Differential effects of active coping and ketamine on stress resilience in females

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Stress is an etiological factor in the onset of depression, anxiety, and post-traumatic stress disorder (PTSD). Therefore, significant efforts have been made to identify factors that produce stress resilience in hopes of mitigating stress outcomes. In a laboratory setting, rats given behavioral control over a stressor (escapable tailshock, ES) are protected from depression and anxiety-like behavioral changes that are present in rats exposed to an equivalent but uncontrollable stressor (inescapable tailshock, IS). Similar to behavioral control, the NMDA receptor antagonist ketamine has gained attention for its ability to prevent outcomes of IS when administered far in advance of the stressor. Despite promising results of preclinical studies performed in male rats, the effects of behavioral control and ketamine in female rats remains unknown.

Here, the effects of behavioral control and ketamine on stress-induced behavioral changes and the neural circuit-level processes that mediate these effects in female rats are explored. We assessed whether behavioral control and ketamine prevent stress-induced dorsal raphe nucleus (DRN) activation and behavioral changes associated with DRN activation. We also assessed whether behavioral control and ketamine engage an inhibitory prelimbic cortex (PL) to DRN (PL-DRN) circuit.

Behavioral control failed to mitigate DRN activation and behavioral outcomes induced by stress, as it does in males. Moreover, behavioral control failed to selectively engage PL neurons that project to the DRN. Pharmacological activation of the PL restored the stress-buffering effects of control. Conversely, ketamine given prior to IS reduced typical stress-induced DRN activation and accompanying behavioral changes. Proactive ketamine altered PL neural ensembles so that

a later experience with IS now activated these cells, which it ordinarily would not. Chemogenetic inhibition of the PL-DRN circuit at the time of stress prevented ketamine-induced stress resilience.

The present experiments provide the first evidence demonstrating a bidirectional influence of ketamine and behavioral control on neural circuits of resilience in females. Ketamine, but not behavioral control, engages an inhibitory PL-DRN circuit and prevents stress-induced behavioral changes. Taken together, this underscores the importance of examining sex differences when studying neural circuits underlying stress resilience and translating these findings to the development of preventative strategies for stress-related disorders.

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Chapter 1:

General Introduction

Part I. General overview

A combination of genetic, neurochemical, and experiential factors influence vulnerability to mood disorders (Charney et al., 2004; Southwick et al., 2005; Duman et al, 2016). Of specific interest is the role that experiential factors such as stress and trauma have on organismic functioning. Many individuals exposed to early-life or ongoing stressors, including acute trauma, go on to develop psychiatric disorders, including major depressive disorder (MDD) and anxiety disorders, such as post-traumatic stress disorder (PTSD) (Kendler et al., 1999; Heim and Nemeroff, 2009). Therefore, it is generally accepted that exposure to stressors, *per se*, is a powerful etiological factor in the onset of a variety of psychiatric disorders. Interestingly, there is significant individual variation in response to seemingly similar adverse events and the majority of individuals exposed to stressors never develop pathological consequences (Russo et al., 2012; Feder et al., 2009; Alleva and Francia, 2009; Charney et al., 2004). For example, while depression and anxiety disorders occur in only a small portion of individuals exposed to stressors, the risk is twice as high in females as in males (Marcus et al., 2005; Marcus et al., 2008; Shansky et al., 2015). Similarly, sex differences are commonly reported in rodent models of stress that are designed to model aspects of depression and anxiety (Korkas et al., 2014). Accordingly, there is great interest in understanding factors that produce "resilience" to the outcomes of stress and sex differences.

For the purpose of the present thesis, resilience is defined as positive adaptations by which most organisms, when faced with significant stress or trauma, maintain normal psychological and physical functioning (Luther and Cicchetti, 2000; Russo et al., 2012). At this point in time, human studies of resilience have focused on perceived self-efficacy, perceived ability to cope, and actual ability to control certain aspects of adverse events as critical psychological and physical factors that modulate the impact of an adverse event (Zimmerman et al., 1999; Yi et al., 2005; Chorpita et al., 1998). The neural processes that underlie these protective factors in humans have received little study.

In the human literature, it is well documented that stress resilience and vulnerability are largely influenced by how an individual appraises an adverse event (Southwick et al., 2005). This encompasses the degree to which an individual can predict, avoid, escape, or otherwise control the adverse event. To that end, resilient individuals have been described as implementing active or control-based coping strategies that directly address the stressor, rather than passive or avoidance-based coping strategies (Southwick et al., 2005). Conversely, lack of control has been proposed to contribute to depression and PTSD (Bosoglu and Mineka, 1992). Unfortunately, a single psychological factor such as active coping is not effective in preventing the consequences of stress in all individuals, and so identification of additional factors that produce resilience is of clinical importance. One such factor that has gained recent attention for its therapeutic potential is the *N*-methyl-D-aspartate (NMDA) receptor antagonist, ketamine. Remarkably, a single subanesthetic dose of ketamine exerts powerful antidepressant and anxiolytic effects in individuals diagnosed with treatment resistant MDD, generalized anxiety disorder, social anxiety disorder, and PTSD (Berman et al., 2000; Zarate et al., 2004; Zarate et al., 2006; Glue et al., 2017). The phenomenological similarities between active coping and ketamine suggest shared neurobiological processes may underlie the actions of these two discrete variables.

In line with clinical evidence, basic research demonstrates that both active coping processes and ketamine produce resistance to the effects of later stress (Amat et al., 2005; Amat et al., 2016). Surprisingly, nearly all of the work dedicated to understanding the neurobiological substrates engaged by these two factors has been performed in male rodents. Therefore, the purpose of this dissertation is to explore the stress-buffering utility of behavioral control over, which is one aspect of coping that can be modeled in rodents, and ketamine in female rats. Here, evidence is presented that ketamine, but not behavioral control, exerts protective stress-buffering effects in female rats. Differential activation of the prelimbic region (PL) of the medial prefrontal cortex (mPFC) to dorsal raphe nucleus (DRN) pathway (PL-DRN) appears to be a critical neural

mechanism underlying the dissimilarity between behavioral control and ketamine on resistance to the outcomes of stress in female rats.

Behavioral control, which is a core feature of active coping, is an aspect of coping that can be studied in a laboratory setting using what has been termed the “triadic design” (Seligman and Maier, 1976; Weiss et al., 1981). Under this arrangement, a series of electric shocks are delivered to the tails of a yoked pair of rats. One rat is allowed to terminate each pseudorandomly delivered tailshock by performing an operant response, which entails turning a wheel mounted to the wall of the chamber in which it is restrained. Each tailshock terminates when the rat responds, and persists if the rat does not respond up to an experimenter-defined limit. Thus, this rat has behavioral control over the duration of each tailshock, and so this treatment is considered controllable stress (escapable stress, ES). Conversely, the yoked subject receives an identical duration, intensity, and temporal pattern of tailshock, yet they have no control over the termination of the shock. For this rat, each shock terminates whenever the ES subject turns the wheel. This treatment is considered uncontrollable stress (inescapable stress, IS). A third group of rats remains undisturbed in the colony room (non-stressed homecage, HC). Under this arrangement, it is possible to study the impact of a psychological variable such as control in an experimental setting in which all other variables are held constant.

At the level of basic research, the first observation of the importance of stressor controllability was demonstrated in an escape learning paradigm in which subjects are required to learn an escape response to terminate footshocks in a shuttlebox (Maier and Seligman, 1967). Interestingly, 24 hours after the initial stressor, subjects that had received ES learned the shuttle escape response as well as did animals that had received only HC, while subjects that had received IS failed to learn the escape response (Maier and Seligman, 1967). Subjects that received IS also went on to develop gastric lesions (ulcers), whereas ES animals did not (Weiss, 1968). Since the initial studies nearly fifty years ago that characterized this phenomenon now known as “learned helplessness”, numerous additional behavioral changes have been identified

in animals previously exposed to IS. These include exaggerated fear conditioning, impaired fear extinction, impaired fight/flight responding, impaired drug extinction, neophobia, reduced social dominance, reduced food and water intake, and reduced juvenile social interaction (JSE) (Christianson et al., 2008; Maier et al., 1998; Baratta et al., 2015; Short and Maier, 1992; reviewed in Maier and Watkins, 2005). Importantly, the environment in which behavioral testing occurs in these experiments is typically distinct from that in which the original stressor occurred, and so behavioral outcomes of IS are considered "trans-situational". Finally, IS-induced behavioral changes are persistent and are typically observed 24 hours to 3 weeks following initial stressor exposure (Grau et al., 1981; Weiss et al., 1981; Weiss JM, 1981; Maier 1990; Baratta et al., 2015).

The use of electric shock in these studies deserves comment since it is an artificial stimulus. Manipulation of stressor controllability requires an aversive event that can be rapidly (sub-second timescale) initiated and terminated so subjects with and without behavioral control can be yoked so as to receive a physically identical stressor. Stimulus equivalency is a requisite component to enable isolation of the impact of controllability of the stressor that cannot be confounded by differences in the stressor. Other stressors that might be used such as predator odor, tail suspension, restraint, pharmacological agents, and social defeat fail to meet this criterion.

Part II. Stress-sensitive brain systems

i. The noradrenergic system

Because IS, but not physically identical ES, produces anxiety-like and depression-like behavioral changes, significant effort has been made to understand how IS produces these sequelae. One method of understanding the differences produce by IS is to identify neurobiological systems activated by IS, but not ES. Several monoaminergic systems have received study in regard to the stressor controllability paradigm. Indeed, disruption of catecholaminergic neurotransmission following exposure to severe, uncontrollable stressors,

such as IS, has been linked to learned helplessness behavior. For instance, Jay Weiss and colleagues first identified transient noradrenergic (NE) deficits in a variety of brain regions following IS, but not ES (Weiss et al., 1970; Weiss et al., 1981). Stress-sensitive NE is primarily produced in the brainstem locus coeruleus (LC) (Chu and Bloom, 1973). Simson and Weiss (1986) suggested that increased LC NE turnover produced by IS sensitizes NE cells in the LC. This sensitization is thought to reduce sensitivity of NE α-2 receptors in the LC. α-2 NE receptors are located on cell bodies and dendrites of LC neurons and are the primary NE autoreceptor. Under normal conditions, stimulation of these receptors by NE release from the LC decreases NE neuronal firing. Therefore, it was hypothesized that stress-induced reductions in activity of these receptors would produce a hyperactive NE response during later behavioral testing (Simson et al., 1986; Weiss et al., 1994). Indeed, it has been shown that microinjection of α-2 receptor antagonists directly into the LC recapitulates some of the behavioral effects of IS, while α-2 receptor agonists prevent these outcomes (Weiss and Simson, 198).

Interestingly, in disagreement with the work by the Weiss group, more recent work by McDevvitt and colleagues (2009) demonstrates that both IS and ES produce similar activation of LC NE cell bodies. This suggests that if the behavioral differences observed between IS and ES animals are due to changes in NE, the changes must be occurring at regions where LC terminals synapse, rather than within the LC itself. Accordingly, IS-induced behaviors can be modulated through manipulation of NE in brain regions that receive LC efferents.

ii. The serotonergic system

One such region that receives intense NE innervation from the LC is the serotonergic (5-HT) dorsal raphe nucleus (DRN) (Peyron et al., 1996). High doses of NE receptor agonists increase the primary metabolite of 5-HT, 5-hydroxyindolacetic acid (5-HIAA) within the DRN (Clement et al., 1992) and inhibition of NE release suppresses DRN 5-HT cell firing (Baraban and Aghajanian, 1981). Previous work from our laboratory demonstrates that the selective α-1

adrenergic receptor antagonist benoxathian prevents IS-induced shuttlebox escape impairments when given prior to IS and exaggerated conditioned fear when given prior to IS or later behavioral testing (Grahn et al., 2002). Together, these data suggest that modulation of the 5-HT system mediates, at least in part, the behavioral sequelae of IS.

Comprised of only ~30,000 5-HT cells, the DRN provides nearly all of the 5-HT input to limbic and forebrain areas involved in a variety of cognitive and emotional processes (Hornung et al., 2010; Jacobs and Azmitia, 1992). Because the DRN gives rise to such diffuse 5-HT projections, 5-HT is implicated in myriad of diverse physiological and behavioral processes including sexual receptivity, aggression, reward, pain, appetite, anxiety, and stress (Lowry et al., 2008; Mendelson, 1992; Almeida and Miczek et al., 2002; Kranz et al., 2010; Abrams et al., 2005; Rueter et al., 1997) In addition, the DRN receives a variety of afferents from regions such as the LC, hypothalamus, bed nucleus of the stria terminalis (BNST), amygdala, and lateral habenula (LHb) (more on these inputs below) (Lowry et al., 2008; Dolzani et al., 2016). These structures are commonly associated with the behavioral response to stress. Taken together, it is apparent that the DRN is uniquely capable of integrating various neural inputs and outputs involved in the production of a variety of behavioral outcomes associated with IS. Indeed, a large body of work suggests that potent stressors, such as IS, sensitize the DRN in a manner whereby subsequent input to the DRN produces an exaggerated release of DRN 5-HT both within the DRN and projection regions, ultimately producing learned helplessness behaviors (*reviewed in* Maier and Watkins, 2005). A variety of converging experimental evidence supports this view.

Remarkably, exposure to IS, but not physically identical ES, produces a massive release (200-500% of baseline) of 5-HT within the DRN (Maswood et al., 1998), as measured by in vivo microdialysis. This effect is quite persistent, as 5-HT levels remain elevated for up to 24 hours following the final tailshock (Amat et al., 2016). Additionally, IS, but not ES, induces greater activation of Fos, an immediate early gene (IEG) used to measure neuronal activation, in 5-HT positive neurons in the DRN (Grahn et al., 1999). Worth noting, DRN activation by IS appears to

be confined to the middle and caudal regions of the DRN. Recurrent axon collaterals release 5-HT within the DRN and it is thought that 5-HT released from the soma, dendrites, and axon collaterals within the DRN produces negative feedback through actions on 5-HT_{1A} somatodendritic autoreceptors (Portas and McCarley, 1994). Thus, the source of DRN 5-HT is the DRN itself and so sampling 5-HT from within the DRN is a valid technique for measuring stress-induced DRN 5-HT release.

As mentioned above, sensitization of the DRN by IS, but not by ES, results in inputs to the DRN producing an exaggerated release of 5-HT both within the DRN, and projection regions such as the ventral hippocampus, mPFC, basolateral amygdala (BLA), dorsal periaquectuctal gray, and the dorsal striatum (Maswood et al., 1998; Amat et al., 1998a; Amat 1998b; Strong et al., 2011). The effect of 5-HT on behavior is specific to the region in which it is released. For instance, Amat et al., (1998b) demonstrated that rats exposed to prior IS showed exaggerated (200% baseline) 5-HT release within the BLA in response to 2 footshocks given 24 hours after the initial stressor. Previous work has shown that exaggerated 5-HT release within the BLA facilitates fear (Graeff et al., 1996) and anxiety-like (Christianson et al., 2010; Dolzani et al., 2016) behavior, and 5-HT release within the BLA is considered a proximal mediator of enhanced shock-elicited freezing and reduced social interaction following IS. Analogously, Strong and colleagues demonstrated that IS sensitizes the release of 5-HT into the dorsal striatum, and blocking 5-HT_{2C} receptors in the dorsal striatum prior to behavioral testing prevented typical stress-induced shuttlebox escape impairments. Others have reported modest increases in 5-HT following tail pinch, restraint, and cold water submersion, however, only intense and potent, prolonged stressors such as IS produce DRN sensitization (Rueter et al., 1997).

To investigate the role of the DRN in IS-mediated learned helplessness, Maier and colleagues performed a series of pharmacological and lesion studies directed at DRN 5-HT cells to assess both the necessity and sufficiency of DRN activation in producing learned helplessness behaviors. Electrolytic lesions of the DRN prior to IS prevented shuttlebox escape deficits and

shock-elicited freezing (Maier et al., 1993). Given that electrolytic lesions affect fibers of passage and often damage surrounding brain areas, a series of pharmacology experiments were later performed to manipulate DRN 5-HT activity. Pharmacological inhibition of the DRN with either the benzodiazepine receptor antagonist, chlordiazepoxide, or the 5-HT_{1A} autoreceptor agonist, 8OH-DPAT, both prevent IS-induced shuttlebox escape deficits and enhanced freezing (Maier et al., 1994; Maier et al., 1995). These same manipulations prevented the behavioral effects of IS when performed 24 hours after the initial IS. Additional evidence demonstrating that activation of the DRN is critical for the behavioral consequences of IS comes from a pharmacological study whereby intra-DRN administration of the benzodiazepine receptor antagonists flumazenil and CGS8216, prior to IS which increase DRN activity by reducing tonic GABAergic inhibition, prevented shuttlebox escape deficits and enhanced fear typically produced by IS (Maier et al., 1995). Finally, in the absence of IS, intra-DRN administration of the benzodiazepine inverses agonist, DMCM precipitates shuttlebox escape deficits and exaggerated fear in a manner similar to IS and the behavioral effects of systemic DMCM are prevented by prior lesions of the DRN (Maier et al., 1995b). Together, these data clearly point to intense activation of the DRN by IS as a core mediator of learned helplessness behaviors.

While IS-induced DRN sensitization was proposed as a critical mediator of the behavioral effects of IS, the exact neurobiological mechanisms underlying DRN sensitization received little study until quite recently. One proposed mechanism whereby DRN sensitization could occur is through transient downregulation or desensitization of 5-HT_{1A} autoreceptors within the DRN. This effectively removes the endogenous inhibitory feedback mechanism that regulates DRN activity under normal conditions. Indeed, others have shown that reduced 5-HT_{1A} autoreceptor expression is associated with increased 5-HT release in DRN projection regions (Richardson-Jones et al., 2010) and high levels of 5-HT_{1A} agonists can desensitize 5-HT_{1A} receptors (Beer et al., 1990). Finally, drugs such as fluoxetine, which increase extracellular 5-HT acutely, are known to desensitize 5-HT_{1A} autoreceptors within the DRN and produce behaviors associated with IS

(Riad et al., 2004; Greenwood et al., 2008). As described above, IS produces prolonged elevation of DRN 5-HT and so the necessary conditions exist for 5-HT_{1A} autoreceptor desensitization. Indeed, Rozeske and colleagues (2011) demonstrated that IS, but not ES, desensitizes 5-HT_{1A} autoreceptors within the DRN at a time course that mirrors the behavioral changes associated with the stressor.

Part III. Uncontrollability and excitatory inputs to the DRN

i. Noradrenergic inputs to the dorsal raphe nucleus

While the DRN is implicated as the primary mediator of IS-induced behavioral changes, the summation of activity from a numerous DRN afferents is necessary for DRN activation to occur. Various studies have demonstrated that removing the influence of specific excitatory inputs to the DRN prevents typical behaviors associated with IS, without affecting the protective effects of ES. A brief description of several of the primary excitatory inputs to the DRN is necessary.

As mentioned above, the LC sends a strong monosynaptic input to the DRN. 5-HT neurons within the DRN are densely populated with α-1 receptors, which upon NE binding, exert near-maximal tonic excitatory influence on DRN activity, a phenomenon that can be prevented by administration of the α-1 receptor antagonist, prazosin (Baraban and Aghajanian, 1980; Bortolozzi and Artigas, 2003). Work performed by Grahn and colleagues (2002) demonstrated that intra-DRN administration of the α-1 receptor antagonist, benoxathian prior to IS, but not prior to behavioral testing 24 hours later, prevents typical IS-induced shuttlebox escape impairments. Interestingly, administration of benoxathian prior to IS or behavioral testing prevented exaggerated fear typically produced by IS. Taking into consideration the results of the studies performed by Weiss et al. (1981) where it was shown that IS, but not ES, activates NE neurons within the LC, it is possible that the LC is a critical input in modulating DRN activation and subsequent IS-induced behavioral changes.

ii. Corticotropin releasing factor inputs to the dorsal raphe nucleus

Corticotropin releasing factor (CRF) is a neuropeptide that is well known for its role in mediating the neuroendocrine, autonomic, and behavioral response to stress (reviewed in Lowry and Moore, 2006). CRF is released from both hypothalamic (Lowry and Moore, 2006) and extrahypothalmic areas such as the bed nucleus of the stria terminalis (BNST) and the central amygdala (CeA) (Peyron et al., 1998; Sakanaka et al., 1983; Ju and Han, 1983). Not surprisingly, the DRN is densely innervated by CRF fibers, with nearly 65% of CRF-positive terminals in the DRN contacting dendrites (Valentino et al., 2001). DRN 5-HT neurons express both CRFR1 and CRFR2 receptors (the two primary CRF receptors), and activation of CRFR2 receptors increases activity of trypthophan hydroxylase-2 (TPH2), the rate-limiting step of neuronal 5-HT synthesis (Day et al., 2004; Donner et al., 2016). Injection of CRF into the caudal DRN is sufficient to produce shuttlebox escape deficits and exaggerated fear conditioning (Hammack et al., 2002). These effects are mediated by activation of CRFR2 receptors, as the CRFR2 agonist, urocortin II, dose dependently increases the release of 5-HT into the BLA and produces learned helplessness behaviors (Amat et al., 2004; Hammack et al., 2003). Activation of CRFR1 receptors fails to produce learned helplessness. Additionally, blocking CRFR2 receptors prior to IS with the antagonist anti-sauvagine-30 prevents learned helplessness behaviors (Hammack et al., 2003).

While it is clear that CRF plays an important role in mediating the consequences of IS, (it is both necessary and sufficient for behavioral outcomes associated with IS) the exact source of the CRF has not been identified. It is possible that CeA CRF influences the DRN during IS, however this is not the most likely source, given that lesioning the CeA prior to IS prevents exaggerated fear conditioning but has no effect on shuttlebox escape impairments (Maier et al., 1993). Instead, the BNST is a likely candidate for the source of DRN CRF, as it is CRF-positive (Peyron et al., 1998) and chemical lesions of the BNST prior to IS prevents the typical behavioral effects of IS (Hammack et al., 2004).

iii. Glutamatergic inputs to the dorsal raphe nucleus

Another major source of excitatory input to the DRN arise from structures that use excitatory amino acid neurotransmitters. A strong glutamatergic projection from the lateral habenula (LHb) to DRN has been noted in many studies (Aghajanian and Wang, 1977; Araki et al., 1988; Kalen et al., 1985; Kalen et al., 1989; Sego et al., 2014). Electrical stimulation of the LHb increases DRN 5-HT cell activity and 5-HT release within DRN projection regions (Ferraro et al., 1996; Kalen et al., 1989). In addition, others have demonstrated increased LHb Fos induction following stress *per se* (Chastrette et al., 1991; Nagao et al., 1993; Wirtshafter et al., 1994; Brown and Shepherd, 2013) and intra-DRN microinjection of the NMDA receptor antagonist, APV prior to IS prevents IS-induced shuttlebox escape impairments and exaggerated fear 24 hours later (Grahn et al., 2000).

Together, the results of these studies led Amat et al. (2001) to examine the precise role of the LHb in modulating the DRN activation during IS. Electrolytic lesions of the habenular complex prevented the typical DRN 5-HT increase during IS and the later behavioral changes produced by IS (Amat at al., 2001). This manipulation also prevented the initial rise in 5-HT observed in ES animals. However, the lesions in the Amat et al., (2001) study often included portions of the medial habenula (MHb), would have damaged fibers of passage, were agnostic to cell type, and it was also not determined whether this same manipulation affects extracellular 5-HT levels during subsequent behavioral testing. In addition, Amat et al. (2001) did not examine LHb activation. A recent study provides a more detailed and definitive understanding in regard to the importance of the LHb-DRN circuit in mediating behavioral responses to IS. We have shown that optogenetic silencing of glutamatergic neurons in the LHb only during the tailshock interval of an IS session prevents the typical IS-induced increase in BLA 5-HT, both during IS and JSI 24 hours later (Dolzani et al., 2016). This same manipulation prevents IS-induced reductions in JSI. Finally, it was demonstrated that both IS and ES activate the LHb-DRN pathway to the same degree. These data are the strongest to date in suggesting that IS and ES provide equivalent excitatory input to the DRN. That is, the pattern suggests that stressors *per se* activate the DRN, without regards to

psychological factors such as controllability, and that the presence of control then actively inhibits the DRN.

It should be noted that the behavioral differences observed following ES and IS does not appear to be influenced by the hypothalamic pituitary adrenal (HPA) axis. This is because both IS and ES produce similar increases in plasma CORT and ACTH following stress and 24 hours later following behavioral testing (Maier et al., 1986).

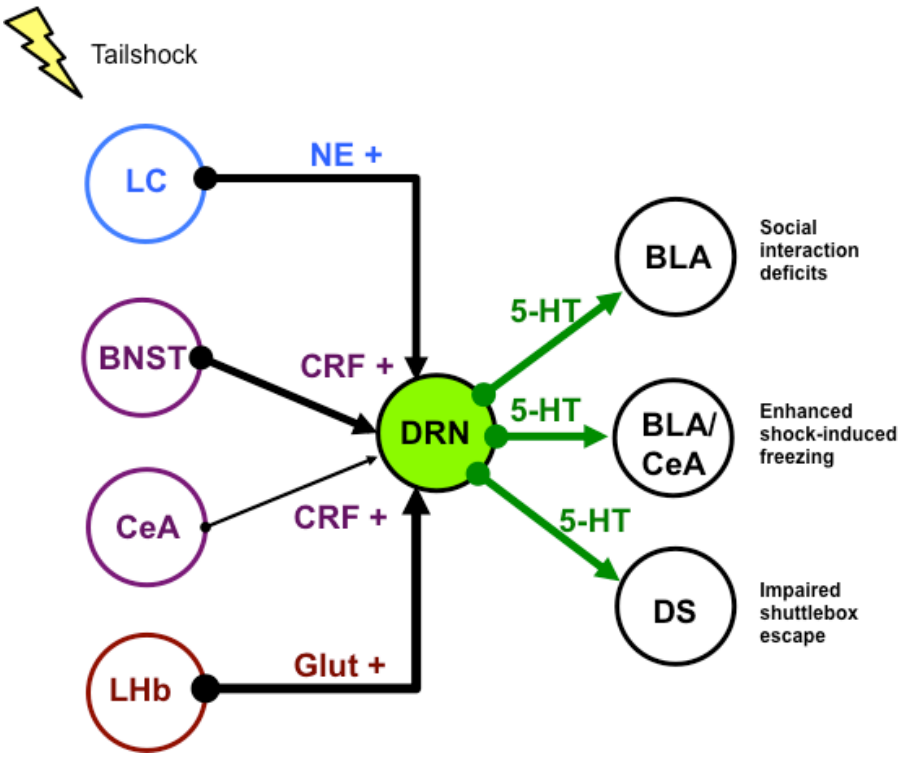

Figure 1.1. Schematic diagram of how tailshock drives activation of the dorsal raphe nucleus and produces dorsal raphe-dependent behavioral changes. In response to tailshock, converging inputs originating from norepinephrine (NE) containing neurons in the locus coereleus (LC), coricotropin releasing factor (CRF) containing neurons in the bed nucleus of the stria terminalis (BNST), CRF containing neurons in the central amygdala (CeA), and glutamate (Glu) containing neurons in the lateral habenula (LHb) drive activation of serotonin (5-HT) containing neurons in the DRN. DRN 5-HT is released within the DRN and into projection regions such as the basolateral amygdala (BLA), CeA, and dorsal striatum (DS) that are proximal mediators of DRN-dependent behavioral changes. Stress-induced DRN 5-HT activation sensitizes the DRN so that future stressors that would not typically activate the DRN now do, resulting in exaggerated release of DRN 5-HT into projection regions.

Part IV. Factors that modulate the neurochemical and behavioral impact of stress

i. Stressor controllability and the medial prefrontal cortex

Given that ES subjects experience a physically identical stressor as do IS subjects, it is remarkable that ES subjects are nearly indistinguishable from non-stressed HC subjects on a variety of behavioral measures. As previously mentioned, an attractive hypothesis that could explain this phenomenon is that during ES an active neural response is engaged that prevents or overrides competing processes that are engaged by tailshock itself. This possibility is suggested by the twin findings that IS, but not ES activates critical DRN 5-HT neurons that mediate the behavioral effects of IS, yet both ES and IS provide equivalent excitatory inputs to the DRN (Grahn et al., 1999; Dolzani et al., 2016).

A discussion of the set of criteria that must be met for a situation to be learned to be controllable is necessary to narrow down the brain regions capable of participating in such a response. A system capable of detecting that an event is controllable must be sensitive to two conditional probabilities. First, the system must be sensitive to the conditional probability of reinforcement given that a response has occurred. Second, the system must be able to detect the conditional probability of reinforcement given that that response has not occurred. The degree of behavioral control is defined by the difference between these two probabilities (Maier and Seligman, 1976). The event is considered uncontrollable if the two probabilities are equal and control increases with the difference between the two probabilities.

To be able to compute the above probabilities a structure would have to receive somatomotor afferents providing information that a behavioral response (wheel turning) has or has not occurred, and somatosensory afferents providing information that reinforcement (tailshock termination) has or has not occurred. The DRN itself is a very small structure devoid of both somatomotor and somatosensory inputs, making it unlikely that detection of control could occur at the level of the DRN. This suggests that the DRN is at the afferent, or downstream, end of the circuit and that the critical structure(s) capable of detecting behavioral control is: 1) distinct

from the DRN and 2) at the efferent, or upstream end of the circuit. This suggests that another structure processes information regarding the controllability of the stressor and uses this information to modulate DRN activity.

Structures receiving somatomotor and somatosensory input that are capable of detecting the degree of control over an event would seem to be of cortical origin. Indeed, learning of instrumental behaviors, action-outcome contingencies, and memory require activation of the mPFC (Ostlund and Balleine, 2005; Goldman-Rakic, 1995; Miller and Cohen, 2001). The DRN receives nearly all of its cortical input from the prelimbic (PL) and infralimbic (IL) regions of the mPFC (Peyron et al., 1998; Vertes et al., 2004; Hoover and Vertes, 2007). Since DRN activation is necessary for the effects of IS, it is possible that the mPFC prevents DRN activation. Efferents from the mPFC synapse primarily on GABAergic dendrites within the DRN (Jankowski and Sesack; 2004) and in response to electrical stimulation of the ventral mPFC (PL/IL) the majority of 5-HT cells recorded in the DRN showed a marked post-stimulus inhibition (Hajos et al., 1998). This effect is specific to stimulation of the mPFC, as stimulation of other cortical regions fails to produce post-stimulus inhibitions within the DRN. Others have demonstrated that GABAergic terminals in the DRN synapse primarily on 5-HT-positive dendrites (Wang et al., 1992). Further, DRN 5-HT cells express GABA receptors (Wirsthafter and Sheppard, 2001) and blockade of GABA receptors with the antagonist bicuculline prevents typical mPFC-induced inhibition of DRN 5-HT cells (Varga et al., 2001). Taken together, it is apparent that the mPFC satisfies the criteria necessary to detect the dimension of control and use this information to inhibit DRN output in the presence of control.

Indeed, the role of the mPFC has been examined extensively in the stressor controllability paradigm. Amat and colleagues hypothesized that if the mPFC detects or uses information regarding the presence of control, then pharmacological inactivation of the mPFC prior to ES should: 1) result in an equivalent increase in DRN 5-HT produced by ES as is observed during IS, and 2) lead ES to produce behavioral changes similar to those observed following IS.

Remarkably, intra-mPFC injection of the $GABA_A$ agonist, muscimol prior to ES led ES to activate DRN 5-HT cells to the same degree as did IS (Amat et al., 2005). DRN 5-HT activation was measured as extracellular DRN 5-HT using in vivo microdialysis and Fos activation in 5-HT labeled cells. Additionally, mPFC inactivation during ES resulted in a variety of behavioral changes typically observed in IS subjects, such as shuttlebox escape impairments (Amat et al., 2005), reduced social interaction (Christianson et al., 2009), potentiated drug reward (Rozeske et al., 2009), and impaired drug extinction (Baratta et al., 2015). Conversely, inactivation of the mPFC during IS had no effect on the behavioral or neurochemical consequences of the stressor – increased DRN 5-HT and IS-associated behavioral outcomes were still present.

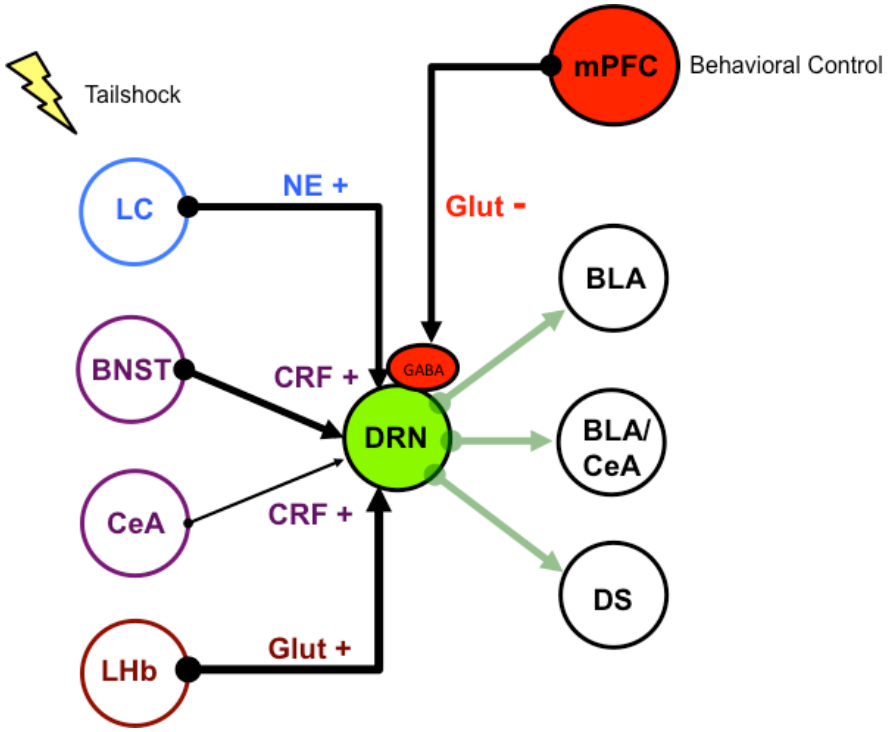

Figure 1.2. Schematic diagram of how behavioral control prevents DRN activation and DRN-dependent behavioral changes. The presence of behavioral control activates a medial prefrontal cortex (mPFC) glutamatergic projection that originates primarily in the prelimbic area (PL) that preferentially synapses on GABAergic interneurons within the DRN. Activation of the PL-DRN projection in response to behavioral control prevents DRN activation, sensitization and subsequent behavioral changes. Thus, the DRN still receives excitatory input from structures such as LC, BNST, CeA, and LHb in the presence of tailshock, however, an active process prevents DRN-mediated outcomes.

ii. Stressor controllability and the corticostriatal system

Detecting and processing the presence of behavioral control and using this information to regulate the DRN are fundamentally distinct processes and therefore one might posit that distinct neural determinants are involved. The results of the studies highlighted above demonstrate that the mPFC is critically involved in regulating the DRN through its descending projections on primarily GABAergic interneurons within the DRN, however none of these studies addressed whether the same system detects or processes the dimension of control. Taking into account the work by Maier and Seligman (1976), a stressor is controllable when either performing or withholding a response increases the probability of stressor alteration relative to the probability of stressor alteration if the response is not performed or withheld. A similar concept has received a great deal of study in the appetitive instrumental learning literature. In this domain, the "act/outcome system" is implicated in encoding the relationship between an action and its consequences (reviewed in Balleine and O'Doherty, 2010). This system is unique in that it is sensitive to the contingency between the action and the response produced by that particular action (Ostlund and O'Doherty, 2005). Reward devaluation is one method of demonstrating this concept. That is, failing to deliver an appetitive reward that was previously associated with a particular action, such as a lever press, results in the subject withholding from that action if the behavior is performed using the act/outcome system (reviewed in Balleine and O'Doherty, 2010). Behavioral control over a stressor requires this type of rapid and relatively flexible action-outcome contingency, whereby the "act" component is defined as the operant behavior (wheel-turn) and the "outcome" is defined as the termination of the stressor. This system relies on encoding of a response-outcome association – i.e. encoding the association between wheel turn and shock termination An additional system called the "habit system" is insensitive to the contingency between an action and its outcome. Under the habit system, action selection is dictated by a learned stimulus-response relationship, without any associative connection between the outcome and the action performed that produced the outcome. This system is insensitive to outcome

devaluation – an organism will continue to lever press even after a lever press no longer yields reinforcement (reviewed in Balleine and O'Doherty 2010). A large body of literature has implicated the PL and dorsomedial striatum (DMS) in the act/outcome system, while the dorsolateral striatum (DLS) (devoid of prefrontal cortical involvement) is implicated in the habit system. In the appetitive instrumental learning literature, it has been shown that excitotoxic lesions and reversible pharmacological inactivation of either the PL or DMS prevents act/outcome associations, without affecting the acquisition of the instrumental response (Yin et al., 2005; Shifflett and Balleine, 2011). Responses are instead acquired using the habit system, and so they are insensitive to changes in the outcome.

Although, conceptually distinct from appetitive instrumental learning, it is plausible that the beneficial effects of behavioral control require an act/outcome system. The PL is obviously involved in control, and one might ask whether sensitivity to the dimension of behavioral control is achieved using the same corticostriatal circuitry that is engaged during appetitive instrumental learning. In order to address this issue, Amat and colleagues (2014) assessed Fos expression in the DMS and DLS of rats exposed to ES, IS, or HC. ES produced a robust increase in Fos protein in the DMS, but not the DLS. Next, the NMDA receptor antagonist AP-5 was injected into the DMS or DLS prior to ES, IS, or HC. Blockade of NMDA receptors within the DMS prior to ES prevented the effect of behavioral control on DRN 5-HT release, so that DRN 5-HT levels now resembled levels typically observed during IS. Additionally, APV injections into the DMS resulted in increased anxiety like behavior 24 hours later. That is, now ES produced behavioral changes typically observed following IS. Thus, DMS inactivation recapitulated both the neurochemical and behavioral effects of pharmacological silencing of the PL. Interestingly, intra-DMS APV had no effect on acquisition or performance of wheel-turn escape, and so the interpretation is that the wheel turn escape response was acquired using the habit system, and learning with this system is devoid of protective effects. Instead, the protective effects of behavioral control require that the controlling response is acquired using the act/outcome system. Under this arrangement, the PL

serves two distinct functions: 1) in conjunction with the DMS, the PL detects the dimension of behavioral control, and 2) the PL uses this information about the presence of control to prevent activation of the DRN (*reviewed in* Maier et al., 2015 and Baratta and Maier, 2017). That is, two separate systems are thought to be involved in the "detect" and "use" components of the stress-buffering effects of behavioral control. Worth noting, the populations of cells projecting from the PL to the DMS and PL to DRN are mutually exclusive. The cells projecting to the DMS and DRN originate in different subregions of the PL (Gabbott et al., 2005) and injection of retrograde tracers into the DRN and DMS produces 2 separate, non-overlapping populations of cells in PL (Baratta and Maier, 2017). To date, no published studies assess functional connectivity between the two circuits. However, using a combination of anterograde and retrograde tracing techniques, our lab has identified a population of DRN-projecting PL neurons that are closely positioned next to axonal projections originating in the medial dorsal thalamic nucleus (MDT), a structure that receives input from the PL (Vertes, 2004). This suggests that the MDT likely serves as a way-station between the PL-DMS and PL-DRN circuits. Future experiments will selectively manipulate these circuits to determine whether the MDT is the critical node linking the detect and use systems. Furthermore, upcoming experiments will address whether the MDT is the site of plasticity for information related to the long-term protective effects of controllability.

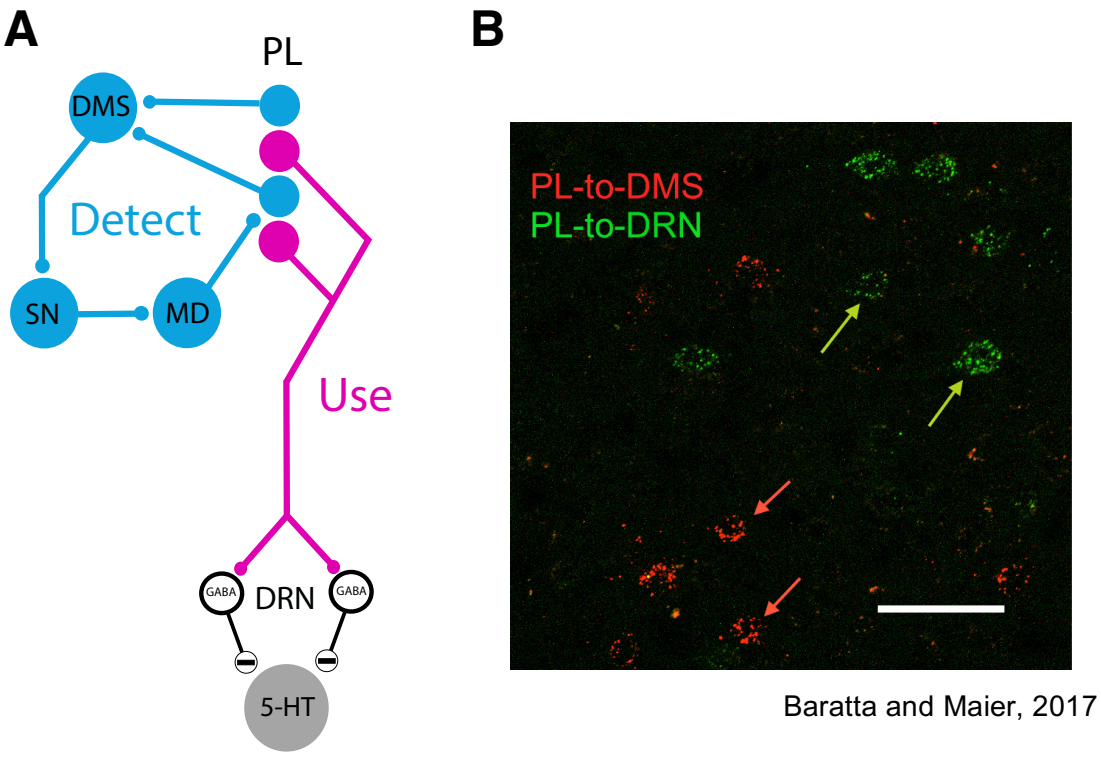

Figure 1.3 Schematic diagram of distinct neural circuits involved in the protective effects of behavioral control. A. Behavioral control is detected by neurons in the corticostriatal (Detect) system. Once control is detected separate DRN-projecting PL neurons (Use) use information regarding control to prevent DRN 5-HT activation, which prevents DRN-mediated behavioral changes. DMS, dorsal medial striatum; SN, substantia nigra; MD, mediodorsal thalamus; PL, prelimbic area; 5-HT, serotonin B. Photomicrograph demonstrating that retrogradely labeled PL neurons projecting to DMS (red) and DRN (green) are neighboring but distinct from each other.

iii. Ketamine and the medial prefrontal cortex

a. Ketamine overview

In addition to behavioral control, the NMDA receptor antagonist ketamine has gained significant attention in recent years for its stress-buffering utility. Surprisingly, until the 1990s, the glutamate system was largely overlooked in regard to behavioral outcomes of stress that are associated with depression and anxiety. This was due to a lack of highly selective compounds to target specific glutamate receptor subtypes and the assumption that compounds that target cathecholaminergic neurotransmission produce therapeutic responses (see Berman et al., 2000 for discussion). In preclinical studies dedicated to understanding the neural mechanisms underlying the effect of antidepressants, Skolnick and colleagues (1996) observed that NMDA receptor antagonists mimic the actions of antidepressants in preclinical studies designed to predict antidepressant efficacy and model behavioral aspects of depression and anxiety. Several years after the initial observation that the glutamatergic system is involved in stress-related behaviors, glutamatergic modulators were found to have antidepressant properties in human clinical trials (Berman et al., 2000; Zarate et al., 2004).

Since the seminal work performed by Berman et al. (2000), numerous preclinical studies have demonstrated that ketamine exerts antidepressant-like and anxiolytic-like effects in rodent models of stress (Maeng et al., 2008; Li et al., 2010; da Silva et al., 2010 Amat et al., 2016; Engin et al., 2009). For example, ketamine has been shown to prevent or reverse many of the behavioral outcomes of stress, such as increased immobility time in the forced swim test (FST) and tail suspension test (TST), reduced social interaction during social investigation (JSI), and corticosterone-induced reductions in sucrose consumption, (Meloni et al., 1993; Layer et al., 1995; Amat et al., 2016).

Several studies demonstrate that stress increases glutamate excitotoxicity within the mPFC, reduces neurotrophic signaling factors, dysregulates pathways involved in cell death and apoptosis, and produces a neuroinflammatory state, all of which are likely to contribute to

neuronal atrophy and neuronal dysfunction associated with depression and anxiety-like behavior (reviewed in Sapolsky, 1996; Machado-Veira et al., 2016; Wang et al., 2015). Ketamine subserves a modulatory role in these component processes within the mPFC (Wang et al., 2015; reviewed in Duman et al., 2016), and therefore the mPFC has received significant interest in regard to the mechanisms underlying the therapeutic effects of ketamine. For the purposes of this thesis, the effects of ketamine on the mPFC glutamate system will be the primary focus and so a comprehensive background on the neural mechanisms of ketamine within the mPFC is necessary.

Ketamine is a cyclohexylamoine that has been used clinically since the 1960s as a general anesthetic, in both humans and animals. The drug was developed for its dual anesthetic and analgesic properties and reduced psychotomimetic effects compared to its predecessor, phencyclidine (Mion et al., 2013). Ketamine administered intravenously results in nearly 100% bioavailability, and this is therefore the most common method of administration (Mion et al., 2013). Ketamine has extensive distribution, owing to its extreme liposolubility, and is rapidly metabolized in the liver, with a plasma half-life of 4 minutes and a terminal half-life of 2.5 hours (Zhao et al., 2012). Ketamine has a high affinity for the NMDA receptor and upon binding blocks NMDA receptor activity (Anis et al., 1983). There are two distinct structural enantiomers of ketamine: *R*-ketamine and *S*-ketamine, which are typically administered together in a racemic mixture (Ebert et al., 1997, Mion et al., 2013). The compound is broken down into the active metabolite, (2S,6S;2R,6R) hydroxynorketamine (HNK) (Zanos et al., 2016). Most studies dedicated to understanding the neural mechanisms of ketamine effectiveness have focused on its actions at the NMDA receptor, although there is increasing interest in its actions at the AMPA receptor and other non-receptor targets.

b. Ketamine increases mPFC glutamate release

Microdialysis and electrophysiological studies performed in rodents indicate a transient "glutamate surge" in the medial prefrontal cortex (mPFC) of animals administered low doses of

ketamine and other NMDA antagonists (discussed in detail below) (Moghaddam et al., 1997; Lorrain et al., 2003). Surprisingly, at higher anesthetic doses, there are no increases in mPFC glutamate. This is particularly interesting because the stress-buffering effects of ketamine only occur at sub-anesthetic doses (Li et al., 2010; Amat et al., 2016), which suggests an important dose-response parallel between the mPFC glutamate surge and the therapeutic properties of ketamine.

The seminal work by Moghaddam et al. (1997) and Lorrain et al. (2003) provides valuable insight into the dynamic actions of ketamine within the mPFC, although behavioral measures were never performed to elucidate whether the glutamate surge is associated with behavioral changes. In light of the recent interest in the therapeutic effects of ketamine, the question has been raised as to whether this increase in glutamate release is related to the more durable effects of ketamine on behaviors associated with depression and anxiety. Chowdhury et al. (2012) utilized ^{1}H-[^{13}C] nuclear magnetic resonance (NMR) spectroscopy to label neuronal glutamate and GABA. These neurotransmitters are released, taken up, and converted to glutamine in astrocytes, and therefore this technique is useful in reflecting changes in neurotransmitter activity in real-time. Using this technique, Chowdhury et al. (2016) demonstrated that sub-anesthetic doses of ketamine produce transient increases in glutamate cycling (glutamate recycling) in the mPFC of rats at a time course that mirrors the onset of behavioral changes produced by the drug. The findings of this study suggest an inverted-U dose-response relationship in the rapid and transient glutamate cycling that correlates with reduced immobility during FST. Higher doses both failed to produce antidepressant effects and also failed to increase mPFC glutamate cycling. Worth noting, the plasma levels of ketamine measured 14 minutes after administration were in the same range as those measured 40 minutes after administration of antidepressant doses of ketamine in humans (Shaffer et al., 2014). The findings of the work by Chowdhury et al. (2016) are striking in regard to the actions of sub-anesthetic doses of ketamine in the mPFC, as they demonstrate a critical

role for increased glutamate signaling within the mPFC in mediating the typical behavioral changes associated with ketamine.

c. Ketamine induces synaptogenesis

Stress induced behavioral changes are sometimes associated with synaptic and dendritic spine alterations in the PL (Li et al., 2011; Shansky and Morrison, 2009; Liston et al., 2006; Fuchikami et al., 2015). Reduced production and release of brain-derived neurotrophic factor (BDNF) is proposed to play a role in these alterations. Interestingly, BDNF deletion attenuates the behavioral response to antidepressants (Chen et al., 2001; Berton et al., 2006; Adachi et al., 2008; Autry et al., 2011) and intra-hippocampal injection of BDNF produces antidepressant-like effects that persist 3-6 days in the forced swim test (FST) (Shirayama et al., 2002). Strikingly, Autry and colleagues (2011) have shown that ketamine results in increased BDNF translation in the mPFC in rodents. Increased glutamatergic signaling is one mechanism by which BDNF levels are upregulated (Abdallah et al., 2016). The paradoxical action of a NMDA receptor antagonist stimulating BDNF transcription and translation may shed light on the unique dose-response relationship of ketamine. Indeed, previous work demonstrates that low dose NMDA receptor antagonists preferentially bind to NMDA receptors localized to GABAergic interneurons in the mPFC, thereby reducing the relative contribution of inhibitory versus excitatory tone (Homayoun and Moghaddam, 2007). Blockade of NMDA receptors located on GABAergic interneurons within the mPFC with low dose NMDA receptor antagonists results in a stimulus-independent glutamate surge followed by activation of AMPA receptors, depolarization, and increased calcium influx (Farber et al. 1998). Depolarization induces BDNF release, as well as mammalian target of rapamycin (mTOR) signaling (Li et al., 2010). At higher doses, presynaptic and postsynaptic NMDA receptors on mPFC glutamatergic neurons and GABAergic interneurons are blocked, which prevents the glutamate surge and the stress-buffering potential of ketamine (Abdallah et al., 2016). Thus, it is apparent that BDNF release is a critical factor in the rapid-acting effects of

ketamine. Ketamine treatment stimulates BDNF release from primary cortical neurons and the blockade of BDNF signaling within the PL with a neutralizing antibody prevents the behavioral actions (reduced immobility during FST) of ketamine (Lepack et al., 2015).

As described above, stress-induced synaptic alterations are largely mediated by reductions in neurotrophic factors, such as BDNF (Nibuya et al., 1998; Rutherford et al., 1998). In the absence of stress, depolarization of presynaptic glutamate cells leads to BDNF release and subsequent binding to post-synaptic TrkB receptors. This binding activates the Akt signaling pathway, which results in mTOR signaling (*reviewed in* Gerhard et al., 2016). mTOR signaling promotes the production of proteins involved in synaptic plasticity and neuronal stability (Hoeffer et al., 2010). Li and colleagues (2010) demonstrated that a single sub-anesthetic dose of ketamine increases phosphorylated and activated levels of Akt and mTOR within the PL. mTOR signaling is required for the protective effects of ketamine, as intracerebroventricular injection (ICV) of the mTOR inhibitor rapamycin prevents the protective effects (Li et al., 2010). Ketamine also reverses stress-induced reductions in several synaptic proteins in the mPFC (Autry et al., 2011). Notably, dendritic spine density in the proximal and distal tuft of Layer V mPFC pyramidal neurons is reduced 24 h after the final stressor in a 3 week chronic unpredictable stress (CUS) paradigm, an effect that is reversed with a single dose of ketamine (Li et al., 2011). Ketamine also increases the population of mushroom, or large diameter, spines in Layer V mPFC that are indicative of spine maturation and synaptic strengthening (Li et al., 2011). mTOR inhibition prevents the reversal of stress-induced dendritic spine deficits typically produced by ketamine. Therefore, ketamine appears to increase mTOR signaling in the mPFC, which prevents stress-induced deficits in synaptic proteins and dendritic spines, and thereby prevents behavioral changes associated with these deficits.

d. AMPA receptor activation and the protective effects of ketamine

Given the results of the aforementioned studies, it is clear that NMDA receptor blockade is not the proximate mediator of the behavioral effects of ketamine (Lorrain et al., 2003). The rapid and sustained stress-buffering actions of ketamine require α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling (Maeng et al., 2008; Koike et al., 2011). Koike et al. (2011) demonstrated that ketamine prevents both acute escape learning deficits during an avoidance learning test and sustained depression-like behavior measured in tail suspension test (TST) 72 hours after an initial ketamine injection. In order to probe the involvement of AMPA receptors in this phenomenon, the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfoamoylbenzo(f)quinoxaline (NBQX) was injected prior to behavioral testing. This completely prevents the acute effects of ketamine in the shuttle box escape test and partially blocks the sustained effects of ketamine in the TST test. Similarly, NBQX administration 24 hr after a single subanesthetic dose of ketamine prevents reduced immobility during the FST (Koike and Chaki, 2014). Therefore, AMPA receptor activation is required for both the rapid and sustained effects of ketamine.

Building on the work of Li et al., (2010), Autry and colleagues (2011) demonstrated that AMPA receptor blockade with NBQX completely prevents increased Akt signaling produced by ketamine, thereby preventing enhanced mTOR signaling and the antidepressant-like effects of ketamine during FST and an avoidance learning test. Others have shown that pretreatment with NBQX prevents the typical upregulation in mTOR and BDNF in the hippocampus and mPFC, as well as ketamine-induced reductions in immobility time during FST (Zhou et al., 2014). Therefore, AMPA receptor activation appears to be necessary for the ketamine-induced enhancements of BDNF that are involved in the production of synaptic proteins necessary for buffering the effects of stress (Autry et al., 2011).

e. Ketamine protects female rodents against stress outcomes

Interestingly, all of the aforementioned mechanistic studies have examined the effects of ketamine in male rats and mice only; however, recent studies have illuminated sex differences in regard to the effects of ketamine on behavior. Recent evidence suggests that female rats and mice are more sensitive than are male rats and mice to the antidepressant-like and anxiolytic-like effects of ketamine (Carrier et al., 2013 Franceschelli et al., 2015) . In rats, ketamine doses (2.5 mg/kg) well below those that are effective in males reduced immobility during FST, reduced latency to feed during NSFT, and increased sucrose preference in females (Carrier et al., 2013). Interestingly, the enhanced sensitivity to ketamine observed in female rats was abolished by ovariectomy (OVX) and was restored in OVX rats following replacement of physiological levels of progesterone and estrogen. While others have demonstrated a critical role for mTOR activation in the antidepressant-like effects of ketamine (Li et al., 2010), the enhanced sensitivity observed in females was not mediated by increased mTOR phosphorylation in the mPFC, as the 2.5 mg/kg dose of ketamine failed to increase phosphorylated mTOR (Carrier et al., 2013). Interestingly, 5.0 mg/kg ketamine, which was effective in reducing depression-like and anxiety-like behavior, increased mTOR in both females and males. Similarly, eEF2 signaling in the hippocampus, which is critical for BDNF translation (Autry et al., 2011), was not responsible for the increased sensitivity to ketamine in female rats (Carrier et al., 2013). Taken together, these data indicate that an alternate mechanism is responsible for increased sensitivity to ketamine in female rodents. One possible explanation for increased ketamine sensitivity in females is heightened levels of ketamine metabolites that exert antidepressant-like effects (Zanos et al., 2016).

As previously discussed, exposure to stress produces structural synaptic changes in the mPFC along with concomitant behavioral changes (Li et al., 2011; Shansky and Morrison, 2009; Liston et al., 2006; Fuchikami et al., 2015). Chronic isolation stress produces depression-like behavior and reductions in dendritic spine density, post-synaptic density protein 95, synapsin-1, and glutamate receptor 1 in the mPFC of both male and female rats in proestrous or diestrous (Sarkar and Kabbaj, 2016). A single dose of ketamine (5 mg/kg) reversed the behavioral and

structural changes associated with isolation stress in male rats. Although the same dose of ketamine reversed the behavioral deficits observed in females, the decrease in synaptic proteins could not be reversed by ketamine. These studies suggest that the mechanisms of action for ketamine in male and female rats are dissimilar, potentially due to fluctuating gonadal hormones in female rats, however, a precise explanation of this phenomenon remains elusive. Thus, it is clear that more research in this domain is needed.

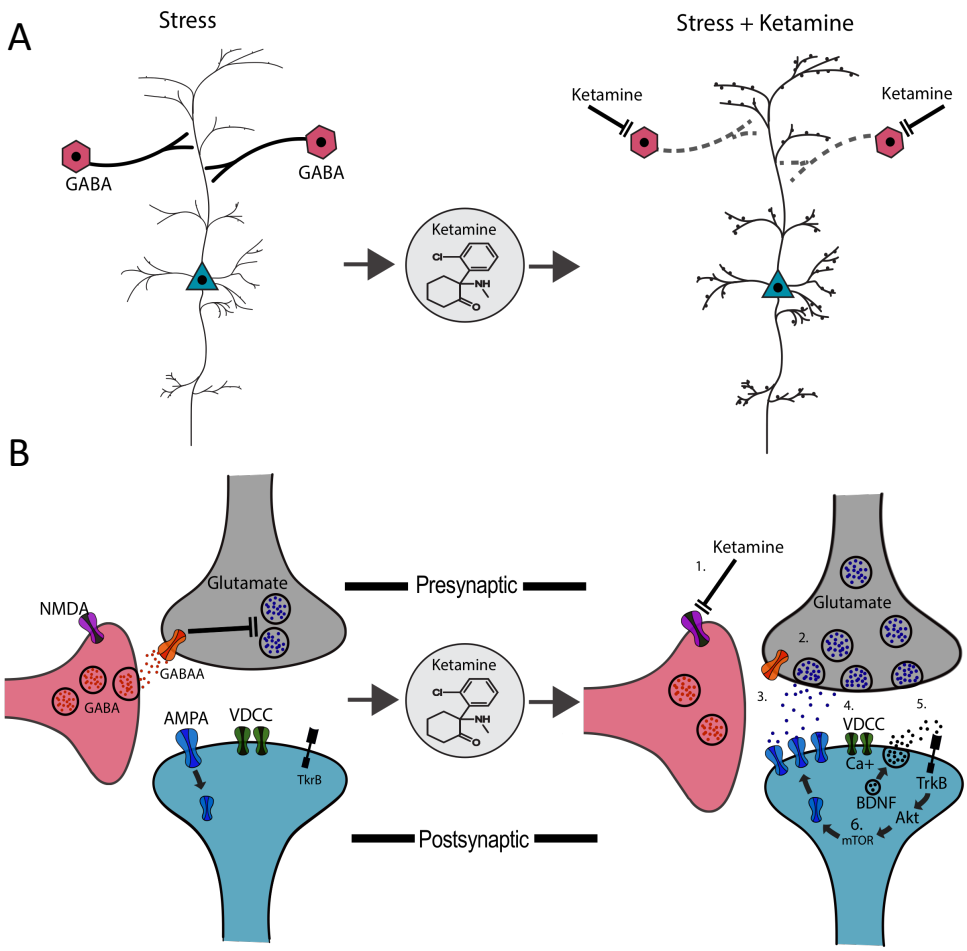

Figure 1.4 Stress decreases, while ketamine increases synaptogenesis: synaptic and intracellular signaling mechanisms. A. Sub-anesthetic ketamine relieves glutamatergic neurons from stress-induced inhibition, resulting in increased glutamate transmission and neurogenesis. B. Sub-anesthetic ketamine preferentially antagonizes NMDA receptors on GABAergic interneurons, resulting in increased glutamate release, voltage dependent calcium channel (VDCC) activation, calcium (Ca^{2+}) influx, and subsequent brain derived neurotrophic factor (BDNF) release. This stimulates receptor tyrosine kinase B (TrkB) activation followed by activation of the Akt and mTOR, resulting in insertion of GluR1 containing AMPA receptors into the

membrane of postsynaptic dendrites. The net result of ketamine is increased dendritic spines and synaptogenesis.

Similarities between ketamine and behavioral control

Nearly all of the published work designed to identify the neural mechanisms underlying the behavioral effects of ketamine has focused on the effect of ketamine administered at two time points in relation to behavioral testing. First, many studies have administered ketamine immediately (30 min-1 hour) prior to behavioral testing and here subjects are likely experiencing direct effects of ketamine. Second, ketamine has been administered after exposure to a period of chronic stress as a restorative or stress-reversing agent. When considering potential similarities between ketamine and behavioral control on the effect of future stressors, the timing of ketamine administration becomes an important consideration. ES prior to later IS protects against the effects of the IS stressor, and so it is apparent that delivering ketamine prior to IS is the appropriate time point to assess potential immunizing properties of the drug (*reviewed in* Maier and Watkins, 2010). Surprisingly, only 3 published studies have examined the effect of ketamine delivered prophylactically (Brachman et al., 2015; Amat et al., 2016; McGowan et al., 2017). Of these studies, only the Amat et al. (2016) group examined the effects of ketamine at a neural (specifically, neural circuit) level. The results of this study are of particular interest for the purpose of the present thesis. First, ketamine delivered 2 hours, 1 week, or 2 weeks prior to IS completely prevented the typical reductions in JSI observed 24 hours later. This manipulation also prevented the neurochemical effect of IS. IS resulted in a ~250% increase in BLA 5-HT, a structure critical for the behavioral effects of IS, in response to IS and this increase was completely prevented by a single injection of ketamine given up to two weeks prior to the stressor. Intra-PL injection (10 ng) of ketamine recapitulated the behavioral and neurochemical effects of systemic ketamine. Furthermore, pharmacological silencing of the PL prior to IS prevented the protective effects of ketamine on later JSI. Finally, ketamine delivered 2 hours prior to IS resulted in IS now activating

the PL-DRN pathway; a phenomenon not typically observed following IS alone. The results of this study are clear in regard to the involvement of the PL in the prophylactic effects of ketamine. Ketamine engages the PL and alters the PL-DRN pathway in a manner whereby a later experience with stress now activates this pathway, thereby preventing the neurochemical and behavioral effects of the stressor.

Specific molecular and cellular changes such as those described above have not been examined at a neural circuit level, however, one might propose that similar changes are occurring at the dendritic spine/synaptic plasticity level in the PL-DRN pathway as those outlined in the initial work designed to identify the neural mechanisms engaged by ketamine (reviewed in Duman et al., 2016). The findings of Amat et al. (2016) are striking in regard to their similarity to previously published stressor controllability work. First, ketamine prior to IS prevented the typical reductions in JSI observed following IS (Christianson et al., 2009; Short et al., 1993; Amat et al., 2016). Second, both ketamine and behavioral control prevent the typical neurochemical changes produced by the stressor, as measured with in vivo microdialysis in the BLA (Amat et al., 2016; Christianson et al., 2010). Additionally, the protective effects of ketamine and behavioral control both require activation of the PL, as pharmacological silencing of the PL with muscimol prevents the protective effects of both manipulations (Amat et al., 2016; Amat et al., 2005). Finally, ketamine and a prior experience with behavioral control both result in a later experience with IS now activating the PL-DRN pathway, suggesting that this pathway is a critical mediator of the behavioral effects of both manipulations (Amat et al., 2016; Baratta et al. 2009). It should be noted that, similar to ES, the effect of ketamine on stress-induced behavior does not appear to be influenced by the hypothalamic pituitary adrenal (HPA) axis, as rats previously injected with ketamine or vehicle yield an identical IS-induced CORT response (Dolzani et al., *unpublished observation*).

Introduction Conclusions and Thesis Organization

From the results of studies designed to identify neural mechanisms through which stressor controllability and ketamine exert protective effects in males, it is apparent that several of the same mechanisms are involved. Behavioral control and ketamine activate an inhibitory PL-DRN circuit, prevent stress-induced DRN 5-HT activation, and prevent behavioral outcomes of a future stressor. Despite promising results from studies designed to identify the neural mechanisms that underlie stress resilience afforded by these two factors in males, very little is known about the effects of these factors in females. Thus, the present thesis seeks to expand our understanding of stress resilience by determining whether: 1) behavioral control over a stressor or ketamine exert similar protective effects in female rats as previously reported in male rats, and 2) what neural mechanisms are involved in the behavioral response to each distinct manipulation.

In Chapter 2 I will explore the behavioral and neurochemical effects of behavioral control on stress outcomes in females. The initial behavioral experiments provide evidence that behavioral control fails to mitigate effects of stress of later JSE, shock-elicited freezing, and shuttlebox escape performance. Subsequent experiments utilizing immunohistochemistry demonstrate that behavioral control fails engage a descending PL-DRN circuit and prevent stress-induced DRN activation. Finally, using a pharmacological approach, I will demonstrate that pharmacological activation of the PL at the time of stress reverses the stress-induced JSE deficits.

In Chapter 3 I will explore the behavioral and neurochemical effects of ketamine in females. The initial behavioral experiment reveals that proactive ketamine prevents typical stress-induced JSE deficits. The subsequent experiments are designed to identify the neural mechanisms through which ketamine exerts its protective effects. Using a combination of immunohistochemistry and retrograde labeling techniques, I demonstrate that ketamine blunts DRN activation and engages a descending PL-DRN circuit. Using a genetically targeted activity monitoring platform called Robust Acivitiy Marker (RAM), I demonstrate that IS activates an ensemble of neurons previously activate by ketamine. Lastly, using a chemogenetic approach I

demonstrate that ketamine-mediated stress resilience requires activation of a descending PL-DRN circuit.

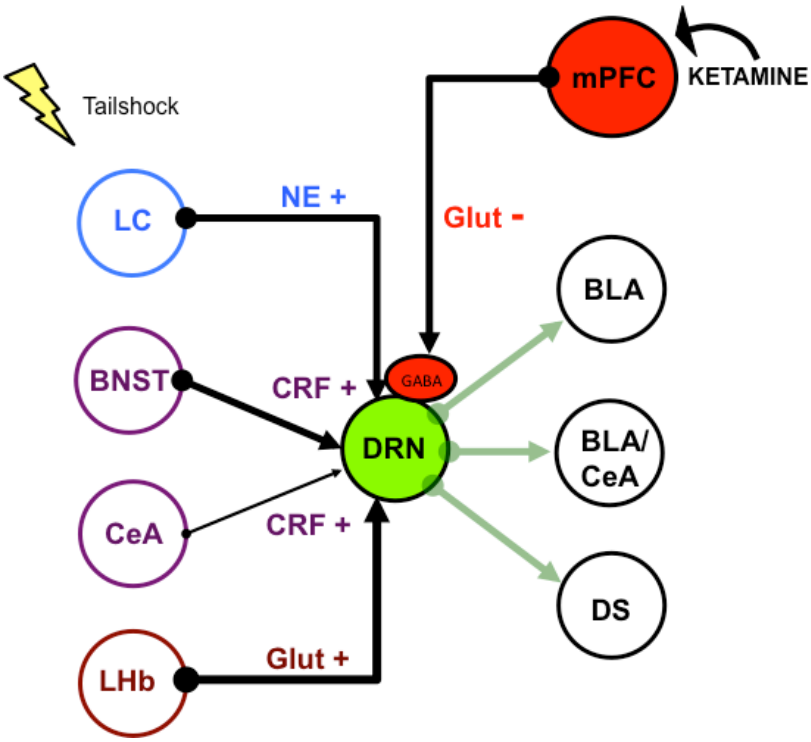

Figure 1.5. Proposed framework for how ketamine prevents DRN activation and DRN-dependent behavioral changes. Proactive ketamine engages a mPFC glutamatergic projection originating in the prelimbic area (PL) that preferentially synapses on GABAergic interneurons within the DRN. Activation of the PL-DRN projection in response to ketamine prevents DRN activation, sensitization and subsequent behavioral changes. Thus, the DRN still receives excitatory input from structures such as LC, BNST, CeA, and LHb in the presence of tailshock, however, ketamine alters the PL-DRN pathway in a manner whereby a later stressor now activates this pathway and prevents DRN-mediated outcomes.

Chapter 2:

Behavioral control fails to engage a descending prefrontal circuit and mitigate stress outcomes in females

ABSTRACT

Stress is a powerful contributor to the development and persistence of depression and anxiety. Females are afflicted at nearly twice the rate of males and so much recent effort has been directed at identifying factors capable of producing stress resilience in females. Mounting evidence indicates that the degree of behavioral control an organism can exert over a stressor is a critical determinant of the neurochemical and behavioral outcome of the stressor. Uncontrollable stressors (inescapable tailshock, IS) produce a variety of neurochemical and behavioral changes that do not occur if the stressor is controllable (escapable tailshock, ES). Research dedicated to understanding the neural mechanisms by which controllable stressors produce resistance/resilience has focused largely on a descending medial prefrontal cortex (mPFC) to dorsal raphe nucleus (DRN) projection. When a stressor is perceived as controllable, mPFC neurons originating in the prelimbic (PL) cortex blunt DRN activation and DRN-mediated behavioral changes. Despite efforts to understand the neural mechanisms that mediate stress resilience, the effects of stressor controllability in female rats have not been examined. Here we present the first series of experiments that examine stressor controllability effects in females. Strikingly, ES fails to prevent stress-induced DRN activation and DRN-mediated behavioral changes, as it does in males. ES fails to engage inhibitory PL-DRN circuit architecture, as in males. Pharmacological activation of the PL rescues female rats from stress-induced behavioral changes. Together, the results of this study demonstrate that, while the necessary resilience circuitry exists, it is not engaged by stressor controllability in females. This highlights the importance of taking sex differences into consideration when studying the neural mechanisms of stress resilience.

INTRODUCTION

Stress-related psychiatric disorders such as depression, anxiety, and post-traumatic stress disorder (PTSD) have a significantly higher prevalence in females than males (Kendler et al., 1999; Blazer et al., 1994; Kessler et al., 1997; Haskell et al., 2010; Kessler et al., 2005). The precise neurobiological processes underlying sex differences in the prevalence of psychiatric disorders remains unclear, however, they may manifest through different coping strategies in response to adverse life events (Ptacek et al., 1994; Kelly et al., 2008; Matud et al., 2004). It is well documented that the outcome of a stressful situation is largely influenced by how an individual appraises the adverse event (Southwick et al., 2005). This encompasses the degree to which an individual can predict, avoid, escape, or otherwise control the adverse event. To that end, resilient individuals have been described as implementing active or control-based coping strategies that directly address the stressor, rather than passive or avoidance-based coping strategies (Southwick et al., 2005). Conversely, perceived lack of control and avoidance-based coping strategies are reported to increase negative affect and vulnerability to depression and PTSD and (Bosoglu and Mineka, 1992; Dunkley et al., 2017).

Perceived or actual control over some aspect of the adverse event is an important feature of coping that can be modeled in a laboratory setting wherein underlying neurobiological processes can be examined and experimentally manipulated. Rats exposed to an uncontrollable stressor (inescapable tailshock, IS), but not an equivalent controllable stressor (escapable tailshock (ES), show a variety of behavioral changes that resemble certain aspects of depression and anxiety, such as exaggerated fear, impaired learning, reduced social behavior, impaired fear extinction. That is, the presence of behavioral control blunts the impact of contemporaneous and future adverse events (Amat et al., 2006; Baratta et al., 2007; Christianson et al., 2009; Christianson et al., 2010; Weiss and Simson, 1986; Maier et al., 1995; see Maier, 2015 for review).

Two lines of work have been dedicated to understanding the neurobiological mechanisms that mediate the behavioral changes that follow IS and ES. One has examined mechanisms

underlying the behavioral changes produced by IS, and the second and has focused on how behavioral control (an escape response) prevents these outcomes. In regard to the former, the behavioral changes produced by IS are mediated, at least in part, by serotonergic (5-HT) neurons located in the mid to caudal dorsal raphe nucleus (DRN). IS and other uncontrollable stressors activate and sensitize these neurons, as evidenced by increased Fos expression in 5-HT labeled cells (Grahn et al., 1999; Takase et al., 2004), and release of 5-HT within the DRN (Maswood et al., 1998) and projection regions such as the basolateral amygdala (BLA), a proximal mediator of IS-induced anxiety-like behavior (Amat et al. 1998; Christianson et al., 2010; Dolzani et al., 2016). Pharmacological inhibition of the DRN during IS prevents the behavioral sequelae of IS (Will et al., 2004; Amat et al., 2006), while pharmacological activation in the absence of IS produces them (Maier et al., 1995; Amat et al., 2008). The intense activation of DRN 5-HT neurons by IS leads to their sensitization (Rozeske et al., 2011), so that later test conditions such as exposure to a non-aggressive juvenile social exploration (JSE) induces the release of exaggerated amounts of 5-HT in projection regions that are the proximate mediators of the behaviors, thereby producing the behavioral changes (Christianson et al., 2010; Dolzani et al. 2016).

As required by the idea that DRN 5-HT activation is critical in mediating the consequences of IS, exactly equal amounts of ES, which do not produce the behavioral changes typically produced by IS, also do not strongly activate DRN 5-HT neurons (Maswood et al., 1998). Differential activation of the DRN by IS relative to ES could be caused by a) excitatory input to the DRN produced by IS but not ES, or b) excitatory input during both but also inhibitory input during ES. A variety of evidence has supported the second of these possibilities (Amat et al., 2005; Dolzani et al., 2016; *reviewed in* Maier, 2015). Indeed, the protective effects of ES are attributed to activation of a subset of neurons in the prelimbic region (PL) of the medial prefrontal cortex (mPFC) that exert top-down inhibitory control over the DRN (Amat et al., 2005; Baratta et al., 2009). In the presence of behavioral control, DRN activation is prevented through activation of PL neurons that project to and synapse primarily on GABAergic neurons within the DRN (Vertes

et al., 2004; Gabbot et al., 2005; Hoover and Vertes, 2007; Hajos et al., 1998; Jankowski and Sesack, 2004). Furthermore, activation of the PL is both necessary and sufficient in mediating protection from the consequences of stress (Amat et al., 2005; Amat et al., 2006; Amat et al., 2008).

While progress has been made in identifying the neural mechanisms that mediate the differential effect of ES relative to IS on the behavioral outcomes of the stressor, nearly all of the work has been conducted using male rodents. This is of significant interest, as prior work has identified sex differences in response to acute stressors, including those that involve the DRN 5-HT system and the mPFC (Iwasaki-Sekino et al., 2008; Kudielka and Kirshbaum, 2004; Yin et al., 2009; Mitsushima et al., 2006; Zohar et al., 2015; Howerton et al., 2014; Shansky et al., 2004). However, all of these studies have implemented uncontrollable stressors and a controllable stress group was never examined. Therefore, it is largely unknown whether controllable stressors afford female rodents protection in a manner similar to that which has been extensively documented in male rodents.

Here we sought to determine whether similar stressor controllability effects are observed in female rodents as those that have been extensively documented in male rodents. We also examine whether the same circuitry engaged by behavioral control in male rats is present and is engaged in female rats granted a controlling response over the stressor.

METHODS

Overall organization

The first experiment examined whether an experience with controllable stress would ameliorate the behavioral consequences of the stressor in female rats. Three separate behavioral measures were assessed following ES, IS, or HC. First, juvenile social exploration (JSE) was measured. A baseline measure of JSE was conducted 24 h prior to rats receiving ES, IS, or HC treatment. Anxiety-like behavior was assessed during a JSE test 24 h after the stressor or HC

treatment. Next, shock-elicited freezing and shuttlebox escape performance were assessed in a separate cohort of rats. In these tests, rats are placed in a shuttlebox 24 hours after ES, IS, or HC. Shock-elicited freezing and shuttlebox escape performance are assessed using parameters described below. These procedures were chosen based on prior work from our lab demonstrating that ES, relative to IS, produces resistance to the behavioral and neurochemical effects of the stressor in male rats (Baratta et al., 2007; Christianson et al., 2008; Christianson et al., 2009; Maier et al., 1990).

Prior work demonstrates that ES prevents DRN activation in male rats (Grahn et al., 1999; Maswood et al., 1998), and so we sought to determine whether an experience with behavioral control would prevent DRN activation in female rats. In the second experiment, DRN activation was assessed using double-label immunohistochemistry to address the following questions: 1) does ketamine reduce overall stress-induced Fos (a marker of cellular activity) expression within the DRN, and, if so, 2) does ketamine reduce Fos expression specifically in 5-HT neurons within the DRN? Fos and 5-HT expression were examined in the rostral, middle, and caudal DRN.

As previously mentioned, the protective effects afforded to male rats by control, are mediated by activation of the PL-DRN pathway (Baratta et al., 2009). Thus, the third experiment examined whether ES activates the PL-DRN pathway in female rats. Briefly, DRN-projecting PL neurons were labeled with the retrograde tracer, Fluorogold (FG), two weeks prior to ES, IS, or HC. Rats were perfused two hours following the last tailshock and Fos expression was examined in the PL-DRN pathway.

Given the striking results of the first set of experiments, the final experiment sought to determine whether pharmacological activation of the PL with the GABA antagonist, picrotoxin, would prevent behavioral outcomes associated with ES and IS. First, rats were implanted with bilateral guide cannula directed at the PL. Following recovery from the surgical procedure, a baseline measure of JSE was conducted 24 hours prior to rats receiving ES, IS, or HC. Thirty

minutes prior to ES, IS, or HC rats received an intra-PL injection of picrotoxin or saline. Anxiety-like behavior was assessed during a final JSE test 24 hours after the stressor of HC treatment.

Rats: Adult female Sprague–Dawley rats (225–300 g; Envigo) were singly housed on a 12-h light–dark cycle (lights on at 06:00 am and off at 06:00 pm). Standard lab chow and water were available *ad libitum*. Rats were allowed to acclimate to colony conditions for 7 days prior to surgical or experimental procedures. All experiments were performed between 9:00 AM and 5:00 PM. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Behavior

Stress procedure: For manipulation of stressor controllability, rats were exposed to either ES or IS in Plexiglas boxes (14x11x17cm) with a wheel mounted on the front wall, as previously described (Baratta et al., 2009). Rats were run in yoked pairs (ES and IS) in 100 trial sessions (approximately 2 h). Shock was delivered to the rat's tail with increasing intensity as the shock session progressed (33 trials at 1.0 mA, 33 trials at 1.3 mA, and 34 trials at 1.6 mA). The average time between shocks was 90-seconds. Tail shock was terminated for both rats (ES and IS) when the ES rat achieved a specific wheel-turn requirement (previously described in Baratta et al., 2009). Therefore, onset, offset, and intensity were identical for both rats in each yoked pair. The initial wheel turn requirement was one quarter of a complete revolution in <5 s. The response requirement increased with 4 successive, successful wheel turns at each required fixed-ratio interval. If the escape requirement was not reached within 30 s, the shock automatically terminated and the escape requirement reverted to the previous, lower response requirement. Rats were removed from PlexiGlas boxes and moved to their home cage immediately after the last tailshock. A single group of rats served as non-stressed homecage controls (HC).

Juvenile Social Exploration (JSE): JSE testing was conducted 24 hours after ES, IS, or HC, as previously described (Amat et al., 2016). Any rats showing signs of injury (including injured hindpaws, forepaws, or toenails) after the stress session were excluded. Social exploration is particularly sensitive to injury and behavior is altered when a subject tends to an injury for an extended period of time during the testing period. Precautions are taken to minimize the frequency of these occurrences, but nonetheless injuries did occur in a small subset of subjects in the present experiments. Briefly, each experimental subject was singly assigned to an empty plastic cage with shaved wood bedding and a wire lid. Experimental subjects remained in the test cage for 1 hour prior to introducing a juvenile (28 ± 2 days old) female conspecific. An observer, blind to treatment, recorded exploratory behavior (allogrooming, sniffing, and pinning) initiated by the experimental subject during a 3-minute test. JSE test scores were reported as percent of social exploration during the 3-minute test, relative to the baseline measure.

Shock-elicited freezing and Shuttlebox escape test: Shock-elicited freezing and shuttlebox escape performance were assessed in shuttleboxes (50.8 X 25.4 X 30.48; Coulbourn Instruments, Holliston MA) using procedures previously described. Twenty-four hours after ES, IS, or HC, subjects were placed into shuttleboxes and following a 5-minute period where rats were allowed to explore, they received two 0.7 mA footshocks delivered on both sides of the grid floor. Under this arrangement, footshocks terminated when the rats successfully crossed from one side of the two-chambered shuttlebox to the other (hereafter referred to as fixed-ratio 1, FR-1). Following the second FR-1 trials, shock-elicited freezing was observed for 20 min by an observer blind to treatment condition. During this period, behavior was assessed every 10 seconds as either freezing or not freezing. Here, freezing is defined as the absence of any movement, aside from that which occurs due to respiration. Previous work from our laboratory demonstrates that freezing is a measure of fear conditioned to the context in which the footshock occurred (Maier et

al., 1990). FR-1 footshock was used rather than a fixed duration footshock, to avoid introducing subjects to an inescapable stressor. Following the 20-min freezing observation period, three additional FR-1 trials occurred. Next, shuttlebox escape performance was assessed during 25 consecutive FR-2 escape trials. During each FR-2 escape trial, the subject must cross from one side of the shuttlebox to the other and then back to the initial side in order to terminate footshock. Footshocks occur pseudorandomly at approximately 60-s intervals. Each footshock terminates if the subject does not achieve the appropriate escape response within 30 seconds. FR-1 and FR-2 escape latencies are recorded using an infrared sensor inside the two sides of the shuttlebox chamber. Data is transferred and recorded to a PC that drives the shuttlebox software program. Escape failures are defined as a escape latency greater than or equal to 25-s.

Surgical Procedures

All stereotactic surgeries were performed under 2.5% isoflurane (Piramal Critical Care, Bethlehem, PA) anesthesia. Rats received preoperative analgesic (meloxicam, 0.5 mg/kg s.c.; Vetmedica, St. Joseph, MO) and antibiotic (Combi-Pen-48, 0.25 ml/kg s.c.; Bimeda, Oakbrook Terrace, IL) prior to surgical procedures. Rats were given two weeks to recover from surgery before experimentation.

Intra-DRN Fluorogold Injection: A small circular window (1x1 mm) was drilled to allow for penetration of a needle (31 gauge, 45 degree tip angle) attached to a 10 µl Hamilton syringe. Using a stereotaxic instrument, the needle was directed to the DRN (AP: -8.0 mm from bregma, DV: -6.7 mm from skull surface, ML: 0.0 mm relative to midline). 200 nl of a 2% solution of Fluorogold (FG) (Fluorochrome, Denver, CO) dissolved in a 0.9% saline buffer was injected over the course of 3 min and allowed to diffuse for an additional 10 min using a microinjection pump (UMP3-1; World Precision Instruments, Sarasota, FL). The scalp incision was closed using Vetbond (3M). Two weeks after FG microinjection, rats received ES, IS, or HC. FG microinjections

were considered successful if expression was visibly confined to DRN in coronal sections of brain obtained after completion of the experiment.

PL Cannula Implantation: Rats were anesthetized under isoflurane anesthesia (2%). Two small windows (1 mm x 1 mm) were drilled into the skull and dual guide cannula were implanted into the PL (AP: +2.6 mm relative to bregma, DV: -1.8 mm from brain surface, ML: ±0.5 mm relative to midline) for drug microinjections (Plastics One, Roanoke, VA). Stainless steel screws and dental cement were used to secure cannula to the skull. Dummy cannula were inserted into each cannula guide and secured with a dust cap (Plastics One). Only rats with accurate cannula placement were used for statistical analyses.

Drug microinfusion: The $GABA_A$ receptor antagonist, picrotoxin (Tocris Bioscience, Bristol, UK), was dissolved in 0.9% sterile saline. Dual microinjectors (33 gauge; Plastics One) attached to PE 50 tubing were inserted through the guides. The other end of the tubing was connected to a 25 µl Hamilton syringe that was attached to a microinjection unit (Model 5000; Kopf Instruments, Tujunga, CA). Animals received 0.5 µl of either picrotoxin (100 ng) or saline in each side of the PL. The volume was injected over a period of 30 s, and the injectors were left in place for 2 min to allow for diffusion.

Immunohistochemistry

Tissue preparation: Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) at 2 hours after the last tailshock or the same time for rats assigned to HC. Rats were transcardially perfused with 100 ml ice-cold 0.9% saline, immediately followed by 250 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH ~7.4). Brains were postfixed overnight in the same fixative and transferred to a 30% sucrose solution in 0.1 M phosphate buffer (PB) then stored at 4 degrees C

until sectioning. Coronal brain sections containing DRN were obtained at 35 μm. DRN tissue used for immunohistochemistry (IHC) was placed directly into a 24 well plate.

Immunohistochemistry for Fos and 5-HT: Staining for Fos and 5-HT was conducted as previously described (Grahn et al., 1999). Staining for Fos was conducted using the avidin–biotin–horseradish peroxidase (ABC) method. Following a series of washes in 0.1 M phosphate-buffered saline (PBS), sections were incubated in a 0.9% hydrogen peroxide solution in order to quench endogenous peroxidases. Then, sections were incubated for 24 h at room temperature (RT) with Fos primary antibody (1:15,000; Santa Cruz Biotechnology) in a blocking solution containing 2% normal goat serum (NGS), 0.5% Triton-X and 0.1% sodium azide. Following the primary antibody incubation, sections were incubated for 2 h at RT in biotinylated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories) in blocking solution. After a series of PBS washes, slices were then incubated in ABC for 1 h at RT. Next, sections were washed in 0.1 m PB and then exposed to a solution containing 3,3-diaminobenzidine, cobalt chloride, nickel ammonium sulfate, ammonium chloride and glucose oxidase in PB. The peroxidase reaction was initiated by the addition of a glucose solution that reacted with the tissue for approximately 7–10 min. The reaction was terminated by washing sections with PBS. Tissue was floated onto slide glass and cover slipped for later analysis. 5-HT staining was conducted using the peroxidase anti-peroxidase (PAP) method. Following a series of washes in PBS, excess background 5-HT staining was prevented by incubating sections in blocking solution for 0.5 h. Tissue was incubated in blocking solution of 5-HT antibody (rabbit polyclonal 1:10,000, ImmunoStar, Hudson, WI) for 48 h at room temperature. Goat anti-rabbit secondary antibody (1:200 Jackson ImmunoResearch, West Grove, PA) was applied to the tissue for 2 h after a series of PBS washes. This step was followed by another series of PBS washes and incubation with PAP antibody (1:200 Jackson ImmunoResearch) for 2 h. Following a series of washes in PBS, tissue was incubated in a solution containing DAB and glucose oxidase. The peroxidase reaction was initiated by addition of glucose

and continued for 15 min. After a final series of PBS washes, tissue was mounted on slides and allowed to dry overnight. Slides were coverslipped with Permount.

Image analysis: Brain sections were observed using a bright field microscope (Olympus BX-61, Olympus America) and analyzed using cellSens software (Olympus America). All digital images were captured using a 20x objective. Images of DRN were taken using parameters similar to those previously described (Grahn et al., 1999). Sections corresponding to an AP coordinate of -1.36, -1.00, and -.70 mm relative to interaural zero were taken for rostral, middle, and caudal DRN, respectively. Fos-positive nuclei in each subregion of the DRN were observed as dark brown or black round/ovoid spots. 5-HT stained cell bodies were observed as light brown particles with and without unstained nuclei. Colocalization of Fos and 5-HT was observed as a light brown cell body with a black stained nucleus. For each subject, two separate counts were taken from different slices within each subregion of the DRN. The two counts for each subregion were averaged and used for statistical analysis.

Estrous cycle determination

Vaginal lavage was performed prior to stress treatment. 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 samples were used to identify the cycle stage: diestrus I/II (presence of nucleated cells and leucocytes), proestrus (presence of nucleated cells), and estrus (presence of anucleated squamous cells).

Statistics

Data analysis was performed with StatView software (SAS Institute, Cary, NC). The effect of treatment was analyzed with repeated-measures (trial block behavioral data), one-way (Stress), two-way (Stress and Drug), or mixed-design (Stress and DRN subregion measures) analysis of variance (ANOVA). Main effects and interactions were considered statistically significant if $p < 0.05$. When appropriate, post hoc analyses and planned comparisons were performed using Fisher's protected least significant difference (PLSD). Values in graphs are represented as mean ± SEM.

RESULTS

Behavioral control is not protective against the behavioral outcomes of stress

Failure to learn the wheel-turn (behavioral control) response is a confounding variable that may influence the behavioral outcome measures. Thus, we examined whether ES rats learned the operant response. The wheel turn requirement was increased as subjects learned the controlling response, using parameters identical to those used in previous male studies from our laboratory (Amat et al., 2005). To determine whether learning of the operant response occurred, the latency to terminate tailshock was measured. Importantly, all ES animals rapidly acquired the wheel-turn response, and so it is unlikely that any behavioral differences observed in females, as compared to prior data from male studies, is not attributable to a failure to acquire the operant behavioral response (Figure 2.1).

We next wanted to answer the question whether behavioral control would prevent various behavioral outcomes associated with stress exposure. Rats underwent a baseline JSE test 24 hours prior to receiving ES, IS, or HC. Similar to the results of previous studies performed in male rats, female rats that received IS showed a dramatic reduction in social exploration when tested again 24 hours after the final tailshock (Figure 2.2). Strikingly, ES led to as great of a reduction in social exploration as IS. A one-way ANOVA revealed main effects of stress ($F_{2,22} = 11.355$, $p < 0.001$, n = 8-9/group). Fisher's post-hoc revealed that ES and IS showed significant reductions in

social exploration, relative to HC, however they did not differ from each other. That is, both ES and IS led to a similar reduction in social exploration.

A separate cohort of rats received two FR-1 trials in a shuttlebox followed by a FR-2 shuttlebox escape test 24 hours after ES, IS, or HC. Freezing to the context was measured for 20 minutes following the two footshocks. Not surprisingly, IS led to exaggerated freezing to the context, relative to rats that received HC (Figure 2.3A). Interestingly, ES failed to mitigate the exaggerated freezing in female rats, unlike the data from previous studies performed in male rats (Maier et al., 1990; Amat et al., 2005). ES subjects were indistinguishable from IS subjects in regard to freezing. Repeated measures ANOVA revealed significant main effects of Stress ($F_{2,26} = 13.239$, $p < 0.001$) and Trial Block ($F_{9,234} = 14.254$, $p < 0.001$, n = 9-10/group). Post-hoc analysis indicated that ES and IS did not differ from each other, but both differed from HC (Fisher's PLSD, ps < 0.05). Next, shuttlebox escape performance was assessed. IS and ES equally disrupted escape performance, as measured by increased escape latencies during FR-2 trials (Figure 2.3B). Repeated measures ANOVA revealed main effects of stress ($F_{2,26} = 5.244$, $p < 0.05$) and Trial Block ($F_{5,130} = 20.126$, $p < 0.001$) and a significant Group X Trial Block interaction ($F_{10,130} = 1.908$, $p < 0.05$). Additionally, IS and ES increased the total number of escape failures during FR-2 trials (Stress: $F_{2,26} = 3.584$, $p < 0.05$) (Figure 3C). Post-hoc analysis revealed that IS and ES subjects had increased escape latencies and number of escape failures, relative to HC controls (ps < 0.05), however they did not differ from each other.

It should be noted that rats in all stages of the estrous cycle (diestrus I/II, proestrus, estrus) were represented in each condition, although the design was not sufficiently powered to conclusively detect the impact of estrous phase. Individual plots of subject data by estrous phase are shown in Figure 2.4.

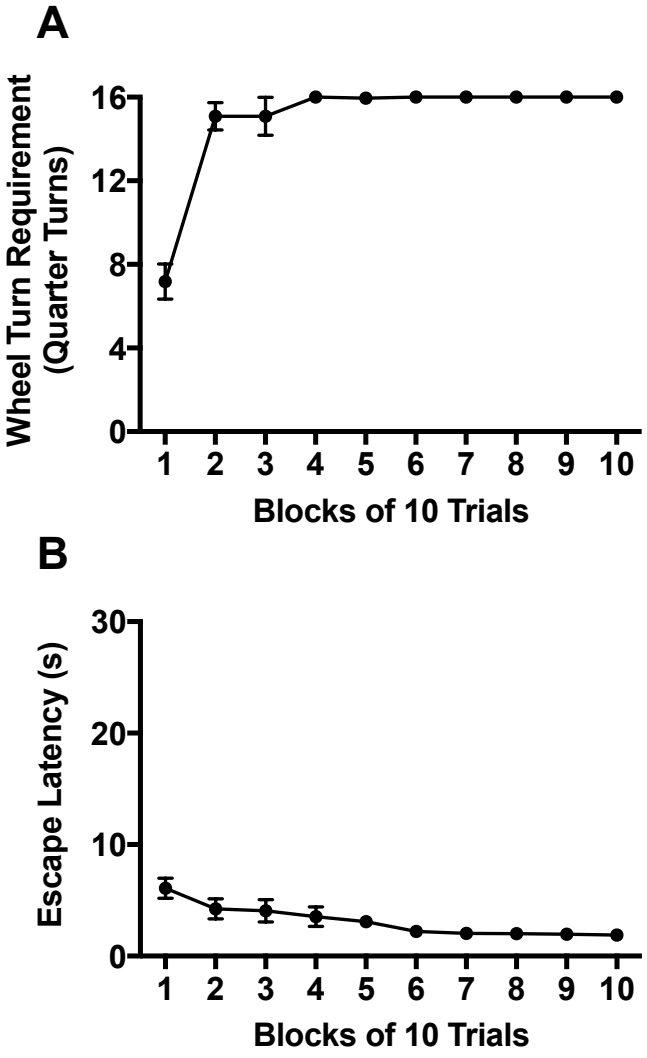

Figure 2.1. Female rats rapidly and reliably learn the operant wheel turn response. (A) Number of quarter wheel turns achieved for the given escape requirement on each trial and (B) latency (s) to escape tailshock (blocks of 10 trials shown). Values are represented as mean ±SEM.

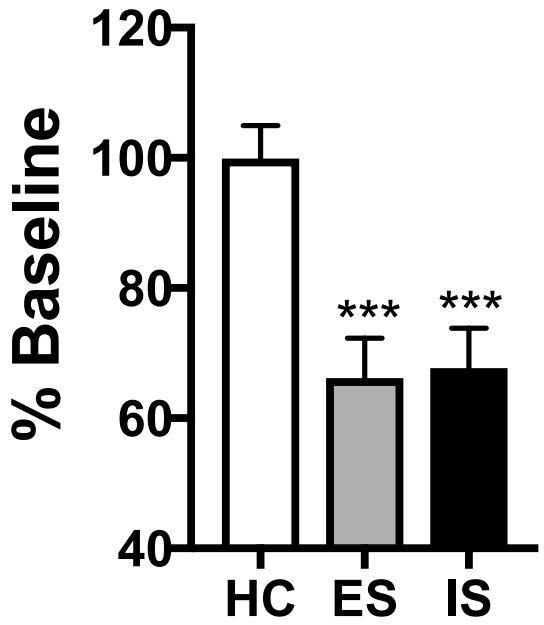

Figure 2.2. Controllable stress fails to protect female rats against later stress-induced juvenile social exploration deficits. (A) Juvenile social exploration measured 24 h after stress or HC treatment (reported as % baseline). Values are represented as mean ±SEM. *$p < 0.001$ compared to HC.

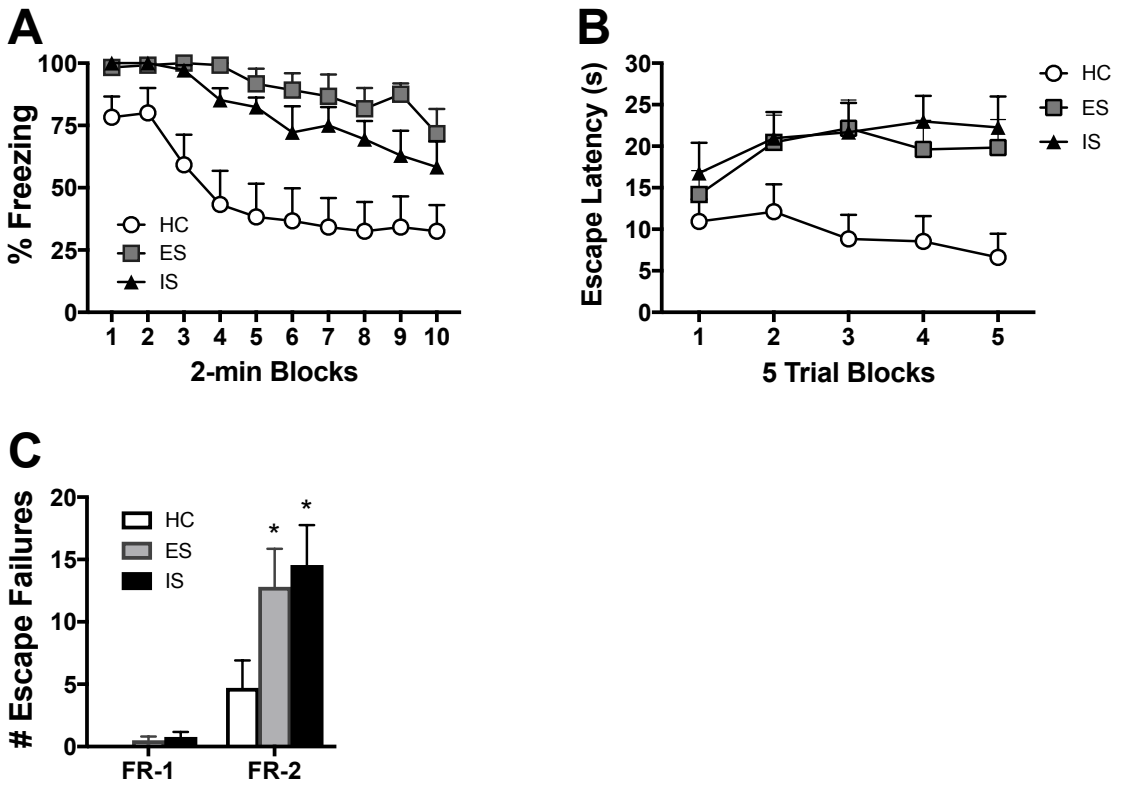

Figure 2.3. Controllable stress fails to mitigate the impact of stress on shock-elicited freezing and shuttlebox escape. (A) Percent freezing following two single footshocks in the shuttlebox apparatus (blocks of 2 min shown). (B) Shuttlebox escape latencies across for FR-2 trials (5 trial blocks shown). (C) Number of FR-1 and FR-2 escape failures. Values are represented as mean ±SEM. *$p < 0.05$ compared to HC.

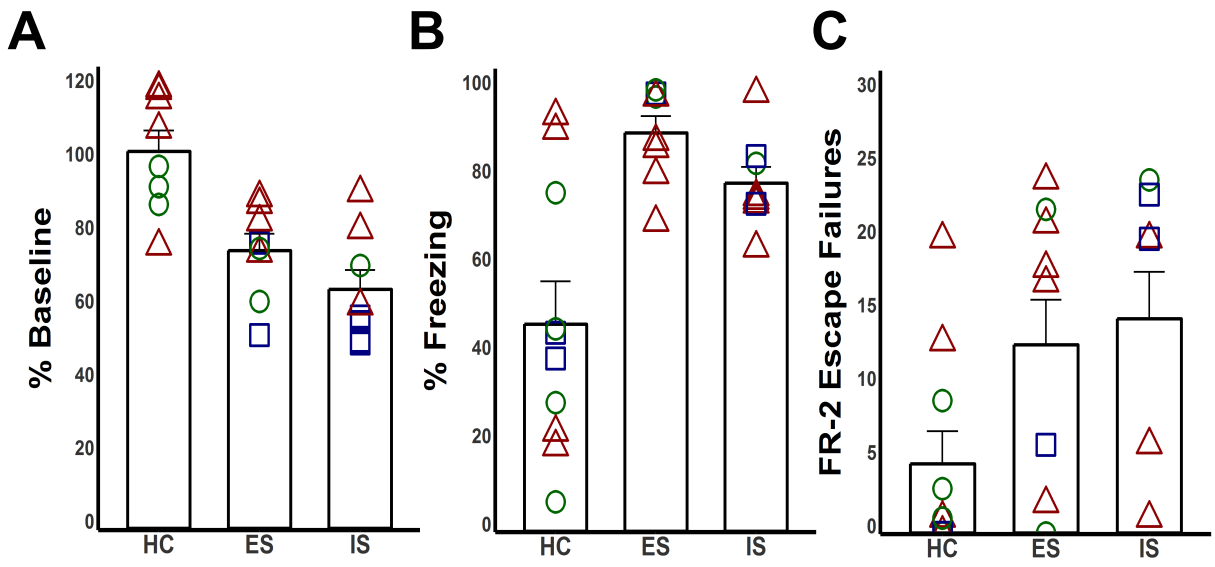

Figure 2.4. Phase of estrous cycle for subjects immediately prior to stress treatment. (A) Percent baseline social exploration adapted from Figure 1, (B) percent freezing adapted from Figure 2A, and (C) number of FR-2 escape failures adapted from Figure 2B. Red triangles = diestrus, blue squares = proestrus, and green circles = estrus. Symbols represent individual values and bars represent the group means ±SEM.

Behavioral control does not prevent stress-induced activation of the dorsal raphe nucleus in female rats

In male rats, behavioral control prevents activation of DRN 5-HT neurons, which is critical for the buffering the effects of the stressor (Grahn et al., 1999; Maswood et al., 1998). Thus, we sought to determine whether ES, which fails to protect female rats from the behavioral consequences of the stressor, would result in activation of 5-HT neurons within the DRN. Rats received ES, IS, or HC and were sacrificed 2 hours later. The total number of Fos cells did not differ between groups (data not shown). A mixed-effects ANOVA revealed a main effect of stress ($F_{2,30}$=14.126 p<0.001, n=11/group), and a significant interaction between Stress and DRN subregion ($F_{4,60}$=5.884, p<0.001) for total Fos expression. Relative to HC, ES and IS increased the total number of Fos-labeled cells in the rostral, middle, and caudal DRN (p's<.001). Fos expression was examined in DRN 5HT neurons (Figure 2.5A). Similar to the total number of Fos-labeled cells, ES and IS increased the percentage of 5-HT cells also expressing Fos (Figure 2.5B). A mixed-effects ANOVA revealed a main effect of stress ($F_{2,30}$ = 13.752, p < 0.001) and a significant interaction between stress and DRN subregion ($F_{4,60}$ = 3.842, p < 0.01). Post-hoc analysis revealed that ES and IS did not differ from each other (p>0.05) however, relative to HC, ES and IS increased the percentage of 5-HT cells expressing Fos (p's<.001).

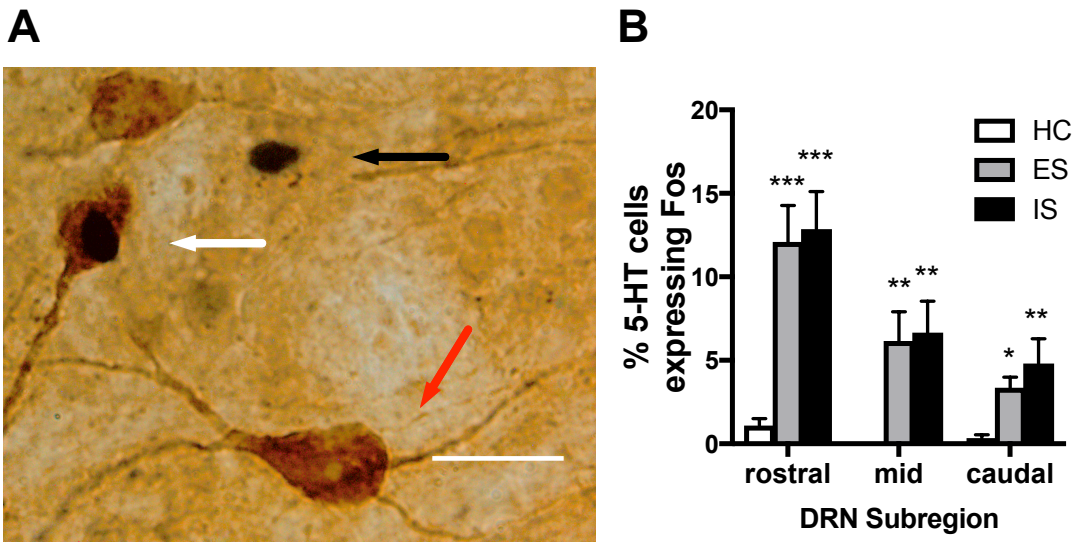

Figure 2.5. Controllable stress has no impact on stress-induced DRN activation. (A) Representative photomicrograph showing a 5-HT immunoreactive cell (red arrow), Fos-positive cells (black arrow), and a double-labeled cell expressing 5-HT and Fos (white arrow). Scale bar = 250 um. (B) Percentage of 5-HT cells also expressing Fos within the rostral, middle, and caudal subregions of the DRN. *p < 0.05, **p < 0.01, ***p < 0.001 compared to HC. Bars represent group means ±SEM.

Behavioral control does not activate the prelimbic cortex-dorsal raphe nucleus pathway in female rats

In male rats, when the PL is activated by an experience with control over tailshock, stress-induced activation of the DRN is inhibited, which prevents DRN-mediated behavioral responses (Amat et al., 2005; Baratta et al., 2009; Amat et al., 2005). Thus, one possible explanation for the lack of protection afforded to female rats by ES is that the PL-DRN pathway is not present in females, as all of the anatomical and behavioral work involving this pathway has been performed in male rodents (Hajos et al., 1998; Vertes, 2004; Hoover and Vertes, 2007). Another possible explanation is that the pathway is not recruited by an experience with stressor controllability. Therefore, we utilized a retrograde tracing technique to determine whether the PL-DRN pathway exists in females, and if so, whether behavioral control fails to activate this pathway. Rats previously injected with FG received ES, IS, or HC treatment and were sacrificed 2 hours later. Importantly, only rats with accurately localized DRN FG injections were included in statistical analysis (Figure 2.6A, representative FG injection) Figure 2.6B shows a representative Fos and FG double-labeling in the PL. Therefore, the PL-DRN pathway is present in female rats. Worth noting, the majority of FG expression was confined to the middle and caudal DRN. The total number of FG-labeled cells in the PL did not differ between groups within the rostral, middle, or caudal DRN. Only rats with accurate injections localized to the DRN were included in statistical analysis. The percentage of PL-DRN neurons that express Fos was examined using a one-way ANCOVA, which yielded no significant main-effects of stress ($F_{2,15}$ = 1.397, p > 0.05, n = 4-8/group), number of FG-positive cells, or interaction between stress and number of FG-positive cells (Figure 2.6C). Taken together, these data demonstrate that while the PL-DRN pathway is present in female rats, it is not engaged by an experience with behavioral control in a manner similar to that which has been extensively documented in male rats.

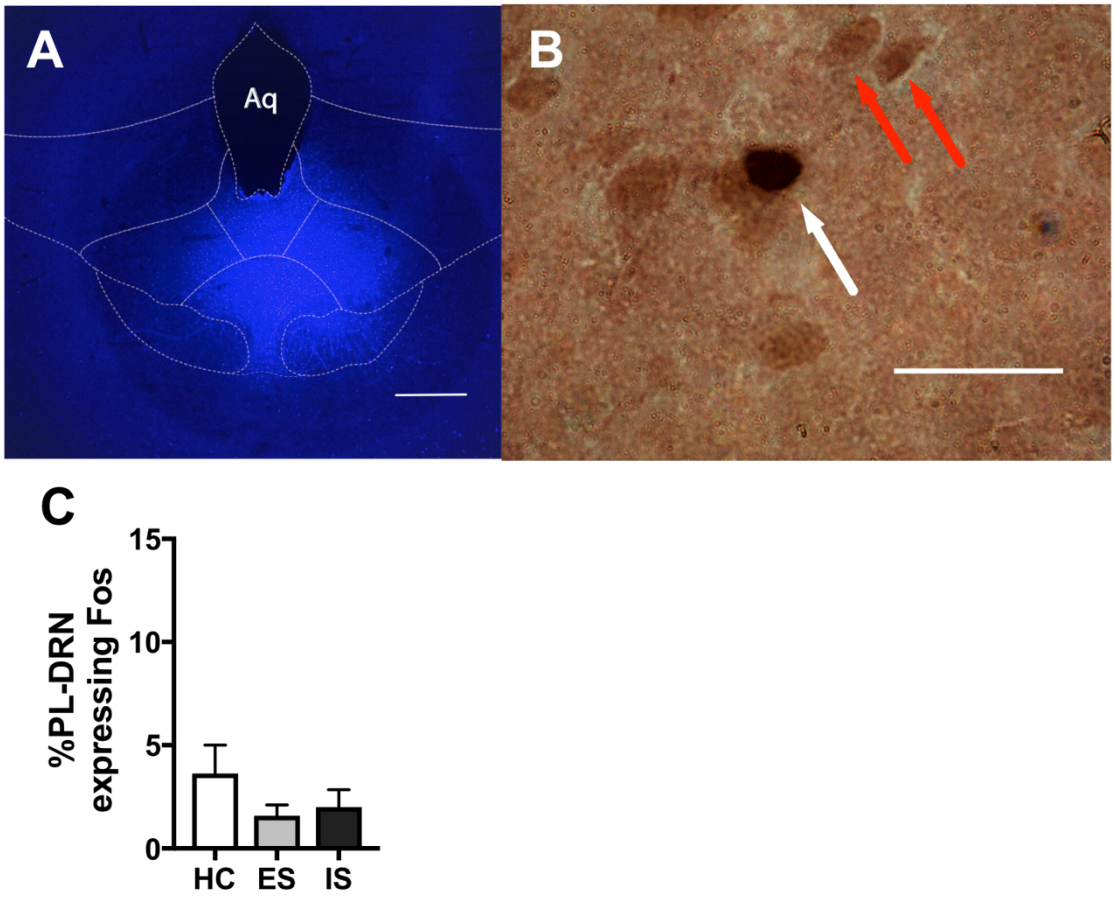

Figure 2.6. Controllable stress has no impact on activation of the PL-DRN pathway. (A) Representative photomicrograph showing a fluorogold (FG) deposit in the DRN. (B) Representative photomicrograph showing a 5-HT positive cells (red arrows) and a double-labeled 5-HT cell coexpressing Fos (white arrow). Scale bar = 250 um. (C) Percentage of PL-DRN cells also expressing Fos. Bars represent group means ±SEM.

Pharmacological activation of the prelimbic cortex during stress produces resistance to the behavioral outcome of the stressor

Based on the results of the pathway tracing experiment where it was revealed that behavioral control does not activate the PL-DRN pathway, we sought to determine whether pharmacological activation of the PL during the stressor would produce resistance to the behavioral outcome of the stressor, as has been shown in male rats (Amat et al., 2008). Twenty-four hours prior to ES, IS, or HC female rats were observed during a baseline measure of social exploration. The following day, rats were received an intra-PL injection of the GABA receptor antagonist picrotoxin (100 ng) 30 minutes prior to ES, IS, or HC treatment. A separate group of rats received intra-PL saline injection (control) prior to stress or HC treatment. Cannula placement was verified following completion of the experiment and only rats with accurate PL cannula implantations were included in the statistical analysis (Figure 2.7A). Similar to results from previous experiments performed in male rats (Amat et al., 2008), intra-PL picrotoxin had no effect on learning the wheel turn response (Figure 2.8). Repeated-measures ANOVA did not indicate any difference between picrotoxin and vehicle-treated ES subjects on response requirement ($F_{1,14} = 0.231$, $p > 0.05$, $n = 8$/group) or escape latency ($F_{1,14} = 0.706$, $p > 0.05$). Twenty-four hours after stress or HC treatment, rats were observed during a final test of social exploration. Both ES and IS dramatically reduced social exploration, relative to HC (Figure 2.7B). Intra-PL injection of picrotoxin had no effect on social exploration in HC subjects, however it prevented the stress-induced social exploration deficits in both ES and IS subjects. ANOVA uncovered significant main effects of Stress ($F_{2,42} = 10.048$, $p < 0.001$, $n = 8$/group) and Drug ($F_{1,42} = 15.770$, $p < 0.001$) and a significant Stress X Drug ($F_{2,42} = 3.573$, $p < 0.05$) interaction. Post hoc analysis revealed that ES-Vehicle and IS-Vehicle differed from all the other groups ($ps < 0.01$), which did not differ among themselves. Thus, intra-PL pictrotoxin prior to either ES or IS prevented the typical stress-induced reductions in social exploration in female rats.

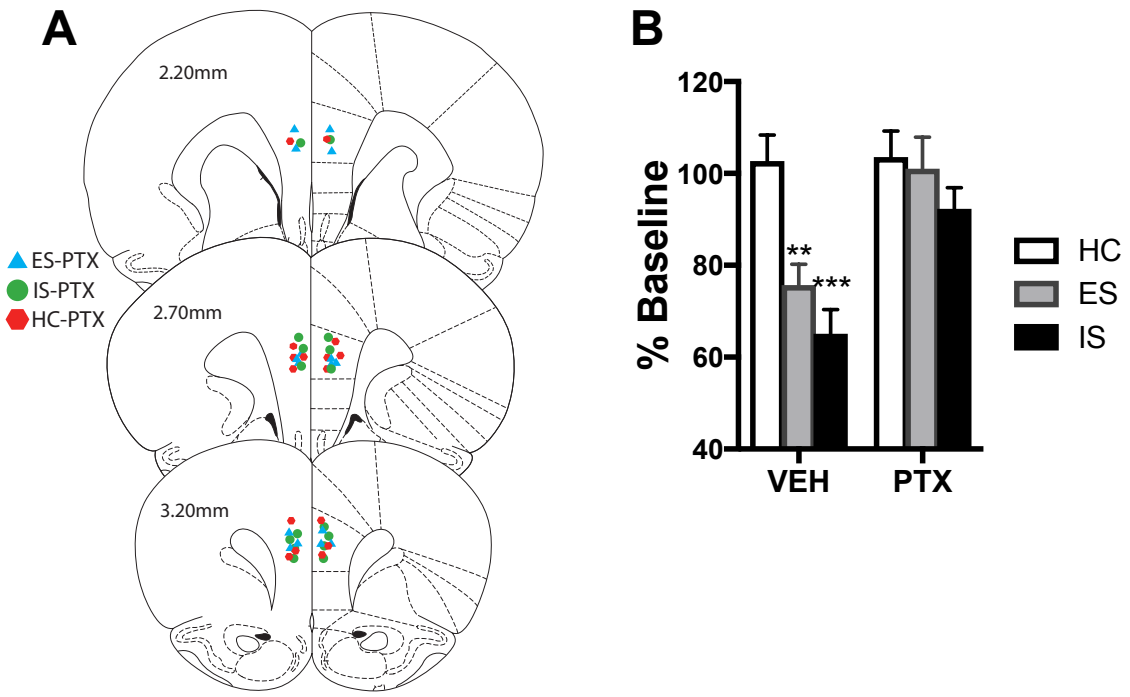

Figure 2.7. Microinfusion of picrotoxin in the prelimbic cortex prior to stressor exposure prevents juvenile social exploration deficits typically caused by the stressor. (A) Location of cannula tips directed to the prelimbic cortex. (B) Juvenile social exploration assessed in rats injected with vehicle or picrotoxin (100 ng) 30 minutes prior to stress or homecage treatment 24 hours prior. Bars represent group means ±SEM.

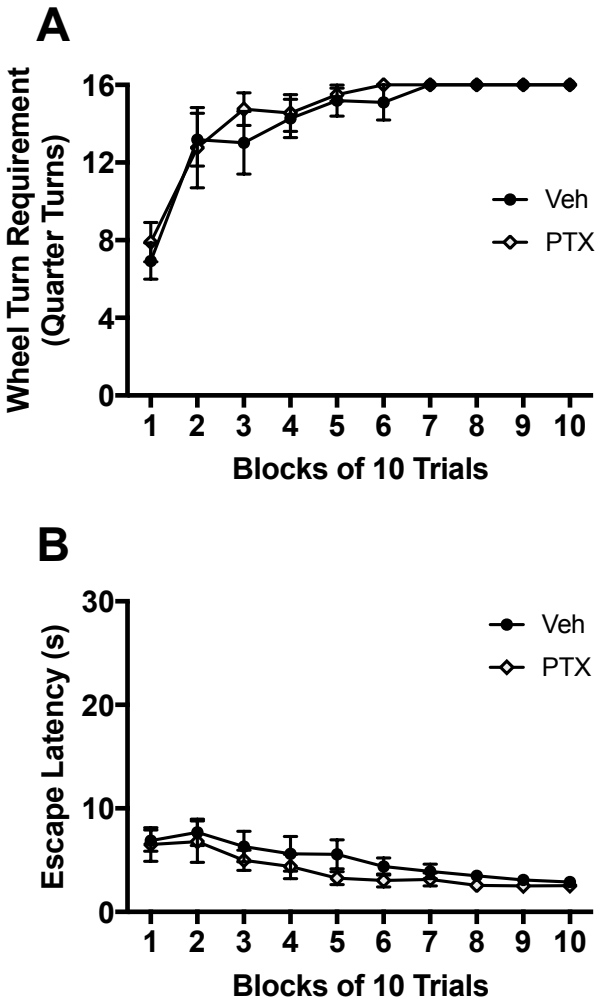

Figure 2.8. Microinfusion of picrotoxin in the prelimbic cortex does not interfere with acquisition of the wheel-turn response. (A) Number of quarter turns of the wheel attained as the escape requirement on each trial and (B) the time (s) to reach escape criterion per trial. Data are expressed as the mean (± SEM).

DISCUSSION

The present study sought to determine whether behavioral control is protective against the typical neurochemical and behavioral outcomes of stress, in a manner consistent with an extensive body of literature derived in male rats (see Maier and Watkins, 2010 for review). Thus, we utilized a number of behavioral and neurochemical measures that have previously been used in studies designed to identify the mechanisms by which behavioral control, or lack thereof produces resilience and vulnerability to the outcomes of the stressor. The results of the initial behavioral experiments were striking and clear. Behavioral control failed to prevent any of the outcomes of the stressor in female rats. That is, ES produced equivalent reductions in social exploration, exaggerated shock-elicited freezing, and impaired shuttlebox escape performance as IS. Thus, we propose that processing of information about behavioral control differs between female and male rats.

The results of these behavioral tests are unexpectedly dissimilar to the results of studies performed in male rats where behavioral control is indeed protective. Thus, we asked what neural processes might mediate the divergent behaviors observed between female and male rats. Various lines of work from our laboratory demonstrate that IS, relative to physically equal ES, results in intense activation of DRN 5-HT neurons (Grahn et al., 1999; Takase et al., 2004) and the subsequent release of 5-HT both within the DRN and into projection regions that mediate the behavioral outcomes of the stressor (Maswood et al., 1998; Amat et al., 1998; Amat et al., 2005; Christianson et al., 2010). Furthermore, activation and release of DRN 5-HT is necessary for reduced social exploration exaggerated shock-elicited freezing, and impaired shuttlebox escape performance (Maier and Watkins, 2005). A significant body of work demonstrates that ES prevents the neurochemical and behavioral outcomes of the stressor by inducing inhibitory control over the DRN via the PL-DRN pathway (Amat et al., 2005; Hajos et al., 1998; Varga et al., 2001; Hoover and Vertes, 2005). Indeed, the PL-DRN pathway is recruited by behavioral control (Amat et al., 2005; Baratta et al., 2009) and activation of the PL is necessary for the protective effects of

behavioral control in male rats (Amat et al., 2005; Amat et al., 2006). Additionally, pharmacological activation of the PL in IS subjects prevents the behavioral outcomes of the stressor, in a manner consistent with ES (Amat et al., 2008). Therefore, we reasoned that the same processes engaged by behavioral control in males were absent in females, which would account for behavioral differences observed following the stressor.

In accordance with the results of the behavioral tests, ES failed to prevent stress-induced DRN activation. Specifically, both ES and IS led to an equivalent increase in Fos protein in 5-HT labeled cells in all DRN subdivisions, relative to HC control subjects. One explanation for the failure of ES to prevent DRN activation and accompanying behavioral changes is that the PL-DRN pathway is not engaged in female rats that receive ES. Indeed, intra-DRN FG injection led to FG-positive cells in the PL, and so the anatomy does exist for the PL-mediated top-down inhibitory control over the DRN. In line with the results of the first two experiments, ES failed to activate the PL-DRN pathway. Unlike male rats, ES produced minimal activation of the PL-DRN pathway compared to HC control treatment (Amat et al., 2005; Baratta et al., 2009). Together, the results of these experiments provide further evidence that processing information about behavioral control differs between female and male rats.

The lack of protection afforded to females by behavioral control is striking and unexpected, given that they rapidly acquired the operant wheel turn escape response and maintained stable responding for the duration of the shock session. Research directed at understanding acquisition of instrumental behavior, mostly derived in the appetitive learning literature, has implicated two separable systems involved in the encoding of instrumental responses (Balleine and O'Doherty, 2010). First, an "act/outcome system" has been identified as a key system that is sensitive to the contingency between a response and a reinforcer (reviewed in Balleine and O'Doherty, 2010). Briefly, this system is sensitive to the difference between the conditional probability of a reward (outcome) in the presense of a response (action) and the conditional probability of a reward in the absence of a response. It is clear that behavioral control requires this type of rapid and relatively

flexible stimulus/response contingency whereby the “act” component is defined as the operant behavior (wheel-turn) and the “outcome” is defined as the termination of the stressor. Worth noting, this system is sensitive to changes in the outcome or reinforcer. An additional system called the “habit system” is insensitive to contingency and changes in the outcome or reinforcer. A large body of literature has implicated a corticostriatal circuit comprised of the PL and dorsomedial striatum (DMS) in the act/outcome system, while the habit system is comprised of the sensorimotor cortex and dorsolateral striatum (DLS). In the appetitive instrumental learning literature, it has been shown that excitotoxic lesions and reversible pharmacological inactivation of either the PL or DMS prevents act/outcome associations, without affecting the acquisition of the instrumental response (Yin et al., 2005; Shifflett and Balleine, 2011). Responses are instead then acquired using the habit system, and so they are insensitive to changes in the outcome. Because the concepts underlying act-outcome contingency learning are identical to that of behavioral control, and the protective effects of behavioral control requires activation of the PL and DMS (Amat et al., 2014), it is possible that females acquire the controlling response preferentially using the habit system, rather than the act-outcome system.

Indeed, previous work demonstrates that DRN activation (Hajos et al., 2003; Dalley et al., 2002) and acute stress-induced norepinephrine (NE) and dopamine (DA) release into the mPFC inhibit mPFC activity, prevent top-down inhibitory control (Puig et al., 2004; Puig et al., 2005; Birnbaum et al., 1999 Arnsten, 2000a; Arnsten et al., 2000b), and strengthen the habit system (Arnsten and Goldman-Rakic, 1998). Furthermore, studies performed in male rats demonstrate that IS, but not ES, increases 5-HT and DA efflux in the mPFC (Bland et al., 2003). Finally, sex differences in basal and stress-induced NE, DA, and 5-HT release have been reported, with the majority of the literature demonstrating higher levels in female rodents (Staiti et al., 2011; Dominguez et al., 2003; Mitsushima et al., 2006). Together, it is apparent that stress-induced neurotransmitter release into the mPFC during ES may play a role in the failure of behavioral control to buffer against stress outcomes in females.

A variety of future experiments will be performed at the level of the mPFC to elucidate the mechanisms underlying the lack of protection afforded by ES in female rats. First, we plan to determine whether neurotransmitter levels, such as 5-HT, NE, and DA, in the mPFC vary between females and males during ES using *in vivo* microdialysis. We will examine whether this might bias control of the wheel turn response towards the habit system, rather than the act-outcome system, or whether increased stress-induced neurotransmitter levels in females are simply taking the mPFC "offline", thereby preventing its ability to regulate DRN activity. The first of these possibilities will be examined by quantifying activity of immediate early genes, such as Fos and Arc, in the DMS and DLS of female and male rats. The second of these possibilities will be examined by blocking 5-HT, NE, or DA receptors in the PL that are likely to be involved reducing mPFC activity. Additionally, we will examine whether females shift from utilization of the act-outcome system to the habit system earlier than that which occurs in male rats. Finally, we will examine whether females simply acquire the wheel turn response at a faster rate than male rats, which might shift responding to the habit system before the PL is able to prevent initial DRN activation and subsequent sensitization. In order to address this possibility, we will reduce the number of ES trials delivered to female and male rats. It should be noted that if one of the proposed experiments identifies a mechanism underlying the lack protection afforded to females by ES, the other experiments may not be necessary.

In support of the possibility that the mPFC is "offline" during ES in female rats, we demonstrated that pharmacological activation of the PL prevents the stress-induced behavioral changes that accompany both ES in female rats, so that ES females now closer resemble ES males. The results of this experiment provide strong evidence that the same neural circuit elements engaged by control in males are not engaged in females exposed to an identical stressor. Given the dissimilarities between the effects of behavioral control in female and male rats, the present study emphasizes the importance of examining both sexes when developing

animal models that are designed to identify neural mechanisms underlying stress-related disorders in humans.

Chapter 3:

Inhibition of a descending prefrontal circuit prevents ketamine-induced stress resilience in females

ABSTRACT

Stress is a potent etiological factor in the onset of major depressive disorder and post-traumatic stress disorder (PTSD). Therefore, significant efforts have been made to identify factors that produce resilience to the outcomes of a later stressor, in hopes of preventing untoward clinical outcomes. The NMDA receptor antagonist ketamine has recently emerged as a prophylactic capable of preventing neurochemical and behavioral outcomes of a future stressor. Despite promising results of preclinical studies performed in male rats, the effects of proactive ketamine in female rats remains unknown. Here we explore the prophylactic effects of ketamine on stress-induced anxiety-like behavior and the neural circuit-level processes that mediate these effects in female rats. Ketamine given 1 week prior to an uncontrollable stressor (inescapable tailshock, IS) reduced typical stress-induced activation of the serotonergic (5-HT) dorsal raphe nucleus (DRN) and eliminated DRN-dependent juvenile social exploration (JSE) deficits 24 hours after the stressor. Proactive ketamine altered prelimbic cortex (PL) neural ensembles so that a later experience with IS now activated these cells, which it ordinarily would not. Ketamine acutely activated a prelimbic cortex (PL) to DRN (PL-DRN) circuit and inhibition of this circuit with Designer Receptor Exclusively Activated by Designer Drugs (DREADDs) at the time of IS 1 week later prevented stress prophylaxis, suggesting that persistent changes in PL-DRN circuit activity are responsible, at least in part, for mediating long term effects associated with ketamine.

INTRODUCTION

Stress-related psychiatric disorders, such as depression and post-traumatic stress disorder (PTSD), affect females at nearly twice the rate of males (Kessler et al., 2005; Steiner et al., 2005) and are among the leading causes of disability worldwide (WHO, 2008; Kessler et al., 1995). Only one third of patients prescribed conventional pharmacotherapies achieve full remission, underscoring the need for more effective therapeutic modalities. (Gaynes et al., 2009). Recently, it has been shown that a single subanesthetic dose (0.5 mg/kg, i.v.) of the nonselective NMDA receptor antagonist ketamine produces rapid and enduring therapeutic effects in individuals with treatment-resistant depression, anxiety, and PTSD (Berman et al., 2000; Zarate et al., 2006; Glue et al., 2017; Price et al., 2009; Feder et al., 2014). Accordingly, a growing body of research has been dedicated to identifying the underlying neurobiological mechanisms by which ketamine produces its effects.

Because of its clinical effectiveness, laboratory work has focused on two paradigms. In one, a single subanesthetic dose (10 mg/kg, i.p.) of ketamine is delivered at various time points before behavioral tests that are thought to reflect depressive or anxiety-related behavior. For example, ketamine delivered minutes to hours prior to behavioral testing prevents typical behavioral changes measured during the forced swim test (Garcia et al., 2008), tail suspension test (da Silva et al., 2010), novelty suppressed feeding test (NSF) (Fuchikami et al., 2015) and the open-field test (Thelen et al., 2016). In the second, ketamine is given after exposure to a stressor to determine whether it would reverse stress effects on behavior. Ketamine delivered shortly after (0-24 hours) exposure to a chronic unpredictable stressor reverses the effects of the stressor on NSF and sucrose preference (Li et al., 2011). Surprisingly, nearly all of the preclinical studies designed to identify the mechanistic actions of ketamine have focused on *male* rats.

There has been a great deal of recent interest in factors that can lead to resilience in the face of adversity (*reviewed in* Baratta et al., 2013), and interestingly, 3 recent reports indicate that single dose of ketamine can blunt the impact of stressors occurring as much as 2 weeks later

(Brachman et al., 2016; Amat et al., 2016; McGowan et al., 2017). Unfortunately, none of these reports employed female subjects.

Thus, we chose to explore the proactive effects of ketamine in female rats, as well as the underlying neural circuit-level processes that mediate such effects. We sought to determine whether ketamine delivered 1 week prior to an uncontrollable stressor (inescapable tailshock, IS) is sufficient to prevent anxiety-like behavior measured during juvenile social exploration (JSE) 24 hours later, in a manner similar to that observed in male rats (Amat et al., 2016). IS-induced behavioral changes are mediated in part by activation of serotonergic (5-HT) neurons within the dorsal raphe nucleus (DRN) (Maier and Watkins, 2005). Specifically, IS activates 5-HT neurons in the mid to caudal DRN (Grahn et al., 1999) leading to 5-HT release in projection regions that are proximal mediators of stress-induced behavioral changes, such as the basolateral amygdala (BLA) (Amat et al. 1998; Christianson et al., 2010; Dolzani et al., 2016). Indeed, blockade of 5-HT2C receptors in the BLA eliminates the reduction in JSE produced by prior IS (Christianson et al., 2010). Therefore, we examined the effect of ketamine on IS-induced Fos activation in DRN 5-HT neurons to determine whether ketamine mitigates IS-induced DRN activation (Amat et al., 2016). Plastic changes in the prelimbic region (PL) of the medial prefrontal cortex (mPFC), a potent regulator of DRN activity (*reviewed in* Maier and Watkins, 2010), are critical for the stress-buffering effects of ketamine (Li et al., 2010; Lepack et al., 2016; *reviewed in* Duman and Krystal, 2016). Thus, we explored whether ketamine alters PL neural ensembles so that later IS now activates the same ensembles. Finally, we examined whether ketamine directly activates the PL-DRN pathway, and if so, whether PL-DRN pathway activation is critical for the protective effects of ketamine at the time of later IS.

METHODS

Experimental design:

The first set of experiments examined whether a single dose of ketamine would mitigate the behavioral and neurochemical effects of IS. Therefore, low dose ketamine (10 mg/kg, i.p.), which is protective against stress outcomes in male rats (Li et al., 2010; Amat et al., 2016), was administered to female rats 1 week (7 days) prior to IS or HC treatment. Separate groups of rats received high dose ketamine (40 mg/kg, i.p.), which is not implicated in stress resistance (Chowdhury et al., 2016), or saline. Anxiety-like behavior was assessed during a juvenile social exploration (JSE) test 24 h after the stressor. Thus, the experiment was a 2 (stress) X 3 (drug) factorial design. Two-way ANOVA was used for statistical analysis. Previous work performed using similar parameters (Dolzani et al., 2016) demonstrates that n's of 9-12/group are sufficient to achieve statistical significance between groups. Three rats were considered statistical outliers (>2.5 SDs from the mean) and were excluded from the statistical analysis. The 1-week time point was selected in order to dissociate the long-term stress-buffering effects of the ketamine from potential acute effects, which are not the focus of the present work. Additionally, previous work from our laboratory demonstrates that ketamine delivered 1-week prior to IS in male rats protects against the typical effects of the stressor (Amat et al., 2016). DRN 5-HT activation was assessed in a separate group of rats using double label immunohistochemistry (IHC) to determine whether ketamine reduces overall stress-induced Fos expression within the DRN, and whether this reduction occurs in 5-HT neurons within the DRN. Fos and 5-HT expression were examined in the rostral, middle, and caudal DRN. Thus, the experiment was a 2 (stress) x 2 (drug) factorial design. Two-way ANOVA was used to separately analyze total 5-HT, total Fos+, and the percent of 5-HT positive cells that also expressed Fos between all possible groups. Previous work from our laboratory (Dolzani et al., 2016) demonstrates that n's of 10-12/group (2 brain slices per rat) are sufficient for detecting statistical differences in immunohistochemical labeling between groups. Four rats were considered statistical outliers (>2.5 SD from group mean for cell counts or insufficient staining for detection) and were removed from the analysis.

The major source of inhibitory control over DRN 5-HT neurons derives from descending PL glutamatergic pyramidal neurons that synapse preferentially on GABA interneurons in the DRN (Jankowski and Sesack, 2004). Interestingly, behavioral control blunts the impact of a stressor by activating this inhibitory pathway (Amat et al., 2005; Baratta et al., 2009). Moreover, the experience of control also has a prophylactic effect in that it blocks the behavioral effects of later uncontrollable stressors such as IS (Amat et al., 2006) and social defeat (Amat et al., 2010). After a prior experience with control an uncontrollable stressor now does not activate DRN 5-HT neurons (Baratta et al., 2009). This occurs because the experience of control alters the PL-DRN pathway so that it is now activated by even uncontrollable stressors such as IS (Baratta et al., 2009). Thus, the second set of experiments sought to determine whether ketamine might engage the same mechanisms as behavioral control and alter PL neurons so that later IS now activates the PL. We utilized the recently developed immediate early gene (IEG) platform Robust Activity Marker (RAM) to interrogate neural activity at two time points: the time of initial ketamine injection (Time 1), and the time of later IS (Time 2). RAM provides a means for labeling neuronal ensembles activated by a particular temporally defined experience or event (see Sørensen et al., 2016 for detailed explanation). When combined with a subsequent IEG labeling technique, such as IHC, RAM allows for interrogation of neuronal activity at multiple time points. RAM utilizes a synthetic activity-dependent promoter (pRAM), which is driven by neuronal specific FOS and NPAS4 activity. pRAM activity drives expression of a tetracycline transactivator domain (tTA), which binds to a tetracycline response element (TRE) and drives expression of the effector gene, mKate2. Temporal control over RAM is achieved using a modified Tet-Off system. Binding of the tTA to the TRE is inhibited in the presence of DOX (DOX+). In the absence of DOX (DOX-), effector gene transcription is enabled. Therefore, we utilized RAM to determine whether ketamine activates PL neurons at the time of injection (Time 1) and whether a later experience with IS (Time 2) now activates the same, or different, neuronal ensembles as did prior ketamine. Importantly, low and high dose ketamine were administered to separate groups of rats to determine whether

IS-induced activation of neural ensembles previously activated by ketamine is specific to a dose of ketamine that protects against behavioral outcomes of stress. Thus, the experiment was a 2 (stress) x 3 (drug) factorial design. Two-way ANOVA was used to separately analyze total RAM, total Fos+, and the percent of RAM cells also expressing Fos between all groups. Previous work by Sørensen et al., (2016) demonstrates that n's of 6-8/group (2 brain slices per rat) are sufficient to detect statistical differences between groups in experiments performed using similar parameters. Five rats were considered outliers (>2.5 SDs from mean for cell counts or failure to detect expression of the injection control (eGFP).

The third experiment examined whether ketamine activates the PL-DRN circuit. Red fluorescent retrogradely transported microspheres (hereafter referred to as retrobeads, RB) were injected into the DRN 2 weeks prior to rats receiving a single injection of ketamine. Two hours later rats were sacrificed and Fos expression was assessed in the PL and PL-DRN pathway. An independent samples t-test was used to examine differences in total RB-positive (RB+) cells, total Fos, and the percent of RB+ cells that also expressed Fos. N's of 6-8/ group (2 brain slices per rat) are sufficient to detect statistical differences between groups. Two rats were considered outliers (>2.5 SD from mean for cell counts) and excluded from analysis. Additionally, 4 rats were excluded from analysis due to inaccurate DRN RB injections.

The final experiment examined whether the PL-DRN circuit is necessary for the protective effects of ketamine. This requires selective inhibition of PL neurons that project to the DRN. Addressing this required the use of a dual viral intersectional genetic strategy to target Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to DRN-projecting PL neurons. A retrogradely transported AAV vector encoding Cre recombinase was delivered into the DRN, and double-floxed AAV vector encoding the inhibitory DREADD receptor, (hM4Di) or mCherry (control virus), was delivered into the PL. This approach enables selective inhibition of neurons following systemic injection of clozapine-*N*-oxide (CNO) (Armbruster et al., 2007; Ferguson et al., 2011; Rogan and Roth, 2011), as verified by reduced expression of *c-Fos* (Ferguson et al., 2011;

Soumire and Sibille, 2014). In order to validate hM4Di-mediated inhibition of the PL-DRN pathway, we used an independent samples t-test to assess total PL-DRN hM4Di/mCherry expression, total Fos, and the percentage of hM4Di/mCherry expressing cells also expressing Fos. In a separate cohort, rats expressing hM4Di or mCherry in the PL-DRN pathway were injected with ketamine or saline, and 1 week later they received CNO injection 30 minutes prior to IS. Anxiety-like behavior was assessed 24 hours later during JSE. Therefore, the experiment was a 2 (virus) x 2 (stress) x 2 (drug) factorial design. Three-way ANOVA was used for statistical analysis. Previous work using similar parameters (Dolzani et al., 2016) demonstrates that n's of 10-12/group are sufficient to detect statistical significance. Three rats were considered statistical outliers (>2.5 SD from mean), and were removed from the statistical analysis. Additionally, two rats were excluded due to stress-related paw injuries, and 6 rats were excluded due to inaccurate or failed viral injections, as determined after completion of the experiment.

Data analysis for all one-way ANOVA, two-way ANOVA, and t-tests was performed using Prism software (Graphpad, La Jolla, CA). Three-way ANOVA was performed using Statview (SPSS, Chicago, IL). All experiments were performed using a between-subjects design and the effect of treatment was analyzed with unpaired t-test (Drug), one-way (Stress), two-way (Stress and Drug), or three-way (Stress and Drug and Virus) ANOVA. Main effects and interactions were considered statistically significant if $p<0.05$. When appropriate, post-hoc analyses and planned comparisons were performed using Tukey's post-hoc method. Values in graphs are represented as mean ± SEM.

Rats: Adult female Sprague–Dawley rats (250–300 g; Envigo) were pair housed on a 12-h light–dark cycle (lights on at 07:00 am and off at 07:00 pm). Rats were housed with free access to food and water and were allowed to acclimate to colony conditions for 7 days prior to surgical or experimental procedures. All stereotactic surgeries were performed under 2.5% isoflurane (Piramal Critical Care, Bethlehem, PA) anesthesia. Rats received preoperative analgesic

(meloxicam, 0.5 mg/kg s.c.; Vetmedica, St. Joseph, MO) and antibiotic (Combi-Pen-48, 0.25 ml/kg s.c.; Bimeda, Oakbrook Terrace, IL) prior to surgical procedures. Rats were given two weeks to recover from surgery before experimentation. All experiments were performed between 9:00 AM and 5:00 PM. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Drug administration: In all experiments, ketamine (Ketalar, Pfizer) was administered at 10 mg/kg or 40 mg/kg intraperitoneally. This dose was based on parameters previously described (Li et al., 2010; Amat et al., 2016; Chowdhury et al., 2016).

Stress procedure: Inescapable tailshock (IS) was delivered as previously described (Amat et al., 2005; Christianson et al., 2013). Briefly, rats were placed in a PlexiGlas restraint tube (8x18 cm, diameter x length). The rat's tail was secured to a Plexiglas post protruding from the rear portion of the box using medical tape and copper electrodes were placed around the tail. Shock was delivered to the rat's tail with increasing intensity as the shock session progressed (33 trials at 1.0 mA, 33 trials at 1.3 mA, and 34 trials at 1.6 mA). Shock was delivered with an average intertrial interval (ITI) of 60 seconds. Rats were removed from the PlexiGlas boxes and placed in their home cage immediately after the last tailshock. Non-shocked homecage control (HC) rats were left undisturbed in the colony.

Juvenile Social Exploration: Juvenile social exploration (JSE) testing was conducted 24 hours after IS or HC, as previously described (Amat et al., 2016). Any rats showing signs of injury (including injured hindpaws, forepaws, or toenails) after the stress session were excluded. Precautions are taken to minimize the frequency of these occurrences, but nonetheless injuries did occur in a small subset of subjects. Each experimental subject was singly assigned to an

empty plastic cage with shaved wood bedding and a wire lid. Experimental subjects remained in the test cage for 45 min-1 hour prior to introducing a juvenile (28 ± 2 days old) female conspecific. An observer, blind to treatment, recorded exploratory behavior (allogrooming, licking, sniffing, and pinning) initiated by the experimental subject during a 3-minute test. JSE test scores were reported as a total time (s) of social exploration during the 3-minute test.

Fluorescent Immunohistochemistry: Staining for Fos was performed using a general immunofluorescence protocol. Following a series of washes in 0.01 M PBS containing 0.5% Triton-X, slices were incubated overnight in a PBS blocking solution containing 0.5% Triton-X (PBST) and 2.5% bovine serum albumin (BSA) at 4 degrees C. Then, slices were washed in PBST and incubated for 24 h at RT in rabbit polyclonal primary antibody (1:1000; Santa Cruz Biotechnology) in blocking solution. After a series of PBS washes, slices were incubated for 2 h at RT in Alexa Fluor 405 goat antirabbit secondary antibody (1:250; Life Technologies). After a series of PBS washes, tissue was floated onto slide glass and coverslipped with Vecta Shield (Vector Labs).

Image analysis for Fluorescent Immunohistochemistry experiments: Brain sections were observed using a Nikon N-SIM structured illumination super-resolution laser scanning confocal microscope (Nikon). Images were captured using NIS Elements (Nikon) and analyzed using FIJI (Image J). All digital images were captured using a 20x objective. For imaging and quantification of PL (taken between AP: +2.5 mm to +3.0 mm relative to bregma) boundaries were based on those previously described (Baratta et al., 2009) with consultation to the brain atlas (Paxinos and Watson, 2011). In the PL-DRN retrograde tracing experiment, RB-positive (RB+) cell bodies in the PL were observed using a 546 nm laser line and were pseudocolored red. Fos+ nuclei were observed using a 405 nm laser line and were pseudocolored blue. RB+ and Fos+ cells in the PL were quantified and recorded separately. Colocalization of RB and Fos was reported when a

red/magenta cell body representing the intermixture of the two fluorophores was observed and verified to be overlapping RB and Fos. In the PL RAM experiment, eGFP labeled neurons (site of viral injection) were observed using a 488 nm laser line and were pseudocolored green. eGFP expression was confirmed in all subjects included in statistical analysis. Following confirmation of accurate viral injection, RAM labeled nuclei were observed using a 546 nm laser line and were pseudocolored red. Fos+ nuclei were observed using a 405 nm laser line and were pseudocolored blue. RAM+ and Fos+ cells in the PL were quantified and recorded separately. Colocalization of RAM and Fos was reported when a magenta nucleus representing the intermixture of the two fluorophores was observed and verified to be overlapping RAM and Fos. In the DREADD-mediated PL-DRN pathway silencing experiment, DREADD or mCherry positive cells were observed using a 546 nm laser line and were pseudocolored red. Fos+ nuclei were observed using a 405 nm laser line and were pseudocolored green. DREADD and Fos positive cells in the PL were quantified and recorded separately. Colocalization of DREADD and Fos was reported when a yellow nucleus representing the intermixture of the two fluorophores was observed and verified to be overlapping DREADD and Fos. For each subject, two separate counts were taken from PL tissue spanning AP: +2.5 mm to +3.0 mm. The two counts for each subregion were averaged and used for statistical analysis.

Effect of ketamine on later juvenile social exploration

Rats randomly assigned to drug and stress treatment received a single systemic injection of ketamine (10 or 40 mg/kg, i.p.) or vehicle 1 week prior to IS or HC. Twenty-four hours after the completion of IS or HC, rats received JSE testing.

Effect of ketamine on stress-induced Fos activation in the dorsal raphe nucleus

Tissue preparation: Rats injected with ketamine 1 w prior to IS or HC were deeply anesthetized with sodium pentobarbital (65 mg/kg) 2 hours after the last tailshock or at the same time for rats

assigned to HC. Rats were transcardially perfused with 100 ml ice-cold 0.9% saline, immediately followed by 250 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH ~7.4). Brains were post-fixed overnight in the same fixative and transferred to a 30% sucrose solution in 0.1 M phosphate buffer (PB) then stored at 4 degrees C until sectioning. Coronal brain sections containing DRN were obtained at 35 µm. DRN tissue used for immunohistochemistry (IHC) was placed directly into a 24 well plate.

Immunohistochemistry for Fos and 5-HT: Staining for Fos and 5-HT was conducted as previously described (Grahn et al., 1999). Staining for Fos was conducted using the avidin–biotin–horseradish peroxidase (ABC) method. Following a series of washes in 0.1 M phosphate-buffered saline (PBS), sections were incubated in a 0.9% hydrogen peroxide solution in order to quench endogenous peroxidases. Then, sections were incubated for 24 h at room temperature (RT) with Fos primary antibody (1:15,000; Santa Cruz Biotechnology) in a blocking solution containing 2% normal goat serum (NGS), 0.5% Triton-X and 0.1% sodium azide. Following the primary antibody incubation, sections were incubated for 2 h at RT in biotinylated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories) in blocking solution. After a series of PBS washes, slices were then incubated in ABC for 1 h at RT. Next, sections were washed in 0.1 m PB and then exposed to a solution containing 3,3-diaminobenzidine, cobalt chloride, nickel ammonium sulfate, ammonium chloride and glucose oxidase in PB. The peroxidase reaction was initiated by the addition of a glucose solution that reacted with the tissue for approximately 7–10 min. The reaction was terminated by washing sections with PBS. Tissue was floated onto slide glass and cover slipped for later analysis. 5-HT staining was conducted using the peroxidase anti-peroxidase (PAP) method. Following a series of washes in PBS, excess background 5-HT staining was prevented by incubating sections in blocking solution for 0.5 h. Next, tissue was incubated in blocking solution of 5-HT antibody (rabbit polyclonal 1:10,000, Jackson Immuno) for 48 h at room temperature. Goat anti-rabbit secondary antibody (1:200 Jackson) was applied to

the tissue for 2 h after a series of PBS washes. This step was followed by another series of PBS washes and incubation with PAP antibody (1:200 Jackson) for 2 h. Following a series of washes in PBS, tissue was incubated in a solution containing DAB and glucose oxidase. The peroxidase reaction was initiated by addition of glucose and continued for 15 min. After a final series of PBS washes, tissue was mounted on slides and allowed to dry overnight. Slides were coverslipped with Permount.

Image analysis: Brain sections were observed using a bright field microscope (Olympus BX-61, Olympus America) and analyzed using cellSens software (Olympus America). All digital images were captured using a 20x objective. Images of DRN were taken using parameters similar to those previously described (Grahn et al., 1999). Sections corresponding to an AP coordinate of -1.36, -1.00, and -.70 mm relative to interaural zero were taken for rostral, middle, and caudal DRN, respectively. Fos-positive nuclei in each subregion of the DRN were observed as dark brown or black round/ovoid spots. 5-HT stained cell bodies were observed as a light brown particles with and without unstained nuclei. Colocalization of Fos and 5-HT was observed as a light brown cell body with a black stained nucleus. For each subject, two separate counts were taken from different slices within each subregion of the DRN. The two counts for each subregion were averaged and used for statistical analysis.

Effect of ketamine on activation of the PL-DRN pathway

Microinjection of retrograde tracer: A small window (1 mm x 1 mm) was drilled into the skull and red fluorescent retrobeads (RB) (Lumafluor) were microinjected into the DRN (AP: -8.0 mm relative to bregma, DV: -6.7 mm from skull surface, ML: ±0.0 mm relative to midline) using a 10 µl Hamilton syringe and a 31-gauge metal needle with a 45 degree beveled tip. The total injection volume (0.3 µl) and flow rate (0.1 µl/min) was controlled with a microinjection pump (UMP3-1; World Precision Instruments). This injection volume was chosen based on pilot experiments in

which robust RB expression was observed within the PL 2 weeks after 0.3 µl injection, while maintaining localized injections. Following injection, the needle was left in place for an additional 10 minutes to allow for retrobead diffusion, after which the needle was withdrawn. The small scalp incision was closed using Vetbond (3M). Pre-operative antibiotic (Combi-Pen-48, 0.25 mL/kg, sc) and a post-operative analgesic (Meloxicam, 2 mg/kg, sc) was administered to all rats. Two weeks after RB microinjection, rats received a systemic injection of ketamine or saline and were sacrificed 2 h later. RB microinjections were considered successful if expression was visibly confined to DRN in coronal sections of brain obtained after completion of the experiment. Only rats with accurate RB injections were used for statistical analysis.

Tissue preparation: Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) at 2 hours following the ketamine or saline injection. Rats were transcardially perfused with 100 ml ice-cold 0.9% saline, immediately followed by 250 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH ~7.4). Brains were post-fixed overnight in the same fixative and transferred to a 30% sucrose solution in 0.1 M phosphate buffer (PB) then stored at 4 degrees C until sectioning. Coronal brain sections containing PL were obtained at 35 µm. PL tissue used for IHC was placed directly into a 24 well plate. DRN tissue was mounted onto glass slides and coverslipped with VectaShield (Vector Labs) mounting medium.

Effect of ketamine on PL neural ensembles at the time of initial injection and at the time of later stress

Virus: Adeno-associated virus (AAV) vectors were used to target the RAM-NLS-mKate2 (RAM) fusion transgene to neurons. RAM cassettes were packaged in AAV vectors serotyped with AAV1 coat proteins (titers: 2.18×10^{13} genome copies/mL) by Virovek (Cambridge, MA). hSYN-eYFP (eYFP) cassettes packaged in AAV vectors serotyped with AAV1 coat proteins were (titers: $3.86\text{x}10^{12}$ genome copies/mL) by University of Pennsylvania (Cambridge, MA). Prior to injection,

RAM was mixed with eYFP (8.5 ul RAM/1.5 ul eYFP). eYFP is expressed in the absence of neuronal activation, and serves as method of determining injection accuracy following completion of the experiment. This ensures that in the absence of RAM expression, injection verification can be achieved.

Viral vector delivery: Rats were placed on doxycycline chow (DOX) (200 mg/kg, BioServ, Flemington, NJ) 24 h prior to surgery as previously described (Sørensen et al., 2016). On the day of surgery, rats were anesthetized and a single unilateral injection of RAM was directed to the PL (AP: +2.5 relative to bregma, DV: -2.0 relative to pial surface, ML: ±1.0 mm) using injection parameters similar to those described above. 1000 nl of RAM was delivered at a rate of 100 nl/min. Following completion, the injection needle was left in place for an additional 10 minutes to allow for diffusion of virus, after which the needle was withdrawn. Post-operative care was performed as described above.

Labeling of neuronal ensembles at the time of ketamine injection and later stress:

Following injection of RAM, rats remained on DOX chow (200 mg/kg) for 96 hours. This period of time is sufficient to prevent basal induction of RAM in the absence of a salient event, such as drug injection. DOX chow was withdrawn and replaced with standard lab chow 96 hours later. Removing DOX chow less than 96 hours prevents RAM expression due to circulating DOX-mediated transgene suppression (data not shown). Rats remained undisturbed in the colony for 96 hours following the withdrawal of DOX. Following the 96 hour DOX- interval, rats randomly assigned to drug and stress treatment were injected with either low-dose ketamine (10 mg/kg, i.p.), high dose ketamine (40 mg/kg, i.p.), or saline. 24 h later, rats were placed back on DOX chow (1000 mg/kg). This time interval allows for robust expression of RAM, while minimizing nonspecific RAM expression. 48 h later, rats received IS or HC and were perfused 2 h later.

Tissue preparation: Rats were deeply anesthetized and perfused as described above. Coronal brain sections containing PL were obtained at 35 µm. PL tissue used for IHC was placed directly into a 24 well plate. Fluorescent IHC was performed as described above.

Effect of PL-DRN inhibition on the stress buffering effects of ketamine

Virus: AAV2-retro-eSyn-eGF-T2A-iCre-WPRE (Cre) cassettes were packaged in AAV vectors serotyped with AAV coat proteins (titers: genome copies/mL) by Vector Biolabs (Malvern, PA). AAV8-hSyn-DIO-hM4Di(G_i)-mCherry (hM4Di) cassettes were packaged in AAV vectors serotyped with AAV8 coat proteins (titers: $4.3x10^{12}$ genome copies/mL) by Addgene (Cambridge, MA). AAV-hSyn-DIO-mCherry (mCherry) cassettes were packaged in AAV vectors serotyped with AAV5 coat proteins were (titers: 2.1×10^{13} genome copies/mL) by Addgene (Cambridge, MA).

hM4Di validation: In order to determine whether CNO delivery is sufficient to prevent activation of PL-DRN neurons, rats targeted with hM4Di to the PL-DRN pathway received a single injection of CNO (3.0 mg/kg, i.p.) or vehicle 30 minutes prior to receiving a single injection of ketamine (10 mg/kg, i.p.). Rats were perfused 90 minutes later. Coronal brain sections containing PL were obtained at 35 µm. PL tissue used for IHC was placed directly into a 24 well plate. Fluorescent IHC was performed and Fos expression was examined in the PL-DRN pathway.

Estrous cycle determination: Vaginal lavage was performed prior to stress treatment. 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 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 samples were used to identify the cycle stage: diestrus I/II (presence of nucleated cells and leucocytes), proestrus (presence of nucleated cells), and estrus (presence of anucleated squamous cells).

PL-DRN silencing: Rats targeted with hM4Di or mCherry to the PL-DRN pathway were randomly assigned to drug and stress treatment. Rats received a single systemic injection of ketamine or vehicle 1-week prior to IS or HC. Rats received a single injection of CNO (3.0 mg/kg, i.p.) 30 minutes prior to IS or HC treatment. Twenty-four hours after the completion of IS or HC, behavior was assessed during JSE. Following completion of behavioral testing, rats were deeply anesthetized and perfused using saline and 4% paraformaldehyde. Coronal sections containing PL and DRN were mounted directly onto slides and accurate viral expression in the PL-DRN pathway was confirmed. Only rats with accurate bilateral expression of hM4Di or mCherry expression were included in the statistical analysis.

Statistics: Data analysis was performed with Prism software (Graphpad, La Jolla, CA). The effect of treatment was analyzed with unpaired t-test (Drug), or one-way (Stress), two-way (Stress and Drug), or three-way (Stress and Drug and Virus) analysis of variance (ANOVA). Main effects and interactions were considered statistically significant if $p < 0.05$. When appropriate, post-hoc analyses and planned comparisons were performed using Tukey's post-hoc method. Values in graphs are represented as mean ± SEM.

RESULTS

Effect of systemic ketamine on JSE and DRN activation

Behavior

Rats received either low dose ketamine (10 mg/kg, i.p.), high-dose ketamine (40 mg/kg, i.p.) or saline (i.p) 7 days prior to IS or HC (n = 9-10/group). Anxiety-like behavior was assessed during JSE 24 hours later. Figure 3.1 shows the total time spent interacting during the 3-minute JSE test. Prior low or high-dose ketamine delivered to HC rats had no effect on JSE, relative to saline injected HC controls. JSE was significantly reduced in rats previously injected with saline or high

dose ketamine that received IS. Importantly, low-dose ketamine delivered 1 week prior completely blocked the effect of IS on JSE. Two-way ANOVA revealed significant main effects of stress ($F_{(1,52)}$=21.13, p<0.0001), drug ($F_{(2,52)}$=12.62, p<0.0001), and a stress by drug interaction ($F_{(2,52)}$=3.97, p=0.025). Tukey's post-hoc method revealed that rats administered saline or high-dose ketamine prior to IS were indistinguishable from each other but differed from all other groups (p's<.05). Rats administered low-dose ketamine and IS differed significantly from rats that received saline and IS or high-dose ketamine and IS, while IS rats previously administered low-dose ketamine were indistinguishable from HC rats that previously received saline, low-dose ketamine, or high-dose ketamine.

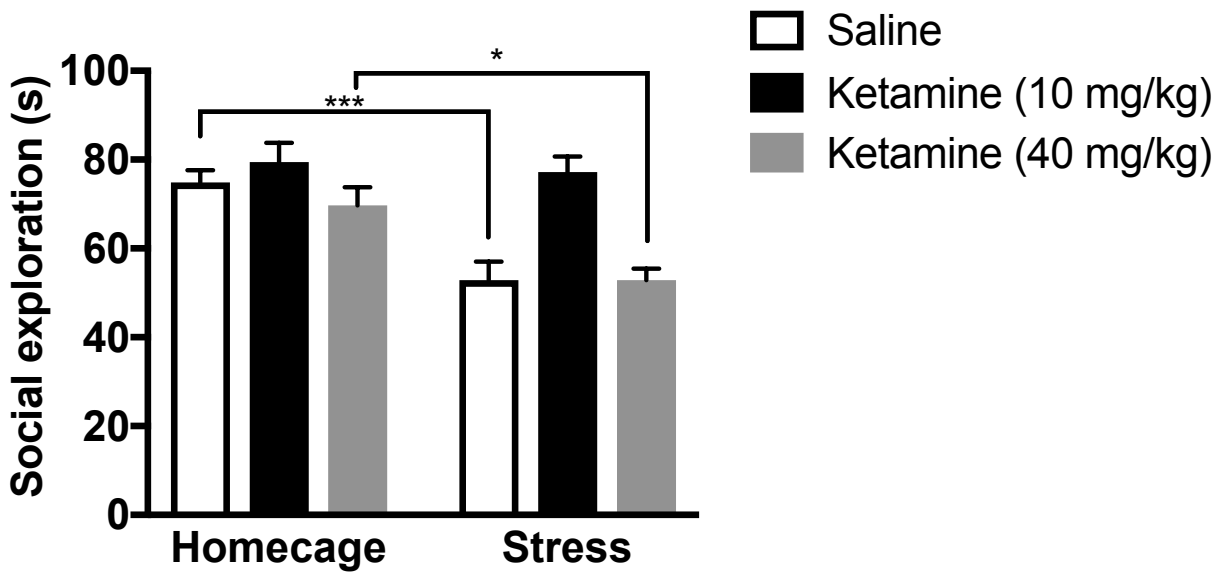

Figure 3.1. Low-dose ketamine protects female rats against later stress-induced juvenile social exploration deficits. (A) Rats received low (10 mg/kg, i.p.) or high (40 mg/kg, i.p.) dose ketamine 1 week prior to stress or HC treatment. Juvenile social exploration was measured 24 h after stress or HC treatment. Values are represented as mean ±SEM. *$p < 0.001$ compared to HC.

DRN 5-HT activation

In order to determine whether ketamine prevents typical IS-induced activation of the DRN, we assessed Fos immunoreactivity (Fos-ir), 5-HT immunoreactivity (5-HT), and the percent of 5-HT cells also expressing Fos (5-HT+Fos) in the rostral, middle, and caudal DRN of rats injected 1-week prior with ketamine or vehicle (Figure 3.2). We focused specifically on these three subregions of the DRN because prior work demonstrates that, in male rats, IS activates the middle and caudal DRN (Grahn et al., 1999). Similarly, the 2-hour time point was chosen based on previous experiments demonstrating stress-induced Fos in the DRN (Grahn et al., 1999). Figure 3.2A shows a representative photomicrograph denoting a 5-HT labeled cell, a Fos labeled nucleus, and a 5-HT labeled cell also expressing Fos. Two-way ANOVA was used to examine whether the total number of 5-HT labeled cells varied between groups within the rostral, middle, or caudal DRN. No differences were observed (p's>0.05) between groups for the rostral, middle, or caudal subregions of the DRN (data not shown). Fos-ir nuclei were examined within each DRN subregion for the different treatment groups (Figure 3.2B). Within the rostral DRN, a two-way ANOVA revealed a main effect of drug ($F_{(1,38)}$=4.29, p=0.046) and a main effect of stress ($F_{(1,38)}$=15.22, p=0.0004). Tukey's post-hoc analysis revealed that IS rats showed increased Fos-ir, relative to rats that received HC (p<.05). Fos-ir was significantly reduced in rats previously administered ketamine that received IS compared to rats administered saline prior to IS (p<.05). Within the middle DRN, stress increased Fos-ir, however this effect was partially prevented by prior ketamine. These observations were confirmed by two-way ANOVA, which revealed main effects of stress ($F_{(1,39)}$=14.94, p=0.0004), drug ($F_{(1,39)}$=4.539, p=0.04), and a stress x drug interaction ($F_{(1,39)}$=4.303, p=0.045). Post-hoc analysis demonstrated that Fos-ir was significantly increased in IS rats, relative to HC rats (p's<.01). Ketamine delivered prior to IS reduced Fos-ir relative to IS rats that received saline (p<.05). The number of Fos-ir cells in the caudal DRN was greater after IS than HC. This was confirmed by two-way ANOVA which revealed a main effects

of stress ($F_{(1,36)}$=9.446, p=0.004). Not surprisingly, IS produced a robust increase in activation of 5-HT cells (5-HT+ Fos) in the rostral, middle and caudal DRN (Figure 3.2B). Within the rostral DRN, a two-way ANOVA revealed main effects of stress ($F_{(1,38)}$=20, p<.0001) and drug ($F_{(1,38)}$=4.255, p=0.046). Compared to HC rats previously administered saline, IS rats that received saline showed enhanced 5-HT activation (p's<.05). 5-HT activation was reduced in IS rats that previously received ketamine compared to IS rats that received saline (p<.05). Within the middle DRN, two-way ANOVA yielded main effects of stress ($F_{(1,39)}$=26.58, p<.0001), drug ($F_{(1,39)}$=5.048, p=0.03), and a stress x drug interaction ($F_{(1,39)}$=5.037, p=0.03). As observed in the rostral DRN, rats previously administered saline or ketamine that received IS showed enhanced DRN activation, compared to rats that received saline or ketamine and HC (p's<.0001). Prior ketamine reduced IS-induced activation of DRN, relative to saline treated rats that received IS (p<.05). Finally, within the caudal DRN, two-way ANOVA revealed a main effect of stress ($F_{(1,36)}$=23.01, p<.0001). Saline treated rats that received IS showed enhanced DRN activation compared to saline and ketamine treated HC rats (p's<.001). In sum, IS activated DRN 5-HT neurons, and this activation was substantially blunted by ketamine administered 7 days earlier.

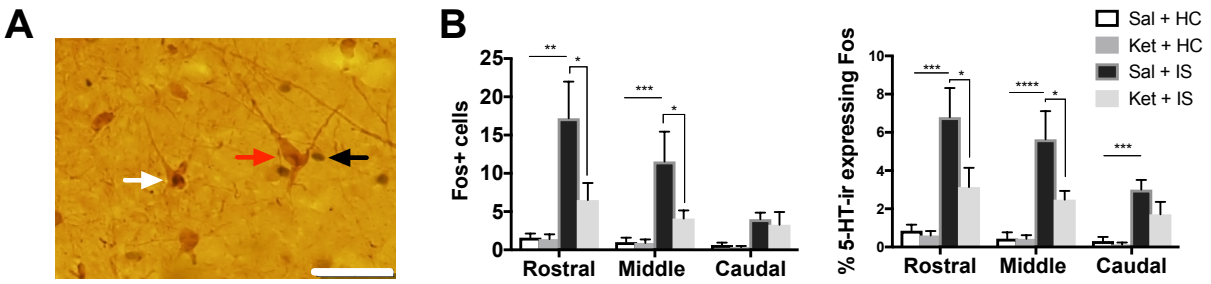

Figure 3.2. Ketamine blunts stress-induced DRN activation. (A) Representative photomicrograph showing a 5-HT immunoreactive cell (red arrow), Fos-positive cells (black arrow), and a double-labeled cell expressing 5-HT and Fos (white arrow). (B) Total number of Fos+ cells (left) and percentage of 5-HT cells also expressing Fos within the rostral, middle, and caudal subregions of the DRN. *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001. Bars represent group means ±SEM.

Effect of ketamine on the activity of PL at the time of stress

Previous work demonstrates a role for deep layer (layer V/VI) PL neurons in the stress-buffering effects of both ketamine and behavioral control (Amat et al., 2016; Amat et al., 2005; Varela et al., 2012). Therefore, we sought to determine whether prior ketamine might induce activation of neural ensembles within the PL, so that a later experience with IS now would activate the PL, in a manner consistent with behavioral control in males (Baratta et al., 2009). Such findings would suggest that the protective effects of ketamine are mediated, at least in part, by enduring changes in a neural node commonly studied with regard to inhibitory control over the DRN (Amat et al., 2005; Baratta et al., 2009). Figure 3.3A shows a schematic diagram of the experimental timeline. First, we sought to determine whether a single injection of ketamine (Figure 3.3B) would induce RAM (RAM+) expression, indicative of PL activation (Figure 3.3C and 3.3D). To optimize activity-dependent RAM expression, we assessed two different time intervals between the removal of DOX and injection of ketamine. Using this approach, we found that rats should be off DOX (DOX-) for 96 hours prior to ketamine injection in order to observe RAM labeling. Worth noting, rats taken off DOX 48 hours prior to ketamine showed no change in RAM, relative to saline treated rats ($p=0.65$), suggesting that DOX was still present 48 h after DOX removal. High-dose ketamine, but not low-dose ketamine or saline, induced robust expression of RAM+ in the PL (main effect of drug, $F_{(2,31)=}10.71$, $p=0.0003$). This was not altered by later IS, as would be expected since the animals were placed back on DOX 24 h after receiving an injection of ketamine. Post-hoc analysis revealed that rats previously injected with high-dose ketamine who later received HC showed enhanced RAM+ expression relative to saline injected rats that later received HC ($p = 0.039$). Similarly, rats previously injected with high-dose ketamine who later received IS showed enhanced RAM+ expression relative to saline injected rats that later received ID ($p = 0.023$). Next, total Fos was examined following IS (Figure 3.3C and 3.3D). As previously described (Baratta et al., 2009), IS increased Fos expression in the PL. ANOVA revealed main effects of stress ($F_{(1,31)}=43.69$, $p<.0001$). Rats previously injected with saline that received later IS showed

enhanced Fos expression in the PL, relative to rats previously injected with saline that received HC (p=0.002). Similarly, rats previously injected with low-dose ketamine that received later IS showed enhanced Fos expression, relative to rats injected with low-dose ketamine that received HC (p's = 0.0004). Finally, the percent of RAM+ cells also expressing Fos was examined to determine whether ketamine-induced experiential ensembles are recruited at the time of later IS (Figure 3.3C and 3.3D). ANOVA yielded main effects of stress ($F_{(1,31)}$=8.55, p=0.0064), drug ($F_{(2,31)}$=16.31, p<.0001), and a stress x drug interaction ($F_{(2,31)}$=5.031, p=0.013). Strikingly, IS produced a profound increase in Fos expression in RAM+ PL neurons previously activated by low-dose, but not high-dose ketamine, compared to rats that received HC and prior saline(p<0.0001) and IS and prior high dose ketamine (p<0.001).

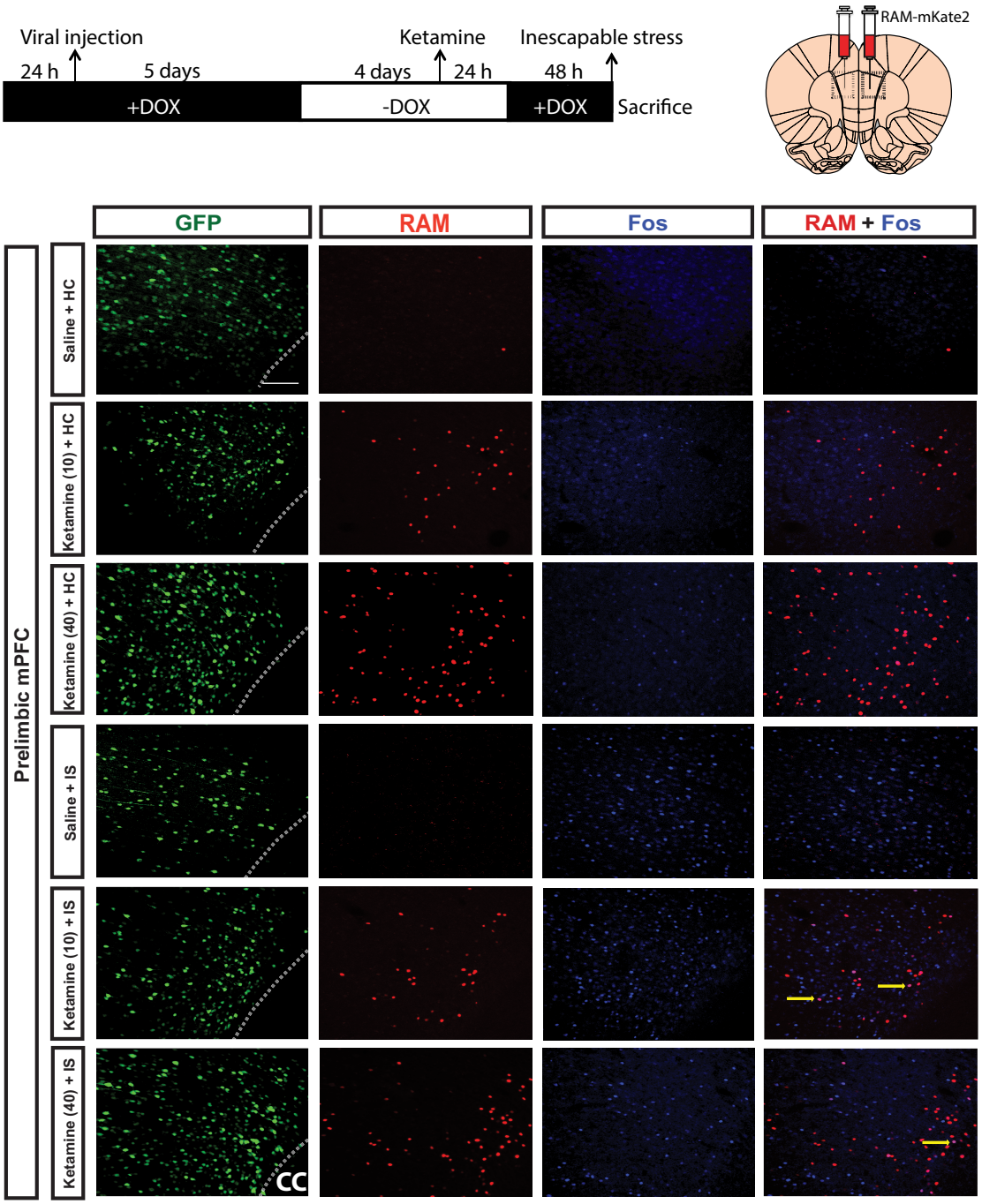
Viral injection
24 h
5 days
+DOX
Ketamine
4 days
24 h
-DOX
Inescapable stress
48 h
+DOX
Sacrifice
RAM-mKate2
GFP
RAM
Fos
RAM + Fos
Prelimbic mPFC
Saline + HC
Ketamine (10) + HC
Ketamine (40) + HC
Saline + IS
Ketamine (10) + IS
Ketamine (40) + IS
CC
Saline
Ketamine (10 mg/kg)
Ketamine (40 mg/kg)
RAM+
Fos+
% RAM+ expressing Fos
Homecage
Stress

Figure 3.3. Ketamine-induced RAM labeling of a transcriptionally active neural ensemble that is later activated by uncontrollable stress. (A) Schematic timeline of the experimental procedure. Rats were injected with AAV-NLS-RAM-mKate2 (RAM). Nine days later rats received a single systemic injection of low dose ketamine (10 mg/kg, i.p.), high dose ketamine (40 mg/kg, i.p.) or saline. Seventy-two hours later, rats were subjected to IS or left undisturbed in their homecage. (B) Schematic diagram of a coronal section of rat brain demonstrating the location of RAM injections into the prelimbic region (PL). Viral injection verification was confirmed with eYFP and RAM + Fos were quantified in the PL subregion denoted with a dashed rectangle. (C) Representative images of the PL showing eYFP (green), RAM (red), Fos (blue), and RAM cells expressing Fos (denoted with yellow arrows in far right panel). Scale bar = 100 um and applies to all images. (D) Enlarged image of the PL showing RAM (denoted with red arrow), Fos (denoted with white arrow), and a RAM cell expressing Fos (denoted with yellow arrow). Scale bar = 50 um. (E) Number of RAM labeled cells (left), Fos+ cells (middle), and percentage of double-labeled RAM+ cells that also express Fos (right) in the PL of rats that received ketamine or saline followed by later IS or HC treatment. $*p < 0.05$, $**p < 0.01$ $***p < 0.001$. Bars represent group means ±SEM.

Effect of ketamine on activity of the PL-DRN pathway

Previous work from our laboratory has demonstrated that the PL-DRN pathway is critically involved in the stress-buffering effects of behavioral control, which shares several common actions with proactive ketamine. (Amat et al., 2005; Baratta et al., 2009; Amat et al., 2014; Amat et al., 2016). The present experiment sought to establish whether prior ketamine might activate the PL-DRN pathway in a manner similar to that described by Baratta et al., (2009). Figure 3.4A shows a schematic diagram of the injection procedure. Fos expression was examined in retrogradely (RB) labeled PL-DRN neurons (Figure 3.4B). As previously described, RB injections in the DRN yielded expression confined primarily to the deep layers (V/VI) of PL and IL, with some expression in the dorsal anterior cingulate (Gabbot et al., 2005; Goncalves et al., 2009; Baratta et al., 2009; Amat et al., 2016). Worth noting, the total number of RB labeled PL neurons did not differ between groups (p=0.496) (Figure 3.4C). To determine whether ketamine activates the PL, we analyzed the total number of Fos-ir cells in rats that received ketamine or saline (n=8 and 7, respectively) (Figure 3.4C). A single injection of ketamine increased Fos-ir in the PL (t_{13}=4.145, p=0.001). Next, we assessed whether ketamine activates the PL-DRN by quantifying the percentage of DRN-projecting PL neurons expressing Fos (Figure 3.4C). Indeed, ketamine produced a robust increase in activation of the PL-DRN pathway (t_{13}=3.453, p=0.004).

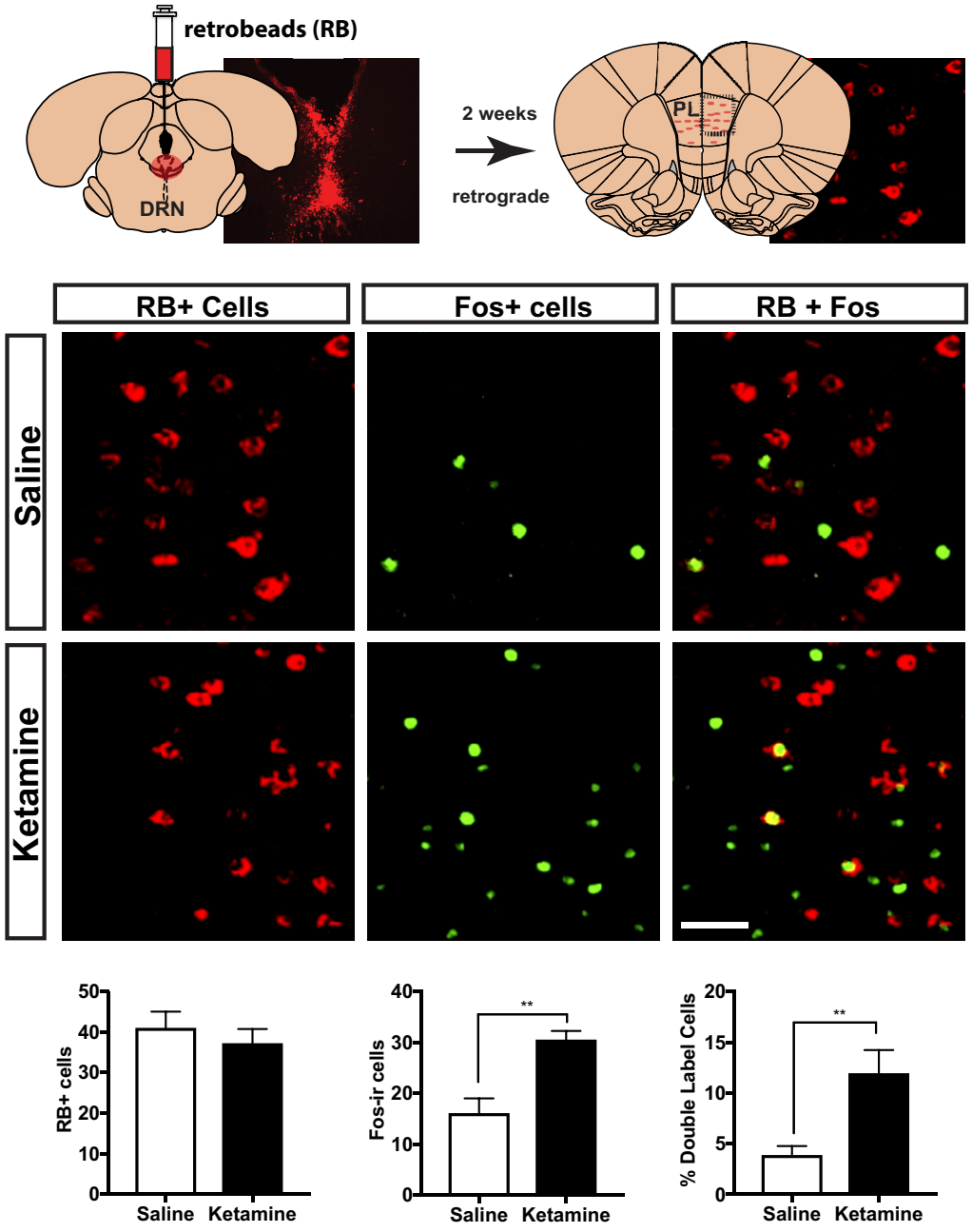

Figure 3.4. Acute ketamine activates the PL-DRN pathway. (A) Schematic diagram of the experimental procedure. Rats were injected with red fluorescent retrobeads (RB) in the DRN. Two weeks later rats received a single systemic injection of ketamine (10 mg/kg, i.p.) or saline. Two hours later rats were sacrificed. RB and Fos expression was assessed in the PL. (B) Representative images of the PL showing RB expression (red), Fos expression (green), and RB + Fos (denoted with white arrows in far right panel). Scale bar = 50 um and applies to all images. (C) Number of RB labeled cells (left), Fos+ cells (middle), and percentage of double-labeled RB labeled cells that also express Fos (right) in the PL of rats that received ketamine or saline. ** $p < 0.01$. Bars represent group means ±SEM.

Effect of DREADD-mediated silencing of the PL-DRN pathway on the stress-buffering effects of ketamine

The fact that after prior ketamine IS now activates a PL-DRN pathway does not imply that activation of this pathway is *necessary* for the protective effects of ketamine. The goal of the present experiment was to assess whether chemogenetic inactivation of the PL-DRN pathway at the time of IS would mitigate the protective effects of prior ketamine. First, the ability of hM4Di to suppress ketamine-mediated PL-DRN pathway activation was validated (Figure 3.5). Figure 3.5A depicts the injection procedure used to target hM4Di to the PL-DRN pathway. Representative images of hM4Di, Fos, and a hM4Di-positive cell also expressing Fos are shown in Figure 3.5B. The total number of mCherry-expressing cells in the PL was quantified (Figure 3.5C). As previously shown, DRN-projecting PL neurons were primarily localized to the deep layers of PL (Gabbot et al., 2005; Goncalves et al., 2009; Baratta et al., 2009). No difference was observed between the two groups (t_8=0.22, p=0.83). In agreement with the results of the PL-DRN retrograde tracing experiment, ketamine significantly increased the number of Fos-positive cells in the PL (Figure 4C). Compared to rats injected with vehicle, CNO significantly reduced ketamine-induced PL Fos expression (t_8=4.337, p=0.003). Next, Fos expression confined to the PL-DRN pathway was examined (Figure 3.5C). Rats injected with CNO showed significantly lower ketamine-induced PL-DRN Fos expression compared to rats injected with saline (t_8=4.051, p=0.004).

In a separate cohort of rats expressing PL-DRN hM4Di or mCherry, ketamine or saline was administered 1 week prior to IS or HC. Thirty minutes prior to IS or HC, all rats were injected with CNO (3.0 mg/kg, i.p.). Therefore, the experiment was a 2 (stress) x 2 (drug) x 2 (virus) factorial design. Twenty-four hours after IS or HC, rats received JSE testing. Figure 3.6 shows the time spent interacting during the JSE test. Three-way ANOVA revealed a main effect of stress ($F_{1,83}$=46.98, p<0.0001), a stress x drug interaction ($F_{1,83}$=8.81, p=0.004), a stress x virus interaction ($F_{1,83}$=16.31, p=0.0001), and a stress x drug x virus interaction ($F_{1,83}$=9.97, p=0.023).

Within the HC groups, neither ketamine nor DREADD-mediated inhibition, or their combination had an effect on social exploration. Consistent with work performed in male (Amat et al., 2016) and female rats (Baratta et al., 2017), IS dramatically reduced social exploration in rats expressing mCherry, relative to mCherry rats that received HC ($p<0.01$). hM4Di had no effect on IS in rats previously injected with saline. That is, rats targeted with hM4Di that saline and IS resembled mCherry-expressing rats that received IS and saline ($p>0.05$). In agreement with the results of Figure 1, prior ketamine, but not saline, administered to rats targeted with PL-DRN mCherry prevented IS-mediated social exploration deficits in rats targeted with PL-DRN mCherry ($p<0.01$). Strikingly, hM4Di-mediated inhibition of the PL-DRN pathway at the time of IS eliminated the protective effects of prior ketamine. Rats that received this treatment now resembled mCherry rats that received saline prior to IS or hM4Di rats that received saline prior to IS ($p's>0.05$).

Worth noting, estrous phase does not appear to influence the behavioral outcome measures associated with stress, as within each phase (diestrus, proestrus, estrus) there was significant variability in behavior (Figure 3.6).

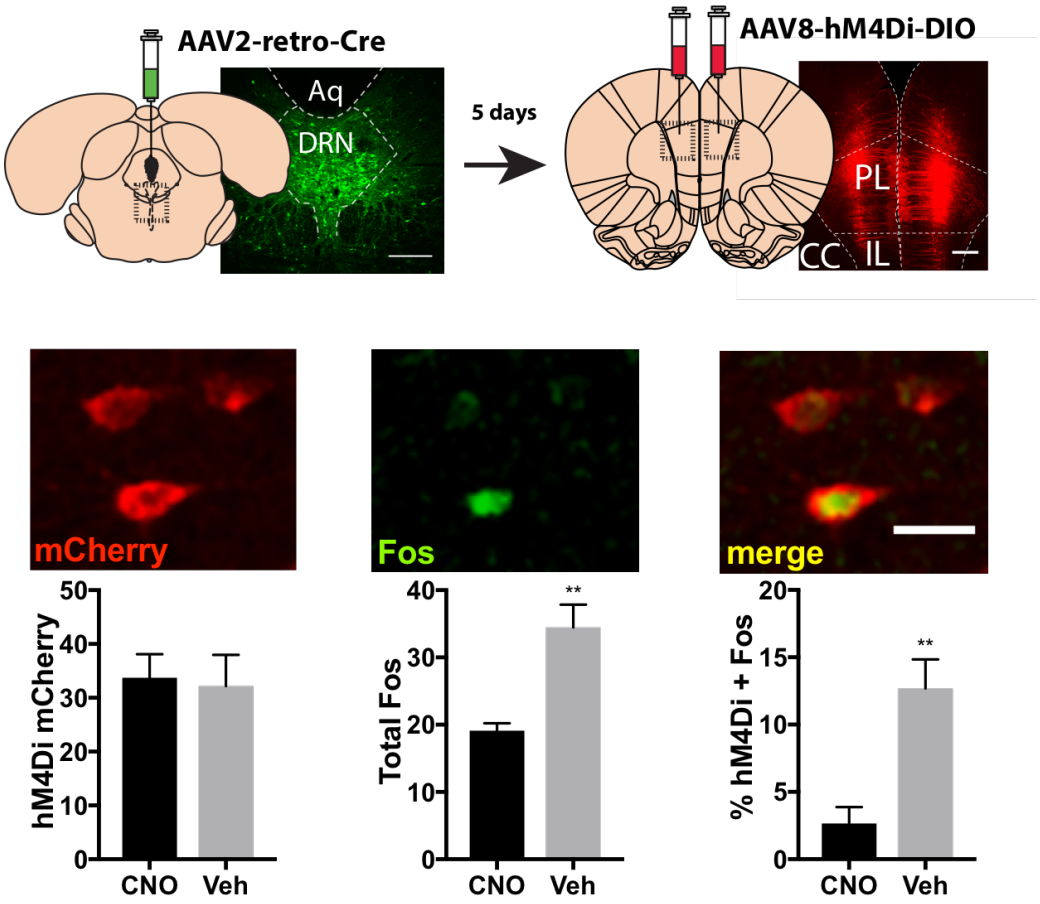

Figure 3.5. hM4Di prevents the effects of prior ketamine on activation of the PL-DRN pathway. (A) Schematic diagram of the injection procedure. Rats were injected with retrogradely transported AAV vectors encoding Cre and eGFP. Five days later, rats were injected with a hM4Di-DIO. (B) Representative images of the PL showing hM4Di-mCherry expression (left), Fos expression (middle), and the colocalization of hM4Di-mCherry and Fos (right). Scale bar = 50 um and applies to all images. (C) Number of hM4Di-mCherry labeled cells (left), Fos+ cells (middle), and percentage of hM4Di-mCherry expressing cells that also express Fos (right) in the PL of rats that received CNO or vehicle. $^{**}p < 0.01$. Bars represent group means ±SEM.

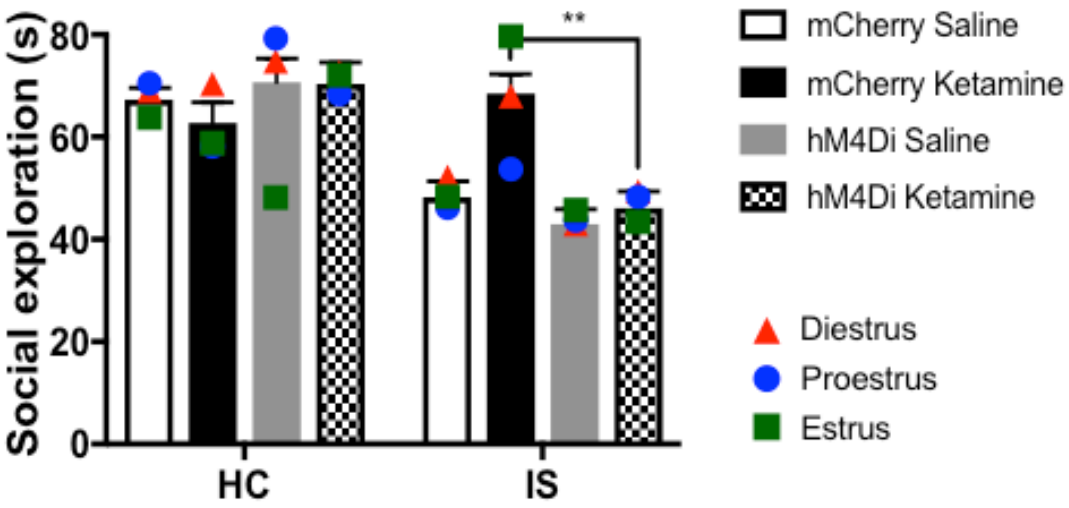

Figure 3.6. hM4Di-mediated inhibition of the PL-DRN pathway prevents the prophylactic effect of prior ketamine on juvenile social exploration. Rats previously injected with hM4Di or mCherry targeted to the PL-DRN pathway received a single injection of saline or ketamine 1 week prior to IS or HC. Juvenile social exploration was assessed 24 hours after IS or HC. Symbols represent mean social exploration for rats in each stage of estrus (diestrus, proestrus, estrus) for each treatment group. $^{**}p < 0.01$. Bars represent group means ±SEM.

DISCUSSION

The present study set out to determine whether the prophylactic effects of ketamine, as well as the neural mechanisms that mediate its effects, are present in female rats in a manner similar to that which has previously been characterized in male rats (Amat et al., 2016). Thus, we assessed various behavioral and neurochemical endpoints that were examined in previous studies conducted using male rats, while expanding on earlier efforts by assessing specific neural ensembles and circuits implicated in the effects of ketamine. The results of the present experiments provide clear evidence that a single injection of low dose, but not high-dose ketamine delivered 1 week prior to uncontrollable stress (IS) blocks the behavioral effects of IS on juvenile social exploration measured 24 hours later. These results are in agreement with studies from our laboratory (Amat et al., 2016) and others (Brachman et al., 2016; McGowan et al., 2017) demonstrating prophylactic effects of ketamine in male rats and mice. However, the present study is the first of its kind to demonstrate ketamine prophylaxis in female rats, and suggests that the prophylactic effects of ketamine on later stress outcomes are generalizable to both sexes. This was especially an issue as behavioral control, which has similarities to the effects of ketamine, is not protective in female rats (Baratta et al., 2018), at least under circumstances identical to those used in males (see below).

A significant body of research, primarily derived in male rodents, indicates that behavioral changes produced by IS, including reduced juvenile social exploration, are mediated by activation of the middle and caudal serotonergic (5-HT) DRN and subsequent release of 5-HT into brain areas such as the basolateral amygdala (BLA) (Takase et al., 2004; Amat et al., 2008; Christianson et al., 2010; Dolzani et al., 2016). This intense activation of the DRN is both necessary (Maier et al., 1993; Maier et al., 1994; Maier et al., 1995) and sufficient (Maier et al., 1995) for behavioral changes associated with IS. Thus, we sought to determine whether ketamine administered prior to IS would mitigate DRN activation typically associated with exposure to IS. Indeed, a single injection of ketamine 1 week prior to IS significantly reduced stress-induced

activation of the rostral and middle DRN. Worth noting, these results are consistent with a recent study by Baratta et al., (2017) that reported DRN 5-HT activation in the rostral and middle DRN of female rats who receive IS. Although not the focus of the present experiments, it is possible that differences in DRN subregion activation between the female and male studies are due to sex-specific differences in DRN anatomy. While females are reported to have higher basal and stress-induced levels of 5-HT (Staiti et al., 2011; Dominguez et al., 2003; Mitsushima et al., 2006), sex differences in DRN anatomy have received little study.

As noted, here we have shown that ketamine blunts both the neurochemical and behavioral consequences of IS when administered 1 week prior to the stressor. To date, no other manipulations have demonstrated an ability to produce these effects in female rats. An extensive body of work has revealed that behavioral control over a stressor prevents the neurochemical and behavioral effects of the stressor in male rats (see Maier et al., 2015 for review). Surprisingly, the protective effects of behavioral control are absent in female rats (Baratta et al., 2018). The stress-buffering effects of behavioral control observed in male rats have been studied in a paradigm in which rats are provided a controlling response (turning a wheel) over termination of tailshocks (escapable tailshock, ES). A separate group of rats receives an identical series of yoked tailshocks, however, they are unable to control any aspect of the tailshock (IS). Behavioral control blunts DRN activation during the controllable stressor and prevents stress-induced behavioral effects typically measured 24 hours later. As previously described, behavioral effects of IS are the result of DRN activation and release of 5-HT into projection regions that are proximal mediators of behaviors associated with the stressor (Grahn et al., 2000; Takase et al., 2004; Amat et al., 2008; Christianson et al., 2010; Dolzani et al., 2016). In male rats, behavioral control activates neurons in the deep layers (V/VI) of the prelimbic (PL) region of the mPFC that preferentially synapse on GABAergic interneurons within the DRN (Hajos et al., 1998; Jankowski et al., 2004; Goncalves et al., 2009). Thus, the net effect of activation of the PL-DRN pathway is reduced DRN 5-HT release and blockade of behavioral effects associated with stressor exposure

(Amat et al., 2005). Additionally, experience with ES prevents DRN activation and the behavioral effects of an exposure IS or another stressor such as social defeat 1 week later (Amat et al., 2006; Amat et al., 2010). Surprisingly, behavioral control fails to activate the PL-DRN pathway in females, and so DRN-mediated behavioral outcomes are not prevented (Baratta et al., 2018). In male rats, the protection afforded by ES against the impact of later uncontrollable stressors is caused by a state change in DRN-projecting PL neurons produced by the initial ES, so that the later experience with IS now activates the PL-DRN pathway (Baratta et al., 2009). Recently, it has also been shown that behavioral control induces plasticity within deep layer (V/VI) PL neurons that are anatomically situated to prevent DRN activation (Varela et al., 2012; Amat et al., 2014; Hajos et al., 1998). Moreover, blockade of plasticity within the PL with protein synthesis inhibitors, glutamate receptor antagonists, and ERK inhibitors prior to ES all prevent ES from protecting against the behavioral effects of later IS (Amat et al., 2006; Amat et al., 2014; Christianson et al., 2014).

Similar to the effects of behavioral control in male rats, a single subanesthetic injection of ketamine enhances synaptic plasticity in PL layer V (Li et al., 2010) and both prevents (Li et al., 2010; Brachman et al., 2016; Amat et al., 2016; McGowan et al., 2017) and reverses (Li et al., 2011) the effects of stressor exposure. Furthermore, ketamine delivered to male rats induces PL Fos activation (Fuchikami et al., 2015; Amat et al., 2016) and alters PL-DRN neurons so that an experience with IS now activates this pathway (Amat et al., 2016). Given the similarities between the effects of behavioral control and ketamine on later outcomes of IS, we sought to determine whether ketamine alters PL activity in a manner whereby a later experience with IS activates the ensemble of PL neurons initially activated by ketamine. Using RAM, we revealed that low dose, but not high dose, ketamine activates ensembles of neurons that are later brought online by IS. This is the strongest evidence to date to suggest that alterations in specific assemblies of PL neurons are involved in the prophylactic effects of ketamine. Others have shown that positive experiences activate neural ensembles in the dentate gyrus and reactivation of these ensembles

is sufficient to reverse depression-like behaviors (Ramirez et al., 2015). Future studies should implement similar methodologies to demonstrate necessity of experiential ensembles in the prophylactic effects of prior ketamine.

Given the effect of ketamine on stress-induced DRN activation and PL activity at the time of drug injection and later IS, we sought to determine whether an acute injection of ketamine activates the PL-DRN pathway and whether activation of this pathway at the time of later IS is necessary for the protective effects of the drug. The results of the retrograde tracing study revealed that an acute injection of ketamine activates the PL-DRN pathway. Using a dual virus intersectional genetic strategy, we found that DREADD-mediated inhibition of the PL-DRN pathway at the time of IS prevents the protective effects of ketamine given 1 week earlier. Others have identified mPFC-hippocampus circuits involved in the acute effects of ketamine given shortly prior to behavioral testing (Carreno et al., 2016), however this is the first study to reveal a precise neural circuit that mediates the prophylactic effects of ketamine in females. It should be noted that although nonspecific neurological and behavioral effects related to CNO metabolism in DREADD experiments have been noted (Gomez et al., 2017), all rats in the present experiment received CNO, and so it is clear that a CNO metabolite is not mediating the observed effects on behavior.

Taken together, the present experiments indicate that ketamine exerts long-lasting prophylactic effects against the deleterious outcomes of stress in female rats. The effects of ketamine persist much longer than the plasma half-life of approximately 2 h in rats (Williams et al., 2004), and so the observed effects cannot be attributed to acute drug effects at the time of behavioral testing. The pattern of the data suggests that the effects of ketamine mirror the long-lasting prophylaxis that occurs in male rats provided with behavioral control. This data suggests that while behavioral control fails to protect female rats, the necessary resilience circuitry does exist and is engaged by ketamine. Therefore, ketamine may prove effective as a therapeutic strategy for female clinical populations likely to experience high levels of stress or trauma.

Chapter 4:

General Discussion

Discussion

A large body of research using male rats reveals that uncontrollable stressors (inescapable tailshock, IS) produce a constellation of neurochemical and behavioral outcomes that are transsituational, persistent, and share similarities with certain aspects of depression and anxiety (Maier and Watkins, 2005). Remarkably, these outcomes are absent in male rats given behavioral control over an aspect of the stressor—the duration of each of the tailshocks (escapable tailshock, ES). Furthermore, exposure to ES produces *resilience* to the outcomes of uncontrollable stressors (IS, social defeat) occurring at a much later time (see Maier and Watkins, 2010 for review). The neural mechanisms underlying the behavioral effects of IS and ES have received significant study. Enhanced serotonergic (5-HT) activity in the dorsal raphe nucleus (DRN) is a critical mediator of outcomes associated with IS. In contrast, activation of a subset of neurons originating in the prelimbic (PL) region of the medial prefrontal cortex (mPFC) that project to the DRN (PL-DRN) and prevent DRN activation (Hajos et al., 1998; Jankowski and Sesack, 2004; Amat et al., 2005) is necessary for the protective effects of ES (Amat et al., 2005).

Interestingly, the NMDA receptor antagonist ketamine, which has powerful antidepressant properties in humans, is also stress-blunting in rodents (Zarate et al., 2006; Krystal et al., 2010; Amat et al., 2016). In male rats, a single subanesthetic injection of ketamine activates the PL, induces top-down inhibitory control over the DRN, and protects against stress outcomes in a manner similar to behavioral control (Fuchikami et al., 2015; Amat et al., 2016). The findings that both behavioral control and ketamine exert similar stress-buffering effects come at a pivotal point in time when increased efforts are being made to identify factors capable of producing resilience in the face of adversity, as opposed to palliative methods initiated after the onset of a pathogenic state.

Strikingly, nearly all of the work dedicated to understanding the neural mechanisms underlying the stress-buffering effects of behavioral control and ketamine has been performed in *male* rodents. This is alarming for a variety of reasons. Of particular importance, females are

afflicted with stress-related disorders at nearly twice the rate of males (Altemus et al., 2006; Shansky et al., 2015). Thus, there has been a recent NIH initiative to increase the study of females in preclinical models designed to identify neural processes involved in human clinical conditions. Indeed, determining whether behavioral control and ketamine are protective in females and identifying the neural mechanisms underlying the behavioral effects of these factors is necessary for developing a more comprehensive understanding of resilience and to shed light on potential drug targets or manipulations that may prevent the onset of human stress-related disorders.

The experiments outlined in this dissertation were designed to recapitulate various aspects of previous experiments carried out in male rodents to determine whether the same stress-buffering factors protect *females*, while also expanding on previous work to provide a detailed understanding of resilience at a *neural circuit* level beyond what is known in males. Chapter 2 revealed that females rapidly learn the operant wheel turn response required to terminate tailshock during ES, yet behavioral control over the stressor fails to mitigate the impact of the stressor on juvenile social exploration (JSE), shock-elicited freezing, and shuttlebox escape performance 24 hours later, as well as coinciding neurochemical changes. Given the results of the behavioral experiments performed in Chapter 2, it is not surprising that both ES and IS activate the DRN to the same degree. Experimental evidence presented here further demonstrates that behavioral control fails to activate a descending inhibitory PL-DRN circuit in females, as it does in males. Thus, stress *per se* drives activation of DRN 5-HT neurons in female rat, resulting in DRN-mediated neurochemical and behavioral outcomes. Pharmacological activation of the PL with picrotoxin rescued female rats from DRN-mediated behavioral changes and produced a behavioral profile that resembled that of non-stressed controls.

In contrast to the results of Chapter 2, the results of experiments performed in Chapter 3 revealed that a single subanesthetic dose of ketamine delivered 1 week prior to IS prevented the impact of the stressor on JSE. Similar to behavioral control in male rats, ketamine reduced stress-induced activation of the DRN. Moreover, ketamine produced a state change in PL neurons so

that a later experience with IS activated the same ensemble of neurons initially activated by ketamine. Finally, ketamine acutely activated the PL-DRN pathway, and activation of this pathway at the time of later IS is critical for protection against behavioral outcomes of the stressor.

The DRN and stress outcomes

The results of the present experiments further support the idea that DRN activation produces a constellation of sequelae that resemble symptoms associated with depression and anxiety (see Maier and Watkin, 2005 for review). In Chapter 2, we observed a variety of DRN-mediated behavioral changes in female rats exposed to ES and IS. These behavioral changes are consistent with previous reports demonstrating a role for stress-induced sensitization of the DRN in behavioral outcomes of stressor exposure (Amat et al., 1998a, Amat et al., 1998b). For instance, studies performed in male rats demonstrate that IS-induced activation of the DRN results in DRN 5-HT_{1A} autoreceptor desensitization, which sensitizes the DRN so that it responds in a hyperactive manner during a later challenge (Rozeske et al., 2011). This leads to exaggerated release of 5-HT in both the DRN and projection regions involved in behavioral outcomes of the stressor, such as the basolateral amygdala (BLA) (Maswood et al., 1998; Amat et al., 1998b; Christianson et al., 2010; Dolzani et al., 2016) Twenty-four hours after IS, various stimuli, including juvenile social exploration, acute morphine, and footshock, result in a sensitized DRN response (Christianson et al., 2010; Dolzani et al., 2016; Bland et al., 2003; Amat et al., 2008). This sensitized response is necessary for the behavioral outcomes of the stressor (Christianson et al., 2010; Dolzani et al., 2016; Bland et al., 2003). Therefore, we hypothesized that DRN activation would occur to a similar degree in females exposed to ES and IS. The effect of ES and IS on DRN 5-HT activation was measured in the rostral, middle, and caudal regions of the DRN using Fos and 5-HT double-label immunohistochemistry (IHC). This experiment was performed using parameters derived from earlier work performed using male rats demonstrating that ES, but not IS, reduces activation of the middle and caudal DRN (Grahn et al., 1999). Indeed, in accordance

with the results of the behavioral experiments, activation of the rostral, middle, and caudal DRN was equivalent in female rats exposed to ES and IS. Interestingly, many of the Fos positive 5-HT cells were localized to the shell of the dorsal raphe dorsal (DRD), which projects strongly to the amygdala and responds to anxiogenic drugs (Lowry et al., 2008). Therefore, it is likely that increased 5-HT release in brain regions such as the BLA mediates the observed behavioral effects (Christianson et al., 2010; Dolzani et al., 2016). Furthermore, the present data suggest, but do not directly demonstrate, that the top-down inhibitory mechanisms in place in male rats that blunt DRN activation during ES are not engaged in female rats.

In contrast to behavioral control, female rats given a single injection of low-dose ketamine 1-week prior to IS were protected against DRN-mediated behavioral deficits. The failure for IS to produce DRN-mediated behavioral changes is consistent with data demonstrating reduced 5-HT release in the DRN and projection regions and a stress-buffered behavioral state in male rats that either receive ketamine or ES up to 2 weeks prior to IS (Amat et al., 2016; Amat et al., 2005). Thus, we examined DRN 5-HT activation using identical IHC parameters as those used in Chapter 2 to determine whether ketamine reduces stress-induced DRN activation. Indeed, ketamine 1-week prior to IS reduced 5-HT activation in the rostral and middle regions of the DRN.

It should be noted that one limitation of the double label IHC experiments performed in Chapter 1 and 2 is the use of Fos as the sole marker for DRN 5-HT activation. Indeed, Fos is an immediate early gene that is used to detect recently activated neurons (Sheng et al., 1990), yet unlike *in vivo* microdialysis where extracellular 5-HT can be directly measured, Fos serves as a proxy for DRN 5-HT activation. Even so, the results of the double-label immunohistochemistry experiments in Chapter 1 and 2 strongly suggest that prior ketamine, but not behavioral control, reduces DRN 5-HT activation at the time of stress.

The results of the double-label immunohistochemistry experiments in Chapter 2 and 3 strongly suggest that prior ketamine, but not behavioral control, reduces DRN 5-HT activation at the time of stress. This indicates that the same “top-down” circuitry that typically prevents DRN

activation in male rats that receive ES is engaged by ketamine, but not behavioral control in females. Furthermore, the results of Chapter 2 and Chapter 3 provide additional evidence that DRN activation at the time of the stress experience is a key player in producing behavioral outcomes associated with depression and anxiety.

The mPFC and stress resilience

The present data are consistent with the work of others demonstrating that the mPFC is critically important for inhibitory control over complex emotional behaviors, rather than simple reflexive responses (Robbins et al., 2000; Miller and Cohen, 2001). Other labs have demonstrated that the mPFC inhibits limbic structures involved in emotions such as fear and anxiety (Quirk et al., 2003; Quirk and Beer, 2006) and appetitive behaviors such as drug use (Nogueira et al., 2006; Peters et al., 2009). Of particular relevance to the present thesis, a growing body of work from our laboratory illustrates that the PL regulates downstream neural circuit elements that are proximal mediators of depression-like and anxiety-like behavior, such as the DRN (Hajos et al., 1998; Maier and Watkins, 2005). The most commonly studied factor capable of inducing top-down inhibitory control over the DRN is behavioral control (turning a wheel to escape tailshock). In the presence of control, the PL is activated, which prevents the excessive release of DRN 5-HT (Amat et al., 2005) and subsequent 5-HT_{1A} autoreceptor desensitization, both during an initial controllable stressor and also during a later experience with IS (Rozeske et al., 2011). Under this arrangement, ES subjects resemble HC subjects at the neurochemical and behavioral level. Interestingly, aversive stimuli during ES drive inputs that activate the DRN (see Dolzani et al., 2016), however, activation of the mPFC, either pharmacologically prior to stressor exposure or via behavioral control, prevents DRN activation, 5-HT_{1A} autoreceptor desensitization, and it is hypothesized that this is the process that also prevents consequent behavioral changes (Amat et al., 2008; Amat et al., 2005; Rozeske et al., 2011).

Much work from our laboratory, and others, suggests that mPFC activity must be linked to some aspect of the stress experience in order to produce resilience to the outcomes of contemporaneous and future stressors. For instance, pharmacological activation of the mPFC with the $GABA_A$ antagonist, picrotoxin, prior to IS prevents typical behavioral outcomes associated with the stressor (Amat et al., 2008). Additional evidence supporting this idea comes from immunization studies in which an initial experience with ES prevents DRN activation and associated behavioral changes at the time of a later experience with IS (Amat et al., 2005; Amat et al., 2006). That is, an initial experience with ES activates an inhibitory PL-DRN circuit and alters the circuit in a manner whereby a later experience with IS now activates the same circuit, which it typically would not do (Baratta et al., 2009). Pharmacological silencing of the mPFC with muscimol prior to ES prevents the protective effects of ES, as well as the immunizing effects of ES on a later experience with IS (Amat et al., 2005; Amat et al., 2006; Christianson et al., 2009). Surprisingly, mPFC activation with picrotoxin in the absence of an aversive stimulus, such as in a homecage setting, fails to protect animals against the outcomes of a later stressor. Together, these data demonstrate that it is not behavioral control *per se* that is the "active ingredient" in protecting against stress outcomes; rather it is activation of the mPFC during aversive stimuli that is required for stress resilience.

Given that activation of the mPFC is necessary for the stress-buffering effects of behavioral control in male rats, and that behavioral control, which fails to activate the mPFC in females also fails to blunt stress outcomes, it is conceivable that a manipulation that activates the PL and produces enduring plasticity within the PL might produce resilience to the outcomes of IS in female rats. Others have shown that subanesthetic doses of ketamine activate the PL (Fuchikami et al., 2015; Amat et al., 2016) and increase the release of mPFC glutamate (Chowdhury et al., 2017), however prior to the experiments outlined in Chapter 3, the effects of an acute low dose of ketamine on persistent PL plasticity in females were not assessed.

Given that acute ketamine produces resistance to the outcomes of IS at a much later time, we sought to determine whether plasticity in the PL might occur following a single injection of ketamine. To address this question, we utilized a novel immediate early gene neural activity-monitoring platform called Robust Activity Marker (RAM) (Sorenson et al., 2016). This technique enabled assessment of PL activity at two distinct time points (immediately after a single injection of ketamine and 72 hours later after IS) Using RAM, we determined that a prophylactic, but not non-prophylactic, dose of ketamine activates an ensemble of PL neurons and alters them in a way whereby a later experience with IS now activates the same group of neurons, which it would not typically do. Although the functional significance of this measure of plasticity was not directly tested, the results of this experiment are striking in regard to their similarity to results of immunization studies performed in males (described in detail above). It should be noted that the present experiment did not examine PL-DRN circuit activity. However, it is possible that a subgroup of the neurons activated by ketamine and later IS are DRN-projecting PL neurons, as the majority of RAM labeled cells were localized to PL Layers V/VI that project to the DRN (Jankowski et al., 2004).

To follow up on this possibility, we examined whether acute ketamine would activate a PL-DRN circuit in females. Indeed, a single prophylactic dose of ketamine activated DRN-projecting PL neurons. Thus, at a *behavioral* and *neural circuit* level, there are similarities between the effects of ketamine administered to female rats and behavioral control in male rats. In males, the protective effects of ES are not context specific and are “transsituational”, as they occur in settings very different from that of the initial ES. Similar to the results of immunization experiments performed in male rats, female rats administered ketamine in one context are immunized to the outcomes of a later IS in a separate context (Amat et al., 2006), and alterations in PL and PL-DRN activity appear to play a role in this process.

Worth noting, the results of the RAM and PL-DRN pathway activation experiment do not imply that PL-DRN pathway activation is *necessary* for stress prophylaxis. Therefore, the purpose

of the final experiment was to assess whether reversible chemogenetic silencing of a descending PL-DRN circuit immediately prior to IS would mitigate the protective effects of ketamine given 1 week prior. Using a dual-virus intersectional genetic strategy, PL-DRN neurons were targeted with inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) that enabled neuronal silencing in the presence of clozapine-*n*-oxide (CNO). Strikingly, PL-DRN silencing at the time of IS completely eliminated the protective effects of prior ketamine on later social exploration. Together, these data suggest that ketamine induces neuroplastic changes in the PL-DRN pathway so that a later experience with IS now activates the PL-DRN and prevents DRN-mediated behavioral outcomes, in a manner similar to behavioral control in males (Amat et al., 2005; Amat et al., 2006; Baratta et al., 2007; Christianson et al., 2009; Varela et al., 2012). Whether these changes occur within the same, or different, PL-DRN neurons remains unknown.

Future studies should utilize pathway-specific activity monitoring strategies, such as Cre-dependent RAM constructs that can be targeted to precise neural circuits when injected upstream from a retrogradely transported Cre recombinase virus. This approach will enable assessment of whether ketamine engages the same neural ensembles in the PL-DRN pathway during later IS in a manner consistent with the work performed by Baratta et al., (2009). Additional RAM constructs that produce DREADD receptors upon neural activation should be utilized to selectively manipulate PL or PL-DRN neural ensembles activated by an initial ketamine injection. These experiments will provide evidence for a functional role for activation of PL and PL-DRN neural ensembles at the time of ketamine and later IS. Additionally, morphological changes should be assessed within PL-DRN neurons to determine whether ketamine produces dendritic spine structural alterations that mediate long-term protection against the effects of a later stressor. Finally, behavioral measures in addition to JSE should be assessed to determine whether the protective effects of ketamine generalize to outcome measures with proximal mediators that are distinct from that of JSE. It should be noted that preliminary data suggests that ketamine reduces shock-elicited freezing and inhibition of the PL-DRN at the time of stress eliminates this effect.

This emphasizes the need for more research in this domain is needed to confirm and expand upon these observations.

Unlike acute ketamine, behavioral control fails to activate the PL-DRN pathway in female rats. Although not directly tested, this suggests that ES results in DRN sensitization and subsequent DRN-mediated behavioral changes. This is surprising because females rapidly acquire the wheel turn response and reliably respond during the entire stress session. Thus, learning deficits are not responsible for the lack of protection afforded by behavioral control. In further support of a critical role for PL activation at the time of stress in protecting against stress outcomes (Amat et al., 2008), pharmacological activation of the PL with picrotoxin prior to stress protects female rats from the behavioral outcomes of ES and IS. One limitation of this experiment comes from the use of picrotoxin to activate the PL. Picrotoxin activation of the PL is not specific to DRN-projecting neurons, and so interpreting the effects of this manipulation on PL-DRN circuit activation is confounded by non-specific actions of picrotoxin on other mPFC circuits. Future studies should implement pathway-specific targeting strategies to determine sufficiency of the PL-DRN pathway in protecting against stress outcomes in female rats. Nevertheless, these data indicate a role for PL-mediated top-down inhibitory control over the DRN in producing stress resilience in females.

The lack of protection afforded to female rats that receive ES requires special comment. Two functionally distinct striatal systems are involved in the acquisition and maintenance of instrumental behaviors, such as turning a wheel to terminate aversive tailshock (reviewed in Balleine and O'Doherty, 2010). Although only recently considered in regard to the stressor controllability paradigm, this concept has received significant study in the appetitive learning literature. It has been shown that the PL and DMS are necessary for acquiring information about "act/outcome" contingencies, but not for performing habitual or instinctive responses (reviewed in Balleine and O'Doherty, 2010). Interestingly, we have shown that the PL and DMS are activated to a greater degree by ES, relative to IS, and these brain areas are necessary for the protective

effects of behavioral control in male rats (Baratta et al., 2009; Amat et al., 2014). In contrast to the PL-DMS act/outcome system, the "habit" system, which is comprised of sensorimotor cortex and the dorsolateral striatum (DLS), is insensitive to the contingency between an action and its outcome. That is, actions performed using the habit system lack an associative link between the response and the resulting outcome. Because concepts underlying contingency learning are formally identical to that of behavioral control (see Chapter 1 for detailed explanation), it is plausible that the failure of behavioral control to activate the PL-DMS act/outcome system is the reason for a lack of protection in female rats. A variety of evidence supports this claim. One proposed mechanism through which this may occur is via enhanced dopamine (DA), norepinephrine (NE), or 5-HT release in the mPFC. Both baseline levels and stress-induced levels of DA, NE, and 5-HT are higher in females (Staiti et al., 2011; Mitsushima et al., 2006) and this is sufficient to impair prefrontal cortical function and strengthen the habit system (Arnsten and Goldman-Rakic, 1998; Arnsten, 2000; Dalley et al., 2003; Yin et al., 2005; Shifflett and Balleine, 2011). Thus, we propose that females are not protected by behavioral control because the wheel-turn response is acquired using the habit system rather than the act/outcome system, due to high levels of neurotransmitter release in the mPFC (Arnsten et al., 1998; Arnsten and Plizscka, 2011; Dalley et al., 2003). In support of this possibility, pharmacological activation of the PL with picrotoxin prevented stress-induced reductions in social exploration in female rats, further supporting the idea that the neural circuitry exists to prevent stress outcomes in females, yet it is not engaged by behavioral control. Future studies should assess ES- and IS-induced neurotransmitter levels in the mPFC using microdialysis to determine whether levels are significantly higher than those previously measured in males (Bland et al., 2003; Amat et al., 2005). Additionally, manipulations that block DA, NE, and 5-HT within the mPFC may prevent stress-induced impairments in cortical function and elucidate the neural mechanisms underlying the lack of protection afforded by behavioral control in females (Arnsten and Goldman-Rakic, 1998; Puig et al., 2004; Hajos et al., 2003)

The results of Chapter 2 highlight a novel mechanism that might explain why females are more likely to suffer from stress-related disorders, such as depression, anxiety, and PTSD. Indeed, the mPFC is implicated in human clinical conditions such as anxiety, depression, and PTSD, where hallmark symptoms include impaired emotional regulation and mPFC activity (Song et al., 2016; Meng et al., 2016; Dodhia et al., 2014). Thus, therapeutic interventions designed to enhance activation of the mPFC by reducing neurotransmitters implicated in taking the mPFC offline might prevent disorders whose genesis is characterized by dysregulated activity of the mPFC and 5-HT system.

Given the proposed role of the mPFC and 5-HT system in the etiology of stress-related disorders (Song et al., 2016; Meng et al., 2016; Dodhia et al., 2014; Mahar et al., 2014), it is surprising that very little research has directly assessed *circuit-level* mechanisms underlying protective factors that influence activity of the mPFC and DRN. Indeed, manipulations such as transcranial magnetic stimulation, active coping, and subanesthetic ketamine are known to modulate activity of the human mPFC (Guhn et al., 2014; Estrelis et al., 2017; Collins et al., 2014) and reduce symptoms of depression, anxiety, and PTSD (Berman et al., 2000; Zarate et al., 2006; Glue et al., 2017; Hartley et al., 2014), however, the effects of these manipulations on downstream neural circuit elements is largely unknown. One proposed mechanism through which these manipulations are thought to be protective is via top-down inhibitory control over limbic and brainstem structures that are proximal mediators of the symptoms that characterize these disorders. Prior to the present studies, whether behavioral control or acute ketamine influence specific mPFC circuits in *females* had not been examined. Here we provide the first evidence demonstrating a bidirectional influence of behavioral control and ketamine on a neural circuit classically studied in regard to stress and resilience in male rats. Indeed, identifying sex differences and similarities in stress vulnerability and resilience is important for addressing clinical phenomena whereby females, who are more likely to use active coping strategies, are afflicted with stress-related disorders at nearly twice the rate of males, yet they respond to antidepressant

treatment at a higher rate (Shansky et al., 2015; Quitkin et al., 2002). One attractive hypothesis is that the relevant neural circuitry exists (as evidenced by the results of the ketamine experiments) but is not engaged by the same factors that are protective in males (as demonstrated in the stressor controllability experiments).

Prior to these experiments, it was not known whether behavioral control or ketamine produce stress resilience in female rats. Here we have expanded our understanding of resilience by demonstrating that factors capable of producing resilience to stress outcomes in one sex may not prove efficacious in the opposite sex. This underscores the importance of taking sex differences into account when studying stress resilience and developing strategies to prevent stress-related disorders.

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