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Vicarious Defeat: A Novel Emotional Stressor in Male Mice

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VICARIOUS DEFEAT: A NOVEL EMOTIONAL STRESSOR IN MALE MICE

By

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ABSTRACT

It is well known that exposure to severe stress increases the risk for developing mood disorders. However, less is known about the complex interactions between witnessing and experiencing traumatic events. While much has been learned from animal models of traumatic stress, current models emphasize physical stressors, while models of emotional stress focus on maternal separation and social isolation paradigms, among others. However, it is common for post-traumatic stress disorder to develop in individuals who simply witness intense violence. Therefore, it is critical to develop animal models that will allow for independent assessment of the neurobiological consequences of emotional stress. This study introduces a novel social stressor that is insulated from the effects of physical stress.

In this study, male C57BL/6J mice witnessed the social defeat of another mouse. Briefly, the home cage of a male CD-1 retired breeder mouse was divided by a Plexiglas divider into two identical adjacent compartments. An adult male C57BL/6J mouse was introduced into the compartment territorialized by the CD-1 mouse where it was repeatedly defeated and demonstrated escape-like behaviors, vocalizations, and submissive posturing, while a second male C57BL/6J mouse witnessed this interaction from the adjacent compartment (i.e., emotional stress: ES). The results demonstrate that 10 days of exposure to ES induces long-lasting deficits in a battery of behavioral assays designed to assess changes in mood. Specifically, ES exposure increases sensitivity to anxiety- and stress-eliciting situations as measured by the social interaction, elevated plus-maze, sucrose preference, and the forced swim tests both 24 h and 1 month after witnessing physical stress. Increases in levels of serum corticosterone, a steroid hormone signaling stress response, accompanied these behavioral deficits. Taken together, these data indicate that witnessing traumatic stress is a potent stressor in adult male mice capable of inducing long-lasting neurobiological perturbations.

CHAPTER ONE

INTRODUCTION

Anxiety disorders are a major problem in the United States. Recent estimates suggest that up to 29% of people in the United State will develop an anxiety disorder sometime in their lifetime (Kessler et al., 2005). Specifically, it is estimated that 7% of the population will develop post-traumatic stress disorder (PTSD), a severe anxiety disorder characterized by a persisting fear of trauma-related stimuli which may emerge following exposure to an intense stressor (Newport and Nemeroff, 2000, Kessler et al., 2009). Surprisingly, the stressor does not have to be directly experienced for an individual to develop PTSD. Instead, PTSD can occur vicariously in individuals who simply witness a traumatic event (Schlenger et al., 2002, Blanchard et al., 2004, Cougle et al., 2009).

Interestingly, individuals suffering from PTSD appear to have changes in functional activity of important limbic brain regions, including the medial prefrontal cortex, hippocampus and amygdala (Protopopescu et al., 2005, Bremner, 2006, Etkin and Wager, 2007, Bonne et al., 2008). These brain areas are essential for physiological stress response, as they are known to interact with the hypothalamus-pituitary-adrenal (HPA) axis (Dedovic et al., 2009). The HPA axis is the primary circuit controlling physiological stress response. When exposed to stress, hypothalamic neurons in the paraventricular nucleus secrete corticotropin releasing factor, which in turn stimulates the anterior pituitary to release adrenocorticotropin (ACTH). Released ACTH stimulates the adrenal cortex to release glucocorticoids (cortisol in humans, and corticosterone in rodents). The medial prefrontal cortex and hippocampus are known to have inhibitory influence, while the amygdala exerts a direct excitatory influence on hypothalamic neurons (Herman and Cullinan, 1997). One important hypothesis explaining anxiety disorders, including PTSD, suggests that there is a decreased inhibitory influence from the hippocampus and medial prefrontal cortex with a corresponding increase in excitatory input from the amygdala (Teicher et al., 2003, Nemeroff et al., 2006). Indeed, when compared to healthy controls, cortisol levels in individuals with PTSD, depression, or anxiety disorders are significantly elevated after stress, demonstrating the disrupted hormonal response that is a hallmark of these disorders (de Kloet et al., 2005, de Kloet et al., 2006). Unfortunately, little is known about the neurobiological

underpinnings of PTSD and effective therapeutic intervention is limited (Nutt, 2005, Ravindran and Stein, 2010). It is imperative, therefore, that appropriate animal models be developed for this condition.

While animal models of stress have been successful in delineating much of the biological basis of stress-response, most current models of traumatic stress focus on physical stressors (i.e., social defeat, chronic mild stress, unpredictable shock, etc.) and neglect the psychological stressors that contribute to risk for PTSD. This is surprising because recent studies in humans have demonstrated that early life emotional abuse/neglect may have significantly more adverse effects than physical abuse alone (Teicher et al., 2006). Unfortunately, animal models of emotional stress are scarce and those that are available are limited. For example, one common emotional stress paradigm is maternal separation. In this model, pre-weanling animals are separated from their mothers for several hours each day. In rats, maternal separation has severe and long-lasting effects on mood-related behavior in adulthood (Levine, 1957, Gutman and Nemeroff, 2002, Lippmann et al., 2007). Importantly, rats exposed to maternal separation as pups also have changes in neuroendocrine activity (i.e., disrupted HPA axis activity) later in life (Plotsky et al., 2005, Lippmann et al., 2007). While this model has led to a significant understanding of the sensitivity of the developing HPA axis to stress, it is limited to very young animals, and cannot speak for the effects of adult exposure to emotional stress. Nevertheless, studies involving maternal separation have yielded substantial support for a view that emotional stress induces long-lasting changes in behavior and neurobiology.

The social defeat paradigm is a model of physical stress that is gaining popularity. In this model, adult male mice are repeatedly forced to intrude into the home cage of a larger, more aggressive male mouse (Kulling et al., 1987, Iñiguez et al., 2010a). The intruder is quickly overpowered and rapidly adopts a submissive posture characterized by rearing, vocalization, and escape-like behaviors. Socially defeated mice have long-lasting deficits in behavioral measures of mood (i.e., increased anxiety-and depression-like behaviors in the elevated plus-maze and forced swim test, respectively) as well as measures of neuroendocrine response to stress, including heightened corticosterone response to forced swim stress (Kudryavtseva et al., 1991, Tsankova et al., 2006, Kinsey et al., 2007, Krishnan et al., 2007, Krishnan et al., 2008).

Interestingly, this paradigm is one of the most useful models of PTSD because socially defeated mice reliably demonstrate social avoidance (i.e., indiscriminately avoid other mice) for weeks after the last social defeat session (Berton et al., 2006). This is important because this effect is robust, reliable, possesses ethological relevance, has strong face validity (i.e., appears to measure what it is supposed to measure), and is easily testable. However, a weakness of this model is its inability to tease apart the different effects of emotional and physical stress, since social defeat is a physical stressor.

Recently, a novel model capable of comparing physical stress to emotional stress has emerged. This model involves using a two chamber apparatus where both chambers are separated by a perforated Plexiglas divider (Pijlman and van Ree, 2002). In one chamber, a rat receives a series of unpredictable electrical shocks and in the second chamber, another rat passively 'witnesses' the first rat being stressed. Using this model, it has been shown that emotional and physical stressors have very disparate effects on behavior. Male rats exposed to the witness condition (i.e., the emotional stressor) are significantly more active in the open field and have increased sensitivity to reward; while physically stressed rats show opposing effects when compared to the control rats [i.e., those rats that did not experience or witness electrical shock] (Ramsey and Van Ree, 1993, Van den Berg et al., 1998, Pijlman et al., 2003). While this model has demonstrated that witnessing despair is a significant stressor, it lacks ethological relevance.

There remains a need for preclinical models that can approximate human risk factors for pathologic condition, including anxiety disorders like PTSD (Nemeroff and Vale, 2005, Nestler and Hyman, 2010). Additionally, there is a need to bridge the gap in the understanding of the effects of emotional stress on behavioral output (van Winkel et al., 2008, Shin and Liberzon, 2010). To address these concerns, in this series of studies a novel emotional stressor which combines the electric-shock-witness and social defeat paradigms, by substituting social defeat for electric shock, was developed. The following set experiments was designed to establish this ethologically relevant model, which is capable of enhancing stressor specificity, and to examine the long-lasting effects of emotional stress on physiological and behavioral measures of mood in adult male mice.

CHAPTER TWO

METHODS AND MATERIALS

Subjects

All mice were housed in clear polypropylene cages containing wood-chip bedding in an animal colony kept between 23-25ºC on a 12 h light/dark (on from 7:00 until 19:00 h) and received food and water ad libitum. Male CD-1 retired breeders (Charles River, NC) were single housed upon arrival. Male C57BL/6J mice were derived from an in-house breeding colony. Litters were culled to 10 pups at postnatal day (PD) 3, weaned at PD21 and separately housed by gender. Only male mice were used in these studies. At PD 60 (adulthood), male C57BL/6J mice were randomly assigned to control stress (CON), emotional stress (ES), or physical stress (PS) conditions. To avoid 'oversampling' or 'within-litter effects,' no more than 1 pup per litter was assigned to a particular condition (Hughes, 1979). ES consisted of a C57BL/6J mouse 'witnessing' the social defeat of another C57BL/6J mouse. Briefly, the home cage (23.5cm x 45.5cm x 15cm) of a CD-1 retired breeder was separated into two compartments by a perforated clear Plexiglas divider, allowing olfactory, acoustic, and visual signals to be shared between the compartments (see figure 1). The ES mouse was placed into the empty compartment adjacent to the CD-1 aggressor, while the PS mouse was placed into the compartment containing the CD-1 aggressor. Each day, the ES- and PS-exposed mice were rotated and stressed such that both mice were novel both to each other and to the CD-1 aggressor. The CON-exposed mice were housed two per cage and were separated by a clear plastic divider. CON-exposed mice were allowed to interact with their cage-mate for 10 min per day. After 10 days, all mice were kept singly housed for the duration of the experiment. Beginning 24 h after the last stress session, their behavioral responses to various emotion-eliciting stimuli were assessed, as described below.

Behavioral and Biochemical Assays

All mice exposed to behavioral testing experienced the social interaction test first (i.e., 24 h after the last exposure to stress). The sequence for the subsequent testing was counterbalanced

across groups such that no mouse was exposed to more than three behavioral tasks, or to the same task twice, with one exception; mice exposed to the social interaction test 1 month after CON, ES, or PS *did* also experience the social interaction test 24 h after the last exposure to stress. Additionally, there was a wait period of at least 24 h between tests. All behaviors were recorded using Noldus Ethovision XT 7.0 and behavioral observations were made by observers with no knowledge of the treatment conditions of the mice.

Corticosterone Enzyme Immuno Assay

Three separate groups of mice were used in these experiments. One group of mice was sacrificed 40 min following a single session of CON, ES, or PS. A second group was sacrificed 24 h after 10 sessions of CON, ES, or PS. The third group was exposed to CON, ES, or PS, and then sacrificed 40 min after the forced swim test. Trunk blood from each animal was individually collected in EDTA lined tubes and kept on ice until use. Whole blood samples were centrifuged at 1500 X g for 30 min at 4°C. Plasma supernatant was decanted for analysis with the corticosterone enzyme immuno assay per manufacturer's instructions (Assay Designs). Briefly, serum was diluted to 10% using the provided buffer and added to the wells of an immuno-lined 96-well plate and allowed to incubate for 2 h with provided antibodies. The plate was washed with a provided wash buffer, developed, and optical density was read using a 96-well plate reader (Biotek). Serum corticosterone was calculated by comparing these values to optical density values obtained from corticosterone standards.

Social Interaction

The social interaction behavioral assay is a test of social avoidance. Briefly, this is a two-session test. In the first session, a mouse is allowed to explore an open field arena (40 cm x 40 cm) for 2.5 min (see figure 2). Along one side of the arena is a wire mesh cage that remains empty during the first trial (no target). The mouse is then removed and a novel CD-1 male mouse is placed into the wire mesh cage. The test mouse is placed back into the arena and the amount of time it spends in the "interaction zone" (an 8 cm wide corridor surrounding the cage) is measured during the 2.5 min trial (target present). Socially defeated mice explore the interaction zone significantly less when another mouse is present (Krishnan et al., 2008). Interestingly, chronic antidepressant treatment alleviates this avoidant behavioral phenotype, but acute treatment does not (Berton et al., 2006, Wilkinson et al., 2009). This makes the social defeat/interaction test a

valid model of antidepressant efficacy and thus a good model to test for depression-like affect, as well as sensitivity to stress-related stimuli (Berton et al., 2006, Nestler and Hyman, 2010).

Elevated Plus-Maze (EPM)

The EPM is a classic test of anxiety-like pharmacologic activity in rodents (Montgomery, 1955). The apparatus consists of two perpendicular intersecting runways, shaped like a plus sign. One runway has no walls (open arms) while the other arm has tall walls (closed arms). The runways are 6 cm wide, 33 cm long and the closed walls are 25 cm tall. The runways are 50 cm from the floor. Mice are placed into the closed arm and allowed to explore for 5 min. Mice tend to prefer the safety of the closed arms, but will eventually begin to explore the open arms. Anxiolytic drugs, such as diazepam, increase time spent in the open arms and decrease time spent in the closed arms (Hogg, 1996). Thus, decreased total time spent in the open arms is interpreted as increased anxiety-like behavior.

Sucrose Preference

The sucrose preference assay has been used extensively to assess motivational state in rodents, including stress-induced anhedonia (Barrot et al., 2002, Bolaños et al., 2003, Bolaños et al., 2008, Iñiguez et al., 2009, Iñiguez et al., 2010b). This test consists of a two-bottle choice paradigm in which mice are given the choice between consuming water and a 1.0 % solution of sucrose. The preference for sucrose over water is used as a measure for rodents' sensitivity to a natural reward (Sucrose Consumption ÷ Total Consumption x 100). Thus, anhedonia is revealed by a reduction in sucrose preference (Papp et al., 1991, Willner et al., 1992).

Forced Swim Test (FST)

The FST is a classic model of depression-like behavior with a high degree of predictive validity that is widely used as a screening tool for antidepressant efficacy as well as a measure of behavioral despair (Lucki, 1997, Porsolt et al., 2001, LaPlant et al., 2010). It was performed according to previously described methods with some modifications (Porsolt et al., 1977, Krishnan et al., 2007). Mice were placed individually into 5L beakers (27 cm x 18 cm) containing 4L of water (23±1 °C) for 6 min. During this time the mouse adopts an immobile posture, characterized by motionless floating and the cessation of struggling. The latency to adopt this posture and total time spent immobile were recorded. In this assay, rodents receiving

antidepressant drugs struggle longer and take more time to adopt the immobile posture, thus pro-depressant behavior is revealed by decreased latency to immobility and increased total immobility (Porsolt et al., 2001, LaPlant et al., 2010).

CHAPTER THREE

RESULTS

Effects of Stress Exposure on Physiological Stress Responses

Corticosterone levels 40 min after a single stress exposure. To determine the acute effects of exposure to CON, ES, or PS on serum corticosterone levels, a group of mice was exposed to a single session of stress and serum corticosterone levels were assessed 40 min later (figure 3A, n = 9-10 per group). Serum corticosterone concentrations varied as a function of stress exposure ($F_{(2, 26)}$= 8.8, p< 0.01). More specifically, ES and PS significantly increased serum corticosterone levels when compared to the CON-exposed mice (p< 0.05, respectively).

Corticosterone levels 24 h after chronic (10 days) stress exposures. To determine the effects of chronic exposure to CON, ES, or PS on serum corticosterone levels, a separate group of mice were exposed to 10 daily stress sessions and serum corticosterone levels were assessed 24 h after the last exposure (figure 3B, n = 10 per group). Serum corticosterone concentrations varied as a function of stress exposure ($F_{(2, 27)}$= 49.7, p< 0.001). More specifically, ES and PS significantly increased serum corticosterone levels when compared to the CON-exposed mice (p< 0.001, respectively).

Weight gain during chronic (10 days) stress sessions. Figure 4 (n = 11-12 per group) shows the effects of chronic CON, ES, or PS exposure on weight gain in adult mice. Repeated-measures ANOVA (for stress day) revealed that stress exposure significantly influenced body weight across days (within-subject main effect: $F_{(18, 558)}$= 14.4, p< 0.0001) and stress exposure (between-subject main effect: $F_{(2, 558)}$= 20.8, p< 0.001). Specifically, exposure to ES and PS reduced weight gain across days, but this effect lost significance within 5 days of the last stress session.

Effects of Stress Exposure on Short-Term Behavioral Measures

Social Interaction. Figure 5 (n = 38 per group) shows the effect of 10 days of CON, ES, or PS exposure on social interaction 24 h after the last stress session. Repeated-measures ANOVA (for presence of CD-1 mouse) revealed that social interaction time varied across testing condition (within-subject main effect: $F_{(1, 111)}$= 12.5, p< 0.001) and by stress exposure (between-subject main effect: $F_{(2, 111)}$= 31.6, p< 0.001). More specifically, both ES and PS exposure reduced the time spent interacting with the CD-1 when compared to the CON-exposed mice (p< 0.05).

Elevated Plus-Maze. To determine the effect of CON, ES, or PS exposure on anxiety-like behavior, one group of mice was exposed to the EPM 48 h after the last stress session. Figure 6A (n = 10 per group) shows that time spent in the open arms of the EPM varied by stress exposure ($F_{(2, 27)}$= 6.3; p< 0.01). Specifically, ES and PS exposure reduced time spent in the open arms of the EPM when compared to CON-exposed mice (p< 0.05). Figure 6B shows that total locomotion did not vary by stress exposure (p> 0.05), indicating that time spent in the open arms was not influenced by altered basal locomotor activity.

Effects of Stress Exposure on Long-Term Behavioral Measures

Social Interaction. One month after the last exposure to the various stress conditions, one group of mice was again exposed to the social interaction test. Figure 7 (n = 12 per group) shows the effect of CON, ES, or PS exposure on social interaction 1 month after the last stress session. Repeated-measures ANOVA (for presence of CD-1 mouse) revealed that interaction time varied across testing condition (within-subject main effect: $F_{(1, 33)}$= 35, p< 0.001) and by stress exposure (between-subject main effect: $F_{(2, 33)}$= 7.9, p< 0.01). Specifically, exposure to ES and PS significantly reduced the time that the mice spent interacting with the CD-1 when compared to the CON-exposed mice (p< 0.05, respectively)**.**

Elevated Plus-Maze. A separate group of mice was exposed to CON, ES, or PS and anxiety-like behavior was assessed 1 month after the last stress session using the EPM. Figure 8A (n = 8 per group) shows that time spent in the open arms of the EPM varied as a function of stress exposure ($F_{(2, 21)}$= 6.1; p< 0.01). Specifically, ES and PS exposure significantly reduced time spent in the open arms of the EPM when compared to the CON-exposed mice (p< 0.05, respectively). Figure 8B shows that distance traveled did not vary by stress exposure (p> 0.05).

Sucrose Preference. Figure 9A (n = 8 per group) shows the effect of CON, ES, or PS exposure on sucrose preference, a measure of natural reward, 1 month after the last stress session. Sucrose preference varied by stress exposure ($F_{(2, 21)=}$ 4.1, $p< 0.05$). Specifically, exposure to ES and PS significantly reduced sucrose preference when compared to the CON-exposed mice ($p< 0.05$, respectively). Figure 9B shows that there were no differences in total liquid consumption (Sucrose + Water) between groups ($p >0.05$).

Forced Swim Test. To assess for long-lasting effects of CON, ES, or PS exposure on behavioral despair, a separate group of mice was exposed to the FST 1 month after the last stress session. An overall one-way ANOVA did not detect an effect of stress exposure on latency to immobility (figure 10A), however a pre-planned comparison revealed that PS exposure decreased latency to immobility (t_{14} = 1.9, $p< 0.05$) when compared to the CON-exposed mice. Nevertheless, figure 10B (n = 8 per group) shows that total immobility did vary as a function of stress exposure ($F_{(2, 21)}= 5.6$, $p< 0.05$). Specifically, exposure to both ES and PS increased total time spent immobile when compared to the CON-exposed mice ($p< 0.05$, respectively). To determine the long-lasting effects of CON, ES, or PS exposure on subsequent endocrine stress response, these mice were sacrificed 40 min after the FST exposure and serum corticosterone was assessed (figure 10C, n = 8 per group). Corticosterone concentrations varied as a function of stress exposure ($F_{(2, 21)}= 6.0$, $p< 0.01$). More specifically, exposure to ES and PS significantly increased serum corticosterone levels when compared to the CON-exposed mice ($p< 0.05$, respectively).

CHAPTER FOUR

DISCUSSION

The goal of this study was to establish an animal model of emotional stress in mice by assessing the neurobiological consequences of exposure to repeated emotional (ES) or physical (PS) stress. It is reported here that both acute and repeated exposure to ES or PS altered serum levels of corticosterone, weight gain, and responsiveness to both rewarding and aversive stimuli in adult male mice. Exposure to these stress conditions also dysregulated serum corticosterone levels in response to subsequent stress (i.e., forced swimming stress) 1 month following cessation of stress exposure. These findings demonstrate that ES is a potent stressor in adult male mice capable of inducing long-lasting alterations in various measures of functional output, and establishes a novel model of stress that is capable of isolating the effects of exposure to ES from those of PS.

Exposure to ES or PS increased serum corticosterone and reduced weight gain in adult mice. Serum levels of the stress hormone corticosterone were measured after either single (1 day) or chronic (10 days) ES or PS exposure to assess whether these manipulations would influence physiological stress response in mice. As described previously, corticosterone is released by the adrenal cortex as an acute reaction to stress. Thus our results strongly suggest that experiencing ES elicits a strong stress response after single or repeated sessions, effects similar to those observed in PS-exposed mice. Chronic ES altered basal corticosterone levels up to 24 h after the last stress exposure, suggesting that corticosterone remained elevated throughout the stress exposure period. It is unknown whether serum corticosterone levels returned to baseline in the days or weeks following cessation of ES treatment, however, previous reports suggest that corticosterone levels may fall below control levels in PS-exposed mice (Krishnan et al., 2007). As a second measure of stress response, the weight of these mice was recorded each day. Decreased body mass has been frequently reported during and following chronic stress protocols and is an excellent indicator that an animal is under stress (Willner et al., 1996, Zelena et al., 1999, Konkle et al., 2003, Krishnan et al., 2007). The decrease in weight gain observed in mice exposed to ES was similar to the decrease seen in mice exposed to PS, further confirming

that this newly developed ES paradigm is a valid stressor. This is surprising, since mice that simply witness, but do not experience PS, have nearly identical changes in stress measures as those that do experience PS. These findings are not influenced by differences in pre-exposure weight, because no significant difference in total body weight was seen between groups on exposure day 0 (see figure 4). The findings in the PS-exposed mice reported here are in agreement with previous reports demonstrating that PS exposure increases corticosterone levels up to 24 h after exposure to either one or repeated stress sessions (Keeney et al., 2006, Krishnan et al., 2007). In addition, these findings further support previous reports showing that repeated PS exposure reduces weight gain in adult mice (Kudryavtseva et al., 1991, Reber et al., 2006), and these findings are now extended to include mice that witness traumatic events.

Importantly, exposure to ES enhances sensitivity to aversive situations 24 h following cessation of the stress regiment. ES exposure decreased social interaction (i.e., target present), a measure of social avoidance, 24 h after the last stress session. As expected, a similar decrease was seen in the PS-exposed mice (Berton et al., 2006, Tsankova et al., 2006, Krishnan et al., 2007). Under normal laboratory conditions, an unexposed mouse is expected to interact more with a novel mouse, since social interaction is rewarding (Insel, 2003). After repeated social defeat, however, mice avoid these interactions (Kudryavtseva et al., 1991, Berton et al., 2006). Reduced interaction with a CD-1 mouse is particularly interesting, since avoidance of trauma-related cues is a hallmark of PTSD (Foa, 2006, Nemeroff et al., 2006). This makes repeated PS useful as a model of PTSD because chronic antidepressant treatment, one of the few effective treatments for PTSD, reverses the avoidant phenotype seen after PS exposure (Berton et al., 2006, Wilkinson et al., 2009). The finding that ES is able to vicariously induce a similar avoidance is particularly striking, since these animals never had physical contact with a CD-1 aggressor, but merely witnessed PS. However, it is currently unknown whether antidepressant treatment will reverse the social avoidance seen after ES. It must also be noted, that social interaction was only measured with aggressive CD-1 mice, but not social interaction with non-aggressive C57BL/6J mice. It is possible that this avoidant phenotype may not be generalized. Exposure to both ES and PS also induced anxiogenic phenotypes 24 h after cessation of stress exposure. More specifically, ES and PS exposure decreased time spent in the open arms of the EPM, a well-known behavioral measure of sensitivity to anxiety-eliciting situations. These

effects are not due to deficits in locomotor activity because no differences in total locomotion were detected during the 5 min trial (see figure 6B). These data are in agreement with previous reports demonstrating that exposure to PS induces social avoidance and increases anxiety within 24 h of the last stress session (Kinsey et al., 2007, Krishnan et al., 2007), and these findings are now extended to mice witnessing stressful events.

Exposure to ES also induced a long-lasting sensitivity to aversive situations, similar to that seen following PS exposure. Mice exposed to ES show significantly decreased social interaction with a CD-1 aggressor mouse 1 month following cessation of stress exposure, suggesting that avoidance of trauma-related cues is persistent. This is important because it suggests that mice that witness violence can vicariously develop a life-long sensitivity to trauma-related stimuli that is similar to that seen in mice which actually experience PS. Additionally, these data indicate that mice that experience either ES or PS develop a long-lasting sensitivity to anxiety-eliciting situations. Mice exposed to ES or PS show reduced time spent in the open arms of the EPM, suggesting that these mice have a long-lasting sensitivity to anxiety-eliciting situations. In addition, mice exposed to ES show an enhanced sensitivity to stressful situations later in life, similar to the PS-exposed mice. Both ES and PS exposure reduced escape-like behaviors in the forced swim test, a frequently used measure of depression-like behavior, in mice tested 1 month following cessation of stress exposure. It is unlikely that the differences seen in these groups are due to changes in locomotor activity, because no differences were seen in total locomotion during social interaction (target not present) or during the EPM (see figure 8B). Taken together, these observations are in agreement with previous reports demonstrating that exposure to PS induces a long-lasting sensitivity to anxiety- and stress-eliciting situations and these findings now expand to include exposure to ES (Berton et al., 2006, Kinsey et al., 2007, Krishnan et al., 2007, Krishnan et al., 2008).

Lastly, chronic exposure to ES results in long-lasting reductions in sensitivity to sucrose, a natural reward, similar to that observed after PS exposure. Specifically, mice exposed to ES and PS showed decreased sucrose preference, a measure of anhedonia, a month after the cessation of stress exposure. Although both ES and PS exposure decreased body weight, which could have influenced sucrose consumption, this is not likely the case because body weight

rapidly returned to normal after the cessation of stress exposure, and preference for sucrose was not tested until 1 month later. Further, if body weight had influenced fluid consumption, differences in total liquid consumption would be expected (see figure 9B). Therefore, decreased sucrose preference is likely due to the influence of stress exposure on brain reward circuitry (Bruijnzeel et al., 2004, Rygula et al., 2005, Krishnan et al., 2007). The neural circuitry controlling reward, currently accepted as being the dopaminergic output from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is critical for regulating responses to rewarding stimuli (Bolaños and Nestler, 2004, Nestler, 2004, Nestler and Carlezon, 2006, Krishnan and Nestler, 2008). Exposure to sweet solutions and other natural rewards activates this circuitry by increasing dopamine release from the VTA into the NAc (Hajnal and Norgren, 2001, Bolaños et al., 2003). Dysregulation of VTA-NAc circuitry, as occurs in depressive-like conditions, results in diminished sucrose preference (Papp et al., 1991, Berlin et al., 1998, Rygula et al., 2005). These results are consistent with reports that exposure to PS decreases interest in sucrose and disrupts the integrity of reward circuitry and now these findings extend to include ES exposure (Krishnan et al., 2007, Iñiguez et al., 2010a).

Together, the findings in PS-exposed mice are in agreement with previous reports: repeated exposure to PS alters sensitivity to both rewarding and aversive stimuli (Berton et al., 2006, Kinsey et al., 2007, Krishnan et al., 2007, Krishnan et al., 2008, Iñiguez et al., 2010a, LaPlant et al., 2010). Interestingly, the present study shows that ES exposure results in similar behavioral phenotypes: increased social avoidance, increased anxiety-like behavior, anhedonia, and increased depression-like behavior. This is contrary to previous reports indicating that exposure to ES has opposing effects to PS (Van den Berg et al., 1998, Pijlman and van Ree, 2002, Pijlman et al., 2003). Inconsistencies between studies exploring ES vs. PS could be due to differences in experimental models (Frick et al., 2000). Previous studies involved a rat model where one rat witnesses another rat being electrically shocked. It must be noted that these rats were housed together and that inescapable shock has been shown to decrease aggression, which would certainly lead to chronic subordination in PS-exposed rats (Corum and Thurmond, 1977, Cuadra and Molina, 1991). Chronic subordination in turn would influence the motivation-related behaviors of rats (Rygula et al., 2005), while repeated victory has been shown to enhance motivation for aggression and alter gene expression within the VTA (Caramaschi et al., 2008,

Fuxjager et al., 2010). Therefore, there is a possibility that the effects seen in the rat studies may be confounded by social hierarchy, a possibility that is ruled out in the present study.

The mechanisms underlying the behavioral effects presented in this thesis are currently unknown. However data in mice exposed to PS provide a number of potential molecular targets that may also be regulated by ES exposure. For example, brain-derived neurotrophic factor (BDNF), DeltaFosB, thymoma viral proto-oncogene (AKT), extracellular signal-related kinase (ERK), and cAMP response element binding (CREB) are proteins that have been implicated in the long-lasting effects of PS exposure (Berton et al., 2006, Tsankova et al., 2006, Krishnan et al., 2008, Wilkinson et al., 2009, Iñiguez et al., 2010a, Vialou et al., 2010). Given the effects of PS on mesolimbic reward circuitry (Krishnan et al., 2008, Miczek et al., 2008, Iñiguez et al., 2010a), it is essential that the biochemical integrity of the VTA-NAc circuitry be assessed following ES. Specifically, examining gene regulation in the VTA will provide an unparalleled view into the dysregulation that occurs in mesolimbic brain areas following chronic ES or PS exposure and will potentially reveal novel targets for future pharmacological intervention for the treatment of PTSD.

In summary, the present study demonstrates that repeated exposure to ES robustly influences stress responses in adult male mice. Further, it shows that repeated exposure to ES induces a negative emotional state characterized by blunted sensitivity to reward and a long-lasting increase in sensitivity to stress- and anxiety-eliciting situations. This study also demonstrates that these ES-induced effects are strikingly similar to deficits seen after PS exposure alone, suggesting that simply witnessing violence/trauma is a potent stressor in male mice. This supports a view that ES exposure is as critical as PS exposure for the long-lasting behavioral and neuroendocrine effects seen after social defeat. Within this context, these findings suggest that repeated ES exposure could be used as a potential animal model for affective disorders, including PTSD.

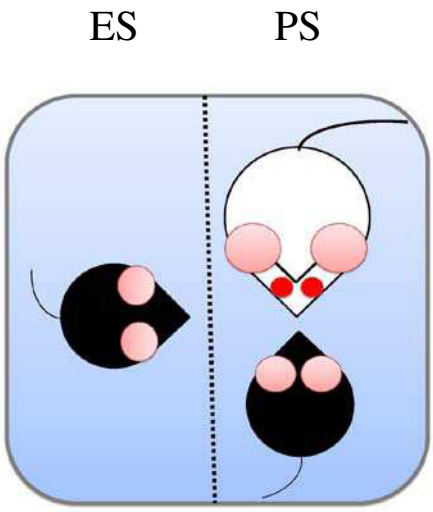

Figure 1. Illustration of the witness model of stress. The mouse depicted in the left-hand side of the cartoon experiences 'emotional stress' (ES), while the identical mouse on the right-hand side of the cartoon experiences physical stress (PS). The CD-1 aggressor mouse is shown in white.

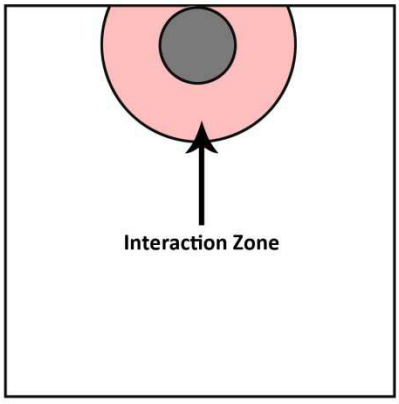

Figure 2. Illustration of the social interaction arena. Mice are allowed to explore the social interaction arena and the amount of time spent in the interaction zone (red circle) is recorded.

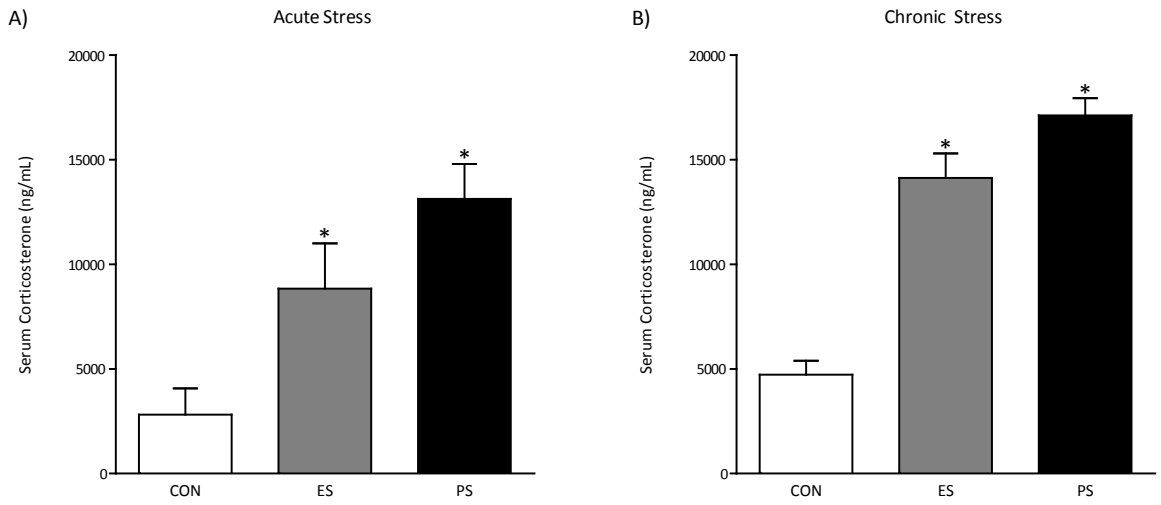

Figure 3. Corticosterone levels following acute or chronic stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on serum corticosterone levels in male mice **(A-B)**. **(A)** Exposure to a single ES or PS session increased serum corticosterone 40 min later. **(B)** Exposure to 10 daily ES or PS sessions increased serum corticosterone 24 h later. *Significantly different than CON-exposed mice, ($p< 0.05$). Data are presented as ng of

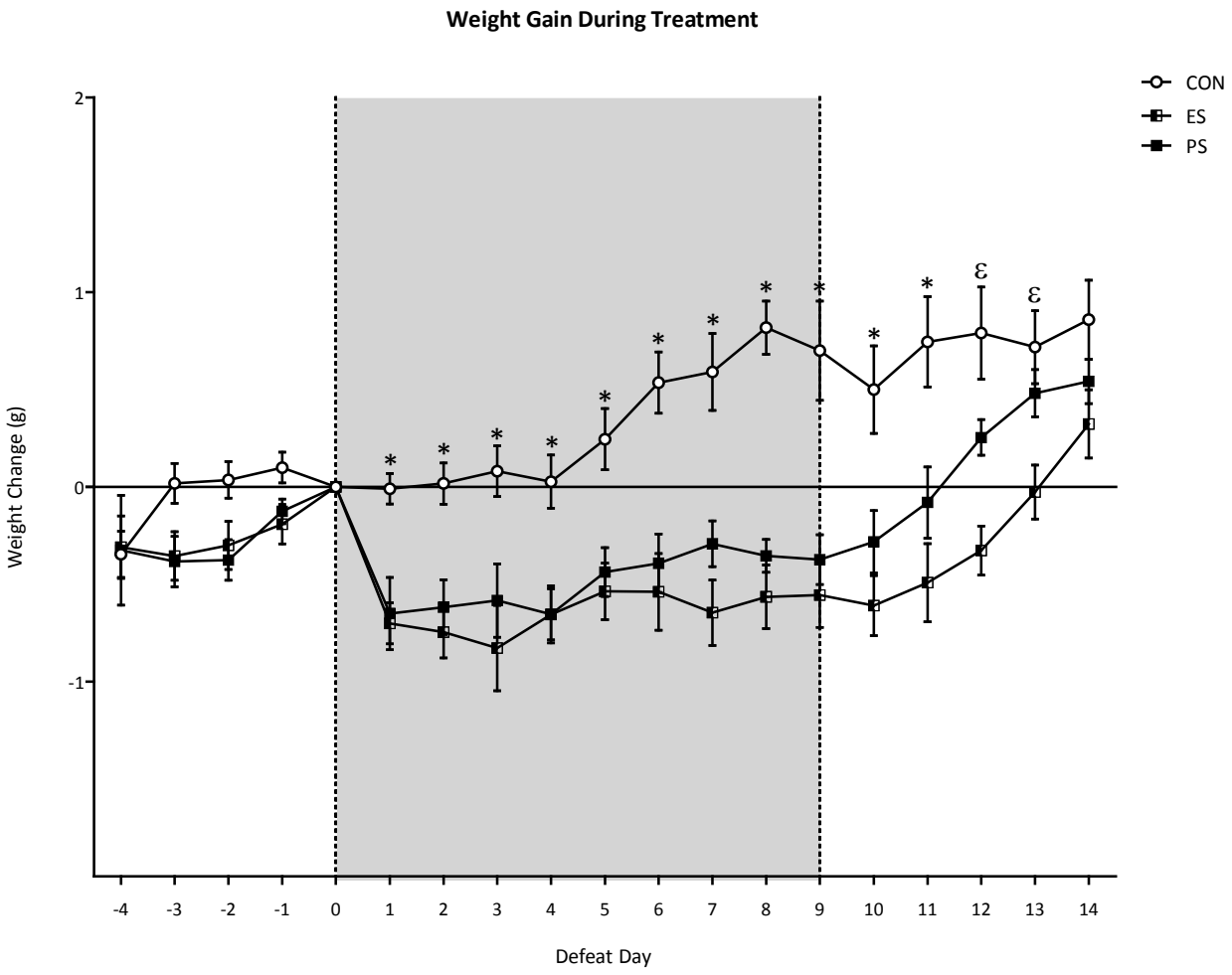

Figure 4. Weight gain during stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on weight gain. Exposure to ES or PS reduced weight gain in adult male mice when compared to the CON-exposed mice. *ES and PS are significantly different from the CON-exposed mice ($p< 0.05$). ES alone are significantly different when compared to the CON-exposed mice ($p< 0.05$). Data are presented as difference from starting weight across defeat day ($p< 0.05$) (mean SEM).

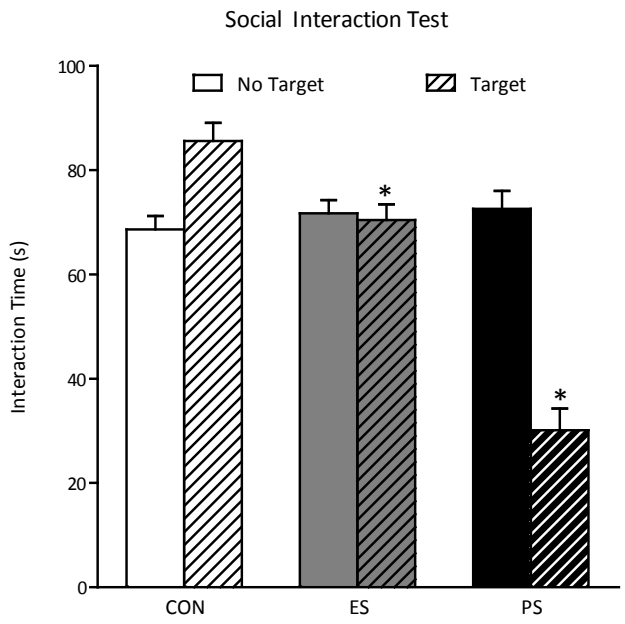

Figure 5. Social interaction 24 h following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on social interaction. When tested 24 h after the last stress session, exposure to ES and PS reduced time spent interacting with a novel CD-1 aggressor mouse (Target). * Significantly different from the CON-exposed mice ($p< 0.05$). Data are presented as time (in seconds) spent in the interaction zone (mean SEM).

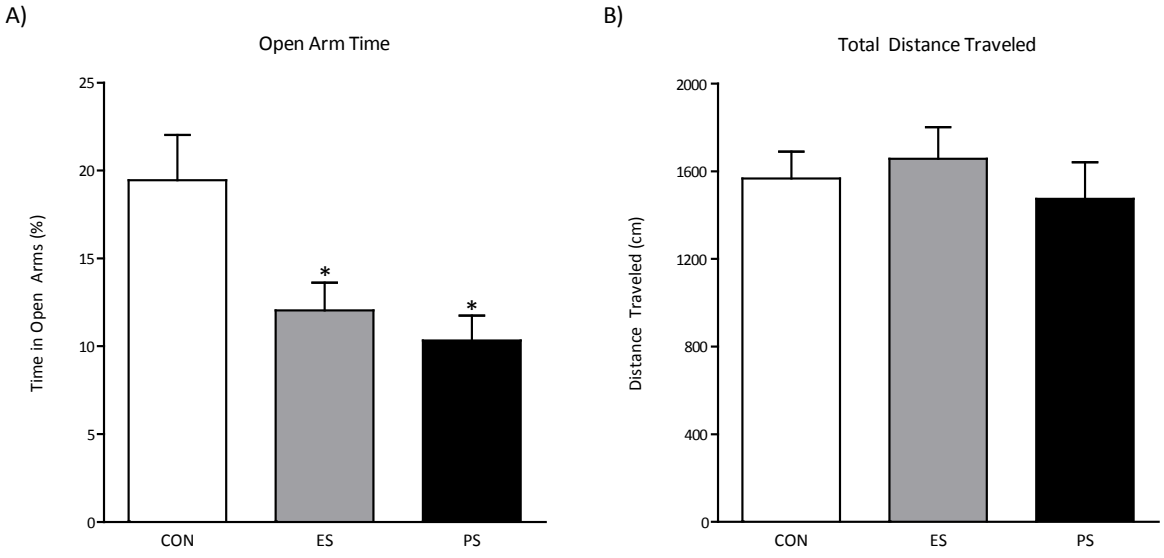

Figure 6. Elevated plus-maze 24 h following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on anxiety-like behaviors in the elevated plus-maze (EPM) **(A-B)**. **(A)** ES and PS exposure reduced time spent in the open arms of the EPM. **(B)** Total distance traveled did not vary by stress exposure. * Significantly different from the CON-exposed mice ($p< 0.05$). Data are presented as percent time spent in the open arms of the EPM and distance traveled in cm (mean SEM).

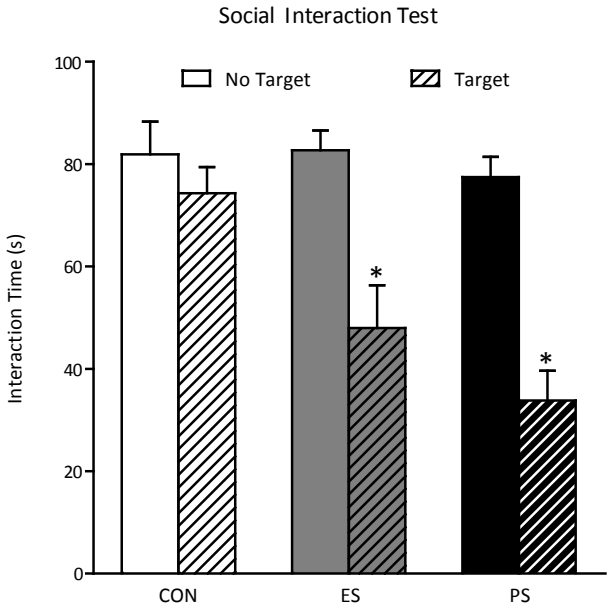

Figure 7. Social interaction 1 month following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on social interaction 1 month after cessation of stress. ES and PS exposure reduced time spent interacting with a novel CD-1 mouse (Target). * Significantly different from the CON-exposed mice ($p < 0.05$). Data are presented as time (in seconds) spent in the interaction zone (mean SEM).

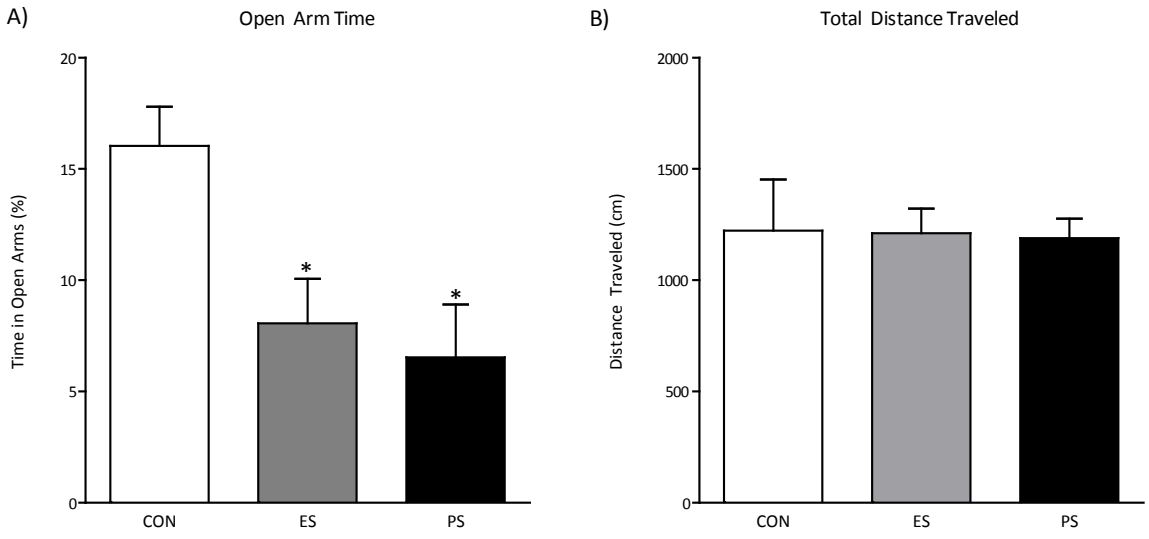

Figure 8. Elevated plus-maze 1 month following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on anxiety-like behaviors in the elevated plus-maze (EPM) 1 month after cessation of stress **(A-B)**. **(A)** ES and PS exposure reduced time spent in the open arms of the EPM. **(B)** Total distance traveled did not vary by stress exposure. * Significantly different from the CON-exposed mice ($p < 0.05$). Data are presented as percent time spent in the open arms of the EPM and distance traveled in cm (mean SEM).

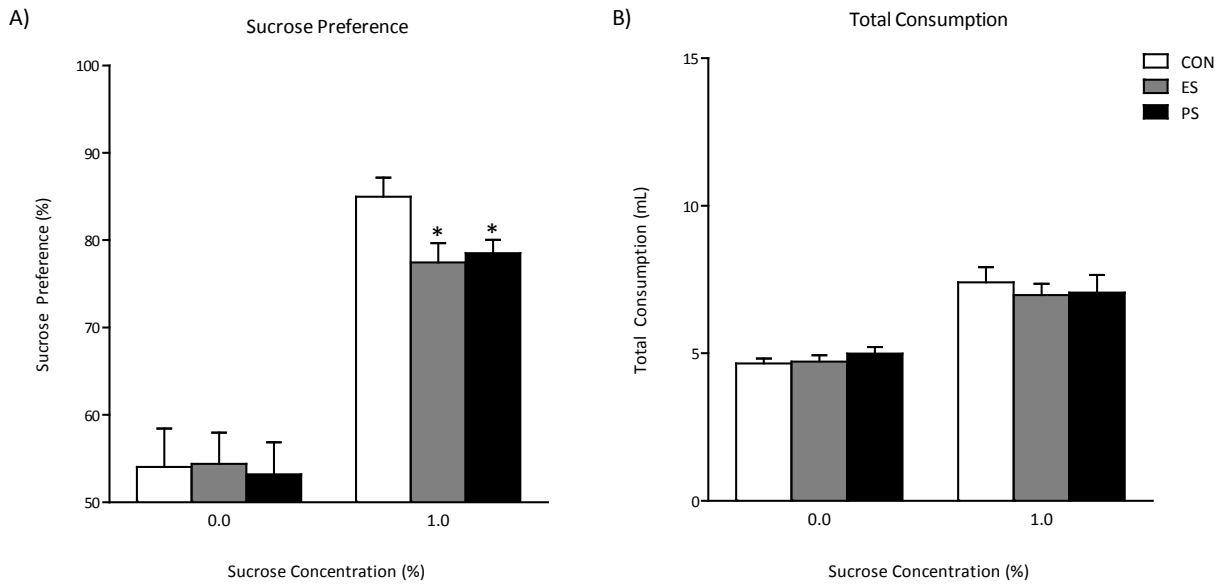

Figure 9. Sucrose preference 1 month following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on sucrose preference 1 month after cessation of treatment **(A-B)**. **(A)** Exposure to ES or PS significantly reduced sucrose preference when compared to the CON-exposed mice. **(B)** No differences in total fluid intake (sucrose + water) were detected. * Significantly different from the CON-exposed mice ($p < 0.05$). Data are presented as percent preference or total mL consumed (mean SEM).

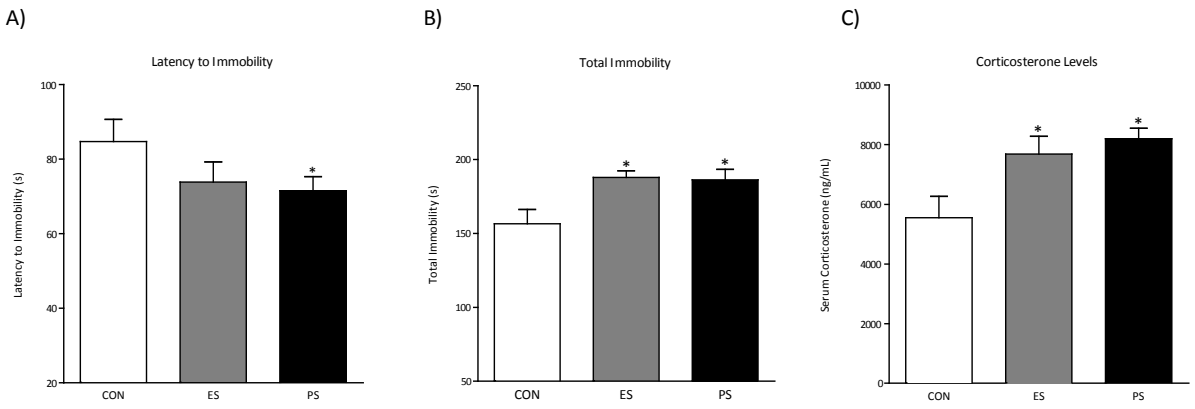

Figure 10. Forced swim test 1 month following stress. Effects of control stress (CON), emotional stress (ES), or physical stress (PS) on responsivity to swim stress 1 month after cessation of treatment **(A-C)**. **(A)** PS, but not ES, exposure significantly reduced latency to become immobile when compared with CON-stressed mice. **(B)** ES and PS exposure increased total immobility when compared to CON-stressed mice. **(C)** ES and PS exposure increased levels of serum corticosterone when compared to the CON-stressed mice 40 min after the FST. * Significantly different from the CON-exposed mice ($p< 0.05$). Data are presented as latencies to become immobile and total immobility (in seconds) and ng of

APPENDIX A

ACUC PROTOCOL APPROVAL

Animal Care and Use Committee (ACUC)
101 Biomedical Research Facility
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Tallahassee, FL 32306-4341
Telephone: 644-4262 Fax: 644-5570
Mail Code: 4341 Email: acucsecretary@mailer.fsu.edu

M E M O R A N D U M

TO: Dr. Carlos Bolanos
Department of Psychology

FROM: Dr. Paul Q. Trombley, Chair PQT
Animal Care and Use Committee

SUBJECT: Protocol **#0923**

DATE: **December 16, 2009**

"YOUR TRIENNIAL PROTOCOL REVIEW HAS BEEN APPROVED"

The Animal Care and Use Committee approved the triennial review of **Protocol #0923 (previously #0608), "*Neurobiology of physical versus emotional stress*"** at the **December 8, 2009** ACUC meeting. Please note that you have agreed to modifications to the protocol as outlined in the attached committee comments; the revised protocol is enclosed. You are approved for the following species and numbers for the proposed protocol approval period.

Species	*Number Animals Approved*	*Protocol Approval Expiration Date*	*Rewrite Due*
Rat (Sprague Dawley)/ *Rattus novergicus*)	820	**December 31, 2012**	**November 1, 2012**
Mice (CD-1)/*Mus musculus*)	500		
Mice (C57)/*Mus musculus*)	1490		

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Peer Reviewed Journal Publications:

1. LaPlant Q, Vialou V, Covington HE 3rd, Dumitriu D, Feng J, **Warren BL**, Maze I, Dietz DM, Watts EL, Iñiguez SD, Koo JW, Mouzon E, Renthal W, Hollis F, Wang H, Noonan MA, Ren Y, Eisch AJ, Bolaños CA, Kabbaj M, Xiao G, Neve RL, Hurd YL, Oosting RS, Fan G, Morrison JH, Nestler EJ (2010). Dnmt3a Regulates Emotional Behavior and Spine Plasticity in the Nucleus Accumbens. *Nature Neuroscience,* 13(9), 1137-43. PMID: 2072844

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Manuscripts in Preparation:

1. **Warren BL**, Iñiguez SD, Bolaños-Guzmán CA. Juvenile administration of *concomitant methylphenidate and fluoxetine alters behavioral reactivity to reward- and mood-related stimuli and disrupts ventral tegmental area gene expression in adulthood.*
2. **Warren BL**, Iñiguez SD, Bolaños-Guzmán CA. *Molecular adaptations following physical versus emotional stress in male mice*.

Published Abstracts

1. **Warren BL,** Iñiguez SD, Nestler EJ, Bolaños-Guzmán CA (2010) *Witnessing physical stress induces an anxiety- and depression-like state in adult mice.* 49th annual meeting *American College of Neuropsychopharmacology* (ACNP), Miami Beach, FL.
2. **Warren BL,** Iñiguez SD, LaPlant Q, Alcantara LF, Weakley S, Nestler EJ, Bolaños-Guzmán CA (2010) *Emotional stress induces an anxiety- and depression-like state in adult mice. Society for Neuroscience*, San Diego, CA.
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