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Evaluating Stressor Controllability Effects on Cognitive Flexibility and Catecholamine Release in Male and Female Rats

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Abstract

Prefrontal cortex dysfunction is implicated in a range of stress related neuropsychiatric disorders, with cognitive flexibility deficits being a major symptom. Clinically, females are much more likely to develop a neuropsychiatric disorder following a traumatic life event. The ability to exert control over stressors has been shown to mitigate the effects of stress in male rats but not in female rats in some behavioral tests. Catecholamines are known to have elevated release within the prefrontal cortex following acute stress and to contribute to prefrontal cortex dysfunction. In this study, male and female rats were subjected to inescapable stress and escapable stress as an animal model of stress coping. One set of groups received attentional set shift testing to evaluate cognitive flexibility following stress. *In vivo* microdialysis during stress was conducted for the second set of groups. Overall, the data showed that escapable stress in male rats blunted both catecholamine release within the ventromedial prefrontal cortex and cognitive flexibility deficits following stress. By contrast, in females, the data showed that escapable stress failed to mitigate both the increase in catecholamine release within the ventromedial prefrontal cortex and cognitive flexibility deficits following stress. Interestingly, the data demonstrated significantly that inescapable stress blunted dopamine release in the ventromedial prefrontal cortex in female rats. Future research needs to be performed with a larger number of subjects due to the lack of statistical significance for portions of this data attributable to a low number of subjects. These experiments provide possible evidence for why female rats lack the protective effects of behavioral control over a stressor. In addition, this research contributes to the growing body of literature on stress controllability consequences, which could be applicable to human neuropsychiatric disorders.

Introduction

The prefrontal cortex (PFC) provides top down regulation of behavior, thought, and emotion. However, uncontrollable stressors may interfere with PFC function, thereby impairing this regulation[19]. Undergoing traumatic experiences is a key contributor to the development of disorders such as post-traumatic stress disorder, anxiety, and depression[1]. Clinically, one of the greatest risk factors for post-trauma related neuropsychiatric disorders is being female.

A large body of literature shows that traumatic life events are consistently associated with the development of neuropsychiatric disease[1,34]. Demographic data indicates that women are over twice as likely to develop PTSD and anxiety disorders than are men and are almost three times more likely to develop substance abuse disorders than males[2]. These disorders can severely hinder quality of life and current treatment options can be ineffective. However, not all people who experience traumatic life events develop these disorders.

Prior studies have shown that factors during the early postnatal stage (including nutrition, environmental enrichment, and hormonal fluctuation) influence vulnerability to trauma related neuropsychiatric disorders[3] . However, the current study is focused on experiential factors that occur during the trauma because these are expected to play a key role in the mitigation and development of the outcome of the experience. In previous studies, it has been shown that the perceived degree of control an individual has over a stressor affects the behavioral outcomes of the stressor[4]. Perceived control is defined as the expectancy that the behavior/stressor is within the individual's abilities to overcome the traumatic situation[35] . Uncontrollable stressors can result in a number of negative effects on humans, such as poor

work performance, anxiety, and depression[5]. Therefore, it is of utmost importance to investigate the neural foundations that contribute to stressor vulnerability.

A vital component of coping with a traumatic experience is the ability to exert control or, have the perception of control over the stressor[6,7]. Maier and Seligman were the first to demonstrate the importance of control, as well as a phenomenon called "learned helplessness" [8]. In phase 1 of this experiment, dogs were restrained in a harness and received a series of tail shocks. One group of dogs was able to terminate each shock by pressing a panel; this was called escapable shock (ES). The other group of dogs were yolked to the ES group and so received the same duration and intensity of shock, but panel pressing had no effect on their own shock. This was called inescapable shock (IS). Thus, the ES subjects had behavioral control over the duration of each of the tail shocks, while the IS subjects did not. During phase 2 of this experiment, the dogs escape behavior was tested in a shuttle box test. During the shuttle box test, a consistent shock was delivered to the feet of the animals. The animals had to move to the other side of the box to terminate each shock. In contrast to the ES condition, the IS dogs passively accepted the shocks in the shuttle box instead of trying to escape because they had learned that shock delivery was independent of responding to the stimulus. These findings were interpreted as learned helplessness due to the failure to attempt to escape the shock. This type of procedure in animals is now utilized to mimic control and lack of control over traumatic life events in humans.

The current study utilized a similar type of procedure using rats as the animal model. In this paradigm, two rats receive identical physical stressors. Both rats are placed in small plexiglass enclosures with a wheel mounted on the front. Their tails extend from the rear of the

box and electrodes are fastened to their tails. For one of the rats, turning the wheel terminates each of a series of tail shocks. Thus, the subject is given behavioral control over the duration of each shock. This rat is the escapable shock (ES) rat. The second rat of the pair (inescapable shock (IS)), is not able to turn the wheel and each tail shock terminates whenever the ES subject turns the wheel. Therefore, the IS subject has no control over the duration of the stressor. A third rat remains in their home cage (HC) as a control and does not receive any tail shock. Given that both the ES and IS animals received the same physical stressor, any neurobiological or behavioral differences between them are due to the ability to exert control over the stressor. This allows researchers to study the various aspects of the effects of having control over a stressor. Behavioral control has been shown to blunt many of the neurobiological and behavioral outcomes of stressor exposure in rats[12]. Overall, the degree of control an organism has over a stressor determines the neurobiological and behavioral outcomes to the stressor[3].

Neural Circuitry

Dorsal Raphe Nucleus (DRN)

Exposure to IS leads to numerous behavioral changes that have been characterized as anxiety-like and depression-like[26]. Importantly, the ability to exert control (ES) prevents the occurrence of all of these behavioral changes in male rats, with ES subjects not differing from HC controls[26]. The DRN is a large serotonergic nucleus that sends extensive projections to the periaqueductal grey (PAG) and the amygdala which are key for the expression of the aforementioned behaviors[27,28]. Increasing serotonin (5-HT) concentration in the amygdala enhances anxiety-like behaviors[29] , and increasing 5-HT concentration in the dPAG alters

depression-like responses[30]. This shows the importance of the DRN serotonergic projections to the PAG and amygdala to generate behavioral reactions to stressors.

It has been shown that IS causes an increase in extracellular serotonin in the DRN and in its projection regions[31]. Pharmacological inhibition of the DRN during IS, via the consequent reduction of serotonin release in projection regions such as the amygdala and PAG, remarkably eliminates the behavioral outcomes that typically follow IS[32]. These findings show the necessity of the DRN and its projection regions to generate the behavioral consequences of IS in rodents. Behavioral control over a stressor inhibits the activation of the DRN and thus of serotonin release in its projection regions[33]. Thus, male ES animals are protected from the behavioral and neurobiological consequences of stress due to this inhibition of the DRN.

Prefrontal Cortex

The PFC plays an important role in regulating our thoughts, emotions, and actions through extensive connections to other regions of the brain. It has been shown that the PFC plays a critical role in inhibiting inappropriate actions and promoting task-relevant processes[13]. This is called top-down regulation by the PFC. PFC processes allow for proper responses to a changing environment, for example, the ability to shift attentional set to relevant new dimensions and alter behavior to seek rewards[14,15]. The PFC also monitors errors and communicates to other brain regions with regard to mistakes and the need to change strategies to achieve goals[18]. Specifically, the ventromedial PFC (vmPFC) has vast connections to subcortical structures such as the amygdala, nucleus accumbens, hypothalamus, and brain stem structures that generate emotional responses and habits[16,17]. The ability of the PFC to perform these functions relies on proper neural connections and are highly sensitive to neurochemical

changes[19]. There are two key neural components underlying stressor controllability effects that have been researched recently: the neural processes responsible for uncontrollable stressors' behavioral effects and how control over physically identical stressors produce resiliency. As described above, the activation of the serotonergic DRN and its projections to its effector regions (*i.e.* PAG) are critical for producing the behavioral consequences of IS[12]. Further research has shown that the ventromedial prefrontal cortex (vmPFC), specifically the prelimbic (PL) subregion that provides top-down inhibitory control over the DRN, is critical for the protective effects of control to occur[12]. Thus, when behavioral control is present, projections from the vmPFC inhibit the stress induced activation of the DRN[12]. Remarkably, it has been recently shown that controllability over a stressor in female rats does not mitigate the adverse behavioral and neurobiological effects of a stressor as it does in male rats[9]. Research has shown that despite successful acquisition of the behavioral action to stop tail shock delivery, and the presence of the vmPFC to DRN circuitry, female rats failed to engage the prelimbic neurons that inhibit the activity of the DRN[9]. This lack of engagement of the protective vmPFC to DRN projection plays a pivotal role in the lack of stressor controllability protective effects in female rats.

Effects of Catecholamines

Catecholamines have been shown to have profound effects on the ability of the PFC to function. The primary sources of norepinephrine and dopamine release within the PFC are the locus coeruleus (LC) and ventral tegmental area (VTA), respectively[41]. There is an inverted U-shaped influence of dopamine and norepinephrine; either too much or too little norepinephrine and/or dopamine impairs function of the PFC[19]. Research has shown that

dopamine and norepinephrine release are increased during exposure to acute, uncontrolled stressors[20,21]. The levels of norepinephrine that are released during normal, alert stages engage α_{2A}-receptors to increase neuronal firing, which optimizes working memory. During stress, high levels of norepinephrine inhibit PFC function by stimulating lower affinity α_1-receptors[22], which decreases neuronal firing in the PFC. Similarly, excessive stimulation of the D1 receptors in the PFC by dopamine decreases the firing rate[23]. The presence of high concentrations of catecholamines in the prefrontal cortex contributes to loss of top down regulation, which promotes the activation of subcortical structures such as the amygdala[24]. In contrast, it has been shown that controllable stressors do not stimulate the same release of dopamine within the PFC as do uncontrollable stressors in male rats[25]. The differential release of catecholamines during IS and ES may contribute to the differential behavioral responses to uncontrollable and controllable stressors.

Hypersecretion of corticotropin-releasing factor (CRF) has been implicated in stress related psychiatric disorders[53]. Previous research has shown that CRF is a key modulator of behavioral stress responses in rats[47]. Specifically, the LC and the VTA are key targets for CRF innervation[47,48]. The VTA in rats receives CRF afferents from the lateral bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the paraventricular nucleus of the hypothalamus[49,50]. The LC in rats receives CRF afferents originating from the central nucleus of the amygdala, paraventricular nucleus of the hypothalamus, Barrington's nucleus, and the nucleus paragigantocellularis[47]. It has been shown that there is a dose dependent effect of CRF on the LC[51]. Moderate levels of CRF within the LC increase mPFC activation and high levels of CRF within the LC inhibit the activation of the mPFC during cognitive flexibility tasks[51].

Additionally, it has been shown that dopamine cells within the VTA receive CRF-positive synapses that are mostly excitatory[52]. Interestingly, it has been shown that the LC neurons of female rats are more sensitive to CRF, with CRF overexpression in female mice increasing LC firing three fold[53]. This is due to differences in receptor trafficking[53].

Female rats with circulating estrogen have greater activation of noradrenergic and dopaminergic actions than do male rats[24]. This is paralleled in human females who catabolize catecholamines less efficiently than males[24]. Additionally, it has been shown that there are larger dopaminergic projections from the VTA to the PFC in female rats[40]. Given that females fail to engage protective neural circuitry during controlled stress[9], the current study seeks to determine whether compared to males, there are differences of catecholamine release in the female PFC during acute stress that may contribute to lack of controllability. There is a lack in the literature pertaining to differential release of norepinephrine in the vmPFC of male rats during IS and ES. There is also a gap in the literature pertaining to the release of catecholamines in the vmPFC in female rats. Differential release of catecholamines between male and female rats could contribute to their different behavioral stress responses. This could also provide evidence for the neural mechanisms related to the overall higher prevalence of stress related neuropsychiatric diseases in females.

Attention Set Shift Test

Cognitive impairment, particularly caused by dysfunction in the circuitry of the PFC, has been implicated in many neuropsychiatric disorders in humans[10]. In general, patients with a range of neuropsychiatric disorders have deficits in their cognitive flexibility[10]. Cognitive flexibility is broadly defined as an executive function that allows for the adaptation of behaviors

in response to environmental changes[36]. Cognitive flexibility can be modeled in animals through an attention set shift test (ASST). In this assay, a reward (usually a cheerio for rats) is buried in 1 of 2 pots. The animal must differentiate between relevant and irrelevant cues to determine which pot the cheerio is buried in. For example, the cheerio may only be buried in a certain type of media with differential odors being a distractor, or the odor may be the relevant cue and the media would be the distractor. An attentional set is inferred when an animal learns that a general set of rules can be applied to more complex stimuli to acquire a reward[11]. For example, an animal will learn to find the reward based on an association with a reward and an odor. The animal applies its attentional set by ignoring the irrelevant cue (*i.e* changing medias) and finding the reward solely based on the odor. A cognitive set is formed when the rat identifies that a single odor (or media type) can be used to find a reward because the rat associates a specific odor with the reward[11]. The reversal stage of the ASST measures cognitive flexibility[11]. During the reversal stage, the previously negative stimuli (*i.e.* the negative odor) becomes the positive one and the animal must learn to ignore the previously positive stimuli (*i.e.* the positive odor) while the irrelevant cue (*i.e.* medium) remains[11]. This challenges the rat's flexibility to maintain the attentional set (*i.e.* odor is the relevant dimension), while altering the cognitive set of the association between the previously positive stimuli and the reward [11] . In other words, the rat must make an intradimensional shift to make a new association between the reward and the new positive stimuli, while maintaining that the irrelevant dimension is still irrelevant. This is a measure of cognitive flexibility because the animal must adapt its behavior based on the stimuli change to find the reward. With cognitive flexibility implicated in neuropsychiatric diseases, it is important to analyze cognitive flexibility relating to the

controllability of a stressor. However, the effects of behavioral control over a stressor on cognitive flexibility for male and female rats have not been studied.

Aims

Despite clinical relevance, the release of norepinephrine and dopamine in the vmPFC of female rats and the release of norepinephrine in the vmPFC of male rats following ES and IS treatment have not been assessed. Additionally, the consequences of controllable and uncontrollable stress on cognitive flexibility have not previously been determined in male or female rats. This thesis aims to further the knowledge on cognitive flexibility deficits of stressors in male and female rats, and the neurobiological mechanisms that could explain the control benefits in male rats and lack of control benefits female rats. Specifically, we will analyze the effects of stressor controllability on a) the performance of male rats on the ASST b) the performance of female rats on the ASST c) the release of dopamine and norepinephrine within the vmPFC in male rats and d) the release of dopamine and norepinephrine within the vmPFC in female rats. The predicted results are a) ES and HC male rats will perform better on the REV stage of the ASST than IS male rats due to the protective effects of control b) Both ES and IS treatment groups for female rats will require equally more trials than the HCC on the REV stage ASST due to the lack of protective effects of control c) There will be increased release of catecholamines within the vmPFC in male IS rats compared to ES male rats due to male rats ability to engage the behavioral control modulated neural circuitry and d) There will be equally elevated release of catecholamines into the vmPFC in ES and IS female rats due to the lack of engagement of behavioral control modulated neural circuitry.

Materials and Methods

Subjects

Adult female and male Sprague Dawley rats were used for all experiments. Animals were housed one per cage on a 12-hour light/dark cycle (lights on at 7:00 A.M and lights off at 7:00 P.M.). Rats were given unlimited access to food and water during the one-week acclimation period prior to experimental procedures. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder, and followed the National Institutes of Health *Guidelines on the Care and Use of Laboratory Animals.*

Stress Paradigm

The rats of this study were place in individual plexiglass boxes. Each box contained a wheel that was mounted on the front. There was a small arch out of the back of the box leading to a Plexiglass rod. The rat was positioned so they were facing the wheel and their tail was extended out of the arch and fastened to the Plexiglass rod. The tail was fastened with two copper electrodes. The shocks were administered simultaneously in 100 trials, with 60 second inter-trial interval, to each ES and IS yoked pair of rats. Initially, the ES rats terminated the tail shock with a ¼ turn of the wheel. When the ES rat reached termination of the shock 3 times in under 5 seconds, the rat was then required to turn the wheel an extra ¼ turn to terminate the shock. In subsequent trials, when the rat terminated the shock 3 times in under 5 seconds, the wheel turning required increased by 50% until the maximum of four-wheel turns was reached. If the ES rat did not reach the minimum wheel turning in 30 seconds, the shock was terminated, and the criteria was reset by a ¼ turn. This procedure is used to ensure that the ES subjects are

learning a true operant response. During the first 30 trials, the shock intensity was 1.0mA. This was then increased to 1.3mA for the next 30 trials, and the final 40 trials used a 1.6 mA current. This procedure was used so that operant escape responding in the ES subjects was maintained. Control animals were kept in their home cage in the colony room. The rationale for this procedure was to produce animals that had undergone identical physical stressors with the independent variable being controllability over the stressor duration.

Canula Placement

During surgery, rats were anesthetized with isoflurane (Webster Veterinary) inhalation (2-5% v/v in 0_2) and were secured to a stereotaxic apparatus. The rats were implanted with cannula guides for microdialysis probes. The tips of the cannula were positioned at the Cingulate 1 to prelimbic junction within the vmPFC: 2.2 mm rostral to bregma, 3 mm ventral from the skull surface and 0.5 mm relative to the midline. A screw cap, from a 15-mL conical centrifuge tube with the central lid portion removed, was fasten to the skull. The threads were exposed through the tube and the tube encircled the cannula guide. This was done to ensure that the assembly on the skull was protected during microdialysis. The rats were given 1-2 weeks to recover before experimentation. All surgical procedures were performed the same day for the animals.

***In Vivo* Microdialysis**

The day before the experiment, a CMA 12 microdialysis probe (0.5 mm in diameter, 2 mm membrane with a 20-kD molecular cutoff), was placed through the cannula guide so that the membranous tip was within the PL region of the PFC. A part of a 15-mL centrifuge tube was screwed onto the cap that was mounted to the skull. This protected the dialysis tubing and the

metal spring during dialysis. Each animal was placed in an individual glass bowl and was infused with artificial CSF solution overnight at a rate of 0.2 μl per minute. At around 9 A.M. the next day, this flow rate was increased to 1.5 μl per minute. This rate was constant throughout the course of the experiment and samples were collected every 20 minutes. The rats were then placed in the plexiglass wheel-turn boxes that were able to accommodate the dialysis tubing. These rats received 100 ES or IS tail shocks. 4 baseline samples, 5 samples during the stress procedure, and 3 post-stress samples were collected in total. The home cage control (HCC) rats remained undisturbed in the dialysis room during the experiment.

Attention Set Shift Test

Procedure Overview

Each rat was placed in an individual ASST chamber. The ASST chamber was a rectangular box with opaque walls. In the middle of the camber there was an opaque, guillotine-style starting gate to separate the waiting area and the testing area. Two ceramic pots were located in the testing area. The pots were filled to the inner rim with the digging medium, and the reward (cheerio) was buried in the medium in one of the two pots. Once the gate separating the testing and waiting area was lifted, the rats were required to discriminate between cues (odor and/or medium) in order to dig in the medium to find the cheerio. Between trials, the gate was added back in place and the rat was brought back to the starting area.

For all tests, the rats were given three trials to dig in the ceramic pot with the wrong cue and go to the other pot and find the reward. This was done in order for the rat to generate an association between the reward and the cue. After the third trial, if the rat dug in the wrong pot, it was brought back behind the opaque gate and the pots were switched. Additionally,

every time the rat dug in the wrong pot, the number of successful trials in a row was reset to zero. For each trial, the pot with the positive cue switched sides, while the rat was behind the opaque gate, in order to avoid association between a location and the reward.

Acclimation

The rats were given a one-week acclimation period upon arrival. During this week, they were given unlimited access to food and water. One-week before beginning the testing (day 7), the rats started on the diet restriction (about 15 g of rat chow/day). The ceramic pots used for the tests were placed with a food reward (a few cheerios) in their home cage in order for the rats to acclimate to the pots and associate the pots with a reward. Food rewards were added to the ceramic pots in the HC every day until the day of testing.

Training

Once starting the diet restriction (Day 8), training for the rats began. The first three days of training (Day 8,9, and 10) required the rats to learn to dig in the pots to find the food reward. For day one of training, two ceramic pots filled with the home cage bedding were placed at the end of the ASST chamber with cheerios in both pots. The cheerio was initially at the top of the bedding and was gradually buried in the bedding in order for the rats to learn to dig for the reward. Once the animal had dug for the reward successfully on three trials, training was concluded for that day. On day 11, only one of the pots filled with bedding had a cheerio, and the rat had to find the cheerio in one of the pots. After the successful completion of six trials, training was concluded for the rats that day.

On day 14, training resumed with the simple discrimination (SD) training with either a medium or an odor. The odor SD training entailed the pots being filled with normal bedding;

however, a different scent was added to each pot. The scent was added by placing 0.1 mL of the essential oil of the odor on the outer rim of the pot using a pipette. One of the scents was associated with a reward and the rat had to learn to pair the scent with the reward. For the media SD, the pots were filled to the inner rim with two different digging mediums. One of the mediums was associated with the reward and the rat had to learn to pair the reward with the specific digging medium. After 6 successful trials of SD in a row, training was concluded for that day. If the rat dug in the wrong pot, then the number of correct trials would go to zero.

On day 15, the rats began training through six consecutive, successful trials of SD. Then, for rats that had undergone odor SD, the pots were filled with two different mediums and the same two scents. The rats were required to ignore the irrelevant cue (medium texture) and maintain that the relevant cue was the one specific odor. For the rats that had undergone media SD, two separate odors were added to the pots with the two different digging mediums. The rats were required to ignore the irrelevant cue (odor) and maintain the relevant cue was the one specific digging medium. This is called compound discrimination (CD). After 6 consecutive, successful CD trials, training was concluded for that day. On day 16, the rats were subject to either: IS, ES, or HCC.

Testing

On testing day, the rats were required to perform 6 consecutive successful trials of SD, CD, and the reversal (REV). The REV stage switches the irrelevant odor to the relevant odor or the irrelevant medium to the relevant medium, while maintaining the same irrelevant cue (media and odor respectively). This required the rats to maintain their attentional set (media or scent is the relevant cue), while altering the positive and negative stimuli. Reaching 6

consecutive correct trials was necessary to move on to the next test of the ASST. The number of trials to complete each different test was recorded.

Dopamine and Norepinephrine Analysis

The extracellular concentration of catecholamines was measured in dialysates from the PL region by HPLC with electrochemical detection. Dialysate (approximately 25 μl) was injected with an autosampler that kept the dialysates at 6 degrees Celsius. External standards were run to quantify dopamine and norepinephrine by means of peak height and retention time, using ESA software. The experimenter was blind to the experimental condition of the rat.

Cannula Dialysis Probe Verification

At the end of the experiment, the rats were administered an overdose of pentobarbital. The brains were removed and frozen on dry ice. A cryostat was used to take 30- μl sections. These sections were stained with Cresyl Violet for cannula placement verification. The dialysis probe was considered successful if at least 70% of the probe was within the PL region of the PFC. If the probe was not in this area, the rats were not included.

Statistical Analysis

Data was analyzed using GraphPad software (Prism). The effect of stress treatment on ASST performance was analyzed using a one-way analysis of variance to determine the effect of stress treatment (ANOVA). A two-way ANOVA was used to analyze the effects of stress treatment and effect of time. Main effects were deemed statistically significant if $p<0.05$. *Post-hoc* analyses were performed using Fisher's least significant difference (LSD) test and Tukey's Honest Significant Difference (Tukey's HSD) test. Graph values are represented as mean ± standard error of the mean (SEM).

Results

Experiment one. Males given ES treatment performed significantly better at the REV stage of the ASST compared to rats given IS treatment.

We investigated whether ES, IS, and HC treatment had an effect on the attention set shift test in male rats. Each treatment group had data from 8 rats. All rats in the ES condition met the fixed ration requirement for wheel turns and learned to effectively turn the wheel. Figure 1 shows the trials to criteria of the ASST for all three treatment groups. A one-way ANOVA did not yield a significant effect for the stress condition (IS,ES, and HC) for simple discrimination or compound discrimination ($p>0.05$). The one-way ANOVA did reveal a significant effect of the stress condition for the reversal stage of the ASST ($p<0.001$). *Post-hoc* analysis using Tukey's HSD test revealed a significant difference between HC and IS conditions ($p<0.05$) and between IS and ES conditions ($p<0.001$). ES and HC were statistically no different from each other, where both groups showed better performance on the reversal task than rats that had received IS.

Experiment two. Male rats did not show a significant interaction between time and the stress condition for norepinephrine release into the vmPFC. Males given ES treatment had significantly less norepinephrine release in the vmPFC at certain time points during stress than did IS treatment rats.

We sought to determine whether there was differential norepinephrine release in the vmPFC between IS and ES conditions in male rats. The IS treatment group had 7 male rats and the ES treatment group had 6. Figure 2 shows the levels of norepinephrine in the vmPFC of male rats represented as percentage of baseline. A two-way ANOVA revealed a main effect of

time ($p<.0001$), did not show a main effect of the stress condition (ES, IS) ($p>0.05$), and did not show a main effect of the interaction of the two ($p>0.05$). Despite lacking a significant interaction between time and stress, a Fisher's LSD *post-hoc* examination was performed. Fisher's LSD *post-hoc* examination revealed that there was a significant difference in norepinephrine release at stress samples 2,3, and 4 ($p<0.5$, $p<0.01$, and $p<0.05$ respectively).

Experiment 3. Male rats did not show a significant interaction between time and the stress condition for dopamine release into the vmPFC. Males given ES treatment had significantly less dopamine release in the vmPFC at certain time points during stress than did IS treatment rats.

We sought to determine whether there was differential dopamine release in the vmPFC between IS and ES conditions in male rats. Each treatment group contained 5 male rats. Figure 3 shows the release of dopamine in the vmPFC of male rats represented as percentage of baseline. A two-way ANOVA revealed a main effect of time ($p<.0001$), did not show a main effect of the stress condition (ES, IS) ($p>0.05$), and did not show a main effect of the interaction of the two ($p>0.05$). Despite lacking a significant interaction between time and stress, a Fisher's LSD *post-hoc* examination was performed. Fisher's LSD *post-hoc* examination revealed that there was a significant difference in dopamine release at stress samples 1,2, and 4 ($p<0.05$, $p<0.05$, and $p<0.01$ respectively).

Experiment 4. Female rats did not show a significant difference between IS, ES, and HC on the ASST, but the data trends toward ES and IS rats performing similarly worse on the REV stage than HC rats.

We investigated whether ES, IS, and HC had an effect on the attention set shift test in female rats. Each treatment group had 4 female rats. All rats in the ES condition met the fixed ration requirement for wheel turns and learned to effectively turn the wheel. Figure 4 shows the trials to criteria of the ASST for all three treatment groups. A one-way ANOVA did not yield a main effect for the stress condition (IS,ES, and HC) for simple discrimination ($p>0.05$), compound discrimination ($p>0.05$), or the reversal ($p>0.05$). The Tukey's HSD *post-hoc* examination did not yield any significant differences between HC and ES, HC and IS, IS and ES ($p>0.05$). However, the data trends towards the ES and IS conditions requiring significantly more trials to complete the REV stage than HC condition. However, with the low number of subjects, statistically all three treatment groups show the same performance on the ASST.

Experiment 5. Females rats had a significant interaction between time and the stress condition for dopamine release into the vmPFC. *Post-hoc* examination revealed significantly less dopamine release in the vmPFC at certain time points during IS compared to ES treated rats.

We sought to determine if there was differential dopamine release in the vmPFC between IS and ES condition in female rats. The IS treatment group contained 6 rats and the ES treatment group contained 4 female rats. Figure 5 shows the release of dopamine in the vmPFC of female rats represented as percentage of baseline release. A two-way ANOVA revealed a main effect of time ($p<.0001$), did not show a main effect of the stress condition (ES, IS) ($p>0.05$), and revealed a significant interaction of the two ($p<0.05$). Fisher's LSD *post-hoc* examination revealed that there was a significant difference in dopamine release at stress samples 1,3, and 4 ($p<0.01$, $p<0.05$, and $p<0.01$ respectively).

Experiment 6. Females rats did not have a significant interaction between time and the stress condition for norepinephrine release into the vmPFC. *Post-hoc* examination revealed significantly less norepinephrine release in the vmPFC at certain time points during IS compared to ES treated rats.

We sought to determine if there was differential norepinephrine release in the vmPFC between IS and ES condition in female rats. Both treatment groups contained 3 female rats. Figure 6 shows the release of norepinephrine in the vmPFC of female rats represented as percentage of baseline release. A two-way ANOVA revealed a main effect of time ($p<.0001$), did not show a main effect of the stress condition (ES, IS) ($p>0.05$), and did not reveal a significant interaction of the two ($p>0.05$). Despite lacking a significant interaction between time and stress, a Fisher's LSD *post-hoc* examination was performed. This t-test revealed that there was a significant difference in dopamine release at stress samples 1 and 4 ($p<0.05$).

Discussion

The present research sought to determine whether behavioral control modulates behavioral and neurobiological stress-induced changes in both female and male rats. Specifically, we investigated whether behavioral control (i) has an effect on the ASST in male and female rats and (ii) influences catecholamine release within the vmPFC of male and female rats. These investigations were based on previous research performed in rats. Previously, it has been shown that the presence of control actively inhibits the behavioral consequences of stressors in male rats, such as in escape decrements from foot shocks in a shuttle box[3]. Secondly, it has been shown that acute uncontrollable stress causes elevated release of catecholamines within the PFC[20,21], and also that male rats subject to IS had higher levels of

mPFC dopamine release compared to ES treated rats[25]. Finally, it has been demonstrated that behavioral control in female rats fails to mitigate the behavioral consequences of stress due to a lack of engagement of the PL top down inhibition of the DRN[9].

Thus, we sought to determine whether behavioral control has an effect on cognitive flexibility through the ASST and if dopamine and norepinephrine release differed between ES and IS treatment groups in male rats. Additionally, we tested female rats to investigate whether the lack of engagement of this behavioral control modulated neural circuity effected cognitive flexibility and if elevated levels of catecholamine release in the PFC could play a role in the lack of behavioral control modulated inhibition of the DRN.

We sought to determine the role of behavioral control in altering later cognitive flexibility in rodents utilizing the ASST as a model. As predicted, ES males performed significantly better than did IS males in the REV stage of the ASST. Past studies have shown that behavioral control in male rats mitigates the behavioral consequences of stress in tests such as: shuttle box escape and juvenile social exploration[3,26]. Additionally, it has been shown that proper function of the PFC is vital for altering behavior to seek rewards (*i.e.* cognitive flexibility) [14,15]. Both ES and IS rats learned the SD and CD stages similarly (Figure 1), trials that do not require cognitive flexibility. However, when reversal of the learned association between behavior and reward was necessary, IS rats required significantly more trials than did HCC and ES rats to complete the REV task (Figure 1). This novel finding suggests that IS causes deficits in cognitive flexibility in male rats and that behavioral control protected from this cognitive flexibility deficit. This result could have implications for human neuropsychiatric disorders that are characterized by deficits in cognitive flexibility such as anxiety related disorders. Also, this

may provide evidence supporting cognitive therapy that emphasizes the importance of generating the perception of control in a patient's life.

Previous research has shown that behavioral control in female rats fails to mitigate the consequences of stress[9]. In agreement with these findings, there was a trend for ES and IS female rats to perform similarly on the ASST. The data trends towards ES and IS females both requiring similarly more trials on average to complete the REV stage of the ASST compared to the HCC rats (Figure 4). However, no conclusion could be drawn until more subjects are included. But, if corroborated, it would provide insight to the high prevalence of anxiety related disorders in women.

Additionally, *in vivo* microdialysis was utilized to measure the release of norepinephrine and dopamine in vmPFC. Proper catecholamine concentration is essential for proper PFC function, with high concentrations decreasing PFC firing rates[19,23]. This was an important topic to investigate because elevated release of catecholamines into the vmPFC could affect the PL top down regulation of the DRN that is essential for behavioral control modulated protective effects in male rats.

There was a significant difference between male IS and ES release of norepinephrine and dopamine into the vmPFC at several time points during stress (Figures 2 and 3), with male rats subject to IS releasing elevated levels of norepinephrine and dopamine compared to ES. This finding may provide support for the previous research that found stressor controllability to modulate dopamine release within the mPFC in male rats[25]. Additionally, this male catecholamine data suggests that either norepinephrine and/or dopamine may play a role in preventing mPFC inhibition of the DRN during IS; and, thus, the lack of catecholamine increase

during ES might allow the mPFC top-down DRN regulation. Previous research has shown that the infusion of a D1 receptor agonist into the PFC impairs working memory function and this impairment was reversed with a D1 receptor antagonist[38]. It has also been shown that the excess norepinephrine in the PFC interferes with working memory function and this can be reversed with an α_1-receptor antagonist[39]. Thus, our data suggests that increased norepinephrine and dopamine release during IS may contribute to the behavioral consequences of IS. The behavioral consequences of IS may be caused by excess catecholamine concentration modulated inhibition of the PL neurons that inhibit the activity of the DRN. Overall, the male catecholamine data provides evidence that the blunted release of norepinephrine and dopamine into vmPFC for ES rats compared to IS might play an important role in the lack of behavioral consequences associated with behavioral control.

By contrast, it has been shown that female rats given behavioral control fail to selectively engage PL neurons that project to the DRN despite the presence of this protective neural circuitry[9]. Additionally, it has been shown that female rats have increased dopaminergic projections from the ventral tegmental area (VTA) to the PFC[40]. This suggests that there may be an elevated release of dopamine in the female rat PFC compared to the male. Due to high levels of catecholamines being implicated in inhibition of the PFC, we predicted that there would be an equally elevated release of dopamine and norepinephrine in the vmPFC compared to baseline for ES and IS rats.

Remarkably, there was a significant increase of dopamine in the vmPFC of female ES rats when compared to IS female rats (Figure 5). The female norepinephrine data followed a similar trend as the dopamine data; ES rats released significantly more norepinephrine into the vmPFC

than IS rats during 2 time points during stress (Figure 6). We expected that there would be an elevated release of catecholamines in the vmPFC in ES female rats, however, it was unexpected to find that there was a blunting effect of IS in catecholamine release. This is an important finding that has never been shown before in physiological stress studies.

The ES data from the female rats suggests that increased catecholamine release within the vmPFC, similar to the IS condition in male rodents, inhibits the PL top down regulation of the DRN, producing the negative consequences of stress. Our data suggests that behavioral control over a stressor in female rats may fail to mitigate catecholamine release into the vmPFC as it does in male rats. The elevated release of catecholamines during ES in female rats may be due to the increased sensitivity to CRF in the LC[53] and the larger dopaminergic projections to from the VTA to the PFC[40]. This data may have important implications regarding the lack of behavioral control modulated protective effects in female rats and the sexually dimorphic neural mechanisms in stress responses.

The escape response learned during the escapable stress portion of this experiment is an instrumental response, and previous research in instrumental learning suggests that there are two separate neural mechanisms involved in the encoding of instrumental responses[42,43]. One system is called the “action-outcome system”. This system encodes the contingency between responses and outcomes and is sensitive to future changes in outcomes, developing an ‘expectation’. This system involves a corticostriatal circuit that involves the PL and the dorsal medial striatum (DMS). On the other hand, the “habit system” encodes the acquisition of fixed, associations between a stimulus and a response that is not sensitive to contingencies or changes in outcome. The habit system involves a path between the sensorimotor cortex and

the dorsal lateral striatum. Previously, it has been shown that the controlling instrumental escape response has to be acquired by the act/outcome learning system for behavioral control over the stressor to be protective[44]. Additionally, it has been demonstrated that high levels of catecholamine release, such as occurs during acute physiological stress, impairs PFC regulation while strengthening the functioning of the habit system[19,45,46]. Thus, our data suggests that female ES rats may selectively use the habit system to encode the controlling response due to the elevated levels of catecholamines causing impairment within vmPFC, while simultaneously strengthening the function of the habit system. This could be an explanation for the lack of protective effects of behavioral control in female rats.

A major limitation to our findings was the number of subjects in each group. Statistical significance was not proven for each data set due to our low number of subjects. In order for the data to be more reliable, it will be important to have additional subjects in future studies.

The main question resulting from this study is: Why is catecholamine release within vmPFC blunted in IS female rats? The blunted catecholamine release into the vmPFC in IS female rats requires future research with a greater number of subjects. It has been shown that social defeat in adolescent rats decreases dopamine activity in the mPFC due to the inactivation of an enzyme (tyrosine hydroxylase) critical for dopamine production in the pre-synaptic terminals[37]. Although this finding was in male, adolescent rats, this mechanism could contribute to the blunted dopamine release into the vmPFC in IS female rats.

A number of other important questions are generated from these findings. What neural mechanisms cause the elevated release of catecholamines into the vmPFC of female ES rats but not ES male rats? Are elevated levels of dopamine and norepinephrine essential for inhibition

of the top down regulation of the PL to the DRN? Do blocking the consequences of dopamine and norepinephrine in the vmPFC restore performance on the ASST?

The primary sources of dopamine and norepinephrine to the PFC are the VTA and the LC, respectively [41]. It has been shown that CRF mediates the activity of the VTA and LC[51,52]. A future experiment could involve measuring the CRF release within the VTA and LC during escapable stress in male and female rats. The purpose of this study would be to determine whether increased CRF concentrations within the LC and VTA in females may contribute to the elevated levels of norepinephrine and dopamine during escapable stress of female rats. This would provide insight to the neural mechanisms contributing to the sex differences of catecholamine release during controllable stress.

In addition, future experiments could inject α_1-receptor and D1 receptor antagonists into the vmPFC in order to block the effects of norepinephrine and dopamine. Then, the rats would be subject to acute stress. In experiment 1, these rats would perform the ASST to determine if blocking the effects of catecholamines restore performance on the ASST. In experiment 2, the activation of 5-HT neurons in the DRN would be visualized using C-Fos. If catecholamines are essential for the inhibition of top down regulation of the PL to the DRN, then the 5-HT neurons in the DRN would show minimal activation.

This thesis provides the first evidence of behavioral control modulated performance on the ASST in male rats and shows data trending towards female rats having cognitive flexibility deficits despite behavioral control. Additionally, this thesis provides novel evidence of sex differences in stress induced catecholamine release during escapable and inescapable stress. Given that cognitive flexibility deficits are implicated in neuropsychiatric diseases in humans

and that women are more susceptible to the development of neuropsychiatric diseases, these findings are of clinical relevance. Continued exploration into sex-based differences in stress responses may lead to improvement of treatment strategies for people suffering from neuropsychiatric diseases.

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Figures

Figure 1. Behavioral control modulates ASST performance in male rats. This graphs shows mean (±SEM) trials to criteria for the ASST. IS treatment resulted in a significant increase of trials compared to the HC controls (*p<0.05) and ES treatment rats (***p<.001).

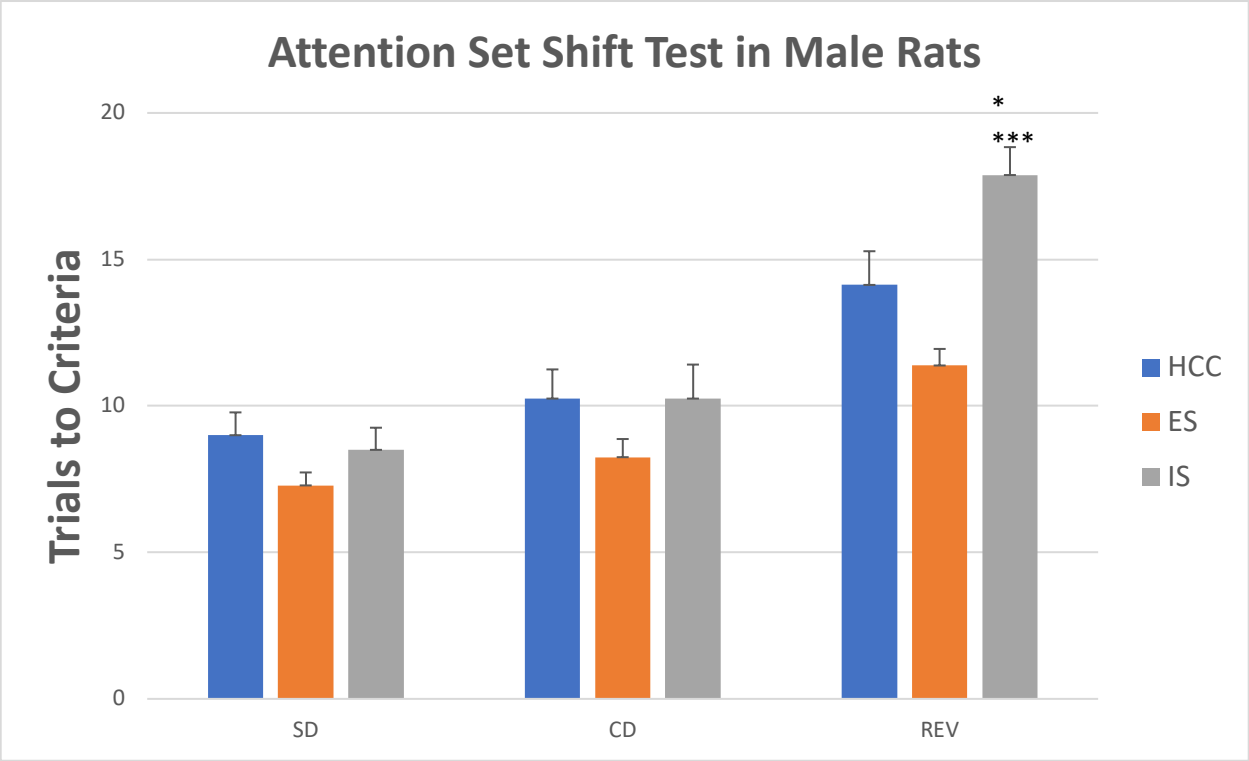

Figure 2. Behavioral control modulated norepinephrine release in the vmPFC at certain points during stress. This graph shows mean (±SEM) percentage of baseline norepinephrine levels. IS rats release significantly more norepinephrine in the vmPFC at stress sample 2,3, and 4 (*p<.05, **p<.001). Norepinephrine levels were expressed as percentage relative to baseline and represent group means.

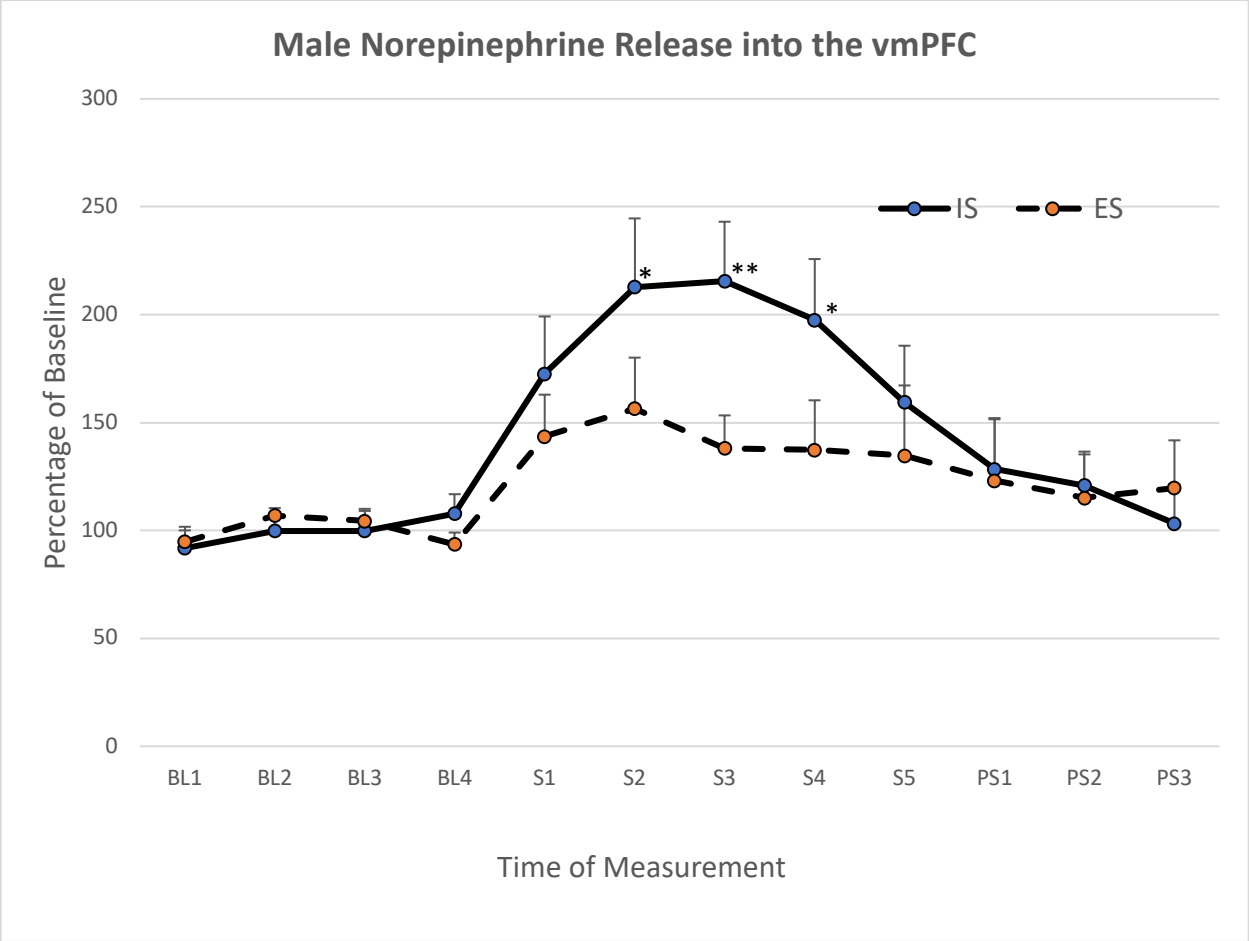

Figure 3. Behavioral control modulated dopamine release in the vmPFC at certain points during stress. This graph shows mean (±SEM) percentage of baseline dopamine levels. IS rats release significantly more dopamine in the vmPFC at stress sample 1,2, and 4 (*p<.05, **p<.001). Dopamine levels were expressed as percentage relative to baseline and represent group means.

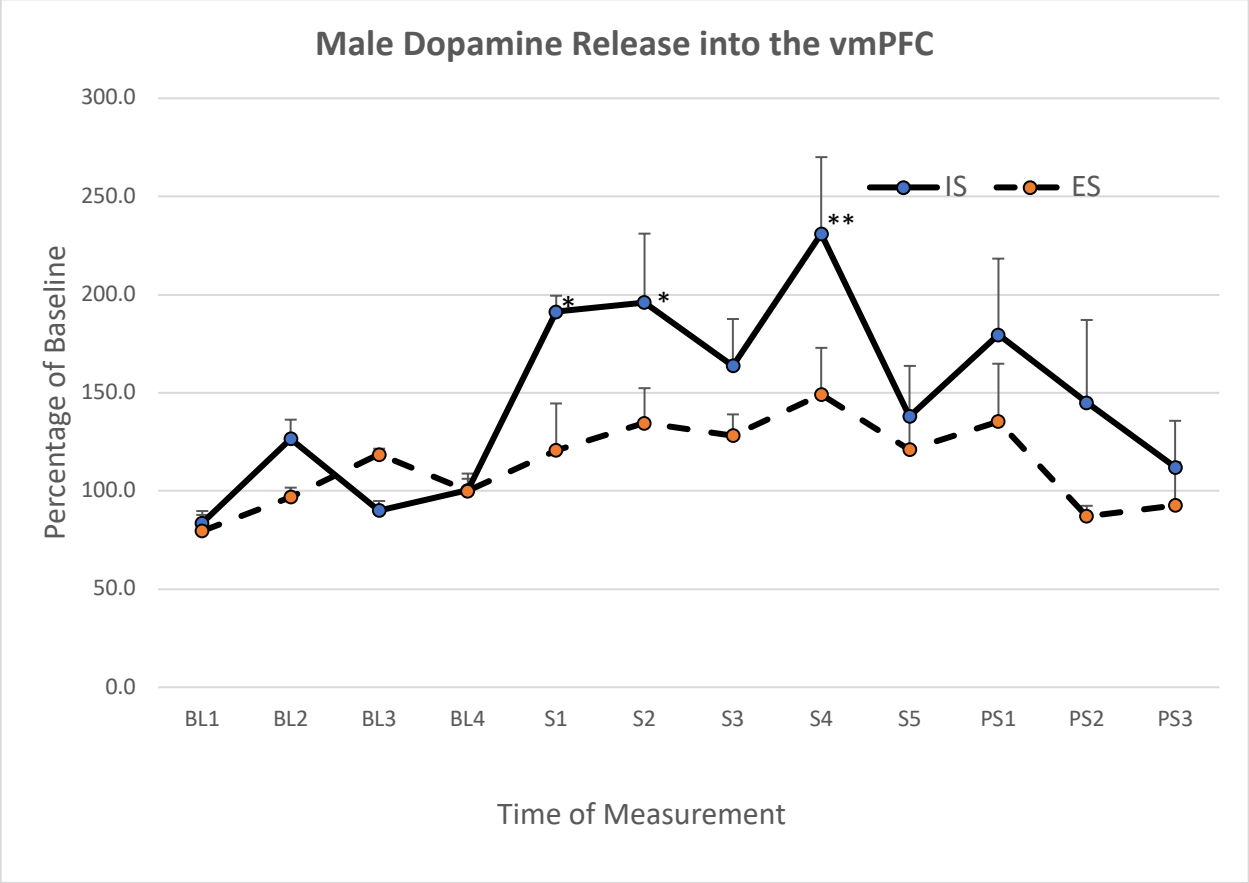

Figure 4. Performance on the ASST did not significantly differ between treatment groups (IS, ES, and HC). This graph shows mean (±SEM) trials to criteria for the ASST for female rats.

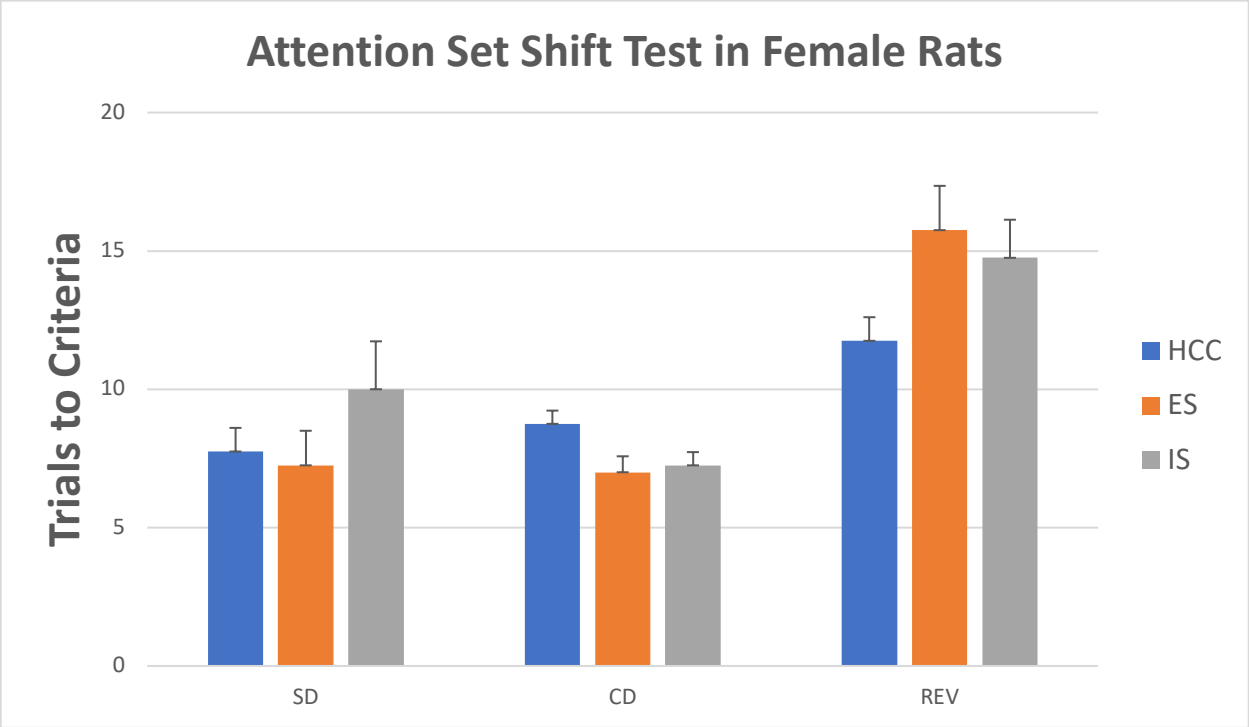

Figure 5. Behavioral control augmented dopamine release in the vmPFC in female rats at certain points during stress. This graph shows mean (±SEM) percentage of baseline dopamine levels. ES rats release significantly more dopamine in the vmPFC at stress sample 1,3, and 4 (*p<.05, **p<.001). Dopamine levels were expressed as percentage relative to baseline and represent group means.

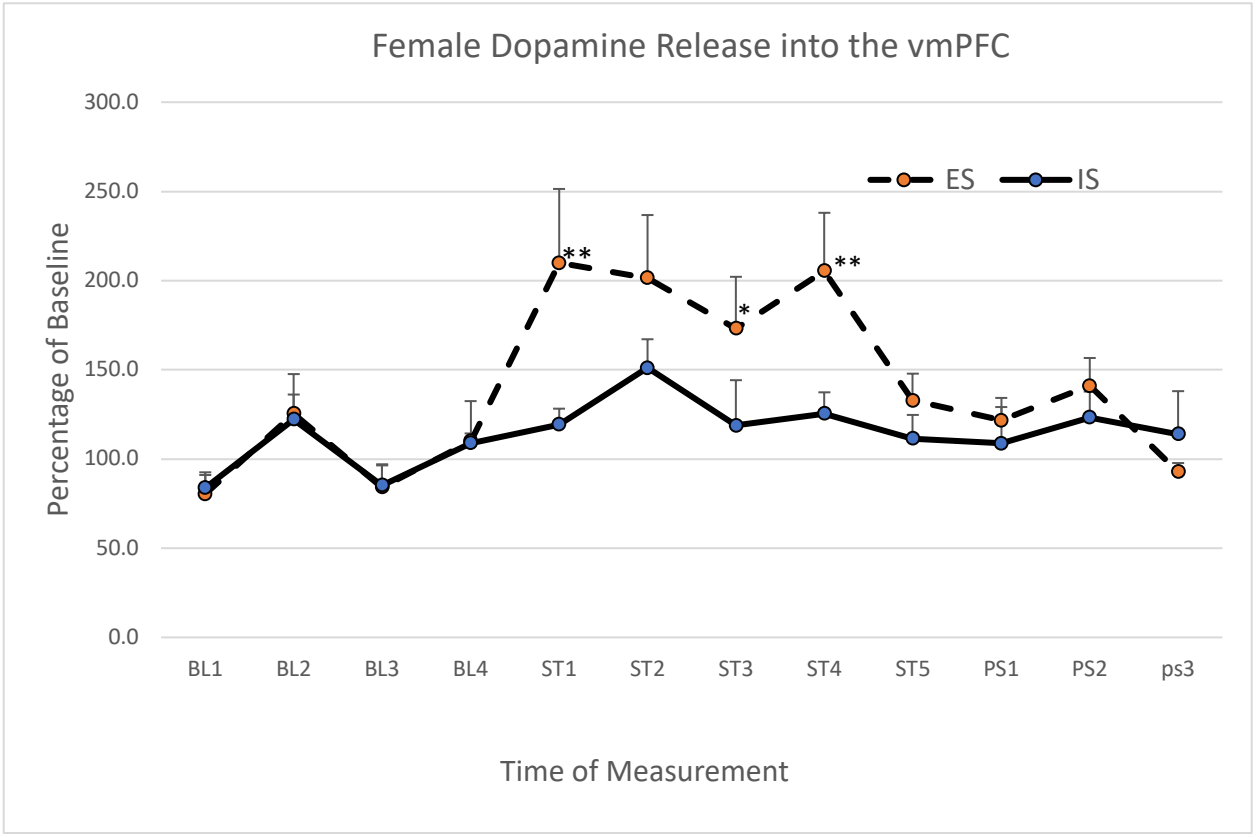

Figure 6. Behavioral control augmented norepinephrine release in the vmPFC in female rats at certain points during stress. This graph shows mean (±SEM) percentage of baseline dopamine levels. ES rats release significantly more dopamine in the vmPFC at stress sample 1 and 4 (*p<.05). Dopamine levels were expressed as percentage relative to baseline and represent group means.