The Time-Course of the Effects of Stress on Behavior in Rodents

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Abstract

Acute stress has been shown to have facilitating effects on memory tasks while chronic stress can enhance the development of psychiatric disorders such as depression and anxiety disorders. It is currently unknown how much stress is required to create these debilitating effects. This study is the first step in examining the time-course of the effects of stress in rodents. Anxiety-like behavior in the Elevated Plus Maze (EPM) and fear memory in Pavlovian conditioning using cued fear conditioning were examined after one or seven days of restraint stress. After seven days of restraint stress there was a decrease in anxiety-like behavior in the EPM, compared to rats exposed to a single session of stress. These results suggest the importance of the time delay between stress and behavioral testing. No significant results were seen in cued fear conditioning. To assess whether this effect is similar in males and females, anxiety-like behavior was measured in female rats after one day of restraint stress. No difference was found between female control and stress rats. These results suggest that females were not more vulnerable to the effects a single restraint stress on the EPM.

Introduction

Depression and anxiety disorders are extremely common in the general population. Lifetime prevalence of the two disorders has been estimated at 20.8% and 28.8% respectively (Kessler, Bergund, Demler, Jin, Merikangas, & Walters, 2005). The 12-month prevalence for anxiety disorders was found to be 18.1%, while depression was less common at 9.5% (Kessler et al., 2005). It is clear that these disorders are quite common in the general population, yet there is much to be learned about the causes of these disorders. Symptoms of depression include depressed mood, anhedonia, altered appetite, nervousness, and irritability (Gregus, Wintink, Davis, & Kalynchuck, 2005). These psychopathologies develop by a complex interaction between genetic predisposition and an adverse environment (Frank et al. 2006 and Pryce et al., 2005).

Stressful, or adverse, life experiences enhance the development of these affective disorders (Pryce et al., 2005, Heim & Nemeroff, 2001). Stress can be real or perceived and is defined as any threat to the homeostasis of an organism (Morilak et al., 2005). This threat can be either physical or psychosocial in nature. In humans, a variety of stressors are experienced daily. Stress can be chronic or acute. Chronic stress is a persistent, long lasting stressor, such as living with a terrible roommate. Acute stress, on the other hand, is a short lasting and one time stressor, such as failing an exam. In addition, stress can be major or minor. This difference will affect the impact that a certain stressor has on an individual. A major stressor would be something like a death in the family, while a minor stressor may be as simple as getting stuck in traffic. The response to a stressor depends on how the individual assesses their environment and the stressor they experience (Kring, Davison, Neale, & Johnson, 2007).

Stress affects the brain both physiologically and chemically (McEwen 2008). When a person decides that a stressor exceeds their ability to handle the situation, homeostasis is thrown off. Allostasis is the term used to refer to how the body responds to stress in order to maintain homeostasis. Allostatic load is the wear and tear produced from the effects of too much stress. As stress increases, wear and tear on the body also increases, producing the behavioral, physiological, and chemical changes in the brain (Figure 1; adapted from Figure 1. McEwen, 2008).

Treatment of psychiatric disorders is aimed at eliminating the debilitating symptoms rather than the cause of the disorder. Common treatments for depression include tricyclic antidepressants, monoamine oxidase inhibitors, and selective serotonin re-uptake inhibitors (SSRIs). Anxiety disorders are often treated with therapy such as cognitive behavioral therapy or SSRIs. Antidepressants may be used in severe cases of anxiety disorders (Kring et al., 2007). Current somatic treatments have numerous side effects such as, memory lapses, difficulty driving, jitteriness, weight gain, and even an interference with sexual functioning (Kring et al., 2007). In response to these side effects, many patients stop taking the medication and therefore relapse. A better understanding the effects of stress on behavior and brain physiology will allow us to determine the factors that lead to these disorders and will lead to the development of more effective treatment (Heim & Nemeroff, 2001).

The effects of stress can be studied using animal models. These models are able to provide useful insights into the behavioral and physiological mechanisms involved in the stress response. The behavioral effects of stress can be studied in rodent models of behavior such as anxiety-like behavior in the elevated plus-maze and fear memory in Pavlovian conditioning. Although useful, these models are not perfect and some inconsistencies exist. As with humans, different stressors in animal models have different effects. In order to eliminate these inconsistencies, further studies are needed. Because of the wide range of effects of stress, behavioral changes as well as physiological and neurobiological changes must be explored (Pryce et al 2005).

Rodent Models of Stress

As with humans, a laboratory rodent can experience many different stressors. Most stressors in humans have both physical and psychological demands and therefore responses to both types of stressors should be examined. Commonly used protocols consist of either physical or psychological stressors. Physical stressors consist of restraint stress, electric footshock, cold swim, or exposure to high intensity noise. Psychological stressors are more social in nature and often consist of social isolation, resident/intruder, maternal separation, or sleep deprivation. Different stressors are selected in an experiment based upon the way previous studies show that they activate the different neurobiological systems involved in the stress response. In addition to the type of stressor used, a non-stress baseline should be achieved in control and experimental animals.

Restraint stress, or immobilization is commonly used because it is less severe than other physical stressors,

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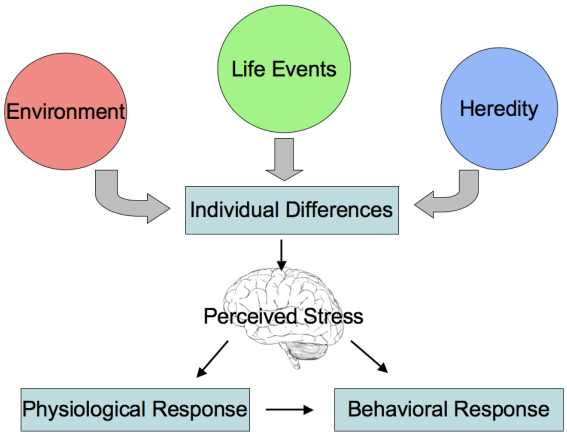

Figure 1: Individual differences in stress. Stress does not affect each individual the same way. A stimulus that may be stressful to one individual may not be stressful to another. Environment, life events, and genetics play a role in an individual's tolerance for stress. When an individual perceives a stimulus as stressful a physiological and behavioral response will be displayed.

such as a footshock, but is still capable of activating the stress response. In this type of stressor, movement is limited by placement in a plexiglass chamber or immobilization bag. Electric footshock is more severe and can be applied using a metal electric grid to shock the foot or applied to the tail. Forced swim at a cold water temperature is another physical stressor. This type of stressor requires physical exertion in order to prevent a passive coping strategy. High intensity noise exposure is also used as a physical stressor. This protocol can be used as a type of environmental stressor to mimic stress in everyday life (Heinrichs & Koob 2005).

Psychological stressors can also be used in the laboratory and are often used to study developmental factors. Social isolation is a common psychological stressor, in which subject is placed in long-term solitary housing. The resident intruder paradigm is a social conflict stress in which an intruder rat is placed in the home cage of a larger territorial rat. Maternal separation is a useful stress model because it mimics an adverse childhood in humans that is commonly associated with the expression of psychiatric disorders later in life (Heinrichs & Koob, 2005). This stressor involves the removal of a pup from the care of its mother for a certain period of time. Sleep deprivation can be used to elicit a stress response as well. This type of stressor consists of denying the subject any opportunity to sleep by placement on a rotating drum. This type of stressor is controversial because it is quite severe (Heinrichs & Koob, 2005).

Stress and the Brain

The endocrine stress response begins with activation of the hypothalamic-pituitary-adrenal axis (HPA axis). Neurons in the hypothalamus release corticotropin-releasing hormone (CRH), which then travels to the anterior pituitary gland, stimulating the release of adrenocorticotropic hormone (ACTH). This hormone then travels through the blood to the adrenal cortex to release glucocorticoids. Corticosterone is released from the adrenal cortex in rats, while cortisol is released in humans (see Figure 2). Glucocorticoids are anti-inflammatory and important for maintaining homeostasis. Cortisol leads to beneficial short-term responses. Long-term exposure to cortisol, however, can cause damage to the hippocampus and is associated with many different psychiatric disorders (Kring et al., 2007). During times of stress the activity of the HPA axis increases resulting in higher glucocorticoid levels, as seen in depression. Several brain regions involved in the stress response, including the amygdala and hippocampus, modify activity of the HPA axis.

The amygdala is important for HPA axis activity. Located just anterior to the hippocampus, the amygdala is activated during fear conditioning as well as in response to emotionally negative pictures, odors and tastes in humans (Shin & Liberzon, 2009). The amygdala is also activated during the coding and retrieval of emotional stimuli (Shin & Liberzon, 2009). Three different regions make up the anatomy of the amygdala. The central amygdala is known to contain CRH neurons (Heim & Nemeroff, 2001; Mikics, 2008) and has extensive connections with the bed nucleus of the stria terminalis (BNST), which then projects to the paraventricular nucleus of the hypothalamus (PVN) and brainstem (Purves et al., 2008). This nucleus of the amygdala is also noted for its relation to the expression of fear. Activation in this area increases after traumatic experiences (Mikics, 2008). The basolateral amygdala (BLA) has been shown to be important during fear conditioning as well. Lesion of the BLA or inactivation with muscimol in rats eliminated fear conditioning (Conrad, Macmillan, Tsekhanov, Wright, Baran, & Fuchs, 2004). The BLA receives inputs from the hippocampal formation as well as the thalamus and sensory cortical areas. The BLA also sends projections to the central amygdala (CeA) and the prefrontal cortex (PFC) (Correll, Rosenkranz, & Grace 2005; Rasia-Filho, 1999). In this way, the BLA is able to associate fear with various sensory information. In contrast, the medial amygdala has not been found to be important for fear conditioning. The amygdala has strong connections with the olfactory bulb and piriform cortex. The medial amygdala likely plays a role in social behavior and processes related to social learning and memory (Fekete, Zhao, Sabino, Vale, & Zorrilla, 2009). Frank et al. (2006) suggest that the medial amygdala also plays a role in aggression.

The hippocampus and PFC have also been noted for their role in the stress response. The hippocampal formation is known to play a role in the encoding and consolidation of declarative memory. Interestingly, the size of the hippocampus in London cab drivers was found to be larger than age-matched controls. Additionally, this size increase correlated positively with the time spent driving the cab (Maguire et al., 2001, as cited in Purves et al., 2008). The hippocampus is sensitive to stressful experiences (Shors, 2006), but changes in the hippocampus following stress are often reversible (McEwen, 2008). An important function of the hippocampus is its regulation of the negative feedback system in the HPA axis (Gregus et al., 2005). High levels of glucocorticoids in the hippocampus lead to downregulation of receptors, which inhibits the ability of the hippocampus to regulate the HPA axis (Gregus et al., 2005).

The role of the PFC has also been studied because of the human abilities of avoidance and cognition (Shin & Liberzon, 2009). The PFC is essential for a higher processing of stressful and emotional stimuli. A stressful stimulus for one individual may not be stressful for another. This inter-individual difference is dependent upon the PFC, more specifically the medial Prefrontal Cortex (mPFC) (Quirk, Likhtik, Pelletler, & Pare, 2003). Correll et al., (2005) found that lesions of the PFC enhanced the response to a train of footshocks, an acute stressor, suggesting a regulatory function of the PFC over the central amygdala after acute stress. In addition, this response was diminished after exposure to chronic cold stress, suggesting the regulatory function of the PFC decreases after chronic stress (Correll et al. 2005).

Effects of Stress on Behavior

Stress has a wide arrange of effects on behavior. These effects vary by age and gender. Various models of stress

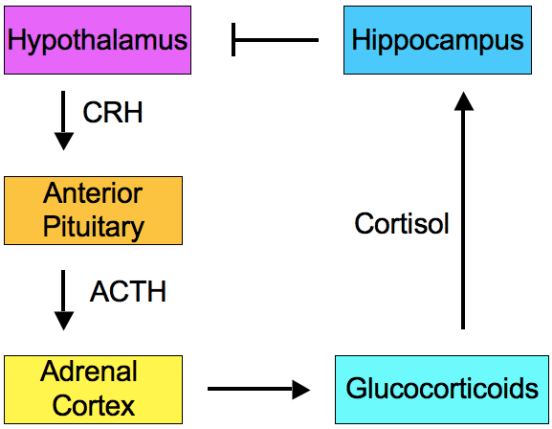

Figure 2: Hypothalamic-Pituitary-Adrenal Axis (HPA). When an individual perceives a stimulus as stressful a physiological response is displayed in the form of the HPA axis. CRH is released from the PVN, which travels to the anterior pituitary gland. From here ACTH is released into the blood stream and reaches the adrenal cortex. Glucocorticoid release is stimulated from the adrenal cortex and travels to the brain. Glucocorticoid receptors can be found in the hippocampus which when activated stimulate inhibitory control to the hypothalamus as a negative feedback system. CRH = Corticotropin-releasing hormone, ACTH = Adrenocorticotropic hormone.

have been shown to have different effects on behavior. In humans, stress has been shown to enhance Pavlovian conditioning of emotional stimuli, such as that seen in Post Traumatic Stress Disorder (PTSD). These patients also exhibit an inability to recall old information but the ability to acquire new information remains intact (Shors, 2006). These types of behaviors, as well as those observed in depression and anxiety disorders, have been studied in numerous animal models.

Anxiety-like behavior in rats is most commonly measured by the open field test or the elevated plus maze (EPM). The open field test measures anxiety by comparing the time spent in the middle of the arena to the time spent close to the walls. The floor of the open field test is divided into zones, with exploration of the inner zones, or those that are in the center of the testing field away from the walls, correlated with less anxiety. Increased anxiety has been shown in rats that underwent one session of footshocks for each of 5 consecutive days. These rats spent less time in the inner zones of the open field test and had fewer zones crossed (Daniels, de Klerk Uys, van Vuuren, & Stein, 2008). They also exhibited increased grooming behavior, which may be a possible coping mechanism in response to stress. Similarly, 10 days of chronic immobilization stress (CIS) increased anxiety-like behavior in the open field test (Vyas & Chattarji, 2004). No effect, however, was observed after social defeat, which results from the resident intruder paradigm mentioned previously (Razzoli, Carboni, Guidi, Gerrard, & Arban, 2007). Chronic unpredictable stress (CUS), which consisted of 10 consecutive days of two alternating forms of stress also failed to produce this effect (Vyas & Chattarji, 2004).

Anxiety-like behavior is also measured using the EPM. Reduced exploration of the open arms of the maze is used as an indicator of anxiety. Anxiogenic factors tend to decrease open arm exploration (Walf and Frye, 2007). Twenty-one consecutive days of CIS has been shown to reduce open arm exploration when compared to unstressed controls (Vyas, Jadhav, & Chattarji, 2006). Social defeat can cause a similar effect in rodents. When combined with social isolation, social defeat produced even more profound effects on anxiety-like behavior (Razzoli et al., 2007). Social isolation is used as to enhance to the effects of social defeat stress. Rats that were housed individually after social defeat spent significantly less time in the open arms of the EPM compared to rats that were housed in pairs after social defeat (Nakayasu & Ishii, 2008). This difference between individually and pair housed rats suggests that a supportive social network may play a possible role in attenuating the effects of stress. Interestingly, studies on adolescent rats have found no difference between stressed rats and non-stressed controls in open arm exploration of the EPM in males, but an increased exploration in female adolescent rats (McCormick, Smith, & Matthews, 2007).

Fear behavior in Pavlovian conditioning is also commonly tested in response to stress. Pavlovian conditioning occurs when a conditioned stimulus, a noise or a context, is paired with an unconditioned stimulus, such as a footshock. Stress has enhancing effects on fear behavior. One 2hr session of restraint stress was enough to enhance freezing time in contextual conditioning when these rats were compared to controls (Cordero, Venero, Kruyt, & Sandi, 2003). This same study found no difference in cued conditioning, which uses an auditory tone as a conditioned stimulus rather than a contextual change. Rats exposed to social defeat also exhibit increased contextual fear (Buwalda et al., 2005).

Fear behavior is also measured after social defeat stress by reexposure to the defeat environment. In this case certain behaviors, other than time freezing in fear conditioning, are measured including risk assessment, defined as a scanning head movement by the rat, as well as grooming, rearing, and social avoidance. After social defeat stress, rats show an increase in risk assessment (Razzoli et al., 2007). Interestingly, the opposite is seen after CIS (Vyas & Chattarji, 2004). Social defeat also led to decreased grooming and rearing (Razzoli et al., 2006, 2007). A decrease in rearing was observed after acute stress by footshock (Daniels et al., 2008). In contrast, the same acute stress caused an increase in grooming behavior. A footshock of .8mA was enough to increase social avoidance even 28 days later in rats (Mikics et al., 2008).

Learning and memory as well as depression-like behavior are also examined in response to stress. Eyeblink conditioning is often used to study learning and memory function. In response to stress, female rats exhibited a decreased conditioned response, while this response increased in males. Control, or non-stressed female rats showed more conditioned responses than males (Hodes & Shors, 2005). The forced swim test is frequently used to examine depression-like behavior. A rat is placed in a tank of water and allowed to swim for 10-15 minutes. A variety of behaviors are measured, such as time spent struggling and time spent immobile to assess depression. The time spent immobile is representative of helplessness, a typical symptom depression in humans. Corticosterone injections increased the time spent immobile during the forced swim test. This effect was not seen after restraint stress (Gregus et al., 2005).

Together, these results highlight a few inconsistencies in the behavioral effects of stress. Different types of stressors elicit different responses in different models of fear and anxiety. Restraint stress increases anxiety-like behavior in the open field test as well as the EPM, but social defeat only increased anxiety in the EPM, not in the open field test. Also, stress enhanced fear conditioning differently in cued and contextual conditioning. This difference is likely due to the brain regions involved in these models. In order to further explore these differences and to eliminate some of the inconsistencies between stress

protocol and behavioral model used, more studies must be conducted.

Additionally, a difference in the effects of stress is highlighted between males and females. Few studies examine the effect of stress on female rats. McCormick et al. (2007), found mixed results in female rats depending on their stage in the estrus cycle. Further studies examining the effects on female rats should be carried out.

Effects of Stress on Brain Anatomy

Neuroimaging studies have shown evidence of consistent changes in humans with certain psychopathologies. Amygdala activity is exaggerated in patients with PTSD (Chung et al., 1996) as well as panic disorder (van den Heuvel et al., 2005). In PTSD amygdala activity has been directly correlated with the severity of the disorder (Armony, Corbo, Clement, & Brunet, 2005). Results of neuroimaging studies show that the amygdala is activated in social phobias. The responsiveness of the amygdala increases during public speaking as well as the anticipation of public speaking (Tillfors, Furmark, Marteinsdottir, & Fredrikson, 2002). Similarly, like PTSD, activity of the amygdala is positively correlated with the severity of anxiety as well as increases in self-reported fear (Tillfors et al., 2001). This effect has also been seen in Generalized Anxiety Disorder. Increased knowledge of how stress affects the amygdala and other brain areas is key to understanding the behaviors associated with these different psychopathologies.

Numerous structural changes have been associated with various brain areas in response to stress. The amygdala undergoes growth during adverse experiences. In response to prolonged immobilization stress, dendrites in the BLA were 45% longer than controls. These lengthened dendrites also had increase of spines spines, which are sites of synaptic input (Vyas et al., 2006). Dendrite lengthening was observed in the BNST, although it did not reach significance. Dendritic arborization was observed in both the BLA and BNST, as evidenced by the number of branch points in dendrites, but not the central amygdala (Vyas et al., 2003, 2006). Chronic cold stress decreased spontaneous firing of CeA neurons, but an increase in firing rate after exposure to a train of footshocks (Correll et al., 2005). Cold stress also increased the responsiveness of BLA neurons (Correll et al., 2005). Additionally, chronic cold stress diminished the regulatory function of the PFC. Vyas et al., (2002) showed that repeated immobilization stress also led to dendritic shortening in the PFC.

In contrast to the amygdala, the hippocampus is reduced in response to stress. A reduction was observed in the volume of the hippocampus in tree shrews in response to stress (Czeh et al., 2001). Stress also reduces the volume of the dentate gyrus of the hippocampus (Gould , Tanapat, McEwen, Flugge, & Fuchs, 1998). This area contains adult stem cells and is a site of neurogenesis (Kozorovitskiy & Gould, 2004). Interestingly, acute stress can enhance the excitability of CA1 pyramidal neurons in the hippocampus (Shors, 2001). It is important to note that these changes are reversible. The hippocampus is noted as one of the most malleable brain regions and changes in this region may not be damage per se, but a form of synaptic plasticity (McEwen, 2008).

Similar to the behavioral effects of stress, physiological and neuroanatomical studies show a few discrepancies in the stress response, most notably, the difference in changes to the hippocampus in response to chronic and acute stress. Chronic stress reduces hippocampal volume, while acute stress has excitatory effects. This difference raises a very important question about the time-course of the effects of stress. How much stress is needed to reach the changes observed in these chronic stress studies? Currently few studies examine this time-course. These studies focus on the time delay between the onset of stress and the time of behavior testing, rather than the length of stress (Razzoli et al., 2007). Additionally, the time-course study was done using social defeat, which as mentioned previously is a psychological stressor. In order to complete the full picture, a physical stressor should be examined as well. It is imperative that the time-course of stress itself be studied. Few studies examine the effects of a single session of stress, but have many consecutive long sessions of stress. It is possible that single stress sessions can be enough to create a behavioral response.

Neurobiological Changes Due to Stress

Many different mechanisms can be studied as underlying the response to stress. This is largely due to the wide range of effects of stress on behavior as well as brain physiology. Certain neurobiological characteristics are descriptive of patients with different psychopathologies. Depressed patients exhibit hyperactivity of the HPA axis as well as exaggerated responses of ACTH and cortisol (Holsboer, 1999). PET scans of patients with panic disorder elucidate decreased binding in serotonin (5-HT) receptors (Nash et al., 2008) as well as in social phobia (Lanzenberger et al., 2007). Altered binding in 5-HT1a receptors is a possible mechanism behind anxiety disorders, such as social phobia and panic disorder (Spindelegger et al., 2009).

Because of its role as the primary modulator of stress, corticotropin-releasing hormone is a major focus of studies on the biological mechanisms of stress. CRH mRNA has been found in the central (Heim & Nemeroff, 2001), medial, and basolateral amygdala (Fekete et al., 2009). Rats that were separated from their mothers, a form of stress, show increased CRH expression in the central amygdala as well as BNST as adults. In addition, early handling, in which the rats are removed from the cage for several minutes, causes an increase in CRH as well as CRH receptor binding in the amygdala (Cratty, Ward, Johnson, Azzaro, & Birkle 1995). Since CRH causes the release of ACTH, which in turn triggers the release of corticosterone, the response of these hormones to stress is studied extensively. Early isolation, or separation from mother and littermates, for 8hrs increased ACTH and corticosterone levels as well as glucocorticoid mRNA levels (Pryce et al., 2005). A single session of restraint stress elevated corticosterone levels, compared to unrestrained controls, 30 minutes after contextual conditioning training (Cordero et al., 2003). Corticosterone levels remain elevated even 60 minutes after restraint stress (Daniels et al., 2008). Social defeat stress has also been shown to affect corticosterone and ACTH levels in rats. Defeated rats and rats that were faced with the threat of a defeat showed elevated corticosterone and ACTH when reexposed to the defeat environment 21 days later (Razzoli et al., 2007). After social defeat, defeated rats have higher levels of both hormones when compared to controls.

The fos protein can also be used to examine the effects of stress. The gene c-fos codes for the protein and is recognized as an immediate early gene. When a cell is stimulated it begins synthesizing the fos protein which then acts as a transcriptional activator. Neuronal growth factors through the ras pathway as well as protein kinase C can activate the fos protein. Activation of these pathways results in neuronal growth and differentiation. Fos was found to be highly colocalized with CRF mRNA in the medial amygdala. In response to stress, fos expression increases in the forebrain, more specifically in the medial amygdala. It is in this brain region that higher fos expression was observed after social defeat stress (Fekete et al., 2009). Expression of c-fos increased in response to psychosocial encounters regardless of stress or no stress experimental conditions

(Mikics et al., 2008). A psychosocial encounter is one in which an opponent rat blocked by a partition or in a plexiglass box is placed in the home cage of the subject. Rats bred for high levels of anxiety-like behavior on the EPM also exhibit increased fos expression after social defeat. Fos expression in these rats increased in the central amygdala, parts of the medial amygdala, and paraventricular nucleus of the hypothalamus (Frank et al., 2006). These data are consistent with evidence of increased dendritic arborization in the amygdala as well as increased levels of glucocorticoids in response to stress.

NMDA receptors became an important area of study in regard to the effects of stress through learning ability in eyeblink conditioning experiments. Deactivation of NMDA during a stressful event prevented an increase in conditioned responses to an air puff, but deactivatio after the event did not (Shors, 2001). This suggests that NMDA receptors are activated during periods of stress. In addition, NR1 hypomorphic mice that have reduced expression of NR1, which is an important subunit of the NMDA receptor, show less aggressive responses when used as residents in the resident-intruder paradigm. These rats also exhibited reduced fos expression in the medial amygdala, BLA, and dentate gyrus (Duncan, Inada, Farrington, Koller, & Moy, 2009). Together, these results suggest that NMDA receptors are important for neuronal activity in response to stress.

Serotonin 5-HT receptors are commonly a target of treatment for many psychopathologies. SSRIs inhibit the reuptake of serotonin in order to increase the amount of serotonin available at the synapse. This reuptake inhibition is done to combat desensitized 5-HT receptors, which is hypothesized to underlie some forms of anxiety. Chronic stress decreases binding to 5-HT1 receptors. Social defeat stress decreases 5-HT receptor sensitivity and also may cause decreased functioning of the receptors in the hippocampus (Buwalda et al., 2005). Studies on early maternal care showed that offspring of mothers that exhibited high licking and grooming, which are measures of good maternal care in rats, had increased turnover in 5-HT activity in the hippocampus. When activated, 5-HT receptors increase the expression of nerve-growth-factor-inducible Factor A (NGFI-A). The gene responsible for glucocorticoid receptors has a binding spot for NGFI-A on exon 1, a promoter region. When NGIF-A binds to this site, expression of glucocorticoid receptors increases in the hippocampus (Meaney & Szyf, 2005). This suggests that serotonin activity in pups can increase a long-term expression of glucocorticoid receptors. One hypothesis is that higher numbers of glucocorticoid receptors can make one more able to deal with the effects of stress and increased levels of glucocorticoids. This is plausible because it will help prevent desensitization of receptors in the hippocampus due to excess glucocorticoids. Amygdalar 5-HT activity is less well studied and has yielded mixed results. Agonists of 5-HT receptors, which increase activity, can be anxiogenic in some tests of anxiety, and anxiolytic in others (Davis, 2000). Interestingly, 5-HT can affect the function of NMDA receptors. Excitatory serotonin receptors (5-HT2a) increase the function of NMDA receptors. On the other hand, inhibitory 5-HT (5-HT1a) has the opposite effect (Bennett, 2010). Given this information, altered 5-HT1a binding in amygdala neurons would decrease the inhibition of NMDA receptors, therefore allowing conditioned fear responses and enhancing the effects of stress.

Other mechanisms have been examined as well. NK1 receptor antagonists have been shown to decrease anxiety and inhibit fear conditioning. In response to a series of footshocks, NK1 receptor mRNA increased. This increase remained significant 28 days later (Mikics et al., 2008). Neuropeptide Y (NPY) has been shown to have anxiolytic effects on the EPM, although after social defeat stress no change in NPY expression was observed (Panksepp, Burgdorf, Beinfeld, Kroes, & Moskal, 2007).

Current Study

As mentioned previously, behavioral and physiological studies examining the effects of stress have highlighted two very important questions. The first is how much stress is needed to observe the debilitating effects that can lead to certain psychopathologies? The second is how are these effects different in males and females? In order to address these questions two different experiments were carried out. Importantly, it should be noted that the models used in these experiments are not models of psychiatric disorders, but a model of stress. These models of stress target the same regions of the brain that are disrupted in various psychiatric disorders.

Experiment I- Duration of Stress

A restraint stress protocol was used to stress rats for either one or seven days. Restraint stress is a well-accepted model of stress that activates the HPA axis and also elicits behavioral effects. Restraint stress was chosen because it is less harmful to the animals than some other models of stress, such as electric footshock or forced swim. Anxiety-like behavior was examined using the EPM. This model is a good assay of levels of anxiety because it has face, construct, and predictive validity (Walf & Frye 2007). Extensive literature exists using the EPM as an assay of anxiety-like behavior in response to stress. Fear memory was examined through cued fear conditioning. This assay of fear memory was chosen because it is a well-studied and supported model of fear behavior in rodents.

This study seeks to examine the time-course of the effects of stress. Chronic stress can increase anxiety-like behavior and enhance cued fear conditioning in rodents. In contrast, a single session of stress can have minimal or protective effects. It remains unclear as to how much stress is needed to produce the negative effects of stress.

Hypothesis

In this experiment, we hypothesized that one day of stress would not affect anxiety-like behavior in the EPM and there would be no enhancement of fear memory when compared to non-stressed controls. Seven days of stress, however, would increase anxiety-like behavior as well as enhance fear memory in cued fear conditioning (Figure 3)

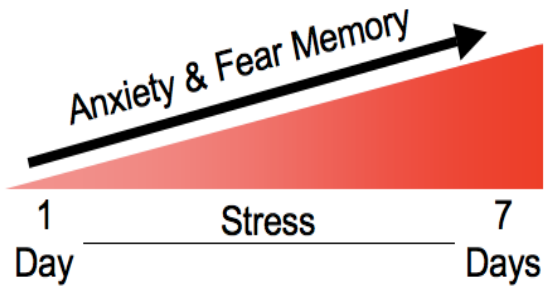

Figure 3. Hypothesis for Experiment I. It is hypothesized that 1 day of stress will have small effects on anxiety-like behavior in the EPM and fear memory in cued fear conditioning. Seven days of stress, however, should be enough to exhibit anxiety-like behavior and enhance fear memory in cued fear conditioning.

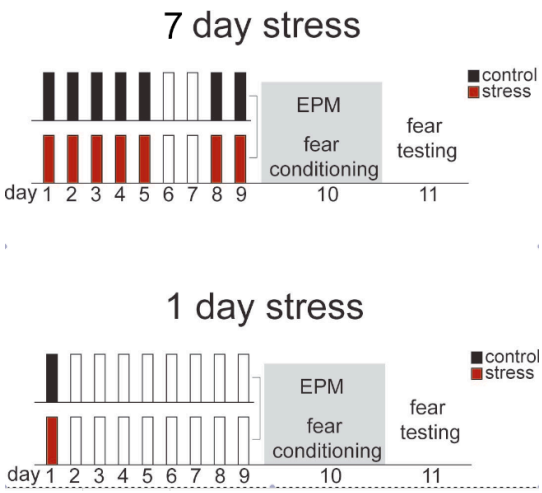

Figure 4. Experimental design. This model describes what was done on each day during the experiment. Red boxes represent restraint stress, while black boxes represent control handling. Rats exposed to 1 day of restraint or control handling were handled on day 1 and then tested on the EPM on day 10. Acquisition training in fear conditioning was also done on day 10 and then fear memory was tested on day 11. Rats exposed to seven days of restraint or control handling underwent 5 consecutive days, 2 days off and then 2 days of more handling. EPM and fear conditioning took place on day 10 and fear testing on day 11.

Experiment II- Female Response to Stress

Studies examining the female stress response have exhibited mixed results (McCormick et al. 2007). Adolescent females increased exploration in the EPM after stress, while adult females have been shown to exhibit increased exploration, as well as decreased exploration of the open arms of the EPM depending on the stage of their estrus cycle. It is clear that more studies are needed to examine the effect of stress on behavior in females. We used one day of restraint stress, as described in Experiment I, as a model of stress, and anxiety-like behavior was measured using the EPM.

Hypothesis

We hypothesized that female rats exposed to stress would experience greater effects of stress than males. This vulnerability to stress will manifest itself through increased anxiety-like behavior in the EPM.

Methods

Experiment I- Duration of Stress Animals

Male Sprague-Dawley rats (Harlan) used in this experiment were approximately 60 days old and typically weighed approximately 300-350 grams at the onset of the experiments. Subjects were housed 2-3 per cage (19 x 10.5 x 8 in.). The housing room was maintained at a constant temperature on a 12h/12h light dark cycle with lights on at 7am. Both food and water were available ad libitum.

Rats underwent 1 or 7 days of restraint stress, were tested for anxiety-like behavior on the elevated plus-maze 10 days after the first stress experience, and tested for fear memory in cued conditioning the following day (Figure 4). Animals were selected for a condition by cage order in the colony room. Animals sharing a cage were in the same condition of the experiment. Four experimental groups were used, including one day stress (n= 17), one day control (n= 18), seven day stress (n=34), and seven day control (n=36). All procedures were performed in accordance with the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Science, and followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Restraint Stress

At the same time each day, rats were removed from their home cage and weighed. They were then placed in a transport cage and taken to the experiment room. Stressed rats were then placed in a plexiglass restraint chamber for 20 minutes (Figure 5A). The chamber was placed on a white plastic platform. Stressed rats were unable to see any other rats for the duration of the stress session. Control rats remained in their transport cage in the experiment room for 20 minutes and were also placed on a white plastic platform. After 20 minutes stressed rats were removed from the restraint chamber, placed in a transport cage, and taken back to their home cage. Control rats after 20 minutes were taken back to their home cage. Stress and control handling occurred at approximately the same time each day, always in the morning between 10:00am and 12:00pm.

Elevated Plus-Maze

The elevated plus-maze (EPM) is made of two open arms and two closed arms of equal size. The arms are arranged so that arms of the same type are opposite each other (Figure 5B). The closed arms have three 30cm high walls that enclose them with the side facing the center of the maze left open. The maze is elevated 75cm off the ground. Rats were individually placed in the center of the maze facing an open arm, away from the experimenter, and allowed to explore the maze for 5 minutes. Entry into an arm was defined as the rat having all four paws in the same arm. The number of entries as well as the time spent in each arm were recorded.

Cued Fear Conditioning

Rats were removed from their home cage in the colony room, placed in a transport cage and taken to the experiment room. The rats were placed individually into a plexiglass chamber (12” X 10” x 8.5” height) with a steel grid floor (Figure 5C). The chamber contained a mounted audio speaker. The test consisted of two phases: training and testing. The training phase took place on the same day and approximately two hours after testing in the EPM. Rats were placed into the plexiglass chamber and allowed to explore for 2 minutes to establish baseline freezing behavior. Freezing is defined as the absence of all movement other than that required for respiration. After two minutes, one of two audio tones (2.8kHz or 300-500Hz) was presented with a footshock (not exceeding 5mA) occurring during the last second of the 20-second tone. The other tone was presented 40 seconds later with no footshock occurring. The process was repeated 50 seconds later until a duration of 10 minutes was reached. Freezing behavior was measured, as either freezing or not freezing, every 10 seconds upon entry into the chamber. The chamber was cleaned after each use with a 70% ethanol solution. The chamber was dry upon entry by each rat. The testing phase occurred 24 hours after the conclusion of the training phase. Rats were placed in a different chamber, without the metal grid floor, during the testing phase. To be sure that the freezing behavior was due to the auditory cue and not the context of the chamber, a different cleaner was used to create a different odor in the chamber, as well as different wall colors. The rats were allowed to explore for two minutes to establish baseline freezing. The same auditory tones presented in training were presented again for 20 seconds. The tones were presented

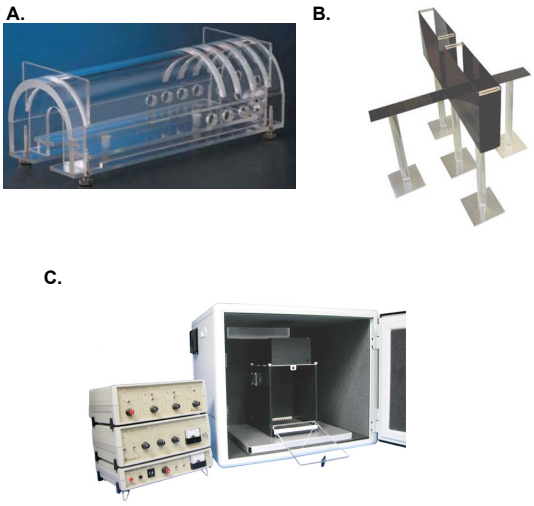

Figure 5. Apparatuses used for stress and behavior testing. A. The plexiglass chamber used for restraint as a stress protocol. Rats are placed inside for 20 minutes. B. The Elevated Plus Maze that is used as an assay for anxiety-like behavior. Rats are allowed to explore the maze freely for 5 minutes. C. Cued fear conditioning chamber used as an assay for fear memory. The chamber has a metal grid floor with a speaker mounted inside.

40 seconds apart and each tone was presented three times. No footshock was associated with either tone during the testing phase. Freezing behavior was scored (freezing or non freezing) every five seconds during the duration of the testing phase except during the presentation of the auditory tones.

Statistical Analysis
Values are presented as means ± SEM. Behavioral data were analyzed with a two-way Analysis of Variance (ANOVA) with stress experience (restraint stress vs. control) and amount of stress (1 day vs. 7 days) as the between-subjects factors. Bonferroni post-hoc tests were used for multiple comparisons. In each analysis a p-value of $p < 0.05$ was defined as statistically significant. Paired t-tests were also used to compare freezing behavior in response to tones in cued fear condition. A p-value of $p < 0.05$ was considered statistically significant.

Experiment II- Female Response to Stress
Animals
Female Sprague-Dawley rats used in the experiment were from the vendor Harlan as well as some that were bred within the animal colony. The animals were approximately 60 days old and typically weighed 150-200 grams at the onset of the experiments. Subjects were housed 2-3 per cage (19 x 10.5 x 8 in.). The housing room was maintained at a constant temperature on a 12h/12h light dark cycle with lights on at 7am. Both food and water were available ad libitum.

Restraint Stress
Female rats were exposed to one 20 minute session of restraint stress as described in Experiment I.

Elevated Plus-Maze.
Female rats were placed facing the same open arm of the EPM and allowed to explore the maze for five minutes as described in Experiment I.

Statistical Analysis
Values are presented as means ± SEM. Behavioral data were analyzed with a student's t-test comparing restraint stress to control rats. In each analysis a p-value of $p < 0.05$ was defined as statistically significant. Additionally, female rats were compared to males using a two-way ANOVA. A p-value of $< .05$ was considered statistically significant.

Results

Duration of Stress

Elevated Plus-Maze
Anxiety like behavior in the elevated plus maze was measured in male rats after one or seven days of stress. The elevated plus maze assesses the rat's natural tendency to explore a novel environment. It is well accepted that anxiogenic factors lead to decreased exploration of the open arms of the EPM. A Two way ANOVA was used to compare the proportion of time in the open arms in relation to total time spent on the maze. There was no main effect of treatment (Control vs. Stress) across time, $F(1, 101) = 1.70$, $p = 0.19$. There was, however, a main effect of time (one vs. seven days) $F(1, 101) = 15.60$, $p < 0.001$. The rats exposed to seven days of stress actually spent more time in the open arms of the maze, and thus exhibited lower levels of anxiety-like behavior, compared to rats exposed to one day of stress. This finding, of course contradicts our hypothesis and its meaning will be fully explored in the discussion section. The treatment by time interaction was not significant, $F(1, 101) = 2.80$, $p = 0.097$. Bonferroni post-hoc comparisons showed that after seven days of stress, rats spent significantly less time in the open arms of the EPM ($M= 0.13$, $SD= 0.11$) when compared to controls ($M= 0.21$, $SD= 0.17$), $p< .05$ (Figure 6.). There was no difference between control and stress rats after one day ($M=0.025$, $SD=0 .04$) and ($M=0.06$, $SD= 0.07$), respectively.

In order to measure overall motor activity in the elevated plus maze, the total number of arm entries was also examined. The number of entries to open and closed arms measures the overall locomotor activity. This measure checks whether or not differences in open arm exploration

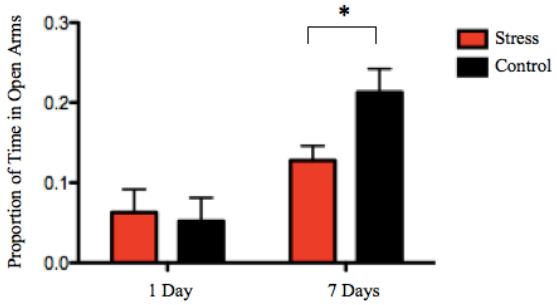

Figure 6: Time Spent in Open Arms for One Day and Seven Day Stressed Rats. A single session of restraint does not significantly alter exploration of open arms in the EPM. This is quantified as the mean proportion of time that the rat spends in the open arm of the EPM during five minutes of free exploration. Repeated restraint stress for seven days reduces exploration of open arms in the EPM. The repeated stress causes a reduction in the mean proportion of time a rat spends in the open arm of the EPM, compared to controls. A reduction of time spent in the open arm is consistent with increased anxiety-like behavior. *($p < 0.01$).

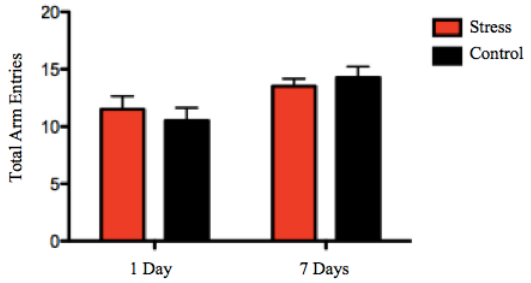

Figure 7: Total Arm Entries for One Day and Seven Day Rats. Stress did not exert a significant effect on the total number of arm entries, compared to control groups. This indicates that the effects of stress on exploration of open arms in the EPM is unlikely to be due to decreased motor activity, or other non-specific effects.

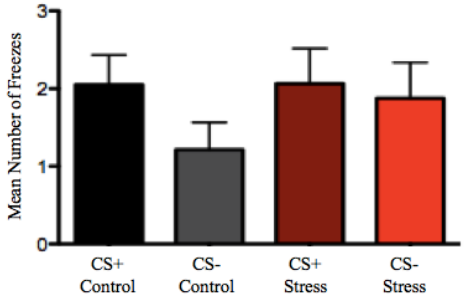

Figure 8: Freezing in response to CS+ and CS- tones in one day stress and control rats. To explore whether stress exerts an effect on Pavlovian conditioning, we examined the differential fear responses between the CS+ and the CS-. The differential fear conditioning was impaired by a one day stress, compared to controls. This result implies that one day of stress may cause fear generalization to non-fearful stimuli. * $p < 0.05$

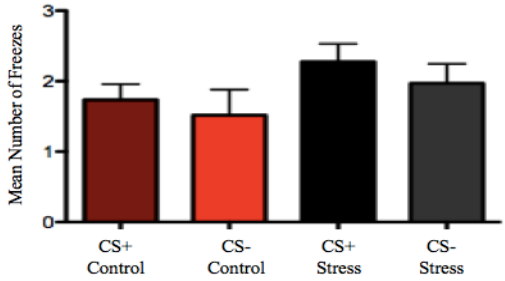

Figure 9: Freezing response to CS+ and CS- tones after seven days. To explore whether stress exerts an effect on Pavlovian conditioning, we examined the differential fear responses between the CS+ and the CS-. Seven days of stress did not significantly impair differential fear conditioning compared to controls.

were due to changes in overall activity in the EPM. A two way ANOVA showed no main effect of treatment (control vs. stress) on total number of arm entries in the EPM, $F (1, 101) = 0.015$, $p > 0.05$. There was a main effect of time (one vs. seven days), $F (1,101) = 10.13$, $p < 0.05$. The treatment by time interaction was not significant, $F (1, 101) = 0.90$, $p = 0.34$. Bonferroni post-hoc comparisons showed no difference between total number of arm entries of control and stress rats at one or seven days (Figure 7).

Cued Fear Conditioning

Fear memory in Pavlovian conditioning was also assessed through fear conditioning in male rats stressed for one or seven days. Memory in fear conditioning can be determined by the difference score during fear testing. This score is calculated by subtracting the mean number of freezes during the tone that was not paired with the footshock (CS-) from the mean number of freezes in response to the tone that was paired with the footshock (CS+). None of the rats exhibited freezing behavior during the first two minutes in the fear-conditioning chamber. A paired t-test showed that one day control rats froze more to the CS+ tone than to the CS- tone, $t(13) = 2.32$, $p < 0.05$. Rats stressed for one day did not exhibit this difference in freezing response to the tones (Figure 8). After seven days of stress, rats showed a trend towards a decreased response to the CS+ tone, but this difference was not significant when compared to controls, $t(45) = 1.59$, $p > 0.05$ (Figure 9).

Female Response to Stress

Anxiety like behavior in the EPM was also assessed in female rats after one day of stress. Female rats exposed to one day of stress or control handling were allowed to explore the EPM, as described previously, for 5 minutes. Anxiety-like behavior was measured by the proportion of time spent in the open arms. There was a trend towards less time spent in the open arms by controls (M= 0.20, SD= 0.11) than stress rats (M= 0.33, SD= 0.18), however, A student's t-test showed that this difference was not statistically significant, $t(11) = 1.39$, $p> 0.05$ (Figure 10.). The total number of arm entries was also measured to assess overall motor activity. A student's t-test showed no difference in total number of arm entries between stress (M= 18, SD= 4.39) and control (M= 22, SD= 11.4), $t(11)= 0.97$, $p >0.05$ (Figure 11).

Additionally, female rats were compared to the male rats exposed to restraint stress and control handling from the previous experiment. A two way ANOVA showed that there was a main effect of gender on the proportion of time spent in the open arms of the elevated plus maze, $F(1, 44) = 22.95$, $p< 0.0001$. There was no main effect of treatment (control vs. stress) and no significant interaction, $F(1, 44) = 2.41$, $p > 0.05$, and $F(1, 44) = 1.71$, $p > 0.05$ respectively (Figure 12A). Similar results were found in total arm entries when compared to males. There was a main effect of gender, $F(1, 44) = 34.72$, $p < .0001$. There was no main effect of treatment, $F(1, 44) = 1.11$, $p > 0.05$ and the interaction was not considered significant, $F(1, 44) = 2.79$, $p > 0.05$ (Figure 12B.)

Discussion

Duration of Stress

Anxiety-Like Behavior

Acute stress can have enhancing effects on memory tasks (Shors 2001), whereas chronic stress is associated with enhancing emotional behavior and possibly leading to the development of various psychopathologies (Pryce et al. 2005). This study sought to answer the question of how much stress is needed to enhance emotional behavior by examining anxiety-like behavior in the EPM as well as fear memory in cued fear conditioning. Statistical analyses showed no difference between one day control and one day stressed rats in the proportion of time spent on the open arms of the EPM. There was a difference observed between

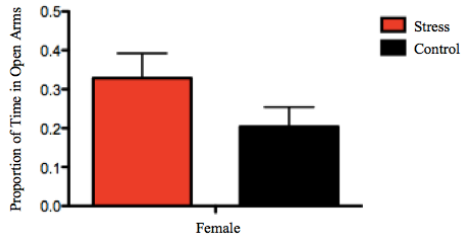

Figure 10: Proportion of time spent in open arms by female rats. A single session of restraint in female rats does not significantly alter exploration of open arms in the EPM. This is quantified as the mean proportion of time that the rat spends in the open arm of the EPM during five minutes of free exploration.

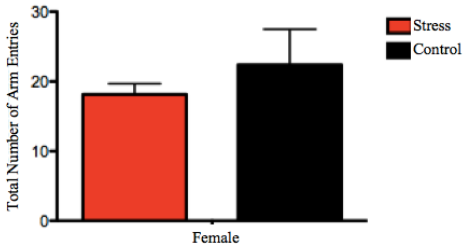

Figure 11: Total number of arm entries by female rats. Stress did not exert a significant effect on the total number of arm entries, compared to control groups in female rats. This indicates that the effects of stress on exploration of open arms in the EPM is unlikely to be due to decreased motor activity, or other non-specific effects.

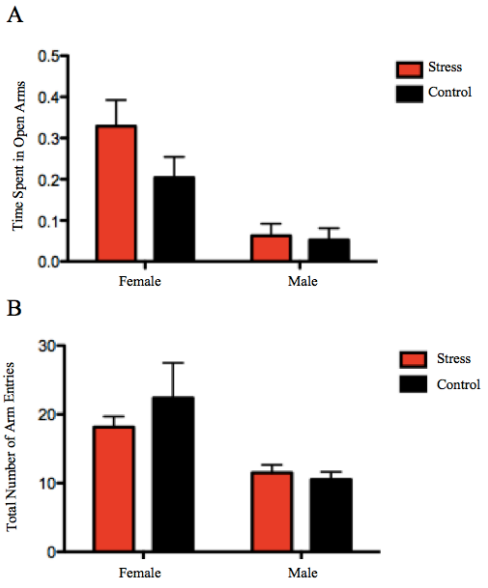

Figure 12: Comparisons between male and female rats. A. A single restraint session did not cause a difference in open arm exploration in the EPM between control and stress rats in males or females. There was, however, a significant increase in open arm exploration by control and stress females when compared to control and stress male rats. B. Total number of arm entries measured locomotor activity in male and female control and stress rats. Control and stress rats did not differ in total number of arm entries in males and females. Female rats, however, entered a significantly greater number of arms in the EPM. This suggests greater locomotor activity in female rats after one day of stress.

control and stress rats after seven days. This difference suggests an increase in anxiety-like behavior after seven days of stress. These results are consistent with previous studies examining the effect of restraint stress on anxiety-like behavior in the EPM (Vyas et al. 2006).

Interestingly, a two way ANOVA revealed a significant main effect of time, or amount of stress, on open arm exploration. Rats exposed to seven days of stress or control handling actually spent more time in the open arms than the one day stress and control rats. This finding could be explained by a number of factors. The first is that because the rats exposed to seven days of stress were handled more, they may have felt more comfortable in the EPM. The second is the time delay between the last session of stress and the time of testing in the EPM. Rats exposed to one day of stress were tested nine days after their last session of stress, whereas the seven day rats were tested the very next day. Interestingly, this delay does seem to make a difference in anxiety-like behavior in the EPM. Mitra, Jadhav, McEwen, Vyas, and Chattarji (2005) found that rats exposed to a single session of stress exhibited increased anxiety-like behavior when tested 10 days later. Additionally, another study, using the same protocol as presented in this experiment, tested rats exposed to a single session of stress the very next day. When tested the day after stress, rats exposed to a single stress did not exhibit this increase in anxiety-like behavior (Rosenkranz, Venheim, and Padivall, 2010). It is quite possible that we are seeing this effect in this experiment as well. This also creates the problem that differences in anxiety-like behavior in the EPM could be due to factors not related to stress. Because the rats exposed to one and seven days of stress were handled differently, it is important to compare them to their matched controls. When this comparison is made, there is no difference between one day stress and control rats, however seven day stressed rats exhibit decreased exploration when compared to their matched controls. It would be interesting to see this time delay effect on seven day stressed rats as well. Interestingly, the one day control rats were very similar in behavior to the one day stressed rats. This effect could be due to a somewhat stressful control handling, or possibly other factors not related to stress in this experiment.

Similar results were found for the total number of arm entries. This measure was used as an assessment of overall motor activity. This measure is important because it allows us to see whether or not a difference, or lack thereof, in the EPM is due to a difference in overall activity on the maze. A two way ANOVA found a significant main effect of duration of stress on total number of arm entries, once again rats exposed to seven days of stress or control handling exhibit greater overall activity on the EPM. This interesting finding could be explained by the same factors discussed in response to the time spent in the open arms. There was no difference between control and stress rats at one or seven days. These results suggest that the decreased open arm exploration by seven day stressed rats, when compared to seven day control rats, is not due to a difference in overall motor activity on the maze.

Anxiety-like behavior was decreased after seven days of stress. This interesting finding could be explained by the time delay of testing in one day stressed rats. This marks the first step in examining the time-course of the effects of stress on anxiety-like behavior. In order to fill in the time-course these effects should also be examined after 3 and 5 days of stress (Figure 13). By looking at the effects of stress at these times we will be able to see when changes in anxiety-like behavior begin.

Fear Memory

Cued fear conditioning, which pairs a conditioned response

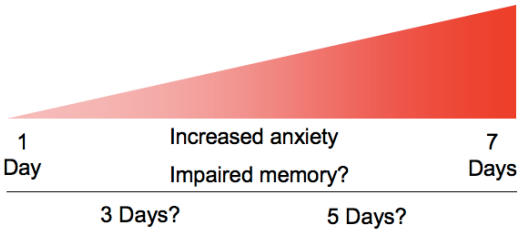

Figure 13. Time-course of the effects of stress on behavior. After seven days of stress and increase in anxiety-like behavior was observed. Nonsignificant trends suggested impaired memory after seven days of stress. In order to fill in the time course and see at what point these effects of stress begin to show, behavioral effects should be measured after 3 and 5 days of stress.

(auditory tone) to an unconditioned response, (footshock) is on that logic we should expect to see an increase in freezing behavior in response to the tone that was paired with the footshock. This result would be expected because of anatomical studies on the amygdala and hippocampus. It has already been observed that amygdala neurons increase dendritic arborization in response to stress, while the opposite effect is seen in the hippocampus. Because of these anatomical studies we can assume that a memory task that is hippocampal-independent should yield enhanced fear memory. Statistical analysis, however, did not support this hypothesis. These results are consistent with those of Cordero, et al. (2003). There was a trend towards decreased fear differentiation after one day of stress. This was supported by the trend for one day stressed rats to freeze similarly to the CS+ tone and the CS- tone. This was in contrast to the one day control rats that showed a trend of greater freezing to the CS+ tone than the CS- tone, although these trends were not statistically significant. These results suggest a possible fear generalization after one day of stress. Seven day rats showed a trend towards decreased freezing in response to the CS+ tone and similar to the one day stressed rats did not exhibit differentiation between the CS+ and CS- tones. These results suggest a possible memory impairment (Figure 13). Interestingly, the seven day control rats exhibited behavior during fear testing that was remarkably similar to the one day stress group. This similarity suggests two things. The first is that these observations are simply due to chance, as none of the results were considered statistically significant. The second is that it suggests our control handling may be considered slightly stressful.

Examining the Stress Protocol

The restraint stress protocol used in this experiment was limited to 20min sessions in order to prevent habituation. A two day break after 5 consecutive days of stress was also used to prevent this effect. This protocol is less than the time of restraint and has fewer days of restraint than seen in other experiments (Vyas et al. 2002, Daniels et al. 2008). Many restraint stress protocols use 6hr sessions of restraint stress for 21 consecutive days. Observation of animals during longer stress sessions tends to show a decreased response to the restraint throughout the session. Even during our 20 minute sessions of stress, by the end of the period the rats exhibit little struggling and seem to adjust to the restraint condition. Our results suggest that the stress protocol was somewhat effective, as is evidenced by the increased anxiety like behavior after seven days of stress, but only when compared to their matched controls. In addition, we were able to replicate the effect of increased anxiety-like behavior in rats exposed to a single session of stress, when tested nine days after the last stress session (Mitra et al., 2005). Other factors could be used to examine the effectiveness of the stress protocol. Levels of glucocorticoids could be measured at the start of the experiment as a baseline measure, after the last stress session to assess the effectiveness of the stress protocol, as well as at the end of the experiment after fear conditioning. Other behavioral factors could be measured as well, such as risk assessment in the EPM (a scanning head movement) and also over the edge exploration in which the rat places its head around the bottom edge of the maze or around the walls of the closed arms, which is indicative of anti-anxiety-like behavior.

Additionally, brain anatomy studies could be done to examine the physiological change in neurons in response to stress. Previous studies have shown dendritic arborization in the amygdala in response to chronic stress (Vyas et al. 2002, 2006). In the current study brain anatomy was not examined due to potential confounds caused by fear conditioning. This process is considered stressful in and of itself and would therefore hinder our ability to say the physiological changes were due to the restraint stress alone. Future studies would benefit by examining glucocorticoids as well as brain anatomy in response to seven days of chronic stress. It would also be interesting to examine the neuronal correlation with these results by looking at dendritic length and spine density in different areas of the brain. Observing the anatomy between one day and seven day stress and control rats would allow us to attribute anatomical changes to the behavioral changes observed in the experiment. Electrophysiology is also of use. This process consists of examining the activity of individual neurons. Electrophysiology has been used in our laboratory, although was not studied in this experiment. Recording from individual neurons could also help to quantify a difference in stress between one and seven days. This could be used to quantify changes in brain activity in different regions of the brain and therefore explain behavioral changes that were observed in the study.

Female Response to Stress

In a second experiment we examined anxiety-like behavior in the EPM in female rats that underwent one day of stress or control handling. This was done to see if females were more vulnerable to effects of stress. Statistical analysis showed no significant results in open arm exploration of the EPM. There was a visible trend, however, towards increased open arm exploration in stressed rats. There was no difference in total number of arm entries. This evidence is consistent with increased open arm exploration in adolescent female rats, although results are mixed when examined in adult female rats (McCormick et al. 2007). It is important to note that both control and stress groups consisted of rather small sample sizes (n= 5 and n=8, respectively). This small sample size could explain the lack of significant results. Another factor that could play a role in anxiety-like behavior would be stage of the estrus cycle. McCormick et al. (2007) found that female rats in the estrus phase spent more time in the open arms of the EPM, while this difference was reversed in diestrus phase females. This factor was not accounted for in this experiment. Another factor that may have played a role in these results is that some of the female rats in the experiment were ordered from a vendor (Harlan), while others were bred within the animal colony. This could create an inherited behavioral difference that we were unaware of. In order to control for this both the stress and control groups had both types of rats within the group. However, considering the small sample size differences between the purchased rats and the bred rats could produce a bias in the results.

The stress protocol could also be an explanation for the lack of significant results. We wanted to see if female rats were more vulnerable to stress than male rats. In order to examine this, a suboptimal stressor must be used. This suboptimal stressor is important because if we used a stressor that was known to have a strong effect, we wouldn't be able to quantify the difference between the males and females. When a suboptimal stressor is used, if female rats are more vulnerable to stress, they will exhibit a response to the stress that would not be observed in males. The one day stress with a time delay serves this purpose quite well in this scenario. It is possible, however, that it is still not enough to observe an effect even if females actually are more vulnerable to the effects of stress. In order to uncover this mystery further, the effects of stress in females will need to be studied with a larger sample size, as well as at various amounts of stress, similar to what was done with the male rats in experiment I. As with the previous experiment, it would be interesting to examine glucocorticoid levels as well as brain anatomy and individual neuron activity to compare the effects in male and female rats.

In addition the female rats were compared to the male rats for proportion of time spent on the open arms, as well as total number of arm entries. Statistical analysis showed a main effect of gender on both of these results. Female rats spent significantly more time in the open arms when compared to males. There was also a difference in total number of arms entered. Female rats exhibited much more activity on the maze than did males. One reason for this difference could be different testing conditions. Female rats were tested on the EPM by an automated system in a room lit with red light. In contrast, male rats were scored on the EPM by human observation and a dimly lit room with white light, which allowed for increased human error in the scoring of the male rats.

Conclusion

The effects of a single session of restraint stress compared to seven days of restraint stress were studied in order to see how much stress is needed to induce increased emotional behavior. Statistical analysis did not support the hypothesis that seven days of stress would be enough to increase anxiety-like behavior in the EPM or fear memory. Rats exposed to seven days of stress actually showed a decrease in anxiety-like behavior in the EPM. In order to verify this effect future studied must be done examining the time delay after stress and before behavioral testing. Fear memory was not enhanced after one day or seven days of stress. There were trends that suggest fear generalization after one day of stress and possible impaired memory after seven days. In order to verify these trends, further studies must be conducted.

Secondly, we sought to study the effects of stress on female rats. Statistical analyses did not support our hypothesis, as stressed rats did not exhibit increased anxiety-like behavior in the EPM when compared to controls. Interestingly, when compared to male rats, females had increased open arm exploration and increased motor activity. Further studies should study these effects in females compared to males at various stress amounts as well as take into account the stage in estrus cycle.

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