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An Ethologically Relevant Animal Model of Post-Traumatic Stress Disorder:  
Physiological, Pharmacological and Behavioral Sequelae in Rats Exposed to  
Predator Stress and Social Instability

by

Phillip R. Zoladz

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
Department of Psychology  
College of Arts and Sciences  
University of South Florida

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*For my loving wife, Meagan. If it were not for you, I would never have made it this far.*

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a debilitating mental illness that results from exposure to intense, life-threatening trauma. Some of the symptoms of PTSD include intrusive flashback memories, persistent anxiety, hyperarousal and cognitive impairments. The finding of reduced basal glucocorticoid levels, as well as a greater suppression of glucocorticoid levels following dexamethasone administration, has also been commonly observed in people with PTSD. Our laboratory has developed an animal model of PTSD which utilizes chronic psychosocial stress, composed of unavoidable predator exposure and daily social instability, to produce changes in rat physiology and behavior that are comparable to the symptoms observed in PTSD patients. The present set of experiments was therefore designed to 1) test the hypothesis that our animal model of PTSD would produce abnormalities in glucocorticoid levels that are comparable to those observed in people with PTSD, 2) examine the ability of antidepressant and anxiolytic agents to ameliorate the PTSD-like physiological and behavioral symptoms induced by our paradigm and 3) ascertain how long the physiological and behavioral effects of our stress regimen could be maintained.

The experimental findings revealed that our animal model of PTSD produces a reduction in basal glucocorticoid levels and increased negative feedback sensitivity to the synthetic glucocorticoid, dexamethasone. In addition, chronic prophylactic administration of amitriptyline (tricyclic antidepressant) and clonidine ( $\alpha_2$ -adrenergic receptor agonist) prevented a subset of the effects of chronic stress on rat physiology and behavior, but tianeptine (antidepressant) was the only drug to block the effects of chronic stress on all physiological and behavioral measures. The final experiment indicated that only a subset of the effects of chronic stress on rat physiology and behavior could be observed 4 months following the initiation of chronic stress, suggesting that some of the effects of our animal model diminish over time. Together, these findings further validate our animal model of PTSD and may provide insight into the mechanisms underlying trauma-induced changes in brain and behavior. They also provide guidance for pharmacotherapeutic approaches in the treatment of individuals suffering from PTSD.

## Chapter One: Background

### *Definition of Post-Traumatic Stress Disorder*

Individuals who are exposed to intense trauma that threatens physical injury or death, such as rape, wartime combat and motor vehicle accidents, are at significant risk for developing post-traumatic stress disorder (PTSD). People who develop PTSD respond to a traumatic experience with intense fear, helplessness or horror (American Psychiatric Association, 1994) and subsequently endure chronic psychological distress by repeatedly reliving their trauma through intrusive, flashback memories (Ehlers et al., 2004; Hackmann et al., 2004; Reynolds & Brewin, 1998; Reynolds & Brewin, 1999; Speckens et al., 2006; Speckens et al., 2007). These intrusions are frequently precipitated by the presence of cues associated with the traumatic event; therefore, PTSD patients make great efforts to avoid stimuli that remind them of their trauma. The re-experiencing and avoidance symptoms of the disorder significantly hinder everyday functioning in PTSD patients and foster the development of several additional debilitating symptoms, including persistent anxiety, exaggerated startle, cognitive impairments, diminished extinction of conditioned fear and pharmacological abnormalities, such as an increased sensitivity to yohimbine (Brewin et al., 2000; Elzinga & Bremner, 2002; Nemeroff et al., 2006; Newport & Nemeroff, 2000; Stam, 2007a).

### *Susceptibility to Post-Traumatic Stress Disorder*

Only about 25% of traumatized individuals develop PTSD (Ozer et al., 2003; Ozer & Weiss, 2004; Yehuda, 2004). While nearly every traumatized person displays re-experiencing, avoidance and hyperarousal symptoms in the acute aftermath of trauma (McFarlane, 2000), only a minority continue to exhibit these symptoms for a period of at least 1 month and fulfill the requirements set forth by the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 1994) for a diagnosis of PTSD (Yehuda & LeDoux, 2007). Thus, in approximately 75% of traumatized individuals, the re-experiencing, avoidance and hyperarousal symptoms subside within a 1-month time frame, and at least one-third of those who continue to display the symptoms at 1-month post-trauma recover within 3 months (Kessler et al., 1995). Thus, the natural response to trauma is recovery, and only a subset of traumatized individuals develops chronic forms of the disorder.

### *Heightened Arousal in Post-Traumatic Stress Disorder*

#### *Elevated Sympathetic Nervous System Activity*

PTSD is characterized by a complex aberrant biological profile involving several physiological systems, one of which is the sympathetic nervous system (SNS). Extensive work has demonstrated that PTSD patients exhibit greater baseline and stress-induced elevations of sympathetic activity than control subjects (Buckley & Kaloupek, 2001; Pole, 2007). In response to traumatic reminders and standard laboratory stressors, people with PTSD display significantly greater increases in heart rate (HR), blood pressure (BP), skin conductance, epinephrine (EPI) and norepinephrine (NE) than do control subjects

(Blanchard et al., 1982; Blanchard et al., 1991; Casada et al., 1998; Kolb & Mutalipassi, 1992; Malloy et al., 1983; McFall et al., 1990; Orr et al., 1998; Pitman et al., 1987; Rabe et al., 2006; Schmahl et al., 2004; Shalev et al., 1993; Veazey et al., 2004). In addition, PTSD patients exhibit significant elevations of baseline HR, systolic BP and diastolic BP (Buckley & Kaloupek, 2001; Pole, 2007), findings that resonate with recent work reporting an association between PTSD and increased risk for cardiovascular disease (Boscarino & Chang, 1999; Kubzansky et al., 2007; Sawchuk et al., 2005).

A vast literature has also implicated increased baseline noradrenergic activity in individuals suffering from PTSD. Several studies have shown that PTSD patients exhibit abnormally high levels of baseline NE (Geraciotti et al., 2001; Kosten et al., 1987; Southwick et al., 1999a; Strawn & Geraciotti, 2008; Yehuda et al., 1998), levels that have been shown to positively correlate with the severity of symptoms in PTSD patients (Geraciotti et al., 2001). Another indication of accentuated sympathetic activity in people with PTSD is the hyperresponsivity they exhibit to the administration of yohimbine, an  $\alpha_2$  adrenergic receptor antagonist which blocks noradrenergic autoreceptors and leads to increased central norepinephrine activity (Rasmusson et al., 2000; Southwick et al., 1993; Southwick et al., 1999c; Southwick et al., 1999a; Southwick et al., 1999b). Southwick and colleagues (Southwick et al., 1993) found that, following yohimbine administration, 70% of PTSD patients experienced panic attacks, and 40% experienced flashbacks. PTSD patients also exhibited significantly greater HR, systolic BP, anxiety-related behavior and acoustic startle responses to the drug (Morgan et al., 1995b).

Bremner and colleagues (Bremner et al., 1997a) suggested that these findings may be related to reduced NE catabolism in PTSD patients. To test this hypothesis, the investigators gave PTSD patients a single bolus of [F-18]2-fluoro-2-deoxyglucose, a compound that is taken up by high-glucose-using cells, immediately prior to intravenous injections of yohimbine. Approximately an hour later, the investigators measured cerebral metabolic activity in participants by employing positron emission tomography (PET). The PET scans revealed that, relative to healthy controls, PTSD patients displayed significantly lower levels of glucose metabolism in several neocortical brain regions that are highly innervated by noradrenergic nerve fibers, suggesting the presence of reduced NE catabolism in people with PTSD. The anxiogenic effects of yohimbine in people with PTSD have also been linked to the presence of fewer and less sensitive  $\alpha_2$  adrenergic receptors and lower levels of plasma neuropeptide Y (NPY) in PTSD patients (Perry et al., 1990; Perry, 1994; Rasmusson et al., 2000). NPY is a peptide neurotransmitter that is colocalized with NE in most sympathetic nerve fibers and within the locus coeruleus, an area of the dorsal pons that contains the major cell bodies of the noradrenergic system. The peptide typically inhibits the release of the neurotransmitter with which it is colocalized. Rasmusson et al. (2000) found that PTSD patients had lower baseline plasma levels of NPY, as well as a smaller increase in NPY levels in response to a yohimbine challenge paradigm. This finding could explain the presence of greater baseline NE levels, as well as greater reactivity to yohimbine, in PTSD patients.

Related to the symptoms of hyperarousal and greater noradrenergic activity, an exaggerated startle response is often presented as a core symptom of PTSD (Grillon et al.,

1996). The startle response is defined as the rapid sequence of flexor motor movements that occurs after the onset of a briefly-presented, intense stimulus (Morgan, 1997). Approximately 85-90% of trauma survivors with PTSD subjectively report having an increased startle response (Shalev et al., 1997). However, empirical investigations examining the startle response in PTSD patients have presented conflicting results. While some studies have found heightened startle in people with PTSD (Butler et al., 1990; Grillon et al., 1998; Morgan et al., 1995a; Morgan et al., 1996; Morgan et al., 1997; Orr et al., 1995; Shalev et al., 1997), others have found no differences between PTSD patients and control subjects (Elsesser et al., 2004; Grillon et al., 1996; Lipschitz et al., 2005; Orr et al., 1997; Siegelaaar et al., 2006). The exaggerated startle response often observed in PTSD patients may not be due to a stable trait of these individuals, but rather an acute state of conditioned fear or anxiety (e.g., anticipatory anxiety) that they experience during the experimental assessment. In support of this hypothesis, several studies have found that manipulations of the experimental context or the presentation of explicit threat cues consistently leads to enhanced startle responses in PTSD patients (Grillon et al., 1998; Grillon & Morgan, 1999; Pole et al., 2003).

Investigators have also faced the challenge of determining whether or not the exaggerated startle response observed in people with PTSD is a secondary effect of the disorder or a predisposing risk factor that increases one's susceptibility to develop the disorder. In a recent study, Guthrie and Bryant (2005) assessed the auditory startle response of firefighters before and after they had been exposed to trauma. Although none of the firefighters who were exposed to trauma developed PTSD during the course of the

study, they did display more symptoms (e.g., intrusive memories, avoidance) of the disorder after the trauma than firefighters who had not been exposed to a traumatic event. More importantly, the investigators found that the magnitude of the pre-trauma startle response predicted the development of acute PTSD symptoms. These findings suggest that an exaggerated startle response may not necessarily be a secondary effect of PTSD; rather, it could be a pre-existing factor that increases one's susceptibility to develop the disorder.

Despite the inconsistent findings on baseline startle responses in PTSD patients, research has reliably shown that upon exposure to briefly-presented, intense stimuli (e.g., loud tones), people with PTSD exhibit significantly greater autonomic reactivity than do controls (Metzger et al., 1999; Orr et al., 1995; Orr et al., 1997; Shalev et al., 1997; Shalev et al., 2000; Siegelaaar et al., 2006). This includes a failure to physiologically habituate to the stimuli, in addition to the elicitation of greater autonomic responses from the onset of the stimuli. In a recent study, Siegelaaar et al. (2006) found that although PTSD patients did not display an exaggerated startle response, relative to control subjects, they did exhibit significantly greater autonomic reactivity, in the form of galvanic skin response, to the test stimuli. The investigators contended that the presence of greater autonomic activity following the presentation of startling stimuli may explain why PTSD patients subjectively report exaggerated startle responses, despite not exhibiting them behaviorally. Ultimately, these findings suggest that while it is unclear whether or not individuals with PTSD exhibit a heightened baseline startle response, they do tend to display exaggerated autonomic reactivity to sudden, intense stimulation.



### *Contribution of the Parasympathetic Nervous System to Sympathetic Overdrive*

The parasympathetic nervous system (PNS), which is metaphorically considered the brakes on the SNS since it reduces SNS activity, makes a significant contribution to the maintenance of HR (Berntson et al., 1993). The vagus nerve, which is an important part of the PNS, innervates the sinoatrial node on the right atrium of the heart, where electrical impulses are generated to trigger cardiac contraction. By modulating the sinoatrial node, the vagus nerve slows HR and helps to maintain a balance between the SNS and PNS. Vagal modulation of HR is important for reactions to and recovery from stressful situations, and has been considered a possible mechanism for the differences in basal HR and changes in HR due to trauma-related cues in individuals with PTSD (Sahar et al., 2001).

Most studies monitoring PNS activity in PTSD patients have utilized heart-rate variability (HRV) as the primary dependent measure. HRV is a measure of beat-to-beat alterations in heart rate, or more specifically, the variability of the intervals between R waves. The two main frequency bands that are examined during HRV assessment are the Low-Frequency (LF) band (0.04 to 0.15 Hz), which is influenced primarily by the SNS, and the High-Frequency (HF) band (0.15 to 0.40 Hz), which is influenced primarily by the PNS (Sahar et al., 2001). Cohen and colleagues (Cohen et al., 1997; Cohen et al., 1998; Cohen et al., 2000a) found that, at rest, PTSD patients displayed greater HR and lower HRV than healthy control subjects. Furthermore, these patients demonstrated lower HF and higher LF components than controls, suggestive of enhanced sympathetic and reduced parasympathetic tone, respectively. Sahar et al. (2001) examined the vagal

modulation of HR in PTSD patients in response to a mental challenge by using respiratory sinus arrhythmia (RSA) as their dependent measure. RSA is the natural fluctuation in heart rate that occurs during the breathing cycle, and changes in RSA have been shown to reflect activity of the vagus nerve (Berntson et al., 1993). Sahar and colleagues (Sahar et al., 2001) found that PTSD patients and traumatized control subjects did not differ on resting levels of parasympathetic activity. However, when faced with a challenging arithmetic task, control subjects showed a significant increase in RSA (which was highly correlated with their HR), while PTSD patients showed no such increase. Thus, vagal mechanisms contributed to HR regulation in control subjects, but not in PTSD patients. These findings suggest that vagal modulation of HR may be impaired in PTSD patients, resulting in poor control of stress-induced changes in HR and increased risk for exaggerated sympathetic tone.

#### *Conclusion on Sympathetic Overdrive in Post-Traumatic Stress Disorder*

Sympathetic overdrive has been hypothesized to contribute to the hyperarousal symptoms observed in PTSD patients. It is also potentially responsible for their enhanced acquisition of conditioned fear and their “over-consolidation” of the original traumatic memory (Cahill et al., 1994; Cahill & McGaugh, 1998; McGaugh et al., 1996; Pitman, 1989). Many researchers have used conditioning theory to explain the development of PTSD (Garakani et al., 2006; Wessa & Flor, 2002). These investigators have speculated that during the trauma, the plethora of cues (CSs) to which an individual is exposed becomes associated with the life-threatening experience (US) that he or she is enduring and eventually elicits feelings of intense fear (CRs) similar to those (URs) experienced

during the original traumatic event. In theory, individuals who are more susceptible to developing the disorder would exhibit more intense fear responses to the trauma, which would then be more strongly associated with the cues from the environment. This would subsequently cause these individuals to exhibit exaggerated physiological and behavioral responses to the presence of trauma-related cues and compel them to avoid reminders of their trauma. One mechanism that could explain the enhanced consolidation of traumatic memories in PTSD patients is excessive adrenergic activity at the time of trauma (Cahill et al., 1994; Cahill & McGaugh, 1998; McGaugh et al., 1996; Pitman, 1989). Decades of animal research has shown that the administration of EPI or NE following learning enhances the storage of emotional memories (Gold et al., 1977; Gold & Van Buskirk, 1975; McGaugh, 2004), and a substantial amount of work in traumatized people has found that those individuals who exhibit greater HR responses to the traumatic event are at a much greater risk of developing PTSD (Bryant et al., 2000; Bryant et al., 2004; Bryant, 2006; Bryant et al., 2007; Shalev et al., 1998; Zatzick et al., 2005). As PTSD is a disorder of memory, in which an individual repeatedly relives his or her trauma through intrusive, flashback memories, an exaggerated sympathetic response to trauma could foster a development of a powerful, unrelenting traumatic memory that in some individuals becomes incapacitating over time.

Although sympathetic activity facilitates a rapid response to threat in one's environment, chronic activation of the system can have detrimental effects on an individual's health (McEwen, 1998; McEwen, 2003; McEwen & Wingfield, 2003). As mentioned above, some studies have indicated that PTSD is associated with increased

risk for cardiovascular disease, including myocardial infarctions and atrioventricular conduction abnormalities (Boscarino & Chang, 1999; Kubzansky et al., 2007; Sawchuk et al., 2005). The finding of reduced PNS activity in PTSD patients could exacerbate this problem. Research has shown that diminished PNS activity is associated with increased susceptibility to cardiac arrhythmias and increased mortality in myocardial infarction patients (La Rovere et al., 1988; La Rovere et al., 1998; Verrier & Dickerson, 1994). Thus, the presence of chronic sympathetic activity and a hyperaroused physiological state can lead to a significant decline in the overall physical health of PTSD patients.

*Abnormal Functioning of the Hypothalamus-Pituitary-Adrenal Axis in Post-Traumatic Stress Disorder*

Stress involves activation of the hypothalamus-pituitary-adrenal (HPA) axis, which entails the paraventricular nucleus of the hypothalamus secreting corticotrophin-releasing hormone (CRH), which travels through the median eminence via the portal vasculature to the anterior pituitary gland. Within the anterior pituitary gland, CRH stimulates the release of adrenocorticotrophin (ACTH), which then circulates through the bloodstream to stimulate the adrenal cortex to synthesize and release corticosteroids (primarily corticosterone in rodents and cortisol in humans). Corticosteroids help coordinate an individual's ability to cope with stress and divert energy to tissues with greater demands (de Kloet et al., 1999). Although corticosteroids are critically involved in the stress response, they also play a role in regulating baseline physiology by influencing metabolism, the immune system and memory consolidation (de Kloet et al.,

1999; Deuschle et al., 1997; Hartmann et al., 1997; Raison & Miller, 2003; Tsigos & Chrousos, 2002).

*Abnormal Baseline Levels of Cortisol and Its Hormone Precursors*

The HPA axis has been one of the most researched biological systems in people with PTSD. Researchers initially considered PTSD to be characterized by hypocortisolism, as a majority of the initial studies in this area of research reported abnormally low levels of baseline cortisol in people with PTSD (for reviews, see Yehuda, 2002; Yehuda, 2005). However, this view has steadily evolved over the past decade, in light of new work that has reported baseline cortisol levels in PTSD patients that are either greater than, or no different from, those of controls (see de Kloet et al., 2006 for a review). Given such a complex set of findings, “it has recently been suggested that there may be no static hypo- or hypercortisolism in PTSD, but a tendency of HPA tone to ‘hyperregulate’ in both [an] upward and downward direction” (Stam, 2007a, p. 536).

Researchers have addressed several factors that could underlie the complexity of baseline cortisol findings in people with PTSD. One factor has been the considerable variability in the characteristics of PTSD patients across studies. According to Yehuda (2005, p. 373), “the absence of cortisol alterations in some studies [implies] that alterations associated with low cortisol...are only present in a *biologic subtype of PTSD*” (italics added for emphasis). The nature of baseline HPA axis alterations in PTSD patients may be dependent, at least in part, on the type of trauma that led to their psychopathology. For instance, a majority of the studies examining people with abuse-related (i.e., sexual or physical abuse, including rape) PTSD have reported greater

baseline cortisol levels in PTSD patients than controls (Bremner et al., 2003a; De Bellis et al., 1999a; Elzinga et al., 2003; Inslicht et al., 2006a; Inslicht et al., 2006b; Lemieux & Coe, 1995), while a majority of the studies examining people with combat-related PTSD have reported lower baseline cortisol levels in PTSD patients than controls (Boscarino, 1996; Kanter et al., 2001; Thaller et al., 1999; Yehuda et al., 1996b; Yehuda et al., 1993a).

Other factors that could have influenced studies examining baseline cortisol levels in people with PTSD are the type (i.e., peripheral vs. central) of cortisol that was assayed and when (i.e., time of day) the assay was performed. Most of the studies in this area of research have used peripheral measures (e.g., urine, saliva, serum) to examine cortisol levels in PTSD patients (for reviews, see de Kloet et al., 2006; Yehuda, 2002; Yehuda, 2005). The only study to assess central levels of cortisol in people with PTSD reported that combat veterans with the disorder exhibited significantly greater CSF cortisol levels than healthy controls (Baker et al., 2005). While cortisol is mostly free (i.e., unbound) and biologically active in CSF, it is largely bound to corticosteroid-binding globulin (CBG) in serum (Dunn et al., 1981; Pardridge, 1981); and, one study found that people with PTSD displayed significantly greater levels of serum CBG than controls (Kanter et al., 2001). Thus, baseline cortisol levels in PTSD patients could vary based on the type of cortisol being measured.

Many of the studies examining baseline cortisol levels in PTSD have collected biological samples for cortisol analysis at a single time point or have pooled the samples over a 12- or 24-hour period. These methodologies could have failed to detect a

difference between PTSD patients and control subjects due to measuring cortisol levels at a time of day when no true differences exist or by masking potential differences through pooling a number of samples spread across the day. To address this issue and study the circadian rhythm of cortisol levels in PTSD patients, Yehuda et al. (1996b) examined baseline levels of cortisol in combat veterans with PTSD at 30-minute intervals over a 24-hour period of bed rest. Their results revealed that individuals with PTSD had lower levels of cortisol than controls during the late evening (i.e., ~10:00 p.m.) and early morning (i.e., ~5:00 a.m.) hours, which appeared to result from a prolonged nadir and short-lived peak response in the cycle of cortisol release. In addition to this finding, several other studies reporting lower levels of cortisol in PTSD patients have collected samples for cortisol analysis in the early morning hours (Brand et al., 2006; Goenjian et al., 1996; King et al., 2001; Lindauer et al., 2006; Rohleder et al., 2004; Seedat et al., 2003; Wessa et al., 2006), suggesting that this may be the time of day when these individuals display hypocortisolism.

In addition to cortisol level abnormalities, investigators have also reported significantly elevated levels of CRH in people with PTSD (Baker et al., 1999; Geraciotti et al., 2001). If most PTSD patients display abnormally low baseline cortisol levels, these findings would create a paradox – that is, how could PTSD patients exhibit lower baseline levels of cortisol if they have significantly elevated levels of CRH? Smith and colleagues (Smith et al., 1989) found that in a CRH challenge paradigm, PTSD patients displayed significantly lower levels of ACTH than healthy control subjects (however, see Kellner et al., 2003; Rasmusson et al., 2001), and Kellner et al. (2000) reported

significantly lower levels of ACTH in PTSD patients following the administration of cholecystokinin tetrapeptide (CCK-4), a potent stimulator of ACTH. In addition, several studies have reported no differences in baseline ACTH levels between PTSD patients and controls (Baker et al., 2005; Duval et al., 2004; Kanter et al., 2001; Liberzon et al., 1999a; Newport et al., 2004; Neylan et al., 2003; Neylan et al., 2006; Otte et al., 2007; Rasmusson et al., 2001; Yehuda et al., 1996a; Yehuda et al., 2004b). One possibility is that PTSD patients have desensitized CRH receptors and/or enhanced negative feedback inhibition at the level of the pituitary, which results in a blunted release of ACTH upon CRH receptor stimulation.

Support for this hypothesis has been provided by studies using the dexamethasone-CRH challenge paradigm (de Kloet et al., 2006). In this paradigm, participants are treated with dexamethasone, a synthetic glucocorticoid, the night before the experiment. Since the HPA axis is regulated through a negative feedback system, the dexamethasone pre-treatment significantly reduces HPA axis activity. On the following morning, the participants are treated with CRH, and their levels of ACTH and cortisol are measured. The advantage of this paradigm is that, since all participants are treated with a relatively high dose of dexamethasone the night prior to the study, by the time of CRH administration, both the PTSD patients and control subjects should display the same amount of dexamethasone-induced cortisol suppression (i.e., the same “baseline”). Of the four studies examining the effects of this challenge paradigm on HPA axis functioning in PTSD patients, two (Rinne et al., 2002; Strohle et al., 2008) have reported that, following CRH administration, participants with PTSD displayed significantly lower ACTH levels



than controls. The other two (de Kloet et al., 2008; Muhtz et al., 2008) reported no significant group differences, which are likely a result of using too high of a dose of dexamethasone (see Strohle et al., 2008). Therefore, given that PTSD patients exhibited significantly less ACTH release upon CRH administration, it would suggest that the disorder is characterized by reduced CRH receptor sensitivity and/or enhanced glucocorticoid negative feedback at the level of the pituitary.

*Mechanisms Underlying Abnormal Hypothalamus-Pituitary-Adrenal Axis Functioning in Post-Traumatic Stress Disorder*

Several hypotheses have been proposed to explain the abnormal HPA axis functioning observed in people with PTSD (de Kloet et al., 2006). As referenced above, one hypothesis has been that PTSD patients display pituitary insufficiency or reduced pituitary sensitivity to CRH stimulation. Although findings have been mixed, the reports above indicating that PTSD patients exhibited lower ACTH levels following CRH administration, relative to controls, support this hypothesis. Another hypothesis has suggested that PTSD is characterized by adrenal insufficiency or reduced adrenal sensitivity to ACTH. However, this scenario seems unlikely, as non-pharmacological challenge paradigms have indicated that PTSD patients exhibit a robust stress-induced increase in cortisol that is greater than that of control subjects (Bremner et al., 2003a; Elzinga et al., 2003). Moreover, if adrenal insufficiency or desensitization were the reason for HPA axis dysfunction in PTSD, one would expect PTSD patients to exhibit lower cortisol levels than controls following an ACTH challenge paradigm. On the contrary, the administration of ACTH has actually been shown to result in significantly

greater cortisol levels in people with PTSD, relative to control subjects (Rasmusson et al., 2001).

Another hypothesis, which has received the most empirical support, is that PTSD patients have enhanced negative feedback inhibition of the HPA axis. When cortisol is released into the bloodstream, it exerts negative feedback on the HPA axis by binding to glucocorticoid receptors throughout the body. Research has shown that PTSD patients have an increased number and sensitivity of glucocorticoid receptors (Rohleder et al., 2004; Stein et al., 1997b; Yehuda et al., 1991; Yehuda et al., 1993a; Yehuda et al., 1995). In addition, studies have reported an increased suppression of cortisol and ACTH in PTSD patients following the administration of dexamethasone, a synthetic glucocorticoid (Duval et al., 2004; Goenjian et al., 1996; Grossman et al., 2003; Newport et al., 2004; Stein et al., 1997b; Yehuda et al., 1993b; Yehuda et al., 1995; Yehuda et al., 2002; Yehuda et al., 2004b). This finding suggests that dexamethasone produces greater negative feedback inhibition of the HPA axis in PTSD patients, which leads to a greater suppression of cortisol and ACTH in these individuals. Some have also observed increased activation of the pituitary gland in PTSD patients following the administration of metyrapone, a glucocorticoid antagonist that blocks the conversion of 11-deoxycortisol to cortisol (or 11-deoxycorticosterone to corticosterone in rodents) (Otte et al., 2006; Yehuda et al., 1996a). Both of these studies found that following the administration of metyrapone, PTSD patients exhibited a significantly greater increase in ACTH and 11-deoxycortisol, two of the primary precursors to cortisol release, relative to controls. Since metyrapone prevents the production of cortisol, it hinders the negative feedback

component of the HPA axis. In theory, PTSD patients in these studies demonstrated greater increases in ACTH and 11-deoxycortisol because metyrapone removed the enhanced negative feedback inhibition initially present in these individuals.

*Structural and Functional Brain Abnormalities in Post-Traumatic Stress Disorder*  
*Smaller Hippocampal Volume*

Investigators have reported smaller hippocampal volume in people who developed PTSD following combat exposure (Bremner et al., 1995a; Gurvits et al., 1996; Hedges et al., 2003; Vythilingam et al., 2005; Woodward et al., 2006a), firefighting (Shin et al., 2004b), police work (Lindauer et al., 2004b; Lindauer et al., 2006), childhood abuse (Bremner et al., 1997b; Bremner et al., 2003b; Stein et al., 1997a), and mixed types of events, such as motor vehicle accidents and assaults (Villarreal et al., 2002; Wignall et al., 2004; Winter & Irle, 2004). In general, these studies have detected smaller hippocampal volume in individuals with PTSD after adjusting for the total brain volume and age of each subject. Nevertheless, numerous other studies have not replicated these findings; they reported no differences in hippocampal volume between individuals diagnosed with PTSD and control subjects (Bonne et al., 2001; De Bellis et al., 1999b; De Bellis et al., 2001; Fennema-Notestine et al., 2002; Jatzko et al., 2006; Pederson et al., 2004; Schuff et al., 2001; Tupler & De Bellis, 2006; Yamasue et al., 2003; Yehuda et al., 2007). The inconsistencies of these findings raise an important issue: is hippocampal volume reduced by trauma, or is a smaller hippocampus a pre-existing risk factor that increases one's susceptibility to develop the disorder?

Work conducted by Gilbertson and colleagues (Gilbertson et al., 2002) had a substantial impact on how the scientific community interpreted smaller hippocampal volume in PTSD patients. These investigators used MRI to measure hippocampal volume of monozygotic twins discordant for trauma exposure, which, in this case, was combat. Consistent with previous findings, those individuals who were exposed to combat and had developed PTSD exhibited smaller hippocampal volume than combat-exposed individuals who did not develop PTSD. The important finding, though, was that the non-exposed twin brothers of those individuals who developed PTSD also displayed smaller hippocampal volume than trauma-exposed individuals who did not develop PTSD. Thus, these individuals had smaller hippocampal volume than controls, even though they were not exposed to a traumatic event. This finding supported the idea that smaller hippocampal volume was a pre-existing familial risk factor that enhanced the likelihood of the combat-exposed brother to develop PTSD.

In another study employing the same strategy, Gilbertson et al. (2007) assessed allocentric (i.e., related to configural relationships among distal stimuli) spatial processing, a hippocampus-dependent task, in monozygotic twins discordant for trauma exposure, which was combat. The investigators found that those individuals who were exposed to combat and had developed PTSD, as well as their twin brothers, made significantly more errors on the spatial task than those individuals who were exposed to combat and did not develop PTSD. These findings extended the earlier report by Gilbertson and colleagues by demonstrating that impaired hippocampal function, in

addition to smaller hippocampal volume, may also be a pre-existing familial risk factor for the development of PTSD.

### *Cognitive Impairments*

Since there is extensive evidence supporting the presence of smaller hippocampal volume in PTSD patients, it is not surprising that numerous studies have reported declarative and working memory impairments, along with deficits in attention, in these individuals as well (Bremner et al., 1993; Bremner et al., 1995b; Bremner et al., 1995a; Gil et al., 1990; Gilbertson et al., 2001; Golier et al., 2002; Jenkins et al., 1998; Moradi et al., 1999; Sachinvala et al., 2000; Uddo et al., 1993; Vasterling et al., 1998; Yehuda et al., 2004a). Bremner and colleagues (Bremner et al., 1993; Bremner et al., 1995b; Bremner et al., 1995a) reported verbal memory deficits in both combat-related and abuse-related PTSD patients. More importantly, Bremner et al. (1995a) found that these verbal memory deficits were significantly associated with the smaller right hippocampus of PTSD patients, suggesting the possibility of a relationship between these two phenomena. Other work has reported that PTSD patients have significant attentional impairments, which are believed to be due to a bias for the processing of emotional information and the persistent intrusiveness of memories related to the traumatic event (Bryant & Harvey, 1997; Buckley et al., 2000; Ehlers et al., 2006; Michael et al., 2005; Moradi et al., 2000; Paunovic et al., 2002). Some studies have reported *enhanced* memory for trauma-related information in PTSD patients (Golier et al., 2003; McNally, 1997), providing support for greater attentional resources devoted to processing emotional, especially trauma-relevant, information in these individuals.

### *Interactions between the Amygdala and Prefrontal Cortex*

Extensive work has implicated involvement of the amygdala, an almond-shaped medial temporal lobe structure, in the acquisition and expression of fear memories (Fanselow & Gale, 2003; LeDoux, 2003; Maren et al., 1996; Maren, 2003; McGaugh, 2002; McGaugh, 2004). Inactivation of the amygdala impairs the acquisition of fear conditioning in rodents (Gale et al., 2004; Maren, 1999; Wallace & Rosen, 2001; Wilensky et al., 1999), and people with lesions of the amygdala have difficulty acquiring conditioned fear (LaBar et al., 1995) and recognizing fearful stimuli (Adolphs, 2002; Scott et al., 1997; Wang et al., 2002). Likewise, neuroimaging studies in humans have consistently reported amygdala activation during fear conditioning (Buchel & Dolan, 2000; Cheng et al., 2003; Knight et al., 2004; LaBar et al., 1998). Investigators have speculated that PTSD patients may display abnormal amygdala functioning, which would lead to an aberrant stress response and an enhanced amygdala-induced augmentation of emotional memories (Elzinga & Bremner, 2002). Several studies have reported amygdala hyperresponsivity in PTSD patients during the presentation of traumatic scripts and stimuli (Driessen et al., 2004; Hendler et al., 2003; Liberzon et al., 1999b; Pissiota et al., 2002; Protopopescu et al., 2005; Rauch et al., 1996; Shin et al., 1997; Shin et al., 2004a), the presentation of non-trauma-relevant emotional stimuli (Rauch et al., 2000; Shin et al., 2005; Williams et al., 2006) and during the acquisition of fear conditioning (Bremner et al., 2005). Others have found a positive relationship between activation of the amygdala and PTSD symptom severity (Armony et al., 2005; Protopopescu et al., 2005; Rauch et

al., 1996; Shin et al., 2004a). These findings suggest an important role of the amygdala in the expression of PTSD symptomatology.

The prefrontal cortex (PFC) is located in the anterior part of the frontal lobe and is involved in working memory processes, attention, and decision making (Braver et al., 1997; Curtis & D'Esposito, 2003; Funahashi & Kubota, 1994; McCarthy et al., 1996; Postle et al., 2000). This area of the brain has been shown to play a major role in more complex cognition, such as the planning and organization of behavior (Koechlin et al., 1999; Koechlin et al., 2000; Tanji & Hoshi, 2001). Reciprocal connections between the PFC and amygdala allow for dynamic interactions between these two brain regions (Amaral & Insausti, 1992; Ghashghaei & Barbas, 2002; McDonald, 1987; McDonald, 1991; Sesack et al., 1989). The PFC allows for the inhibition of inappropriate cognitive and emotional responses that are mediated in part by the amygdala (Elzinga & Bremner, 2002). Such a role of the PFC has led researchers to speculate that PTSD patients may have impaired PFC functioning and that such an impairment may allow for hyperactivity of the amygdala and exaggerated emotional responsiveness. In agreement with this hypothesis, several studies have shown that PTSD patients have a smaller volume of major regions of the PFC (e.g., anterior cingulate cortex, medial frontal gyrus) (Carrion et al., 2001; De Bellis et al., 2002; Fennema-Notestine et al., 2002; Rauch et al., 2003; Woodward et al., 2006b; Yamasue et al., 2003) and perform more poorly on tasks dependent upon an intact PFC (Koenen et al., 2001).

In addition, the PFC is involved in the extinction of fear memories. Animal studies have shown that lesions of the medial PFC impair the extinction of conditioned

fear (Lebron et al., 2004), while stimulation of this area facilitates this process (Milad et al., 2004). Research has shown that PTSD patients are impaired at extinguishing fear (Orr et al., 2000; Peri et al., 2000) and demonstrate reduced activity of PFC regions during extinction trials (Bremner et al., 2005). Moreover, these individuals exhibit less activity of, or a complete failure to activate, PFC brain regions during the presentation of trauma-relevant stimuli (Bremner, 1999; Bremner et al., 1999; Britton et al., 2005; Lanius et al., 2001; Lindauer et al., 2004a; Shin et al., 1999; Shin et al., 2004a). In theory, reduced activation of the PFC, in conjunction with amygdala hyperactivity, could promote the intrusive emotional thoughts and memories that PTSD patients often experience. Such a system could lead to greater governance of behavior by lower brain areas, such as the amygdala and hypothalamus, rather than the prefrontal areas, which allow for adaptation, behavioral flexibility and coherent cognitive processing.

### *Pharmacotherapy for Post-Traumatic Stress Disorder*

#### *Selective Serotonin Reuptake Inhibitors*

The fact that a subset of people with PTSD exhibit significant improvement in some of their symptoms following treatment with selective serotonin reuptake inhibitors (SSRIs) suggests a role of the serotonergic system in this disorder (Asnis et al., 2004; Davidson, 2003; Davis et al., 2006; Hidalgo & Davidson, 2000; Ipser et al., 2006; Stein et al., 2006). Research has shown that several SSRIs, such as fluoxetine, fluvoxamine and citalopram, exert positive effects on people with PTSD and lead to significant improvements in quality of life (Brady et al., 2000; Brady et al., 1995; Cavaljuga et al., 2003; Connor et al., 1999; Davidson et al., 2001; De Boer et al., 1992; English et al.,



2006; Escalona et al., 2002; Figgitt & McClellan, 2000; Friedman et al., 2007; Londborg et al., 2001; March, 1992; Martenyi et al., 2002a; Martenyi et al., 2002b; Martenyi & Soldatenkova, 2006; McRae et al., 2004; Meltzer-Brody et al., 2000; Neylan et al., 2001; Robert et al., 2006; Schwartz & Rothbaum, 2002; Seedat et al., 2001; Smajkic et al., 2001; Van der Kolk et al., 1994). However, the response rates to SSRIs in PTSD patients rarely exceeds 60%, and full remission from the disorder is achieved following SSRI treatment only 20-30% of the time (Stein et al., 2002). In addition, SSRIs tend to blunt only the depressive components of PTSD, while having little effect on the memory- and anxiety-related symptoms of the disorder (Asnis et al., 2004; Boehnlein & Kinzie, 2007; Brady et al., 2000; Van der Kolk et al., 1994). Some forms of PTSD, such as combat-related PTSD, are incredibly resistant to SSRI treatment (Jakovljevic et al., 2003; Rothbaum et al., 2008; Stein et al., 2002). SSRIs are also anxiogenic early in the treatment phase and only exert anxiolytic effects after a substantial delay (Browning et al., 2007; Burghardt et al., 2004; Humble & Wistedt, 1992). Given the numerous caveats to the efficacy of SSRIs in treating PTSD, there is a need for additional research in people with PTSD and in animal models of the disorder to facilitate the development of more effective treatments for PTSD.

#### *Tricyclic Antidepressants and Monoamine Oxidase Inhibitors*

Tricyclic antidepressants, named for their three-carbon ring molecular structure, inhibit the reuptake of serotonin and NE to varying degrees. They also antagonize, to a lesser extent, dopaminergic, histaminergic, adrenergic and cholinergic receptor sites, which often produces an array of adverse secondary side effects (Albucher & Liberzon,

2002). Few randomized, placebo-controlled studies have been conducted to assess the effects of tricyclic antidepressants on PTSD, but those that have been performed have reported positive effects on PTSD symptomatology (Bisson, 2007). Some studies found that amitriptyline (Davidson et al., 1990; Davidson et al., 1993) and imipramine (Burstein, 1984; Frank et al., 1988; Kosten et al., 1991) significantly reduced global scores of PTSD severity and were particularly effective in ameliorating the avoidance, intrusion and re-experiencing symptoms in PTSD patients. Another study found that desipramine effectively reduced symptoms of depression in PTSD patients but had no effect on the anxiety-related symptoms that are specific to PTSD (Reist et al., 1989).

Monoamine oxidase inhibitors (MAOIs) prevent the enzyme monoamine oxidase from breaking down monoamine transmitter substances. This leads to a significant increase in the synaptic release of monoamines, such as dopamine, norepinephrine, epinephrine and serotonin. Several studies have shown that MAOIs, such as phenelzine (Kosten et al., 1991; Shestatzky et al., 1988), brofaromine (Baker et al., 1995; Katz et al., 1994) and moclobemide (Neal et al., 1997), are effective in reducing avoidance, intrusion and hyperarousal symptoms associated with PTSD, and MAOIs appear to be more effective in treating PTSD than tricyclic antidepressants (Albucher & Liberzon, 2002).

Despite the positive effects of tricyclic antidepressants and MAOIs on PTSD symptoms, these agents are rarely used as the first line of treatment for PTSD and, instead, are typically only employed when SSRIs are ineffective (Albucher & Liberzon, 2002). Due to the numerous side effects of both drug classes, the dropout rates for these agents are very high (e.g., 30-50%). Additionally, patients who take MAOIs must adhere

to a special low tyramine diet to avoid a potential life-threatening hypertensive crisis. Thus, tricyclic antidepressants and MAOIs, although effective treatments for some PTSD symptoms, are difficult to tolerate and therefore frequently avoided.

#### *Noradrenergic Modulators*

People with PTSD have significantly elevated baseline levels of NE and EPI and demonstrate adverse reactions (e.g., panic attacks, flashbacks) to agents that increase adrenergic activity, such as yohimbine. These adrenergic abnormalities are believed to contribute to the hyperarousal, intrusion and avoidance symptoms, as well as the sleep disturbances, that are often reported in PTSD patients (Boehnlein & Kinzie, 2007; Strawn & Geraciotti, 2008). Thus, recent work has begun testing the effects of pharmacological agents that reduce adrenergic activity on PTSD symptomatology. Some studies have found that propranolol, a  $\beta$ -adrenergic receptor antagonist, may be effective in preventing the disorder's development (Pitman et al., 2002; Taylor & Cahill, 2002; Vaiva et al., 2003). For instance, Vaiva et al. (2003) found that propranolol treatment shortly after experiencing a traumatic event significantly reduced the incidence and symptoms of PTSD in individuals 2 months later, and Pitman and colleagues (Pitman et al., 2002) reported that post-trauma administration of propranolol ameliorated sympathetic responses to traumatic reminders at a 1-month follow-up visit. Additional work has shown that propranolol can effectively reduce PTSD symptoms if administered following the re-experiencing of a trauma (Taylor & Cahill, 2002), suggesting that it may be effective at preventing the reconsolidation of the traumatic memory (Brunet et al., 2008). These findings suggest that propranolol may be an effective treatment for PTSD if

administered immediately after the traumatic event or after the re-experiencing of a traumatic event.

Other work has shown that clonidine, an  $\alpha_2$ -adrenergic receptor agonist, and prazosin, an  $\alpha_1$ -adrenergic receptor antagonist, can significantly ameliorate symptoms of heightened anxiety and hyperarousal in people with PTSD (Boehnlein & Kinzie, 2007). Clonidine works by facilitating  $\alpha_2$ -adrenergic autoreceptors, which ultimately leads to decreased NE levels. Though many studies have examined the effects of clonidine on PTSD and found it to be effective at reducing intrusive memories and hyperarousal (Harmon & Riggs, 1996; Porter & Bell, 1999; Viola et al., 1997), no randomized, placebo-controlled studies of clonidine's effects on PTSD have been performed (Boehnlein & Kinzie, 2007). Prazosin, on the other hand, works by inhibiting post-synaptic  $\alpha_1$ -adrenergic receptors, which, similar to clonidine, leads to a reduction of NE activity. Recent work has shown that prazosin is an effective treatment for hyperarousal symptoms, intrusive thoughts, recurrent distressing dreams and sleep disturbances in PTSD (Brkanac et al., 2003; Peskind et al., 2003; Raskind et al., 2002; Raskind et al., 2003; Taylor & Raskind, 2002; Taylor et al., 2006). Collectively, these studies suggest that the reduction of adrenergic activity in PTSD patients is an effective approach to ameliorating many of the disorder's debilitating symptoms.

#### *The Antidepressant Tianeptine*

Tianeptine is most commonly known to exert antidepressant effects and ameliorate symptoms of MDD, but it has been shown to have beneficial effects in treating PTSD as well (Onder et al., 2006). Early studies on tianeptine's mechanism of

action showed that the drug led to significantly lower extracellular levels of serotonin, a finding that was hypothesized to result from enhanced serotonin reuptake (Fattaccini et al., 1990; Labrid et al., 1992; Mennini et al., 1987; Mocaer et al., 1988). However, tianeptine's effects on the serotonergic system may be an indirect consequence of the drug's influences on an alternative neurotransmitter system because later studies failed to show any direct effects of tianeptine on serotonergic neurotransmission (Pineyro et al., 1995a; Pineyro et al., 1995b). Additionally, research has shown that tianeptine does not alter the density or affinity of any serotonin receptor subtype, and tianeptine's affinity for the serotonin transporter is very low (Kato & Weitsch, 1988; Svenningsson et al., 2007). Some have also contested the validity of the original studies on tianeptine's mechanism of action based on technical limitations that were present at the time (Malagie et al., 2000).

Recently, extensive work has suggested that tianeptine's therapeutic effects are more associated with modulation of the glutamatergic system (Brink et al., 2006; Kasper & McEwen, 2008; Zoladz et al., in press). Glutamate is the primary excitatory neurotransmitter of the central nervous system, and one of its roles is to regulate calcium influx by acting on postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors (Riedel et al., 2003). Extensive work has implicated hyperactivity of the glutamatergic system in the deleterious effects of stress on brain structure and function. Experiments conducted primarily on the hippocampus have shown that stress significantly increases glutamate levels (Bagley & Moghaddam, 1997; Lowy et al., 1993; Lowy et al., 1995; Moghaddam,

1993; Reznikov et al., 2007), inhibits glutamate uptake (Yang et al., 2005), increases the expression and binding of glutamate receptors (Bartanusz et al., 1995; Krugers et al., 1993; McEwen et al., 2002) and increases calcium currents (Joels et al., 2003).

Accordingly, researchers have shown that administration of NMDA receptor antagonists blocks the effects of stress on behavioral, morphological and electrophysiological measures of hippocampal function (Kim et al., 1996; Magarinos & McEwen, 1995; Park et al., 2004).

Tianeptine appears to protect the hippocampus and prefrontal cortex from the deleterious effects of stress by normalizing the stress-induced modulation of glutamatergic activity. Researchers have also shown that tianeptine inhibits the acute stress-induced increase in extracellular levels of glutamate in the amygdala (Reznikov et al., 2007). In addition to its glutamatergic modulation, tianeptine reduces the expression of CRH mRNA in the amygdala and the bed nucleus of the stria terminalis, a brain region that is highly innervated by amygdala fibers (Kim et al., 2006). CRH neurotransmission in both of these regions has been implicated in the expression of anxiety-like behaviors (Holsboer, 1999; Strohle & Holsboer, 2003). These findings suggest that tianeptine could be an effective pharmacological treatment for PTSD.

### *Animal Models of Post-Traumatic Stress Disorder*

#### *Existing Models of Post-Traumatic Stress Disorder in Rodents*

Preclinical researchers have used several types of stressors to model aspects of PTSD in rodents (see Stam, 2007b for a review). Such stressors have included electric shock (Garrick et al., 2001; Li et al., 2006; Milde et al., 2003; Pynoos et al., 1996; Rau et

al., 2005; Sawamura et al., 2004; Servatius et al., 1995; Shimizu et al., 2004; Shimizu et al., 2006; Siegmund & Wotjak, 2007a; Siegmund & Wotjak, 2007b; Wakizono et al., 2007), underwater trauma (Cohen et al., 2004; Richter-Levin, 1998), stress-restress paradigms and single prolonged stress paradigms (Harvey et al., 2003; Khan & Liberzon, 2004; Kohda et al., 2007; Liberzon et al., 1997; Takahashi et al., 2006) and exposure to predators (Adamec, 1997; Adamec et al., 2007; Adamec et al., 1999; Adamec et al., 2006; Adamec & Shallow, 1993; Blanchard et al., 1998; Park et al., 2001) or predator-related cues (Cohen et al., 2000b; Cohen et al., 2004; Cohen et al., 2006; Cohen et al., 2007; Cohen & Zohar, 2004). The stressors employed in these studies typically produced increased behavioral signs of anxiety, and in some cases, exaggerated startle, cognitive impairments, enhanced fear conditioning and reduced social interaction. Although these studies have reported physiological and behavioral changes resembling those observed in people with PTSD, most have utilized only a small set of assessments, such as stress-induced changes in anxiety, without assessing other measures common in people with PTSD, such as an impairment in cognition. Moreover, many of these studies have evaluated stress-induced changes in responses for a relatively short period of time. Thus, while these studies have provided insight into how stress or fear conditioning changes aspects of behavior and physiology, the field would benefit from an animal model of PTSD that takes into account how traumatic stress produces long-lasting PTSD-like changes in rats given multiple behavioral and physiological diagnostic tests.

*Our Laboratory's Recently Developed Animal Model of Post-Traumatic Stress Disorder*

Our laboratory has developed an animal model of PTSD in which rats are exposed to a cat (predator stress) on two separate occasions, in conjunction with daily social stress, and tested 3 weeks after the second cat exposure (Zoladz et al., 2008). We found that rats stressed in this paradigm exhibited reduced growth rate, greater adrenal gland weight, reduced thymus weight, heightened anxiety, an exaggerated startle response, impaired hippocampus-dependent memory, greater cardiovascular and corticosterone reactivity to an acute stressor and an exaggerated physiological and behavioral response to yohimbine. Importantly, all of these physiological and behavioral abnormalities are commonly observed in people with PTSD.

Our animal model of PTSD was developed to expose rats to conditions which, based on DSM-IV criteria, are analogous to conditions that produce PTSD in people. Specifically, a subset of the DSM-IV criteria for the diagnosis of PTSD includes the following three conditions: (1) PTSD can be triggered by an event that involves threatened death or a threat to one's physical integrity; (2) a person's response to the event involves intense fear, helplessness or horror; and (3) in the aftermath of the trauma, the person feels as if the traumatic event were recurring, including a sense of reliving the experience (American Psychiatric Association, 1994).

The behaviors that rats exhibit in response to forced exposure to a cat are consistent with the first two components of the DSM-IV criteria for PTSD. That is, rats exhibit an intense fear response when exposed to a predator, which is a condition that is a threat to their survival. In addition, we have observed that rats typically direct their



posture away from the cat's gaze, which provides the rat with an element of control over its confrontation with the cat. As control critically influences the expression of the stress response, in general (Kim & Diamond, 2002), and a loss of control exacerbates behavioral and physiological responses to stress conditions (Amat et al., 2005; Bland et al., 2006; Bland et al., 2007; Kavushansky et al., 2006; Maier et al., 1993; Maier & Watkins, 2005; Shors et al., 1989), we immobilized the rats during predator exposure. The immobilization component of our animal model, therefore, may provide a rodent analogue to the sense of helplessness and a loss of control which feature prominently in the DSM-IV criteria for PTSD.

Another component of our model is that rats are exposed to the cat on two occasions, separated by 10 days. PTSD develops in some people only after they have repeated traumatic experiences (Resnick et al., 1995; Taylor & Cahill, 2002), and prolonged exposure to trauma increases the likelihood of developing symptoms of PTSD (Gurvits et al., 1996). Therefore, the repeated inescapable cat exposure was designed to increase the likelihood that the manipulations would produce effects in the rats that could be broadly applied to people who develop PTSD as a result of multiple traumatic experiences. In addition, people who develop PTSD in response to only a single trauma experience powerful episodes of anxiety and panic as a result of their repeated reliving of the trauma through intrusive, flashback memories (Reynolds & Brewin, 1999). As mentioned above, the repeated reliving of the original experience through disturbing intrusive memories is a criterion for the diagnosis of PTSD. The second exposure of the rats to the cat forced them to re-experience the original stress experience, which can be

considered analogous to how people with PTSD report that they feel as if they relive their original trauma when they have an intrusive memory of the experience.

The second reason why the rats were re-exposed to the cat pertained to the issue of predictability. The first predator exposure occurred during the light cycle and the second predator exposure occurred during the dark cycle, thereby adding an element of unpredictability as to when the rats might re-experience the traumatic event. A lack of predictability in one's environment is a major factor in the development of PTSD, as a means with which to increase the susceptibility of a subset of people to develop PTSD in response to trauma, as well as to influence the later expression of PTSD symptoms (Orr et al., 1990; Regehr et al., 2000; Solomon et al., 1989; Solomon et al., 1988).

Lastly, McEwen and colleagues observed increased spine density on dendritic arbors of amygdala neurons 10 days after a single immobilization experience (Mitra et al., 2005). Therefore, the second stress session reinforced stress-induced changes in brain and behavior which were presumably initiated by the first stress session. In theory, the reinforcement of morphological plasticity in the amygdala through a reminder of the original experience would augment the PTSD-like syndrome in psychosocially stressed rats. The strengthening of plasticity in the amygdala, which may be expressed in a number of different ways, such as dendritic hypertrophy (Fuchs et al., 2006; McEwen & Chattarji, 2004; Mitra et al., 2005; Vyas et al., 2002; Vyas et al., 2003; Vyas et al., 2006) or as stress-induced long-term potentiation (Kavushansky & Richter-Levin, 2006; Manzanares et al., 2005; Vouimba et al., 2004; Vouimba et al., 2006), lends itself to experimentation via pharmacological manipulations of the reconsolidation process, which

is likely to occur in response to traumatic memory recall (Cai et al., 2006; Debiec et al., 2002; Debiec & LeDoux, 2004; Debiec & LeDoux, 2006; Maroun & Akirav, 2008; Nader et al., 2000; Przybylski et al., 1999; Przybylski & Sara, 1997; Sara, 2000; Suzuki et al., 2004).

In addition to the two acute cat exposures, we included chronic unstable housing conditions in the psychosocial stress paradigm to mimic the lack of social support and chronic mild stress experienced by people with PTSD (Andrews et al., 2003; Boscarino, 1995; Brewin et al., 2000; Solomon et al., 1989; Ullman & Filipas, 2001). We hypothesized that the daily anxiety produced by unstable housing would exacerbate any adverse effects on the rats induced by predator exposure, alone. This hypothesis was supported by our finding that the combination of two cat exposures with social instability produced greater anxiogenic effects on rat behavior than either manipulation in isolation. Chronic social instability, alone, had no negative effects on behavior and may have even been beneficial for rats, as it led to a small increase in growth rate and significantly greater motor activity on the elevated plus maze.

In sum, the primary goal of this preliminary work was to develop an animal model of PTSD based on the factors that are known to be involved in the etiology and persistence of PTSD symptoms in people. To accomplish this goal, we combined a life-threatening stress experience (i.e., unavoidable predator exposure) with a re-experiencing of the trauma and chronic social instability, all of which are well-described risk factors for PTSD. This approach enabled us to produce an animal model that targets the subset of people who actually develop PTSD in response to trauma and affords us the opportunity

to explore the mechanisms responsible for the effects of traumatic stress on brain and behavior.

*Purpose of the Present Experiments*

The purpose of the present experiments was to further examine the neurobiological mechanisms responsible for the PTSD-like sequelae induced by our laboratory's animal model and to explore the longevity of the effects induced by our chronic psychosocial stress paradigm. Specifically, the present set of experiments were designed to 1) test the hypothesis that our animal model of PTSD would produce abnormalities in glucocorticoid levels that are comparable to those observed in people with PTSD, 2) examine the ability of antidepressant and anxiolytic agents to ameliorate the PTSD-like physiological and behavioral symptoms induced by our laboratory's paradigm and 3) ascertain how long the physiological and behavioral effects of our laboratory's stress regimen could be maintained.

## Chapter Two: Experiment One

### *Chronic Psychosocial Stress Produces a Reduction in Basal Glucocorticoid Levels in Rats: Further Validation of an Animal Model of PTSD*

Although findings have been mixed, extensive work has reported abnormally low baseline levels of cortisol in people with PTSD (for reviews, see de Kloet et al., 2006; Yehuda, 2002; Yehuda, 2005). Additionally, some (Bremner et al., 2003a; Elzinga et al., 2003), but not all (Geraciotti et al., 2008), studies have reported significantly greater stress-induced elevations of cortisol in PTSD patients, relative to control subjects. Therefore, to further validate our laboratory's animal model of PTSD, Experiment One was designed to examine the effects of chronic psychosocial stress, composed of two acute predator exposures and daily social instability, on baseline and stress-induced serum corticosterone levels in rats. While previous studies in our laboratory have examined rat serum corticosterone levels following the proposed stress paradigm (Zoladz et al., 2008), these studies did not obtain *undisturbed*, baseline measures of corticosterone from psychosocially stressed animals. In each case, the rats were transported to the laboratory, and in some cases injected, prior to blood sampling, which could have induced a stress response in the rats. Moreover, the rats in these studies had been exposed to several behavioral assessments on the days prior to blood sampling. Both of these factors could have hindered an accurate interpretation of the data. Therefore, in order to obtain undisturbed, baseline measures of corticosterone, the rats in the present study were

exposed to only one endpoint manipulation, which was blood sampling, and the first blood sample was obtained immediately after removing the rats from their housing rooms. In light of the PTSD literature, I hypothesized that rats exposed to chronic psychosocial stress would display significantly lower baseline, but significantly greater stress-induced, corticosterone levels than control (i.e., unstressed) animals.

### *Methods*

#### *Rats*

Experimentally naïve adult male Sprague-Dawley rats (225-250 g upon delivery) obtained from Charles River laboratories (Wilmington, Massachusetts) were used for the present experiment. The rats were housed on a 12-hr light/dark schedule (lights on at 0700) in standard Plexiglas cages (two per cage) with free access to food and water. The colony room temperature and humidity were maintained at  $20\pm 1^{\circ}\text{C}$  and  $60\pm 3\%$ , respectively. Upon arrival, all rats were given 1 week to acclimate to the housing room environment, as well as cage changing procedures, before any experimental manipulations took place. All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

#### *Psychosocial Stress Procedure*

*Acute Stress Sessions.* Following the 1-week acclimation phase, rats were brought to the laboratory, weighed and assigned to “psychosocial stress” or “no psychosocial stress” groups (N = 10 rats/group). Rats in the psychosocial stress group were immobilized in plastic DecapiCones (BainTree Scientific; Braintree, MA) and placed in a perforated wedge-shaped Plexiglas enclosure (BainTree Scientific; Braintree, MA; 20 x

20 x 8 cm). Then, the rats, still immobilized in the plastic DecapiCones within the Plexiglas enclosure, were taken to the cat housing room where they were placed in a metal cage (24 x 21 x 20 in) with an adult female cat for 1 hour. The Plexiglas enclosure prevented any contact between the cat and rats, but the rats were still exposed to all non-tactile sensory stimuli associated with the cat. Canned cat food was smeared on top of the Plexiglas enclosure to direct cat activity toward the rats. An hour later, the rats were returned to the laboratory. Rats in the no psychosocial stress group remained in their home cages in the laboratory for the 1-hour stress period. Rats were exposed to two acute stress sessions, which were separated by 10 days. The first stress session took place during the light cycle, between 0800 and 1300 hours, and the second stress session took place during the dark cycle, between 1900 and 2100 hours.

*Daily Social Stress.* Beginning on the day of the first stress session, rats in the psychosocial stress group were exposed to unstable housing conditions for the next 31 days. Rats in the psychosocial stress group were still housed two per cage, but every day, their cohort pair combination was changed. Therefore, no rat in the psychosocial stress group had the same cage mate on two consecutive days during the 31-day stress period.

#### *Assessment of Basal and Stress-Induced Glucocorticoid Levels*

*Preparation.* Twenty days after the second stress session, rats in the psychosocial stress and no psychosocial stress groups were brought to the laboratory and weighed. Then, the hind legs of all rats were shaved to allow access to their saphenous veins. The rats were then taken back to the housing room and left undisturbed for the remainder of the day. The hind legs of all rats were shaved 1 day prior to blood sampling to minimize

the amount of time it took the experimenter to obtain baseline blood samples on the following day.

*Blood Sampling and Post-Mortem Dissection.* Twenty-four hours later, rats were brought, one cage (i.e., 2 rats) at a time, to a nearby procedure room for blood sampling. Petroleum jelly was applied to each rat's hind leg, and the saphenous vein of each rat was punctured with a sterile, 27-gauge syringe needle. A 0.2 cc sample of blood was then collected from each rat in a microcentrifuge tube. The first blood sample was considered a "baseline" measure of corticosterone and was collected within 2 minutes after the rats were removed from the housing room. After obtaining this sample, the rats were immobilized in plastic DecapiCones for 20 minutes. Then, the rats were removed from the DecapiCones, and another 0.2 cc sample of blood was collected in a microcentrifuge tube via saphenous vein venipuncture. This blood sample served to examine the hormonal responses of rats to acute immobilization stress. After collecting this sample, the rats were returned to their home cages. An hour later, one last blood sample (trunk blood) was collected following rapid decapitation. This sample was collected to examine the recovery of corticosterone levels following acute immobilization stress. Following rapid decapitation, the adrenal and thymus glands were removed and weighed. Once all of the blood had clotted at room temperature, it was centrifuged (3000 rpm for 8 minutes), and the serum was extracted and stored at -80° C until assayed by Monika Fleshner at the University of Colorado at Boulder.

Most studies have reported abnormal cortisol levels in PTSD patients in the early morning hours, when the levels of cortisol reach their peak in people (Brand et al., 2006;



Goenjian et al., 1996; King et al., 2001; Lindauer et al., 2006; Rohleder et al., 2004; Seedat et al., 2003; Wessa et al., 2006). Since rats are nocturnal, their circadian rhythm is reversed (Meaney et al., 1992). Rats exhibit very low morning corticosterone levels that slowly rise throughout the day and peak in the early evening hours (e.g., around 1800 hours). Thus, in order to avoid a floor effect and allow room for between-group differences in basal corticosterone levels, as well as to relate the present findings to the PTSD literature, all blood sampling for this study took place between 1700 and 2000 hours.

#### *Statistical Analyses*

*Experimental Design.* The present study utilized a single factor, between-subjects design. The between-subjects factor was psychosocial stress (psychosocial stress, no psychosocial stress).

*Growth Rate, Adrenal Gland Weight and Thymus Weight.* Growth rates, expressed as grams per day (g/day), were calculated for all rats by dividing their total body weight gained during the course of the experiment by the total number of days in the experiment (i.e., 31 days). The adrenal glands and thymuses were weighed and expressed as milligrams per 100 grams of body weight (mg/100 g b.w.). Independent samples *t*-tests were used to compare the growth rates, adrenal gland weights and thymus weights between the psychosocial stress and no psychosocial stress groups.

*Corticosterone Levels.* Since the purpose of the present experiment was to examine whether the proposed animal model of PTSD would produce reduced baseline glucocorticoid levels, a planned comparison (independent samples *t*-test) was used to

compare the baseline corticosterone levels of the psychosocial stress and no psychosocial stress groups. Additionally, a mixed-model ANOVA was employed to analyze the corticosterone levels of the psychosocial stress and no psychosocial stress groups from all three time points. In the ANOVA, psychosocial stress served as the between-subjects factor, and time point (baseline, stress, return-to-baseline) served as the within-subjects factor.

For all statistical analyses, alpha was set at 0.05, and Holm-Sidak post hoc comparisons were employed when necessary.

### *Results*

#### *Growth Rates (see Table 1)*

The psychosocial stress group tended to display a reduced growth rate, relative to the no psychosocial stress group, but this difference did not reach statistical significance,  $t(18) = 2.02, p = 0.058$ .

#### *Adrenal Gland Weights (see Table 1)*

The psychosocial stress group exhibited significantly larger adrenal glands than the no psychosocial stress group, indicative of chronic stress-induced adrenal hypertrophy,  $t(16) = 4.26, p < 0.001$ .

#### *Thymus Weights (see Table 1)*

The psychosocial stress group exhibited significantly smaller thymuses than the no psychosocial stress group, indicative of chronic stress-induced suppression of the immune system,  $t(16) = 2.12, p = 0.05$ .

Table 1

*Growth Rates, Adrenal Gland Weights and Thymus Weights ( $\pm$  SEM) for the Groups in Experiment 1*

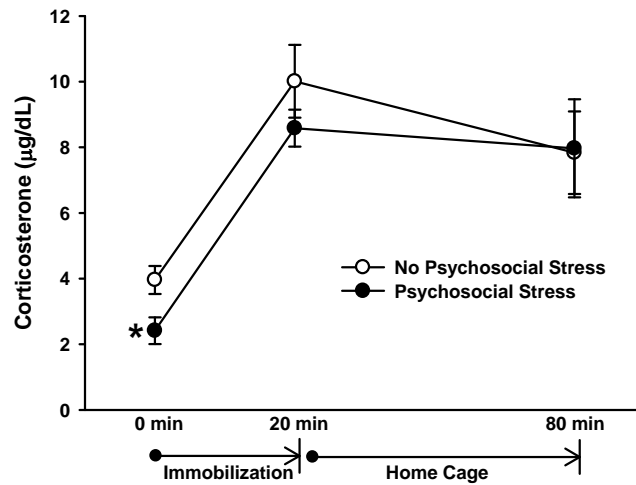
	Growth Rate (g/day)	Adrenal Gland Weight (mg/100 g b.w.)	Thymus Weight (mg/100 g b.w.)
No Psychosocial Stress	4.75 $\pm$ 0.26	7.45 $\pm$ 0.64	115.18 $\pm$ 7.31
Psychosocial Stress	3.72 $\pm$ 0.43	10.66 $\pm$ 0.44	96.47 $\pm$ 4.98

*Corticosterone Levels (see Figure 1)*

A planned comparison indicated that the psychosocial stress group (2.42  $\pm$  0.41  $\mu$ g/dL) displayed significantly lower baseline corticosterone levels than the no psychosocial stress group (3.96  $\pm$  0.43  $\mu$ g/dL),  $t(17) = 2.60$ ,  $p < 0.05$ .

The mixed-model ANOVA revealed a significant main effect of time point,  $F(2,32) = 23.19$ ,  $p < 0.001$ . Post hoc tests indicated that both groups demonstrated a significant increase in corticosterone levels following 20 minutes of acute immobilization stress and that these levels remained significantly elevated, relative to baseline, 1 hour later ( $p$ 's  $< 0.05$ ). There was no significant main effect of psychosocial stress,  $F(1,16) = 0.85$ , and the Time Point x Psychosocial Stress interaction was not significant,  $F(2,32) = 0.38$  ( $p$ 's  $> 0.05$ ).

### Chronic Psychosocial Stress Produced a Reduction of Basal Glucocorticoid Levels



*Figure 1.* Chronic psychosocial stress produced a reduction of basal glucocorticoid levels in rats. The data are presented as mean corticosterone levels ( $\mu\text{g/dL}$ )  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

#### *Discussion of Findings*

As expected, psychosocially stressed rats exhibited significantly larger adrenal glands, significantly smaller thymuses and a marginally significant reduction in growth rate, relative to control rats. Yet, the most important finding of the present experiment is that rats exposed to chronic psychosocial stress exhibited significantly lower baseline levels of corticosterone than control animals. This difference was observed in the early evening hours (i.e., 1700-2000 hours), a time when serum corticosterone levels begin to rise in rats, and is comparable to much of the literature in PTSD patients. Several studies have reported abnormally low baseline levels of cortisol in people with PTSD in the early morning hours, when levels of cortisol begin to rise in humans (Brand et al., 2006; Goenjian et al., 1996; King et al., 2001; Lindauer et al., 2006; Rohleder et al., 2004;

Seedat et al., 2003; Wessa et al., 2006). However, as indicated above, the findings regarding baseline levels of cortisol in PTSD patients have been mixed, and the presence of abnormally low levels of baseline cortisol may only be present in a biologic subtype of PTSD (for reviews, see de Kloet et al., 2006; Yehuda, 2002; Yehuda, 2005). If this is the case, then it is important to consider what subtype of PTSD our laboratory's chronic psychosocial stress paradigm is modeling. Since abnormally low levels of baseline cortisol have predominantly been reported in patients with combat-related PTSD (Boscarino, 1996; Kanter et al., 2001; Thaller et al., 1999; Yehuda et al., 1996b; Yehuda et al., 1993a), it is possible that our stress regimen models a subtype related to that which is caused by exposure to wartime combat. However, future work must clarify what specific biologic subtypes of PTSD exist before such a conclusion can be drawn with certainty.

Many animal models have reported that chronic stress, such as daily restraint stress (6 hours/day for 21 days), results in significantly elevated baseline glucocorticoid levels (Blanchard et al., 1993; Kant et al., 1987; Lepsch et al., 2005; Marin et al., 2007; Mizoguchi et al., 2001; Patterson-Buckendahl et al., 2001; Touyarot & Sandi, 2002). Few, however, have been shown to produce abnormally low baseline glucocorticoid levels similar to those reported here. Those animal models that have reported significantly reduced baseline glucocorticoid levels have employed either the single prolonged stress paradigm or a stress-restress paradigm consisting of situational reminders of the original stress experience (Diehl et al., 2007; Harvey et al., 2003). The single prolonged stress paradigm involves exposing rats to 2 hours of restraint, followed

by 20 minutes of swim stress, which is then terminated with exposure to ether vapors until anesthesia is induced. Investigators have reported that, up to 1 week later, such a paradigm results in abnormally low baseline glucocorticoid levels and enhanced negative feedback of the HPA axis, among other behavioral impairments (e.g., heightened anxiety, cognitive impairments, exaggerated startle response) (Diehl et al., 2007; Harada et al., 2008; Harvey et al., 2003; Iwamoto et al., 2007; Kohda et al., 2007; Liberzon et al., 1997; Takahashi et al., 2006; Wang et al., 2008). The present experiment therefore extends these findings by demonstrating that similar HPA axis abnormalities can be produced in rats by exposure to acute predator stress and daily social instability more than 4 weeks after the initial stress experience.

Psychosocially stressed rats did not display a greater acute stress-induced increase in corticosterone levels than control animals. This null effect could potentially be due to the time of day during which the blood samples were collected. Previous work has shown that the stress-induced increase in rodent corticosterone levels is not as robust during the dark cycle as it is during the light cycle (Kant et al., 1986; Yamada & Iwasaki, 1994). Therefore, it is possible that psychosocially stressed animals were limited in the extent to which their corticosterone levels could be increased by immobilization. Additionally, this null finding, although unexpected, is consistent with some of the PTSD literature reporting a blunted stress-induced increase in cortisol levels in PTSD patients. For instance, Geraciotti et al. (2008) found that combat veterans with PTSD, despite reporting significantly greater levels of anxiety, exhibited a significant reduction of CSF CRH levels and peripheral cortisol levels while watching a trauma-related film. Importantly,

this effect was not observed when the same combat veterans were exposed to a neutral film about oil painting. Some animal models of PTSD have also reported a blunted glucocorticoid response to acute stress in animals that have developed PTSD-like behaviors. Harvey et al. (2006) found that previously-stressed rats exhibited significantly lower corticosterone levels than control animals following 20 minutes of acute swim stress. In addition, Louvart et al. (2005) reported that previously-shocked animals displayed a smaller increase in corticosterone levels in response to a situational reminder of the shock than controls. Investigators have contended that these findings are a result of stress-induced changes in HPA axis function that results in enhanced negative feedback inhibition. Thus, in these referenced studies, the same acute stress-induced increase in corticosterone levels that was observed in control animals would theoretically result in significantly greater glucocorticoid receptor occupancy in previously stressed animals and, ultimately, lead to a much greater suppression of their corticosterone levels.

The findings of Experiment One indicate that the our laboratory's animal model of PTSD, composed of two acute predator exposures and daily social instability, produces changes in HPA axis functioning that are comparable to those observed in people with PTSD. Specifically, rats exposed to this chronic psychosocial stress paradigm exhibited significantly lower baseline glucocorticoid levels than control animals, and this effect was observed at a time of the circadian rhythm during which similar effects have been reported in PTSD patients. Therefore, this study provides further validation of our laboratory's animal model of PTSD and promotes its use to further investigate the mechanisms underlying trauma-induced changes in brain and behavior.

## Chapter Three: Experiment Two

### *Chronic Psychosocial Stress Results in Enhanced Suppression of Corticosterone Levels following Dexamethasone Administration: Evidence for Enhanced Negative Feedback of the Hypothalamus-Pituitary-Adrenal Axis*

Extensive work has suggested that people with PTSD may exhibit abnormally low baseline levels of cortisol due to the presence of enhanced negative feedback of the HPA axis (de Kloet et al., 2006). Several studies have found that PTSD patients have an increased number and sensitivity of glucocorticoid receptors (Rohleder et al., 2004; Stein et al., 1997b; Yehuda et al., 1991; Yehuda et al., 1993a; Yehuda et al., 1995). In addition, a majority of the PTSD literature has reported an increased suppression of cortisol and ACTH in people with PTSD following the administration of dexamethasone, a synthetic glucocorticoid (Duval et al., 2004; Goenjian et al., 1996; Grossman et al., 2003; Newport et al., 2004; Stein et al., 1997b; Yehuda et al., 1993b; Yehuda et al., 1995; Yehuda et al., 2002; Yehuda et al., 2004b). These findings suggest that dexamethasone results in greater negative feedback inhibition of the HPA axis, presumably due to the presence of more glucocorticoid receptors, in PTSD patients, which leads to a greater suppression of cortisol and ACTH in these individuals. Taking these findings into consideration, Experiment Two was designed to examine the effects of chronic psychosocial stress, composed of two acute predator exposures and daily social instability, on the corticosterone response in rats to the dexamethasone suppression test. I hypothesized that



psychosocially stressed rats would exhibit a significantly greater suppression of corticosterone levels than control rats following the administration of dexamethasone.

### *Methods*

#### *Rats*

The same weight range and strain of rats, as well as the housing conditions, that were employed in Experiment One were used in the present experiment. Upon arrival, all rats were given 1 week to acclimate to the housing room environment and cage changing procedures before any experimental manipulations took place. All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

#### *Psychosocial Stress Procedure*

Following the 1-week acclimation phase, rats were brought to the laboratory, weighed and assigned to “psychosocial stress” or “no psychosocial stress” groups (N = 40 rats/group). Afterwards, each group of rats was exposed to the same respective manipulations that were utilized in Experiment One. That is, rats in the psychosocial stress group were given two acute cat exposures in conjunction with daily social stress, while rats in the no psychosocial stress group were given two laboratory exposures (remaining in their home cages) and had the same cage mates throughout the duration of the experiment.

#### *Assessment of Post-Dexamethasone Basal and Stress-Induced Glucocorticoid Levels*

*Preparation.* Twenty days after the second stress session, rats in the psychosocial stress and no psychosocial stress groups were brought to the laboratory and weighed. As

in Experiment One, the hind legs of all rats were then shaved to allow access to their saphenous veins. The rats were then taken back to the housing room and left undisturbed for the remainder of the day.

*Pharmacological Manipulations.* On the following day, between 1100 and 1400 hours, rats were taken to the procedure room, one cage at a time, where they were administered subcutaneous (s.c.) injections of dexamethasone (10 µg/kg, 25 µg/kg, 50 µg/kg) or vehicle at a volume of 1 ml/kg. These doses were chosen because previous work indicated that they produced a modest suppression of corticosterone levels in control rats (Lurie et al., 1989). Ten rats from each of the psychosocial stress and no psychosocial stress groups were randomly assigned to receive s.c. injections of one of the three doses of dexamethasone or the vehicle solution, for a total of 10 rats per group. Dexamethasone (Sigma-Aldrich, St. Louis, MO) was dissolved in a vehicle solution consisting of sodium sulfite (1 mg/ml) and sodium citrate (19.4 mg/ml), which were both dissolved in distilled water. Immediately following the administration of dexamethasone or vehicle, the rats were returned to the housing room until the commencement of blood sampling.

*Blood Sampling and Post-Mortem Dissection.* Six hours following dexamethasone or vehicle administration, three blood samples (baseline, stress and return-to-baseline) were obtained from all rats, following the procedures utilized in Experiment One. Following rapid decapitation, the adrenal and thymus glands were removed and weighed. All blood sampling took place between 1700 and 2100 hours. Once all of the blood had clotted at room temperature, it was centrifuged (3000 rpm for 8

minutes), and the serum was extracted and stored at -80° C until assayed by Monika Fleshner at the University of Colorado at Boulder.

### *Statistical Analyses*

*Experimental Design.* The present study utilized a between-subjects, 2 x 4 factorial design. The between-subjects factors were psychosocial stress (psychosocial stress, no psychosocial stress) and dexamethasone (vehicle and 10 µg/kg, 25 µg/kg or 50 µg/kg of dexamethasone).

*Growth Rate, Adrenal Gland Weights and Thymus Weights.* Growth rates, expressed as grams per day (g/day), were calculated for all rats by dividing their total body weight gained during the course of the experiment by the total number of days in the experiment (i.e., 31 days). The adrenal glands and thymuses were weighed and expressed as milligrams per 100 grams of body weight (mg/100 g b.w.). Two-way ANOVAs were used to analyze the growth rates, adrenal gland weights and thymus weights, with psychosocial stress and dexamethasone serving as the between-subjects factors in each case.

*Corticosterone Levels.* A mixed-model ANOVA was employed to analyze the corticosterone levels of all groups from the three time points. In the ANOVA, psychosocial stress and dexamethasone served as the between-subjects factors, and time point (baseline, stress, return-to-baseline) served as the within-subjects factor.

For all statistical analyses, alpha was set at 0.05, and Holm-Sidak post hoc comparisons were employed when necessary. Since dexamethasone administration took place on the final day of the experiment, it was predicted that the drug would have no

effect on growth rate and adrenal gland or thymus weights. As this was confirmed via the statistical analyses below, Table 2 presents the predicted effects of psychosocial stress on growth rate and adrenal gland and thymus weights, collapsed across all drug conditions.

### *Results*

#### *Growth Rates (see Table 2)*

The growth rate analysis revealed a significant main effect of psychosocial stress,  $F(1,72) = 9.67, p < 0.01$ , indicating that the psychosocial stress group had a significantly lower growth rate than the no psychosocial stress group. There was no significant main effect of dexamethasone,  $F(3,72) = 1.80$ , and the Psychosocial Stress x Dexamethasone interaction was not significant,  $F(3,72) = 1.01 (p's > 0.05)$ .

#### *Adrenal Gland Weights (see Table 2)*

The analysis of adrenal gland weights revealed a significant main effect of psychosocial stress,  $F(1,72) = 8.42, p < 0.01$ , indicating that the psychosocial stress group had significantly larger adrenal glands than the no psychosocial stress group. There was no significant main effect of dexamethasone,  $F(3,72) = 1.56$ , and the Psychosocial Stress x Dexamethasone interaction was not significant,  $F(3,72) = 0.03 (p's > 0.05)$ .

#### *Thymus Weights (see Table 2)*

The analysis of thymus weights revealed a significant main effect of psychosocial stress,  $F(1,70) = 35.89, p < 0.001$ , indicating that the psychosocial stress group had significantly smaller thymuses than the no psychosocial stress group. There was no significant main effect of dexamethasone,  $F(3,70) = 2.67$ , and the Psychosocial Stress x Dexamethasone interaction was not significant,  $F(3,70) = 1.77 (p's > 0.05)$ .

Table 2

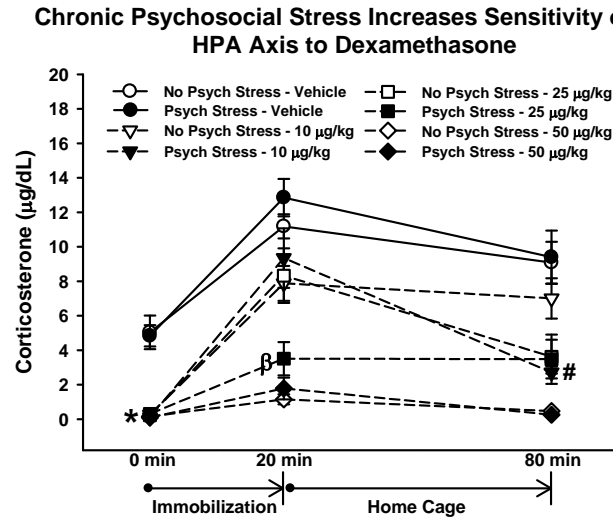
*Growth Rates, Adrenal Gland Weights and Thymus Weights ( $\pm$  SEM) for the Psychosocial Stress and No Psychosocial Stress Groups (collapsed across all dexamethasone conditions) in Experiment 2*

	Growth Rate (g/day)	Adrenal Gland Weight (mg/100 g b.w.)	Thymus Weight (mg/100 g b.w.)
No Psychosocial Stress	5.39 $\pm$ 0.18	9.70 $\pm$ 0.39	113.77 $\pm$ 3.34
Psychosocial Stress	4.65 $\pm$ 0.16	11.32 $\pm$ 0.39	90.08 $\pm$ 2.59

*Corticosterone Levels (See Figures 2 and 3)*

The mixed-model ANOVA revealed a significant main effect of time point,  $F(2,126) = 102.31, p < 0.001$ . Post hoc tests indicated that, overall, rats demonstrated a significant increase in corticosterone levels following 20 minutes of acute immobilization stress and that these levels significantly declined, yet remained elevated relative to baseline, 1 hour later ( $p$ 's  $< 0.05$ ). There was also a significant main effect of dexamethasone,  $F(3,63) = 67.47, p < 0.001$ . Post hoc tests revealed that, as expected, dexamethasone led to a significant reduction in circulating corticosterone levels. More specifically, the rats treated with 10  $\mu$ g/kg or 25  $\mu$ g/kg of dexamethasone displayed significantly lower corticosterone levels than the rats treated with vehicle, and the rats treated with 50  $\mu$ g/kg of dexamethasone exhibited significantly lower corticosterone

levels than the rats treated with vehicle or the two lower doses of dexamethasone. There was no significant main effect of psychosocial stress,  $F(1,63) = 1.28, p > 0.05$ .

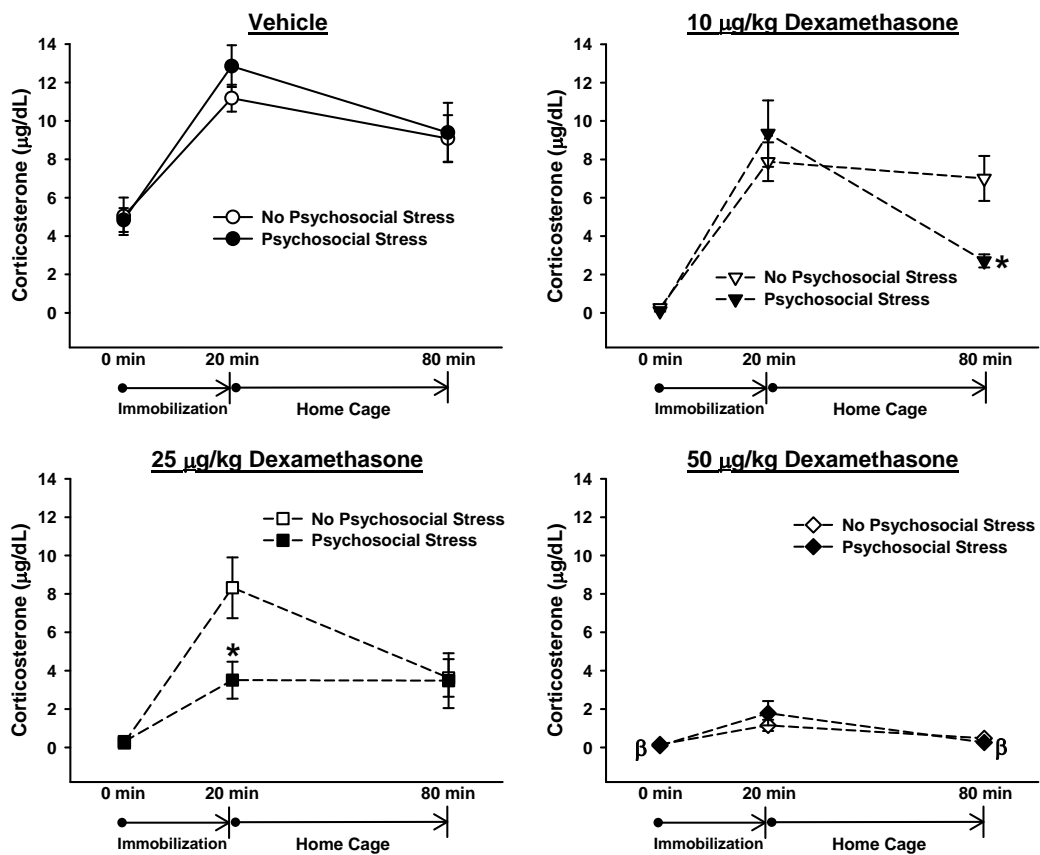


*Figure 2.* Chronic psychosocial stress increases sensitivity of the HPA axis to dexamethasone. The data are presented as mean corticosterone levels ( $\mu\text{g/dL}$ )  $\pm$  SEM. \* =  $p < 0.05$  (all dexamethasone-treated groups relative to the vehicle-treated groups);  $\beta = p < 0.05$  relative to 25  $\mu\text{g/kg}$  dexamethasone-treated no psychosocial stress group; # =  $p < 0.05$  relative to 10  $\mu\text{g/kg}$  dexamethasone-treated no psychosocial stress group.

The Time Point x Dexamethasone,  $F(6,126) = 8.68$ , and Time Point x Psychosocial Stress x Dexamethasone,  $F(6,126) = 4.12$ , interactions were significant ( $p$ 's  $< 0.001$ ). Post hoc tests indicated that 10  $\mu\text{g/kg}$  of dexamethasone did not prevent the acute stress-induced increase in corticosterone levels in either the psychosocial stress or no psychosocial stress groups; however, it did lead to a greater suppression of post-immobilization corticosterone levels in the psychosocial stress group. The administration of 25  $\mu\text{g/kg}$  of dexamethasone prevented the acute stress-induced increase in corticosterone levels in the psychosocial stress group only. Finally, 50  $\mu\text{g/kg}$  of

dexamethasone tended to produce lower baseline and post-immobilization corticosterone levels in the psychosocial stress group, relative to the no psychosocial stress group, although these comparisons did not achieve statistical significance ( $p$ 's = 0.07). The Time Point x Psychosocial Stress,  $F(2,126) = 0.92$ , and Psychosocial Stress x Dexamethasone,  $F(3,63) = 1.38$ , interactions were not significant ( $p$ 's > 0.05).

**Effects of Chronic Psychosocial Stress on Corticosterone Responses following Different Doses of Dexamethasone Administration**



*Figure 3.* Effects of chronic psychosocial stress on corticosterone responses following different doses of dexamethasone. The data are presented as mean corticosterone levels (µg/dL) ± SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group; β =  $p = 0.07$  relative to the no psychosocial stress group.

### *Discussion of Findings*

In contrast to Experiment One, vehicle-treated psychosocially stressed rats did not display significantly lower baseline corticosterone levels than vehicle-treated control animals. This null effect is likely due to the fact that rats in the present experiment were not left undisturbed for the entire day leading up to blood sampling, as was the case in Experiment One. Rats in the present experiment were injected 6 hours prior to blood sampling, which could have potentially influenced baseline HPA axis functioning for the remainder of the day.

As expected, dexamethasone-treated animals, in general, displayed significantly lower baseline corticosterone levels than vehicle-treated animals. In addition, relative to vehicle, the two higher doses of dexamethasone significantly blunted the immobilization-induced increase in corticosterone levels and led to significantly lower corticosterone levels in all rats an hour later. As in Experiment One, psychosocially stressed rats also exhibited significantly larger adrenal glands, significantly smaller thymuses and a significant reduction in growth rate, relative to control rats.

The most important finding of the present experiment, however, is that chronic psychosocial stress, involving two acute predator exposures and daily social instability, resulted in enhanced negative feedback sensitivity to the synthetic glucocorticoid, dexamethasone. Psychosocially stressed animals displayed a greater suppression of post-dexamethasone corticosterone levels in a dose- and time-dependent manner. In response to 10  $\mu\text{g}/\text{kg}$  of dexamethasone, psychosocially stressed rats exhibited a greater recovery of corticosterone levels than control animals an hour following exposure to 20 minutes



of immobilization. The 25 µg/kg dose of dexamethasone prevented the acute immobilization-induced increase in corticosterone levels in only the psychosocial stress group, and the 50 µg/kg dose led to marginally lower corticosterone levels in the psychosocial stress group, relative to controls, at baseline and an hour following the 20 minutes of immobilization. These findings suggest that the stress regimen employed in this experiment results in enhanced negative feedback inhibition of the HPA axis. More specifically, since the doses of dexamethasone that were used in this study do not cross the blood-brain barrier (Meijer et al., 1998; Schinkel et al., 1995), the findings indicate that this enhanced negative feedback occurs at the level of the pituitary gland.

These findings are consistent with a majority of the PTSD literature. A number of studies have reported that PTSD patients have an increased number and sensitivity of glucocorticoid receptors (Rohleder et al., 2004; Stein et al., 1997b; Yehuda et al., 1991; Yehuda et al., 1993a; Yehuda et al., 1995) and display an increased suppression of cortisol and ACTH following the administration of dexamethasone (Duval et al., 2004; Goenjian et al., 1996; Grossman et al., 2003; Newport et al., 2004; Stein et al., 1997b; Yehuda et al., 1993b; Yehuda et al., 1995; Yehuda et al., 2002; Yehuda et al., 2004b). Some studies have also observed increased activation of the pituitary gland in PTSD patients following the administration of metyrapone, which investigators believe to be due to the fact that metyrapone removes the enhanced negative feedback inhibition initially present in these individuals (Otte et al., 2006; Yehuda et al., 1996a). Collectively, these findings have implicated enhanced negative feedback inhibition in the HPA axis abnormalities observed in people with PTSD.

Rarely have investigators tested for the presence of enhanced negative feedback inhibition of the HPA axis in animal models of PTSD. Only two studies have conducted such assessments. Liberzon et al. (1997) found that rats exposed to a single prolonged stress paradigm subsequently (i.e., 1 week later) exhibited a blunted restraint stress-induced increase in ACTH levels following the administration of cortisol. In addition, similar to the present findings, Kohda et al. (2007) reported that rats exposed to a single prolonged stress paradigm subsequently (i.e., 1 week later) exhibited a blunted restraint stress-induced increase in corticosterone levels following the administration of dexamethasone. Both of these findings suggest that the single prolonged stress paradigm produces changes in HPA axis function that resemble enhanced negative feedback inhibition and are comparable to the present set of data.

The commonalities in HPA responses between psychosocially stressed rats from the present studies and traumatized people with PTSD further validate the use of this chronic psychosocial stress paradigm to explore the mechanisms underlying emotional trauma-induced changes in brain and behavior. Nevertheless, future work concerning the neurobiological bases of the present effects should examine other markers of enhanced negative feedback inhibition of the HPA axis, such as enhanced glucocorticoid receptor expression in key areas of the brain (e.g., anterior pituitary gland, hippocampus). Future studies will also need to explore the effect of metyrapone or dexamethasone-CRH challenge paradigms on pituitary function (e.g., ACTH release). Our laboratory already has preliminary data indicating that rats exposed to the psychosocial stress regimen exhibit significantly greater baseline levels of CRH mRNA in the paraventricular nucleus

of the hypothalamus than control animals (unpublished findings). This finding suggests that psychosocially stressed rats might also display abnormally high CRH levels, which would also be consistent with the PTSD literature. Future work, however, must be conducted to verify this hypothesis.

## Chapter Four: Experiment Three

### *Differential Effectiveness of the Pharmacological Agents Amitriptyline, Clonidine and Tianeptine in Blocking the PTSD-Like Physiological and Behavioral Sequelae in Rats*

A subset of people with PTSD exhibit significant improvement in their symptoms following treatment with SSRIs (Asnis et al., 2004; Davidson, 2003; Davis et al., 2006; Hidalgo & Davidson, 2000; Ipser et al., 2006; Stein et al., 2006). At this time, the SSRIs sertraline and paroxetine are the only two medications that have been approved by the Food and Drug Administration (FDA) for the treatment of PTSD (Albucher & Liberzon, 2002; Barrett et al., 2005; Van der Kolk, 2001; Vaswani et al., 2003). However, SSRIs tend to blunt only the depressive components of PTSD, while having little effect on the memory- and anxiety-related symptoms of the disorder (Asnis et al., 2004; Boehnlein & Kinzie, 2007; Brady et al., 2000; Van der Kolk et al., 1994). In addition, some forms of PTSD, such as combat-related PTSD, are incredibly resistant to SSRI treatment (Jakovljevic et al., 2003; Rothbaum et al., 2008; Stein et al., 2002). They can even produce severe adverse side effects, including sleep disruption, headache, abdominal pain, sexual dysfunction, agitation, nausea and weight gain, which significantly interfere with an individual's daily life (Asnis et al., 2004; Boehnlein & Kinzie, 2007; Brady et al., 2000; Van der Kolk et al., 1994). Thus, there is a need for additional pharmacological research in people with PTSD and in animal models of the disorder to facilitate the development of more effective pharmacotherapy for PTSD patients.

The purpose of Experiment Three was to ascertain whether chronic prophylactic administration of amitriptyline, clonidine and tianeptine would ameliorate the physiological and behavioral sequelae induced by chronic psychosocial stress in rats. Amitriptyline is a tricyclic antidepressant that has been reported to significantly ameliorate many symptoms of PTSD, especially those related to intrusion, avoidance and re-experiencing (Davidson et al., 1990; Davidson et al., 1993). Clonidine is an anxiolytic and  $\alpha_2$  adrenergic receptor agonist that, via the facilitation of adrenergic autoreceptor activity, leads to a significant reduction in NE levels throughout the central nervous system. As PTSD is characterized by abnormally high levels of NE, researchers have contended that clonidine should ameliorate many of the symptoms of PTSD, and especially those related to hyperarousal (Boehnlein & Kinzie, 2007). However, as of now, there have been no randomized, placebo-controlled studies on the efficacy of clonidine in treating people with PTSD. The final experimental treatment was tianeptine, an antidepressant. While this agent is most commonly known to exert antidepressant effects and ameliorate symptoms of major depression, it has been shown to have beneficial effects in PTSD patients as well (Onder et al., 2006). Moreover, numerous studies in rodents have provided support for tianeptine's use in treating stress-related psychopathologies, as it blocks the adverse effects of stress on cognitive, electrophysiological, morphological and molecular measures of hippocampal functioning (Diamond et al., 2004; Kasper & McEwen, 2008; McEwen et al., 2002; McEwen & Olie, 2005; Uzbay, 2008). To emphasize that the design of the present experiment had clinical relevance, administration of the pharmacological agents did not begin until the day after

the stress paradigm commenced. Treatment beginning 24 hours after exposing rats to an intense stressor is potentially relevant to treatment applications begun in people within 24 hours of a traumatic experience and may highlight the importance of quickly beginning a treatment regimen soon after experiencing intense trauma.

### *Methods*

#### *Rats*

The same weight range and strain of rats, as well as the housing conditions, that were employed in Experiments One and Two were used in the present experiment. Upon arrival, all rats were given 1 week to acclimate to the housing room environment and cage changing procedures before any experimental manipulations took place. All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

#### *Psychosocial Stress Procedure*

PTSD is a disorder of memory, and individuals suffering from PTSD experience chronic psychological distress by repeatedly reliving their trauma through intrusive, flashback memories (Ehlers et al., 2004; Hackmann et al., 2004; Reynolds & Brewin, 1998; Reynolds & Brewin, 1999; Speckens et al., 2006; Speckens et al., 2007).

Therefore, to incorporate a rat analog of a traumatic memory into our animal model of PTSD, we developed a paradigm to quantify the memory for the acute cat exposures that are a part of the psychosocial stress procedure (Halonen et al., 2006). This paradigm was included in the psychosocial stress procedure in the present experiment to assess, during behavioral testing, the long-term memory of the acute cat exposures.

Following the 1-week acclimation phase, rats were brought to the laboratory and then assigned to “psychosocial stress” or “no psychosocial stress” groups (N = 60 rats/group). Afterwards, rats from each group were exposed to a chamber for 3 minutes. During the last 30 seconds of the 3-minute chamber exposure, a 74-dB, 2500 Hz tone was presented to the rats. The chamber (25.50 x 30 x 29 cm; Coulbourn Instruments; Allentown, PA) consisted of two aluminum sides, an aluminum ceiling, and a Plexiglas front and back. The floor of the chamber consisted of 18 stainless steel rods, spaced 1.25 cm apart. The rats were not exposed to footshock at any time in the chamber. The sole purpose of exposing rats to the chamber was to allow rats in the psychosocial stress group to associate the chamber (contextual fear conditioning) and tone [auditory (cue) fear conditioning] with the acute stress experience (i.e., immobilization plus cat exposure) and measure their memory for the experience (via an assessment of immobility in the chamber) during behavioral testing. Locomotor activity in the chamber was measured during the acute stress sessions and behavioral testing by a 24-cell infrared activity monitor (Coulbourn Instruments; Allentown, PA) mounted on the top of the chamber, which used the emitted infrared body heat image (1300 nm) from the animals to detect their movement. Immobility was defined as periods of inactivity lasting at least seven seconds. Following the 3-minute chamber exposure, rats in the psychosocial stress group were exposed to 1 hour of immobilization during cat exposure (as per the methods in Experiments One and Two), while rats in the no psychosocial stress group remained in their home cages in the laboratory for a yoked period of time. Both groups of rats were weighed following the 1-hour period and then returned to their housing rooms. As per

Experiments One and Two, this entire process (i.e., acute stress session) was repeated 10 days later (during the dark cycle on Day 11), and beginning with the day of the first stress session, rats in the psychosocial stress group were exposed to unstable housing conditions for the next 31 days.

### *Pharmacological Agents*

Twenty-four hours after the first stress session (i.e., Day 2), all rats began receiving daily intraperitoneal (i.p.) injections of amitriptyline (5 or 10 mg/kg), clonidine (0.01 or 0.05 mg/kg), tianeptine (10 mg/kg), or vehicle (distilled water). The injections occurred every day throughout the 31-day period of psychosocial stress and also during behavioral testing. Drug administration was continued during behavioral testing to prevent withdrawal effects from influencing rat behavior. The injections were always administered in the morning (between 0900 and 1200 hours) at a volume of 1 ml/kg. Ten rats from each of the psychosocial stress and no psychosocial stress groups were randomly assigned to each of the drug conditions, for a total of 10 rats per group. Amitriptyline and clonidine were obtained from Sigma-Aldrich (St. Louis, MO), while tianeptine was provided by Servier Pharmaceuticals (France).

### *Behavioral Testing*

Three weeks after the second stress session (Day 32), rats were given tests to measure their fear memory, anxiety, startle, learning and memory, cardiovascular activity and corticosterone activity. The 3-week delay from the second stress session to behavioral testing was based on comparable time periods employed in other studies on the effects of stress on brain and behavior (Adamec & Shallow, 1993; Cook & Wellman,



2004; Magarinos et al., 1996; McLaughlin et al., 2007; Park et al., 2001; Watanabe et al., 1992a; Watanabe et al., 1992c; Watanabe et al., 1992b). Additionally, work from our laboratory has previously shown that the chronic psychosocial stress paradigm employed in the present experiment produces significant changes in rat physiology and behavior that can be detected 3 weeks following the second stress session (Zoladz et al., 2008). On the first 4 days of behavioral testing (Days 32-35), all rats were taken to the procedure room across from the rat housing rooms, where they received i.p. injections of the drug appropriate to the condition to which they had been assigned. Then, they were taken to the laboratory and left undisturbed for 30 minutes before testing began. All behavioral testing took place during the light cycle, between 0800 and 1500 hours.

#### *Behavioral Apparatus*

*Contextual and Cue Fear Memory.* On Day 32, rat behavior in response to the chamber (context test) and tone (cue test) that were previously paired with the acute stress sessions was examined. Rats were placed in the same chamber that they were exposed to during each of the two stress sessions for 5 minutes, and their immobility was recorded, as per the methods described above. An hour after the 5-minute context test, the rats were placed in a novel chamber that had different lighting, walls and flooring from that of the chamber in which they were placed during each of the two stress sessions. The rats were placed in the novel chamber for a total of 6 minutes (cue test). Three minutes into the cue test, the rats were presented with a 74-dB, 2500 Hz tone that continuously played for the remainder of the 6-minute testing period. The amount of immobility recorded during the first 3 minutes of the cue test (i.e., no tone) provided a measure of the

general fear of a novel place, while the amount of immobility recorded during the last 3 minutes of the cue test (i.e., tone) provided a measure of the fear response to the cue that was, in the psychosocial stress group, specifically associated with the two acute cat exposures.

*Elevated Plus Maze.* The elevated plus maze (EPM) is a routine test of anxiety in rodents (Korte & De Boer, 2003) and consists of two open arms (10.80 x 50.17 cm) and two closed arms (10.80 x 50.17 cm) that intersect each other to form the shape of a plus sign. On Day 33, the rats were placed on the EPM for 5 minutes, and their behavior was scored by 48 infrared photobeams (located along the perimeter of the open and closed arms), which were connected to a computer program (Motor Monitor, Hamilton-Kinder, San Diego, CA). The primary dependent measures of interest were the amount of time rats spent in the open arms and the number of ambulations made by each rat. An arm entry was scored by the computer program only when a rat's entire body had moved from one arm into a new arm (e.g., the entire body of the rat moved from the closed arms into an open arm). Thus, the computer program would begin tallying open arm time only after a rat had completely entered an open arm. An ambulation was scored by the computer program each time a rat crossed a photobeam sensor. Thus, the ambulations score consisted of the total number of beam breaks made by each rat during the 5-minute trial and served as a measure of motor activity. Between each testing session, the EPM was wiped down with a 25% ethanol solution.

*Startle Response.* One hour after the EPM assessment, acoustic startle testing was administered. The rats were placed inside a small Plexiglas box (18.50 x 9.75 x 9.75 cm),

which was inside a larger startle monitor cabinet (Hamilton-Kinder; San Diego, CA; 35.56 x 27.62 x 49.53 cm). The small Plexiglas box within this cabinet contained a sensory transducer on which the rats were placed at the beginning of the trial. The sensory transducer was connected to a computer (Startle Monitor computer program; Hamilton-Kinder; San Diego, CA), which recorded the startle responses by measuring the maximum amount of force (in Newtons) that rats exerted on the sensory transducer for a period of 250 ms after the presentation of each auditory stimulus. To control for any differences in body weight, the sensitivity of the sensory transducer was adjusted prior to each trial via a Vernier adjustment with a sensitivity range of 0-7 arbitrary units. The startle trial began with a 5-minute acclimation period, followed by the presentation of 24 bursts of white noise (50 ms each), eight from each of three auditory intensities (90, 100, and 110 dB). The noise bursts were presented in sequential order (i.e. eight bursts at 90 dB, followed by eight bursts at 100 dB, followed by eight bursts at 110 dB), and the time between each noise burst varied pseudorandomly between 25 and 55 seconds. Upon the commencement of the first noise burst, the startle apparatus provided uninterrupted background white noise (57 dB).

*Novel Object Recognition.* The novel object recognition (NOR) task was a modified version of that which was employed by Baker and Kim (2002). On Day 34, the rats were placed in an open field (Hamilton-Kinder, San Diego, CA – 40 x 47 x 70 cm) for 5 minutes to acclimate to the environment. Their behavior was monitored by a Logitech camera that was mounted on the ceiling overlooking the open field. This camera was connected to a computer program known as ANY-Maze (Stoelting; Wood Dale, IL),

which scored rat behavior. Twenty-four hours later (Day 35), the rats were placed in the same open field with two identical (plastic/metal) objects for 5 minutes. The objects were in opposite corners of the open field and secured to the flooring to prevent the rats from displacing them. The objects were counterbalanced across rats, as were the corners in which the objects were placed. Three hours later, the rats were returned to the open field for a final 5-minute test trial, but this time the open field contained a replica of the object that had been there before and a novel object. During this testing session, greater time spent by the rats in proximity to the novel versus familiar object was an indication of intact memory for the familiar object. The time that each rat spent with the objects during training and testing was quantified by specifying a 16 cm<sup>2</sup> zone around the objects for the ANY-maze software to score the duration of investigatory behavior.

*Preparation for Blood Sampling.* Immediately following the 3-hour object recognition test, the hind legs of all rats were shaved to allow access to their saphenous veins, as per Experiments One and Two.

*Blood Sampling and Cardiovascular Activity.* On the final day of behavioral testing (Day 36), rats were brought, one cage at a time, to a nearby procedure room for blood sampling. Then, baseline and post-stress samples of blood were collected from the rats, as per the methods employed in Experiments One and Two. Immediately after collecting the post-immobilization blood sample, the rats were placed in Plexiglas tubes within a warming test chamber to increase their body temperature. This enhanced blood flow to their tails, and allow HR and BP to be assessed using a tail cuff fitted with photoelectric sensors (IITC Life Science; Woodland Hills, CA). Once their body

temperature reached approximately 32° C, three HR and BP recordings were obtained from each rat (these three recordings were averaged to create single HR and BP data points for each rat). An hour later, one last blood sample (trunk blood) was collected following rapid decapitation. Then, the adrenal glands and thymuses were removed and weighed. Once all of the blood had clotted at room temperature, it was centrifuged (3000 rpm for 8 minutes), and the serum was extracted and stored at -80° C until assayed by Monika Fleshner at the University of Colorado at Boulder.

### *Statistical Analyses*

*Experimental Design and General Analyses.* The present study utilized a between-subjects, 2 x 6 factorial design. The independent variables were psychosocial stress (psychosocial stress, no psychosocial stress) and drug (vehicle, amitriptyline – 5 and 10 mg/kg, clonidine – 0.01 and 0.05 mg/kg, tianeptine – 10 mg/kg). In most cases, two-way, between-subjects ANOVAs were used to analyze the data from the physiological and behavioral assessments, with psychosocial stress and drug serving as the between-subjects factors. Planned comparisons (independent samples *t*-tests) were also conducted between groups that were predicted to differ *a priori*. For all analyses, alpha was set at 0.05, and Holm Sidak post hoc tests were employed when necessary.

*Fear Memory.* The amount of immobility from each chamber exposure (Stress Session 1, Stress Session 2, Context Test, Cue Test – No Tone, Cue Test – Tone) was analyzed separately. The number of fecal boli that rats produced in the chamber was also analyzed for the Context and Cue Tests. For each assessment, two-way, between-subjects

ANOVAs were used to analyze behavior. Psychosocial stress and drug served as the between-subjects factors in each case.

*Elevated Plus Maze.* The amount of time that rats spent in the open arms of the EPM was calculated as a percent of the total trial time. The percent time that rats spent in the open arms, as well as the number of ambulations that rats made on the EPM were analyzed with two-way, between-subjects ANOVAs. Each of these analyses was performed for the entire 5-minute testing trial and for the first minute of the testing trial, with psychosocial stress and drug serving as the between-subjects factors in each case.

*Startle Response.* Startle responses to each of the three auditory stimulus intensities (90, 100 and 110 dB) were analyzed separately. In each case, two-way, between-subjects ANOVAs were employed to analyze the data, with psychosocial stress and drug serving as the between-subjects factors.

*Novel Object Recognition.* For habituation, a two-way, between-subjects ANOVA was used to compare overall locomotor activity across all groups, with psychosocial stress and drug serving as the between-subjects factors. The amount of time that rats spent in each area of the open field during the habituation phase was also analyzed to assure that the rats did not display a preference for one area of the open field over another. For the analysis, the open field was divided into four square quadrants via the ANY-Maze computer program. The amount of time that rats spent in each of the quadrants was analyzed with a mixed-model ANOVA, with psychosocial stress and drug serving as the between-subjects factors and time spent in each quadrant serving as the within-subjects factor. For training, paired samples *t*-tests were first conducted to

determine whether the rats within each group spent a comparable amount of time with each object replica (to rule out object preference effects). Then, the total time that rats spent with both object replicas during training was compared across groups by using two-way, between-subjects ANOVAs, with psychosocial stress and drug serving as the between-subjects factors. For testing, a “ratio time” score was calculated for each group by taking the time that rats spent with the novel object and dividing it by the time that rats spent with the familiar object (i.e., ratio time = time with novel object / time with familiar object). The ratio times were compared across groups by utilizing two-way, between-subjects ANOVAs, with psychosocial stress and drug again serving as the between-subjects factors. This was performed for the entire 5-minute testing trial and for the first minute of the testing trial.

*Corticosterone Levels.* A mixed-model ANOVA was used to analyze corticosterone levels at the three time points. Psychosocial stress and drug served as the between-subjects factors, and time point (baseline, stress, return-to-baseline) served as the within-subjects factor.

*Heart Rate and Blood Pressure.* The HR, systolic BP and diastolic BP data were analyzed with two-way, between-subjects ANOVAs, with psychosocial stress and drug serving as the between-subjects factors.

*Growth Rates, Adrenal Gland Weights and Thymus Weights.* Growth rates, expressed as grams per day (g/day), were calculated for all rats by dividing their total body weight gained during the course of the experiment by the total number of days in the experiment (i.e., 31 days). The adrenal glands and thymuses were weighed and

expressed as milligrams per 100 grams of body weight (mg/100 g b.w.). Two-way, between-subjects ANOVAs were used to analyze the growth rates, adrenal gland weights and thymus weights, with psychosocial stress and drug serving as the between-subjects factors in each case.

## Results

### Fear Memory

*Stress Session One* (see Figure 4). For the analysis of immobility during the 3-minute chamber exposure during stress session one, there were no significant main effects of psychosocial stress,  $F(1,104) = 0.48$ , or drug,  $F(5,104) = 0.84$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,104) = 1.13$  ( $p$ 's > 0.05).

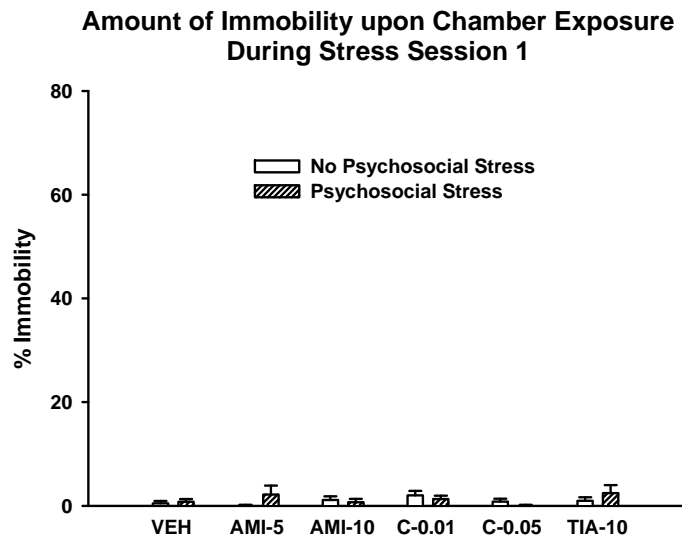
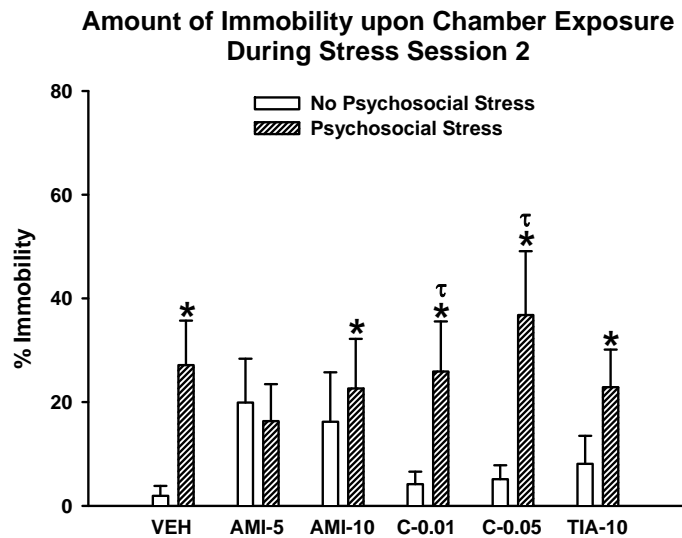


Figure 4. Amount of immobility during the 3-minute chamber exposure during stress session one. The data are presented as mean percent immobility  $\pm$  SEM.

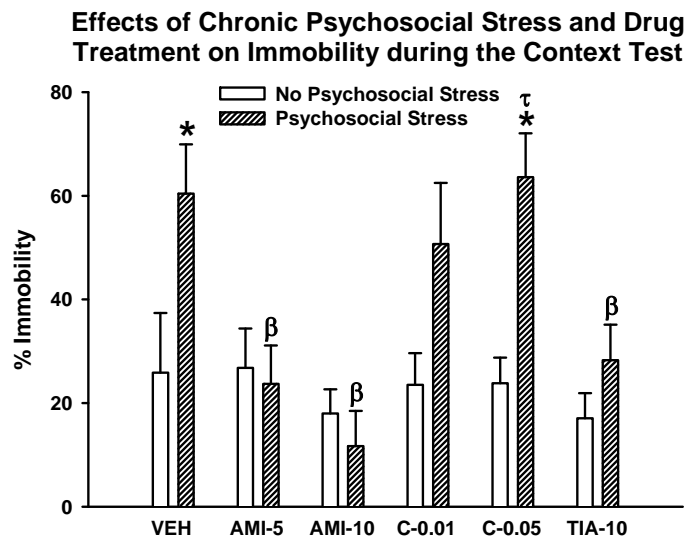
*Stress Session Two* (see Figure 5). For the analysis of immobility during the 3-minute chamber exposure during stress session two, there was a significant main effect of



psychosocial stress, indicating that the psychosocial stress groups spent significantly more time immobile than the no psychosocial stress groups,  $F(1,103) = 7.55, p < 0.01$ . There was no significant main effect of drug,  $F(5,103) = 0.48$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,103) = 0.96 (p's > 0.05)$ . Planned comparisons were also conducted between groups that were predicted to differ *a priori*. The vehicle-treated psychosocial stress group spent significantly more time immobile than the vehicle-treated no psychosocial stress group,  $t(17) = 2.73, p < 0.05$ . Groups of psychosocially stressed rats that were treated with 0.01,  $t(18) = 2.19$ , or 0.05,  $t(16) = 2.26$ , of clonidine were the only other psychosocial stress groups that displayed significantly greater immobility than their respective control groups ( $p's < 0.05$ ).



*Figure 5.* Amount of immobility during the 3-minute chamber exposure during stress session two. The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.



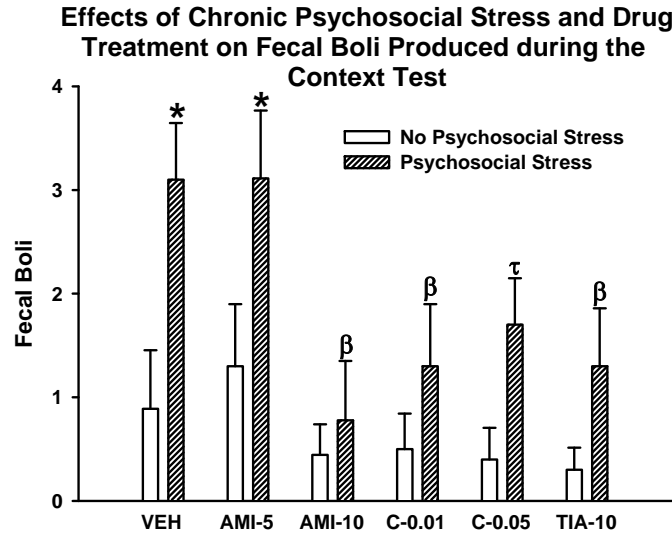
*Figure 6.* Effects of chronic psychosocial stress and drug treatment on immobility during the 5-minute context test. The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Context Test Immobility (see Figure 6).* For the analysis of immobility during the 5-minute context test, there were significant main effects of psychosocial stress,  $F(1,97) = 11.96$ , and drug,  $F(5,97) = 3.90$ , and the Psychosocial Stress  $\times$  Drug interaction was significant,  $F(5,97) = 2.56$  ( $p$ 's  $< 0.05$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group spent significantly more time immobile than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced increase in immobility, as evidenced by significantly less immobility than the vehicle-treated psychosocial stress group and a lack of statistical significance relative

to each of the group's respective drug-treated no psychosocial stress group. The group of psychosocially stressed rats treated with 0.01 mg/kg of clonidine also did not exhibit significantly greater immobility than its respective drug-treated control group; however, the amount of immobility displayed by this group was not statistically different from that of the vehicle-treated psychosocial stress group.

*Context Test Fecal Boli (see Figure 7).* The analysis of fecal boli produced during the context test revealed significant main effects of psychosocial stress,  $F(1,97) = 15.45$ , and drug,  $F(5,97) = 4.09$  ( $p$ 's  $< 0.01$ ). The psychosocial stress groups produced significantly more fecal boli than the no psychosocial stress groups, and groups that were treated with 5 mg/kg of amitriptyline produced significantly more fecal boli than groups that were treated with 10 mg/kg of amitriptyline or tianeptine. The Psychosocial Stress x Drug interaction was not significant,  $F(5,97) = 0.87$ ,  $p > 0.05$ . Planned comparisons were also conducted between groups that were predicted to differ *a priori*. The vehicle-treated psychosocial stress group produced significantly more fecal boli than the vehicle-treated no psychosocial stress group,  $t(17) = 2.81$ ,  $p < 0.05$ . Chronic treatment with 10 mg/kg of amitriptyline, 0.01 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the stress-induced increase in fecal boli, as evidenced by the presence of significantly fewer fecal boli deposits than the vehicle-treated psychosocial stress group and a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress groups. While the group of psychosocially stressed rats treated with 0.05 mg/kg of clonidine did not defecate more than the vehicle-treated control animals,  $t(17) = 1.14$ ,  $p > 0.05$ , they did produce

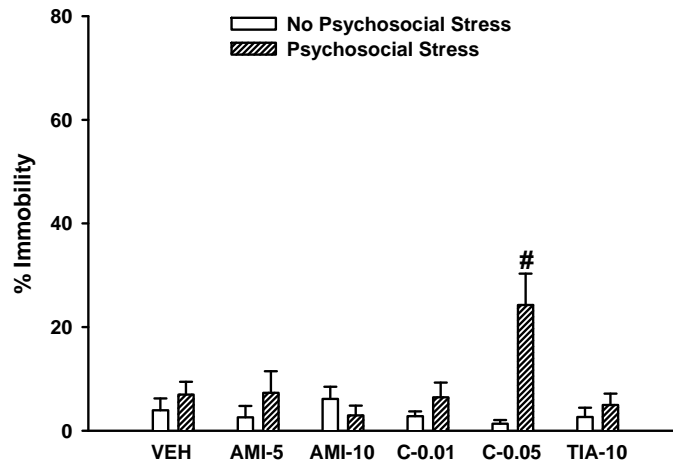
significantly more fecal boli than their respective drug-treated controls,  $t(18) = 2.40$ ,  $p < 0.05$ .



*Figure 7.* Effects of chronic psychosocial stress and drug treatment on fecal boli produced during the 5-minute context test. The data are presented as mean number of fecal boli  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Cue Test Immobility – No Tone (i.e., Novel Environment) (see Figure 8).* For the analysis of immobility during the first 3 minutes of the cue test, there were significant main effects of psychosocial stress,  $F(1,100) = 9.40$ , and drug,  $F(5,100) = 2.72$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,100) = 5.73$  ( $p$ 's  $< 0.05$ ). Post hoc tests indicated that chronic treatment with 0.05 mg/kg of clonidine in rats that were psychosocially stressed led to significantly greater immobility than all other groups.

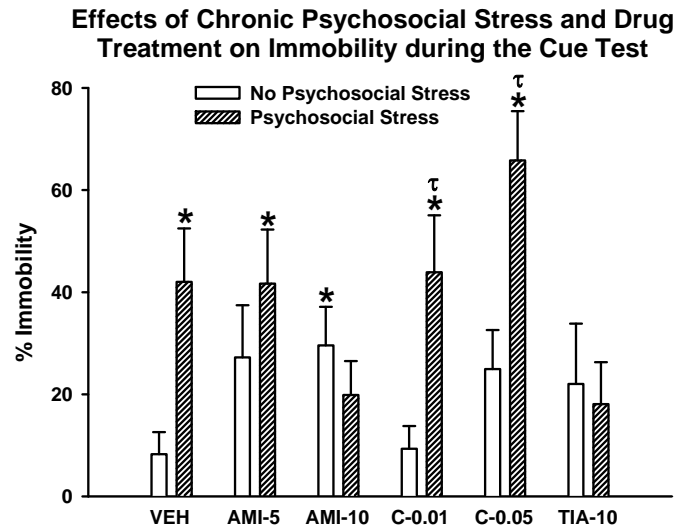
**Effects of Chronic Psychosocial Stress and Drug Treatment on Immobility in a Novel Environment**



*Figure 8.* Effects of chronic psychosocial stress and drug treatment on immobility during the first 3 minutes of the cue test. The data are presented as mean percent immobility  $\pm$  SEM. # =  $p < 0.05$  relative to all other groups.

*Cue Test Immobility – Tone* (see *Figure 9*). For the analysis of immobility during the tone, there was no significant main effect of drug,  $F(5,100) = 2.02$ ,  $p > 0.05$ . There was a significant main effect of psychosocial stress,  $F(1,100) = 12.04$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,100) = 2.71$  ( $p$ 's  $< 0.05$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group spent significantly more time immobile than the vehicle-treated no psychosocial stress group. Additionally, chronic treatment with 10 mg/kg of amitriptyline in groups that were not psychosocially stressed led to significantly greater immobility than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-

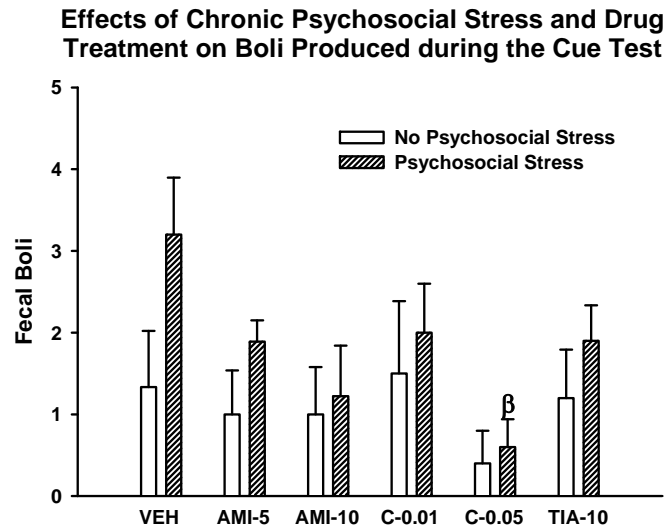
induced increase in immobility, as evidenced by a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.



*Figure 9.* Effects of chronic psychosocial stress and drug treatment on immobility during the tone. The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Cue Test Fecal Boli* (see *Figure 10*). The analysis of fecal boli produced during the cue test revealed a significant main effect of psychosocial stress,  $F(1,100) = 7.34$ . The psychosocial stress groups produced significantly more fecal boli during the cue test than the no psychosocial stress groups. There was no significant main effect of drug,  $F(5,100) = 2.19$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,100) = 0.57$  ( $p$ 's  $> 0.05$ ). Planned comparisons were also conducted between groups that were predicted to differ *a priori*. There was no significant difference between the vehicle-treated psychosocial stress group and the vehicle-treated no psychosocial stress group,

$t(17) = 1.90, p > 0.05$ . The psychosocial stress group that was chronically treated with 0.05 mg/kg of clonidine produced significantly less fecal boli than the vehicle-treated psychosocial stress group,  $t(18) = 3.36, p < 0.01$ .

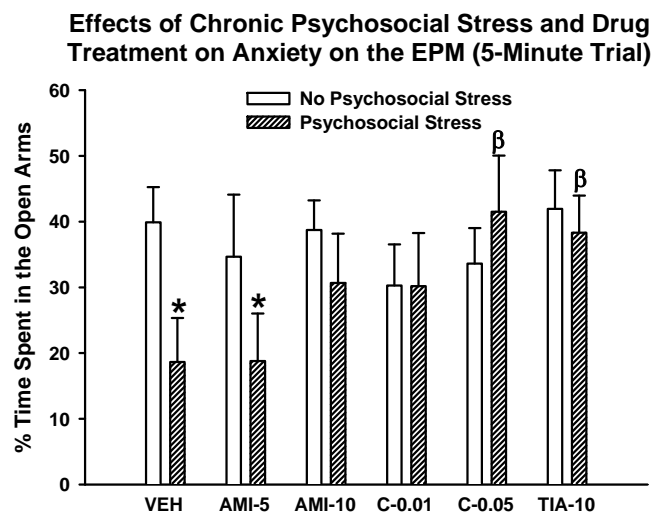


*Figure 10.* Effects of chronic psychosocial stress and drug treatment on fecal boli produced during the 6-minute cue test. The data are presented as mean number of fecal boli  $\pm$  SEM.  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group.

#### *Elevated Plus Maze*

*Percent Time in Open Arms, 5-Minute Trial (see Figure 11).* For the analysis of percent time in the open arms during the 5-minute trial on the EPM, there were no significant main effects of psychosocial stress,  $F(1,100) = 2.89$ , or drug,  $F(5,100) = 1.14$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,100) = 1.16$  ( $p$ 's  $> 0.05$ ). Planned comparisons were also conducted between groups that were predicted to differ *a priori*. The vehicle-treated psychosocial stress group spent significantly less time in the open arms of the EPM than the vehicle-treated no psychosocial stress group,  $t(15)$

= 2.44,  $p < 0.05$ . Chronic treatment with 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced decrease in open arm exploration, as evidenced by the presence of significantly greater percent time spent in the open arms than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.

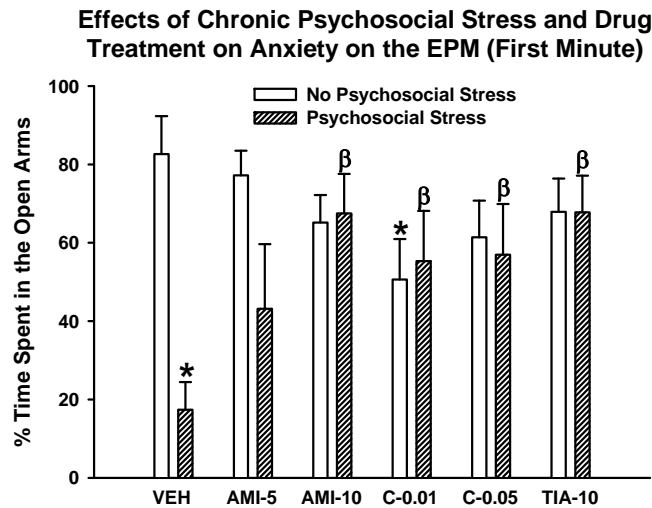


*Figure 11.* Effects of chronic psychosocial stress and drug treatment on percent time spent in the open arms during the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta$  =  $p < 0.05$  relative to the vehicle-treated psychosocial stress group.

*Percent Time in Open Arms, First Minute (see Figure 12).* For the analysis of percent time in the open arms during the first minute of the 5-minute trial on the EPM, there was a significant main effect of psychosocial stress,  $F(1,102) = 4.79$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,102) = 3.03$  ( $p$ 's  $< 0.05$ ). The

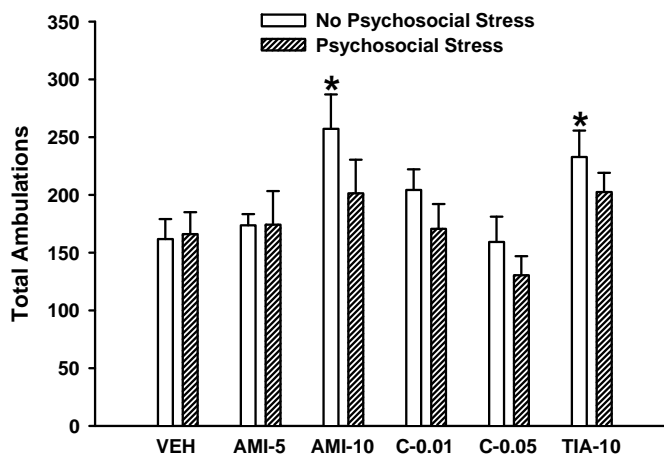


vehicle-treated psychosocial stress group spent significantly less time in the open arms of the EPM than the vehicle-treated no psychosocial stress group. Chronic treatment with 0.01 mg/kg of clonidine in the group that was not psychosocially stressed led to significantly less open arm exploration on the EPM, relative to the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced decrease in open arm exploration, as evidenced by the presence of significantly greater percent time spent in the open arms than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.



*Figure 12.* Effects of chronic psychosocial stress and drug treatment on percent time spent in the open arms during the first minute of the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group.

**Effects of Chronic Psychosocial Stress and Drug Treatment on Motor Activity on the EPM (5-Minute Trial)**



*Figure 13.* Effects of chronic psychosocial stress and drug treatment on ambulations made during the 5-minute trial on the elevated plus maze. The data are presented as mean number of ambulations  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group.

*Ambulations, 5-Minute Trial (see Figure 13).* For the analysis of ambulations made during the 5-minute trial on the EPM, there was no significant main effect of psychosocial stress,  $F(1,100) = 3.70$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,100) = 0.52$  ( $p$ 's  $> 0.05$ ). There was a significant main effect of drug,  $F(5,100) = 4.48$ ,  $p < 0.001$ . Planned comparisons were also conducted between groups that were predicted to differ *a priori*. There was no significant difference between the number of ambulations made by the vehicle-treated psychosocial stress group and the vehicle-treated no psychosocial stress group on the EPM,  $t(16) = 0.16$ ,  $p > 0.05$ . Chronic treatment with 10 mg/kg of amitriptyline,  $t(15) = 2.67$ , or 10 mg/kg of tianeptine,  $t(16) = 2.38$ , in groups that were not psychosocially stressed led to a significantly greater number

of ambulations on the EPM, relative to the vehicle-treated no psychosocial stress group ( $p$ 's < 0.05).

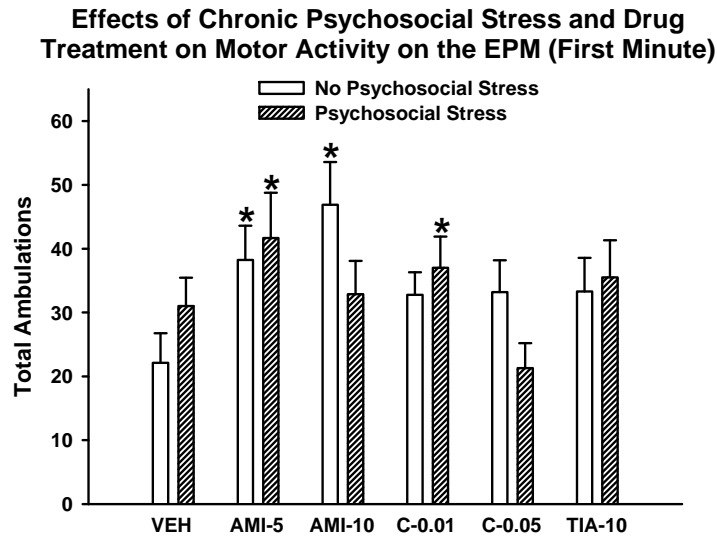
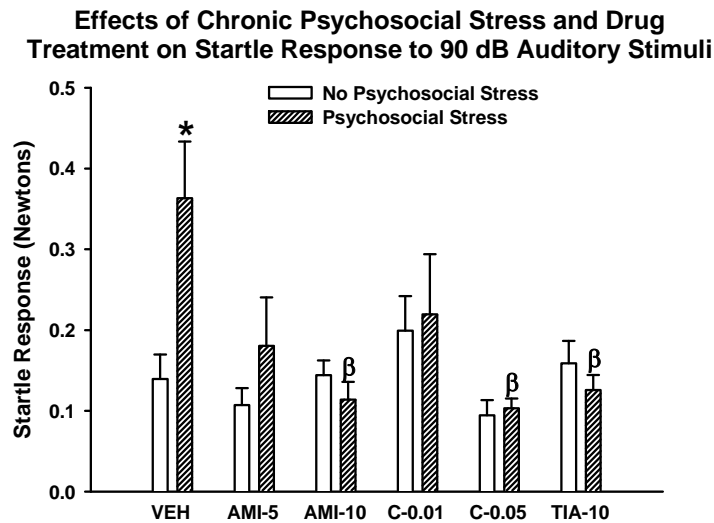


Figure 14. Effects of chronic psychosocial stress and drug treatment on ambulations made during the first minute of the 5-minute trial on the elevated plus maze. The data are presented as mean number of ambulations  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group.

*Ambulations, First Minute* (see Figure 14). For the analysis of ambulations made during the first minute of the 5-minute trial on the EPM, there was no significant main effect of psychosocial stress,  $F(1,100) = 0.16$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,100) = 1.57$  ( $p$ 's > 0.05). There was a significant main effect of drug,  $F(5,100) = 2.43$ ,  $p < 0.05$ . Planned comparisons were also conducted between groups that were predicted to differ *a priori*. There was no significant difference between the number of ambulations made by the vehicle-treated psychosocial stress group and the vehicle-treated no psychosocial stress group on the EPM,  $t(16) = 1.37$ ,  $p >$

0.05. Chronic treatment with 5 mg/kg,  $t(15) = 2.24$ , or 10 mg/kg,  $t(15) = 2.96$ , of amitriptyline in groups that were not psychosocially stressed and 5 mg/kg of amitriptyline,  $t(15) = 2.24$ , or 0.01 mg/kg of clonidine,  $t(16) = 2.16$ , in groups that were psychosocially stressed led to a significantly greater number of ambulations on the EPM, relative to the vehicle-treated no psychosocial stress group ( $p$ 's < 0.05).



*Figure 15.* Effects of chronic psychosocial stress and drug treatment on startle responses to the 90 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group.

### *Startle Response*

*90 dB Auditory Stimuli (see Figure 15).* For the analysis of startle responses to the 90 dB auditory stimuli, there was no significant main effect of psychosocial stress,  $F(1,102) = 3.40$ ,  $p > 0.05$ . There was a significant main effect of drug,  $F(5,102) = 3.84$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,102) = 2.74$  ( $p$ 's <

0.05). Post hoc tests indicated that the vehicle-treated psychosocial stress group exhibited significantly greater startle responses than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced increase in startle response, as evidenced by the presence of significantly lower startle responses than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.

*100 dB Auditory Stimuli (see Figure 16).* For the analysis of startle responses to the 100 dB auditory stimuli, there was no significant main effect of drug,  $F(5,98) = 1.39$ ,  $p > 0.05$ . There was a significant main effect of psychosocial stress,  $F(1,98) = 7.29$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,98) = 2.32$  ( $p$ 's  $< 0.05$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group exhibited significantly greater startle responses than the vehicle-treated no psychosocial stress group. Chronic treatment with 10 mg/kg of amitriptyline, 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced increase in startle response, as evidenced by the presence of significantly lower startle responses than the vehicle-treated psychosocial stress group. The psychosocial stress groups treated with 5 mg/kg of amitriptyline or 0.01 mg/kg of clonidine did not exhibit significantly greater startle responses than the vehicle-treated control group; however, neither of the groups displayed significantly lower startle responses than the vehicle-treated psychosocial stress group, and the psychosocial stress

group treated with 5 mg/kg of amitriptyline demonstrated significantly greater startle responses than its respective drug-treated control group.

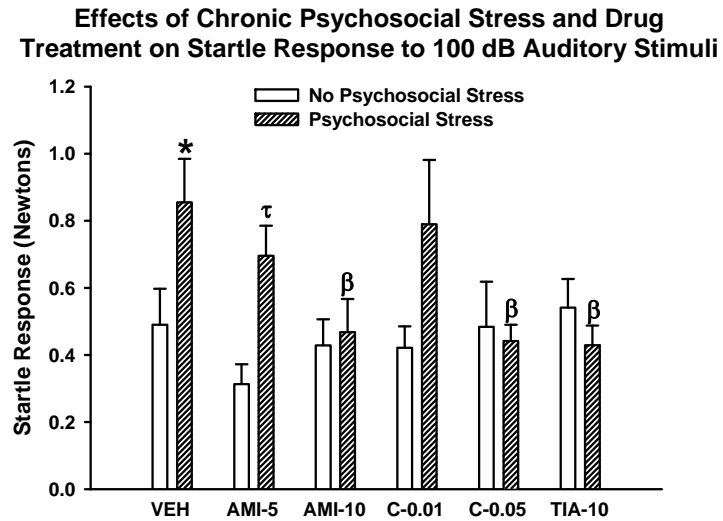
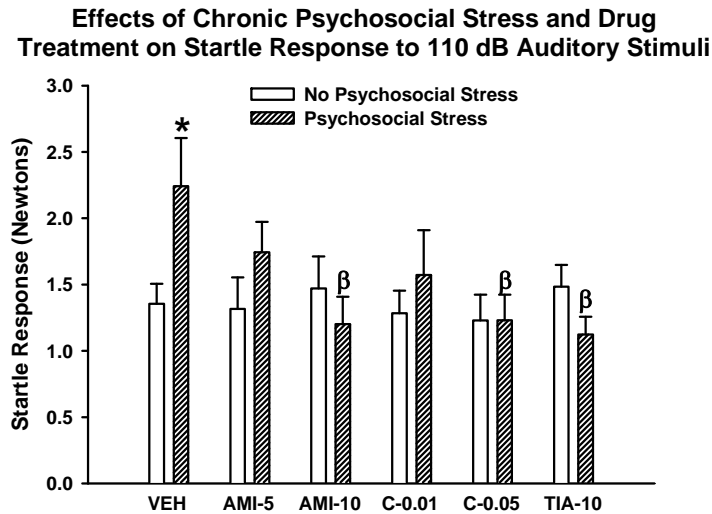


Figure 16. Effects of chronic psychosocial stress and drug treatment on startle responses to the 100 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

110 dB Auditory Stimuli (see Figure 17). For the analysis of startle responses to the 110 dB auditory stimuli, there were no significant main effects of psychosocial stress,  $F(1,100) = 1.42$ , or drug,  $F(5,100) = 1.49$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,100) = 1.92$  ( $p$ 's  $> 0.05$ ). Planned comparisons were also conducted between groups that were predicted to differ *a priori*. The vehicle-treated psychosocial stress group tended to exhibit greater startle responses than the vehicle-treated no psychosocial stress group, although this difference did not achieve statistical

significance,  $t(16) = 2.06$ ,  $p = 0.056$ . Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented this marginally significant, chronic stress-induced increase in startle response, as evidenced by the presence of significantly lower startle responses than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.

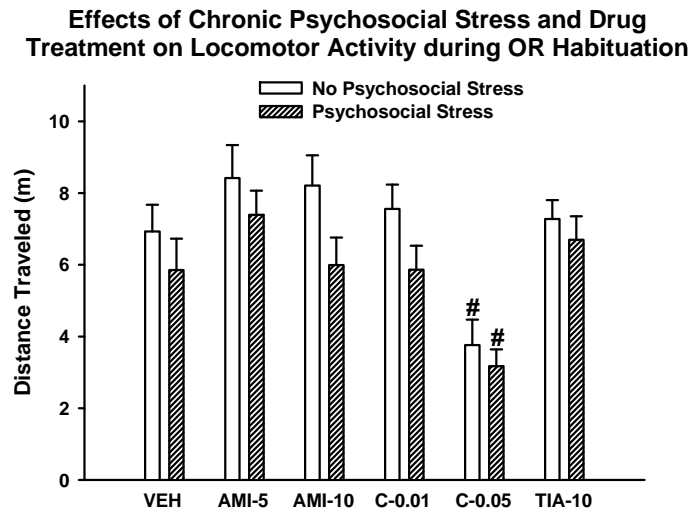


*Figure 17.* Effects of chronic psychosocial stress and drug treatment on startle responses to the 110 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM. \* =  $p < 0.056$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group.

### *Novel Object Recognition*

*Habituation (see Figure 18).* The analysis of locomotor activity in the open field during the 5-minute habituation phase revealed significant main effects of psychosocial stress,  $F(1,104) = 8.25$ , and drug,  $F(5,104) = 9.27$  ( $p$ 's  $< 0.01$ ). In general, rats that had

been psychosocially stressed traveled significantly less distance in the open field than rats that had not been psychosocially stressed. In addition, rats that were treated with 0.05 mg/kg of clonidine, independent of psychosocial stress, traveled significantly less distance than all other groups. The Psychosocial Stress x Drug interaction was not significant,  $F(5,104) = 0.40$ ,  $p > 0.05$ .



*Figure 18.* Effects of chronic psychosocial stress and drug treatment on locomotor activity during the 5-minute object recognition habituation period. The data are presented as mean distance traveled (m)  $\pm$  SEM. # =  $p < 0.05$  relative to all groups that were not treated with 0.05 mg/kg of clonidine.

The analysis of time that the rats spent in each area of the open field revealed no significant main effect of quadrant,  $F(3,309) = 1.26$ , psychosocial stress,  $F(1,103) = 0.54$ , or drug,  $F(5,103) = 1.00$ , and the Quadrant x Psychosocial Stress,  $F(3,309) = 2.54$ , Quadrant x Drug,  $F(15,309) = 1.33$ , Psychosocial Stress x Drug,  $F(5,103) = 0.43$ , and Quadrant x Psychosocial Stress x Drug,  $F(15,309) = 1.61$ , interactions were not significant ( $p$ 's  $> 0.05$ ; data not shown).



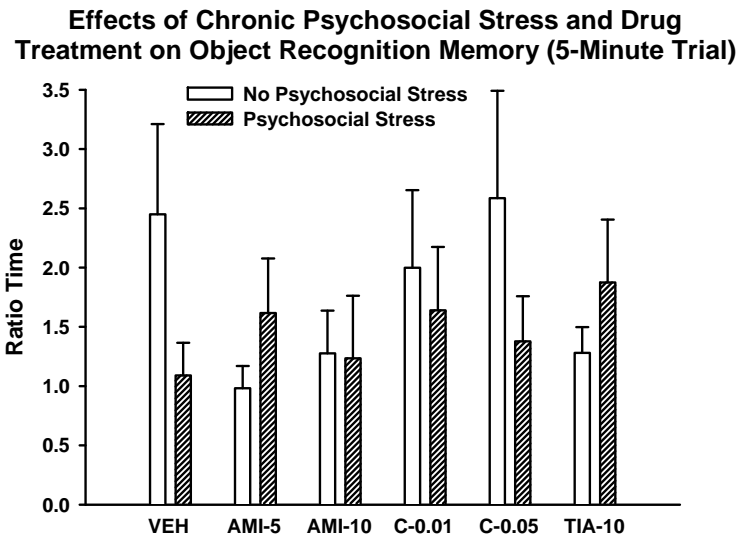
Table 3

*Time (seconds  $\pm$  SEM) Spent with Each Object during Object Recognition Training for all Groups in Experiment 3*

	Object 1	Object 2	Paired <i>t</i> -test * = <i>p</i> < 0.05
<b>No Psychosocial Stress</b>			
Vehicle	10.54 $\pm$ 1.89	9.87 $\pm$ 1.19	<i>t</i> (9) = 0.03
5 mg/kg Amitriptyline	5.87 $\pm$ 0.88	6.26 $\pm$ 1.50	<i>t</i> (9) = 0.37
10 mg/kg Amitriptyline	9.03 $\pm$ 2.37	7.77 $\pm$ 1.29	<i>t</i> (9) = 0.46
0.01 mg/kg Clonidine	11.85 $\pm$ 1.94	7.13 $\pm$ 0.85	<i>t</i> (9) = 2.78*
0.05 mg/kg Clonidine	6.70 $\pm$ 1.89	9.55 $\pm$ 2.90	<i>t</i> (9) = 0.83
Tianeptine	8.07 $\pm$ 1.50	9.76 $\pm$ 0.98	<i>t</i> (9) = 0.84
<b>Psychosocial Stress</b>			
Vehicle	6.31 $\pm$ 1.38	13.88 $\pm$ 5.32	<i>t</i> (9) = 1.34
5 mg/kg Amitriptyline	5.90 $\pm$ 0.91	9.46 $\pm$ 1.63	<i>t</i> (9) = 1.86
10 mg/kg Amitriptyline	8.41 $\pm$ 1.12	9.53 $\pm$ 2.82	<i>t</i> (9) = 0.36
0.01 mg/kg Clonidine	7.80 $\pm$ 1.30	6.32 $\pm$ 0.87	<i>t</i> (9) = 1.02
0.05 mg/kg Clonidine	12.20 $\pm$ 3.30	5.05 $\pm$ 1.91	<i>t</i> (9) = 1.55
Tianeptine	7.40 $\pm$ 1.49	6.38 $\pm$ 0.96	<i>t</i> (9) = 0.93

*Training (see Table 3).* Within-group comparisons indicated that most groups spent a comparable amount of time with each of the objects that were placed in the open

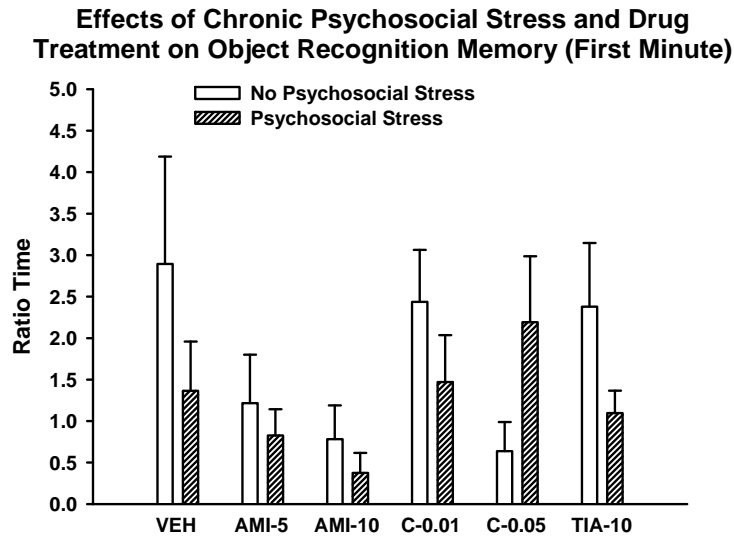
field during object recognition training (see Table 3), suggesting that no object preference effects were present. Only one group, the 0.01 mg/kg clonidine-treated no psychosocial stress group, spent more time with one object than the other. A between-groups comparison of the total amount of time spent with both objects during training revealed no significant main effects of psychosocial stress,  $F(1,103) = 0.27$ , or drug,  $F(5,103) = 0.82$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,103) = 0.99$  ( $p$ 's > 0.05; data not shown). These findings indicated that all groups spent a comparable amount of time with both objects during training.



*Figure 19.* Effects of chronic psychosocial stress and drug treatment on object recognition memory during the entire 5-minute testing trial. The data are presented as mean ratio time  $\pm$  SEM.

*Testing, 5-Minute Trial (see Figure 19).* The analysis comparing the ratio times of all groups during the 5-minute object recognition testing session revealed no significant

main effects of psychosocial stress,  $F(1,87) = 0.80$ , or drug,  $F(5,87) = 0.52$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,87) = 1.17$  ( $p$ 's > 0.05).



*Figure 20.* Effects of chronic psychosocial stress and drug treatment on object recognition memory during the first minute of the testing trial. The data are presented as mean ratio time  $\pm$  SEM.

*Testing, First Minute (see Figure 20).* The analysis comparing the ratio times of all groups during the first minute of the testing trial revealed no significant main effects of psychosocial stress,  $F(1,68) = 1.56$ , or drug,  $F(5,68) = 1.34$ , and the Psychosocial Stress x Drug interaction was not significant,  $F(5,68) = 1.44$  ( $p$ 's > 0.05).

*Corticosterone Levels (see Figure 21)*

For the analysis of serum corticosterone levels, there was no significant main effect of psychosocial stress,  $F(1,97) = 0.02$ ,  $p > 0.05$ . There was, however, a significant main effect of time point,  $F(2,194) = 487.29$ ,  $p < 0.001$ . Post hoc tests indicated that rats demonstrated a significant increase in corticosterone levels following 20 minutes of acute

immobilization stress and that these levels declined, but remained significantly elevated relative to baseline, 1 hour later.

### Effects of Chronic Psychosocial Stress and Drug Treatment on Serum Corticosterone Levels

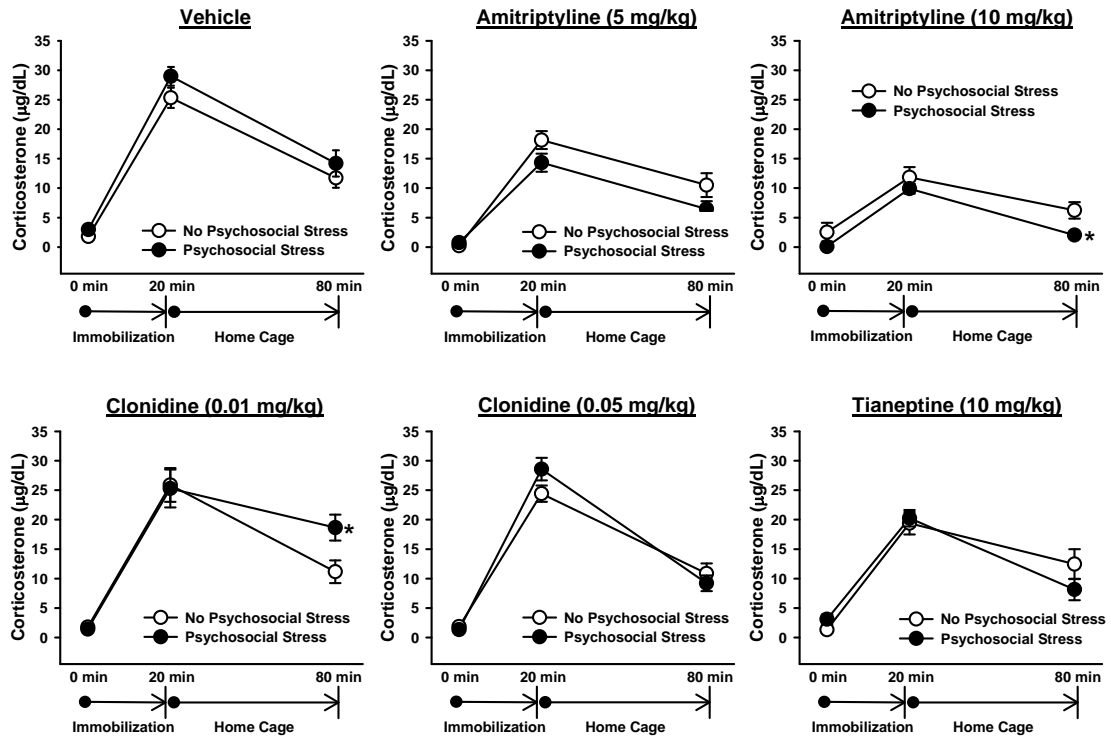


Figure 21. Effects of chronic psychosocial stress and drug treatment on serum corticosterone levels. The data are presented as mean serum corticosterone levels ( $\mu\text{g/dL}$ )  $\pm$  SEM. \* =  $p < 0.05$  relative to the respective no psychosocial stress group.

There was also a significant main effect of drug,  $F(5,97) = 24.00$ ,  $p < 0.001$ . Post hoc tests indicated that rats treated with 5 mg/kg of amitriptyline displayed significantly lower corticosterone levels than all other groups except for those treated with tianeptine or 10 mg/kg of amitriptyline. In addition, rats treated with 10 mg/kg of amitriptyline exhibited significantly lower corticosterone levels than all other groups, except for those

treated with 5 mg/kg of amitriptyline. Lastly, rats that were treated with tianeptine had significantly lower corticosterone levels than rats treated with vehicle or 0.01 mg/kg of clonidine.

The Time Point x Psychosocial Stress interaction was not significant,  $F(2,194) = 0.34, p > 0.05$ . However, the Time Point x Drug interaction was significant,  $F(10,194) = 8.91, p < 0.001$ . Post hoc tests indicated that both doses of amitriptyline, particularly the 10 mg/kg dose, significantly blunted the acute immobilization-induced increase in serum corticosterone levels. Tianeptine had a similar effect, although not as pronounced as that of amitriptyline. The Psychosocial Stress x Drug,  $F(5,97) = 2.70$ , and Time Point x Psychosocial Stress x Drug,  $F(10,194) = 2.21$ , interactions were also significant ( $p$ 's  $< 0.05$ ). Post hoc tests revealed that psychosocially stressed rats treated with 10 mg/kg of amitriptyline exhibited significantly lower corticosterone levels than the controls treated with 10 mg/kg of amitriptyline an hour following 20 minutes of immobilization. In contrast, 0.01 mg/kg of clonidine prevented the reduction in corticosterone levels an hour following immobilization in the psychosocial stress group only.

#### *Cardiovascular Activity*

*Heart Rate (see Figure 22)*. For the analysis of heart rate, there were no significant main effects of psychosocial stress,  $F(1,81) = 0.05$ , or drug,  $F(5,81) = 1.15$  ( $p$ 's  $> 0.05$ ). The Psychosocial Stress x Drug interaction was significant,  $F(5,81) = 3.99$  ( $p < 0.01$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group exhibited significantly greater heart rate than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of

clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced increase in heart rate, as evidenced by the presence of significantly lower heart rate than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.

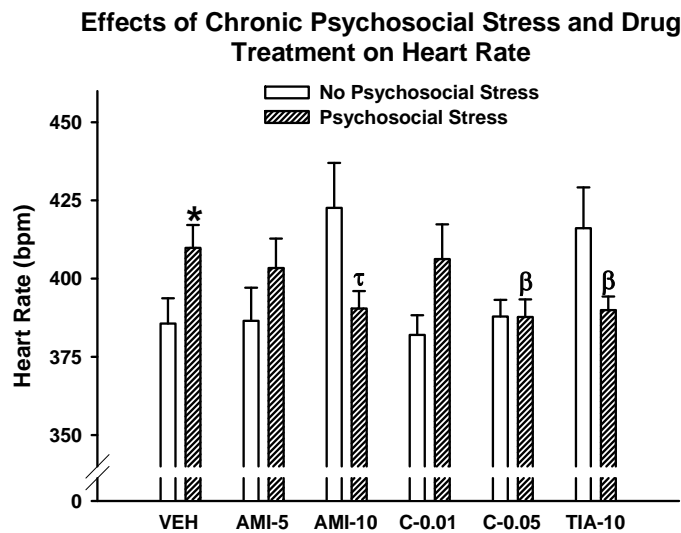
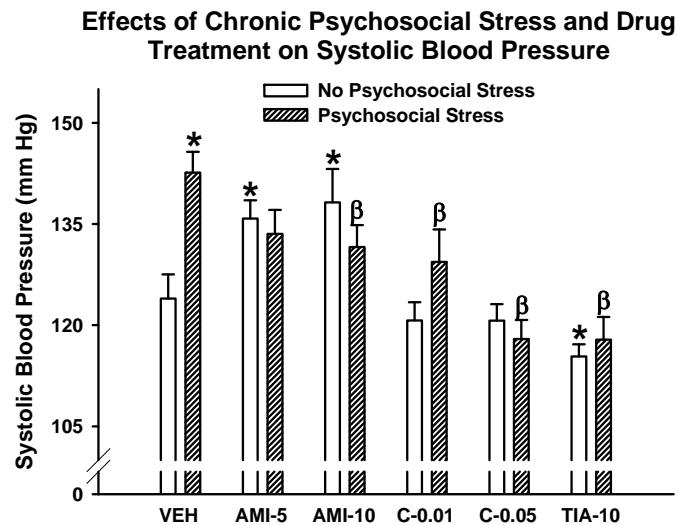


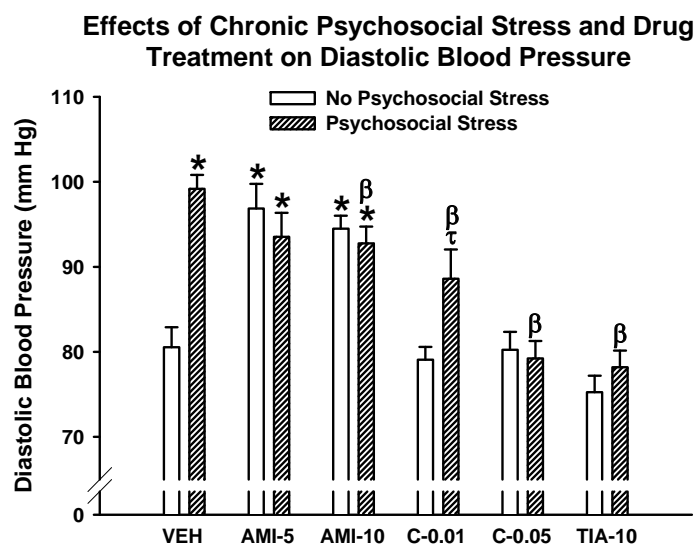
Figure 22. Effects of chronic psychosocial stress and drug treatment on heart rate. The data are presented as mean heart rate (bpm)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Systolic Blood Pressure* (see Figure 23). For the analysis of systolic blood pressure, there was no significant main effect of psychosocial stress,  $F(1,86) = 1.90, p > 0.05$ . There was a significant main effect of drug,  $F(5,86) = 11.80$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,86) = 3.60$  ( $p$ 's  $< 0.01$ ). Post hoc tests

indicated that the vehicle-treated psychosocial stress group had significantly higher systolic blood pressure than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline in groups that were not psychosocially stressed led to significantly greater systolic blood pressure than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced increase in systolic blood pressure, as evidenced by the presence of significantly lower systolic blood pressure than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group.



*Figure 23.* Effects of chronic psychosocial stress and drug treatment on systolic blood pressure. The data are presented as mean systolic blood pressure (mm Hg)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group.



*Figure 24.* Effects of chronic psychosocial stress and drug treatment on diastolic blood pressure. The data are presented as mean diastolic blood pressure (mm Hg)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta$  =  $p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau$  =  $p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Diastolic Blood Pressure* (see Figure 24). For the analysis of diastolic blood pressure, there were significant main effects of psychosocial stress,  $F(1,86) = 9.67$ , and drug,  $F(5,86) = 21.78$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,86) = 6.11$  ( $p$ 's  $< 0.01$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group had significantly higher diastolic blood pressure than the vehicle-treated no psychosocial stress group. Additionally, chronic treatment with 5 or 10 mg/kg of amitriptyline in groups that were not psychosocially stressed led to significantly greater diastolic blood pressure than the vehicle-treated no psychosocial stress group. Chronic treatment with 10 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of



tianeptine in psychosocially stressed rats led to significantly lower diastolic blood pressure than the vehicle-treated psychosocial stress group. However, psychosocially stressed rats that were treated with 10 mg/kg of amitriptyline still displayed significantly greater diastolic blood pressure than the vehicle-treated no psychosocial stress group, and psychosocially stressed rats that were treated with 0.01 mg/kg of clonidine still exhibited significantly greater diastolic blood pressure than its respective drug-treated no psychosocial stress group.

*Growth Rates (see Figure 25)*

For the analysis of growth rate, there was no significant main effect of psychosocial stress,  $F(1,100) = 0.61$ ,  $p > 0.05$ . There was a significant main effect of drug,  $F(5,100) = 11.78$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,100) = 13.42$  ( $p$ 's  $< 0.001$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group had a significantly lower growth rate than the vehicle-treated no psychosocial stress group. Additionally, chronic treatment with 5 or 10 mg/kg of amitriptyline or 0.05 mg/kg of clonidine in groups that were not psychosocially stressed led to significantly lower growth rates than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 or 10 mg/kg of amitriptyline, 0.01 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced reduction of growth rate, as evidenced by the presence of significantly greater growth rates than the vehicle-treated psychosocial stress group or a lack of statistical significance relative to each of the group's respective drug-treated no psychosocial stress group. However, the psychosocial stress group treated with 5 mg/kg

of amitriptyline still exhibited a significantly lower growth rate than the vehicle-treated no psychosocial stress group.

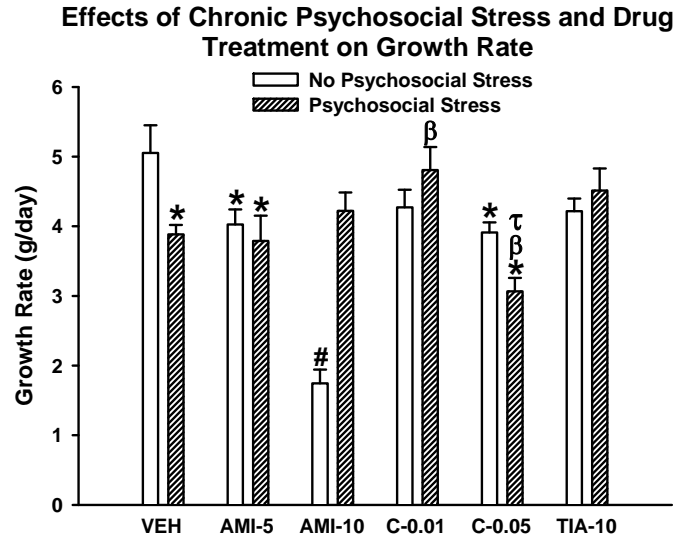
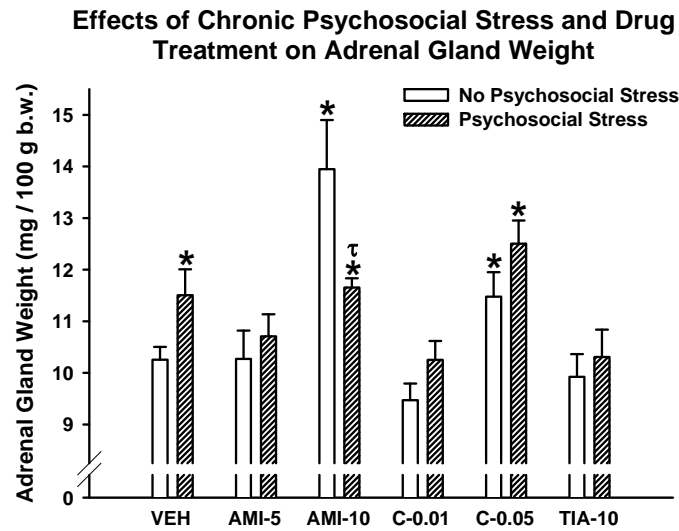


Figure 25. Effects of chronic psychosocial stress and drug treatment on growth rate. The data are presented as mean growth rate (g/day) ± SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group; # =  $p < 0.05$  relative to all other groups;  $\beta$  =  $p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau$  =  $p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Adrenal Gland Weights (see Figure 26)*

For the analysis of adrenal gland weights, there was no significant main effect of psychosocial stress,  $F(1,97) = 0.89$ ,  $p > 0.05$ . There was a significant main effect of drug,  $F(5,97) = 10.53$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,97) = 3.26$  ( $p$ 's  $< 0.01$ ). Post hoc tests indicated that the vehicle-treated psychosocial stress group had significantly larger adrenal glands than the vehicle-treated no psychosocial stress group. Additionally, chronic treatment with 10 mg/kg of amitriptyline or 0.05

mg/kg of clonidine in groups that were not psychosocially stressed led to significantly larger adrenal glands than the vehicle-treated no psychosocial stress group. Chronic treatment with 5 mg/kg of amitriptyline, 0.01 or 0.05 mg/kg of clonidine or 10 mg/kg of tianeptine in groups that were psychosocially stressed prevented the chronic stress-induced hypertrophy of the adrenal glands, as evidenced by a lack of a statistically significant increase in adrenal gland weight relative to each of the group's respective drug-treated no psychosocial stress group. Interestingly, the psychosocial stress group treated with 10 mg/kg of amitriptyline exhibited significantly lower adrenal gland weights than its respective drug-treated control group.



*Figure 26.* Effects of chronic psychosocial stress and drug treatment on adrenal gland weight. The data are presented as mean adrenal gland weight (mg/100 g b.w.)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

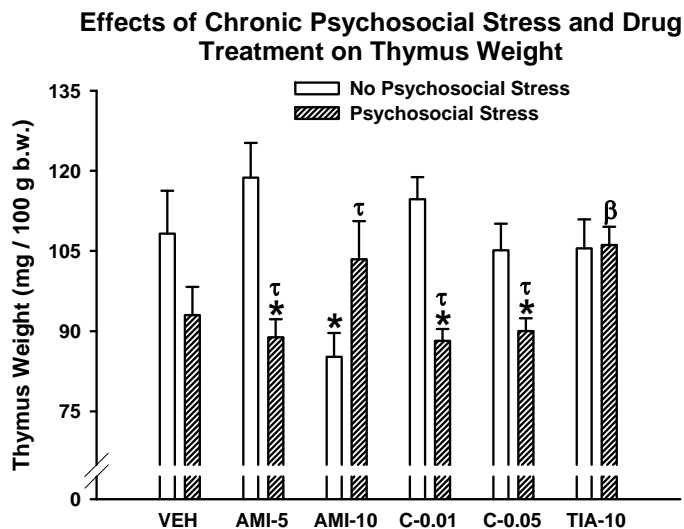


Figure 27. Effects of chronic psychosocial stress and drug treatment on thymus weight. The data are presented as mean thymus weight (mg/100 g b.w.)  $\pm$  SEM. \* =  $p < 0.05$  relative to the vehicle-treated no psychosocial stress group;  $\beta = p < 0.05$  relative to the vehicle-treated psychosocial stress group;  $\tau = p < 0.05$  relative to the respective drug-treated no psychosocial stress group.

*Thymus Weights (see Figure 27)*

For the analysis of thymus weights, there was no significant main effect of drug,  $F(5,91) = 1.42, p > 0.05$ . There was a significant main effect of psychosocial stress,  $F(1,91) = 17.12$ , and the Psychosocial Stress x Drug interaction was significant,  $F(5,91) = 6.49$  ( $p$ 's  $< 0.001$ ). Post hoc analyses indicated that the vehicle-treated psychosocial stress group tended to exhibit smaller thymuses than the vehicle-treated no psychosocial stress group, yet this difference did not reach statistical significance. Additionally, chronic treatment with 10 mg/kg of amitriptyline in groups that were not psychosocially stressed led to significantly smaller thymuses than the vehicle-treated no psychosocial

stress group. Chronic treatment with 5 mg/kg of amitriptyline or 0.01 or 0.05 mg/kg of clonidine in groups that were psychosocially stressed led to significantly smaller thymuses than the vehicle-treated no psychosocial stress group. Chronic treatment with 10 mg/kg of amitriptyline or 10 mg/kg of tianeptine prevented the slight decrease in thymus weight induced by chronic psychosocial stress, as evidenced by the presence of significantly greater thymus weights than the vehicle-treated psychosocial stress group and/or a lack of a statistically significant decreases in thymus weight relative to each of the group's respective drug-treated no psychosocial stress group.

#### *Discussion of Findings*

Consistent with our previous work (Zoladz et al., 2008), vehicle-treated rats that were exposed to chronic psychosocial stress, composed of two acute predator exposures and daily social instability, exhibited reduced growth rate, greater adrenal gland weight, heightened anxiety, an exaggerated startle response, greater blood pressure reactivity to an acute stressor and intact memories for the context and cue that were associated with the two cat exposures. In contrast to our prior findings, however, the vehicle-treated psychosocially stressed rats did not display a significant impairment of object recognition memory or significantly reduced thymus weights, relative to vehicle-treated control (i.e., unstressed) animals. Nonetheless, it is important to note that these effects were in the hypothesized direction. That is to say, vehicle-treated psychosocially stressed rats spent less time with the novel object and had smaller thymuses, albeit both non-significantly, than vehicle-treated control animals. One possible explanation for the lack of statistical significance is that the chronic injections in the present study acted as a chronic mild

stressor in control rats, which added considerable variability to their data on these measures. Studies in rodents have reported that chronic mild stress in the form of repeated injections can significantly alter the morphology of neurons in the prefrontal cortex (Weaver et al., 2005; Wellman, 2001). Therefore, chronic injections in the present study could have adversely influenced rat physiology and behavior.

Another finding that is in conflict with our previous work is that the vehicle-treated psychosocially stressed rats demonstrated significantly greater HR than vehicle-treated control rats following exposure to an acute stressor on the final day of testing. Previously, we reported that the present psychosocial stress paradigm resulted in significantly *lower* HR, compared to controls, following acute stress on the final day of testing (Zoladz et al., 2008). Nevertheless, the HR exhibited by psychosocially stressed rats in the present study ( $409.85 \pm 7.30$  bpm) was very similar to the HR exhibited by psychosocially stressed rats in our previous work ( $413.25 \pm 9.93$  bpm). What appears to be the cause of the inconsistent effects between the findings of the present study and those of our previous work is the HR exhibited by the control animals in each case. Vehicle-treated control rats displayed much lower HR in the present study ( $385.61 \pm 8.11$  bpm) than that which was displayed by controls in the prior study ( $462.88 \pm 11.43$  bpm). In theory, vehicle-treated controls could have exhibited much lower HR in the present study because the chronic mild stress of repeated injections protected them against responding as strongly to the acute stressor as the more naïve animals that were utilized in our prior work.

### *Amitriptyline*

Both doses of the tricyclic antidepressant amitriptyline blocked the expression of fear-related behaviors in psychosocially stressed rats in response to the context and cue that were paired with the two cat exposures. These fear responses served as a measure of memory for the acute stress experiences and rat analogs of a traumatic memory in humans. As traumatic memories are a source of psychological distress in people with PTSD, these findings suggest that amitriptyline could serve to effectively reduce the strength of traumatic memories and consequentially diminish the intrusion and re-experiencing symptoms endured by PTSD patients. One caveat to this interpretation, however, is that extensive work has reported amitriptyline-induced memory impairments in both humans (Kerr et al., 1996; Liljequist et al., 1978; Mattila et al., 1978; Spring et al., 1992; van Laar et al., 2002) and rodents (Everss et al., 2005; Gonzalez-Pardo et al., 2008; Kumar & Kulkarni, 1996), findings that may be related to the drug's anti-cholinergic side effects (Pavone et al., 1997). Therefore, the attenuation of contextual and cue fear conditioning in psychosocially stressed rats treated with amitriptyline could simply be due to its amnesic side effects, rather than a specific amelioration of the chronic stress-induced behavioral sequelae. On the other hand, studies reporting amitriptyline-induced memory impairments have administered the drug prior to learning. In the present experiment, amitriptyline treatment did not begin until 24 hours after the first pairing of the context and cue with the cat exposure. Therefore, a more likely explanation of the present findings is that amitriptyline blunted the augmentation of contextual and cue fear conditioning in psychosocially stressed rats that occurred in

response to the second cat exposure on Day 11 of the paradigm. Another possible explanation of these findings is that amitriptyline increased general locomotor activity, thus reducing overall immobility. Amitriptyline-treated control animals did display a significantly greater amount of motor activity on the EPM than vehicle-treated animals, but the same effect was not observed during the open field habituation period on the following day. In addition, the finding that amitriptyline, at least at the higher dose, led to significantly fewer fecal boli deposits in psychosocially stressed rats during the context test supports the notion that the observed effects were not by-products of drug-induced changes in locomotor activity.

Both doses of amitriptyline were at least partially effective in preventing the chronic stress-induced increase in startle responses, but only the 10 mg/kg dose of amitriptyline blocked the effects of chronic psychosocial stress on anxiety, as measured by rat behavior on the EPM. These findings are consistent with other work in the rodent literature reporting that amitriptyline exerts anxiolytic effects in control animals (Bodnoff et al., 1988; Zajackowski & Gorka, 1993) and blocks stress-induced increases in anxiety-like behavior and startle (Orsetti et al., 2007; Poltyrev & Weinstock, 2004; West & Weiss, 2005). Research in humans has also shown that amitriptyline significantly blunts startle responses (Phillips et al., 2000). Thus, amitriptyline appears to have potent anxiolytic effects that may effectively ameliorate the hyperarousal symptoms related to PTSD.

Amitriptyline also led to significantly lower serum corticosterone levels in rats and was particularly effective in blunting the immobilization-induced increase in these



levels on the final day of testing. This finding is consistent with several studies in the rodent literature reporting that chronic amitriptyline administration results in significantly reduced basal and stress-induced levels of ACTH and corticosterone in rats (Barden, 1999; Reul et al., 1993). Amitriptyline appears to accomplish these effects by enhancing the negative feedback inhibition of the HPA axis. Investigators have shown that chronic amitriptyline administration leads to an up-regulation of glucocorticoid receptor expression and enhanced glucocorticoid receptor binding in several brain regions (Barden, 1999; Pariante & Miller, 2001; Przegalinski & Budziszewska, 1993; Reul et al., 1993). Interestingly, in the present study, there was an additive effect of amitriptyline and psychosocial stress on the recovery of serum corticosterone levels following immobilization. Psychosocially stressed rats that were treated with amitriptyline, particularly the 10 mg/kg dose, exhibited significantly lower corticosterone levels at the 80 minute time point than amitriptyline-treated controls. In theory, chronic amitriptyline and psychosocial stress synergistically facilitated the production of enhanced negative feedback of the HPA axis, which led to a more rapid recovery of stress-induced serum corticosterone levels in these rats.

Despite the positive effects of amitriptyline on the chronic stress-induced physiological and behavioral sequelae in rats, there were adverse side effects of the drug that should be considered. For instance, chronic amitriptyline treatment resulted in significantly greater stress-induced increases in systolic and diastolic blood pressure than vehicle. Most work in both humans and rodents has reported that chronic amitriptyline treatment results in increased heart rate, postural hypotension and increased

cardiotoxicity (Balcioglu et al., 1991; Fiedler et al., 1986; Hong et al., 1974; Joubert et al., 1985; Kopera, 1978; Low & Opfer-Gehrking, 1992; Yokota et al., 1987). The results presented here are novel in that they reveal that chronic amitriptyline treatment has unfavorable effects on *stress-induced* changes in cardiovascular activity.

Amitriptyline also led to a significant reduction in growth rate in rats, particularly at the higher dose of the drug. Although counterintuitive, 10 mg/kg of amitriptyline significantly reduced growth rates in the control rats, an effect that was reversed by exposure to chronic psychosocial stress. Similar findings were observed with regards to adrenal gland and thymus weights. The higher dose of amitriptyline led to significantly larger adrenal glands and significantly smaller thymuses than vehicle in control animals, and exposure to chronic psychosocial stress significantly blunted each of these effects. These findings suggest that, in the present experiment, there was an interaction between amitriptyline treatment and chronic psychosocial stress, in which the physiological consequences of the drug were more prominent in those rats that were unstressed. Another finding of interest is that upon dissection of these animals, there were a large number of adhesions observed on the internal organs, such as the liver, intestines and spleen, and the mortality rate for rats chronically treated with amitriptyline (3 out of 40, or 7.5%) was greater than the mortality rates for rats chronically treated with clonidine (0 out of 40, or 0%) or tianeptine (0 out of 20, or 0%). Thus, despite its ability to prevent the effects of psychosocial stress on anxiety-like behavior and startle and the development of a powerful traumatic memory, the adverse physiological side effects of amitriptyline could be a major limitation to its use in the treatment of people with PTSD.

### *Clonidine*

Neither dose of clonidine prevented the expression of fear-related behaviors in psychosocially stressed rats in response to the context and cue that were paired with the two cat exposures. Although psychosocially stressed rats chronically treated with 0.01 mg/kg of clonidine did not display significantly greater immobility during the context test than vehicle-treated control rats ( $p = 0.15$ ), the within-drug contrast (i.e., clonidine-treated psychosocial stress group vs. clonidine-treated controls) was marginally significant ( $p = 0.06$ ). These findings should be interpreted cautiously, however. Since clonidine is an  $\alpha_2$ -adrenergic receptor agonist and significantly reduces central noradrenergic activity, it can have sedative side effects at higher doses (Millan et al., 2000). In the present study, the higher dose of clonidine did result in a significant reduction of locomotor activity in the open field during OR habituation. On the other hand, clonidine had no significant effects on motor activity on the EPM, and psychosocially stressed rats treated with 0.05 mg/kg of clonidine still produced significantly more fecal boli during the context test than the no psychosocial stress group treated with 0.05 mg/kg of clonidine. One study also reported that clonidine's sedative effects are not observed until doses greater than 0.1 mg/kg are employed (Millan et al., 2000). Therefore, the data support the notion that clonidine is ineffective in blunting the expression of a traumatic memory in rats.

Both doses of clonidine, and in particular the 0.05 mg/kg dose, blocked the effects of psychosocial stress on anxiety and startle, as well as cardiovascular responses to acute immobilization. However, the higher dose of clonidine led to a significantly reduced

growth rate in controls, which was exacerbated by chronic psychosocial stress. It also resulted in significantly increased adrenal gland weights in control animals. Lastly, neither dose of clonidine prevented the chronic psychosocial stress-induced decrease in thymus weights. Thus, despite its amelioration of the chronic stress-induced behavioral and cardiovascular sequelae, clonidine was ineffective at preventing the remaining physiological changes induced by our laboratory's stress regimen.

Since people with PTSD have significantly elevated baseline NE levels and demonstrate adverse reactions (e.g., panic attacks, flashbacks) to agents that increase these levels (e.g., yohimbine), pharmacological agents that reduce noradrenergic activity could be effective treatments for people with PTSD (Boehnlein & Kinzie, 2007; Strawn & Geraciotti, 2008). Some studies have reported that propranolol, a  $\beta$ -adrenergic receptor antagonist, may be an effective treatment for PTSD if administered immediately after the traumatic event or after the re-experiencing of a traumatic event (Pitman et al., 2002; Taylor & Cahill, 2002; Vaiva et al., 2003). Other work has found that prazosin, an  $\alpha_1$ -adrenergic receptor antagonist, reduces hyperarousal symptoms, intrusive thoughts, recurrent distressing dreams and sleep disturbances in PTSD (Brkanac et al., 2003; Peskind et al., 2003; Raskind et al., 2002; Raskind et al., 2003; Taylor & Raskind, 2002; Taylor et al., 2006). However, despite the case of clonidine's use in treating PTSD, no randomized, placebo-controlled studies of clonidine's effects on PTSD have been performed. The present findings suggest that clonidine may be particularly effective in ameliorating the anxiety, hyperarousal (e.g., exaggerated startle response) and

cardiovascular components of PTSD. They also highlight the need for clinical research addressing the effectiveness of clonidine as a treatment for the disorder.

### *Tianeptine*

In the present experiment, tianeptine was the only pharmacological agent to prevent the effects of chronic psychosocial stress on all physiological and behavioral measures. Tianeptine completely blocked the expression of fear-related behaviors in psychosocially stressed rats in response to the context and cue that were paired with the two cat exposures. It also prevented the effects of psychosocial stress on anxiety, startle, cardiovascular reactivity to an acute stressor, growth rate, adrenal gland weight and thymus weight. These findings suggest that tianeptine could be a premier treatment for PTSD.

The present findings may be related to previous work reporting that chronic, but not acute, administration of tianeptine significantly impairs the acquisition and expression of conditioned fear in rats (Burghardt et al., 2004). These effects appear to be more related to the anxiolytic, rather than memory-impairing, properties of tianeptine, as numerous studies have shown that tianeptine treatment enhances, rather than impairs, hippocampus-dependent learning and memory (Jaffard et al., 1991; Meneses, 2002; Munoz et al., 2005). Interestingly, the same investigators reporting tianeptine's effects on fear conditioning found that acute administration of the SSRI citalopram *enhanced* the acquisition of auditory fear conditioning, while chronic treatment with the drug impaired the acquisition and expression of conditioned fear. Thus, tianeptine appears to

demonstrate long-term anxiolytic effects that are similar to SSRIs, without having the acute anxiogenic effects typically found with these agents.

In the present study, tianeptine significantly attenuated the immobilization-induced increase in serum corticosterone levels. This finding is consistent with previous work indicating that tianeptine reduces stress-induced activation of the HPA axis (Delbende et al., 1991). Additionally, tianeptine, relative to vehicle, resulted in significantly lower systolic BP in control animals following 20 minutes of immobilization. Few studies have examined the effects of tianeptine on cardiovascular activity, but those that have investigated the phenomenon have typically reported no effects of the drug on HR or BP (Juvent et al., 1990; Lasnier et al., 1991). On the other hand, one study did report that tianeptine resulted in significantly reduced diastolic BP (Lechin et al., 2006). The effects of tianeptine on cardiovascular activity in the present study are not likely due to acute effects of the drug. Our laboratory has preliminary data indicating that tianeptine does not prevent an acute stress-induced increase in BP (unpublished findings). In this particular study, rats were treated with 10 mg/kg of tianeptine or vehicle 30 minutes prior to a 15-minute exposure to predator stress. Animals that were exposed to the cat for 15 minutes exhibited significant elevations of systolic and diastolic BP, regardless of whether or not they had received tianeptine. In other words, tianeptine was ineffective in preventing the acute stress-induced increase in blood pressure. Thus, the ability of tianeptine to prevent the effects of chronic psychosocial stress on cardiovascular reactivity to an acute stressor is most likely attributable to its effects on general anxiety. Although speculative, tianeptine theoretically enabled the

psychosocially stressed rats to cope better with the daily mild stress of social instability and also with future acute stressors, such as the 20-minute exposure to immobilization on the final day of testing.

Chronic treatment with tianeptine also prevented the effects of chronic psychosocial stress on all of the other physiological endpoints, including growth rate, adrenal gland weight and thymus weight. Previous work in rodents has reported that tianeptine had no effect on the chronic stress-induced adrenal gland hypertrophy or reduction in growth rate (Magarinos et al., 1999; Watanabe et al., 1992b). However, these studies utilized a different stressor (restraint stress, 6 hours per day for 21 days) than that which was employed here, which could account for the apparent discrepancies. The finding that tianeptine prevented any chronic stress-induced atrophy of the thymus is consistent with work demonstrating that tianeptine significantly interacts with the immune system. For instance, several studies have shown that tianeptine prevents the adverse effects of cytokines on brain biochemistry and peripheral measures of inflammation in the rat (Castanon et al., 2001; Plaisant et al., 2003b; Plaisant et al., 2003a). Thus, an interesting avenue of future research would involve exploring the contribution of tianeptine-immune system interactions to its anti-stress effects on rat physiology and behavior.

Early studies on tianeptine's mechanism of action showed that the drug led to significantly lower extracellular levels of serotonin, a finding that was hypothesized to result from enhanced serotonin reuptake (Fattaccini et al., 1990; Labrid et al., 1992; Mennini et al., 1987; Mocaer et al., 1988). However, tianeptine's effects on the

serotonergic system may be an indirect consequence of the drug's influences on an alternative neurotransmitter system because later studies failed to show any direct effects of tianeptine on serotonergic neurotransmission (Pineyro et al., 1995a; Pineyro et al., 1995b). Additionally, research has shown that tianeptine does not alter the density or affinity of any serotonin receptor subtype, and tianeptine's affinity for the serotonin transporter is very low (Kato & Weitsch, 1988; Svenningsson et al., 2007). Some have also contested the validity of the original studies on tianeptine's mechanism of action based on technical limitations that were present at the time (Malagie et al., 2000).

Recent work has suggested that its therapeutic effects may be more associated with modulation of the glutamatergic system (Brink et al., 2006; Kasper & McEwen, 2008; Zoladz et al., in press). Extensive work has implicated hyperactivity of the glutamatergic system in the deleterious effects of stress on brain structure and function (Bagley & Moghaddam, 1997; Bartanusz et al., 1995; Joels et al., 2003; Kim et al., 1996; Krugers et al., 1993; Lowy et al., 1993; Lowy et al., 1995; Magarinos & McEwen, 1995; McEwen et al., 2002; Moghaddam, 1993; Park et al., 2004; Reznikov et al., 2007; Yang et al., 2005), and tianeptine appears to protect brain regions that are highly susceptible to stress, such as the hippocampus and prefrontal cortex, from the deleterious effects of stress by normalizing the stress-induced modulation of glutamatergic activity. For instance, tianeptine has been shown to prevent stress-induced increases in NMDA channel currents, as well as the ratio of NMDA:non-NMDA receptor currents, in the CA3 region of the hippocampus (Kole et al., 2002). It also inhibits the acute stress-induced increase in extracellular levels of glutamate in the basolateral amygdala (Reznikov et al.,



2007). In addition to its glutamatergic modulation, tianeptine reduces the expression of CRH mRNA in the amygdala and the bed nucleus of the stria terminalis, a brain region that is highly innervated by amygdala fibers (Kim et al., 2006). CRH neurotransmission in both of these regions has been implicated in the expression of anxiety-like behaviors (Holsboer, 1999; Strohle & Holsboer, 2003). Thus, tianeptine's effects on glutamatergic and CRH activity in these various brain regions may play an important role in its ability to reverse the effects of chronic stress on the expression of anxiety-like behaviors.

Uzbay and colleagues found that tianeptine reduced the intensity (Ceyhan et al., 2005) and delayed the onset (Uzbay et al., 2007) of pentylenetetrazole-induced seizures in rodents. The latter effect was blocked by the administration of caffeine, a nonspecific adenosine receptor antagonist, and 8-cyclopentyl-1,3-dipropylxanthine, an A<sub>1</sub> receptor-specific antagonist. However, administration of the A<sub>2</sub> receptor-specific antagonist, 8-(3-chlorostyryl) caffeine, had no effect on the tianeptine-induced delay of seizure onset, suggesting that tianeptine's anticonvulsant properties are dependent upon activation of A<sub>1</sub> adenosine receptors. Since previous work has shown that activation of A<sub>1</sub> adenosine receptors has anxiolytic effects (Florio et al., 1998; Jain et al., 1995; Prediger et al., 2004; Prediger et al., 2006), this specific category of adenosinergic receptors could be responsible, at least in part, for tianeptine's anxiolytic effects in rodents (Burghardt et al., 2004; File et al., 1993; File & Mabbutt, 1991; Pillai et al., 2004) and in the depressed population (Defrance et al., 1988; Wilde & Benfield, 1995).

In sum, tianeptine was the only pharmacological agent to prevent the effects of chronic psychosocial stress on all physiological and behavioral measures. Extensive

preclinical research has shown that exposure to stress results in a significant increase in glutamate activity, and tianeptine's antidepressant properties have been attributed to its ability to normalize this hyperactivity of the glutamatergic system. It is therefore likely that at least some of the behavioral changes observed in our animal model of PTSD are a result of stress-induced alterations in glutamate function. Furthermore, it is possible that abnormalities in glutamatergic function also underlie the pathology of PTSD (Chambers et al., 1999; Nair & Singh, 2008; Reul & Nutt, 2008), in which case tianeptine would certainly be an optimal choice to treat individuals with the disorder.

#### *Limitations and Future Research*

The design of this experiment does not distinguish between the acute and chronic effects of amitriptyline, clonidine and tianeptine on rat physiology and behavior. Rats were administered these pharmacological agents not only on Days 2-31 of the chronic psychosocial stress paradigm, but throughout behavioral testing as well. The drug administration continued during behavioral testing to prevent withdrawal effects from influencing rat behavior. Importantly, our laboratory does have preliminary data indicating that chronic tianeptine treatment prevents the effects of the current stress regimen on all physiological and behavioral measures even if it is administered only during Days 2-31 and discontinued at the commencement of behavioral testing. Nevertheless, future work should examine the effects of the present compounds on the stress-induced changes in rat physiology and behavior when they are administered during the chronic stress period only and during behavioral testing only.

*Summary and Application to Pharmacotherapy for Post-Traumatic Stress Disorder*

A subset of people with PTSD show significant improvement in their symptoms following treatment with SSRIs (Asnis et al., 2004; Davidson, 2003; Davis et al., 2006; Hidalgo & Davidson, 2000; Ipser et al., 2006; Stein et al., 2006). However, SSRIs tend to blunt only the depressive components of PTSD, while having little effect on the memory- and anxiety-related symptoms of the disorder (Asnis et al., 2004; Boehnlein & Kinzie, 2007; Brady et al., 2000; Van der Kolk et al., 1994). In addition, some forms of PTSD, such as combat-related PTSD, are incredibly resistant to SSRI treatment (Jakovljevic et al., 2003; Rothbaum et al., 2008; Stein et al., 2002). These agents also have anxiogenic effects early in the treatment phase and only exert their antidepressant effects after a substantial delay (Browning et al., 2007; Burghardt et al., 2004; Humble & Wistedt, 1992). Thus, there is an urgent need to develop alternative pharmacotherapeutic interventions for the treatment of PTSD.

The present experiment examined the ability of amitriptyline, clonidine and tianeptine to prevent the development of PTSD-like sequelae in rats exposed to chronic psychosocial stress. The tricyclic antidepressant amitriptyline was effective in reducing the memories for the context and cue that were associated with the acute cat exposures, and it ameliorated the stress-induced increase in anxiety and startle. However, this agent had adverse side effects, as it significantly increased cardiovascular reactivity in control animals and led to adverse physiological reactions, including reduced growth rate, increase adrenal gland weight and internal adhesions. Thus, despite its positive effects,

the adverse physiological side effects of amitriptyline could be a major limitation to its use in the treatment of people with PTSD.

Clonidine also blocked the effects of chronic psychosocial stress on anxiety and startle, but in contrast to amitriptyline, prevented the stress-induced changes in cardiovascular reactivity to an acute stressor as well. However, it did not prevent the expression of fear-related behaviors in psychosocially stressed rats upon exposed to the context and cue that were paired with the acute cat exposures. Clonidine also had some adverse side effects of its own, including a significant reduction in growth rate and a significant increase in adrenal gland weight. Thus, clonidine may be particularly effective in ameliorating the anxiety, hyperarousal (e.g., exaggerated startle response) and cardiovascular components of PTSD, but have little effect on the strength of a traumatic memory.

Lastly, tianeptine was the only pharmacological agent to prevent the effects of chronic psychosocial stress on all physiological and behavioral endpoints. It completely blocked the expression of fear-related behaviors in psychosocially stressed rats in response to the context and cue that were paired with the two cat exposures and prevented the effects of psychosocial stress on anxiety, startle, cardiovascular reactivity to an acute stressor, growth rate, adrenal gland weight and thymus weight. Collectively, these findings illustrate the differential effectiveness of these three treatments in blocking the PTSD-like sequelae in rats, and the profile of tianeptine as the most effective agent provides guidance for pharmacotherapeutic approaches in the treatment of individuals suffering from PTSD.

## Chapter Five: Experiment Four

### *Temporal Dynamics of the Physiological and Behavioral Sequelae Induced by Chronic Psychosocial Stress*

People with chronic PTSD display physiological and behavioral symptoms of the disorder years after the original trauma took place. These symptoms develop acutely after experiencing the trauma and progressively worsen to eventually produce full-blown PTSD. A valid animal model of PTSD should be able to demonstrate PTSD-like effects on physiology and behavior long after the initial stress exposure. Therefore, the purpose of Experiment Four was to examine whether or not the stress regimen employed in the previous experiments would produce physiological and behavioral changes in rats that would be present for a longer period of time. It was also designed to explore the contribution of an additional, third, acute stress session and irregular, rather than just daily, social instability to the maintenance of the PTSD-like effects for this extended period of time.

#### *Methods*

##### *Rats*

The same weight range and strain of rats, as well as the housing conditions, that were employed in Experiments One, Two and Three were used in the present experiment. Upon arrival, all rats were given 1 week to acclimate to the housing room environment and cage changing procedures before any experimental manipulations took place. All

procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

### *Psychosocial Stress Procedure*

Following the 1-week acclimation phase, rats were brought to the laboratory and then randomly assigned to one of four groups (see Figure 28; N = 10 rats per group). In contrast to Experiments One through Three, each of the four groups was exposed to three, as opposed to two, acute stress sessions. During each stress session, as in Experiment Three, the rats were exposed to a chamber for 3 minutes (with a 30-second tone presented at the end of the 3-minute period). Then, the rats were either immobilized and exposed to a cat or placed back in their home cages for 1 hour. As before, the first stress session occurred during the light cycle, between 0800 and 1300 hours, while the second stress session occurred 10 days later during the dark cycle, between 1900 and 2100 hours. The third and final stress session took place 3 weeks following the second stress session during the light cycle, between 0800 and 1300 hours.

Of the four groups in the present experiment, three were exposed to chronic psychosocial stress and one was a control, no psychosocial stress, group. Each of the three psychosocial stress groups was exposed to the same manipulations until the third stress session. That is, these groups were exposed to the chamber followed by immobilization plus cat exposure during the first and second stress sessions, as well as daily randomized housing throughout the 31-day period leading up to the third stress session. During the third stress session (Day 32), rats in “psychosocial stress group 1” were exposed to the chamber for 3 minutes followed by a 1-hour exposure to their home

cages. Rats in “psychosocial stress group 2” and “psychosocial stress group 3” were exposed to the chamber for 3 minutes followed by 1 hour of immobilization during cat exposure. These procedures are illustrated in Figure 28.

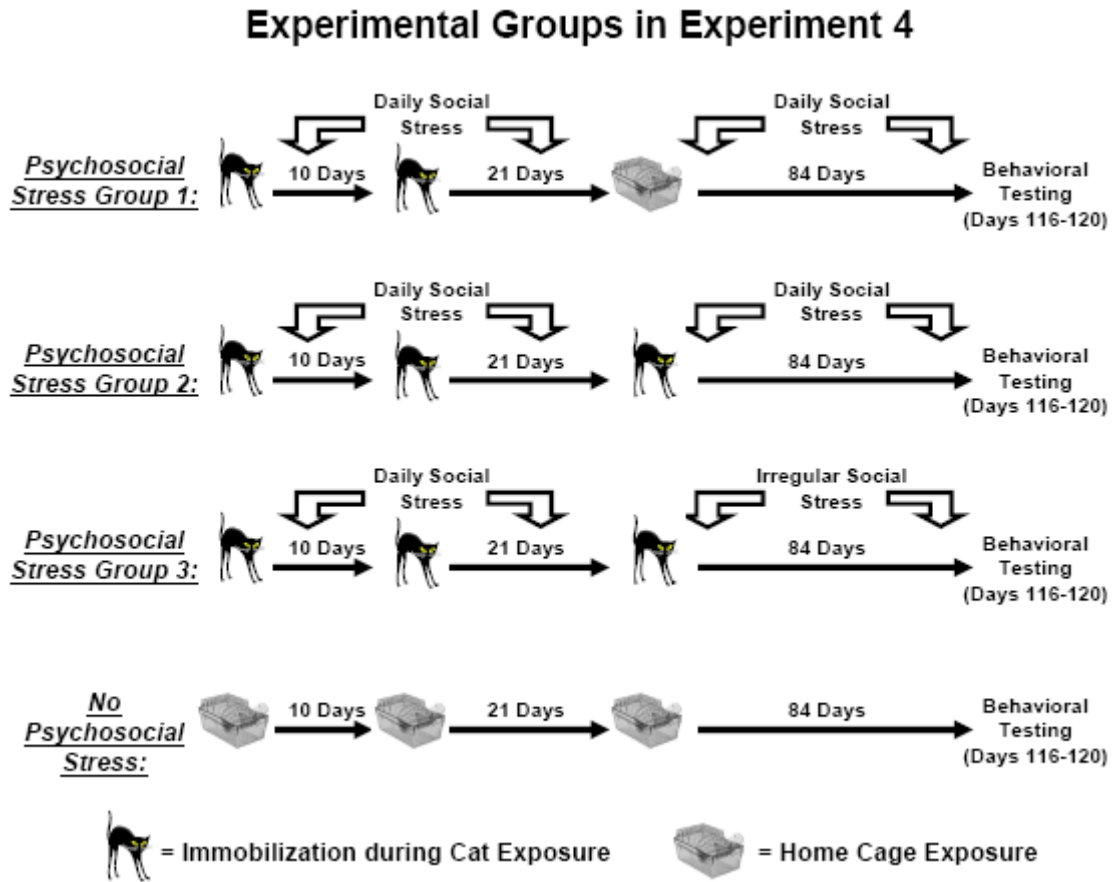


Figure 28. Experimental groups in Experiment 4.

Following the third stress session, each of the three psychosocial stress groups was exposed to randomized housing. As in the previous experiments, psychosocial groups 1 and 2 were exposed to daily randomized housing for the 12 weeks following the third stress session. In contrast, psychosocial stress group 3 was exposed to irregular randomized housing for the next 12 weeks. In other words, the cage mates in this group

were randomized every 1-4 days. The strategy behind this manipulation was to add an additional element of unpredictability to the stress experience. The typical daily randomized housing procedure, although effective for the 31-day paradigm, is somewhat predictable in that it occurs at approximately the same time every day, and if continued for an extended period of time, could eventually become ineffective.

### *Behavioral Testing*

Twelve weeks after the third stress session (i.e., Day 116), rats were given tests to measure their fear memory, anxiety, startle, learning and memory, cardiovascular activity and corticosterone activity. On the first 4 days of behavioral testing (Days 116-119), all rats were brought to the laboratory and left undisturbed for 30 minutes before testing began. All behavioral testing took place during the light cycle, between 0800 and 1500 hours.

### *Behavioral Apparatus*

All rats in the present experiment were exposed to the same physiological and behavioral testing procedures that were employed in Experiment Three. Thus, these procedures will only be briefly addressed here.

*Contextual and Cue Fear Memory.* On Day 116, rat behavior in response to the chamber (context test) and tone (cue test) that were previously paired with the acute stress sessions was examined. Testing adhered to the procedures employed in Experiment Three.



*Elevated Plus Maze.* On Day 117, the rats were placed on the EPM for 5 minutes, and their behavior was scored according to the procedures employed in Experiment Three.

*Startle Response.* One hour after the EPM assessment, acoustic startle testing was administered according to the procedures employed in Experiment Three.

*Novel Object Recognition.* On Day 118, the rats were placed in an open field for 5 minutes to acclimate to the environment. Their behavior was monitored and scored according to the procedures employed in Experiment Three. Twenty-four hours later (Day 119), the rats were given novel object recognition training and testing according to the procedures employed in Experiment Three.

*Preparation for Blood Sampling.* Immediately following the 3-hour object recognition test, the hind legs of all rats were shaved to allow access to their saphenous veins, as per Experiments One through Three.

*Blood Sampling and Cardiovascular Activity.* On the final day of behavioral testing (Day 120), three blood samples, as well as measures of heart rate and blood pressure, were collected, according to the procedures utilized in Experiment Three. Following rapid decapitation, the adrenal and thymus glands were removed and weighed. Once all of the blood had clotted at room temperature, it was centrifuged (3000 rpm for 8 min), and the serum was extracted and stored at -80° C until assayed by Monika Fleshner at the University of Colorado at Boulder.

### *Statistical Analyses*

*Experimental Design and General Analyses.* The present study utilized a single factor, between-subjects design. The independent variable was psychosocial stress (psychosocial stress group 1, psychosocial stress group 2, psychosocial stress group 3, no psychosocial stress). In most cases, one-way, between-subjects ANOVAs were used to analyze the data from the physiological and behavioral assessments, with psychosocial stress serving as the between-subjects factors. Planned comparisons (independent samples *t*-tests) were also conducted between each of the psychosocial stress groups and the no psychosocial stress group. For all analyses, alpha was set at 0.05, and Holm-Sidak post hoc tests were employed when necessary.

*Fear Memory.* The amount of immobility during each chamber exposure (Stress Session 1, Stress Session 2, Stress Session 3, Context Test, Cue Test – No Tone, Cue Test – Tone) was analyzed separately. For each test, one-way, between-subjects ANOVAs were used to analyze behavior. Psychosocial stress served as the between-subjects factor in each case.

*Elevated Plus Maze.* The amount of time that rats spent in the open arms of the EPM was calculated as a percent of the total trial time. The percent time that rats spent in the open arms, as well as the number of ambulations that rats made on the EPM were analyzed with one-way, between-subjects ANOVAs. Each of these analyses was performed for the entire 5-minute testing trial and for the first minute of the testing trial, with psychosocial stress serving as the between-subjects factor in each case.

*Startle Response.* Startle responses to each of the 3 auditory stimulus intensities (90, 100 and 110 dB) were analyzed separately. In each case, one-way, between-subjects ANOVAs were employed to analyze the data, with psychosocial stress serving as the between-subjects factor.

*Novel Object Recognition.* For habituation, a one-way, between-subjects ANOVA was used to compare overall locomotor activity across all groups, with psychosocial stress serving as the between-subjects factor. The amount of time that rats spent in each area of the open field during the habituation phase was also analyzed to assure that the rats did not display a preference for one area of the open field over another. For the analysis, the open field was divided into four square quadrants via the ANY-Maze computer program. The amount of time that rats spent in each of the quadrants was analyzed with a mixed-model ANOVA, with psychosocial stress serving as the between-subjects factor and time spent in each quadrant serving as the within-subjects factor. For training, paired samples *t*-tests were first conducted to determine whether the rats within each group spent a comparable amount of time with each object replica (to rule out object preference effects). Then, the total time that rats spent with both object replicas during training was compared across groups by using one-way, between-subjects ANOVAs, with psychosocial stress serving as the between-subjects factor. For testing, a “ratio time” score was calculated for each group by taking the time that rats spent with the novel object and dividing it by the time that rats spent with the familiar object (i.e., ratio time = time with novel object / time with familiar object). The ratio times were compared across groups by utilizing one-way, between-subjects ANOVAs, with psychosocial stress again

serving as the between-subjects factor. This was performed for the entire 5-minute testing trial and for the first minute of the testing trial.

*Corticosterone Levels.* A mixed-model ANOVA was used to analyze corticosterone levels at the three time points. Psychosocial stress served as the between-subjects factor, and time point (baseline, stress, return-to-baseline) served as the within-subjects factor.

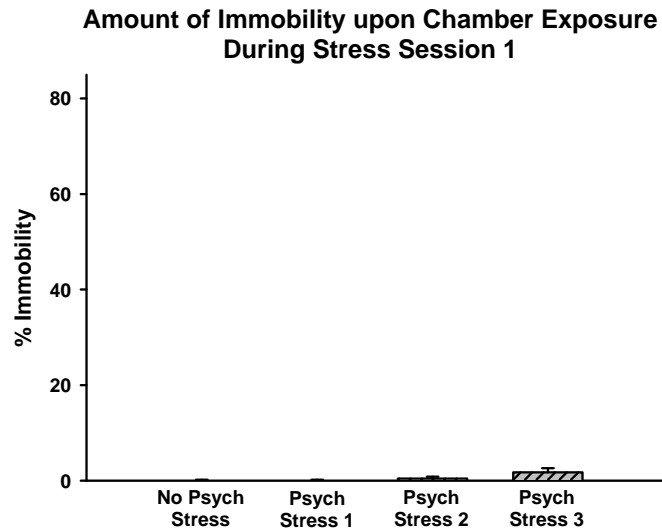
*Heart Rate and Blood Pressure.* The HR, systolic BP and diastolic BP data were analyzed with one-way, between-subjects ANOVAs, with psychosocial stress serving as the between-subjects factor.

*Growth Rates, Adrenal Gland Weights and Thymus Weights.* Growth rates, expressed as grams per day (g/day), were calculated for all rats by dividing their total body weight gained during the course of the experiment by the total number of days in the experiment (i.e., 115 days). The adrenal glands and thymuses were weighed and expressed as milligrams per 100 grams of body weight (mg/100 g b.w.). The growth rate, adrenal gland weights and thymus weights were analyzed with one-way, between-subjects ANOVAs, with psychosocial stress serving as the between-subjects factor.

## *Results*

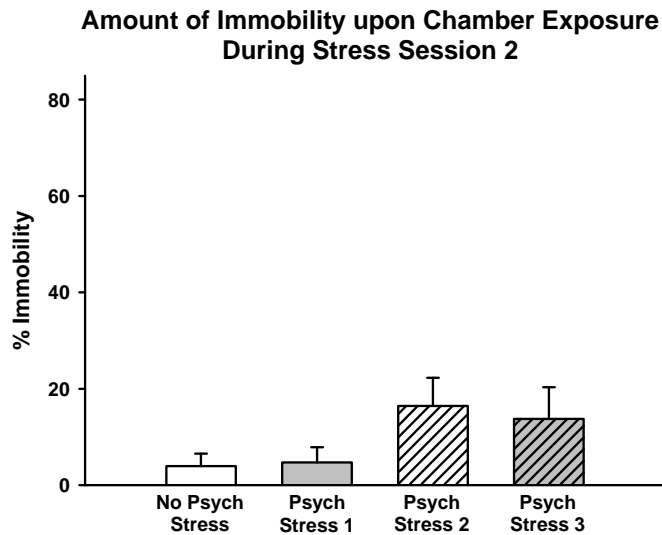
### *Fear Memory*

*Stress Session One (see Figure 29).* The analysis of immobility during the 3-minute chamber exposure during stress session one revealed a significant main effect of psychosocial stress,  $F(3,35) = 3.07$ ,  $p < 0.05$ . However, post hoc analyses did not indicate any significant differences between the groups.



*Figure 29.* Amount of immobility upon chamber exposure during stress session one. The data are presented as mean percent immobility  $\pm$  SEM.

*Stress Session Two* (see *Figure 30*). The analysis of immobility during the 3-minute chamber exposure during stress session two revealed no significant main effect of psychosocial stress,  $F(3,35) = 1.79, p > 0.05$ .



*Figure 30.* Amount of immobility upon chamber exposure during stress session two. The data are presented as mean percent immobility  $\pm$  SEM.

*Stress Session Three* (see Figure 31). The analysis of immobility during the 3-minute chamber exposure during stress session three revealed no significant main effect of psychosocial stress,  $F(3,33) = 2.63, p = 0.067$ . However, planned comparisons indicated that psychosocial stress group 1,  $t(16) = 2.31$ , and psychosocial stress group 2,  $t(16) = 2.85$ , spent significantly more time immobile than the no psychosocial stress group ( $p$ 's  $< 0.05$ ).

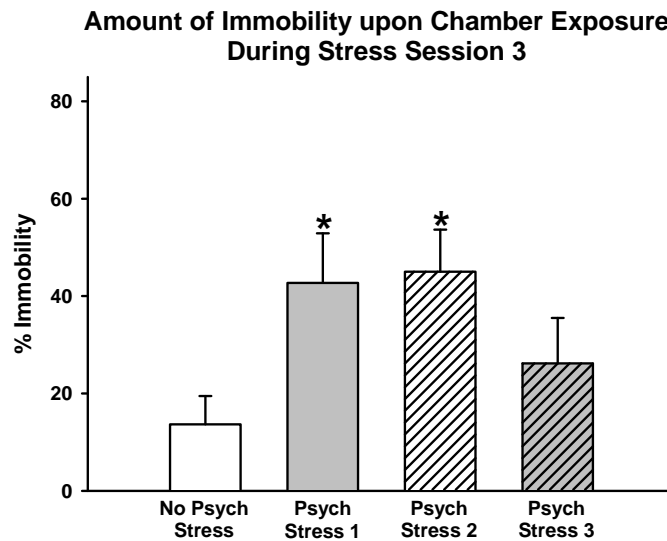
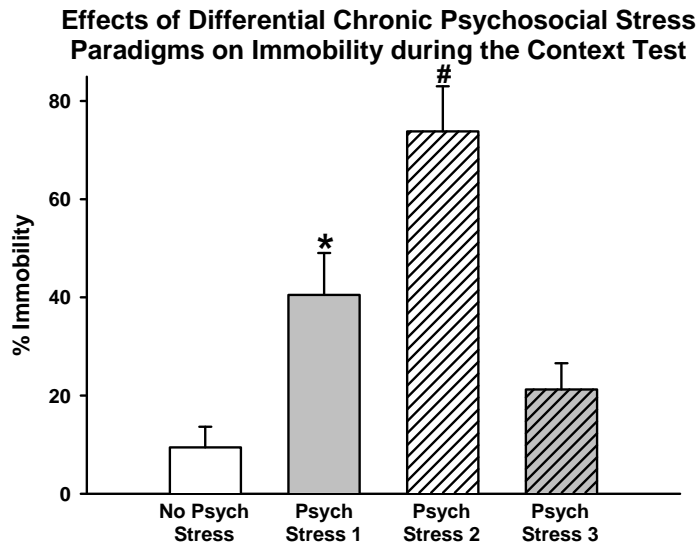


Figure 31. Amount of immobility upon chamber exposure during stress session three.

The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

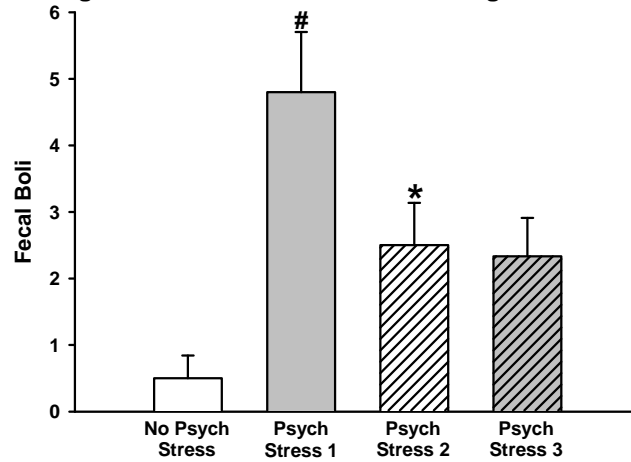
*Context Test Immobility* (see Figure 32). The analysis of immobility during the 5-minute context test revealed a significant main effect of psychosocial stress,  $F(3,34) = 14.79, p < 0.001$ . Post hoc tests indicated that psychosocial stress group 1 spent significantly more time immobile than the no psychosocial stress group, and psychosocial stress group 2 spent significantly more time immobile than all other groups.



*Figure 32.* Effects of differential chronic psychosocial stress paradigms on immobility during the 5-minute context test. The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group; # =  $p < 0.05$  relative to all other groups.

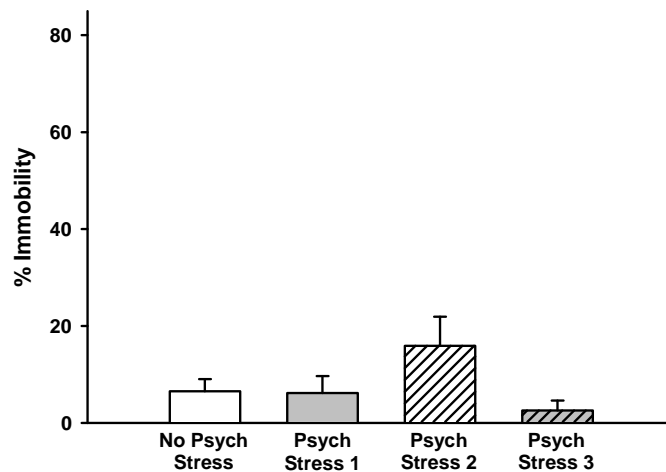
*Context Test Fecal Boli* (see *Figure 33*). The analysis of fecal boli produced during the 5-minute context test revealed a significant main effect of psychosocial stress,  $F(3,35) = 7.51, p < 0.001$ . Post hoc tests indicated that psychosocial stress group 2 produced significantly more fecal boli than the no psychosocial stress group, and psychosocial stress group 1 produced significantly more fecal boli than all other groups.

**Effects of Differential Chronic Psychosocial Stress Paradigms on Fecal Boli Produced during the Context Test**



*Figure 33.* Effects of differential chronic psychosocial stress paradigms on fecal boli produced during the 5-minute context test. The data are presented as mean number of fecal boli  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group; # =  $p < 0.05$  relative to all other groups.

**Effects of Differential Chronic Psychosocial Stress Paradigms on Immobility in a Novel Environment**

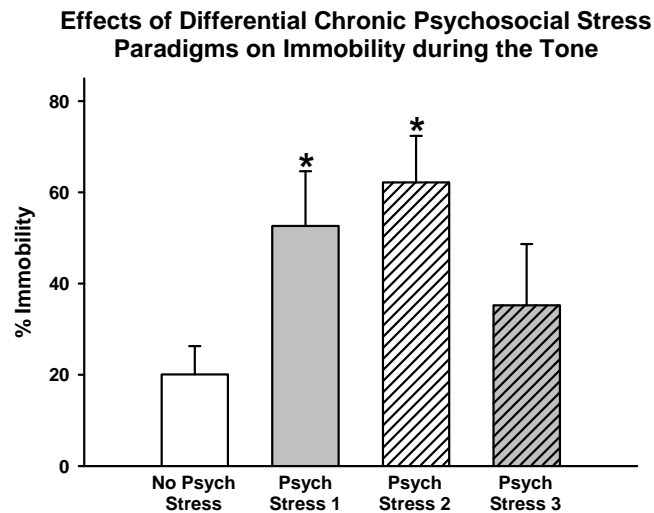


*Figure 34.* Effects of differential chronic psychosocial stress paradigms on immobility during the first 3 minutes of the cue test. The data are presented as mean percent immobility  $\pm$  SEM.



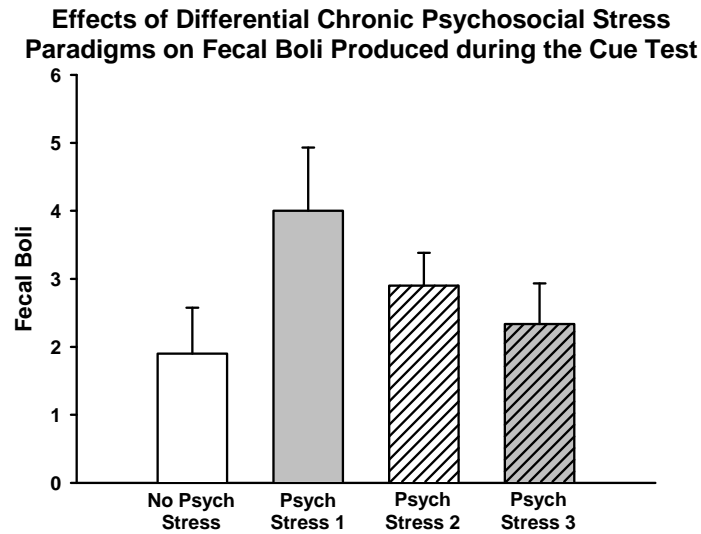
*Cue Test Immobility – No Tone (i.e., Novel Environment) (see Figure 34).* The analysis of immobility during the first 3 minutes of the cue test revealed no significant main effect of psychosocial stress,  $F(3,34) = 2.09, p > 0.05$ .

*Cue Test Immobility – Tone (see Figure 35).* The analysis of immobility during the last 3 minutes of the cue test revealed no significant main effect of psychosocial stress,  $F(3,33) = 2.73, p = 0.059$ . However, planned comparisons indicated that psychosocial stress group 1,  $t(16) = 2.23$ , and psychosocial stress group 2,  $t(16) = 3.30$ , spent significantly more time immobile than the no psychosocial stress group ( $p$ 's < 0.05).



*Figure 35.* Effects of differential chronic psychosocial stress paradigms on immobility during the tone. The data are presented as mean percent immobility  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

*Cue Test Fecal Boli (see Figure 36).* The analysis of fecal boli produced during the 6-minute cue test revealed no significant main effect of psychosocial stress,  $F(3,35) = 1.73, p > 0.05$ .



*Figure 36.* Effects of differential chronic psychosocial stress paradigms on fecal boli produced during the 6-minute cue test. The data are presented as mean number of fecal boli  $\pm$  SEM.

#### *Elevated Plus Maze*

*Percent Time in Open Arms, 5-Minute Trial (see Figure 37).* The analysis of percent time spent in the open arms during the 5-minute trial on the EPM revealed no significant main effect of psychosocial stress,  $F(3,33) = 0.43, p > 0.05$ .

*Percent Time in Open Arms, First Minute (see Figure 38).* The analysis of percent time spent in the open arms during the first minute of the 5-minute trial on the EPM revealed a significant main effect of psychosocial stress,  $F(3,31) = 5.28, p < 0.01$ . Post hoc tests indicated that each of the psychosocial stress groups spent significantly less time in the open arms than the no psychosocial stress group.

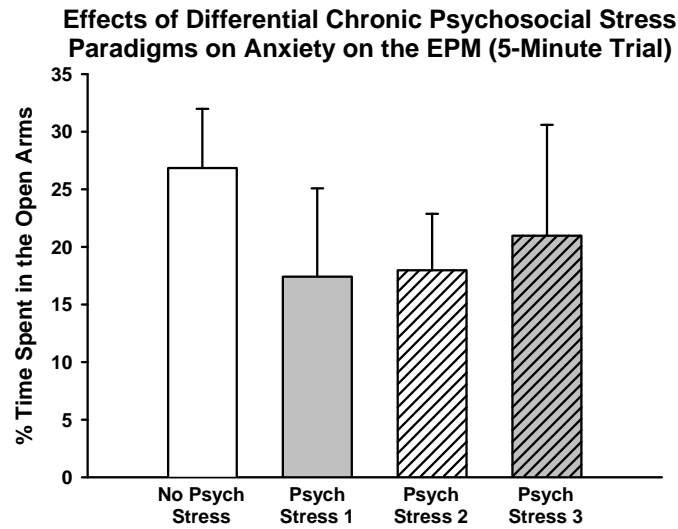


Figure 37. Effects of differential chronic psychosocial stress paradigms on percent time spent in the open arms during the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM.

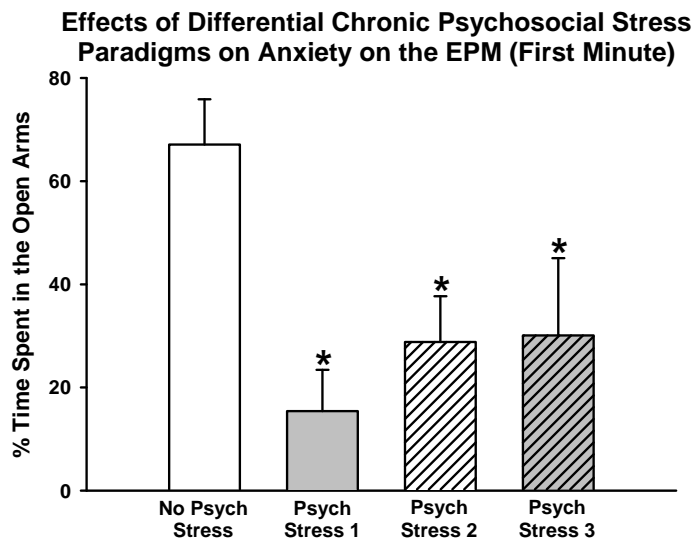
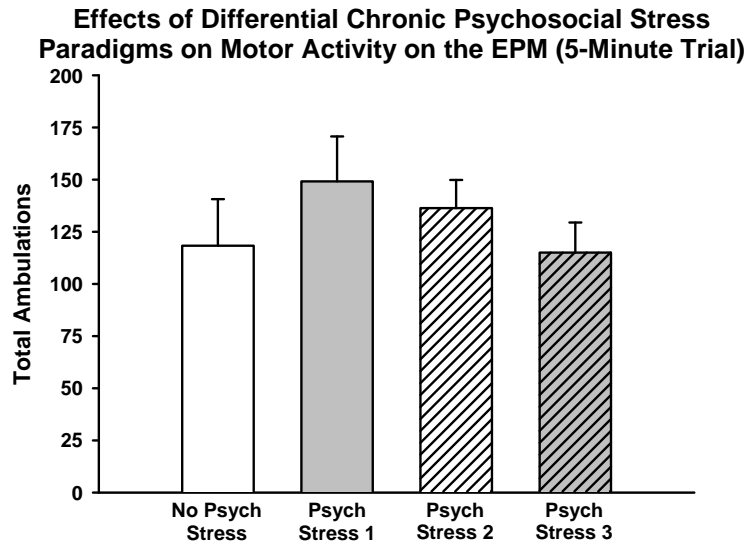


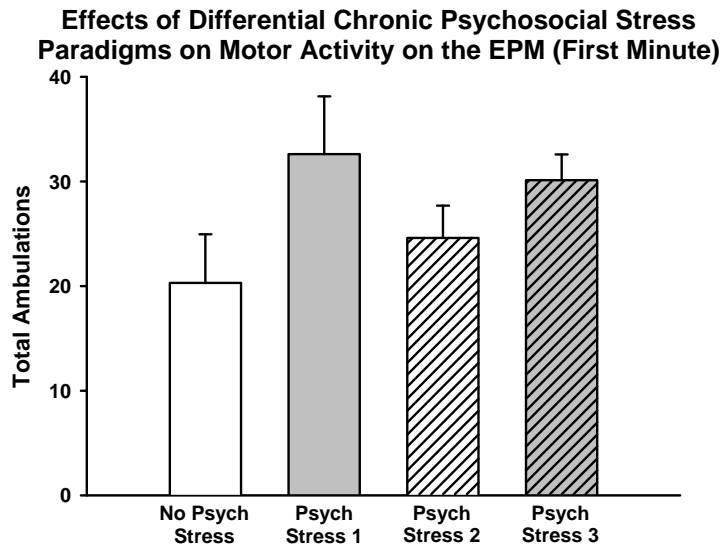
Figure 38. Effects of differential chronic psychosocial stress paradigms on percent time spent in the open arms during the first minute of the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

*Ambulations, 5-Minute Trial (see Figure 39).* The analysis of ambulations made during the 5-minute trial on the EPM revealed no significant main effect of psychosocial stress,  $F(3,35) = 0.73, p > 0.05$ .

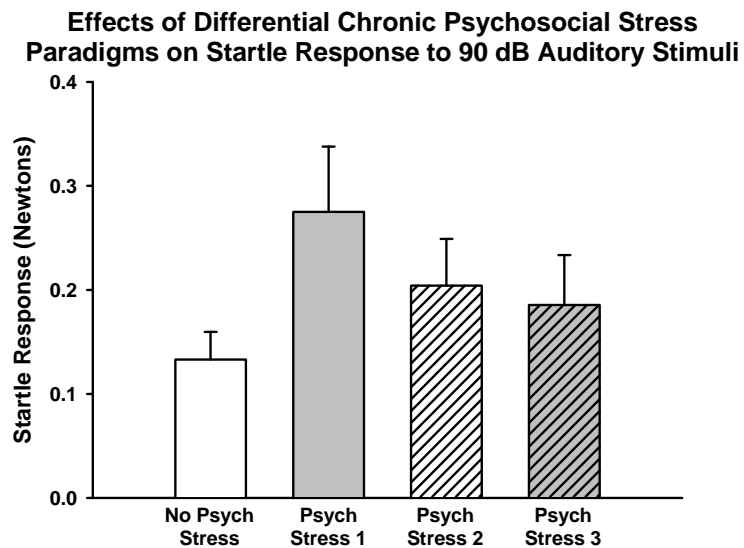


*Figure 39.* Effects of differential chronic psychosocial stress paradigms on ambulations made during the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM.

*Ambulations, First Minute (see Figure 40).* The analysis of ambulations made during the first minute of the 5-minute trial on the EPM revealed no significant main effect of psychosocial stress,  $F(3,35) = 1.77, p > 0.05$ .



*Figure 40.* Effects of differential chronic psychosocial stress paradigms on ambulations made during the first minute of the 5-minute trial on the elevated plus maze. The data are presented as mean percent time spent in the open arms  $\pm$  SEM.

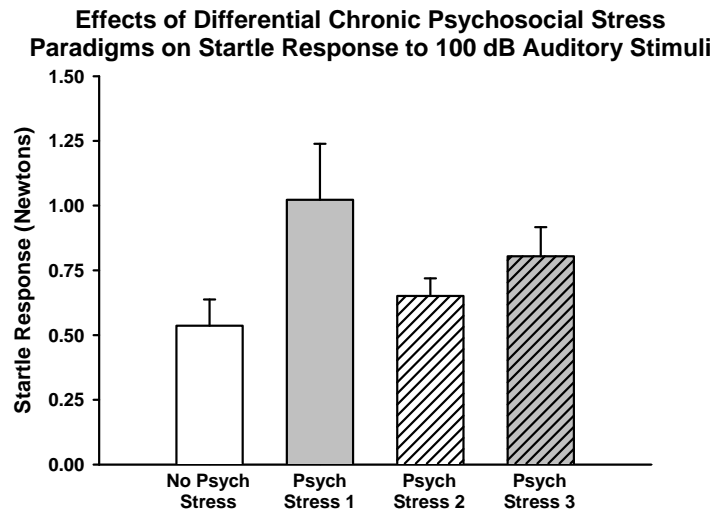


*Figure 41.* Effects of differential chronic psychosocial stress paradigms on startle responses to the 90 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM.

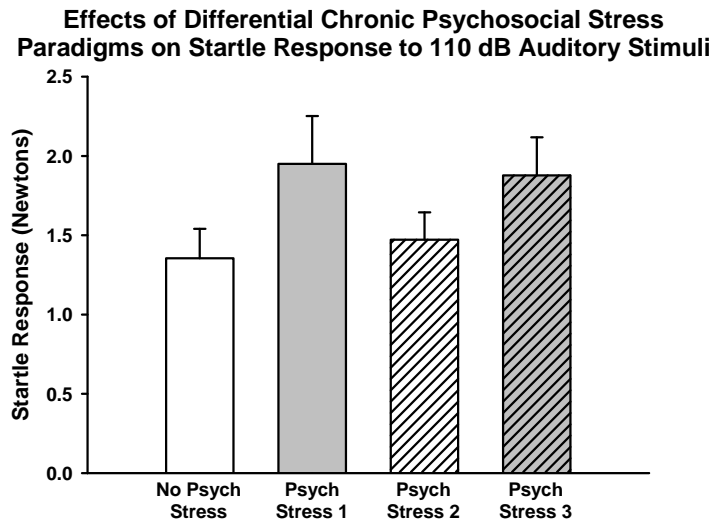
### *Startle Response*

*90 dB Auditory Stimuli (see Figure 41).* The analysis of startle responses to the 90 dB auditory stimuli revealed no significant main effect of psychosocial stress,  $F(3,35) = 1.57, p > 0.05$ .

*100 dB Auditory Stimuli (see Figure 42).* The analysis of startle responses to the 100 dB auditory stimuli revealed no significant main effect of psychosocial stress,  $F(3,34) = 2.30, p > 0.05$ .



*Figure 42.* Effects of differential chronic psychosocial stress paradigms on startle responses to the 100 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM.



*Figure 43.* Effects of differential chronic psychosocial stress paradigms on startle responses to the 110 dB auditory stimuli. The data are presented as mean startle response (Newtons)  $\pm$  SEM.

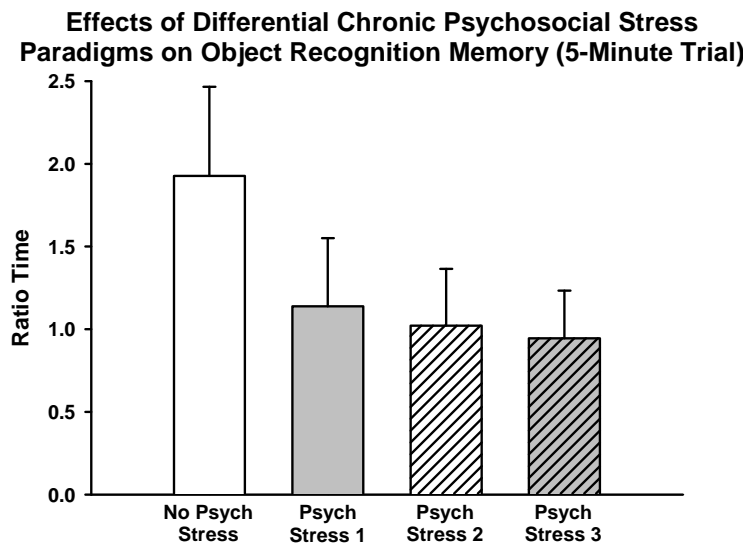
*110 dB Auditory Stimuli (see Figure 43).* The analysis of startle responses to the 110 dB auditory stimuli revealed no significant main effect of psychosocial stress,  $F(3,34) = 1.62, p > 0.05$ .

#### *Novel Object Recognition*

*Habituation.* The analysis of locomotor activity in the open field during the 5-minute habituation phase revealed no significant main effect of psychosocial stress,  $F(3,35) = 0.68, p > 0.05$  (data not shown). The analysis of time that the rats spent in each area of the open field revealed no significant main effects of quadrant,  $F(3,105) = 1.05$ , or psychosocial stress,  $F(3,35) = 0.91$ , and the Quadrant x Psychosocial Stress interaction was not significant,  $F(9,105) = 0.40 (p's > 0.05; data not shown)$ . These findings

indicated that the rats did not display a preference for one area of the open field over another.

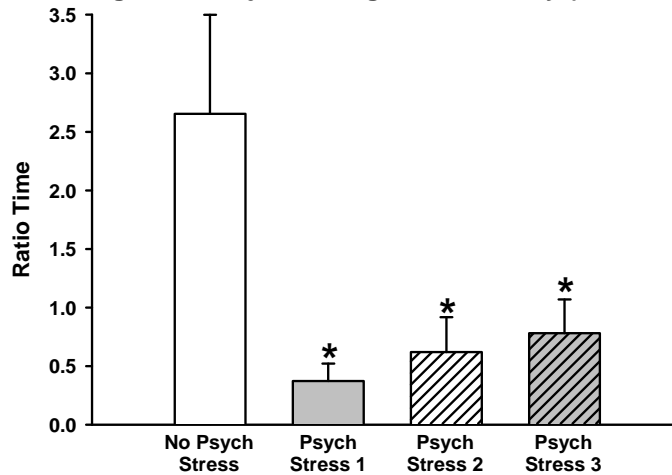
*Training.* Within-group comparisons showed that the no psychosocial stress group,  $t(9) = 0.81$ , psychosocial stress group 1,  $t(9) = 0.72$ , psychosocial stress group 2,  $t(9) = 1.30$ , and psychosocial stress group 3,  $t(8) = 0.91$ , spent a comparable amount of time with each of the objects that were placed in the open field during object recognition training ( $p$ 's  $> 0.05$ ; data not shown), indicating that no object preference effects were present. Moreover, a between-groups comparison of the total amount of time spent with both objects during training revealed no significant main effect of psychosocial stress,  $F(3,35) = 1.44$ ,  $p > 0.05$ , indicating that all groups spent a comparable amount of time with both objects during training (data not shown).



*Figure 44.* Effects of differential chronic psychosocial stress paradigms on object recognition memory during the entire 5-minute testing trial. The data are presented as mean ratio time  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.



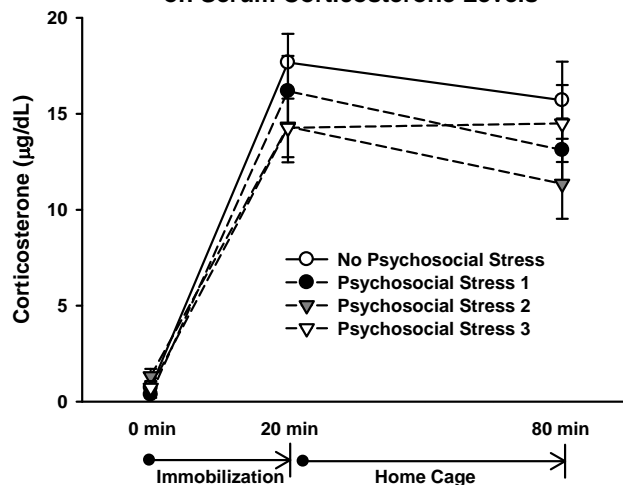
**Effects of Differential Chronic Psychosocial Stress Paradigms on Object Recognition Memory (First Minute)**



*Figure 45.* Effects of differential chronic psychosocial stress paradigms on object recognition memory during the first minute of the testing trial. The data are presented as mean ratio time  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

*Testing.* The analysis comparing the ratio times of all groups during the 5-minute object recognition testing session revealed no significant main effect of psychosocial stress,  $F(3,29) = 1.23$ ,  $p > 0.05$  (see Figure 44). The analysis comparing the ratio times of all groups during the first minute of the testing trial revealed a significant main effect of psychosocial stress,  $F(3,20) = 6.29$ ,  $p < 0.01$  (see Figure 45). Post hoc contrasts indicated that each of the 3 psychosocial stress groups exhibited significantly lower ratio times than the no psychosocial stress group ( $p$ 's  $< 0.05$ ).

**Effects of Differential Chronic Psychosocial Stress Paradigms on Serum Corticosterone Levels**



*Figure 46.* Effects of differential chronic psychosocial stress paradigms on serum corticosterone levels. The data are presented as mean corticosterone levels ( $\mu\text{g/dL}$ )  $\pm$  SEM.

*Corticosterone Levels (see Figure 46)*

The analysis of corticosterone levels revealed a significant main effect of time point,  $F(2,62) = 138.41, p < 0.001$ . Post hoc tests indicated that all groups displayed significantly elevated serum corticosterone levels, relative to baseline, following 20 minutes of acute immobilization stress and that these levels remained elevated an hour later ( $p$ 's  $< 0.05$ ). There was no significant main effect of psychosocial stress,  $F(3,31) = 1.11$ , and the Time Point  $\times$  Psychosocial Stress interaction was not significant,  $F(6,62) = 0.93$  ( $p$ 's  $> 0.05$ ).

*Cardiovascular Activity*

*Heart Rate (see Figure 47).* The analysis of heart rate revealed no significant main effect of psychosocial stress,  $F(3,22) = 0.17, p > 0.05$ .

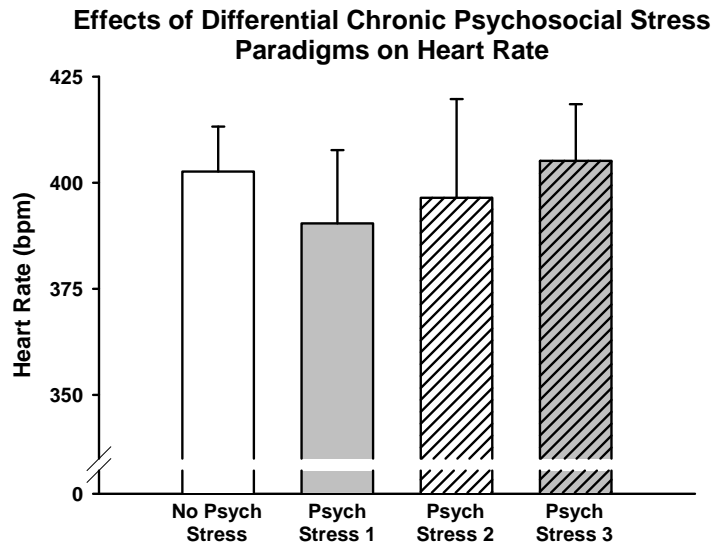


Figure 47. Effects of differential chronic psychosocial stress paradigms on heart rate. The data are presented as mean heart rate (bpm)  $\pm$  SEM.

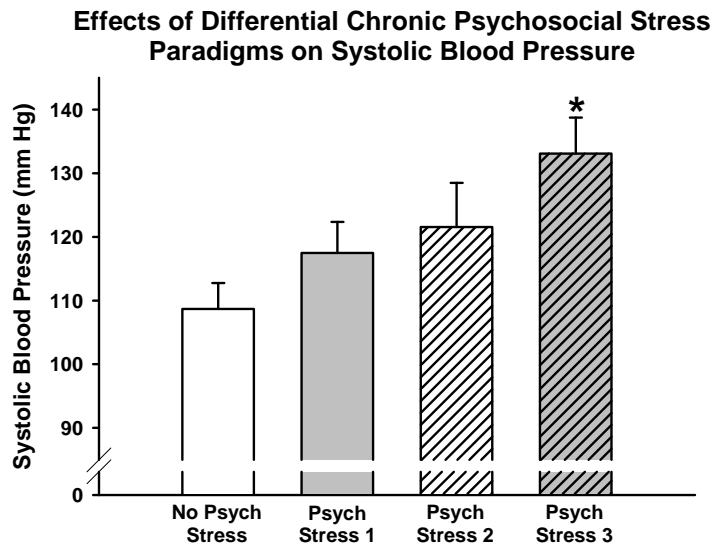
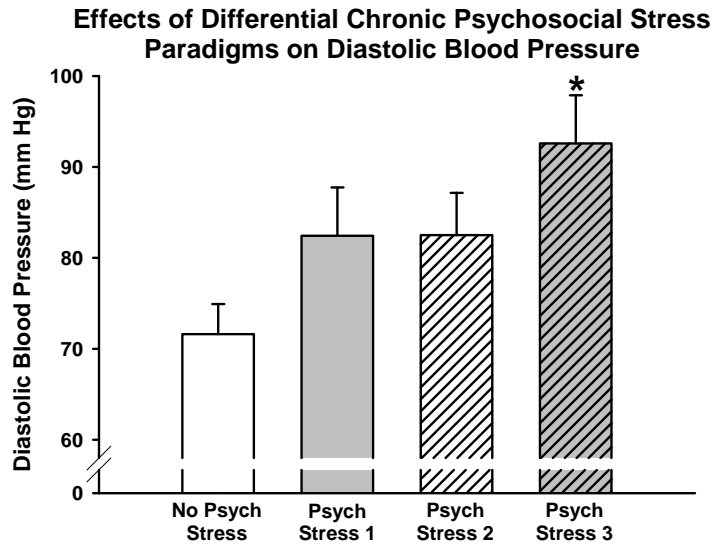


Figure 48. Effects of differential chronic psychosocial stress paradigms on systolic blood pressure. The data are presented as mean systolic blood pressure (mm Hg)  $\pm$  SEM. \* =  $p < 0.05$  relative to the no stress group.

*Systolic Blood Pressure* (see Figure 48). The analysis of systolic blood pressure revealed a significant main effect of psychosocial stress,  $F(3,22) = 3.46, p < 0.05$ . Post hoc tests indicated that psychosocial stress group 3 exhibited significantly greater systolic blood pressure than the no psychosocial stress group.

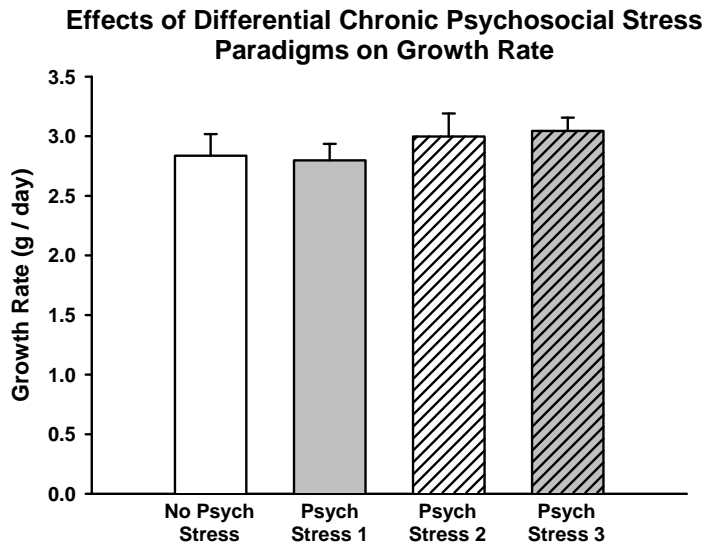


*Figure 49.* Effects of differential chronic psychosocial stress paradigms on diastolic blood pressure. The data are presented as mean diastolic blood pressure (mm Hg)  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group.

*Diastolic Blood Pressure* (see Figure 49). The analysis of diastolic blood pressure revealed a significant main effect of psychosocial stress,  $F(3,22) = 3.13, p < 0.05$ . Post hoc tests indicated that psychosocial stress group 3 exhibited significantly greater diastolic blood pressure than the no psychosocial stress group.

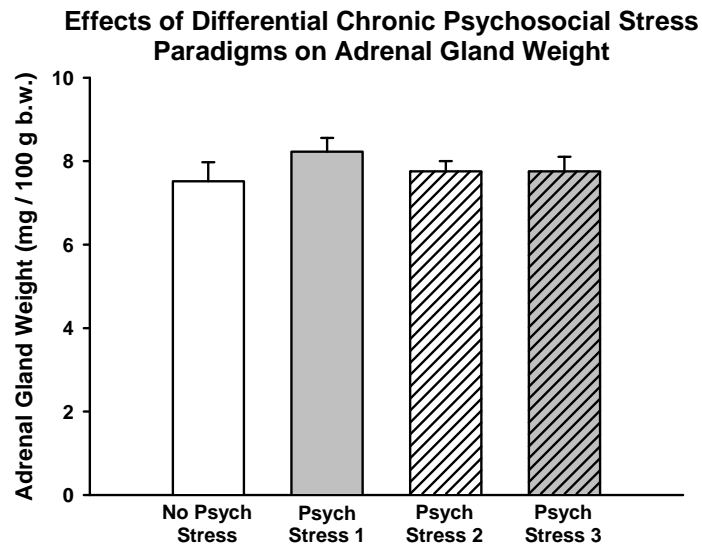
*Growth Rates* (see Figure 50)

The analysis of growth rate revealed no significant main effect of psychosocial stress,  $F(3,34) = 0.54, p > 0.05$ .



*Figure 50.* Effects of differential chronic psychosocial stress paradigms on growth rate.

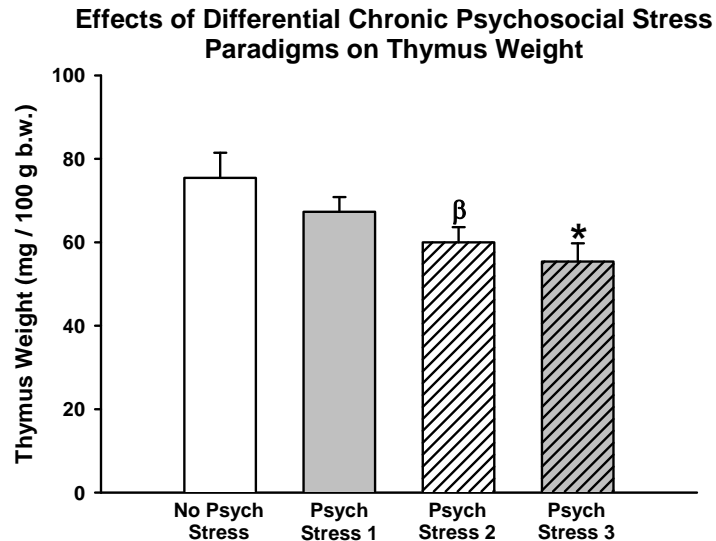
The data are presented as mean growth rate (g/day)  $\pm$  SEM.



*Figure 51.* Effects of differential chronic psychosocial stress paradigms on adrenal gland weight. The data are presented as mean adrenal gland weight (mg/100 g b.w.)  $\pm$  SEM.

*Adrenal Gland Weights (see Figure 51)*

The analysis of adrenal glands weight revealed no significant main effect of psychosocial stress,  $F(3,34) = 0.73, p > 0.05$ .



*Figure 52.* Effects of differential chronic psychosocial stress paradigms on thymus weight. The data are presented as mean thymus weight (mg/100 g b.w.)  $\pm$  SEM. \* =  $p < 0.05$  relative to the no psychosocial stress group;  $\beta = p = 0.057$  relative to the no psychosocial stress group.

*Thymus Weights (see Figure 52)*

The analysis of thymus weight revealed a significant main effect of psychosocial stress,  $F(3,35) = 3.76, p < 0.05$ . Post hoc tests indicated that psychosocial stress group 3 exhibited significantly smaller thymuses than the no psychosocial stress group. Moreover, psychosocial stress group 2 tended to display smaller thymuses than the no psychosocial stress group, although this difference did not reach statistical significance.

### *Discussion of Findings*

The most important finding of the present experiment is that at least some of the PTSD-like physiological and behavioral effects induced by the chronic psychosocial stress paradigm employed in Experiments One through Three could be maintained for at least 4 months following the initial stress session. Psychosocial stress group 1, which was given two acute cat exposures and daily social instability throughout the entire experiment, displayed significantly greater fear responses to the context and cue that were paired with the acute cat exposures, heightened anxiety on the EPM and impaired object recognition memory, relative to the no psychosocial stress (i.e., control) group. However, psychosocial stress group 1 did not exhibit an exaggerated startle response to any auditory stimulus intensity or reduced growth rate, larger adrenal glands or smaller thymuses than the controls. These findings suggest that some of the effects of our chronic stress regimen, and in particular the physiological effects, diminish over time, even with the continued presence of daily social instability.

Psychosocial stress group 2 exhibited physiological and behavioral effects (i.e., significant fear responses to the context and cue tests, heightened anxiety on the EPM, impaired object recognition memory) that were very similar to those observed in psychosocial stress group 1. Like psychosocial stress group 1, psychosocial stress group 2 did not exhibit an exaggerated startle response to any auditory stimulus intensity or a reduced growth rate, larger adrenal glands or smaller thymuses than the controls. One major difference between these two groups, however, was that psychosocial stress group 2 displayed a significantly greater fear response, at least with regards to immobility, to

the context that was paired with the acute cat exposures than all other groups. These findings indicate that the additional cat exposure resulted only in a stronger contextual fear memory for the acute cat exposures and did not reinforce the physiological and behavioral changes that were lacking in psychosocial stress group 1.

Similar to psychosocial stress groups 1 and 2, psychosocial stress group 3 exhibited heightened anxiety on the EPM and impaired object recognition memory. Interestingly, however, psychosocial stress group 3 displayed some physiological and behavioral effects that were markedly different from those of the other psychosocial stress groups. First, psychosocial stress group 3 did not exhibit a significant fear response to the context or tone that was paired with the acute cat exposures. Moreover, psychosocial stress group 3 was the only psychosocial stress group to display significantly greater blood pressure and smaller thymuses than the control group. Since the only difference between psychosocial stress groups 2 and 3 was the type of social instability to which each was exposed, these findings suggest that the irregular social instability resulted in stronger effects on contextual and cue fear memory, as well as the physiological responses of rats to an acute stressor, than daily social instability.

#### *Conclusions and Limitations*

The results of this final experiment provide insight into the temporal and social factors that mediate the length of time that trauma-induced changes in rat physiology and behavior last. This is the first study to report physiological and behavioral changes in rats subjected to a chronic psychosocial stress paradigm more than 4 months after the stress regimen began. The findings of this experiment indicate that some of the PTSD-like



behavioral changes (i.e., intact fear memory, heightened anxiety, cognitive impairments) produced by our laboratory's stress paradigm can be maintained up to 115 days post-stress. One caveat to these findings is that in every psychosocial stress group, the social instability manipulation continued until the beginning of behavioral testing. Thus, the observed effects may have been caused, at least in part, by the presence of social instability until behavioral testing.

Perhaps the most interesting finding of the present experiment was that irregular social instability led to an impairment of the memories for the context and cue that were previously paired with the acute cat exposures and exacerbated the stress-induced physiological changes in rats. Psychosocial stress group 3 was the only stress group to display significantly greater cardiovascular reactivity to an acute stressor and a significantly smaller thymus than the no psychosocial stress group. Most studies, and even those from our own laboratory, that have employed unstable housing conditions in a stress paradigm have used *daily* social instability to demonstrate its adverse effects on rat physiology and behavior (Baran et al., 2005; Baranyi et al., 2005; Gerges et al., 2004; Haller et al., 2004; Lemaire et al., 1997; Park et al., 2001). The strategy behind using the irregular social stress in the present experiment was to make the unstable housing more unpredictable. The typical daily randomized housing procedure, although effective for the 31-day paradigm, is somewhat predictable in that it occurs at approximately the same time every day, and if continued for an extended period of time, could eventually become ineffective. Therefore, I reasoned that the irregular social stress could exacerbate the physiological and behavioral effects of the daily social instability and increase the

likelihood that rats would exhibit PTSD-like abnormalities for a longer period of time.

The present findings demonstrate that the element of predictability in day-to-day stressors may play a major role in the development of chronic PTSD.

## Chapter Six: Concluding Remarks

PTSD is a debilitating mental illness that is characterized by the repeated reliving of a life-threatening traumatic event through intrusive, flashback memories. People with PTSD display an array of physiological and behavioral symptoms, including persistent anxiety, exaggerated startle, heightened autonomic activity, impaired HPA axis functioning, cognitive impairments and impaired extinction of conditioned fear. Despite scientific advances over the past couple of decades, the neurobiological mechanisms underlying the development and maintenance of PTSD remain unclear. Moreover, there are currently no pharmacological agents that effectively treat both the dynamic memory (e.g., intrusive memories, re-experiencing symptoms) and stable trait (e.g., anxiety, hyperarousal) components of the disorder. Thus, the need for a valid animal model of PTSD to use for preclinical research has become an issue of growing importance.

Many of the symptoms of PTSD (e.g., heightened anxiety, exaggerated startle response, cognitive impairments, etc.) can be experienced by people suffering from other mental illnesses such as major depressive disorder, panic disorder or generalized anxiety disorder. Thus, an animal model of PTSD should produce physiological and behavioral changes that are unique to the disorder as it is observed in humans. Some of the symptoms of PTSD that set it apart from other disorders include the presence of a powerful and intrusive memory of the traumatic event, abnormally low levels of glucocorticoids and enhanced suppression of glucocorticoid levels following the

administration of dexamethasone. While other mental illnesses have also been characterized by abnormal HPA axis functioning, PTSD is the only disorder to be characterized by abnormal *reductions* in basal cortisol levels and an enhanced suppression of cortisol in the dexamethasone suppression test (Marshall et al., 2002; Yehuda, 2002; Yehuda, 2005; Yehuda et al., 1993a). For instance, major depressive disorder is characterized by chronically elevated glucocorticoid levels and reduced glucocorticoid suppression following the administration of dexamethasone (Pariante & Lightman, 2008). In contrast to people with PTSD, patients suffering from MDD also display an abnormally low number of glucocorticoid receptors. Previous work from our laboratory has already demonstrated that the present psychosocial stress regimen produces heightened anxiety, an exaggerated startle response, cognitive impairments, heightened cardiovascular reactivity and hyperresponsivity to yohimbine (Zoladz et al., 2008). The present work has extended these findings by demonstrating that it also results in a powerful memory for the two isolated stress experiences and produces HPA abnormalities that are commonly observed in people with PTSD. In conjunction with our previous work, these findings further support that this psychosocial stress regimen produces physiological and behavioral changes that specifically model those found in PTSD.

Experiment Three also indicated that this model demonstrates predictive validity. That is to say, compounds that were predicted to ameliorate stress-induced changes in rat physiology and behavior and have led to improvements in some symptom clusters of PTSD were shown to ameliorate some of the physiological and behavioral sequelae

induced by our chronic psychosocial stress paradigm. Thus, this paradigm could be used in future research to guide the development of new pharmacotherapeutic approaches to the treatment of PTSD. The differential effectiveness of the pharmacological agents examined in Experiment Three, particularly the profile of tianeptine as the most effective agent, suggests that abnormal glutamatergic functioning may be involved in this regimen's stress-induced changes in rat physiology and behavior.

Finally, the last study provided evidence that some of the effects induced by our chronic psychosocial stress paradigm could be maintained for at least 4 months following the first stress exposure. As PTSD is often a chronic disorder that affects people for most of their lives, such a finding indicates that this paradigm could serve to examine the neurobiological correlates of chronic PTSD as well. The reason why the exaggerated startle response, along with some of the other effects that we normally detect (e.g., reduced growth rate, increase adrenal gland weight, etc.), was not observed in our typical psychosocial stress paradigm at 4 months post-stress should be examined further in future work.

Collectively, these studies have provided insight into the mechanisms underlying trauma-induced changes in brain and behavior and should advance our understanding of the biological basis of PTSD. Yet, much remains to be known regarding the neurobiological underpinnings of the present effects. Future studies should examine the effects of the present psychosocial stress manipulations on neuroplasticity within brain regions that play a major role in PTSD, such as the PFC, amygdala and hippocampus and

whether or not these changes correlate with the observed alterations in rat physiology and behavior.

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