Dissection of ventral medial prefrontal cortex projections in two distinct stress-buffering models: stressor controllability and prophylactic ketamine

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Abstract

It is critical to develop an understanding of the neuronal circuitry elements that underly resilience in order to develop a better understanding of how the brain copes with later stressors. Thus our lab has taken a circuit level approach in understanding how exposure to an escapable stressor aids in future coping events. Recent work from our lab has identified that female rodents do not develop resilience from escapable stress (ES) as do their male counterparts, which introduces an interesting model to dissect the neuronal circuitry underlying resilience. In addition to this, the role of the infralimbic cortex (IL) in ES mediated attenuation of fear is of interest due to previous work within our lab. Thus, within this study we work to develop a greater understanding of the neuronal mechanisms underlying stress and resilience through two experiments. In this study we 1) aim to understand the role of infralimbic cortex (IL) projections to the basomedial amygdala (BMA) in ES mediated fear attenuation, and 2) work to determine the effectiveness of ketamine as a protective agent in females and to also dissect the role of prefrontal cortex (PFC) projections to both the dorsal medial striatum and the dorsal raphe nucleus (DRN) in females. Through this work, we identify a possible role of the IL-BMA projection in ES mediated fear attenuation and also identify that prophylactic ketamine induces a profound protective effect in females and selectively activates the PFC-DRN pathway.

Introduction

The psychiatric illnesses of depression and anxiety are critical problems being faced by society. Although there are a plethora of variables which may contribute to the development of these disorders, it is well documented that experiential factors such as exposure to adverse life events, including stress and trauma, are important in the etiology of depression and anxiety in humans (DiCorcia et al., 2011; Duman et al., 2016; Southwick et al., 2005). Stress resilience and vulnerability are largely mediated by coping. For instance, an individual's perceived level of control over the initial stressor has been shown to be correlated with their ability to cope with future adverse events (Basoglu, 1992; DiCorcia et al., 2011; Southwick et al., 2005; Weiss et al., 1986). Conversely, people who perceive themselves as lacking control over a traumatic stressful event demonstrate an increased prevalence of stress-induced psychiatric disorders (Basoglu, 1992; Duman, 2014; Southwick et al., 2005). With this clinical relevance, it is pertinent to develop and understanding of how perceived situational control leads to the protective effects of resilience.

In order to better understand the underlying neuronal factors that contribute to this stressor controllability effect, our lab implements a triadic stressor controllability design. Under this design, a pair of rats receive a series of electric tail shocks. One rat is granted behavioral control over the termination of the tail shock. This rat can turn a wheel positioned inside the conditioning chamber which will terminate the shock for the rat and its "yoked" partner. The yoked partner, although placed in a similar testing chamber, is not granted the ability to terminate the tail shock. In this design, the rat granted access to a controlling response receives "escapable stress" (ES) while the yoked partner receives inescapable stress (IS). Throughout the experiment,

a third rat remains undisturbed in the colony room (homecage, HC). Following IS, a variety of behaviors associated with symptoms of depression and anxiety occur. Collectively, these behaviors are considered "learned helplessness" behaviors and include: decreased juvenile social interaction, heightened fear response, and impaired fight/flight responding, impaired drug extinction (Amat et al., 1998; Baratta et al., 2007; Baratta et al., 2015; Christianson et al., 2010; Greenwood et al., 2003; Kenneth Kubala et al., 2012; K. Kubala et al., 2012). Remarkably, rats exposed to ES are protected from the adverse behavioral effects of both prior and future stressors (Amat et al., 1998; Williams et al., 1977). This protective effect observed in ES animals is often termed as "stress resilience" and has been shown to be trans-situational and persistent. (Amat et al., 1998; K. H. Kubala et al., 2012; Williams et al., 1977) Because these observations indicate a profound stress-buffering behavioral effect of ES, it is of particular interest to understand the neural processes underlying the protective effects of behavioral control in the ES model.

In the journey to identify the neuronal factors leading to stress resilience, it was found that the dorsal raphe nucleus (DRN) played a critical role. The DRN is a small serotonergic nucleus which resides in the caudal region of the rodent brain. Studies have identified that serotonin (5-HT) levels are increased in specific regions of the brain, such as the amygdala and ventral hippocampus, in rodents who have undergone IS conditioning (Amat et al., 1998; Amat et al., 2001). Interestingly, the DRN diffuses projections throughout the brain and is known to facilitate amygdala activation after exposure to IS (Amat et al., 2005; Christianson et al., 2010; Graeff et al., 1997; Maswood et al., 1998). Additionally, studies have identified that selective inhibition, or lesion, of the DRN prevents many of the behavioral effects associated with IS

(Maier et al., 1993; Maier et al., 1995; Maier et al., 1994). Thus, the DRN has been implicated as a critical component in the development of learned helplessness. Despite the notable role of the DRN in the development of learned helplessness, it is of interest to understand how stressor controllability interacts with the DRN to provoke resilience.

The DRN has been noted to receive virtually all of its cortical inputs from the pre-frontal cortex (PFC) (Maswood et al., 1998; Peyron et al., 1997; Vertes, 2004). Thus there is an emphasized interest on the PFC in relation to DRN mediation. Consequently, it was identified that the PFC was a necessary component for the protective effects observed in ES animals (Amat et al., 2005; Baratta et al., 2009). Injection of muscimol, a selective GABA agonist, into the infralimbic cortex (IL) of the ventral medial PFC (vmPFC) prior to ES conditioning results in learned helplessness behaviors regardless of actual stressor controllability (Amat et al., 2005; Christianson et al., 2009). Thus, the IL has been indicated to be a primary participant in the top-down regulation of the DRN (Amat et al., 2005; Maier, 2015). The IL projections to the DRN appear to be excitatory projections that synapse primarily on GABAergic interneurons, resulting in an overall inhibitory effect (Hajós et al., 1998). Thus, activation of this PFC-DRN pathway inhibits the DRN's activity and has been observed to be critical for the protective effects of ES. However, despite this mechanism for ES induced PFC modulation of the DRN, the problem still stands to identify how the PFC recognizes when a stressor is controllable.

When considering the recognition of stressor controllability, the PFC to dorsal medial striatum (DMS) circuit (PFC-DMS) is of particular interest due to its necessary role in act/outcome contingency learning (Balleine et al., 1998; Balleine et al., 2010; Liljeholm et al., 2011). Studies utilizing the ES/IS model have demonstrated that the DMS must be active in order for ES

to exert stress-buffering in male rodents (Amat et al., 2014). In addition to the necessity of the DMS in ES resilience, it has been demonstrated that the pre-limbic cortex (PL) region of the vmPFC must also be active for ES resilience to be obtained (Amat et al., 2014). Therefore, the communication between the DMS, a necessary structure for act/outcome contingency, and PL, a structure with prominent projections to the DRN, appears to be directly correlated with the detection of control and ultimately the development of stressor resilience within the ES model.

Using this ES/IS paradigm, we can continue to identify the underlying neuronal circuitry of learned helplessness. Our lab has sought to identify novel circuitry in relation to the previously mentioned circuitry elements. Through this work, we were led to develop two questions that expand upon the foundation of this learned helplessness model. First, we became interested in identifying the neural circuit elements that may be involved in the top-down regulation of fear in rodents. Second, we began to explore the stressor controllability phenom in females and identified that females are not protected from ES treatment as are males. Thus, we sought to investigate which neuronal circuits that female rodents may be engaging in contrast to their male counterparts.

It has been observed that both ES and IS have an effect on fear expression in rodents. Specifically, animals with a prior experience of ES exhibit a significant reduction of fear response relative to HC (see figure 1; Baratta et al., 2007), while IS has been demonstrated to show a significant increase in fear response relative to HC (see figure 1; Baratta et al., 2007). It was found that activation of the IL region reduces conditioned fear (Thompson et al., 2010). However, despite these observations the neural processes underlying this ES/IL mediated attenuation of fear have not been identified (Cho et al., 2013; Likhtik et al., 2008; Ozawa et al.,

2017; Pinard et al., 2012). We suggest two possible mechanisms for which how prior ES results in reduced expression of fear. It is possible that this reduction in fear response is due to: 1) increased inhibition of the amygdala in animals that previously underwent ES, or 2) increased activation of the amygdala in rats with previous exposure IS.

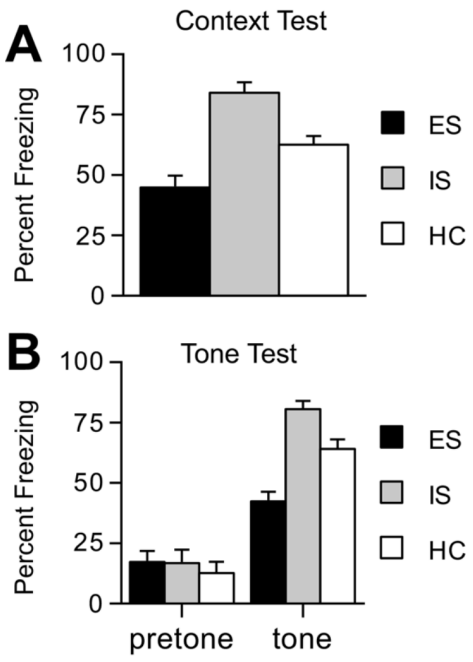

Fig. 1. Figure adapted from Baratta et al. 2007. **(A)** demonstrates the mean difference between HC/ES (P<.001) and HC/IS (P<.001) in fear response of context. **(B)** Demonstrates the mean difference between HC/ES (P<.001) and HC/IS (P<.001) in fear response of tone. Additionally, it is shown that there is no difference among groups pretone.

Recent work by Adhikari et al. has demonstrated the existence of an IL-BMA circuit, which plays a role in the top-down mediated control of fear and anxiety-like behavior in rodents (Adhikari et al., 2015). Adhikari et al. suggests that the PFC may regulate fear and anxiety expression in rodents via BMA projections to the Intercalated Nuclei of the Amygdala (ITA) (Adhikari et al., 2015). Recent work has indicated that the ITC receive input from the IL of the mPFC and that activation of the ITA reduces amygdala output thereby reducing expression of fear (Cho et al., 2013; Likhtik et al., 2008; Ozawa et al., 2017; Pinard et al., 2012). This is of interest given the suggested role of IL mediated suppression of fear in ES (Baratta et al., 2007; Thompson et al., 2010). Although the stressor controllability model was not directly utilized by Adikari et al., the findings led our lab to question if the IL-BMA pathway may be involved in the regulation of stress induced fear response.

Through interest in the effects of ES mediated stress resilience in females, our lab has observed that female rodents are not protected from prior ES conditioning (Unpublished work from Baratta et al.). However, although ES does not offer any protective effects in females, it has been observed that the administration of ketamine, an NMDA receptor antagonist, offers similar protectives effects as ES to both males and females (Sarkar et al., 2016). In a clinical setting, ketamine has been demonstrated to be a powerful anti-depressant and possesses anxiolytic effects. Thus it was of not much surprise to find that ketamine reverses many of the stress-induced behavioral effects in animal models (Brachman et al., 2016; Duman, 2014; Duman et al., 2016; Duman et al., 2012). Through work on the neuronal elements underlying the anxiolytic effects of ketamine, it has been identified that the PFC is a critical region for the drug's protective effects (Amat et al., 2016; Brachman et al., 2016; Duman et al., 2012). Further, it has been identified that, within males, ketamine acts to prevent 5-HT release in regions such as the baso-lateral amygdala (Amat et al., 2016). Although much of the current work has focused on the effect of ketamine administered following a traumatic stressor, recent findings have demonstrated a profound prophylactic effect of ketamine in male rodents (Amat et al., 2016; Brachman et al., 2016). This is of interest because our lab has been studying factors that prevent the impact of later stressors. Accordingly, our lab has sought to determine why ketamine is effective in females. Preliminary evidence suggests that ketamine delivered 1 week prior to IS prevents the detrimental behavioral effects of stressors in females, as has been observed in males (Dolzani et al.; in preparation; Amat et al., 2016; Brachman et al., 2016). Therefore, we sought to determine whether ketamine engages the PFC-DRN and PFC-DMS circuits in females in a similar method in which ES does in males.

In order to further understand the neural processes by which distinct factors, such as behavioral control and ketamine, buffer against the effects of stress our lab has taken a circuit level approach. In this study, we examined two distinct factors implicated in stress resilience in order to potentially identify circuits that mediate developed resilience. First, our lab sought to analyze the possible role of the IL-BMA pathway in mediating ES-induced attenuation of later fear expression in male rats. Second, our lab aimed to understand the role of the PL-DMS and PL-DRN pathways in mediating protective effects of ketamine in female rodents (see supplemental figure 1 for visual representation of the proposed neural circuitry being investigated in this study). Together, these studies work to advance the understanding of underlying neuronal mechanisms involved in stress resilience in both male and female rodents.

Aims

1) Examine the role of the IL-BMA pathway in ES-mediated attenuation of fear.
2) Identify the neural circuits that may contribute to the protective effects of ketamine administered prior to inescapable stress in female rats.

Materials and Methods

Proposed PFC-BMA circuit and its role in ES mediated diminished fear acquisition organization

Male Sprague Dawley rats were initially injected with retrogradely transported red florescent microspheres (retrobeads, RB) into the BMA following the "tracer microinjection procedure" described below. Rats were then caged for fourteen days in order to provide ample time for the retrobeads to be taken up by any PFC-BMA projecting neurons. Following this fourteen day

period, rats were separated into ES, IS, and HC groups and underwent the respective treatments as is described below in "stress procedure." ES (n=6), IS (n=7) , and HC (n=7) animals were assigned in such a way that rats were housed with animals of their same treatment group (e.g., HC with HC, ES with ES). After seven days, all subjects underwent fear conditioning following the "Fear procedure" described below. Rats were caged 24 hours after conditioning, and then placed into a novel context for fear expression testing as described in the "Fear procedure" below. Two hours after testing, animals were perfused and tissue was collected. This study was conducted with strict adherence to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

Neural circuitry elements underlying the protective effects of ketamine administered prior to inescapable stress in female rats organization

Female Sprague Dawley rats were used for this portion of the study. To first identify if ketamine had the same protective effects on females as has been observed in male rats, a cohort of female rodents was injected with ketamine (n=10) and saline (n=10) 1 week prior to testing in accordance with the "Drug administration" procedure described below. These animals then underwent IS conditioning as described below in "Stress procedure." Twenty-four hours following IS, these rodents participated in Juvenile Social Interaction testing as described in "Juvenile Social Interaction" below. After this, there were two additional cohorts of animals used to explore the pathways utilized by ketamine to induce stress resilience. For the first cohort, designated the DRN cohort, female rats were injected with retrobeads into the DRN. For the

second cohort, designated DMS cohort, female rats were injected with retrobeads into the DMS. Both cohorts were injected in accordance to the "Tracer microinjection" procedure described below. After this, both cohorts were caged for fourteen days to allow ample time for retrobead uptake. In the DRN Cohort, animals were separated into either saline (n=8) or ketamine (n=7) groups. In the DMS cohort, animals were similarly separated into either saline (n=8) or ketamine (n=8). Animals were then injected in relation to their saline or ketamine designation 2 hours before tissue collection in accordance to the "Drug administration" procedure described below. Animals were perfused and tissue was collected. This study was conducted with strict adherence to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

Subjects

The animals utilized in the BMA study consisted of Male Sprague Dawley rats while the animals used in the ketamine study were Female Sprague Dawley rats (Harlan Laboratories). In both studies, the rats weighed from 250 g to 300 g during the injection phase of the study. Rats were housed two per cage, each with a same-sex cagemate, on a constant 12 hour light dark cycle (on at 7:00 A.M. and off at 7:00 P.M.).

Tracer microinjection

Rats were anesthetized under inhaled isoflurane (2–5% v/v in O2) and then oriented into a stereotaxic frame. Through adjustment of the rat's incisor, it was ensured that bregma and

lambda were in the same horizontal plane. With coordinates dependent on the study, a small craniotomy (1.0 mm in diameter) was made to allow for penetration of a needle (31 gauge, 45° bevel) attached to a 10 l Hamilton syringe. Injections were then made using a microinjection pump (UMP3-1; World Precision Instruments). For the BMA study, the needle was placed stereotaxically in the BMA (AP, -2.0 mm from bregma; DV, -8.4 mm from Brain surface; ML, ± 3.6 mm from bregma). Within the ketamine study, the needle was stereotaxically placed in the DRN (AP, -8.0 mm from bregma; DV, -6.7 mm from skull surface; ML, 0.0 mm from bregma) and the DMS (AP, +0.1 mm from bregma; DV, -3.7 mm from brain surface; ML, ±2.0 mm from bregma). In all injections, three hundred nano-liters of a 1:3 concentration of retrobeads in saline were unilaterally injected over the course of 3 minutes. In both studies, the injections were allowed to diffuse for an additional 10 minutes after completion before the injection needle was removed.

Stress procedure

As described previously, animals were yoked together in ES and IS treatments (Amat et al., 2005). Rats were placed into a plexiglas box (14 x 11 x 17 cm) with a front mounted wheel. For animals that were designated as ES subjects, the wheel mounted to the front of the box had the ability to turn. During a shock trial, turning of this wheel would terminate shock and lead to escape of the stressor. The wheel within the IS animals' boxes did not have the ability to turn and thus could not terminate shock. All ES animals had an individual IS animal yoked to them, therefore shocks initiated and terminated simultaneously for both rats in a pair. This method of yoked stress for the IS animal produces IS effects that are similar to fixed-duration tail shocks.

Stress treatments consisted of 100 trials with an interval of 60 s between them. The first thirty trials consisted of 1.0 mA tail shocks, the following thirty consisted of 1.3 mA, and the final forty consisted of 1.6 mA. For the ES animal, initially the shock was terminated by a successful quarter of a wheel turn. The requirement to terminate shock was increased by a one-quarter turn every time an ES animal successfully terminated the shock within three consecutive trials with a 5 s latency. If an animal's latency was less than 5 s, the wheel turn requirement was increased by 50% up to a total of four full turns. If the ES animal did not terminate shock within a 30 s interval, the shock was automatically terminated and the wheel turn requirement reduced to a single quarter turn. Wheel turn response was analyzed (Supplemental figure 2) to show that ES animals learned the wheel turn response. For IS only animal cohorts, rodents were placed into loose Plexiglas restraint tubes (8 cm diameter, 18 cm length) yolked together. IS only animals then underwent a similar shock procedure as described above with the exception that they could not terminate shock and they had a lower shock duration; 100 trials, 5 s long shock period, 60 s interval between shocks, 30 shocks at 1.0 mA, 30 shocks at 1.3 mA, and 40 shocks at 1.6 mA.

Fear procedure

Prior to testing, rats were removed from the colony room and transported to an illuminated isolated room. Animals were then placed in two identical plexiglass chambers (26 x 21 x 24 cm). The removable floor of these chambers consisted of 22 stainless-steel rods that were 0.5 cm in diameter and spaced 1.75 cm from each rod's center (Coulbourn Instruments, Allentown, PA, USA; model E63- 23-MOD001). The rods were wired to a shock generator and scrambler

(Coulbourn Instruments; model H13-16). These chambers were positioned each in individual sound-attenuating Igloo ice boxes with white interiors. Both ice box had a speaker and a 6 W clear light bulb mounted to their ceilings. For conditioning, animals were placed in the plexiglass chambers and a tone was played for 2 minutes from the mounted speaker. The mounted speaker delivered a 76 dB, 2000 Hz tone. After this, a 2 s 1 mA shock was given to the rats through the metal floor. After conditioning, rats were immediately removed from the conditioning chamber and returned to the colony. Animals were then tested for fear expression 24 hours following this fear conditioning. Fear response was measured by observation of rodents placed into a novel context while playing the same 76 dB tone that was played during conditioning. The amount of time each rodent spent frozen was noted and interpreted to represent a fear response. The rods and floor of the chambers were cleaned with water before each animal was conditioned or tested.

Juvenile Social Interaction

Juvenile Social interaction (JSI) testing was conducted 24 h after stress treatment as described previously (Christianson et al., 2010). Although strenuous effort was made to prevent injury during the stress procedure, some injuries did occur and any injured animal was excluded from JSI tests since injury can affect the level and type of interaction. Test rats were placed individually into a clean test cage and allowed to acclimate for 45 min. After this, a ~28 day old juvenile rat of the same sex as the test rat was introduced to the cage for 3 min. An observer, blind to treatment, timed exploratory behaviors (such as sniffing, pinning, and allogrooming) initiated by the adult. Although Juveniles were used for multiple tests, any juvenile rat was never used twice with one adult rat.

Drug administration

Rats were intraperitoneally injected with either ketamine (10 mg/kg, dilluted in saline) (Ketaset; Boehringer Ingelheim) or saline. Both ketamine and saline injections were ~0.3 mL in total volume, and injected using a 26 gauge (45° bevel) needle.

Statistical analysis

Data was analyzed by two-way ANOVA with multiple comparisons test followed by Fisher's protected least significant difference test (PLSD) *post hoc* comparison (set at 0.05).

Tissue preparation

To prepare tissue for analysis, rats were given an overdose of sodium pentobarbital (60 mg/kg, i.p.). After no response from the rat, rats were perfused transcardially with 100 ml of ice-chilled 0.9% saline and then by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, brains were removed and postfixed overnight in the previously described paraformaldehyde fixative. Brains were then transferred to a 30% sucrose solution in 0.1 M PB and then stored at 4°C until sectioning. Brains were sectioned through the coronal plane. Regions of interest, which varied based on the study, were taken at 35 mm. All PFC tissue, from both studies, used for immunohistochemistry was placed in wells and stored at 4°C in cryoprotectant. DRN, DMS, and BMA sections were mounted directly onto Superfrost Plus slides (Fisher Scientific) and cover slipped with Vectashield (Vector Laboratories) mounting medium.

Immunohistochemistry

In order to stain for FOS, PFC tissue was first put through a series of washes with 0.01 M PBS and a solution consisting of 0.5% Triton X-100 and 0.01 M PBS. Tissue was then incubated overnight in a 0.01 M PBS blocking solution consisting of 0.5% Triton X-100 and 2.5% bovine serum albumin at 4°C . Slices were then washed in PBS and incubated for 24 h at room temperature in rabbit polyclonal primary antibody (1:2000; Santa Cruz Biotechnology) in blocking solution. Following this, slices were incubated for 2 h at RT in Alexa Fluor (AF) dependent on the study (488 goat anti-rabbit secondary antibody for PFC-BMA study and 405 for ketamine studies (1:250; Life Technologies)). After a series of PBS washes, tissue was floated onto slide glass and coverslipped.

Image analysis

Brain sections were observed using a Nikon N-SIM structured illumination super-resolution and A1 laser scanning confocal microscope and captured using NIS Elements software (Nikon). All digital images of the PFC were captured using a 20 x objective (numerical aperture 0.8) in monochrome coloring. Regions within the brain were identified based off reference to the rat brain atlas (Paxinos and Watson 1998). FOS was observed under a 488 nm (for AF 488) or 405 nm (for AF 405) wavelength and then pseudo-colored green for analysis. Retrobeads where observed under 561 nm wavelength and then pseudo-colored red for analysis. After images were taken, they were coded in a random fashion in order to blind the person who quantified the images. Colocalization of Fos and retrobeads was marked by identifying cell bodies where the two fluorophores overlapped. For each subject, two separate counts were taken from the PFC and averaged. The location of retrobead deposits in the BMA, DRN, and DMS were observed using a 5 x objective (numerical aperture 0.16). Retro bead deposits where observed under 561 nm

wavelength. Images were taken in order to asses injection accuracy. Injection accuracy was determined through reference with the rat brain atlas (Paxinos and Watson 1998).

Results

Study 1: Analysis of the proposed PFC-BMA circuit and its role in ES mediated diminished fear acquisition

Effect of ES/IS on IL-BMA pathway activation

Coronal sections of the BMA from the PFC-BMA cohort of male rats was observed in order to ensure animals had accurate injection deposits of red retrobeads. Animals that had inaccurate injections were excluded from the study (see figure 2 for a representation of what was considered as accurate). Ultimately, twenty animals were identified to have accurate deposits within the BMA (Figure 2). PFC slices from animals with accurate BMA injections were then imaged and

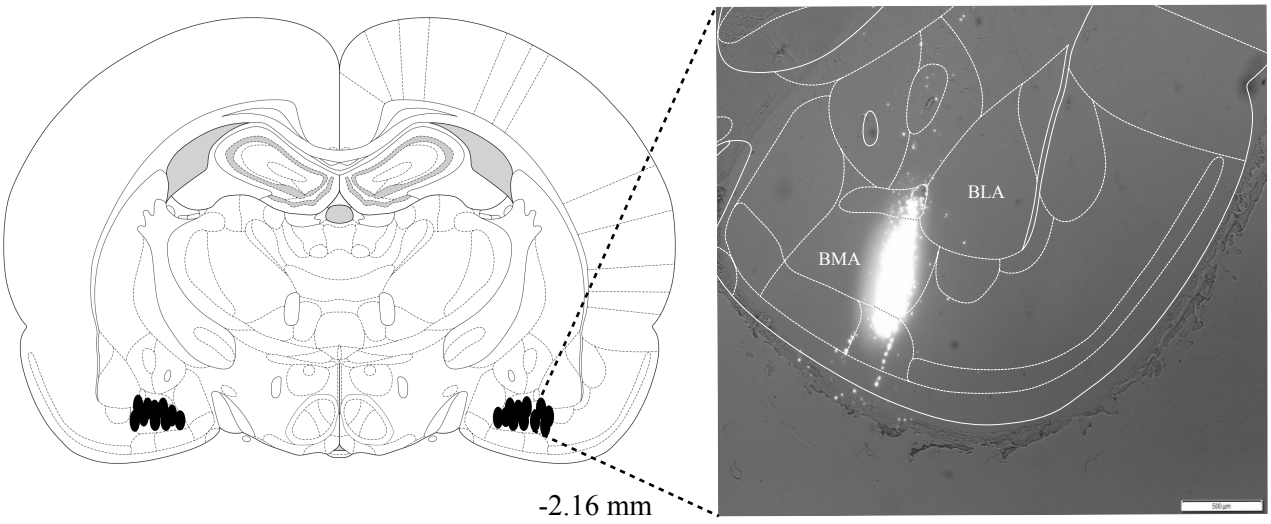

Fig. 2. (left) Coronal slice of rat brain -2.16mm from bregma. Black marks represent locations of accurate bead deposits in the BMA. (right) Representative image shows an accurate injection (5x magnification) of retrobeads in the BMA. Note that the scale bar in the representative image indicates a 500 µm distance.

analyzed by an observer blind to treatment. Quantification of retrobead cell counts in the IL region of the PFC identified that there was no significant difference in the number of cells containing retrobeads across groups (Figure 3 d). It was also identified that there was a significant (P=0.0012) increase of Fos activation in ES animals in comparison to HC within the IL (Figure 3 c). Co-localized cells were quantified from the same slices that Fos and retrobead counts were quantified from (see figure 3 a for representative of how co-localization was identified). Through this analysis, it was identified that the average number of cells with co-localization of retrobeads and Fos from animals in the ES group was significantly ($p<0.0001$) increased in comparison to both HC and IS (Figure 3).

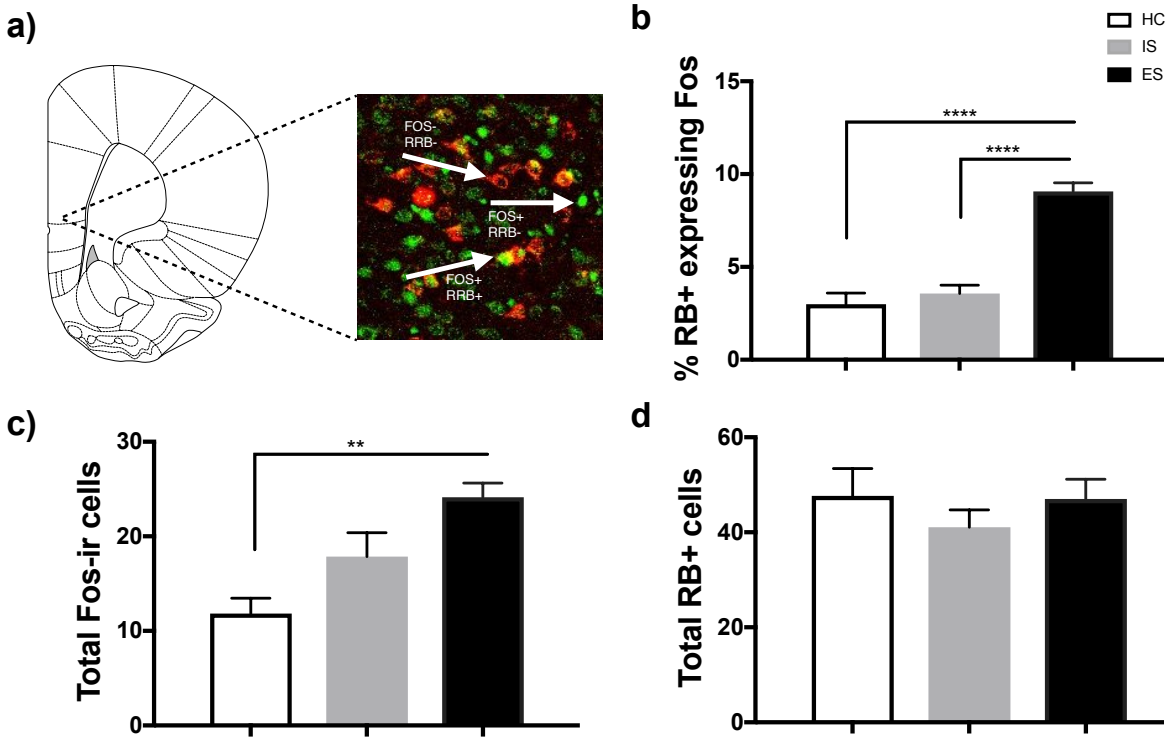

Figure 3. **(a)** Coronal section of rat PFC representing the region that was quantified for BMA study. The representative photo demonstrates how retrobead positive (RB+), Fos positive (FOS+), and co-localized (RB+/FOS+) cells were identified **(b)** Figure demonstrates that there is a significant increase of co-localized cells in ES in comparison the other groups ($P<0.0001$) and that there is no difference between retrobead/Fos co-localization between HC and IS groups. **(c)** Demonstrates a significance increase in total Fos positive cells between ES and HC ($P=0.0012$) and that there is no significant difference of Fos activation between IS and the other groups. **(d)** Demonstrates that there was no significant difference in retrobead positive cells found in the PFC across groups.

Behavioral effect of ES/IS on fear expression

After fear expression data was collected, fear expression from the PFC-BMA study was analyzed in several ways. First, fear expression was analyzed in accordance to the same method utilized in Baratta et al. 2007 (See figure 1 for the Baratta et al. representation of fear expression). Using a similar comparison, animals' total amount of freezing was graphed (See Figure 5 for statistical data concerning this representation, see figure 4 for graph). This comparison indicated that there was no level of significance between the groups in either the pre nor post tone tests. As an additional measure to ensure that data representing an increase in fear expression was represented as relative to each group, the data was compared as a percent increase from baseline. This graph was created by utilizing the pre-tone measurement as a group's baseline and tone freezing to represent an increase (See Figure 5 for statistical data concerning this representation, see figure 4 for graph). This method of analysis indicated a significant increase (p=0.0246) in fear expression of the IS group in comparison to the HC/ES groups.

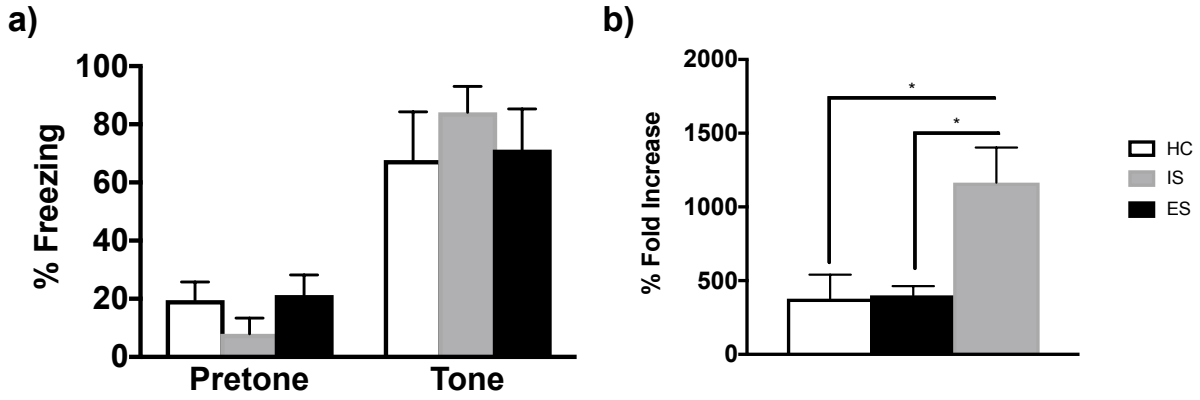

Figure 4. **(a)** Shows the average percent freezing of animals who underwent Home Cage (HC), Inescapable Shock (IS), and Escapable Shock (ES) treatments and then were fear conditioned. Pre-tone represents the pre-conditioning values levels of freezing while tone represents the post-condition values of freezing. **(b)** Demonstrates a significant difference in increased fear response when comparing animals to their base-line level fear response.

Raw freezing data									
Average Freezing instances	ES			IS			HC		
	Pre-tone	Tone	Total Possible	Pre-tone	Tone	Total Possible	Pre-tone	Tone	Total Possible
	4.83	13.83	19	2.43	16.14	19	4.00	13.14	19
Total pre-tone and tone statistical data									
	ES			IS			HC		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Pre-Tone	21.3	6.94	6	7.94	5.40	6	19.44	6.28	7
Tone	71.3	14.01	6	84.13	8.94	6	67.59	16.60	7
Percent of Baseline statistical data									
Percent fold increase	3.50	0.63	6	10.82	2.61	6	4.59	1.58	7

Figure 5. This figure demonstrates the statistical fear expression data collected from animals in the BMA study. The first row "raw freezing data" demonstrates the averages in freezing counted for each group, where 1 was the lowest score possible and 19 was the highest. The second row "Total pre-tone and tone statistical data" represents that statistical information that was used to analyze the groups in accordance to the Baratta et al. 2007 study. The third row titled "Percent of Baseline statistical data represents the statistical data that was used to analyze the groups in accordance to an increase from baseline fear expression.

Study 2: Neural circuitry elements underlying the protective effects of ketamine administered prior to inescapable stress in female rats

Juvenile social interaction

In the first phase of the ketamine experiment, female rodents were injected with either saline or ketamine and then 1 week later subjected to IS treatment. Home cage (HC) animals

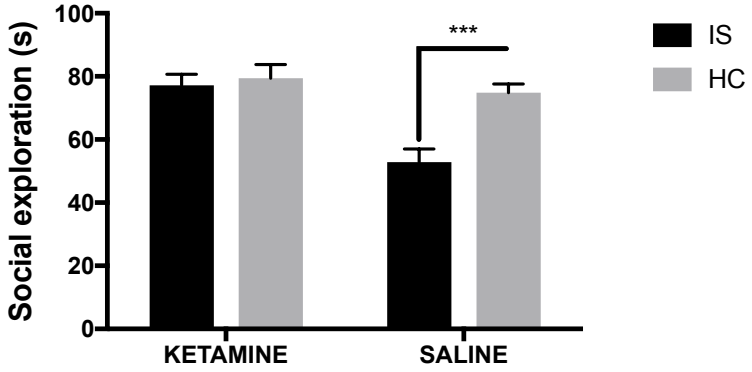

Figure 6. This figure represents female rats that were injected with either ketamine or saline and then subjected to IS conditioning. The significant reduction in juvenile social interaction observed in saline injected animals is rescued when female rats were instead injected with ketamine.

were neither subjected to IS nor injected with either substance. It was observed that the female rodents that were injected with ketamine, who later received IS, failed to exhibit juvenile social interaction deficits that were observed in the group of rats injected with saline who also received IS (Figure 6).

Ketamine induced pathway activation

Coronal sections of the both the DRN and DMS was observed in order to ensure animals had accurate injection deposits of red retrobeads. Animals that had inaccurate injections were excluded from the study (see supplemental figure 3 for a representation of what was considered as accurate). First, the DMS cohort of ketamine injected females was analyzed. Images of PFC slices were taken and a reviewer blind to treatment counted cells expressing retrobeads, Fos, and any co-localized cels. It was identified that there was no significant difference in retrobead positive cells between the saline or ketamine groups (Figure 8). However, there was a significant increase ($P=0.0044$) of Fos positive cells within the ketamine group in contrast to the saline group (Figure 8). However, of significant interest there was no difference between co-localized cells within the DMS cohort (Figure 8). The second group of animals, the DRN cohort, were analyzed in the same way as the first. In the DRN cohort, it was observed that the number of retrobeads was consistent among the two groups (Figure 9). Additionally there was a significant increase ($P=0.0012$) in the number of Fos positive cells within the ketamine group in comparison to the saline group (Figure 9). In contrast to the DMS cohort, there was an increase of co-localized cells in the animals injected with ketamine of the DRN cohort (Figure 9). See supplemental 5 for statistical data representing the two different groups.

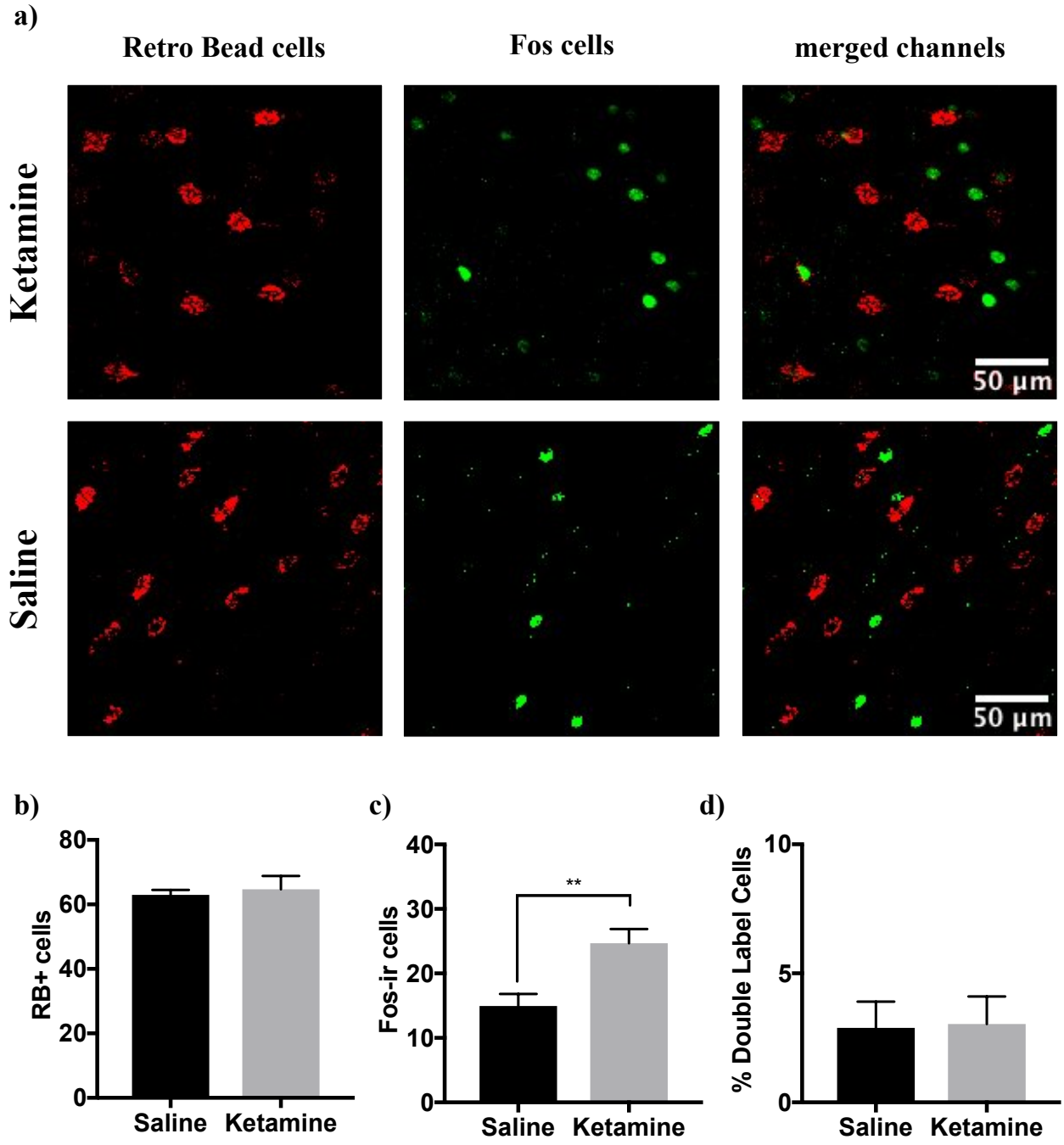

Figure 8. **(a)** Representative images taken of retrobead deposits in the DMS cohort of female ketamine study animals. The first two images represent retrobead positive cells and Fos positive cells retrospectively. The final image is both channels merged, showing any co-localized cells.**(b)** Figure represents that there is no significance in the number of retrobead cells within the PFC from animals in either group **(c)** Demonstrates that there is a significant difference between the number of Fos positive cells (P=0.0012) between the ketamine and saline groups. **(d)** Demonstrates that there is no significance in co-localized cells between ketamine and saline groups.

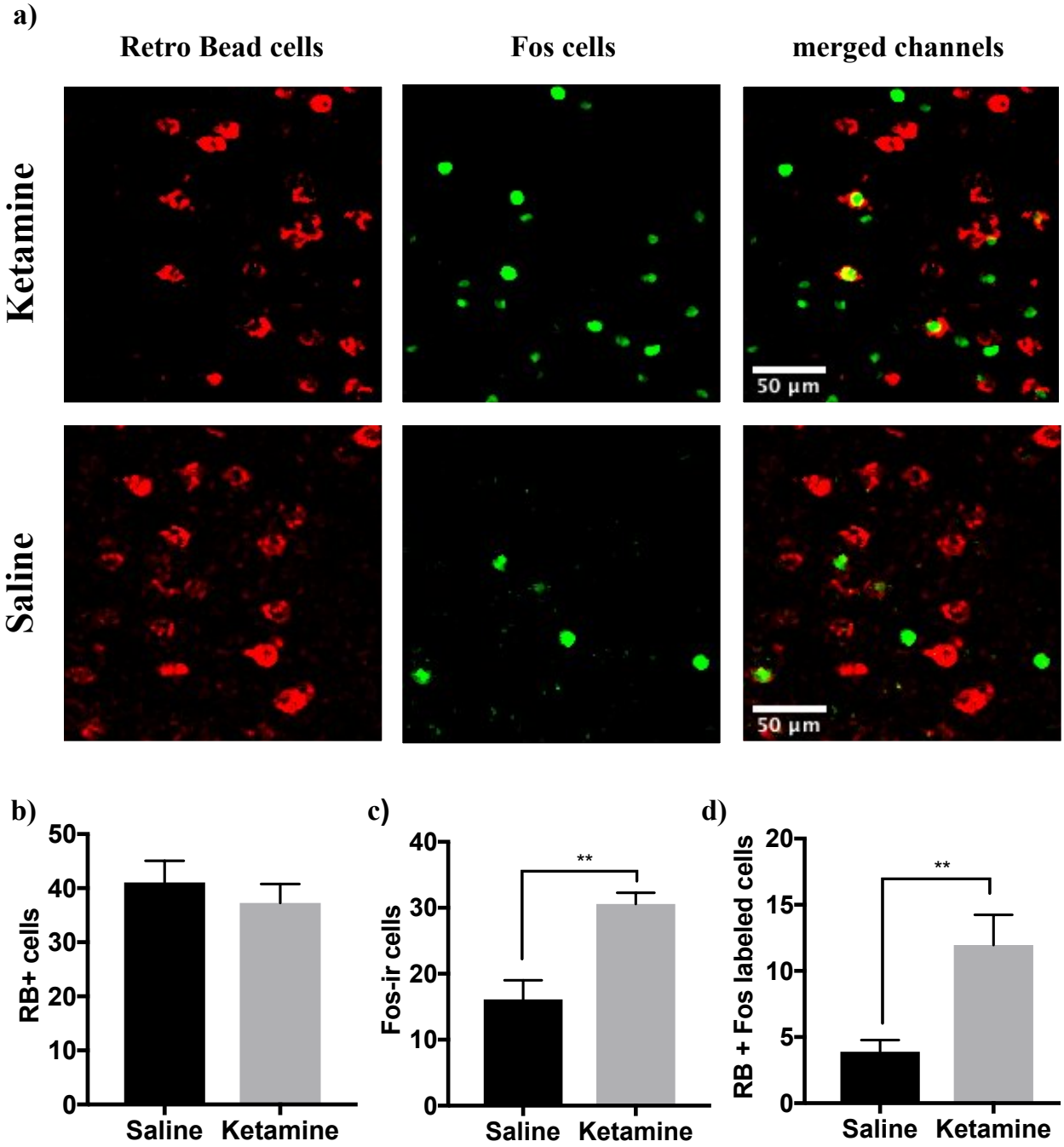

Figure 9. **(a)** Representative images taken of retrobead deposits in the DRN cohort of female ketamine study animals. The first two images represent retrobead positive cells and Fos positive cells retrospectively. The final image is both channels merged, showing any co-localized cells. **(b)** Figure represents that there is no significant difference in the number of retrobead positive cells within the PFC from animals in either group **(c)** Demonstrates that there is a significant difference between the number of Fos positive cells (P=0.0012) between the ketamine and saline groups. **(d)** Demonstrates a significant (P=0.0043) difference in co-localized cells between ketamine and saline groups.

Discussion

Study 1: Analysis of the proposed PFC-BMA circuit and its role in ES mediated diminished fear acquisition.

Deposits of retrobeads in the BMA confirm that accurate injections were made into the BMA. Further, the observation of retrobead positive cells within the IL region of the PFC is indicative that IL neurons do in fact extend projections to the BMA. These observations confirm the existence of a IL-BMA pathway. When looking at the data collected from the PFC slices, it is significant that the total number of retrobead cells did not differ significantly between any of the three cohorts (Figure 3 d). This suggests that equal numbers of IL neurons took up the retrobeads within each group and eliminates the possibility that differences in co-localized cell counts existed due to variable injections or variable neuronal uptake of the retrobeads. In continuation, it is of interest that the total Fos cell count in the IL was significantly increased in ES in contrast to HC (Figure 3 c). This is indicative that prior ES increases activation within the IL following a later fearful situation. Although IS animals appeared to show an increase of Fos activation relative to HC, any increase was not significant and could ultimately be attributed to heightened neuronal activation due to IS treatment. These findings are important because they coincide with current work that suggests that ES induces a heightened activation of PFC neurons within male rats, induces neural plasticity within the PFC, and results in heightened activation within the PFC following later stressors (Amat et al., 2005). Despite these findings, the most pertinent observation to our PFC-BMA study was the increase in co-localized Fos/retrobead cell counts within ES in comparison to IS or HC (Figure 3 b). The observation of co-localization of

retrobeads and Fos is indicative that the cell was activated during fear testing and also possessed projections to the BMA. The observed increase of co-localized cells in the ES cohort is significant because animals who underwent previous ES demonstrate heightened use of this neuronal pathway. Additionally, since HC and IS demonstrate no significant difference between co-localized cell counts, it can be inferred that IS fails to induce plasticity that leads to later activation in the IL. These conclusions are significant because they indicate a possible role of the IL-BMA pathway in stress resilience and may help explain the findings from Baratta et al. 2007 at the neuronal circuit level. However, these results can become somewhat conflicting when the behavioral data is taken into account.

Since fear conditioning and testing has been performed within our lab regularly, it is important to note that the results demonstrated in Figure 1 are what is expected after prior ES/IS animals are subjected to fear conditioning and testing (Baratta et al., 2007). However, despite our use of similar methods to the Baratta et al. 2007 work being used to condition, test, and analyze fear expression there was no significant difference between IS, HC, or ES behavioral data within the IL-BMA study (Figure 4 a). After initial analysis of fear expression, we were curious if the lack of expected behavior was due to fluctuation in baseline levels of fear expression among the different groups. This led us to analyze the percent freezing data as a delta from baseline, thus analyzing a percent increase within each cohort (Figure 4 b). This comparison did demonstrate a significant increase in IS fear response relative to HC/ES, but still lacked the important demonstration of decreased ES fear response relative to HC. These inconsistencies in the behavioral data between the present study and the Baratta et al. (2007) study introduce several difficulties into interpreting the data collected in the immunohistochemistry phase of the study.

Although some may suggest that this confounding behavioral data casts doubt on the circuit level analysis of the IL-BMA pathway, we have several interpretations for the confounding phenomenon. First, recently our lab has been performing experiments on both male and female rodents. Although every effort to clean lab testing areas is made, it is possible that female presence within testing/conditioning chambers altered the behavioral expression in males. However, this is not the most promising explanation as to why the expected behavioral data was not observed because recent work within our lab has been able to replicate the expected fear expression in different cohorts. This leads to the second explanation, which is that it is possible that the seven day waiting period after fear conditioning resulted in an attenuated fear response. Although the Baratta et al. 2007 paper also waited seven days after initial fear conditioning, recent work within our lab that has been successful in replicating this fear response has been performing fear testing much sooner than seven days (typically 72 hours). Thus it is possible that fear expression is not as robust in our cohort as was in the Baratta et al. 2007 cohort, and thus required fear expression to be tested sooner than we did. A final explanation to the lack of fear suppression in ES animals relative to HC is that the ES animals may have had difficulty learning the ES task. As demonstrated in supplemental figure 2, the rodents demonstrated a lower wheel turn requirement in bins 32-6 (trials 30-60). This along with the increased wheel turn latency are indicative of a possible hindrance in learning the ES wheel turn response. Although, the rodents learn the wheel turn response by the end of the experiment, the variable results obtained in bins 3-6 is abnormal. It would be expected that once rodents reach 16 quarter wheel turns, that they stay at this level for the entirety of the conditioning session and that their wheel turn latency would decrease throughout the session.

Ultimately, it is difficult to place the responsibility of the unexpected fear expression on one factor. It is possible that there were numerous factors, and even factors in combination, that contributed to the lack of observed fear expression in the subjects. However, despite this unexpected behavioral data, it is important to note that all cohorts demonstrated a relative increase in fear expression during the test. Additionally, it is significant that when animals were compared as a percent from baseline that ES animals appeared to be similar to HC while IS animals demonstrated a heightened level of fear expression. Therefore, even though the expected behavioral data was not observed, considering our explanations above, it is possible that there was still a IL-BMA effect of ES mediated fear response. Together, these data demonstrate that there is an increase in IL activation in fear expression within animals given prior ES exposure.

Study 2: Neural circuitry elements underlying the protective effects of ketamine administered prior to inescapable stress in female rats

The stress-induced reduction in JSI that is observed in saline injected females demonstrates known effects of IS on JSI. The fact that ketamine injected females had no significant difference in JSI relative to HC is demonstrative of a ketamine mediated prophylactic protective effect against later stress in female rodents. This is important for two reasons. First, as was mentioned previously, most of the current work focuses on the recovery effects of ketamine administered after IS. This work, in contrast, demonstrates that there is indeed a prophylactic protective effect of ketamine. This is important because it identifies that, by whatever mechanism is utilized by ketamine, the drug exerts protective effects in such a way that it can either rescue or protect animals from learned helplessness. The second significant point is that this protective

effect demonstrates that ketamine exerts similar stress-buffering effects in females as it does males. To date, this is the only manipulation performed in our laboratory that produces similar stress-buffering effects in both female and male rats. These data together create a unique model that enables us to study what pathways may be used differently or shared in ketamine-induced resilience in both males and females.

The second phase of the study identified critical observations in regard to circuit utilization in females. As was previously mentioned, it has been noted by unpublished work that females do not obtain the same protective effects of ES as do their male counterparts but that ketamine does offer an equal protective effect between the sexes. Additionally, the time point that was chosen to administer ketamine, and to take the brains, is significant because it aids in identifying whether ketamine is activating these pathways acutely. However, because of this chosen time point we could not run behavioral experiments since Fos is a transient immediate early gene and thus has a short life span within cells.Thus, waiting to examine Fos expression following behavior would fail to provide information on whether ketamine activates the PL-DRN and PL-DMS circuits acutely. Ultimately, the immunohistochemical data collected within this study begins to identify the acute effects of ketamine on precise neural circuits. The first observation of immunohistochemical data was that, in both the DMS and DRN cohorts respectively, there was no difference between the average number of retrobead positive cells between ketamine or saline injected animals. As stated previously, this is significant because it indicates that any difference in double label cell counts were not due to variable retrobead injections or neuronal uptake. Further, within both the DRN and DMS cohorts there was a significant increase of Fos positive cells within ketamine injected animals in comparison to the

saline groups. This is important because it suggests that ketamine is inducing heightened PFC activity within female rats (see Supplemental figure 4 for a comparison between the Fos cells of the two groups). This aligns with other work within male rodents that demonstrates that ketamine, and ES, results in increased neuronal activity within the PFC (Fuchikami et al., 2015). However, some of the most significant data for this study was identified through analyzation of the differences in co-localized cells in the DMS and DRN cohorts.

A significant increase of co-localized cells relative to HC was observed in the PFC-DRN animals. This is a pertinent observation because it suggests that ketamine induces heightened activity within the PFC-DRN pathway. Consequently, this suggests that ketamine is acting through the PFC-DRN pathway as a mechanism to induce stress resilience in females. Given that it is known that the PFC-DRN pathway is critical for the neuroprotective effects of ES in males, it is possible that this pathway is not only utilized by ketamine to induce protective effects in females but in males as well. However, in contrast to the DRN cohort, it was identified that there was no significant difference between the number of co-localized cells within the ketamine or saline animals within the DMS cohort. This lack of activation of PFC-DMS neurons in comparison to HC suggests that this pathway is not activated in female rats through the ketamine-induced prophylaxis of stress. This is and interesting observation considering the important role that the DMS has been implicated in within ES resilience in male models. These two observations together generate several interesting interpretations.

One of the first interpretations of these data relates to the role of the PFC-DRN circuit. In males, the PFC-DRN circuit has been known to be critical in the development of stress resilience in ES. Thus, the observation that ketamine induces activation of PFC-DRN is significant, since it

suggests that there is a similar pathway used by both sexes in the development of stress resilience. Additionally, since the PFC-DMS circuit has been implicated as a critical region for the recognition of "act-outcome" actions, these data suggest that ketamine bypasses the cognitive recognition of "act-outcome" and rather directly initiates the protective circuit of the PFC-DRN in females. Not only does this demonstrate a circuit level method of ketamine action, but it also offers a possible explanation as to why females are not protected by ES. Since the PFC-DRN pathway appears to be selectively activated by ketamine within females, this pathway can be implicated as a critical region in the development of stress resilience in females. In addition, PFC mediated DMS activation is suggested to not be required for the development of stress resilience via ketamine in females. Thus, by putting these two facets together, it can be suggested that female rodents are not protected from traditional ES because they may not engage the DMS pathway during the experience of behavioral control. Although more work would need to be performed to confirm these suggestions, it is regardlessly significant to note that the PFC-DRN pathway offers a possible critical circuit in the development of stress resilience.

Together, these observations develop several important conclusions in regard to the role of ketamine induced resilience in females. First, it is demonstrated that the effects of ketamine administration prior to IS in female rats is effective in producing stress resilience. In addition, it is demonstrated that ketamine selectively activates the PFC-DRN pathway while the PFC-DMS pathway appears not to be activated. The lack of PFC-DMS activation suggests that ketamine bypasses the act-outcome circuit, which has been implicated in ES-induced resilience, and even suggests an explanation as to why ES does not protect female rats.

Conclusion

Both of these studies introduce novel neuronal circuitry elements underlying the development of resilience. The IL-BMA pathway was identified to be activated in male ES rats who were exposed to fear conditioning in later fear tests. The PFC-DRN pathway was identified to be activated in female rats who had received ketamine, while the PFC-DMS pathway appeared to remain inactive. These conclusions are significant in that they advance or current understanding in regard to the underlying neuronal factors that lead to stress resilience in both males and females. In addition, the results gathered in the female ketamine experiment may aid in beginning to determine why females are not protected from ES. Understanding these pathways that are activated in the development of resilience, can lead to a greater understanding of the methodology behind the development of anxiety-like behaviors in rodents and how resilience takes effect. This work, although not immediately applicable to humans, can aid in the development of an understanding surrounding the neuronal pathways within humans and may ultimately lead to a deeper understanding about the neuronal processes underlying depression and anxiety.

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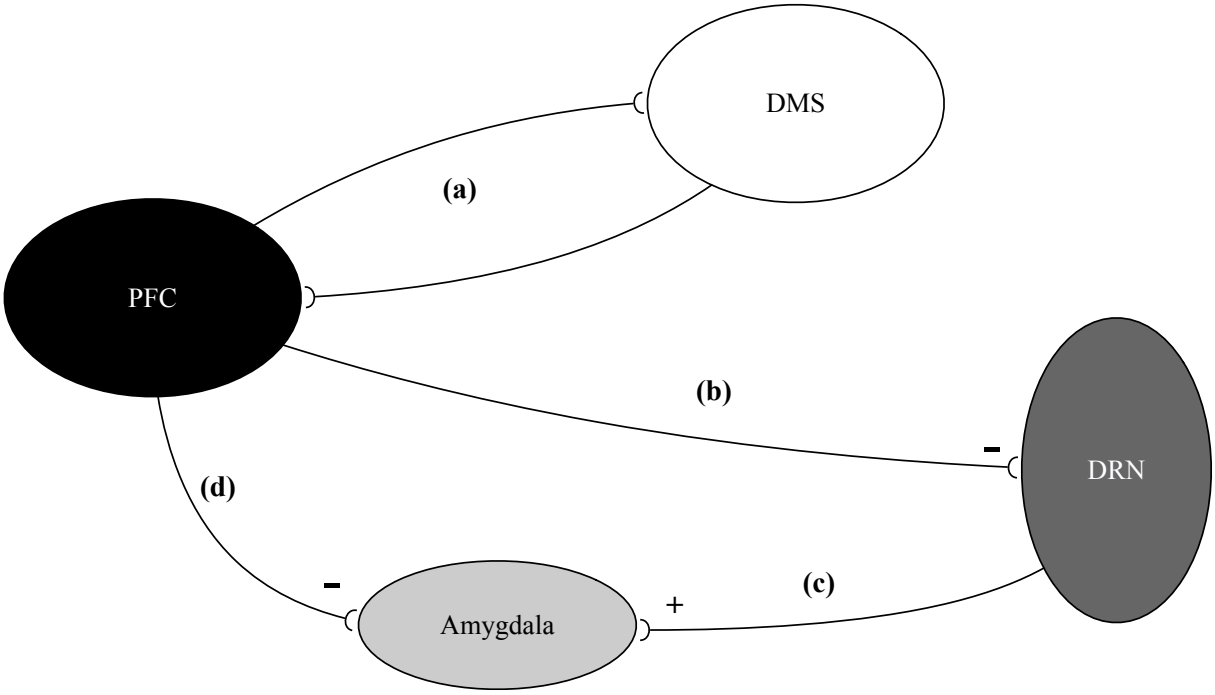
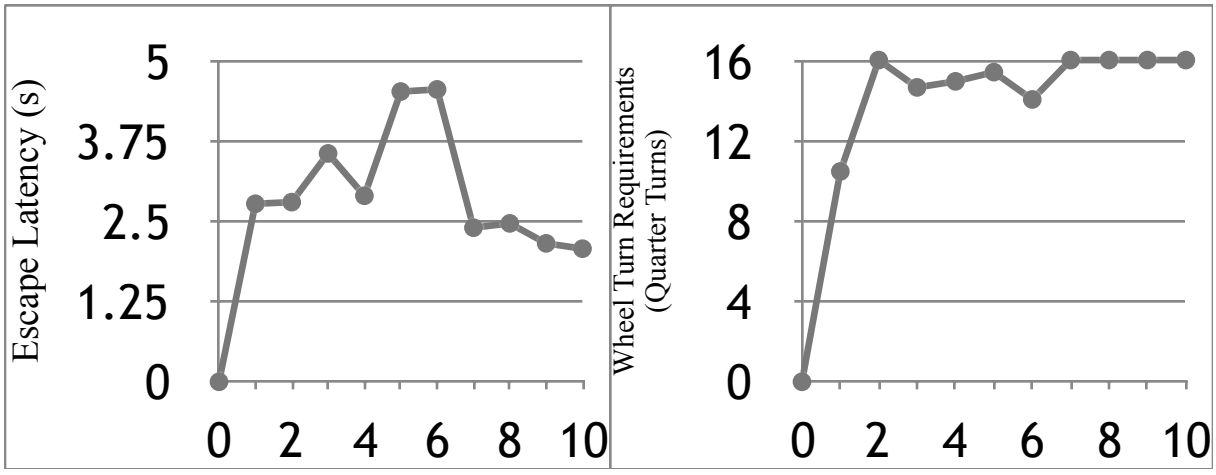
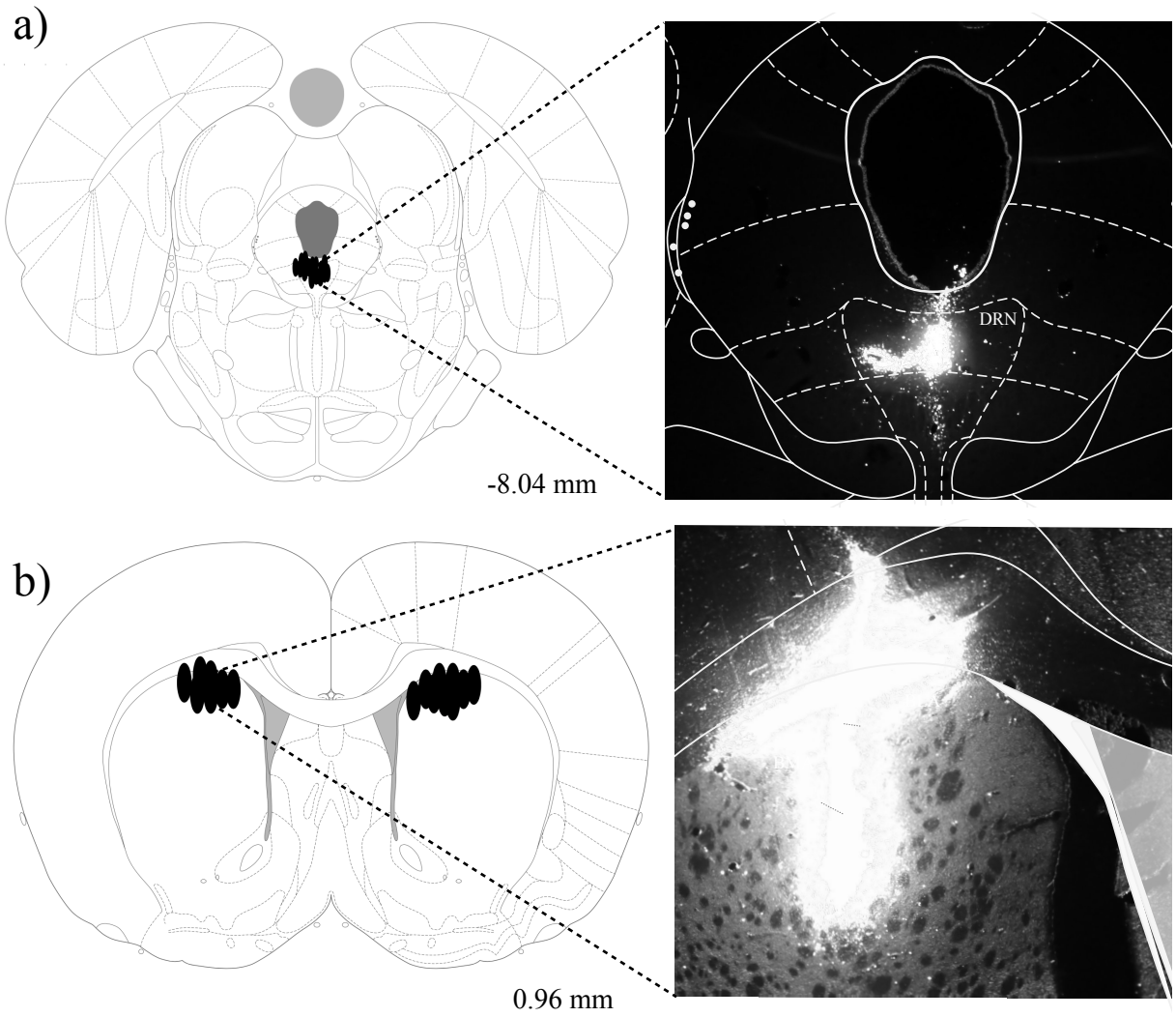


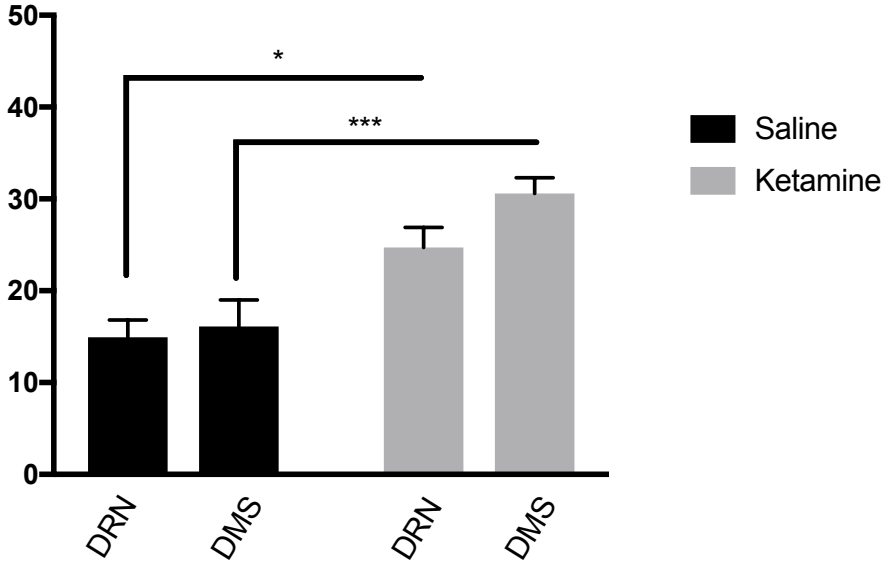
Supplemental figure 1. This figure demonstrates the suggested neuronal circuity involved in ES/ ketamine mediated stress resilience. **(a)** This is the PL-DMS circuit which may be contributed to the behavioral control detected by males during ES. **(b)** This is the PFC-DRN pathway which inhibits the serotonergic DRN. **(c)** The DRN-Amygdala circuit which is utilized by the DRN to activate the amygdala. **(d)** This is the proposed PFC-BMA circuit which may be utilized to induce resilience, and suppress fear, within the ES model.



Supplemental figure 2. This figure represents data collected during ES conditioning of the "Analysis of the proposed PFC-BMA circuit and its role in ES mediated diminished fear acquisition" experiment. 100 trials were broken down into 10 "bins" with each bin consisting of the average of 10 of the 100 trials from all ES animals used in the study (e.g. bin 2 represents trials 11-20). (left) demonstrates the wheel turn latency of ES animals through the progress of the ES conditioning. (right) demonstrates the number of required wheel turns to terminate shock through the progress of the ES conditioning (in quarter turns).



Supplemental figure 3. **(a)** (left) Coronal slice of rat brain -8.04 mm from bregma. Black marks represent locations of accurate retrobead deposits in the DRN. (right) Representative image indicating an accurate injection (5x magnification) of retrobeads in the DRN. **(b)** (left) Coronal slice of rat brain 0.96 mm from bregma. Black marks represent locations of accurate bead deposits in the DMS. (right) Representative image indicating an accurate injection (5x magnification) of retrobeads in the DMS.



Supplemental figure 4. This figure demonstrates a 2way ANOVA with multiple comparisons. On the X axis, DRN signifies the DRN cohort of the female ketamine study and DMS signifies the DMS cohort. There is a significant increase (DRN comparison P=0.0007, DMS comparison P=0.0209) of Fos activation in ketamine injected females from both ketamine groups in comparison to their perspective Saline groups.

PFC-DRN statistical data									
	Retrobead			Fos			Co-localized		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Ketamine	37.29	3.50	7.00	30.57	1.72	7.00	11.95	2.29	7.00
Saline	41.06	4.00	8.00	16.13	2.88	8.00	3.89	0.89	8.00
PFC-DMS statistical data									
	Retrobead			Fos			Co-localized		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Ketamine	64.69	4.13	8.00	24.69	2.19	8.00	3.04	1.07	8.00
Saline	62.94	1.55	8.00	14.94	1.87	8.00	2.89	1.02	8.00

Supplemental figure 5. This figure demonstrates the statistical fear expression data collected from animals in the ketamine study. The first row "PFC-DRN statistical data" demonstrates the averages in retrobeads, Fos, and co-localized cells between the ketamine and saline groups. The second row "PFC-DMS statistical data" represents the same information but for the animals within the DMS cohort.