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The Effects of Restraint Stress on Anxiety-Like Behavior in Adolescent and Adult Male Sprague-Dawley Rats: An Examination of the Effects of Early-Life Stress on the Development of Psychopathologies

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Abstract

Several lines of research on human and rodent subjects have demonstrated that early-life stress results in multiple negative outcomes, including increased incidence of psychopathologies. The current study sought to further the research on adolescent versus adult rats on anxiety-like reactions to chronic stress. The purpose of the current study was to assess whether 7 days of chronic restraint stress (20 minutes/day) results in higher anxiety-like profiles on the elevated plus maze and increased stress-induced neuroendocrine adaptations in adolescent versus adult rats. This type of research is critical for the prevention and treatment of psychopathologies stemming from early-life stress/maltreatment. There were no significant differences in anxiety-like behavior on the elevated plus maze or between age groups on neuroendocrine measures of stress. However, non-significant trends were observed in the anticipated directions, such that adolescent stressed rats spent less time on the open arms of the elevated plus maze. Implications and suggestions for future research are discussed.

Introduction

Psychopathologies: An Epidemic
Psychological disorders are widespread, somewhat enigmatic, problems facing our society today. Andrade, Walters, Gentil, & Laurenti (2002) estimated that the lifetime prevalence of at least one disorder, or the percentage of subjects in that population who experienced one incident of a disorder in their lifetime, was 45.9%. Two of the most common disorders are anxiety and mood disorders (e.g. Andrade et al., 2002; Kessler, Chiu, Demler, & Walters, 2005). Anxiety disorders are marked by excessive fear or worry about objects or events that interfere with optimal functioning (Kring, Davison, Neale, & Johnson, 2007). These disorders can manifest themselves in a variety of forms: social and specific phobias (e.g. agoraphobia, arachnophobia), panic disorder, generalized anxiety disorder, and posttraumatic stress disorder, to name a few. Sheehan and Sheehan (1994) estimated the 12-month prevalence of anxiety disorders in the United States at over 13% for individuals aged 18-54. More recently, Kessler et al. (2005) estimated that the 1-year prevalence for anxiety disorder in the United States has increased to 18.1%. Mood disorders, on the other hand, encompass mild to severe emotional disturbances on both ends of the affective spectrum: extreme sadness and intense elation and mania (Kring et al., 2007). Andrade et al. (2002) reported that mood disorder was the second most prevalent lifetime disorder in a Brazilian population of 1,464 respondents following nicotine dependence. Kessler et al. (2005) estimated the 12-month

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prevalence of mood disorder in the United States in individuals aged 18-54 to be 9.5%.

In a review published in 2007, Ronald Kessler compared the European Study of the Epidemiology of Mental Disorders (ESEMeD) with several previous findings and estimated that the median age of anxiety disorder onset was only 15, while the onset for mood disorders was 26 years. Kessler's review found that afflicted individuals had increased absences at work, higher rates of unemployment and underemployment, lower levels of work performance, and more instances of social isolation and interpersonal problems. Kouzis and Eaton (1994) used data from the Eastern Baltimore Mental Health Survey to analyze data from 3,481 respondents regarding the condition of their mental health and days of missed work. Fully 44% of the sample diagnosed as having either depressive disorder of anxiety disorder (DSM-III, 6 month prevalence) reported at least one missed day in the previous three months resulting from emotional problems. Furthermore, they found that suffering from a major depressive episode made respondents 27 times more likely to experience impairment connected to work activities. Wang, Simon, & Kessler (2003) reported that diminished work productivity accounts for $33 billion of the $53 billion economic cost of depression annually in the United States alone, while the remainder is comprised of explicit care costs and increased mortality rate costs due to suicide. Even more distressing is the finding that the lifetime prevalence of both anxiety and mood disorders are increasing, which suggests that more people will experience some kind of disorder in their lifetime (Kessler, 2007). With the World Health Organization predicting that depression will be the second leading cause of disability in 2020 next to heart disease (Holden, 2000) and in light of the enormous financial burden caused by mental illnesses, the importance of understanding the etiology of these illnesses and factors affecting their onset is crucial.

Findings of Early Life Stress & Psychopathology
Early life stress is considered to play an instrumental role in the development of certain psychopathologies, including anxiety and depression (Heim & Nemeroff, 2001; Thornberry, Ireland, & Smith, 2001). Studies of abused children consistently report high rates of negative outcomes in later life (Gibb, Butler, & Beck et al., 2003; Harkness & Wildes, 2002). Thornberry et al. (2001) reported that early life maltreatment was associated with increased aggression, academic problems, higher rates of teen pregnancies in females, more suicide attempts, and short-term cognitive impairments. In addition to increased risks for psychopathologies, early maltreatment puts individuals at risks for a litany of somatic problems, including cardiac disease, lung disease, diabetes, and autoimmune disease (Gillespie & Nemeroff, 2007). For instance, using a sample of nearly 2000 women, Nemeroff (2004) found that women with a history of sexual or physical abuse were four times more likely to develop depression and had an increased risk for drug abuse and suicide. The same trend of results is attained when both genders are considered; Johnson et al. (1999) found that individuals with a history of physical abuse or neglect are four times more likely than non-abused and neglected individuals to develop a personality disorder in adulthood.

To fully understand the impact of early life stress on development, one must consider a variety of factors

related to stressor-specificity and the time-course of stressful experiences. In particular, does the type of early maltreatment (sexual, physical, emotional abuse, or neglect) affect the type of negative consequence rendered in later life? Are there certain periods of development when humans are particularly vulnerable to maltreatment? Several research teams have investigated these topics in human populations, which have yielded a variety of results (Gibb et al., 2003; Harkness & Wilde, 2002; Johnson et al., 1999; Thornberry, Ireland, & Smith, 2001).

Harkness and Wilde (2002) examined whether stressor-specificity influences the nature of maladaptive outcomes in women with major depressive disorder (MDD) and one of two types of co-morbidity (anxiety or dysthymia—a lesser form of MDD that is marked by prolonged sadness, low self-esteem, and anhedonia) (Kring, 2007). Specifically, they examined the relationship between the co-occurring disorder (anxiety or dysthymia) and type of childhood adversity experienced. Harkness and Wilde hypothesized that histories of abuse perceived as threatening or dangerous (i.e., physical or sexual abuse) would be preferentially correlated to anxiety co-morbidity in MDD. In contrast, individuals with a history of parental antipathy and indifference, with themes of worthlessness and low self-regard, would be more associated with dysthymia co-morbidity in major depressive disorder. To examine these relationships, Harkness and Wilde recruited a sample of 74 women with MDD from the Northwestern United States who completed an interview to assess childhood maltreatment, which was classified into 4 categories: childhood physical abuse, sexual abuse, psychological abuse, and antipathy and indifference.

Consistent with their expectations, they found that sexual abuse was significantly correlated with anxiety co-morbidity and *not* dysthymia. Inconsistent with their hypothesis was the finding that physical abuse was significantly related to dysthymia co-morbidity, which is surprising since the associated cognitive schemas of physical abuse were thought to foster feelings more closely resembling anxiety. Parental antipathy and indifference showed no preferential type of co-morbidity as it was significantly related to both anxiety and dysthymia co-morbidity (Harkness & Wilde, 2002). Therefore, the Harkness & Wilde data provide partial support for the notion that the type of stressor can predict the psychopathological outcome. Consistent with this ambiguous nature of stressor-specificity data, other studies have reported somewhat varied effects of stressor-specificity.

Gibb et al. (2003) analyzed self-reports of past abuse from 552 psychiatric outpatients with regards to psychiatric outcome and similarly found preliminary indications of a stressor-specificity effect. The types of maltreatment under consideration were sexual abuse, emotional abuse, and physical abuse. Gibb et al. found that reports of emotional abuse were more strongly correlated with current diagnoses of depression than anxiety, which is consistent with Harkness and Wilde's (2002) model proposing that themes of worthless correspond to maladaptations to self-esteem. Somewhat contrary to Harkness and Wilde's findings, Gibb et al. found that physical abuse predicted outcomes of anxiety, but sexual abuse showed no effect of specificity. These results taken together suggest a role of stressor-specificity in the development of maladaptive behaviors, which may be an indication that separate physiological coping mechanisms are associated with certain types of stressors; however, more consistent data are needed to support serious consideration of this proposition.

Thornberry et al. (2001) examined the effect of the timing of maltreatment on the severity of adverse consequences in later life. They conducted a longitudinal study of 738 adolescents using the Rochester Youth Development Study, whereby adolescents and caretakers were interviewed in waves every 6 months for several years regarding instances of maltreatment and developmental outcomes. The maltreatment data consisted of substantiated instances of maltreatment documented by the local social services agency. Based on these data, participants were classified into maltreatment categories: early-childhood only, late-childhood only, adolescence-only, persistent, and no substantiated maltreatment. The researchers then looked for correlations between age of maltreatment and several negative developmental outcomes (Thornberry et al., 2001).

Thornberry et al. (2001) found compelling evidence for a temporal effect of maltreatment on the severity of problematic outcomes. Their data suggested that adolescence-only maltreatment yielded the most damaging behavioral consequences in later life. For each of the outcomes analyzed (general delinquency, drug use, alcohol-related problems, depressive symptoms, internalizing problems, externalizing problems, and multiple problems), adolescent-only maltreated individuals were at an increased risk for each of them. Additionally, subjects who were maltreated in early-childhood only were at no increased risk, compared to non-maltreated individuals, for self-reports of alcohol consumption, depressive symptoms, delinquency, or drug use. These authors suggested that *childhood* maltreatment is not the strongest indicator of later behavioral disruptions, but rather that adolescence is the period of vulnerability where maltreatment has its most damaging effects. This study not only serves to make a distinction between adolescent and childhood maltreatment, but is also compelling evidence from the human literature that adolescence is the critical period of vulnerability for maltreatment, which coincides nicely with neurobiological data of the time-course of brain development which will be reviewed next.

Development of the Brain: A Vulnerable Time for Adolescents
Adolescence in humans and nonhuman animals is commonly thought to be a critical time period for growth and maturation because of the myriad of changes taking place throughout the brain and body (Andersen, 2003; Crews, He, & Hodge, 2007; de Graaf-Peters & Hadders-Algra, 2006; Heim & Nemeroff, 2001; Holmes et al., 2005). Indeed, this corresponds with previously cited findings that early life stress corresponds to a wide array of psychological and physical illnesses (Gillespie & Nemeroff, 2007; Heim & Nemeroff, 2001; Thornberry et al., 2001). In humans and rats, the adolescent period is marked by large increases in receptor and synapse production followed by a period of both pruning and competitive termination, which occurs throughout the brain in different regions at distinct time points (Andersen, 2003). Furthermore, higher-level mental processes (e.g., selective attention, problem solving, working memory) appear to develop concurrently with synaptic pruning and myelination in the frontal cortex during adolescence (Crews et al., 2007). Researchers believe that high levels of pruning and myelination are indicative of mature brains that are capable of faster processing (Andersen, 2003; Crews et al., 2007).The magnitude of the development of the brain during adolescence makes the brain particularly vulnerable to maltreatment. The following sections will outline the physiological stress response and implicate the Hypothalamic-pituitary adrenal (HPA) axis as a plastic mechanism that is likely responsible for certain negative outcomes of chronic stress.

Stress Mechanisms
One theory regarding the development of certain psychopathologies posits that early life stress induces sensitization of the Central Nervous System (CNS) stress response circuits, such as the HPA-axis and the sympathetic nervous system, which increases susceptibility to depression and anxiety (Heim, Meinlschmidt, & Nemeroff, 2003). As seen in Figure 1, when a stress response is initiated, the hypothalamus secretes corticotropin-releasing factor (CRF) to the anterior pituitary, situated just below the hypothalamus, which in turn secretes adrenocorticotropin hormone (ACTH) (Nemeroff, 2004). This ACTH then travels to the adrenal cortex, or the outer layer of the adrenal glands, and signals a secretion of glucocorticoids, namely corticosterone in animals and cortisol in humans (Breedlove, Rosenzweig, Watson, 2007). Through a negative-feedback system, the glucocorticoids travel back to the hypothalamus, pituitary, and hippocampus (among other brain structures), to terminate the stress response by signaling a shutoff of the CRF (De Bellis et al., 1999). Chronic triggering of the stress response produces maladaptive physiological and behavioral outcomes most likely resulting from hypersecretion of glucocorticoids (Heim et al., 2003) One of the mechanisms of this hypersecretion is proposed to be a result of downregulation, whereby the body reduces the number of glucocorticoid receptors in certain brain regions, namely the hypothalamus, which has already been established as partially responsible for termination of the stress response. This downregulation likely results in elevated levels of glucocorticoids in a stressed organism's system due to an inability to properly regulate and turnoff the hormonal stress response. This dysregulation manifests itself in elevated basal levels of circulating glucocorticoids or in the processes of sensitization and habituation, which are reviewed next.

HPA-axis Dysregulation and Ramifications
One prevailing theory concerning the maladaptive outcomes corresponding to early life stress, as we have just discussed, posits that exposure to early life stress results in lasting physiological changes to systems that mediate the stress response. One of the most studied mechanisms is the HPA axis, which plays a key role in the stress response by secreting stress hormones, such as corticotropin-releasing hormone. Studies on humans with a history of maltreatment support the hypothesis that early life stress results in sensitization of the HPA-axis, such that stressors encountered later produce a heightened response of the hormones secreted by the HPA axis (e.g., Heim et al., 2001). Another possibility is that exposure to early maltreatment results in an increase in basal levels of stress hormones, which result in heightened stress responses, but studies on this topic have yielded conflicting results (for support, see Renard, Suárez, Levin, & Rivarola, 2005; Sandstrom & Hart, 2005; for dissent, see Mathews, Wilton, Styles, & McCormick, 2008). Ultimately, studies of HPA-axis and neural dysregulation resulting from repeated stressors provide evidence that habituation and sensitization are most likely instrumental in the long-term changes of the stress response. While these changes may be adaptive when that stressor is frequently present, and may contribute to the ability of the animal to cope with the stress, these long-term changes may be maladaptive in other environments.

Heim et al. (2001) assessed cortisol levels, adrenocorticotropin hormone (ACTH), and heart rate in a cohort of 49 women after a mild psychosocial stressor, which consisted of public speaking and mental math problems. These women were classified into four groups: women with no history of abuse and no DSM-IV diagnosis of current major depression, women with no history of abuse and a

current diagnosis of major depression, women with a history of abuse and no diagnosis of current major depression, and women with a history of abuse and a current diagnosis of major depression. Women who were abused and currently had a diagnosis of major depression exhibited higher levels of cortisol than all other groups, higher average maximum heart rate, and a more than 6-fold increase in maximum ACTH levels (all after baseline). Perhaps the most compelling finding was that women with a history of past abuse with *and* without a current diagnosis of major depression exhibited significantly higher concentrations of ACTH after start of the stressor than the control group and non-abused group with a current major depression diagnosis (Heim et al., 2001). This supports the notion that early life stress results in lifetime changes in the stress response, and specifically the HPA-axis.

Sandstrom and Hart (2005) reported similar results in studies using animal subjects. Sandstrom and Hart exposed 15-21 day-old rats to either 6 hours social isolation per day or control handling, which consisted of being lifted from the cage, handled and then immediately returned to the cage. They found that baseline levels of corticosterone measured in adulthood (> 3 months old) were significantly higher as compared to controls. This supports data from Heim et al.'s (2001) human study and suggests that early stress produces changes in the HPA-axis. In contrast, however, Mathews et al. (2008) reported no significant difference in basal corticosterone levels between their stress and control subjects following 15 days of chronic social stress, which consisted of 1-hour isolation followed by a new cage mate each day. The difference in the social stress regime could account for the differences found, but minimally we know that generally speaking social stress does *not* always increase basal levels of glucocorticoids.

An interesting experiment by Weinberg et al. (2009) also lends support to the notion that chronic stress induces dysregulation of the HPA-axis via sensitization and habituation. They examined whether repeated ferret odor exposure induced homotypic (same) stressor habituation and heterotypic (novel) stressor sensitization in the HPA-axis, as well as in neuronal activity, measured via immediate-early gene expression in the paraventricular nucleus of the hypothalamus (PVN). They found that after 14 days of ferret odor exposure rats exhibited habituated responses to the homotypic-stressor (ferret odor) evidenced through reduced corticosterone levels and decreased c-fos expression in the PVN. Moreover, they reported sensitization to a novel stressor (restraint), such that corticosterone levels were elevated and c-fos expression in the PVN was higher than that of naïve animals following the novel stressor (Weinberg et al., 2009).

Weinberg et al. (2009) also reported distinct time-courses for habituation and sensitization to stressors. They tested for HPA-axis and neural activation as described above after 0, 2, 7, and 14 days of ferret odor exposure to isolate the temporal relationship between stressor and stress adaptation. They found that endocrine and neural habituation to ferret odor appeared much sooner than sensitization—after the third and eighth day of ferret odor animals were exhibiting diminished neural and endocrine responses to the stressor, respectively. The time-course of sensitization to the heterotypic stressor was much more gradual, such that substantial differences in HPA activation (comparing adrenocorticotropin hormone levels) were only markedly evident at the end of the two week experiment. This suggests that these two adaptive responses to stress operate independently of one another and have different time courses (Weinberg et al., 2009).

The aforementioned research collectively illustrates that chronic stress results in dysregulation of the
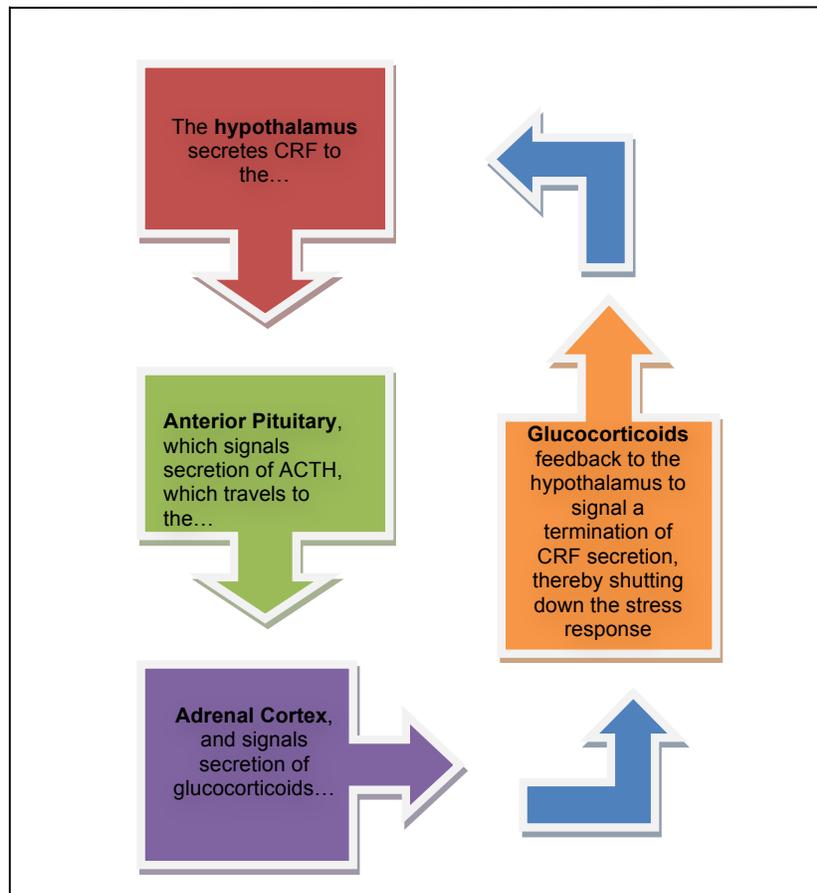
Figure 1 is a diagram of the HPA-axis response to stress and the negative feedback system. Abbreviations: Corticotropin-releasing factor (CRF), Adrenocorticotropin hormone (ACTH)

HPA-axis in animal and human cohorts via increased sensitivity to novel stressors or increased basal levels of glucocorticoids. These findings are particularly important because of the deleterious mental and physiological outcomes that have been associated with elevated glucocorticoid levels. Elevated glucocorticoid levels have been associated with several negative outcomes including hippocampal atrophy, which results in learning deficits (Isgor, Kabbaj, Akil, & Watson, 2004; Kim, Koo, Lee, & Han, 2005) and enhancements in fear conditioning in animals, which may be related to changes in the amygdala and indicate a priming for conditioning of specific phobias (Conrad, LeDoux, Magariños, & McEwen, 1999). Regardless of the role hormonal dysregulation plays in an organism's maladaptive stress response, a combination of genetic factors, early stressful experiences, and ongoing stress most likely all come together to determine the occurrence of pathologies (Hammen et al., 1992).

Stress and Learning
Stress exerts a powerful impact that influences various physiological and cognitive mechanisms, including learning. In animal models, the influence of stress on learning has been shown to depend on a wide variety of factors, such as: age, sex, type of stressor, length of stressor, and type of learning (see Shors, 2006 for a detailed review).

The effects of gender and stress on learning are often observed in opposite directions and these effects have been attributed to the variety of sex hormones that affect these processes. Wood and Shors (1998) used male and female Wistar rats in a paradigm of restraint and intermittent tail shock to stress their experimental groups. Then, they trained all groups on a classical conditioning eyeblink task, followed by a test for acquisition of the learned eyeblink response. They reported that stressed males exhibited enhanced classical conditioning, while stressed females show impaired acquisition of the conditioned stimulus. In an extension to the study, Wood et al. (1998) compared ovariectomized females to sham controls in the same experimental design and showed that removal of the ovaries eliminated the learning deficits observed in stressed females. Wood et al. explicitly implicated estrogen as a key factor in the stress-induced disruption in learning by examining the effects of an estrogen antagonist on the associative learning procedure and finding that stressed females receiving the estrogen antagonist did not display the learning deficits. These powerful results not only revealed the sex differences in learning, but also implicated mechanisms by which these effects occur.

The perception of a stressor also seems to play a role determining how stress will affect learning. Leuner, Mendolia-Loffredo, & Shors (2004) examined whether controllability of the stressor may influence classical conditioning to the eyeblink task in male and female Sprague-Dawley rats. Controllability was manipulated by yoking two rats in separate shuttle-boxes, such that both rats

received a foot-shock at the same time, but one rat (in the controllable stress condition) could turn off the shock by running through a doorway, which terminated the shock in the other rat's box as well. Using this method, the rat in the uncontrollable condition does not receive more shocks than his yoked partner, but he has no control over when his stressor is terminated. Controllability in this study reversed the effects of stress on learning in both males and females. Namely, having controllability over a stressor eliminated the impaired learning observed in females and eliminated the enhanced conditioned responses in males. These results suggest that the *perception* of stress may differentially affect the physiological mechanisms underlying stress and learning in males and females.

To explain the various effects of stress on learning in male rats, researchers have turned to glucocorticoids as an explanation. de Quervain, Roozendaal, & McGaugh (1998) were able to determine that impairment on a spatial memory task, namely finding a hidden platform in a water maze, depended on the amount of time elapsed since the stressor. Specifically, rats that received foot-shock two minutes or four hours prior to being tested on the maze exhibited superior memory for the submerged platform than did rats that received the stressor 30 minutes before the test. de Quervain et al. (1998) suggested that the presence of glucocorticoids, which were elevated after the maze testing in the 30 minute group but not the others because of the slow time-course of glucocorticoid elevation which peaks after about 20 minutes, may be partially responsible for the differences in memory retrieval. Indeed, after administering metyrapone, a drug that decreases the production of corticosterone, rats stressed 30 minutes prior to testing did not show the increased corticosterone levels and did not show the same deficits in learning the spatial water maze. These are convincing data that corticosterone somehow modulates learning and, in this case, retrieval of information.

Clearly, stress has complex effects on learning, such that the slight alterations of the learning context can change the direction or magnitude of the learning. The next section focuses on some behavioral responses to chronic stress that can be indicative of psychopathologies.

Stress and Behavior
The literature on stress and behavior in animals indicates that stress can reliably produce depressive behavior in rodents, while only sometimes producing anxiety-like behaviors (Bondi et al., 2008; Rygula et al., 2005; Kompagne et al., 2008; Matuszewich et al., 2007). Various stress paradigms have been shown to increase anxiety-like behavior (Bondi et al., 2008; Rygula et al., 2005), decrease anxiety-like behavior (Kompagne et al., 2008), or have no effect on anxiety-like behavior in rodent models (Matuszewich et al., 2007). These disparate findings may be attributable to differences in the type of stressor and time between the stressor and subsequent assessment of anxiety. Gender also plays an important role in moderating the effects of stress on depressive and anxiety-like behaviors.

In an elaborate study of the effects of chronic social stress on subsequent depressive and anxiety-like behaviors, Rygula et al. (2005) reported a wide variety of behavioral indices indicative of depression and anxiety. Male Wistar rats were subjected to the resident-intruder paradigm of social subordination and defeat daily for five weeks. In this model, the stressed subject is placed into a foreign male's cage (the resident), effectively becoming an intruder. The resident will likely engage the intruder in a physical altercation and defeat him. Once the intruder has submitted to the resident, he remains safely behind a mesh divider for the remainder of an hour, where he can still fully sense the dominant male, but cannot be injured.

Rygula et al. (2005) assessed sucrose preference at baseline before stress and weekly throughout the stress paradigm. Decreased sucrose consumption is a measure of anhedonia, a common characteristic of depression. They found that the stressed rats' preferences for sucrose significantly decreased from baseline after the third week of social stress, indicating a loss of pleasure or anhedonia. Stressed rats exhibited further indications of depressive behaviors in that they spent significantly more time immobile in the forced swim test than controls when tested 24 hours after their final social stress exposure. Finally, this stress paradigm resulted in decreased locomotor and exploratory behavior in the stressed rats to their own baseline and controls after one and five weeks of social stress. These results suggested that five weeks of chronic social stress, using the resident-intruder paradigm, produced depressive behaviors in the form of anhedonia and loss of motivation, and anxiety-like behaviors in the form of diminished exploratory behavior.

Other stress paradigms, including maternal separation and chronic unpredictable stress, have failed to induce high anxiety profiles. Matuszewich et al. (2007) suggested that this occurs because stress enhances conditioned anxiety-like behavioral responses, but not unconditioned anxiety-like behavioral responses. To illustrate the differences between these behavioral responses, consider the defensive burying test and the elevated-plus maze. In the defensive burying test, the animal must learn that a rigged wooden dowel shocked them and then react behaviorally to it; they *naturally* do not fear the wooden probe, so they must develop a conditioned behavioral response. Alternatively, in the elevated plus maze, the animal comes equipped with an innate (unconditioned) fear of open spaces; hence no additional learning is required to produce an anxiety-like response. Matuszewich et al. (2007) tested this hypothesis by exposing rats to 10 days of chronic unpredictable stress (described more fully in next paragraph) and then testing them with the defensive burying test and the elevated plus maze, a conditioned and unconditioned response test, respectively. Results from the study confirm their hypothesis in that chronic unpredictable stress increased burying of the probe and immobility in a defensive burying test compared to controls, but no effect of stress was observed on the elevated plus maze.

In spite of Matuszewich et al.'s findings, Bondi et al. (2007) reported that chronic unpredictable stress *can* induce increased anxiety-like behavior on the elevated plus maze. Bondi et al. used a chronic stress procedure that consisted of 30 minute restraint stress, the resident-intruder paradigm, warm and cold water swim, electric foot-shock, tail pinch, shaking-crowding, and high density housing as unpredictable stressors over a 14 day period. Their data revealed that stressed animals spent significantly less time in the open arms, with lower open to total arm entries and lower open arm to total time, than control rats. Bondi et al.'s stress procedure was similar to Matuszewich et al.'s in that it included a variety of stressors, but critically different in that Matuszewich et al.'s (2007) procedure in that it only lasted 10 days and seemed to include less direct physical stressors, such as lights on overnight, food and water deprivation, and wet bedding, to name a few. These methodological differences may explain the differences in producing anxiety-like behavior on the elevated plus maze and may be an indication that the elevated plus maze is very sensitive to rat history and context.

Kompagne et al. (2008) conducted a study that supports the notion that chronic stress can increase

Figure 2: Pictured left is a typical Elevated Plus Maze that researchers use to assess anxiety levels in rats.
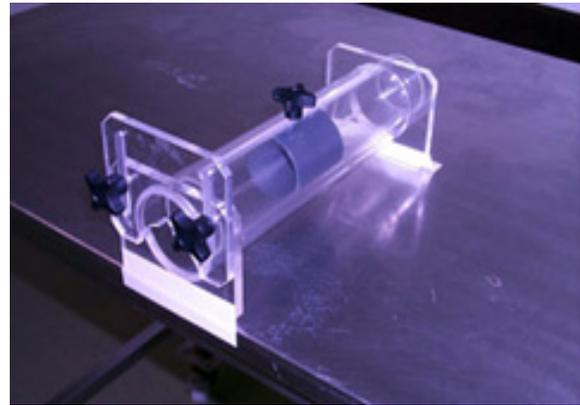**Figure 3:** Pictured right is depicted a restraint chamber. The rat would be placed inside for 20 minutes of restraint.

depressive behaviors and have ambiguous effects on anxiety-like behaviors in rats. Kompagne et al. used a chronic mild stress model, which resembles the stress paradigms used by Matuszewich et al. (2007) and Bondi et al. (2007) described above. Over the course of three weeks, Kompagne et al. included tilted cage, lights on at night (2 hours), loose restraint, housing with an unfamiliar male, platform stress, crowded housing, swimming, wet bedding, and isolation, to name a few. Their results were similar to Rygula et al. (2005) on measures of depressive behavior—specifically, the chronic mild stress protocol decreased sucrose consumption from baseline in stressed rats and increased the time spent immobile in the forced swim test in stressed rats compared to controls.

However, the measures of anxiety assessed in Kompagne et al.'s experiment were harder to decipher. Stressed rats spent *more* time in the open arms of the elevated plus maze than their control counterparts, which is indicative of *decreased* anxiety. On the social avoidance test, however, when rats had the choice to spend time in a portion of a plastic cage that visibly exposes them to another unfamiliar male rat or a portion where they cannot see the 'opponent', the stressed rats spent significantly less time in the in the portion exposing them to the 'opponent' and made less entries into the portion of the cage where they were exposed. These results support Matuszewich et al.'s (2007) interpretation of conditioned and unconditioned anxiety models, since fear of an unknown male could be considered an unconditioned response.

Current Study
Researchers have established that early life stress reliably predicts a variety of negative outcomes, including mental and physiological pathologies. Given that more than 1,000,000 children experience severe neglect or abuse, sexually or physically, each year (Gillespie et al., 2007), this realization is particularly poignant. The long-term negative outcomes, such as anxiety and mood disorders, are likely due to physiological dysregulation that occurs at the stress response level. Specifically, disruptions in the HPA-axis may cause elevated glucocorticoid levels, which may alter subsequent stress responses and learning, ultimately resulting in maladaptive behavioral consequences. There are still a variety of questions that need to be answered regarding exactly how HPA dysregulation occurs, how it disrupts learning and emotionality, what kind of role genetics

play in moderating an organism's stress response, and what age is most crucial with regards to stressors and maltreatment.

The current study was designed to assess the impact of age at the time of stressor (adolescent or adult) on certain behavioral and neuroendocrinological outcomes. This study differs from many studies in that it targets adolescence, whereas many existing animal studies have focused on weanlings and rat pups. Additionally, this study focuses on determining differences in behavior when the stress is experienced at different time points in life: adolescent or adulthood. Following a period of restraint stress, we examined behavioral markers of anxiety using the elevated plus maze and adrenal-to-body weight ratios as an index of neuroendocrinological activity. Adolescent stressed rats are expected to exhibit the highest amount of anxiety-like behavior and stressed-induced changes because adolescence is such a critical time for development and brain plasticity.

Hypotheses
Previous research suggests that the elevated plus maze (see Figure 2 above) is an anxiogenic stimulus and that administering anxiolytic drugs, such as benzodiazepines, prior to administration of the elevated plus maze increases time spent in the open arms, indicating that these behaviors on the elevated plus maze are indicative of blunted anxiety (Walf & Frye, 2007). Accordingly, in the current experiment, we expect that the controls will spend more time in the open arms of the maze, but that both groups will make similar total arm entries. Conversely, we expect the stressed groups, adult and adolescent, to spend less time on the open arms of the elevated plus maze. Additionally, we predict that stressed rats classified as adolescents will exhibit a greater stress response to the task than their adult stressed counterparts because of extensive evidence indicating that adolescence is a vulnerable time to encounter stress.

There is considerable evidence suggesting that chronic stress causes increases in adrenal weight as well as decreases of the thymus (Karst & Joëls, 2002; Rygula, et al., 2005; Sterlemann et al., 2008). Accordingly, we expect that the stressed animals will have a greater adrenal-to-body weight ratio than controls, and that the largest ratio will belong to stressed adolescents. For this experiment we have no data regarding changes to the thymus. Finally, we expect the stressed animals to gain less weight over the treatment

period than their control counterparts to validate the effectiveness of our stress paradigm.

Results

Elevated Plus Maze (Time Spent in Arms)
The elevated plus maze is used as an assay of anxiety-like behavior in rodents. Time spent on the open arms was computed because it is accepted as a measure of anti-anxiety like behavior (Walf & Frye, 2007). Figure 4 (below) displays the mean time, in seconds, spent on the open arms of the elevated plus maze as a function of age and treatment group. A one-factor independent-samples analysis of variance (ANOVA) revealed no significant main effect of age, $F_{(1,59)}=.361$; $p>.05$, or condition, $F_{(1,59)}=1.252$; $p>.05$. Group averages revealed non-significant trends indicating that the stressed groups spent less time on the open arms (M=38.27, S.D.=37.52 sec) than control groups (M=50.60, S.D.=44.75 sec) and that adolescents spent less time *on average* (M=40.96, S.D.=33.73 sec) than adults (M=47.47, S.D.=47.46 sec) on the open arms of the elevated plus maze. There was also no significant interaction of age and condition on time spent in the open arms, $F_{(1,59)}=.087$; $p>.05$.

Time spent on the closed arms of the maze was compared between groups using a one-factor independent-samples ANOVA. The ANOVA revealed no significant main effects of age or condition, $F_{(1,59)}=.235$; $p>.05$ and $F_{(1,59)}=1.411$; $p>.05$, respectively. Group averages revealed that none of the treatment groups differed greatly from one another on time spent in the closed arms; adolescent stressed (M=221.21, S.D.=38.31 sec), adolescent controls (M=213.00, S.D.=44.52 sec), adult stress (M=221.81, S.D.=44.64 sec), and adult control

(200.25, S.D.=61.46 sec). Furthermore, there was no significant interaction between treatment and age on time spent in the closed arms, $F_{(1,59)}=.284$; $p>.05$.

Elevated Plus Maze (Arm Entries)
Similar to time spent on the open arms of the elevated plus maze, number of entries made into open arms is considered anti-anxiety like behavior. Figure 5 (above) displays the average number of open arm entries across the different treatment groups. A one-factor independent-samples ANOVA revealed no significant main effect of age or condition on the number of entries into open arms, $F_{(1,59)}=.068$; $p>.05$ and $F_{(1,59)}=1.514$; $p>.05$, respectively. Group averages showed that none of the treatment groups differed substantially in their number of entries made into the open arms; stress groups (M=2.73, S.D.=2.10) and control groups (M=3.5, S.D.=2.37). The ANOVA also failed to reveal a significant interaction between age and condition on the number of entries into the open arms of the elevated plus maze, $F_{(1,59)}=1.832$; $p>.05$.

Entries into closed arms of the elevated plus maze, on the other hand, are considered a measure of overall locomotor activity. A one-factor independent-samples ANOVA compared the number of entries made into closed arms between treatment groups. The ANOVA revealed no significant main effect of age or condition on entries made into the closed arms, $F_{(1,59)}=.414$; $p>.05$ and $F_{(1,59)}=.613$; $p>.05$, respectively. Also, the ANOVA did not yield a significant interaction between age and condition on entries into the closed arms, $F_{(1,59)}=.527$; $p>.05$. The group averages indicated that there were no discernable differences between the groups on the number of closed arm entries made; stress group (M=11.67, S.D.=4.04) and control group (M=10.87, S.D.=4.30).

Average Time Spent in Open Arms
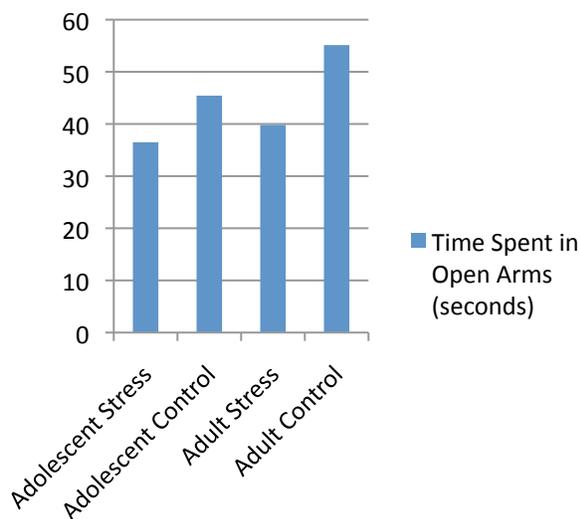
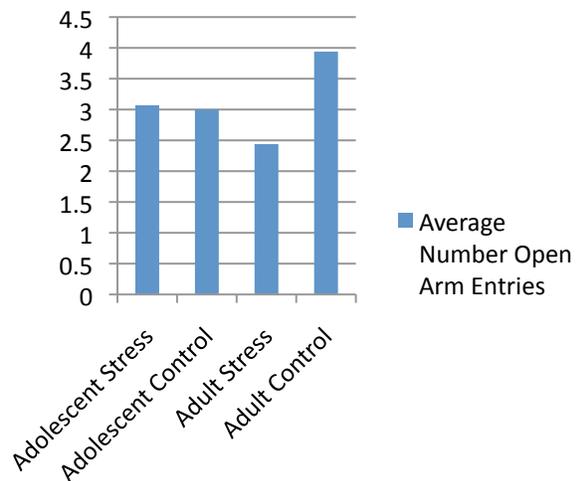**Average Number Open Arm Entries Across Age & Conditions**

Figure 4 (above left) displays the average time, in seconds, spent on the open arms of the elevated plus maze across treatment group and age. It illustrates a trend towards stressed groups spending less time on the open arms than their control, age-matched counterparts.

Figure 5 (above right) displays the average number of open arm entries and illustrates the minimal difference across all groups on average number of open arm entries made.

Adolescent or Adult	Stress or Control	Mean	Std. Deviation	N
Adolescent	Stress Group	.7374	.12772	14
	Control Group	.7100	.14840	14
	Total	.7237	.13657	28
Adult	Stress Group	.7394	.14879	16
	Control Group	.6675	.20485	16
	Total	.7034	.17986	32
Total	Stress Group	.7384	.13698	30
	Control Group	.6873	.17901	30
	Total	.7129	.16012	60

Table 1 (above) displays the average proportion of time each age and treatment group spent in the closed arms of the elevated plus maze.

Overall activity on the elevated plus maze was further assessed by comparing total entries into arms between treatment groups using a one-factor independent-samples ANOVA. The ANOVA revealed no significant main effects of age or condition on total arm entries, $F_{(1,59)}=.485$; $p>.05$ and $F_{(1,59)}=.013$ $p>.05$, respectively. The ANOVA also revealed no significant interaction between age and condition on total arm entries, $F_{(1,59)}=1.656$; $p>.05$.

Elevated Plus Maze (Percentage Time Spent in Arms)
The ratio of time spent in open and closed arms of the elevated plus maze offers a standardized measure of an animal's anxiety-like behavior, weighted by their overall tendencies. Figure 6 displays the average percentage of time spent in the open arms of the elevated plus maze as a function of age and treatment group. A one-factor independent-samples ANOVA revealed no significant main effect of either age or condition, $F_{(1,59)}=.361$; $p>.05$ and $F_{(1,59)}=1.252$; $p>.05$, respectively. Group averages indicated a nonsignificant trend in the anticipated direction, such that stressed animals spent a smaller percentage of their time in the open arms (M=.128, S.D.=.125) than control animals (M=.169, S.D.=.149). The ANOVA revealed no significant interaction between age and condition on percentage of time spent in the open arms, $F_{(1,59)}=.087$; $p>.05$.

Table 1 displays the average percentage of time spent in the closed arms across age and treatment groups. A one-factor independent-samples ANOVA revealed no significant main effects of either age or condition on ratio of time spent in the closed arms, $F_{(1,59)}=.235$; $p>.05$ and $F_{(1,59)}=1.411$; $p>.05$, respectively. The ANOVA also revealed no significant interaction of age and condition on percentage of time spent in the closed arms, $F_{(1,59)}=.284$; $p>.05$.

Elevated Plus Maze (Ratio Entries into Arms)
Figure 7 displays the average percentage of entries into open arms as a function of age and condition. A one factor independent samples ANOVA revealed no significant main effects of either age or condition on the ratio of open arm to total arm entries, $F_{(1,59)}=.138$; $p>.05$ and $F_{(1,59)}=.911$; $p>.05$, respectively. Group averages indicate small trends in the anticipated directions, such that adolescent stress made less percentage of entries (M=.228, S.D.=.126) than adolescent control rats (M=.244, S.D.=.184) and adult stress rats made less percentage of entries into open arms (M=.186, S.D.=.205) than adult control rats (M=.253, S.D.=.145). The ANOVA yielded no significant interaction

between age and condition on ratio of open arm entries to total arm entries, $F_{(1,59)}=.350$; $p>.05$.

Table 2 displays the average percentage of entries made into closed arms across age and treatment groups. A one factor independent samples ANOVA revealed no significant main effects of age or condition on the percentage of entries into closed arms, $F_{(1,59)}=.138$; $p>.05$ and $F_{(1,59)}=.911$; $p>.05$, respectively. The ANOVA also revealed no significant interaction between age and condition on percentage of closed arm entries, $F_{(1,59)}=.350$; $P>.05$.

Adrenal-to-Body Weight Ratio
Relative adrenal weights were collected to assess the efficacy of the stressors, because higher relative adrenal weights are associated with chronic triggering of the HPA-axis. Figure 8 displays the average adrenal-to-body weight ratio across age and treatment groups. A one factor independent samples ANOVA revealed a significant main effect of age on adrenal-to-body weight ratio, $F_{(1,14)}=13.843$; $p<.05$. Post-hoc Sidak analysis revealed that adolescent rats had significantly higher adrenal-to-body weight ratios (M=.120, S.D.=.009) than adults (M=.088, S.D.=.022). However, there was no significant main effect of condition on adrenal-to-body weight ratio, $F_{(1,15)}=1.942$; $p>.05$. In addition, there was no significant interaction between age and condition on adrenal-to-body weight ratio, $F_{(1,15)}=1.082$; $p>.05$.

Weight Gain over the Treatment Period
The amount of weight gained over the stress period was another measure used to assess to effectiveness of the stress protocol. Figure 9 displays the average weight gain over the treatment period across age and treatment group. A one-factor independent-samples ANOVA revealed a significant main effect of age on weight gain over the treatment period, $F_{(1,59)}=83.036$; $p<.05$. Post-hoc Sidak tests revealed that adolescents gained significantly more weight (M=55.79, S.D.=5.05 g) than adults (M=36.13, S.D.=10.24 g). The ANOVA revealed no significant main effect of condition and no significant interaction between age and condition on weight gain over the treatment period, $F_{(1,59)}=.340$; $p>.05$ and $F_{(1,59)}=.268$; $p>.05$, respectively.

Discussion

The statistical analyses failed to reject the null hypothesis in all of the anxiety-related conditions above. Oftentimes, however, the nominal trend in the data supported the notion

Average Percentage of Time Spent in the Open Arms Across Groups
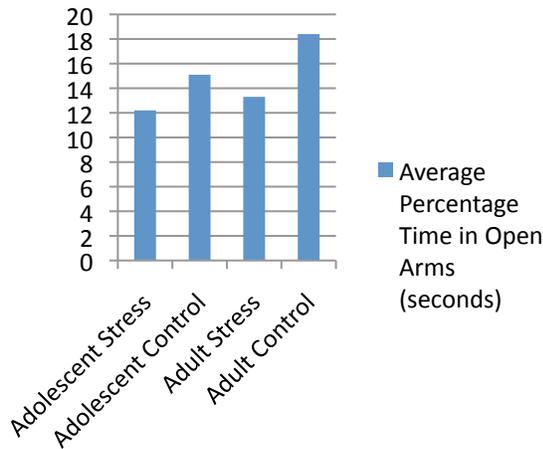
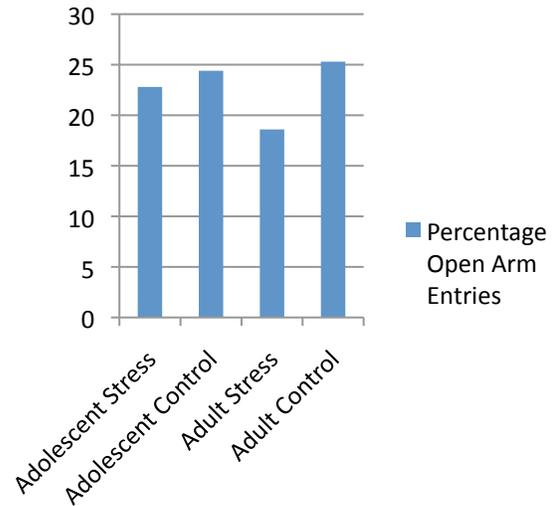## Percentage of Open Arm Entries Across Groups

Figure 6 (left) displays the average percentage of time spent on the open arms of the elevated plus maze, illustrating a standardized measure of each animal's time on the open arms.

Figure 7 (right) displays the ratio of open arm entries to total arm entries as a function of age and condition.

Table 2: Average Percentage of Entries into the Closed Arms

Adolescent or Adult	Stress or Control	Mean	Std. Deviation	N
Adolescent	Stress Group	.7722	.12575	14
	Control Group	.7564	.18355	14
	Total	.7643	.15459	28
Adult	Stress Group	.8142	.20509	16
	Control Group	.7468	.14484	16
	Total	.7805	.17798	32
Total	Stress Group	.7946	.17117	30
	Control Group	.7513	.16117	30
	Total	.7729	.16627	60

Table 2 (above) displays the average ratio of closed arm entries to open arm entries across age and treatment groups.

that stress had an effect on anxiety-like behavior. Specifically, the trends suggest that stress increased anxiety-like behavior on the elevated plus maze. However, the experiment was not powerful enough to report any significant findings—with 60 subjects, there were only about 15 subjects per condition and the variability for each dependent measure was often high (note the high standard deviations reported). It is also possible that the stressor manipulation (restraint stress) was not strong enough to induce an anxiety-like profile or that the control condition constituted a significant enough stressor to eliminate any stress-induced anxiety-like differences in the animals

(see below for a more detailed discussion).

General Discussion
The data from this experiment failed to support the hypothesis that stressed rats would exhibit more behavioral anxiety on the elevated plus maze and show larger relative adrenal weights compared to control counterparts. More specifically, the data failed to support the hypothesis that stressed adolescent rats would display the greatest anxiety-like profiles across groups. These results, however, should not be interpreted as an invalidation of the aforementioned hypotheses. There are several potential considerations to

Adrenal-to-Body Weight Ratio
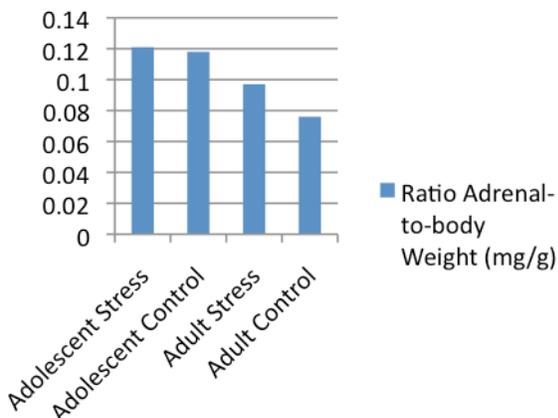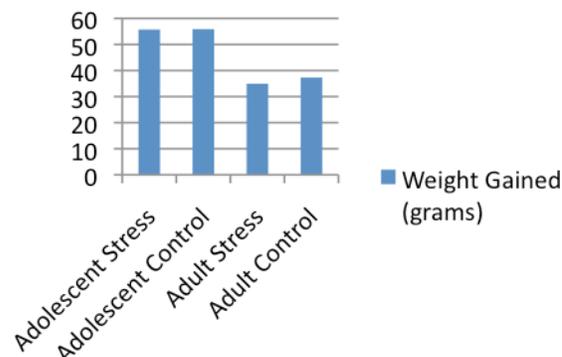

Average Weight Gained Over the Treatment Period

Figure 8 (above left) displays the average ratio of adrenal-to-body weight across a subgroup of subjects from the treatment groups. The graph shows a trend toward the stress groups having slightly higher ratios than their control counterparts.

Figure 9 (above right) displays the average weight (in grams) gained over the treatment period and shows that adolescents gained the most weight overall, and that treatment across the age groups did not induce differences in weight gain.

explain the observed results including the effectiveness of the treatment manipulations, the nature of the results, and the utility of the behavioral measure.

The first explanation regarding the lack of expected results is the possibility that the stress paradigm did not effectively stress the animals. There are certain indications that the stress protocol may have not been optimally effective. The fact that stressed animals, either adolescent or adult, did not gain less weight than their age-matched controls suggests that the stress paradigm was not effective because several studies have demonstrated that stressed animals gain less weight (Lehmann et al., 1999; Matuszewich et al., 2007; McLaughlin et al., 2007). Furthermore, the most compelling evidence that the stress protocol may have been less than effective is that there were no significant differences in relative adrenal weight between the stress and control groups. It is possible that the 20-minute stress period for 7 days was not enough time to induce significant behavioral or neuroendocrine changes. McLaughlin et al. (2007) found that 6 hours of restraint stress each day for 21 days was the only duration of stress that was effective in producing stress-related changes in the hippocampus and that 2 hours a day for 10 days was not sufficient to produce these changes. This study indicates that a longer duration of stress may have been necessary to produce larger behavioral and physiological effects of stress.

In addition to the possibility that the stress protocol was ineffective is the possibility that the handling procedure constituted a mild to moderate stressor in itself. The same rationale regarding the lack of significant differences between the stress and control groups on measures of weight gain and relative adrenal weight apply to this logic. The handling manipulation consisted of removing the animal from its' home cage and mate(s) and placing it in a new room and cage for 20 minutes for 7 days. This protocol is similar to models of isolation stress, in that the animal is isolated from their cage mates for varying periods of time each day (Lukkes, Mokin, Scholl, & Forster, 2008; Matuszewich et al., 2007; Sandstrom et al., 2005)—although, admittedly, the shortest duration of isolation housing used in the studies mentioned was 6 hours a day for

7 days (Sandstrom et al., 2005). Regardless, the possibility that the 'control' animals were mildly stressed is important to acknowledge. Perhaps there was an interplay between the manipulations, such that the stress protocol was not effective enough and the control condition was stressfully-arousing enough to reduce the size of the observed effects. This possibility is particularly worth noting when we look at the trends in the results.

A closer look at the results, although primarily insignificant, shows trends in the anticipated directions. On average, the adolescent stressed group tended to spend the *least* amount of time on the open arms of the elevated plus maze, the least percentage of time in the open arms, and had slightly larger relative adrenal weights, all of which are in line with our hypotheses. The failure of these results to reach significance is likely due to the high standard deviations and relatively low sample sizes in each condition. For example, the average time spent on the open arms of the elevated plus maze for adolescent stressed rats was 36.5 seconds with a standard deviation of 26.53, while control adolescents spent on average 45.43 seconds with a standard deviation of 40.20. This pattern of results was fairly consistent across the data, suggesting that our failure to reject the null hypothesis stems from small group average differences and large variability throughout the groups.

The final consideration when interpreting these results concerns the effectiveness of the elevated plus maze in assessing behavioral anxiety. Although the elevated plus maze has construct and predictive validity, in that anxiogenic drugs reduce time spent on the open arms and behavior on the elevated plus maze can predict behavior in an open field (Walf et al., 2007), studies of stress have resulted in a wide variety of outcomes. A variety of stress paradigms have been shown to decrease anxiety-like behavior (D'Aquila, Brain, & Willner, 1994; Kompagne et al., 2008), increase anxiety-like behavior (Bessa et al., 2009; McCormick et al., 2007; Pohl et al., 2007) or have no effect on anxiety-like behavior (Matuszewich et al., 2007) on the elevated plus maze. However, 7 out of the 10 articles reviewed regarding the relationship between the elevated plus maze and stress indicated that stress *did* increase anxiety-like behaviors. This

suggests that generally the elevated plus maze is a reliable measure of anxiety, but that it can be vulnerable and sensitive to minor methodological changes.

Another problem concerning the elevated plus maze concerns a lack of resources. Many experiments that use the elevated plus maze have a camera hanging above the maze so that the behavior can be recorded later and verified with multiple observers (Walf et al., 2007). Unfortunately, in the current study, there was no camera and the observation was done by a single, trained (but not 'blind') observer as it occurred. This introduces potential problems regarding experimenter bias and error, especially since the observer was not blind to the condition; however, this certainly did not 'improve', with regard to the hypotheses, the outcome of the results. Regardless, the fortunate aspect of this situation is that the observer remained the same for all the subjects, so any experimenter error or bias should be comparable throughout the experiment.

The methodological considerations (e.g. the stress protocol and assessment of anxiety), small n, and high variability all are likely contributors to our failures to reject the null hypotheses in this study. Furthermore, the inclusion of several measures of anxiety would have been most advantageous in order to best assess the animal's anxiety profiles post-stress. In a replication of this study, additional and varied stress protocols may enhance the stress experience of the animals. In particular, the resident-intruder paradigm is an intriguing model of social defeat and subordination that aptly models the day-to-day human experiences with social stressors (Rygula et al., 2005).

A follow-up to this experiment should address the methodological problems and include more measures of anxiety that would tap into both conditioned and unconditioned behavioral responses. For instance, a more effective stress period should last at least 21 days and include longer durations of the chosen stressor. Anxiety measures would include the elevated plus maze, the defensive burying test, and the open-field test. Another area that could be strengthened is the neuroendocrine measures. Blood samples should be taken from different cohorts before the start of stressors, immediately after the last stressor, at baseline 24-hours after the last stressor, and at varying time points during the anxiety tests. This would allow for assessment of any changes to the rats' baseline glucocorticoids resulting from stressor exposure and their neuroendocrine responsiveness to novel stressors (the anxiety tests). This, however, would require an extremely large number of animals since they would likely be sacrificed in order to collect their blood. Regardless, a study of this magnitude would answer a multitude of questions regarding the impact of stressors on differing types of anxiety behavior and neuroendocrine stress responses.

In addition, subsequent experiments should look at the effects of early-life stress on anxiety-like behaviors at different time-points. For example, research should examine the differences in anxiety-like behavior in adolescence and adulthood in rats that have been stressed in adolescence. This would allow researchers to more fully understand the long-term effects of early-life stress on anxiety-like behaviors.

An area that needs further attention in conjunction with early-life stress leading to increased vulnerability to psychopathologies is the field of drug-addiction. Early-life stress has been shown to alter the levels of circulating catecholamines in individuals (De Bellis, 2005; McEwen, 2003), which are thought to be key to an organism's reward system (Breedlove et al., 2007). Thus, it follows that the chemical alterations associated with early-life stress may make an organism more vulnerable to psychostimulants. As such, research should focus on the age-dependent effects of stress on drug-seeking behavior with a variety of drugs of abuse.

Another area of study rife with anticipation for research is that of the effects of stress on various types of learning. Stress is known to enhance fear conditioning, which is an amygdala-dependent task, and impair spatial memory tasks, which are hippocampal-dependent. Therefore, we know that the stress response differentially affects regions of the brain responsible for different types of learning. An assessment of glucocorticoid levels during different phases of the learning process (acquisition, retrieval, etc.) should be included to create a fuller picture of the mechanisms required for learning, or the disruption thereof. Currently, this lab is conducting an experiment examining the age-dependent effects of restraint stress on cued fear conditioning.

The implications of this experiment speak mostly to methodological concerns regarding designing an effective stress/anxiety protocol. One of the primary suggestions regards scope: effective anxiety/stress experiments should be *large* in scope. They should have a large sample size, include a wide variety of anxiety assessments, and a wide variety of stress measurements. If there were an infinite amount of resources for this experiment it could have included the open-field test for anxiety, the forced swim test for depression, and the novelty-induced suppression of feeding test for anxiety to cover a wide variety of tests for comparison and thoroughness.

Methodology

Subjects were 60 Sprague-Dawley rats housed in groups of 2 or 3 in 47.1 cm x 25.4 cm plexiglas cages with corn cob bedding on a 12-hour light/dark cycle (lights on at 0700). Rats were allowed access to food and water *ad libitum* except during treatments and tests. Typically rats were given at least 7 days to acclimate to their new surroundings before undergoing any manipulation, but in some cases the acclimation period was only 5 days. Animals were chosen in a pseudorandom fashion, such that 30 were placed in the control condition and 30 were placed in the stress condition. Rats were considered adolescent if they received treatment and testing between postnatal day (PND) 26 and PND 45, closely resembling estimates of rat adolescence from Crews et al. (2007). Any rats receiving treatment *and* testing after PND 45 were considered adults. In the stress condition 14 animals were classified as adolescents (mean PND at start= 31.25; range 27.5-36.5) and 16 were classified as adults (mean PND at start=61.13; range 52-79). Similarly, in the control condition 14 rats were classified as adolescents (mean PND at start= 30.86; range 27.5-36.5) and 16 rats were classified as adults (mean PND at start=59.28; range 49.5-79).

Design & Procedure
Animals in the stress condition were put on a 5-2-2 schedule of restraint, such that they received 5 days of stress, 2 days without treatment, and another 2 days of stress. The elevated plus maze and adrenal weights were assessed the day following an animal's last treatment. On treatment days, animals were taken from their home cages and, after being weighed, placed into a smaller transport cage measuring 28.1 cm x 18.4 cm with wood chip bedding. The animals were then transported to a different room where restraint took place. The counter on which restraint took place and the restraint chambers (see Figure 3 below) were cleaned with a 50% solution of Lemon Lysol before and after each animal received treatment to create a consistent restraint setting for the rats. Animals were immediately placed in one of three adjustable-length plexiglas restraint chambers depending on their weight. Rats were placed in these holding chambers for 20 minutes a day between 0800 and 1600 hours on stress days. Upon completion of the stress regimen, rats were placed back into the transporter cage and returned to the holding room and their home cages.

Animals in the control condition were treated identically to the animals in the stress condition with the exception of restraint. The animals were taken from their home cages, weighed, and moved into transport cages. They were transported to the same room where restraint occurred for the stressed animals and remained in the transport cages for 20 minutes. Similarly, the counter their cages

were placed on was cleaned with a 50% Lemon Lysol solution before and after each treatment. After the duration of handling, the animals were returned to their home cages.

The elevated plus maze was administered to rats one day after their last day of treatment, stress or control. The elevated plus maze was cleaned with a solution of 25% Simply Green before and after each animal was tested in order to remove any trace of previous animals that had been tested. Animals were moved from their home cages into transport cages and brought into a new room where the elevated plus maze was located. A trained observer placed the rat in the center of the maze facing an open arm and immediately started a timer. The rat's motor behavior was observed for a duration of 5 minutes, during which the most robust effects of the elevated plus maze have been cited (Walf & Frye, 2007). Rats were only considered to have entered an arm if all four paws were in that section of the elevated plus maze. The observer recorded the number of entries into specific arms and the amount of time spent in each arm. Upon completion of the behavioral assay, animals were weighed in the holding room and then returned to their home cages. At this point, some animals were used for a tangential electrophysiology experiment, while the remaining were used for further neuroendocrine assays.

In order to ascertain the extent of the stress effects on the neuroendocrine system, some rat's adrenal glands were removed to provide an adrenal-to-body weight ratio, which has been shown to increase as a result of chronic stress (Karst & Joëls, 2003; Rygula et al., 2005). Within 3 to 4 hours following elevated plus maze testing, animals were heavily sedated with a large dose of chloral hydrate (8%, >400 mg/kg) and sacrificed via rapid decapitation. Immediately upon death, the animals were cut open and their adrenal glands were removed and weighed. Adrenal weights were expressed as a ratio to body weight.

In order to assess the efficacy of the stress paradigm weight gain over the treatment period was also measured by subtracting the rat's weight at the time of testing with weight at the start of treatment. Chronic stress has been shown to decrease the amount of weight gain during the stress period (McLaughlin, Gomez, Baran, & Conrad, 2007; Rygula et al., 2005), so lower weight gain should be preferentially associated with the stressed subjects.

Acknowledgements

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