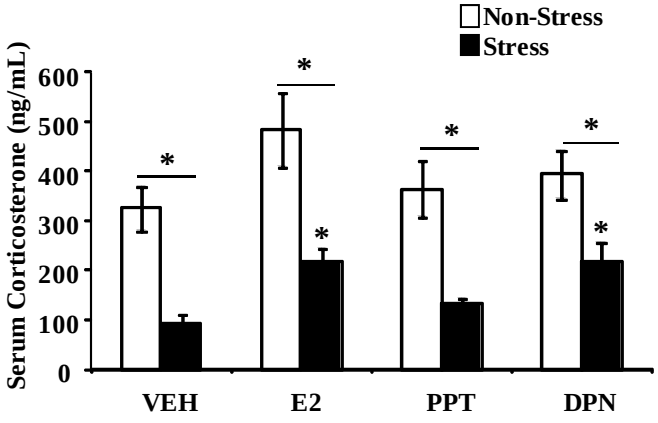
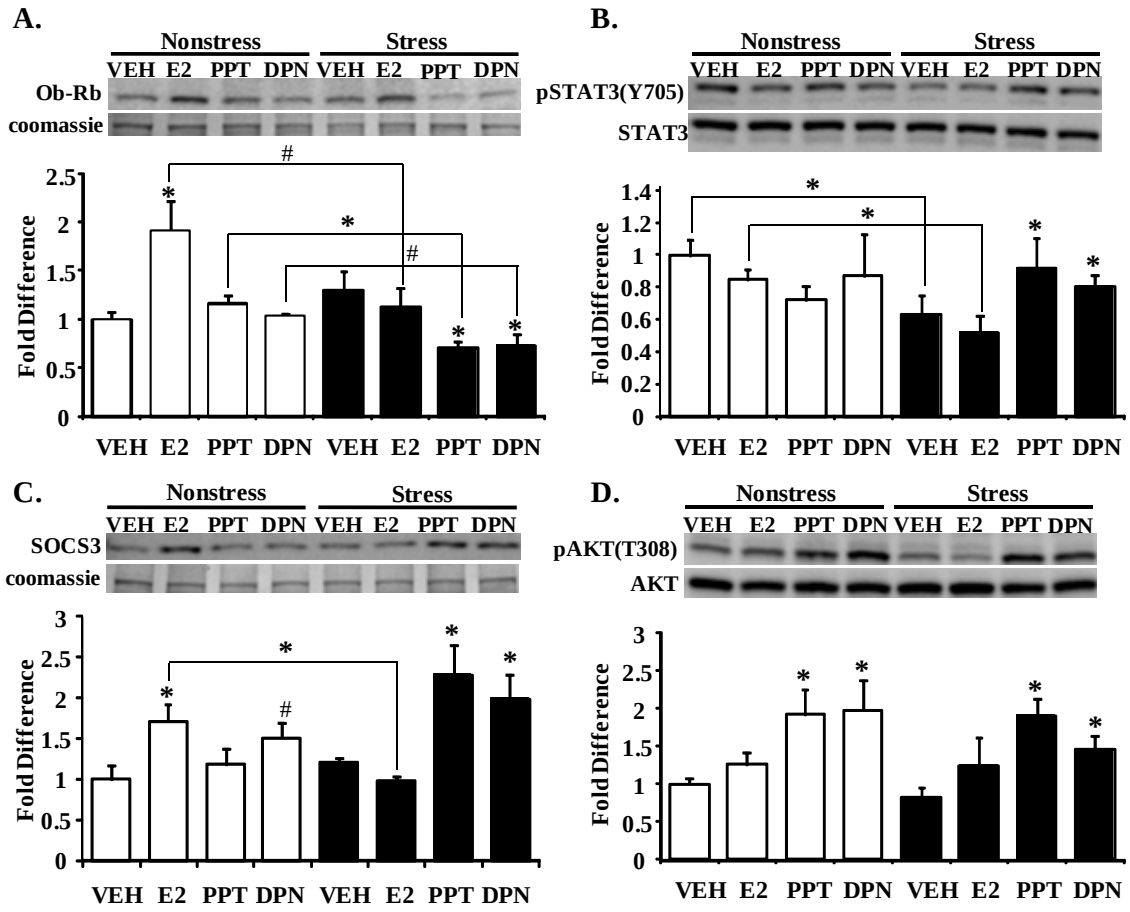

Non-Stress
Stress
Serum Corticosterone (ng/mL)
600
500
400
300
200
100
0
*
*
*
*
*
VEH
E2
PPT
DPN

Figure 6


A.
Nonstress
Stress
VEH E2 PPT DPN VEH E2 PPT DPN
Ob-Rb
coomassie
Fold Difference
B.
pSTAT3(Y705)
STAT3
C.
SOCS3
D.
pAKT(T308)
AKT

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CHAPTER 6

DISCUSSION AND CONCLUSIONS

Stress is a normal response that allows the body to react to threatening situations. If properly coordinated, the stress response is transient and the body's physiological systems are retuned to pre-threatening conditions. However if that threat is perceived by an individual to be too intense or chronic, it may lead to disabling disorders. PTSD is one such disorder brought on by exposure to a traumatic stressor. The etiology of PTSD is currently unknown and contributing to this knowledge gap is the lack of a valid animal model to study the disease. None to date consider the neuroendocrine aspect of PTSD. The primary goal of this study was to determine whether chronic immobilization stress in a rat can reliably mimic PTSD symptoms (Table 2). In this study, we examined the gaps in this stress model. Furthermore, we evaluated a novel pharmacotherapy for prevention of the disabling factors associated with this disorder.

In order to understand the etiology of PTSD, an animal model must fulfill certain criteria that parallel the human disease. These are depicted in Table 2. The chronic rat immobilization model has many of the characteristics of PTSD but remain to be further developed. To further test the model, we used the OVX Sprague-Dawley rats that were subjected 1 hour daily immobilization stress paradigm using a finger-like Centrap cage over a 22 day time period. The timing of the immobilization stress and all testing occurred during the early dark phase of the rat's circadian rhythm when the animal is active [1-2]. To strengthen the predictive value validity of this animal model for PTSD, several conditions must be met: (1) CORT levels must not be different or lower than CORT levels in nonstressed (control) rats, (2) Animals must display heightened anxiety, (3) Animals must also express exaggerated startle response, In addition, there are other

parameters that are important to considered to include decreased activity, decreased body weight gain, and increased acute pain thresholds.

Our study shows that CORT levels were significantly decreased from baseline by the late-phase of stress (days 14-22). Interestingly, there appears to be a developmental change in the circulating CORT levels that is dependent on the length of exposure to the immobilization stressor. During the early-phase (7 days) CORT levels were elevated in agreement with other studies [3-4]. CORT levels then returned to baseline in the mid-phase (10-14 days), again consistent with other studies [5-7]. To our knowledge, this is the first report correlating this HPA response to daily immobilization stress lasting 1 hour over a period of 22 days in the rodent and that CORT levels reflect a development response to the stressor in that is time-dependent. Decreased and normal levels of cortisol have been found in clinical studies with patients suffering from PTSD [8-9]. Stress hormone levels enabled the evaluation for convergent validity of the repeated daily restraint stress model in accordance with a putative neuroendocrine marker of PTSD. This is believed to occur from either an enhanced inhibition of the HPA axis [10 11] or from a decrease in sensitivity of the pituitary and adrenals when stimulated [12].

To strengthen the predictive validity of our stress paradigm, anxiety responses were measured in the late period of the experimental phase. Stressed animals displayed more anxiety like behavior when compared to non-stressed animals. This suggests that this stressor is sufficient enough to change the animal's emotionality. These results are in agreement with other studies that have used repeat immobilization stress [13 14]. A core feature of PTSD, anxiety like behavior represents symptoms of numbing and avoidance [15]. Likely due to altered regions of the brain particularly the amygdala, hippocampus and

prefrontal cortex [16-19]. However, further studies are needed to elucidate their exact role and influence as well as determining a possible mechanism linking of anxiety and stress together.

A major characteristic of PTSD is hypervigilance which is reflected in the laboratory as exaggerated startle [20-21]. Elicitation of this stimulus in both rats and humans has occurred using identical stimulus parameters therefore allowing results of animals studies be generalized to humans [22]. In the present study, stressed animals showed exaggerated startle in response to the acoustic stimulus at all time points tested. This finding suggests that animals did not habituate to the immobilization stressor over time. Studies using restraint stress, have suggested that habituation may occur if repeated stress last longer than 10 days[23]. Accounting for this difference is that immobilizations stress is a psychological stressor and restraint stress is a physical stressor still allowing the animal to maintain a measure of control within the restrainer such as barrel rolling. This finding showcases the criteria that this stressor induces biological alterations that persist over time. In addition to the exaggerated startle, we found that chronic immobilization stress reduces the effects of PPI. This suggests stressed animals have an altered sensorimotor gating and pre-attentive information processing [24].

The general activity level of the animal was examined with locomotor testing over the three timepoints (early, mid and late phases). Activity was decreased in late phase but not the early and mid phases of stressed animals suggesting that lower CORT levels may influence general activity levels. This is in line with the concept of homeostasis and the importance that stress hormones have in promoting replenishment of the body's energy reserves [25-26]. Furthermore reduced activity during locomotor/open field testing

can indicate a depressive state [27] [28] which is a frequent comorbid disorder associated with PTSD [29]. In addition, a study specifically addressing physical activity in patients with PTSD found a significant reduction in physical activity following the onset of the disease [30].

The concept of pain and the role body weight are additional areas of interest addressed in the PTSD literature. Both are altered by chronic stress [31-34]. When exposed to an acute thermal pain stressed rats had an increase in pain tolerance. Elevation of circulating plasma β-endorphin levels despite hormone treatment is one possible explanation for the improved pain thresholds. Reduced pain sensitivity in the animals that were stressed is consistent with clinical observations reported in combat veterans suffering from PTSD [35-36]. Body weight has been implicated in symptom severity for patients suffering from PTSD [37-39]. We found a reduction in body weight gain in stressed animals which is a common occurrence in animal models of both acute and chronic stress [34, 40]. It appears stress plays a role on body weight gain and regions of the amygdala and alterations in leptin receptors are involved but further study is needed.

Manipulation of ERs, which co-located in many brain regions associated with stress and behavior, with E2 and ER agonist offered a novel site of manipulation [41]. Our results showed a reduction in locomotor and non-locomotor (stereotypy) across all time points, reduced anxiety-like behavior and improved PPI during EPM testing, reduced startle response, and enhanced pain tolerance. Improvements in behavior from estradiol treatment appear to be independent of corticosterone levels as there was no change back to baseline. E2 has been shown to alleviate anxiety and depression, a frequent comorbidity of PTSD, in humans [42-44]. While results from rodent studies remained

mixed, with some studies indicating that E2 increases anxiety and others showing that E2 decreases anxiety [45-46], our results indicate under these conditions ER stimulation, more specifically ERα, is protective. Activation of ERα also appears to be responsible for the reduction in startle response across all timepoints under nonstress and stress conditions. The mechanism of action for this improvement remains to be elucidated but influence in serotonergic [47-52], dopaminergic [53-55] and cholinerestase [56-57] pathways might be involved. The reduction in locomotor activity was surprising and not fully understood. It is postulated that E2 may have an inhibiting effect on dopamine receptors which are centrally located in motor function regions (mesostriatal and mesolimbic systems) of the brain [58]. E2 increased pain thresholds but specifically ERα stimulation, in conditions of nonstress and stress. Stimulation of ERβ only increased pain thresholds in stress conditions suggesting a possible glucocorticoid interaction. In addition, rats treated with E2 or PPT had reduced body weight and consumed less food than VEH and DPN treated animals. While reduced body weights have been associated with increased PTSD symptom severity, this did not occur E2 and PPT reduced anxiety and startle response. The chronic immobilization stress model appears to be consistent with symptoms associated with the human condition. Furthermore, findings fall within many of the criteria established by Rachel Yehuda and Seymour Antelman[59] for a useful animal model to study PTSD. Our studies are consistent with some criteria while others will need to be further developed. In the present study, the immobilization stress is brief and easily managed in a manner to result in biological and behavioral changes consistent with PTSD symptoms. These changes included decreased plasma CORT level, exaggerated ASR and increased anxiety. These biological and behavioral changes occurred in a

developmental fashion some persisting over time and others becoming more pronounced. Long term studies are needed to establish how far the noted behavioral changes would occur or if extinction, decline or disappearance in detrimental response, would take place. Our results, after exposure to immobilization stress, showed biobehavioral alterations cycling between excitatory and inhibitory changes. Stressed animals displayed exaggerated ASR, and increased anxiety levels but at the same time had reduced activity levels. While our results utilizing standard behavioral assays, further studies using modified anxiety and cognitive measures are needed specifically for PTSD studies along with anatomical approaches to determine the neurocircuitry involved. Finally, these studies pave the way for future studies on individual differences. These may be accomplished using a comparison of known genetic strains of animals that may have varying vulnerabilities to anxiety, such as the Long Evans and the Kyoto Wistar strains. Furthermore, these studies also pave the way for epigenetic studies by examining vulnerabilities to stress(es) using epigenetic models.

Establishment a valid animal model for PTSD has proved to be very difficult because of the complexity of its etiology and expression [59-60]. Allostasis maintains homeostasis by creating new setpoints through continual reevaluation of bodily systems to anticipate needs. Physiologic and behavioral responses are then adjustment of to meet those needs. It is hypothesized past experiences influence what is anticipated therefore influencing new setpoints [25]. It is likely the trauma suffered by PTSD patients influence what is perceived as stressful, alter anticipated needs, and ultimately deviate response systems to far from homeostasis. Disruption of the HPA axis appears to be an important physiologic system involved in PTSD. Important findings with regards to alterations in

plasma CORT levels and behavior following chronic immobilization stress have added increasing the predictive validity of this model. Additional studies are needed to elucidate the mechanisms of actions responsible for these findings. Studies confirming structural changes in specific brain regions are also needed. The role of estrogen receptor stimulation as a preventive treatment for PTSD has shown some promising results but additional studies are needed to elucidate likely mechanisms of action and rule out any detrimental effects.

Table

	Human PTSD	Rodent Immobilization (Acute)	Rodent Immobilization (Chronic)
Body Weight	Weight loss [224-226]	Decreased weight gain and decreased feeding[227]	Decreased weight gain and decreased feeding [228-229] (Cruthirds (et al)
Heart Rate	Elevated basal heart rate [230-231]	Elevated heart rate & mean arterial pressure post stressor [232]	Unknown
Glucocorticoid	Decreased/no change in cortisol levels [109, 233]	Elevations in corticosterone levels [41]	Reduction in corticosterone levels (Cruthirds (et al)
Norepinephrine	Elevations of urine and CNS norepinephrine levels [233-235]	Elevated microdiasylate norepinephrine levels in the medial amygdala[236]	Elevated plasma norepinephrine [237]
Hippocampus	Decrease in size of Hippocampus [83-85, 238]	No change in apical dendrites of the CA3 pyramidal neurons [239]	Atrophy of apical dendrites of the CA3 pyramidal neurons [20-21]
Amygdala	Increased activation of the amygdala after symptom provocation [238]	Reduced dendritic arborization posterodorsal medial region [240]	Enhanced dendritic arborization in basolateral region[21]
Anxiety	Elevated anxiety levels [241]	Elevated anxiety levels [242]	Elevated anxiety levels [242] (Cruthirds (et al)
Memory	Logical memory deficits [112, 243] Enhanced declarative memory [244]	Impaired spatial memory [245-246]	Unknown
Startle	Exaggerated Startle Response [32, 247]	Exaggerated Startle Response [22]	Exaggerated Startle Response (males only)[22] (Cruthirds (et al)
Pain	Increased pain thresholds to thermal pain [248]	Tail flick hypoanalgesic [249] Thermal hot plate hypoanalgesic [250-253]	TMJ/Tail Flick-hyperalgesia [242, 249] Increased thermal pain threshold (Cruthirds (et al)

Table 1: Comparison of PTSD Symptoms to Acute and Chronic immobilization stress induced dysfunction in rodents

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