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Evaluating Stressor Controllability Effects in Female Rats

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Abstract

Traumatic life events play a critical role in the development of neuropsychiatric disease. Clinical research has shown that one of the greatest risk factors for developing post trauma related disease is being female. Therefore, understanding why females are more susceptible to the development of post trauma related disorders such as anxiety and depression is of clinical relevance. Notably, perceived or actual ability to exert control over the stressor is a potent modulator of the stressor's outcome. Prior research has demonstrated that behavioral control over adverse events provides protection from the outcomes of adverse events. However, prior investigation of neural mechanisms underlying stressor controllability phenomena has almost entirely been conducted in male rats. This thesis seeks to investigate whether the stress-buffering effects of behavioral control, and the underlying neural mechanisms, are present in female rats. Understanding sex-based differences in stress and coping processes may lead to the improvement of treatment strategies for neuropsychiatric disorders in women.

Introduction

The experience of traumatic life events may provoke the development of neuropsychiatric diseases such as anxiety, depression, substance use disorders, and post-traumatic stress disorder [1]. Some individuals are vulnerable to the psychological and behavioral consequences of these instances, whereas the majority are resistant to disease development. In a clinical setting, one of the greatest risk factors for developing post trauma related neuropsychiatric disease is being female. However, the neural mechanisms responsible for this predisposition are unknown. The purpose of the present research is to improve our understanding of the development of post trauma related neuropsychiatric disease in women.

In the scientific literature, traumatic life events have been consistently associated with the development of neuropsychiatric disease. A traumatic event is described as the witness or experience of life threatening, seriously injuring, or physically or mentally invading occurrences [2]. However, a traumatic event can also encompass a broader variety of experiences such as the divorce of one's parents or the loss of a loved one. Based on a demographic of individuals who have experienced one or more instances of trauma, studies have reported that anywhere from 28% to 65% will develop PTSD, and the risk doubles for women [3, 4, 5]. In fact, a current meta-analysis reported that women are over twice as likely to develop PTSD and anxiety disorders, and nearly twice as likely to develop a mood disorder than are males [6]. Millions of Americans currently suffer from post trauma related diseases and current treatment strategies are not always effective [1]. With or without treatment, neuropsychiatric disease can severely dampen an individual's quality of life and is incredibly expensive for the healthcare industry to manage [7]. Despite the relationship between trauma and disease development, not all individuals exposed to

such instances develop these disorders. One strategy to help attenuate the incidences of post trauma related disease is to establish how individuals differentially adapt to trauma.

Prior investigation has revealed that experiential factors occurring in the early postnatal environment (levels of nutrition, handling, and enrichment) or non-experiential factors (polymorphism, hormonal fluctuation) are expected to influence susceptibility *prior* to the occurrence of the event [8]. The present research is interested in experiential factors occurring *during* trauma, such as the level of control an organism has over the event, as these are expected to play a profound role in the development and mitigation of the stressor's outcome. In humans, it is understood that an individual's appraisal of an adverse event (e.g., perceived efficacy, degree of control) is key to their ability to cope [9, 10]. Furthermore, clinical data suggests that the use of differential coping strategies may strongly influence the pathogenesis of post-trauma disease in women and in men [11].

Coping is a term used to describe how people adapt their emotion, behavior and cognition to manage pressures caused by stress. Coping strategies can be grouped into two general categories: emotion-focused coping and problem-focused coping [12]. Emotion-focused coping describes the case in which an individual disengages and changes their emotional reaction towards a stressor. Problem-focused coping describes the situation where an individual exerts a change in behavior or cognition in order to reduce or extinguish the stressor. Generally, problem-focused coping has been described as more effective than emotion-focused coping due to its strong associations with good health and wellbeing [13, 14, 15].

A fundamental element in effective coping strategies (i.e. problem focused strategies that encourage recovery and resilience against future stressors) is the ability for an individual to perceive control or to actually exert control over a stressor [16, 17]. Coping is a difficult

phenomenon to experimentally exhibit and manipulate in animals so that the underlying neural mechanisms can be explored. However, a controlling response (e.g. turning a wheel) over some aspect of the adverse event (e.g. its termination) can be provided to experimental animals. Prior research has shown that the degree of control an organism has during an adverse event actually dictates the severity of the neurochemical and behavioral outcomes [8]. Maier and Seligman were the first to develop an experimental method in which they could investigate the impact of behavioral control in animals [18]. The procedure is designed to mimic a traumatic life event in which the affected has the option to engage an instrumental response that will terminate it.

In this paradigm two subjects (typically rats) receive physically identical stressors. The rats are each placed in wheel-turn boxes that has a wheel mounted on the front. Their tails extend out the rear of the box so that an electrode can be secured to their tail. One of the rats is given control over the termination of the stressor (e.g. via turning the wheel) and the other has no control. The rat with control (ES, escapable shock) can terminate each of a series of tail shocks by spinning the wheel. Termination comes from meeting a fixed ratio requirement of wheel turns. The second rat (IS, inescapable shock) is yoked with the ES rat so that it receives tail shocks of the same duration and intensity as determined by their partner. However, turning the wheel for the IS rat has no consequence. A third rat is placed in their home cage (HC) as a control and does not receive any tail shock. Given that ES and IS subjects receive identical stressors (i.e. shock intensity and duration), differences in behavioral and neurochemical outcomes can only be due to the degree of control. This allows the researcher to detect if endocrine, neurophysiological, or behavioral processes during stress can be modulated by controllability.

In the past, stressor controllability effects on brain and behavior has only been examined in male rats. That is, although extremely clinically relevant, there is little mechanistic understanding of why females are more susceptible to the development of neuropsychiatric disease. Fortunately, given that there is a comprehensive understanding of the neural components that modulate the beneficial effects of control in males, we are in a good position to understand what contributes to increased susceptibility in females. The work presented in this thesis applies the stressor controllability paradigm to explore the relationship between control over adverse events and the behavioral and neurochemical consequences in females.

Stressor controllability phenomena

The presence or absence of control during stress is an important variable in the behavioral outcome following stress. Although it is difficult to model or measure a neuropsychiatric condition in rat, certain aspects of the human disorder can be modeled in animals. For example, performance on the juvenile social investigation (JSI) test and quantification of freezing behavior during shuttle box escape learning can be used as indices of anxiety-like behavior [19, 20]. Social avoidance during the juvenile social investigation test in rats can be used to model anxiety-like behavior in humans because avoidance of social situations in which unfamiliar people are involved is a diagnostic criterion for social anxiety disorder [21]. The second example involves quantification of 'freezing', a fear behavior observed in both humans and rats in response to perceived danger. Anxiety in humans can be defined as a fear response that is intensified beyond what is considered adaptive to the external threat [2]. Although freezing is a natural fear response, perpetual freezing without the arrival of danger can be indicative of anxiety-like behavior because the fear remains present in the absence of an external cue.

The juvenile social investigation test developed by File and Hyde [20] can be used to model social avoidance, an aspect of anxiety behavior in humans. In the juvenile social investigation test, the rat is placed in Plexiglas cage where a novel juvenile stimulus rat is later introduced. The length of investigative behaviors including sniffing, allogrooming, and pinning are measured. Novel juvenile rats are used because there is less variance in the amount of time an adult rat spends investigating the juvenile in comparison to an adult. Passive behavior (i.e. decreased interaction) can therefore be assessed. The duration of interaction is quantified before and after stress treatment (ES, IS, HC) in each rat. Adult male rats exposed to IS avoid interaction with the unfamiliar juvenile and have a significantly reduced duration of investigative behaviors relative to baseline and in comparison to HC control and ES conditions. In contrast, adult male rats exposed to ES will not exhibit a reduction in the duration of investigative behaviors. In fact, ES rats explore the juvenile rats before and after stress similarly to HC control animals. Controllability during stress provides the rat with protection from IS-induced effects on social interaction behavior.

The shuttle box escape learning task can be used to measure freezing behavior and escape failures. In the shuttle box escape learning task, a rat is placed in a Plexiglas box that contains two chambers connected by a small archway through which the rat can pass. The bottom of the chamber is lined with rods that provide a consistent mild foot shock until the rat learns to cross over to the other side (fixed ratio 1, FR-1). Once the rat has passed over to the other side, the shock is terminated. The rats are given two FR-1 trails separated by a 1-minute interval. After the two FR-1 trails, freezing behavior is assessed. ‘Freezing’ is natural physiological response to threatening stimuli and one of many fear behaviors observed in mammals. Fear behaviors are part of a system of adaptive responses that motivate and prepare an animal to escape or defend

against a threat [19]. Thus, freezing behavior in rats can be activated by both learned (e.g. a context that signifies shock) and unlearned (e.g. detection of a predator) indications of danger [22, 23]. After a male rat is exposed to IS, freezing behavior duration in the shuttle box escape leaning task is significantly increased relative to rats in the ES and HC control conditions [24], an outcome that is present up 2-3 days after IS [22]. Male rats previously exposed to ES, although subjected to the same shock intensity and duration as rats exposed to IS, have a freezing duration similar to rats in the HC control condition. This effect is remarkable because behavioral control during tail shock determines how long the rat will freeze or express fear behavior. Moreover, control-induced attenuation of freezing behavior during shuttle box escape learning can be observed for up to ~56 days [25]. That is, male rats that are exposed to ES are resilient even when future stressors occur in a different situation.

The shuttle box escape learning task can also be used measure escape failures. After freezing behavior is measured in the shuttle box escape learning task, animals receive 3 more FR-1 escape trails followed by 25 FR-2 escape trials. The FR-2 escape trial requires the animal to first cross over to the other side, and then come back to the initial side in order to terminate the shock. If the rat does not learn to escape the shock within 30 seconds, the shock is terminated and considered an escape failure. Male rats exposed to IS prior to the shuttle box escape learning will fail to escape significantly more times than rats previously exposed to ES and HC control conditions. This phenomenon was first observed by Maier and Seligman and originally termed 'learned helplessness'[18]. Rats who were previously exposed to IS are thought to have learned helplessness because prior IS confirmed that their actions are independent from the outcome (tail shock). Therefore, these rats will be less likely to attempt escape. This phenomenon has also been identified in humans where students given unsolvable problems (lack of control) will show

anagram deficits similar to depressed patients [26]. Some other clinical symptoms of depression (e.g. loss of interest, weight loss, sleep problems) have been recapitulated experimentally in both rats and humans after a prior experience of an uncontrollable adverse event [27, 26, 28]. Consistent with other behavioral tests, the ability to control a stressor prior to shuttle box escape learning prevents escape failures. Specifically, ES male rats who retain control over the stressor prior to shuttle box escape learning will have a similar amount of escape failures as HC control animals. As the stressor controllability paradigm models stress and coping with stress, stress and coping are likely to be involved with a lot of human disorders.

In addition to the behavioral outcomes discussed above, IS will impair fear extinction and potentiate fear conditioning [29], produce immobility in the forced swim task [30], and increase stereotypy in response to a subsequent amphetamine challenge [31]. Male rats who retain control over tail shock are consistently blunted from the behavioral effects of IS and adapt to the experience in a way that protects them from these consequences of future challenges [32].

The role of the dorsal raphe nucleus

As described above, behavioral outcomes following IS include enhanced freezing behavior, poor escape behavior, reduced juvenile social interaction, and potentiated fear conditioning. Structures mediated by the dorsal raphe nucleus (DRN), such as the periaqueductal grey (PAG) and the amygdala, are key anatomical sites for the expression of these behaviors [33, 34]. For example, rats who receive lesions in the central nucleus of the amygdala will show impaired freezing after fear conditioning [35]. Additionally, excitation of the periaqueductal grey will induce somatic and autonomic responses characteristic of defense and immobility in rats [36]. The DRN is the largest nucleus of 5-hydroxytryptamine (5-HT, serotonin) cells in the mammalian brain and projects widely to forebrain structures such as the amygdala and PAG. An

increase in 5-HT concentration in the amygdala will enhance the association of defensive fear behavior and external stimuli (i.e. enhanced fear conditioning) [37]. Additionally, an increase in 5-HT concentration in the dPAG will inhibit innate fear response [38] .

As determined by microdialysis and co-quantification of Fos and 5-HT, IS has been shown to evoke a substantial rise in extracellular 5-HT in the DRN and in projection regions like the amygdala [39]. C-fos is an immediate early gene that is expressed directly after depolarization of a neuron. Thus, increased Fos, the protein product of the c-fos gene, in a cell that contains 5-HT signifies neuronal activity in 5-HT cells. Pharmacological inhibition of the DRN through the activation of 5-HT_{1A} inhibitory auto-receptors in target regions of the DRN (amygdala and PAG) during IS actually eliminates the typical behaviors outcomes that follow IS [40]. These findings reveal that excitation of the DRN is both necessary and sufficient to produce the behavioral consequences of IS.

Interestingly, extracellular increases in DRN 5-HT after IS only persists for a few hours, whereas the behavioral consequences of IS can be observed for a few days. Prior research has shown that a mild foot shock of an animal who received IS a full day before will result in activation of the DRN [41]. In comparison, male animals that did not undergo IS will not show activation in the DRN. These two lines of evidence suggest that IS induces DRN sensitization, or hyperactivity, for a number of days. It has been suggested that a down regulation of inhibitory auto-receptors on the soma and dendrites of 5-HT neurons may be the mechanism by which the DRN loses its ability to receive negative feedback from rises in 5-HT. Using an ex vivo extracellular single-unit recording in the DRN, it has been shown that an increases in extracellular 5-HT concentration impairs 5-HT_{1A} receptor-mediated inhibition of the DRN [42]. This evidence is consistent with the idea that IS will induce DRN sensitization.

The introduction of behavioral control over the stressor (ES) has an opposing effect on DRN activity and its projection regions [43]. ES does not lead to activation of the DRN and thus the behavioral consequences that come with the activation of downstream limbic structures. Animals in the ES condition are deemed protected from the behavioral consequences of IS. It seems likely that evolutionarily recent neural constituents (e.g. the neocortex) are involved in the silencing of DRN neurons. In order to achieve this, the structure must be sensitive to contingency or the association between an action (wheel turn) and the predicted outcome (termination of shock). It must also be in a position to regulate limbic and brainstem circuits (i.e. neural pathways involved in the detection of behavioral control and modulation of the DRN-to-limbic structure circuitry).

The role of the medial prefrontal cortex

The medial prefrontal cortex (mPFC), an anatomically distinct portion of the neocortex, is a key site for contingency learning and executive function [44]. These attributes are relevant because the stressor controllability paradigm is dependent on contingency learning; the turning of the wheel is associated with the predicted outcome (termination of the shock). Research in male rats has shown that the mPFC is a key player in control detection and modulation of downstream brain structures. Examination of DRN cortical projections has revealed that the majority of DRN input comes from the prelimbic (PL) region of the mPFC [45]. Activation of the glutamatergic pyramidal PL neurons via electrical stimulation leads to inhibition of DRN activity. This result its mediated by GABAergic interneurons that receive input form PL terminals and synapse on DRN cell bodies [46, 47]. Therefore, the DRN receives selective top down inhibitory control through the PL region of the mPFC.

The PL region of the mPFC has been shown to be preferentially activated in males who received ES treatment. This is consistent with the observation that the DRN is less active in ES animals. Fos positive cells in the prelimbic region that had been retrogradedly labeled from the DRN with fluorogold (FG) are increased ES animals as compared to IS and HC [48]. In other words, the PL to DRN pathway is sensitive to the presence or absence of control. Amat et al. showed that pharmacological blockade of the PL during ES causes ES animals to be no longer protected from the behavioral outcomes of acute stress. This manipulation produced stress behavior in ES rats was indistinguishable from IS [49]. Conversely, pharmacological activation of the PL using the GABA antagonist picrotoxin reduces DRN 5-HT activation in IS animals. Under this pharmacological condition, IS animal behavior is indistinguishable from ES behavior [50]. These experiments strongly suggest that protection from the behavioral outcomes of stress is dependent on the activation of the PL region of the mPFC and thus inhibition of the DRN.

A profound effect of behavioral control in male animals is the behavioral and neurochemical protection from future stressful events can last up to 56 days. This includes exposure to novel stressors [25]. This evidence suggests animals who are able to control the stressor develop prolonged neuronal adaptations (immunization) that allow the animals to be resilient to stressors that induce DRN sensitization.

Aims

Despite the clinical demand, behavioral and neurochemical consequences of escapable and inescapable shock have never been determined in female rats. Given the profound effects of ES in male rats, this thesis seeks to investigate the stress-buffering effects of behavioral control, and the underlying neural mechanism, in female rats. Specifically, we will examine the effects of controllability on a) behavioral outcomes such as juvenile social investigation, shuttle box

escape, and fear behavior and b) neurochemical measures such as DRN 5-HT activation and PL-to-DRN pathway activation. If controllability effects are present in females we would expect to observe a) similar durations of juvenile investigative behavior, number of shuttle box escape failures, and fear behavior between ES and HC conditions where both differ from the effects IS on these behaviors and b) reduced DRN 5-HT activation and increased PL-to-DRN pathway activation in ES animals relative to IS and HC conditions. If controllability effects are absent in females then we would expect to observe a) similar durations of juvenile investigative behavior, number of shuttle box escape failures, and fear behavior between IS and ES conditions where both differ from behaviors observed in the HC control condition and b) increased DRN 5-HT activation and decreased PL-DRN pathway activation in ES and IS animals relative to HC controls.

Materials and Methods

Subjects

Adult female Sprague Dawley rats (230-270 g; Harlan) were used for all experiments and juvenile male and female Sprague Dawley rats (90–100g) were used for experiments involving the juvenile social investigation method. Animals were housed two per cage on a 12-hour light/dark cycle (lights on at 7:00 A.M and lights off at 7:00 P.M.). All rats received unlimited access to food and water and were given a one-week acclimation period prior to experimental procedures. Experiments were conducted between 9:00 A.M. and 4:00 P.M. All Animals 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*.

Wheel-Turn escapable/yoked inescapable shock procedure

Controllable and uncontrollable tail shock administration followed methods previously described [49]. Rats were placed in their own box comprised of Plexiglas (14 X 11 X 17 cm). Each box contained a wheel that was mounted on the front and contained a small archway at the bottom rear of the box that led to an extended Plexiglas rod. The rat's tail extended out of the small arch way and rested on the Plexiglas rod where two copper electrodes could be secured to the tail. Shock was administered simultaneously in 100 trials, with a 60s inter-trial pause, to each ES and IS yoked pair. The ES rat initially terminated the shock with a single quarter turn. If the rat successfully terminated the shock three times each under 5s, the criteria to terminate the shock increased by a quarter turn. Subsequent latencies under 5s led to a 50% increase in wheel turn requirement until the maximum of four wheel turns was met. The shock was terminated if the rat did not meet the requirement within 30s where by the criteria was reset to a single quarter turn. For the first 30 trials a shock intensity of 1.0mA was used which was increased to 1.3mA for the next 30 trails. The last 40 trials used a 1.6 mA shock intensity. This design ensured the learning of an operant response. Control animals that received no shock treatment were kept in their home cage in the colony room during the stress procedure.

Juvenile social investigation

The juvenile social investigation test was conducted using methods identical to those previously described [49]. 24 hours before the adult rats were given stress treatment, they were taken to a well-lit behavioral testing room where they were each placed in their own cage. The cages were comprised of clear plastic tubs capped with a wire lid and were stocked with fresh shaved wood bedding prior to testing. They contained no food or water. A 1-hour acclimation period was given before the addition of a 2-3 week-old juvenile stimulus rat. Adult rats were

observed for 3 min by a blind experimenter. During this time, the length of investigative behaviors such as sniffing, allogrooming, and pinning were measured with a stop watch. At the end of the 3-min time period, the adult rats were placed back in their home cage. 24 hours after stress treatment, the adult rats were tested in the same way they were tested 24 hours before stress. The same juvenile stimulus rats were used for more than one test, however, none of the adult rats were exposed to the same juvenile twice. The length of investigative behaviors was expressed as a percent of the baseline measurements.

Shuttle box escape learning

Shuttle box procedures followed methods previously described [24]. 24 hours after stress treatment, rats were placed into their own shuttle box measuring 46.0 cm X 20.7 cm X 20.0 cm. An archway of Plexiglas separated the two sides of the apparatus. On each side, the bottom of the box was lined with metal bars that conduct current at an intensity of .6 mA. The animals were given two fixed ratio (FR-1) escape trials. In each FR-1 trail, the foot shock was terminated if the animal crossed over to the other side of shuttle box by passing through the small archway. Each trial was separated by a 1-minute interval. The animal failed to escape the shock if they did not cross over within 30 seconds; at which point the shock was terminated. Prior to the first FR-1 escape trial, animals were given 5 minutes to become familiar with the environment. After the completion of 2 FR-1 escape trials, an experimenter blind to the groups scored freezing behavior (i.e. the absence of any motion except for what is required to breathe) every 10 sec for 20 min. After freezing behavior was scored, the animals received 3 more FR-1 escape trails followed by 25 FR-2 escape trials. The FR-2 escape trial required the animal to first cross over to the other side, and then come back to the initial side in order to terminate the shock.

Microinjection of retrograde tracer

Injection of the retrograde tracer fluorochrome was performed using the same procedure previously described [48]. Rats were anesthetized with isoflurane (Webster Veterinary) inhalation (2-5% v/v in O_2) and secured to a stereotaxic frame. Prior to the first incision, the animals were given a subcutaneous injection of the analgesic Loxicam (.5 mg/kg; Norbrook Laboratories) and the antibiotic penicillin (0.25 ml/kg; Combi-Pen 48; Agrilabs). A minor craniotomy (1.0mm diameter) was made using a drill, dorsal to the DRN. A 31-gauge needle with a 45º bevel was attached to a 10µl Hamilton syringe and positioned via stereotaxic adjustments directly into the DRN (AP, -8.0 mm from bregma; DV, -6.7mm from the skull surface, at midline). 200 nanoliters of a solution comprised of 2% fluorochrome in 0.9% saline buffer was injected into the DRN at a rate of 75 nL/min using a microinjection pump (UMP3-1; World Precision Instruments). The solution was allowed to diffuse for 10min. Rats were given 1-2 weeks to recover from surgery before experimentation.

Cannula placement and microinjection

Cannula placement and microinjection procedures are identical to those described in [50]. Rats were anesthetized with isoflurane (Webster Veterinary) inhalation (2-5% v/v in O_2) and secured to a stereotaxic frame. Prior to the first incision, the animals were given a subcutaneous injection of the analgesic Meloxicam (.5 mg/kg; Norbrook Laboratories) and the antibiotic penicillin (0.25 ml/kg; Combi-Pen 48; Agrilabs). Each rat was implanted with a single dual guide cannula (26g, 1 mm center-to-center distance; Plastics One, Roanoke, VA) that would guide the bilateral injector tips to the infralimbic and prelimbic cortices (AP +2.9, LM +/− 0.5, DV −2.9). Cannulae were held in place on the skull with three screws and dental cement. Microinjections

were made by gently restraining the rat in a towel and extending a microinjector 1mm beyond the cannula tip (33 g; Plastics One, Roanoke, VA) joined to a cannula connector. 0.5 µl of picrotoxin, 100 ng/side, in 0.9% saline or saline alone was injected at a rate of 1µl/min through PE-50 tubing by a 25µl Hamilton syringe and a Kopf micromanipulator. 2 min were allowed to permit diffusion. At the end of the experiment brains were processed for cresyl violet verification of cannula placement using standard procedures.

Tissue preparation

All rats were deeply anesthetized with isoflurane (Webster Veterinary) two hours after the last tail shock. Rats were then transcardially perfused with 100ml of ice-cold. 9% saline solution containing .1% heparin, followed by 250 ml of 4% paraformaldehyde in .1 M phosphate buffer (PB) pH ~ 7.4. Brains were extracted and postfixed overnight in the same fixative. After overnight fixation, brains were the transferred to a 30% sucrose solution and stored at 4℃ until sectioning. Brains were frozen in isopentane at ~ -40℃ and coronally sectioned in a −20°C cryostat at 35µm. Sections were stored free-floating in cryoprotectant until staining (FG/Fos mPFC and 5-HT/Fos DRN) or placed directly on charged microscopic slides (FG DRN).

DAB immunohistochemistry (5-HT/Fos)

A 3,3'-diaminobenzidine (DAB) oxidation reaction was used to visualize 5-HT and Fos. The stain was conducted using procedures previously described [39]. The tissue sections of the DRN were chosen to represent rostral, medial, and caudal regions. The stains were conducted consecutively and Fos was stained for first. Tissue was washed in a 0.01M phosphate buffered saline (PBS) solution three times for 10 min each prior to a 30-min incubation in PBS-T containing 1% H_2O_2. Following another series of 10 min washes in 0.01M PBS, the tissue was

then incubated for two hours in the primary antibody, rabbit α-fos (Santa Cruz Biotechnology, sc-52), diluted 1: 15,000 in blocking solution. The blocking solution was comprised of 1% normal goat serum, 1% bovine serum albumin, and 0.25% Triton-X in 0.01M PBS. The tissue was then washed three times for 10 min each in 0.01M PBS solution before a two-hour incubation in the secondary antibody, biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-065-003), diluted 1:200 in blocking solution. After the incubation, the avidin-biotin-horseradish peroxidase complex (ABC) solution was prepared during another series of .01M PBS washes (30mins in advance) using the Vectastain Elite ABC kit. The tissue was then incubated in the ABC solution for one hour. After another series of washes in .1M PB, the tissue was placed in the DAB solution comprised of nickelous ammonium sulfate, cobalt chloride, ammonium chloride, and 3,3'-diaminobenzidine (DAB), in .1 M PB. The reaction was activated upon the addition of Glucose Oxidase and proceeded for 12 min at which point Fos staining was evident. The reaction was terminated with three 10 min washes in PBS.

The tissue was stained for 5-HT using the peroxidase anti-peroxidase (PAP) method. Prior to the primary antibody incubation, the tissue was placed in blocking solution for 30 min. Then, the samples were incubated for 48 hours in rabbit a-5-HT IgG (ImmunoStar, 20080) diluted 1: 10,000 in blocking solution. Tissue was given a series of 0.01M PBS washes before it was placed into the secondary antibody, a solution containing a 1:200 dilution of non-biotinylated goat a-rabbit IgG (Jackson Laboratories) in blocking solution. The tissue was then washed three times in 0.01M PBS before a two-hour incubation in a solution containing a 1:500 dilution of rabbit peroxidase anti-peroxidase soluble complex (Sigma, P1291) in 0.01M PBS. After the incubation, the tissue was washed 3 times for 10 min each in 0.1M PB and then placed in the DAB solution containing a DAB tablet and 0.1M PB. The solution was activated with the

addition of glucose oxidase and proceeded for 12 min at which point 5-HT staining was evident. The reaction was terminated with three 10 min washes in PBS. The stained tissue samples were then floated onto gelatin coated slides, dehydrated with progressive ethanol baths, defatted with Histoclear, and coverslipped with Permount.

DAB immunohistochemistry (FG/Fos)

The method used for Fos identification was identical to the Fos method described above (5-HT/Fos). Tissue sections of the mPFC were taken to represent the middle region of the mPFC. FG was stained for following Fos identification. This method has been described previously [48]. Sections were incubated in rabbit polyclonal antibody directed against FG (fluorochrome) with a dilution factor of 1:50,000 in PBS for 48 hours at 4°C. Slices were then incubated with a non-biotinylated goat αrabbit IgG (Jackson ImmunoResearch Laboratories) with a dilution factor of 1 :200 in blocking solution for 2 hours at RT. This was followed by an incubation step with peroxidase–antiperoxidase diluted 1:500 in PBS for 2 hour. The tissue was given a series of .1 M PB washes before the chromogen was developed with a NovaRED substrate kit for peroxidase (Vector Laboratories). This yields a red reaction product. The stained tissue samples were then floated onto gelatin coated slides and allowed to dry overnight. They were then dehydrated with progressive ethanol baths, defatted with Histoclear, and cover-slipped with Permount.

Image analysis

5-HT/Fos

Tissue sections were visualized through a bright-field microscope (Olympus BX-61, Olympus America) by a blind observer. Cells were quantified using Olympus Suite Software (Olympus America). The number of 5-HT-stained cells and the number of cells double-labeled

for both 5-HT and Fos were counted. Fos-stained nuclei were distinguished by dark brown or black ovoid particles. 5-HT-stained cells were identified by larger reddish-tan particles, with or without Fos-stained nuclei. An anteroposterior coordinate of -7.64mm from bregma approximated rostral DRN sections, -8mm from bregma for middle DRN sections, and -8.3mm from bregma for caudal DRN sections.

Fos/FG

Tissue sections were visualized through a bright-field microscope (Olympus BX-61, Olympus America) by a blind observer. Cells were quantified using Olympus Suite Software (Olympus America). The number of FG-stained cells and the number of cells double-labeled for both FG and Fos were counted in the IL and PL regions of the mPFC. Fos-stained nuclei were distinguished by dark brown or black ovoid particles. FG-stained cells were identified by larger reddish-tan particles, with or without Fos-stained nuclei. An anteroposterior coordinate of +2.7 mm from bregma approximated mPFC sections.

Estrous cycle determination

Prior to stress treatment, vaginal smears were taken for each animal. A blunt-tipped eyedropper filled with a small amount of 0.9% sterile saline was inserted into the vagina. Fluid was quickly expelled 2-3 times to gently wash off and collect vaginal cells (approximately 0.25-0.5 mL). A drop was placed onto a glass slide and immediately examined with a 40x objective lens. Characteristic changes in the cytological appearance of the smears were used to identify the cycle stage: diestrus, proestrus, and estrus.

Statistical analysis

Data was analyzed using StatView software (SAS institute, Cary, NC). The effect of stress treatment on wheel-turn performance was analyzed using a repeated-measures analysis of variance (ANOVA). A mixed-design ANOVA was used to analyze the effect of stress treatment (between subjects) and effect of brain sub region (within subjects). A one-way ANOVA was used to analyze and effect of stress treatment and a two-way ANOVA was used to analyze the effects of drug and stress treatment. Main effects were deemed statistically significant if $p < .05$. Post hoc analyses and planned comparisons were performed where appropriate using Fisher's protected least significant difference (PLSD). Graph values are represented as mean ± SEM.

Results

Experiment one: Females given ES and IS treatment exhibited reduced juvenile investigation time relative to baseline and differ significantly from HC controls

We investigated whether ES, IS, and (HC) had an effect on the length of juvenile investigative behavior in female rodents. Rats in the ES condition learned met the fixed ratio requirement for wheel turns across all trial blocks (Figure 1A) and learned to turn the wheel efficiently (Figure 1B). Figure 1C shows the percent change of investigative behavior time, measured in seconds, relative to baseline. A one-way ANOVA revealed a significant effect of stress condition (IS, ES, and HC) ($F_{2, 22} = 11.355$, $p < 0.001$). Post hoc analysis using a fisher's PLSD test revealed a significant difference between HC and each ES and IS conditions ($p < 0.001$). ES and IS conditions did not differ from each other. ES animals were statistically no different than IS animals, where both groups showed a reduction in juvenile social investigation times relative to baseline and relative to controls.

Experiment two: Females given ES and IS treatment had longer freezing latencies and more escape failures than HC controls

Here, we sought to determine whether the treatment condition (ES, IS, HC) differentially predicted freezing duration and escape failure in female rodents during the shuttle box escape task. Figure 2A shows the percent of time spent freezing in a 2-min trial block for each experimental group. A repeated measures ANOVA revealed a main effect of stress condition (ES, IS, HC) ($F_{2,23} = 13.238$, $p = 0.0001$) and trial block ($F_{9, 207} = 13.725$, $p < 0.0001$). Fisher's PLSD *post hoc* analysis revealed no significant difference between ES and IS conditions, however, HC freezing duration was significantly shorter than the freezing duration in both ES and IS conditions across all trial blocks ($p < 0.05$).

Figure 2B shows shuttle box test performance for all groups. The mean escape latencies for each group are displayed in blocks of five trials. A repeated measures ANOVA showed a reliable effect of stress condition (ES, IS, HC) ($F_{2, 23} = 3.801$, $p < 0.05$) and effect of trial block ($F_{5,115} = 17.256$, $p < 0.0001$). Fisher's PLSD *post hoc* analysis revealed no significant difference between the ES and IS conditions however both ES and IS had significantly longer escape latencies in comparison to the HC condition across all FR-2 trail blocks but not the FR-1 trails ($p < .05$).

Figure 2C shows escape failures for each of the stress conditions. Animals in the ES and IS conditions failed to escape significantly more often than animals in HC condition. The number total number of escape failures did not differ between ES and IS animals. A one-way ANOVA revealed significant effect of stress condition ($F_{2,26} = 3.801$, $p < 0.05$). Fisher's PLSD *post hoc* evaluation revealed that, in terms of the number of escape failures, the ES and IS conditions are likely to have come from the same population. Both ES and IS conditions were

significantly different than the HC condition ($p < 0.05$). Stress condition, independent of control, predicted a larger number of escape failures relative to HC controls.

Experiment three: Females given ES and IS had a larger percentage of 5-HT neurons that expressed Fos in all regions of the DRN relative to HC controls

We sought to determine if female rodents in the ES, IS, and HC conditions produced Fos activation specific to 5-HT cells in the DRN differentially from one another. Figure 3A shows the percent of 5-HT containing neurons that also express Fos for each ES, IS, and HC conditions. Figure 3B displays DRN tissue with Fos stained (black), 5-HT stained (red), and Fos/5-HT doubled labeled cells (black on top of red). Table 1 shows the mean number of Fos positive and 5-HT positive cells in rostral, medial, and caudal regions of the DRN for each condition. The total number of 5-HT cells in each region did not differ between the groups. A mixed design ANOVA revealed a main effect of stress condition (ES, IS, HC) ($F_{2,2} = 21.562$, $p < .001$), a main effect of brain region (rostral, middle, caudal) ($F_{2,2} = 14.469$, $p < 0.001$), and an interaction between the two ($F_{2,4} = 3.066$, $p < .05$). Fisher's PLSD *Post hoc* analysis for each rostral, middle and caudal regions yielded similar results; ES and IS were significantly different than HC ($p < 0.001$) and ES and IS did not differ. There was a significant and consistent effect of stress on the percent of 5-HT neurons co-expressing Fos throughout all sub regions of the DRN.

Experiment four: Females given ES and IS treatment had a similar number of FG cells co-expressing Fos

Given the lack of DRN inactivation by ES, we examined Fos in PL neurons that project selectively to the DRN to determine the strength and level of activation of this pathway in females in all conditions. Figure 4A shows the percent of FG positive cells that also express Fos

for each stress condition. Figure 4B displays mPFC tissue with FG positive cells (red) and FG/Fos positive cells (black on top of red). Figure 4C displays the DRN injection sight for FG. For total PL Fos, a one-way ANOVA revealed no significant main effect ($F_{2,15} = 2.675$, $p > 0.05$). However, Fisher's PLSD *post hoc* revealed a significant difference between ES and HC conditions ($p < 0.05$). A one-way ANOVA was conducted on percent of FG cells double labeled with Fos and revealed no significant main effect ($F_{2,15} = 1.397$, $p > 0.05$). Fisher's PSLD *post hoc* analysis showed ES IS and HC conditions contained an amount of double labeled FG/Fos cells that did not significantly differ from one another. ($p > 0.05$). ES did not preferentially induce Fos activation in PL-to-DRN pathway in comparison to IS.

Experiment five: Picrotoxin rescued the stress induced investigative behavior reduction seen in animals in the ES and IS conditions

In order to determine if the PL-to-DRN pathway could have inhibitory action, we applied a pharmacological approach to artificially induce activation of the PL in female rodents during stress treatment (ES, IS, and HC) and used the length of juvenile investigation as a metric of its effect. Figure 5 shows the percent change of investigative behavior time relative to baseline for each condition combined with drug or vehicle. A two-way ANOVA revealed a main effect of stress condition (ES, IS, and HC) ($F_{2,1} = 7.726$, $p < 0.001$), a main effect of drug (picrotoxin and vehicle) ($F_{2,1} = 7.405$, $p < 0.001$), and an interaction between the two ($F_{2,2} = 3.350$, $p < 0.05$). Fisher's PLSD *post hoc* examination revealed that animals in the ES-veh and the IS-veh conditions were significantly different than HC-veh ($p < 0.01$ and $p < 0.001$ respectively). HC animals that received intra-PL picrotoxin had a significantly higher percent interaction time when compared to each ES-veh and IS-veh percent interaction times individually ($p < 0.01$ and $p < 0.001$ respectively). Both ES and IS vehicle groups had a significantly lower percent

interaction when compared their pictoroxin counterparts (ES-Veh/ES-PTX $p < 0.05$, IS-Veh/IS-PTX $p < 0.05$) and animals in the ES picrotoxin condition had a significantly higher interaction time than the animals in the IS vehicle condition ($p < 0.01$). Both stress conditions that received intra-PL picrotoxin, controllable and uncontrollable, showed increased juvenile interaction times relative to their respective stress conditions not given picrotoxin (vehicle).

Discussion

The present research sought to determine whether behavioral control modulates behavioral and neurochemical processes in female rodents. Specifically, we asked, does behavioral control (i) regulate juvenile social interaction behavior (ii) influence shuttle box escape learning and freezing behavior and (iii) reduce activation of 5-HT cells in the DRN iv) engage the same mPFC-to-DRN circuitry previously characterized in male rodents. The inquiries were based on three previous findings in male rodents. The first finding in male rodents is that engaging behavioral control over acute tail shock will cause an animal to have juvenile social interaction times that are significantly longer in duration than IS animals [22]. Secondly, in male rodents, ES will prevent IS induced failure to escape in the shuttle box escape learning task [49]. ES will also prevent the shock-induced enhancement of freezing behavior, relative to IS, after 2 FR1 trials in shuttle box escape learning. The third finding in male rodents is that ES engages the PL region of the mPFC, providing 'top down' inhibition if the DRN [49]. Thus, we explored whether the characteristics of ES, observed in male rodents, could also be observed in female rodents.

Remarkably, ES females did not significantly differ from IS females in the juvenile social investigation test. It has been previously demonstrated in male rodent models that IS

significantly reduces an animal's juvenile social investigation time relative to baseline [50]. Male rats given ES treatment are deemed 'protected' from this behavioral outcome as there is no observed reduction in juvenile social investigation time. This phenomenon was absent in ES females revealing that behavioral control does not modulate this behavior in females the same way that it does in males. Wheel turn efficiency and escape latency data (Figure 1A and 1B) during escapable shock for all experiments revealed that females learned the escape behavior and fulfilled requirements efficiently. It has been shown previously that the estrus cycle phase determines extinction recall [51] so we made note of the estrous cycle phase in animals in ES, IS and HC conditions. Two findings led us to rule out any modulation of post ES, IS and HC JSI interaction times by the estrous cycle phase. First, juvenile social investigation times in ES animals had little variation (Figure 1C). Second, there was no obvious pattern observed between estrus cycle stage, determined prior to stress, and juvenile social investigation time.

We examined shuttle box escape and freezing latencies to determine the role of behavioral control in "learned helplessness" (inability to escape shock in a different situation) and fear behavior. Behavioral control did not modulate freezing behavior or shuttle box escape learning in female rodents. In males, animals given ES will exhibit significantly shorter escape latencies relative to IS animals during the FR2 trails. Males ES rats are protected from future stressors, even ones that don't resemble the same context [49]. Here, ES female rats were indistinguishable from IS female rats across all trial blocks and both groups had significantly longer escape latencies relative to HC across all FR2 trails (Figure 2B). This finding is consistent with the JSI test results in which the impact of stress was not modulated by control.

Freezing behavior was assessed after the first two FR1 trails of the shuttle box escape learning task and did not significantly differ between ES and IS groups (Figure 1A). In other

words, behavioral control did not modulate fear behavior. There was no effect of controllability on female behavior, a consistent and surprising result. It is important to note that the estrus cycle stage showed no apparent pattern between shuttle box escape latencies or freezing behavior.

Previous research using male rats has shown that IS but not ES treatment in males potentiates DRN activity during shuttle box escape learning [41]. If we assume this neuronal circuitry is similar in female rats, the increased shuttle box escape and freezing latencies would suggest that the DRN remains in an active state in both ES and IS groups. Thus, we predicted influential mPFC activity is absent; control does not promote the mPFC to engage inhibitory action on the DRN in a way that is behaviorally detectable in female rodents. The next experiments sought to address these neurochemical inquiries.

Not surprisingly, Fos and 5-HT co-expression in the DRN did not significantly differ between ES and IS females (Figure 3A). ES and IS conditions both produced an increase in 5-HT cells expressing Fos relative to controls where the number of 5-HT positive cells did not differ between the groups (Table 1). These results suggest that the behavioral sequelae of IS and ES in females are related to DRN activation as it is in IS males. This evidence is also consistent with the prediction that the mPFC is not engaging inhibitory action on the DRN. However, there is a possibility that the mPFC-to-DRN pathway is not as robust in females or does not exist at all.

To determine whether the mPFC-to-DRN pathway exists in female rodents, FG was injected into the DRN to retrogradely label neurons that project from the mPFC. The number of FG cells co-expressing Fos in both the prelimbic (PL) region of the mPFC did not significantly differ between ES, IS, and HC females (Figure 4A). In other words, the mPFC was not preferentially activated in females who had control. However, total Fos in the PL of ES animals was significantly higher in comparison to HC animals indicating stress has an effect on total Fos

activation independent of the PL-to-DRN pathway. Comparatively, prior investigation in male rats has shown that the PL region of the mPFC is preferentially activated animals that retained control over the stressor (ES) [48]. The average number of FG cells were similar to the average number of FG cells in male rats. This reveals that the mPFC-to-DRN pathway exists in females and is comparable in magnitude to males.

Picrotoxin, a GABA receptor antagonist, was injected into the mPFC to pharmacologically activate it during ES and IS. A GABA receptor antagonist will induce activation due the removal of any inhibitory input from GABA releasing neurons. Astonishingly, female rats given picrotoxin during ES and IS were 'rescued' from the behavioral effects of acute tail shock. ES and IS females given picrotoxin had significantly increased juvenile social interaction times as compared to ES and IS animals given vehicle (Figure 5). This effect has also been demonstrated in IS male rats. Specifically, IS males given picrotoxin also exhibited significantly longer juvenile interaction times relative to IS animals given vehicle [50]. This strongly suggests that activation of the mPFC during escapable shock was sufficient to inhibit the DRN and thus behavioral ramifications of acute tail shock.

We conclude that the mPFC-to-DRN pathway exists in females but is not being engaged in animals who have control. Perhaps the mPFC does not detect control and therefore does not implement the same action (i.e. mPFC induced inhibition of the DRN). To appreciate how detection and implementation may coordinate in the female rat brain I will discuss neuronal circuitry characterized in male rodents. Work done by Maier et al. [52] suggests there are two distinct circuits involved in the (i) detection of control and the (ii) modulation of behavioral and neuronal activity in response to detection. The initial detection of control is thought to be regulated by a corticostriatal circuit consisting of the PL region of the mPFC and the DMS. The

modulatory circuit is comprised of a connection between the PL region of the mPFC and the DRN via inhibitory GABAergic interneurons. In this arrangement, the PL, upon detection of control (DMS), provides top down inhibition of limbic structures through inhibitory projections to the DRN [47].

The DMS's involvement in the detection circuit draws from one of two distinct systems that have been experimentally shown to contribute to instrumental learning. The first is the act-outcome system, responsive to contingency, or the relationship between an action and the probability of an outcome. In other words, evaluation of the difference in likelihood of gaining a reward if an action is executed and the likelihood of gaining a reward if no action is executed. It is dependent on the PL region of the mPFC and the DMS and has been shown to be the primary contributor to instrumental learning during ES in male rats. The other instrumental learning system is referred to as the habit system and is not sensitive to contingency. It depends on different circuitry, the IL region of the mPFC and the dorsolateral striatum (DLS) [53]. It can be conceptualized as an association that is strengthened by a reward (or punishment) following a stimulus and is independent of any evaluation of the action. It is understood that the activation of the act-outcome system in male rodents (i.e. when the animal turns the wheel and terminates the shock) prompts the modulation of limbic structures. Amat et al. demonstrated that engagement of the act-outcome system is necessary for the protective effects of ES seen in male rats. Injection of the NMDA antagonist AP5 into the DMS, but not the DLS, during ES eliminates the protective effects of control [52]. AP5 will inhibit or take the DMS offline because it removes excitatory glutamatergic activation in the DMS. This will force the organism to use the DLS (habit system) to learn the escape response. Females may preferentially engage the habit system

during escapable tail shock. Use of the habit system would mean activation of the DLS and not the DMS during instrumental learning and thus failure to engage the PL-to-DRN pathway.

Do females have a functional PL to DRN pathway? If so, does the activation of this pathway rescue IS induced reduction in JSI behavior? Given that females are not protected from control, are they preferentially using the habit system to learn the wheel turn response? Would biasing the brain to use the PL-DMS act-outcome circuitry protect females from the effects of IS?

In order to address the former, future studies could use a dual viral strategy that combines the use of cre-expressing canine adenovirus-2 (CAV-2) and designer receptors activated by a designer drugs (DREADDs) to transiently activate the specific PL to DRN pathway during ES and IS in female rodents. DREADD is a receptor genetically designed to selectively bind colazapine-N-oxide (CNO). Using DREADD and CAV-2 technology would be advantageous because these tools would provide pathway specific activation in a way that mimics natural neuronal signaling. Activation of DREADD receptors is more precise than traditional pharmacological methods because CNO will only bind to the designer receptors. Activation of the PL to DRN pathway using DREADD would provide causal evidence of a functional mPFC-to-DRN pathway in females. The experiment would require insertion of two viral constructs, CRE-recombinase expressing CAV-2 and an adeno-associated virus that contains the inverted floxed sequence of the excitatory (DREADD) (AAV-hSyn-DIO-hM3D(Gq)-mCherry) activated selectively by CNO. CAV-2 injected into the DRN would travel retrogradely to the PL region of the mPFC. Another injection of the adeno associated virus containing the excitatory designer receptor into the PL would drive designer receptor expression only in neurons that project to the DRN. Injection of CNO 30mins prior to stress treatment would target activation of the PL to

DRN projections. It would be expected that the activation of this pathway during ES and IS would yield increased juvenile social interaction times relative to animals who did not receive CNO.

Future experiments would benefit by using an optogenetic approach to determine if female rodents are preferentially engaging the habit system over the act-outcome system while learning the instrumental response. The advantage of using optogentics would be the ability to change neuronal activity with temporal precision. Pharmacological approaches or DREADD technology take time to produce a physiological effect and theses effects would last too long for the purpose of this experiment. Optogenetics can be used in vivo where membrane expression of an opsin channel will, upon excitation with a particular electromagnetic frequency, allow the flow of positive or negative ions into the neuron. Insertion of an adeno-associated virus containing an inhibitory halorhodopsin (AAV-hSyn-NpHR-EYFP) into the DLS would allow for temporal inhibition of the DLS during ES treatment. Inhibition of the DLS during ES would require the animal to learn the instrumental response using the act-outcome system. It would be expected that this would produce juvenile social interaction times that are significantly greater in comparison to animals who do not receive light stimulation. This result would provide evidence that learning the instrumental response through the act-outcome system is sufficient to provide female rodents with behavioral protection from the negative outcomes of acute tail shock.

This thesis provides the first evidence of sex-base differences in stress and modeled coping processes in rats. Specifically, behavioral control during stress has no effect on the behavioral on neurochemical consequences in female rats. Given that, in humans, women are more susceptible to the development of neuropsychiatric disease, these findings are of clinical

relevance. Understanding sex-base differences in stress and coping my lead to the improvement of treatment strategies for neuropsychiatric disorders in women.

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Figure/table legends

Figure 1. Behavioral control does not modulate juvenile investigative behaviors in female rodents. **(A)** Mean (±SEM) percent time spent exploring a juvenile stimulus rat during a 3-min encounter. Post treatment times are expressed as a percent relative to baseline and represent group means. ES and IS treatment resulted in a significant reduction in investigation time relative to (HC) controls, *** $p < 0.001$) **(B)** Mean (±SEM) of wheel-turn escape behavior during exposure to ES in 20 blocks of 5 trails. The 'state' indicates the mean number of one-quarter turns required to achieve an escape. **(C)** Mean (±SEM) of wheel turn escape latencies for animals exposed to ES.

Figure 2. Both ES and IS conditions revealed significantly increased freezing, escape latency, and escape failure in comparison to HC controls. **(A)** Mean (±SEM) time spent freezing during a 10sec observation period after two FR1 trials in the shuttle box. Values represent group means and are expressed in blocks of 2 mins. ES and IS treatments resulted in significantly longer times spent freezing than (HC) controls, $p < 0.05$. **(B)** Mean (±SEM) escape latencies during one block of 5 FR1 trials and 5 blocks of 5 FR2 trails. ES and IS treatments resulted in significantly longer escape latencies across all trial blocks, $p < 0.05$. **(C)** Mean (±SEM) number of escape failures during the shuttle box escape learning task for each stress treatment. ES and IS treatment resulted in significantly more escape failures than (HC) controls, * $p < 0.05$.

Figure 3. 5-HT containing cells that co-express Fos in the DRN did not significantly differ between ES and IS females **(A)** Mean (±SEM) percent of 5-HT positive cells expressing Fos for each sub region of the DRN. ES and IS treatments resulted in a significantly higher percent of 5-

HT neurons expressing Fos in rostral, middle, and caudal regions relative to (HC) controls. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$) **(B)** Photomicrograph of a coronal DRN section at -8mm from bregma. The white arrow points to a 5-HT/Fos double labeled cell, the black arrow points to a Fos positive cell, and the red arrow points to a 5-HT positive cell. The scale bar represents 25µm.

Figure 4. Percent of FG cells co-expressing Fos in the PL region did not significantly differ between ES and IS conditions **(A)** Mean (±SEM) percentage of FG cells co-expressing Fos in the PL neurons that project to the DRN. **(B)** Bright field photomicrograph of the PL region of a coronal mPFC section at +2.5mm from bregma. The red arrow points to a FG positive cell and the black arrow points to a 5-HT/FG double labeled cell. The scale bar represents 25µm. **(C)** Florescent photomicrograph showing a FG deposit in the dorsal raphe nucleus. The scale bar represents 500 µm.

Figure 5. Picrotoxin infused into the PL region during stress produced juvenile social investigation times in the IS and ES animals that were significantly longer than investigative times in the respective vehicle conditions. Mean (±SEM) of percent time spent exploring a juvenile stimulus rat during a 3-min encounter. Post treatment times are expressed as a percent relative to baseline and represent group means. ES and IS groups given vehicle had significantly shorter times investigating the juvenile in comparison to ES and IS groups given picrotoxin, ** $p< 0.001$, *** $p< 0.001$.

Table 1. Total number of 5-HT cells did not differ for each sub region of the DRN. The table expresses average and ±SEM of 5-HT and Fos cells in each sub region of the DRN for each stress treatment.

Figures/tables

Figure 1.

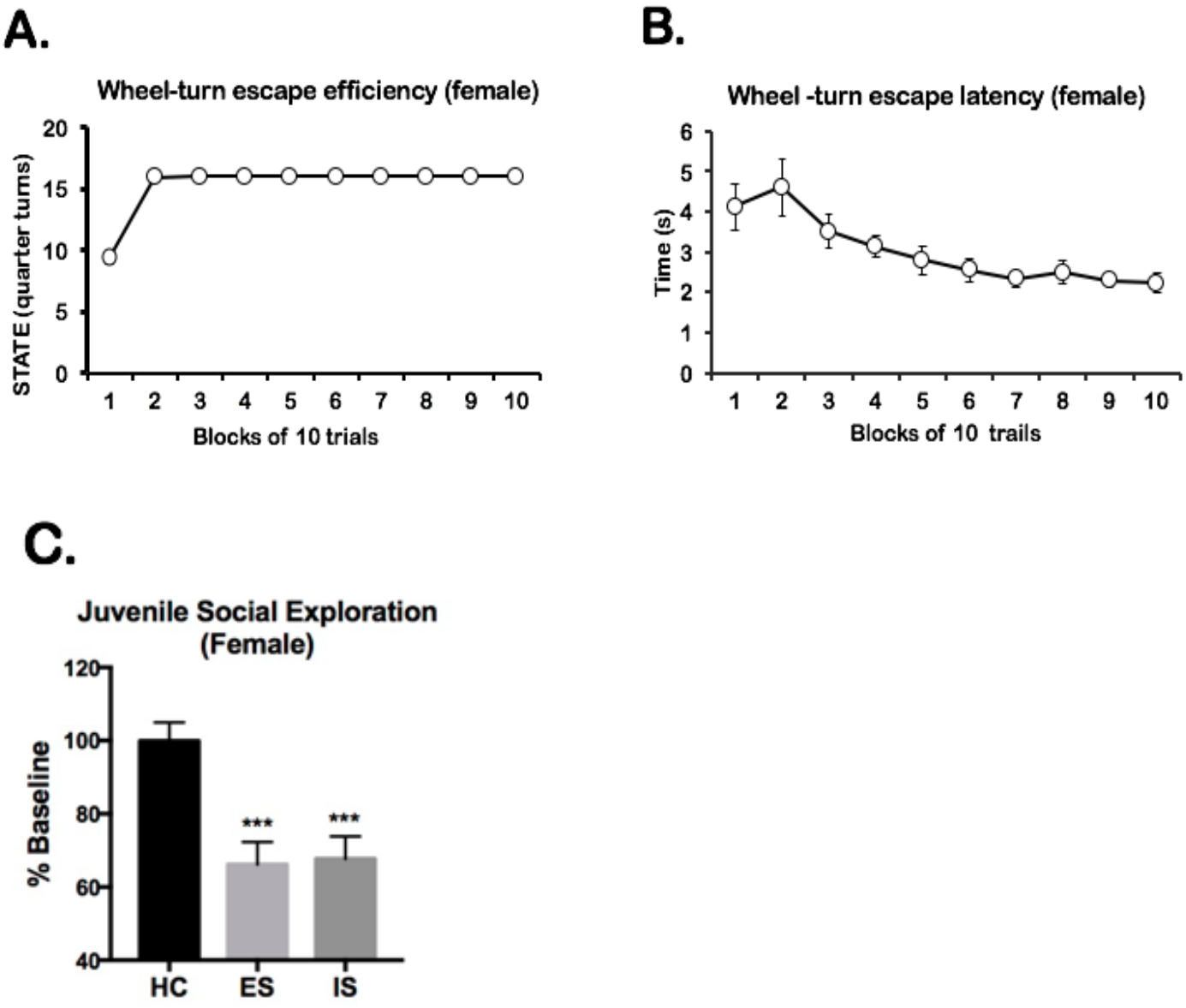
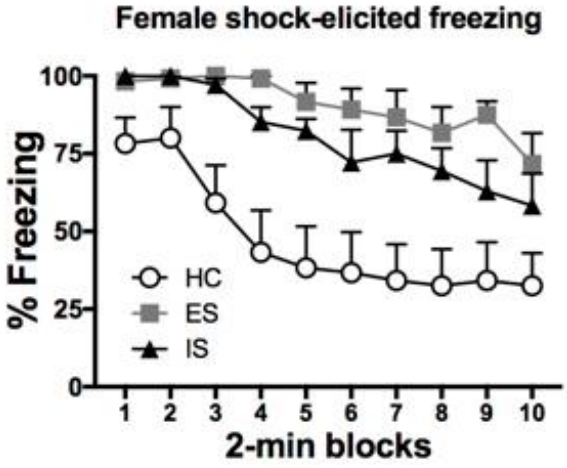
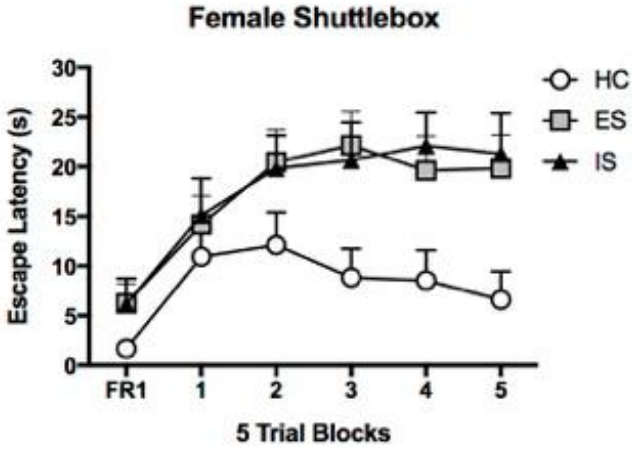


Figure 2.

A.


Female shock-elicited freezing
% Freezing
100
75
50
25
0
HC
ES
IS
1 2 3 4 5 6 7 8 9 10
2-min blocks

B.


Female Shuttlebox
Escape Latency (s)
30
25
20
15
10
5
0
HC
ES
IS
FR1 1 2 3 4 5
5 Trial Blocks

C.

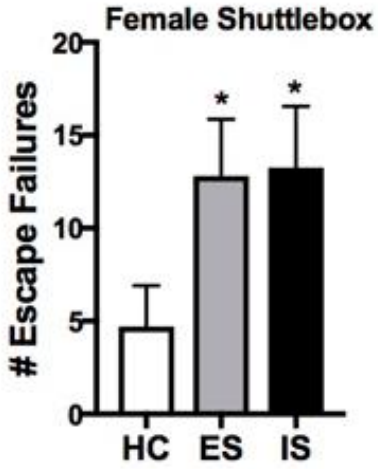
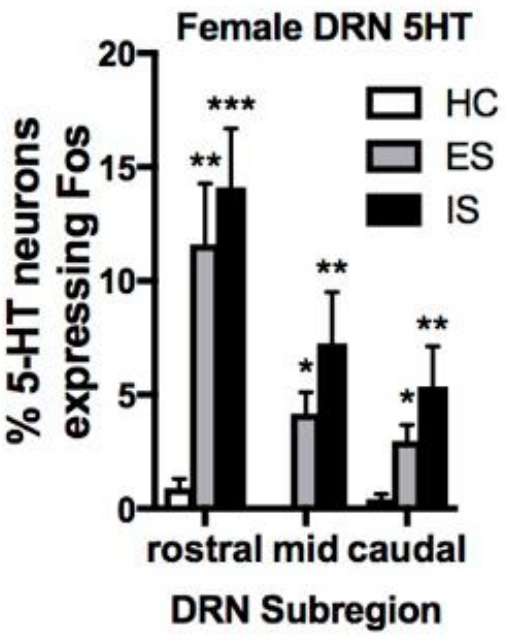

Female Shuttlebox
Escape Failures
20
15
10
5
0
*
*
HC ES IS

Figure 3.

A.


Female DRN 5HT
% 5-HT neurons expressing Fos
20
15
10
5
0
HC
ES
IS
***
**
**
*
**
*
rostral mid caudal
DRN Subregion

B.

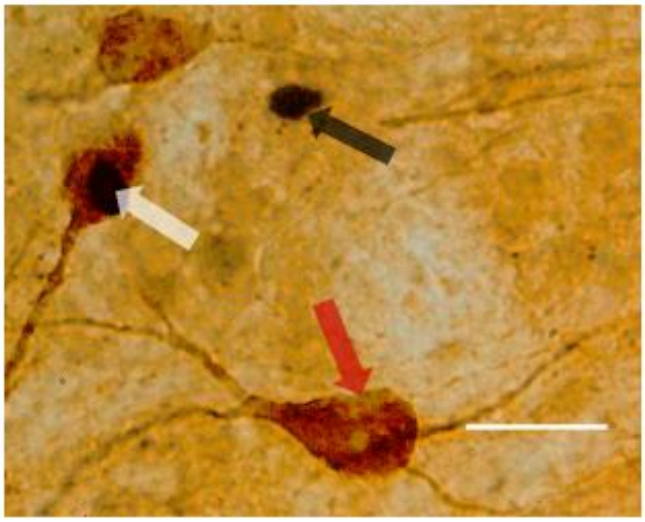


Figure 4.

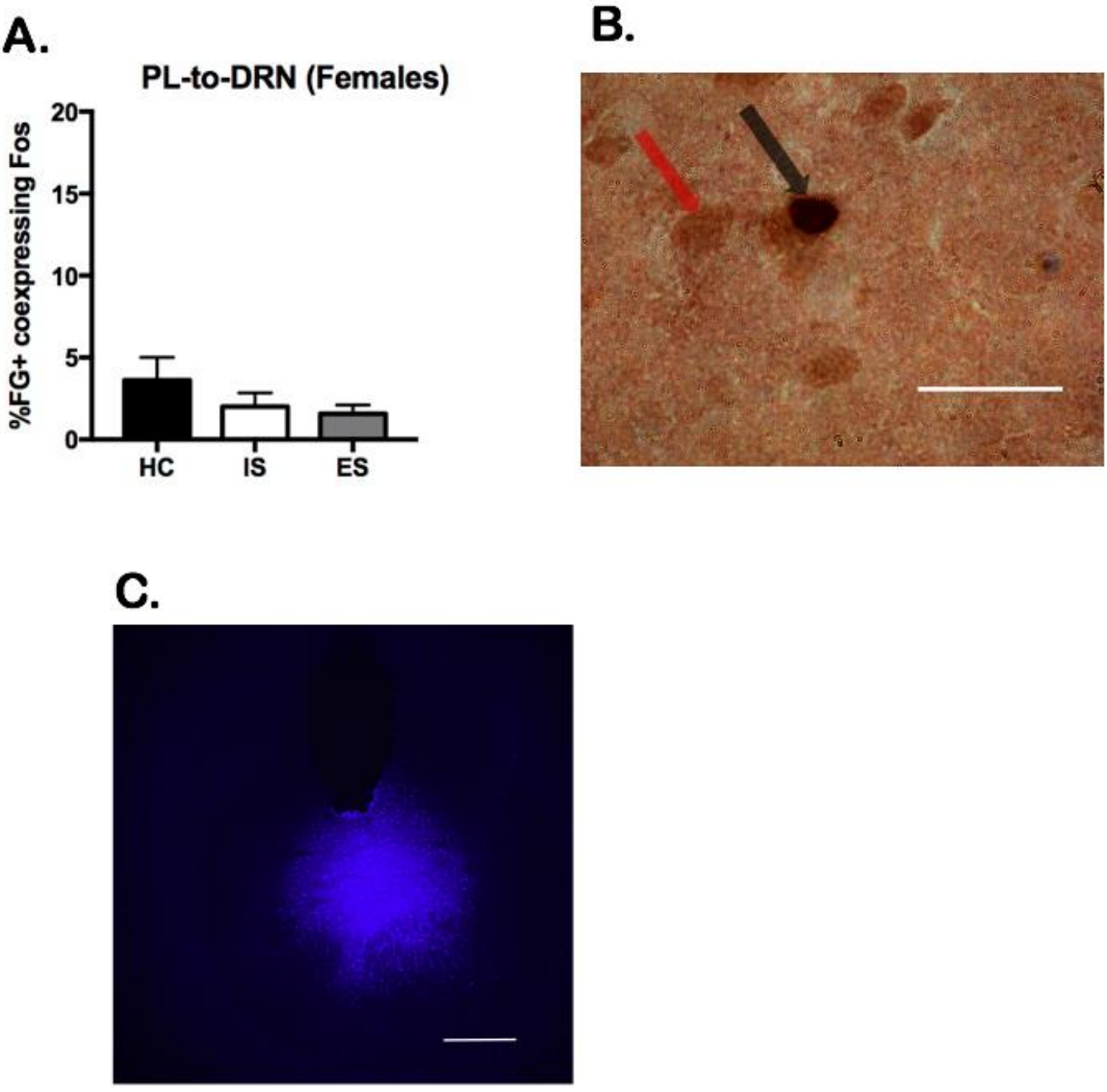


Figure 5.

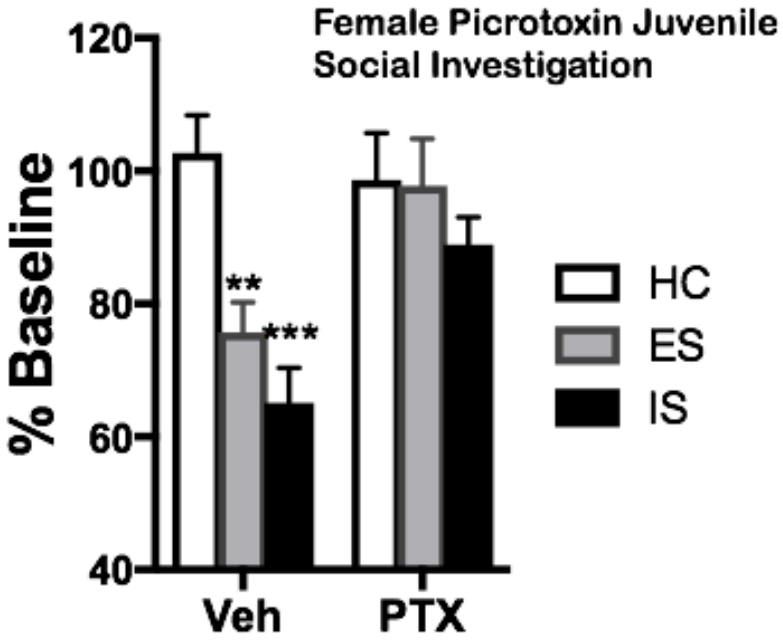

Female Picrotoxin Juvenile
Social Investigation
% Baseline
120
100
80
60
40
**
***
Veh
PTX
HC
ES
IS

Table 1.

Average number of cells in the DRN

DRN Sub region	5-HT			Fos		
	Group	**Mean**	**SD**	**Group**	**Mean**	**SD**
Rostral	ES	116.33	20.97	ES	43.67	11.21
	IS	76.33	9.48	IS	56.83	15.65
	HC	118.25	17.97	HC	1.00	1.00
Middle	ES	133	6.65	ES	18.33	4.32
	IS	126.33	17.90	IS	26.5	6.76
	HC	145.00	21.55	HC	0.00	0.00
Caudal	ES	107.17	17.25	ES	10.33	3.03
	IS	74.80	16.55	IS	12.3	3.65
	HC	82.5	21.55	HC	0.25	0.25