THE STRESSED FEMALE BRAIN: DISSOCIATING THE PRELIMBIC AND INFRALIMBIC REGIONS OF THE MEDIAL PREFRONTAL CORTEX IN THE SUPPRESSION OF LEARNING AFTER ACUTE STRESS

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

For the degree of

Doctor of Philosophy

Graduate Program in Psychology

Written under the direction of

Dr. Tracey J. Shors, PhD

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New Brunswick, New Jersey

October 2013

ABSTRACT OF THE DISSERTATION

The Stressed Female Brain: Dissociating the prelimbic and infralimbic regions of the medial prefrontal cortex in the suppression of learning after acute stress

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Women are nearly twice as likely as men to suffer from depression, which may be the result of a greater vulnerability to stress in females (Tolin & Foa, 2006). A profound sex difference in the response to stress is also observed in laboratory animals. Acute stress exposure disrupts associative learning in female rats but enhances learning in male rats (Wood & Shors, 1998). These sex differences in response to stress are mediated by different brain regions. For example, neuronal activity in the medial prefrontal cortex (mPFC) during the stressor is necessary to modify learning in females but not to modify learning in males (Maeng et al., 2010). The medial prefrontal cortex can be divided into different subregions: the prelimbic (PL) and the infralimbic (IL). There are structural and functional differences between the two areas. For instance, the prelimbic cortex projects more heavily to limbic structures such as the basolateral amygdala; in contrast, the infralimbic cortex projects more to sites involved in visceromotor processes (Vertes, 2004). Because the stress effect on learning in females relies on communication between the mPFC and the basolateral amygdala (Maeng et al., 2010), it was hypothesized that neural activity within the PL during the stressor would be necessary in order to

suppress learning, whereas neural activity within the IL would not be necessary. To test this hypothesis, two separate experiments were conducted. In the first experiment, the prelimbic subregion of the mPFC in adult female rats was bilaterally inactivated with $GABA_A$ agonist muscimol. In the second experiment, the infralimbic area of the medial prefrontal cortex was bilaterally inactivated with muscimol. The animals were then exposed to acute inescapable swim stress or left unstressed. One day later, all subjects were trained with classical conditioning of the eyelid response, using a white noise conditioned stimulus paired with an eyelid stimulation unconditioned stimulus. They were trained with 100 paired trials a day for four consecutive days. Interestingly, females without neuronal activity in the PL during the stressor were able to learn well. The percentage of learned responses was significantly different from that expressed by females in which IL activity was suppressed; these females did not learn well after the stressor. Together, these data suggest that stress exposure critically engages the prelimbic, but not infralimbic, subregion of the mPFC to suppress learning in females. Moreover, because the suppressed learning after stress depends on communication between the mPFC and the amygdala, this communication must be via the prelimbic region. Together, these data suggest that neuronal communication between the prelimbic cortex and the amygdala mediates the enhanced vulnerability to stress in females. This circuit may be especially responsive in women who develop depression triggered by stressful life events.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Tracey J. Shors, for her guidance and support during my graduate career. I would also like to thank Drs. George Wagner, Mark West, and Dipak Sarkar for taking the time out of their busy schedules to serve on this committee and appreciate their helpful insight. I would like to express my gratitude to my undergraduate assistants, colleagues, and labmates who have been great friends and were so supportive throughout the years. Last, but certainly not the least, I thank my family for their loving support.

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GENERAL INTRODUCTION

Stress and mental illness: sex differences

Stressful life events can disrupt cognitive processes such as learning and memory. In some humans, these events can induce or exacerbate the symptoms of depression and other stress-related mental illness such as post-traumatic stress disorder (PTSD) and generalized anxiety disorder (Kendler et al., 1995, 1999; Brown, 1998; Kessler, 1997; Lupien & Lepage, 2001; Lupien et al., 2009; McEwen, 2005; Shors, 2004; Yehuda, 2002; O'Donnell et al., 2004). Emotion dysregulation has been implicated in these psychopathologies and is being applied to clinical intervention practices (Mennin et al., 2007; Gross & Munoz, 1995; Nolen-Hoeksema et al., 2008; Campbell-Sills & Barlow, 2007). Dysregulation of negative mood or emotions has also been included in the newly proposed criteria for PTSD in the fifth revision of the American Psychological Association's (APA) Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Specifically, it has been theorized that the inability to downregulate negative emotions can increase the risk for or lead to the development of various stress and anxiety disorders (Nolen-Hoeksema, 1991; Campbell-Sills & Barlow, 2007; Mennin et al., 2005).

Women are especially at risk as they are twice as likely as men to suffer from depression and anxiety disorders (Breslau et al., 1997; Nolen-Hoeksema & Girgus, 1994; Kessler et al., 2005; Foa & Street, 2001; Tolin & Foa, 2006). There is evidence attributing this sex difference to the different strategies used by men and women to regulate their emotions (Nolen-Hoeksema, 2012). The coping strategy in which profound sex differences are most often observed is rumination, the incessant thought or attention to one's negative emotions and the causes and consequences of one's distress (Tamres et al., 2002; Lopez et al., 2009). In a meta-analysis examining more than 2,000 subjects across 10 different studies, self-reports revealed a

significantly greater inclination in women than men to ruminate when in distress (Tamres et al., 2002). The greater use of this rumination strategy by women corresponded to greater levels of depression and anxiety in women than men (Nolen-Hoeksema & Aldao, 2011). Because of its strong link to increased symptoms of depression and anxiety in women and adolescent girls, ruminative coping style is considered as one of the vulnerability factors that can lead to depression in the presence of stress; rumination can facilitate recall of negative or stressful life events that can maintain or worsen depression (Hyde et al., 2008; Hankin & Abramson, 2001; Hankin et al., 2007).

Sex differences observed in the incidence of depression as well as other stress-related mental illness tend to arise during or after puberty and are maintained throughout adulthood until women reach menopause; these trends indicate a time period during which women are most vulnerable to stress (Nolen-Hoeksema & Girgus, 1994; Kessler et al., 1993; Hyde et al., 2008; Sonnenberg et al., 2000; Silberg et al., 1999). This time course is similarly observed in a model of the stress response in female rodents. In this model, rats are trained with classical eyeblink conditioning 24h after acute stress exposure. In the absence of the stressor, animals emit a well-timed eyeblink conditioned response when they learn to associate a neutral white noise conditioned stimulus with an eyelid stimulation unconditioned stimulus. Exposure to a stressor 24h before training induces deficits in conditioned responding in female rats, whereas learning in males is enhanced. These sex differences in the effects of stress on this associative learning task do not appear until after puberty. In pubescent rats, exposure to an acute stressor (stimulations to the tail) enhanced learning in both males and females; however, in adulthood, stress impaired this type of learning in the females, whereas learning was facilitated in males (Hodes & Shors, 2005). Furthermore, the stress-induced impairment in learning in adult female rats was absent in

aged females that were no longer cycling (Hodes & Shors, 2007). These findings in rodents are consistent with what is observed in the clinical population, and thus, experiments using this rodent model may provide insight on what is observed in humans. They both suggest that the activational effects of ovarian hormones may confer a greater sensitivity to stress to females that can make them more susceptible than males to developing depression and stress-related mental disorders. Because of the complexity of the factors involved and their interactions, it is difficult to explain the alarming disparity between the sexes in the incidence of various stress-related psychopathologies. Behavioral and neuroanatomical data in both human and non-human animals suggest that further investigation is needed to examine the higher vulnerability to the negative consequences of stress in women. This may involve identifying the neural circuitry associated with dysregulated emotional responding to stress.

The neurobiological mechanisms involved in stress and emotion regulation have been studied extensively and are implicated in the etiology of depression and stress-related mood disorders. The idea that the brain regions involved in stress physiology, learning, and memory are dysregulated and/or become dysfunctional in response to stress is being increasingly explored in humans and rodents (Figure 1). One such brain region is the hippocampus. It is well established that the hippocampus modulates learning and memory processes in humans and nonhuman animals (for review, see Squire, 1992; Eichenbaum et al., 1992). Like the hippocampus, the medial prefrontal cortex is also critical for memory and learning processes such as working memory and decision-making (Fuster, 1973; Goldman-Rakic, 1996). Acute and chronic stress exposure can alter the structure and function of both the hippocampus and medial prefrontal cortex, usually impairing behaviors mediated by these brain structures (Cook & Wellman, 2004; Arnsten, 2009; McEwen, 2005; Watanabe et al., 1992; Luine et al., 1994; de

Quervain et al., 1998). Another region of interest is the amygdala because it regulates emotional and fear responses and is important for fear learning and memory, which can also be disrupted by stress (LeDoux, 2000; Conrad et al., 1999; McGaugh, 2002; Miranda et al., 2003). Structural and functional alterations due to stress and psychopathology have been reported in these brain regions. Reductions in hippocampal and prefrontal volume and amygdalar hyperactivity have been characterized in patients with depression and PTSD (Bremner et al., 1997; Coffey et al., 1993; Shin et al., 2005; Lebron-Milad et al., 2012; Tang et al., 2012). Interestingly, in both human and nonhuman animals, these brain regions not only subserve cognition and emotional processing, but they are also differentially activated between the sexes during stress and learning (Bangasser & Shors, 2010; Baran et al., 2010; Goldstein et al., 2010; Lebron-Milad et al., 2012).

The medial prefrontal cortex is of particular importance because of its critical involvement in behaviors that are disrupted in the symptomology of the aforementioned mental disorders. For example, the medial prefrontal cortex plays a critical role in behavioral control and flexibility as well as perseveration, which is the lack of behavioral inhibition (Ragozzino et al., 1999; Aron et al., 2004; Thompson-Schill et al., 2002; Squire et al., 2003). These are important processes that help us respond appropriately to certain situations by inhibiting behaviors that are not relevant to the task. It also enables us to adjust or adapt these responses in order to survive in an ever-changing environment.

The mPFC is heavily interconnected with the amygdala, which is known to regulate emotion (Vertes, 2004, 2006; Hoover & Vertes, 2007). Specifically, it has been reported that the mPFC exerts an inhibitory control over the amygdala (LeDoux, 2000; Likhtik et al., 2005; Rosenkranz and Grace, 2001; Sotres-Bayon et al., 2004). Because the mPFC and the processes it regulates are greatly and rapidly impaired by stress (Arnsten, 2009), stress-induced disturbances

in the mPFC can weaken or disrupt its control of the amygdala. Therefore, these data in rodents suggest that it is possible that the medial prefrontal cortex is involved in the ruminative behaviors described in humans and their inability to downregulate negative thoughts and emotions as a result of stressful experience. These behaviors presumably can lead to psychopathology in women especially.

The mPFC may be a major mediator of the sex differences in stress-related mental disorders; it is strongly implicated in human imaging studies in psychiatric illnesses known to differ between the sexes (Drevets, 1999, 2000; Shin & Liberzon, 2010). In one functional magnetic resonance imaging (fMRI) study, it was reported that rumination induces increased activation of the mPFC as well as the amygdala in 14 subjects (both men and women) diagnosed with major depressive disorder compared to 14 healthy controls (Cooney et al., 2010). Although sex differences were not analyzed in this study, rumination, as previously mentioned, is a stress-coping strategy predominantly used by women who are more likely to be depressed. Because rumination is also strongly associated with the development and maintenance of negative mood states, this finding indicates that the mPFC and amygdala may be critical brain structures in women who become depressed, possibly contributing to a difficulty regulating their responses to stress. In parallel, it has been demonstrated that these brain regions and the communication between them are essential for the stress-induced impairment of eyeblink conditioning in female rats. Cooney et al. (2010) also reported greater activation of the dorsolateral prefrontal cortex during rumination in major depressive disorder patients compared to controls. The dorsolateral prefrontal cortex of humans and nonhuman primates has been considered to be homologous in terms of function to the rodent prelimbic subregion of the mPFC (Vertes, 2004, 2006; Hoover & Vertes, 2007; Farovik et al., 2008; Kolb, 1984). Therefore, the prelimbic area of the mPFC may

be mediating the female stress effect on learning. To address this possibility, subregional differences in the mPFC were explored in the present study.

Sex differences in the way that the amygdala and prefrontal cortex communicate with one another may be critical to understanding the substantial differences between males and females in the prevalence of depression and other stress-related disorders such as PTSD. One approach is to target behaviors characteristic of these psychopathologies that are sex-specific and identify the neurobiological mechanisms that mediate them. Then, these mechanisms can be examined in other paradigms in which we find sex-specific effects. In the experiments conducted here, we focus on the medial prefrontal cortex, its subdivisions, and its role in the suppression of learning after a stressful life event, an effect which occurs preferentially in females.

The medial prefrontal cortex and learning

The medial prefrontal cortex is critically involved in numerous systems of learning and performance of higher-order cognitive tasks such as those involving decision-making, attention-shifting, trace eyeblink conditioning, extinction learning, goal-directing, behavioral flexibility, and working memory (Fuster, 1973; Goldman-Rakic, 1996; Eichenbaum et al., 1983; Kronforst-Collins & Disterhoft, 1998; Morgan et al., 1993; Miller & Cohen, 2001; Ongur & Price, 2000; Kolb, 1990; Kesner et al., 1996; Ragozzino et al., 1998; 2002; Bai et al., 2012). For instance, in one study, male rats had to choose one arm in an elevated T-maze to get to either a low food reward (LR arm) of two food pellets or a high food reward (HR arm) of four food pellets (Walton et al., 2002). There were barriers that they had to climb over in order to obtain the rewards in both arms; however, the barrier was higher in the HR arm and thus, the animals needed to work harder in order to reap the greater food reward, which they did. Following

bilateral excitotoxic lesions of the medial prefrontal cortex, these rats changed their choices and entered the LR arm with more frequency, reducing their pre-lesion number of HR arm entries of 317 to 11 post-lesion HR arm entries. These data suggest that loss of function in the medial prefrontal cortex may make an individual less likely to engage in effort-based decision-making (Walton et al., 2002). This response may reflect behavioral flexibility, which is also altered by lesions of the mPFC (de Bruin et al., 1994; Ragozzino et al., 1999; Dias & Aggleton, 2000). Behavioral flexibility is the ability to shift to a different strategy and inhibit the use of a previously learned strategy that is no longer applicable as they adapt to changes in their environment or situation (Kolb, 1990). Excitotoxic mPFC lesions impaired strategy switching or behavioral flexibility in male rats that were trained on a matching-to-place task in the T-maze; they were not able to make correct responses when they had to switch from their natural tendency to use a non-matching-to-place strategy (Dias & Aggleton, 2000). These findings in rodents are consistent with those observed in humans, illustrating the importance of the medial prefrontal cortex in behaviors that are often compromised in individuals suffering from depression and stress-related disorders.

In humans and nonhuman primates, the dorsolateral prefrontal cortex, which may be homologous to the rat mPFC, is important in the performance of tasks that assess behavioral flexibility or perseverative tendencies such as attentional set-shifting (Verin et al., 1993; Robbins et al., 1996; Berman et al., 1995; Dias et al., 1996). For example, patients with damage to the prefrontal cortex perform poorly on the Wisconsin card sorting test (Anderson et al., 1991; Robbins et al., 1996). In this test, the subjects are presented with a set of cards and told to match them without instruction on how to do so. With each pair, they are told whether they are right or wrong. Based on this response, the subjects modify their rules for matching the cards. Therefore,

this test measures their cognitive flexibility in changing strategies to answer correctly. The crucial role of the medial prefrontal cortex in these behaviors suggests a role for this brain area in behaviors observed in depression and PTSD, such as the inability to control recurring and intrusive distressing thoughts or negative emotions, indecisiveness, lack of concentration, and difficulty learning new information (American Psychiatric Association [DSM-IV-TR], 2000; Bremner et al., 1995).

In the learning paradigm used in the present study, classical eyeblink conditioning, the subject is presented with a neutral tone or white noise conditioned stimulus (CS) that is paired with an airpuff or eyelid stimulation unconditioned stimulus (US). The subject naturally emits an eyeblink unconditioned response (UR) when presented with the US, but after many trials of training, learns the relationship between the paired stimuli and blinks in anticipation of the impending airpuff or periorbital stimulation US. This eyeblink response is called the conditioned response (CR) and is used to assess how well the animal has learned the CS-US association. In the trace version of this task, the two stimuli are separated by a stimulus-free time interval (trace interval), thus making it more difficult for the animal to predict the onset of the airpuff or periorbital stimulation; in delay eyeblink conditioning, on the other hand, the two stimuli are not separated by time but overlap and coterminate and is considered much easier to learn.

The medial prefrontal cortex is distinctly necessary for acquisition of the more cognitively demanding trace eyeblink conditioning task but is not necessary to learn delay conditioning. Kronforst-Collins and Disterhoft (1998) report deficits in the acquisition of a conditioned response in trace eyeblink conditioning in female rabbits with lesions by aspirations of the caudal medial prefrontal cortex, which has been suggested to be homologous to the primate dorsolateral prefrontal cortex. These animals expressed significantly fewer conditioned

responses during 10 sessions of training and extinguished much slower during extinction compared to the controls and rostral mPFC-lesioned rabbits. However, the caudal mPFC-lesioned animals were able to quickly acquire the conditioned response within two sessions of delay eyeblink conditioning, indicating the absence of a deficit in the version of the task that requires less effort to learn. Together, these data demonstrate and are supported by other studies that the medial prefrontal cortex is essential in the circuitry for complex associative learning and is not for learning simpler associations as in delay (Weible et al., 2000; Powell et al., 2005; McLaughlin et al., 2002). Similar to this finding in female rabbits, muscimol inactivation of the medial prefrontal cortex in male rats before training each day significantly impaired CR acquisition in trace eyeblink conditioning but not in delay conditioning (Takehara-Nishiuchi et al., 2005). In humans, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies reveal increased activation of the mPFC that was specific to learning during the same classical eyeblink conditioning task (Blaxton et al., 1996; Molchan et al., 1994). These findings delineate a role for the mPFC that is consistent with those observed in effort-based decision-making. Notably, they demonstrate the disruptive effects that mPFC lesions have on performance in tasks that are more difficult to learn, such as trace eyeblink conditioning. In an effort-based decision-making task, animals with lesions to the mPFC use a strategy that takes less effort over another strategy that would require more effort but would result in a greater food reward (Walton et al., 2002). Therefore, activity within the mPFC is necessary for complex and effortful but not simple learning.

The mPFC is involved in not only the acquisition of learning tasks such as trace eyeblink conditioning, but also in other phases of learning and memory, i.e. memory consolidation, recall, extinction, etc. For example, lesions of the medial prefrontal cortex via aspirations in rats

severely impair remote, but not recent, memory retention of a previously learned trace eyeblink conditioning task (Takehara et al., 2003). In this study, the animals were trained until they reached more than 60% adaptive conditioned responding and then were received lesions of the hippocampus, mPFC, or the cerebellum one day, one week, two weeks, and four weeks after reaching learning criterion. Animals with aspirations of the medial prefrontal cortex emitted slightly fewer conditioned responses than sham rats when lesioned one day after acquisition and were significantly more impaired by four weeks from learning. These data suggest that the mPFC is more critically involved in the post-learning retention of a remotely acquired response. This differed from animals with lesions of the hippocampus, which is a brain structure known to be necessary for trace eyeblink conditioning (Weiss et al., 1999; Beylin et al., 2001). Rats with hippocampal lesions one day after acquisition emitted significantly fewer conditioned responses than the control animals that received lesions of the cortex overlying the hippocampus. However, the hippocampal lesion group did not differ from the control group in adaptive conditioned responding at one, two, or four weeks after learning, supporting the idea that the hippocampus is only critical for recently acquired memory retention and not for more remote memories, i.e. long-term trace memory (for review, see Squire, 1992; Kim et al., 1995). Aspirations of the cerebellum impaired both recent and remote memory, significantly reducing conditioned responding regardless of how long after acquisition the lesion was made. This result is not surprising as there is a comprehensive amount of research on the importance of the cerebellum in eyeblink conditioning (for review, see Thompson et al., 1997). Therefore, the mPFC is highly integrated within a network of other brain structures that modulate learning. It can interact with the hippocampus, and its critical role can be temporally specific; the mPFC becomes necessary

for memory retention at a time during which the hippocampus is not in trace eyeblink conditioning (Takehara et al., 2003).

There is also evidence that the mPFC interacts with the amygdala during these learning processes. Although rats without an mPFC were impaired in the recall of the trace eyeblink conditioned response at all post-operative intervals, they were able to relearn the task, increasing the number of emitted conditioned responses across the three sessions of post-lesion training (Takehara et al., 2003). A similar study that supports these findings demonstrated that rabbits that received electrolytic lesions to the mPFC six hours, 24 hours, one week, two weeks, and four weeks after reaching learning criterion did not perform as well as the sham controls with the greatest deficit at one week (Simon et al., 2005). Again, there was evidence of reacquisition across three sessions of post-operative testing, suggesting that there may be a compensatory mechanism, perhaps in other brain structures like the amygdala, that becomes active when the mPFC is not. The findings by Oswald et al. (2009) could provide insight into a potential explanation for learning that occurs in the absence of a functional mPFC. This study demonstrated that the role of the medial prefrontal cortex in trace eyeblink conditioning depends on the type and intensity of unconditioned stimulus used. For instance, rabbits that received lesions of the mPFC did not display the deficits in trace eyeblink conditioning when an eyelid shock US was employed. However, these mPFC-lesioned animals were impaired on the same task when trained with an airpuff US, which is presumably less aversive (Oswald et al., 2006). The differential effects of periorbital shock vs. airpuff US on mPFC lesions in trace eyeblink conditioning have also been observed in other studies (Kronforst-Collins & Disterhoft, 1998; McLaughlin et al., 2002; Powell et al., 1996). Furthermore, Oswald et al. (2009) demonstrated that although a mild eyelid shock US disrupted trace eyeblink conditioning, a higher intensity

and most likely more aversive eyelid shock did not. Together, these results suggest that when the emotional valence of the unconditioned stimulus is high, additional brain structures such as those involved in limbic circuitry may be critically engaged to facilitate learning without input from the mPFC. One such structure may be the amygdala. The amygdala is implicated in learning that has an emotional component (i.e. aversive eyelid stimulation US in the aforementioned studies) and is heavily connected to the mPFC (LeDoux, 2000; Vertes, 2004). Therefore, when the emotional aspect of a learning task is highly aversive, mPFC control of this task seems to shift, and the amygdala may be recruited to maintain learning in the absence of mPFC function. This mPFC-amygdala connection is critical for emotional learning.

Numerous studies have also demonstrated that the medial prefrontal cortex is critical for successful extinction learning (Morgan & LeDoux, 1995; Milad & Quirk, 2002; Mickley et al., 2005; Maroun et al., 2012; Kronforst-Collins & Disterhoft, 1998). In extinction, a conditioned stimulus is no longer paired with the unconditioned stimulus, the animal learns that the previous outcome will not follow the CS and decreases its anticipatory conditioned behavior accordingly. Without an intact mPFC, the subject is unable to make this new association and continues to perform the previously acquired behavior. For example, lesions of the ventral mPFC do not affect the ability to learn the association between a tone and footshock in a fear conditioning task, but they disrupt the extinction of conditioned fear in rats (Morgan et al., 1993). Aspiration lesions of the rostral medial prefrontal cortex in rabbits also displayed an impairment in extinction of trace eyeblink conditioning, unable to suppress the previously learned conditioned response (Weible et al., 2000).

As mentioned previously, these mPFC-mediated cognitive processes are often compromised in individuals diagnosed with depression and other stress-related disorders. Fear

conditioning is similar to symptoms of PTSD in which trauma-related stimuli elicit a fear response and is also associated with impairments in fear extinction (Milad et al., 2006; Rauch et al., 2006). It is evident that the medial prefrontal cortex is not only essential itself, but it is also integrated in various neural networks for the proper functioning and control of many behaviors important to life. This is one reason why it has been challenging to classify and understand its specific functions.

Stress effects on the medial prefrontal cortex and learning

In addition to learning, the medial prefrontal cortex is implicated in stress and emotion regulation, responding to stress both functionally and structurally (Arnsten, 2009; Cerqueira et al., 2007; Cerqueira et al., 2008; Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006; Brown et al., 2005; Luine, 2002). Its role in stress may be in part due to the anatomical connections between the mPFC and limbic and brainstem structures that critically participate in the physiological and behavioral responses to stress and anxiety (Vertes, 2004). Indeed, stress induces pronounced morphological changes such as dendritic remodeling (evidence for structural plasticity) within the medial prefrontal cortex in rats (Liston et al., 2006; Shansky & Morrison, 2009; Brown et al., 2005; Radley et al., 2006). Because most of these projections are in the ventral part of the mPFC, much of the stress literature refers to the mPFC as the ventromedial prefrontal cortex, or vmPFC (prelimbic and infralimbic subregions). I will refer to the prelimbic and infralimbic subregions together as the medial prefrontal cortex in this context.

Dendrites are important sites for synaptic transmission and connectivity between neurons. Alterations in dendritic arborization such as in spine density, branching, and shape can translate to changes in neural activity and function in that area of the brain and thus behaviors mediated

by that region (Mainen & Sejnowksi, 1996). Dendritic remodeling has also been implicated in learning and memory and is sensitive to stress in various brain structures (Yang et al., 2009; Leuner et al., 2004; Leuner & Shors, 2004; Shors et al., 2001; Dalla et al., 2009). 21 days of restraint stress caused a 20% reduction in apical dendritic material and an 11% decrease in apical dendritic branching in the medial prefrontal cortex of rats; this stress-induced change in dendritic morphology was also accompanied by impairments in attention-shifting (Liston et al., 2006). Briefly, in the mPFC-dependent attention set-shifting task, rats were trained to dig in bowls for food rewards and perform a series of discriminations (pairings of different odors, mediums in the bowls, and the textures covering the bowls) to find which bowls were baited and not baited with the food reward. Therefore, this task assessed how well the animal could shift its attention between different dimensions of the stimuli in order to be able to discriminate them. Exposure to chronic restraint stress disrupted this ability in rats, an effect that also occurred after lesions of the mPFC (Birrell & Brown, 2000). Reductions in mPFC volume, possibly from dendritic atrophy, after chronic uncontrollable stress (daily exposure to a random variety of stressors) in rats were accompanied with deficits in working memory as well as in behavioral flexibility, which was assessed in a reversal learning task (Cerqueira et al., 2007). Chronic stress exposure also enhances perseverative behaviors, which is consistent with these findings of impairment in behavioral flexibility (McLaughlin et al., 2009; Baran et al., 2010). In essence, the structural changes induced by stress in the medial prefrontal cortex impair mPFC-dependent learning, similar to the behavioral effects produced by damage to this brain region. This is an interesting connection that has important clinical relevance as alterations in mPFC structure have been correlated with learning and memory deficits in patients with stress-related disorders.

As noted, the response to stress is a major risk factor for depression, PTSD, and other anxiety disorders (Brown & Harris, 1989; Kessler, 1997; McEwen, 1998; Sapolsky, 1996; Charney & Manji, 2004; Mazure & Maciejewski, 2003). Stress-related mental illness is also associated with reductions in mPFC volume and functional activity in humans (Bremner et al., 1999; Drevets, 2000; Rajkowska, 2000; Shin et al., 2005; Tang et al., 2012; Kroes et al., 2011). Individuals suffering from these psychiatric disorders also often report difficulties in attention, learning, and memory (Bremner et al., 1993; Uddo et al., 1993). Differences in activity in the medial prefrontal cortex were observed during presentations of traumatic images and sounds to Vietnam combat veterans with PTSD; results of positron emission tomography revealed that those with PTSD had decreased blood flow or neural activity in this area, whereas those subjects without PTSD did not (Bremner et al., 1999). Similarly, the most significant changes in regional cerebral blood flow (rCBF) were reported in the medial prefrontal cortex as measured by PET with a reduction in depressed patients with cognitive impairments compared to those that were not cognitively impaired (Dolan et al., 1992). This difference in rCBF in the mPFC was specific to whether or not depression was accompanied by cognitive impairment; the pattern of rCBF in depressed patients compared to normal control subjects illustrated differences centered in the dorsolateral prefrontal cortex (Dolan et al., 1992). This is interesting in that there is differential activity not only in depressed patients with or without impairments in cognition, but these changes also occur in a different brain region than that is typically altered in depressed patients compared to non-depressed individuals. However, cognitive ability in this study was assessed using a battery of tasks that were not necessarily dependent on the mPFC. In a recent study, Hanson et al. (2012) examined a cohort of adolescent boys and girls, finding a correlation between cumulative life stress, decreased PFC volume, and impaired spatial working memory.

Together, there are compelling nonhuman animal and human data that demonstrate that stress-induced structural and functional alterations in the medial prefrontal cortex can influence behaviors mediated by this brain region, suggesting a role for the mPFC in vulnerability to stress-related disorders.

Sex differences in the medial prefrontal cortex in stress and learning

Sex differences have been demonstrated to exist in various types of learning and behaviors, and interestingly in those dependent on the medial prefrontal cortex such as trace eyeblink conditioning, fear conditioning, and extinction learning (Gupta et al., 2001; Ribeiro et al., 2010; Baran et al., 2010; for review, see Dalla & Shors, 2009). For example, females tend to perform better than males in trace eyeblink conditioning, learning it faster and timing the conditioned eyeblink response better (Wood & Shors, 1998; Wood et al., 2001; Dalla et al., 2009). Conversely, females do not perform as well as males in fear conditioning and extinction learning (Ribeiro et al., 2010; Baran et al., 2009, 2010). For example, male rats trained in a plus maze to avoid an aversive arm (bright light and loud noise) made more entries into the arm when the aversive stimuli were no longer present during extinction; however, female rats decreased aversive arm exploration, indicating that they were not able to extinguish the behavior (Ribeiro et al., 2010).

The sex differences in learning may be due to how the mPFC functions or is engaged during the behavior that may differ between the sexes. In one study, male rats performed better than females during acquisition of a fear conditioning task in which a tone was paired with footshock. During extinction after a one-hour delay, males with and without mPFC lesions and sham females reduced freezing to the tone, whereas female rats with lesions of the mPFC

continued to freeze to the tone and failed to acquire extinction (Baran et al., 2010). This effect persisted even after a 24-hour delay in the mPFC-lesioned females, whereas sham females and mPFC-lesioned male rats decreased freezing to the tone after the initial presentations and were able to reacquire extinction. In this study, the mPFC was necessary for extinction recall in males and for extinction acquisition in females (Baran et al., 2010). Therefore, these data indicate that the medial prefrontal cortex is used differently by males and females in extinction, learning that is dependent on this brain region. Evidence in humans also suggests that men and women engage different brain areas to perform the same task but also may be using the same regions differently. A functional MRI study revealed that women and men activate different regions of the prefrontal cortex despite similar performance on a difficult auditory and verbal working memory task (Goldstein et al., 2005). There were also changes in neural activity in both sexes within the dorsolateral prefrontal cortex and interestingly, differed in the magnitude of signal intensity changes with greater activation in women than men during performance of the task (Goldstein et al., 2005). These data corroborate the idea that males and females use different neural areas to execute the same behaviors, but also may be differentially engaging the same regions to do so as demonstrated in the medial prefrontal cortex.

Stress can also affect learning differently as a function of sex. In both humans and animals, males and females engage different neural substrates to perform certain tasks in response to stress, and they may underlie the sex differences in the effects of stress on learning (Bangasser & Shors, 2008; Lebron-Milad et al., 2012; Baran et al., 2009; Maeng et al., 2010). For instance, acute inescapable stress facilitates classical eyeblink conditioning (both trace and delay) in male rats, but impairs learning in females (Wood & Shors, 1998; Wood et al., 2001; Bangasser & Shors, 2010; Waddell et al., 2008; Maeng et al., 2010). The hippocampus and

basolateral amygdala are critical in both sexes for the stress-induced enhancement in males and suppression of learning after stress in females (Bangasser & Shors, 2007; Waddell et al., 2008). Activity within the bed nucleus of the stria terminalis during the stressful event is necessary for enhanced associative learning in males but not for the stress-induced impairment in females (Bangasser et al., 2005). On the other hand, the medial prefrontal cortex is critically engaged in the stress effect on learning specifically in females but not males (Figure 2; Maeng et al., 2010). Therefore, there appears to be divergent circuitry between the sexes that is activated during stress to improve learning in males but produce a learning deficit in females. In humans, sex differences in stress response circuitry have been described; greater blood oxygenation level-dependent (BOLD) signal (measure of brain activity) changes in response to negative vs. neutral images in the medial prefrontal cortex as well as other regions in men compared to women (Goldstein et al., 2010). These regions coincide with those found to be sexually dimorphic in the pattern of activation during fear conditioning and extinction (Lebron-Milad et al., 2012). Investigating these sex-specific targets, and the mPFC in females in particular, may provide insight as to why women are more likely to develop a mental illness after a stressful or traumatic experience.

Stress and learning in the female medial prefrontal cortex

Substantially more studies investigating specific brain mechanisms and circuitry involved in mPFC-mediated learning have been conducted in males. This also appears to hold true in research on stressed mPFC function, despite evidence to suggest that the mPFC may function differently between the sexes and may be of particular importance in females. Neural activity within the mPFC, as well as its morphology, is especially sensitive to stress in females, and the

responses to stress in this structure are influenced by fluctuating levels of estrogen (Gerrits et al., 2006; Garrett & Wellman, 2009; ter Horst et al., 2009). For example, chronic stress exposure induces structural alterations in the medial prefrontal cortex that differ between the sexes. The morphology of the mPFC is naturally sexually dimorphic; the layer II-III pyramidal neurons in female rats have smaller apical dendritic arbors than those in males. Restraint stress for three hours a day for seven days decreased apical dendritic arborization in male rats, a reduction that has been similarly observed in other studies (Izquierdo et al., 2006; Cook & Wellman, 2004; Brown et al., 2005). However, chronic restraint stress had the opposite effect in females and increased dendritic arbor and length (Garrett & Wellman, 2009). It was further demonstrated that the stress-induced increase in the number and length of apical dendritic branches in the medial prefrontal cortex is prevented by ovariectomy, and thus, may be dependent on estrogen (Garrett & Wellman, 2009).

The effects of stress on mPFC-mediated behaviors, the performance of which is disrupted by lesions of the medial prefrontal cortex, appears to rely on the presence of female gonadal hormones such as estrogen. Shansky and colleagues (2006) trained male and female rats on an mPFC-dependent delayed alternation task to assess working memory, attention, and behavioral inhibition after acute restraint stress. In cycling rats, they demonstrated that 60-minute restraint stress impairs performance of the task during proestrus and elevated levels of estrogen. This was not observed in males, or females in estrus with low levels of circulating estrogen (Shansky et al., 2006). In our laboratory, we have also demonstrated that exposure to acute stress during diestrus 2, the phase of the estrous cycle in which estrogen levels are low and increasing in transition to proestrus, disrupts performance in an associative learning task (Shors et al., 1998). Furthermore, this effect of stress on learning is dependent on activity within the medial prefrontal cortex in

female rats but not in male rats (Maeng et al., 2010). There is a similar influence of fluctuating ovarian hormone concentration levels that occurs naturally with the menstrual cycle on the response to stress in women (Goldstein et al., 2005, 2010; Milad et al., 2006, 2009; Nielsen et al., 2013). Women in the early follicular menstrual phase (low estrogen and progesterone) did not significantly differ from men in blood oxygenation level-dependent signal changes when presented with negative vs. neutral visual stimuli (Goldstein et al., 2010). However, there were significantly greater BOLD signal changes in men compared to women in the late follicular-midcycle menstrual phase (high estrogen and progesterone) with the greatest differences in the ventral and medial prefrontal cortices. Moreover, hormonal contraceptives have also been reported to alter extinction learning and the neural regions that are involved and overlap with those affected in the aforementioned studies (Graham & Milad, 2012; Merz et al., 2011). Therefore, the mPFC may be a site in which cognitive and emotional processing under the influence of estrogen converge to modulate behavior after stress, specifically in females.

Prelimbic vs. infralimbic cortex: morphological and functional differences

The medial prefrontal cortex is a heterogenous structure with anatomically and functionally distinct subdivisions. One of the challenges of comparing research on the medial prefrontal cortex is the inconsistency in terminology across studies regarding which subregions are included in the region we generally refer to as the medial prefrontal cortex. This may be due in part to the ambiguity of these subdivisional borders and thus difficulty of specifically targeting these areas but also differences across species (Ongur & Price, 2000). For example, de Visser et al. (2011) report that reversible inactivation of the mPFC enhanced the expression of anxiety behaviors in the elevated plus maze and impaired performance in a decision-making task in male

rats. However, review of their methods revealed that their target site was mainly within the prelimbic subregion of the medial prefrontal cortex. Other studies describe effects of mPFC lesions that often aimed at the prelimbic area but extended into the infralimbic cortex as well as other surrounding subregions.

In the present study, we focused on what is commonly referred to as the ventromedial prefrontal cortex, especially in stress literature, which is comprised of two main subregions, one positioned dorsally and the other more ventrally. These subregions are identified as the prelimbic and infralimbic areas of the mPFC, respectively (Figure 4). Though more commonly studied in conjunction, many studies have demonstrated that the roles for each subregion are quite different (Vertes, 2004; Heidbreder & Groenewegen, 2003; Cerqueira et al., 2008). For instance, the infralimbic area has been noted to be more involved in visceral or autonomic functioning, whereas the prelimbic area has been more closely associated with limbic and cognitive functioning (Vertes, 2004).

The functional differences between the two subregions are likely due to differences in the distribution of their projections throughout the brain. The major projections of the prelimbic cortex include those to the basolateral amygdala, nucleus accumbens, paraventricular, mediodorsal, and reuniens nuclei of the thalamus, and those to the dorsal and median raphe brainstem nuclei (Sesack et al., 1989; Vertes, 2004). These regions are involved in emotion and stress regulation and reward. In contrast, the infralimbic subdivision of the mPFC projects more heavily to the bed nucleus of the stria terminalis (BNST), hypothalamic nuclei, lateral septum, medial and central amygdala, and the nucleus of the solitary tract (NTS) in the brainstem (Hurley et al., 1991; Vertes, 2004). The BNST is a major output of the amygdala and connects with the limbic-hypothalamo-pituitary-adrenal stress system. The central amygdala also serves as an

output for the expression of fear. Furthermore, the NTS is involved in autonomic modulation circuits and visceral function. Therefore, these differing targets of the PL and IL may explain why they function differently and the infralimbic area, and not the prelimbic area, is more involved in visceromotor responses to stressful stimuli.

In addition to these topographical differences, there is evidence indicating that the prelimbic and infralimbic areas of the mPFC differentially modulate performance during learning of various tasks. As noted previously, the mPFC is critical for working memory and behavioral flexibility. mPFC lesions of both the prelimbic and infralimbic regions in male rats significantly impair working memory for visual objects, suggesting a role for both areas in behavioral flexibility (Ragozzino et al., 1999; 2002). However, studies report that lesions of only the prelimbic cortex, keeping the IL intact, can disrupt attention and behavioral flexibility in working memory processes (Granon et al., 1994; Granon & Poucet, 2000). Lidocaine inactivation of the prelimbic subregion impairs performance in delayed response tasks in male rats (Floresco et al., 1997). These animals were trained to enter baited arms in a radial arm maze and then were removed for a delay period of five to 30 minutes until they were returned to the maze and had to remember which arms to enter. In another study reporting a specific effect in only the PL, infusions of the psychostimulant methylphenidate, also known as Ritalin, into the prelimbic cortex but not into the infralimbic area enhance performance in a working memory task (Spencer et al., 2011). Further illustrating the dissociable roles for the PL and IL cortices within the same learning paradigm, electrolytic lesions of the infralimbic cortex, but not the prelimbic cortex, disrupt performance of a passive avoidance task (Jinks & McGregor, 1997).

The infralimbic cortex is critically involved in extinction processes and thus studied more extensively for its role in extinction learning (Milad & Quirk, 2002; Thompson et al., 2010;

Laurent & Westbrook, 2009). Moreover, microstimulation of the prelimbic and infralimbic cortices, in which pulses of current were passed through an implanted electrode after a tone, simulated neural activity in this region evoked by the tone and produced different effects on behavior in male rats (Vidal-Gonzalez et al., 2006). Neural activity induced in the prelimbic area increases the expression of conditioned fear (freezing behavior) and impairs extinction learning; however, microstimulation of the IL does the opposite and decreases conditioned fear expression, facilitating fear extinction (Vidal-Gonzalez et al., 2006). These data describe not only different but opposite functions within the same learning paradigms. Consistent with these findings, preventing neuronal activity via muscimol inactivation of the infralimbic subregion attenuates acquisition of extinction learning and memory but has no effect on the expression of fear, whereas inactivation of the prelimbic region disrupts fear expression but not extinction (Sierra-Mercado et al., 2011). Together these data are interesting because they provide evidence of fear modulation by the same brain region that is bidirectional based on subregion-specific neural activation. Coincidentally, there are also sex differences in these types of mPFC-dependent learning as mentioned previously; females do not perform as well as males in fear extinction learning (Dalla & Shors, 2009). Some of these differences between the sexes appear to be mediated by sex hormones, namely estrogen, which at high levels can improve extinction and impair extinction at low levels (Lebron-Milad et al., 2012; Milad et al., 2009, 2010; Goldstein et al., 2010; Chang et al., 2009; Graham & Milad, 2012). Therefore, it is possible that PL and IL activity and function differ during various types of learning and may be doing so via gonadal hormones to produce sex differences in these behaviors. Moreover, circulating estrogen levels can differentially influence how stress affects learning; therefore, fluctuating levels of estrogen

may confer different roles to the prelimbic and infralimbic areas in how stress modifies learning in females.

Stress effects on prelimbic and infralimbic cortices: implications for learning

Stress induces both morphological and functional alterations in the medial prefrontal cortex that differ not only between the sexes but also between the prelimbic and infralimbic subregions of the mPFC. The medial prefrontal cortex is critically involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis and is affected by the subsequent release of glucocorticoids, stress hormones released by the adrenal cortex, in response to emotional stress (Diorio et al., 1993). Concurrently, there are sex differences in stress-induced HPA activity in nonhuman animals and humans (Kudielka & Kirschbaum, 2005; Seeman et al., 2001). In laboratory studies, it has been generally reported that females have greater levels of glucocorticoid or corticosterone release than in males following stress or HPA activation (Handa et al., 1994; Galea et al., 1997; Bland et al., 2005; Shors et al., 1998). Furthermore, the effects of stress and HPA modulation are different in the PL and IL areas of the mPFC.

Not only do the prelimbic and infralimbic subregions of the medial prefrontal cortex have different targets in the stress circuitry (Gabbot et al., 2005; Sesack et al., 1989; Hurley et al., 1991; Vertes, 2004), but possibly as a result, they also have opposite influences on the physiological responses to stress as well as HPA axis activity (Tavares et al., 2009; Herman et al., 2005). For instance, temporary inhibition of local synapses with synaptic blocker $CoCl_2$ in the PL during acute restraint stress exposure further elevated stress-induced increases in heart rate; however, inhibition of neurotransmission by $CoCl_2$ injections into the IL significantly decreased the restraint stress-induced heart rate increases (Tavares et al., 2009). Therefore, tachycardia

(increased heart rate) induced by acute restraint stress is enhanced by neurotransmission during stress in the infralimbic area but is reduced by synaptic activity in the prelimbic area. This supports the idea that the infralimbic cortex modulates autonomic and visceromotor responses to stress, and it has been suggested that these effects may be related to increased parasympathetic activity and/or decreased sympathetic activity (Tavares et al., 2009). Similarly, lesions of the prelimbic area increase the HPA response to stress, whereas lesions of the infralimbic area decrease it, suggesting an inhibitory role of the prelimbic cortex and an excitatory role of the infralimbic cortex in the physiological response to stress and stress-induced HPA activity (Herman et al., 2005). Furthermore, acute activation of the prelimbic cortex with bicuculline, a $GABA_A$ receptor antagonist, reduces the hypothalamic-adrenal-pituitary (HPA) axis response to restraint stress (Jones et al., 2011). Together, these data suggest that the PL inhibits, whereas the IL enhances, physiological responses to psychological stress in rats.

This pattern of subregional effects is oddly inconsistent with what is observed in the behavioral responses to stress. Neural activation of the PL reduces, whereas IL increases, the physiological responses to stress. Thus, it could be predicted that a similar direction of effects (reductions by PL activation and enhancement by IL activation) would be observed in learning behaviors mediated by these regions after stress exposure. On the contrary, numerous fear conditioning and extinction studies demonstrate that the PL enhances conditioned fear expression, whereas the IL exerts more of an inhibitory influence and enhances extinction or inhibition of fear (Vidal-Gonzalez et al., 2006; Sierra-Mercado et al., 2011). This inconsistency may be something that should be considered, although it may just be due to differences in PL and IL function in two distinct systems: one that regulates the autonomic or physiological responses to stress and another separate system that modulates fear learning. One study that suggests this

reports that lesions centered on the prelimbic mPFC in rabbits impairs trace eyeblink conditioning but does not affect the accompanying conditioned bradycardia (decelerated heart rate conditioned responses) that is typically observed during eyeblink conditioning (Powell et al., 2001). This finding suggests that prelimbic function during eyeblink conditioning may be independent from its function in autonomic associative learning. Because prelimbic activity also reduces HPA responses to stress, it may be possible that glucocorticoids may actually play a smaller part than commonly thought in the modulation of learning by stress.

As is evident in the previously reviewed data, most research investigating the effects of stress on mPFC-mediated learning have examined the medial prefrontal cortex without distinguishing between the PL and IL subregions (Maroun, 2006; Amat et al., 2005; Baratta et al., 2009; Maeng et al., 2010). This is also the case for studies researching the role of the mPFC in anxiety and fear, the responses of which are affected by stress (Blanco et al., 2009; Lebron-Milad et al., 2012; Shin & Liberzon, 2010). For example, electrolytic lesions of the medial prefrontal cortex (both the PL and IL) in male rats that were subjected to 3h of restraint stress and assessed in an elevated T maze 24h later attenuated passive avoidance and escape behaviors that are typically observed after inescapable stress exposure (Blanco et al., 2009). These data suggest that the mPFC is critically involved in enhanced expression of fear and anxiety behaviors following exposure to uncontrollable stress, an effect of stress that has been observed across other fear learning paradigms (Maier, 1990).

Interestingly, activity within the mPFC is necessary to process the controllability of a stressor, which can alter how stress influences subsequent learning (Amat et al., 2005; Baratta et al., 2008). Stressor controllability is the ability to control the onset and/or termination of a stressor. Uncontrollable stress disrupts subsequent fear conditioning and escape learning in male

rats, whereas escapable, or controllable, stress does not (Maier & Watkins, 2010; Maier et al., 2006). In this paradigm, male rats that had control over the stressor and could terminate the duration of a tail shock by wheel-turning were yoked to animals that did not have control but endured the same durations of tail shocks as their partners. Animals with mPFC inactivation of both the prelimbic and infralimbic areas responded to escapable stress as if it were uncontrollable, performing poorly on an escape learning task and displaying an exaggerated fear response (Amat et al., 2005). Similarly, inescapable stress was treated as controllable stress by animals with mPFC activation, which prevented the deficits in learning induced by uncontrollable stress (Amat et al., 2008). Stressor controllability also influences the effects of stress on classical trace eyeblink conditioning, which have been demonstrated to differ between the sexes (Leuner et al., 2004). Acute inescapable stress exposure, either tail shock or swim stress, enhances eyeblink conditioned responding 24h later in males, whereas the same stressor decreases the percentage of learned responses emitted by female rats. In this study, yoked pairs of animals were placed into shuttle boxes, and when one animal received a foot shock and was able to escape to the other side, the yoked rat also received a shock but was not able to escape it. Having control over the stressor prevented both the stress-induced facilitation of associative learning in male rats and impairment in learning after stress in female rats. The neural mechanisms involved were not examined in this study, although based on the findings in fear conditioning, the mPFC may be involved. Similarly in humans, controllability over the presentation of snake videos in women who were snake phobic induced greater activation of the ventromedial mPFC during anticipation of snake images compared to when they had no control over the presentation of the snake videos (Kerr et al., 2012). However, as an increasing number of studies are revealing distinct, and sometimes opposite, roles for the prelimbic and infralimbic areas of the mPFC, it is important to

consider their contributions separately in mediating phenomenon like the ability to detect controllability of a stressor as it influences how stress affects learning. This is especially worth exploration in females because being able to control the stressor can prevent the stress-induced deficits in associative learning.

Prelimbic and infralimbic distinctions have been made in processes of stress, anxiety, and fear learning in males, but these data are not always consistent and are lacking in females. PL inactivation does not appear to affect conditioned fear acquisition but reduces the expression of fear in male rats (Corcoran & Quirk, 2007). Stern and colleagues (2010) demonstrated that inactivation of the prelimbic area increases open-arm entries in the elevated plus maze indicating a decrease in anxiety. This conflicts with other findings that report that lesions of the PL increase the expression of fear and anxiety in male rats (Jinks & McGregor, 1997; Morgan & LeDoux, 1995). The inconsistency in identifying the role of the PL in the modulation of fear and anxiety may be due to differences in the span and placement of lesions vs. inactivation, as well as behavioral measures. As noted previously, the infralimbic area plays a critical role in extinction and has been important to study for its role in stress influences on extinction learning (Izquierdo et al., 2006; Farrell et al., 2010; Sotres-Bayon & Quirk, 2010; Akirav & Maroun, 2007). For example, the IL is more sensitive to acute inescapable swim stress, which induces dendritic retraction in this region, but has no effect in the PL; this morphological alteration due to stress may underlie the impairment in extinction (IL-mediated) learning after the stressor (Izquierdo et al., 2006). This study was conducted in only male rats, and thus, it is uncertain whether this effect would be observed in females, or whether sex differences exist within these cortical subregions in response to stress.

Prelimbic vs. infralimbic cortices and stress in females

Although the high incidence rates of stress-induced mental illness in women persist, the majority of studies conducted to examine this phenomenon have been focused on males and thus more research is needed in females. It is difficult to conclude distinct roles for the prelimbic and infralimbic cortices in mPFC-mediated effects of stress on learning from the findings in males, which also makes it challenging to understand their functions in females. In the limited studies, most do not investigate the effects of their manipulations in both the PL and IL regions but report effects in only one or the other, making direct comparisons difficult. One study that did focus on females found that mPFC lesions that were more damaging to the prelimbic area prevented stress-induced open field behaviors after chronic footshock stress (Gerrits et al., 2003). This result suggests that in female rats, activity in the prelimbic cortex is necessary for the effects of stress on behavior. However, these females were not matched for the phase of the estrous cycle; therefore, the possible effects of ovarian hormones were not accounted for. This is important to include in the analysis because animals respond differently to stress as their hormone levels fluctuate.

A greater sensitivity to stress on learning in female rats during low levels of estrogen and progesterone has been reported (Shors et al., 1998). For instance, in ovariectomized female rats treated with estrogen, exposure to chronic restraint stress (two hours/day for 10 days) increased dendritic branching and spine density, which may be associated with increased connectivity or function, only in neurons in the IL of the mPFC that projected to the basolateral amygdala (Shansky & Morrison, 2010). On the other hand, there were no reported morphological alterations in these BLA-projecting infralimbic neurons in ovariectomized females without estrogen replacement. In males, there was stress-induced dendritic retraction in IL neurons that

did not project to the BLA, whereas those that did project to the BLA were unaltered. These data indicate that exposure to stress affects the IL-BLA pathway differently between the sexes, and it may mediated by estrogen.

Our laboratory has repeatedly observed that acute uncontrollable stress suppresses eyeblink conditioning in cycling adult virgin female rats, whereas males learn this task better after exposure to stress (Wood & Shors, 1998; Wood et al., 2001; Waddell et al., 2008; Bangasser & Shors, 2007; Maeng et al., 2010; Maeng & Shors, 2012). This profound stress effect on learning is dependent on neural activity within the medial prefrontal cortex and its communication with the basolateral amygdala in females but not males (Maeng et al., 2010). Muscimol inactivation of the mPFC (both PL and IL) prevented the decremented conditioned responding in stressed female rats. However, activation of the mPFC via bilateral infusions of GABA antagonist picrotoxin alone without stressor exposure was not sufficient to induce this stress effect. Contralateral excitotoxic lesions to the mPFC and BLA, which disconnected communication between the two areas, prevented the stress-induced learning suppression in female rats; ipsilateral lesions to these regions kept communication in one hemisphere intact and had no effect on the suppression of learning after stress (Figure 3). Therefore, activation of both the medial prefrontal cortex and the basolateral amygdala and communication between them during stress is a critical circuit mediating the effects of stress on learning specifically in females.

The connection between the medial prefrontal cortex and amygdala has also been implicated in depression, PTSD, and other stress-related disorders (Berkowitz et al., 2007; Bremner et al., 1999; Shin et al., 2005; Milad et al., 2007; Koenigs & Grafman, 2009). As mentioned previously, increased activity in the mPFC and amygdala during rumination, a coping strategy commonly used by depressed women, was reported in 14 men and women diagnosed

with major depressive disorder compared to healthy controls (Cooney et al., 2010). There is also increased functional connectivity between the mPFC and amygdala during extinction recall, which is impaired in people suffering from PTSD (Milad et al., 2007). Some studies describe hypoactivity in the mPFC and hyperactivity in the amygdala in PTSD patients (Liberzon et al., 1999; Rauch et al., 2006; Etkin & Wager, 2007). Hyperactivity in the amygdala is thought to be caused by a loss of inhibitory control by a hypoactive medial prefrontal cortex and can lead to emotion dysregulation. In light of these data, it is evident that the mPFC-BLA circuit is of great significance in stress- and anxiety-related psychopathologies. Thus, sex differences in this circuit should also be explored as it may be integral to understanding why women are more at risk than men to develop stress-induced mental illness.

As evidenced in the literature, it is possible that the prelimbic and infralimbic subregions of the mPFC may be contributing differently to modify learning after a stressful experience in females. This question is addressed in the present study. Differences in projection sites appear to be an important influence in the functional dissociation of the PL and IL in stress and learning. In contrast to the infralimbic cortex, the prelimbic cortex has robust projections to the basolateral amygdala (McDonald et al., 1996; Vertes, 2004). This connection may be especially sensitive to stress in females. As noted previously, it has been demonstrated that only the IL neurons that project to the BLA are structurally altered by stress in the presence of estrogen (Shansky & Morrison, 2010). However, it was not investigated whether PL neurons respond similarly to these IL neurons. Perhaps due to its dense connections to the BLA, there may be effects of stress on neurons in the PL that are also specific to a PL-BLA circuit and may explain the learning deficit in females after stress.

Based on these data and observations, it was hypothesized that there are distinct roles for each subregion in this phenomenon and that the prelimbic cortex-BLA circuit may be specifically mediating the effects of stress on eyeblink conditioning in females. In order to test our hypotheses, two experiments were conducted. In the first experiment, the PL was bilaterally inactivated with muscimol in female rats during inescapable swim stress and trained with four days of Pavlovian eyeblink conditioning 24h later (Figure 5). In the second experiment, the IL was inactivated during the stressor, and training ensued as in the first experiment. It is expected that if the prelimbic cortex is necessary for the suppression of learning after stress, inhibiting PL neuronal activity during the stressful event would prevent the stress effect. On the other hand, if the infralimbic cortex is critically involved, then females that have an inactivated IL during stress would be expected to learn well.

GENERAL METHODS

Subjects

Cycling adult female Sprague Dawley rats (90-120 days of age) were bred and obtained from a breeding facility at Rutgers University. They were housed in groups of 3–4 until surgery. After surgery, rats were singly housed in standard plastic "shoebox" home cages (44.5 cm long, 21.59 cm wide, and 23.32 cm high). All animals were maintained on *ad libitum* access to rat chow and water on a 12 h light and 12 h dark schedule. The current experiments were conducted with full compliance to the rules and regulations specified by the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Surgery

All animals were anesthetized with sodium pentobarbital (50mg/kg) and received trace amounts of isoflurane throughout the surgery to maintain anesthetization for the entire duration of the procedure. After the scalp was shaved and scrubbed with betadine, an incision was made with a scalpel. To implant bilateral cannula into the prelimbic subregion of the medial prefrontal cortex, guide cannulas (Plastics One, Inc.) were placed into sites at the following locations relative to bregma at a 15˚ angle: +2.90mm AP, ±1.60mm ML, and -2.80mm DV from the surface of the brain. For bilateral cannulation of the infralimbic area, the following coordinates were used at a 30˚ angle and relative to bregma: +2.80mm AP, ±3.10mm ML, and -3.80mm from dura. After holes were drilled into the skull at these sites, cannula tips were lowered and allowed to settle for 1 minute. The holes were then covered with bone wax. 4 insulated wires attached to a headstage were implanted through the periorbital muscles of the eyelid to administer stimulation to the eyelid and record electromyographic (EMG) activity. Acrylic dental cement

was applied to the skull and anchored by skull screws to secure the headstage and cannulas in place. To prevent occlusion, obturators were placed into the cannula after implantation.

Vaginal cytology

5-7 days following surgery, phases of the estrous cycle were monitored daily via vaginal smears. Loose vaginal cells were obtained with a cotton-tipped applicator dipped in sterile 0.9% saline that was inserted into the vaginal canal. Samples were placed onto slides and stained with 1% toluidine for estrous phase identification. Dense clusters of non-nucleated blue cornified cells are characteristic of the estrus phase, and dark purple-stained nucleated epithelial cells of the proestrus phase. Diestrus 1 is identified by a combination of leukocytes and few cornified epithelial cells and diestrus 2 by very sparse leukocyte and nucleated epithelial cell types. All animals began experimentation during the diestrus 2 phase of the estrous cycle because it has been reported that the stress effect on learning is most pronounced during this phase (Shors et al., 1998).

Drug microinfusions

Following an acclimation period in the conditioning chambers, the animals were transferred to another room. The obturators were removed, and injectors (with projections 1mm past the guide cannula) were inserted into cannula. All females were bilaterally infused with either 0.5µl artificial cerebrospinal fluid (aCSF) vehicle or 0.5µg (1µg/µl) of γ-aminobutyric acid ($GABA_A$) receptor agonist muscimol. All infusions were administered at a rate of 0.125µl/minute for 4 minutes for a total infusion volume of 0.5µl. After 1-2 minutes to allow diffusion, the obturators were replaced.

Stress procedure

Immediately following the microinfusions of either aCSF or muscimol, animals in the stressed groups were placed into another room (a different context from conditioning and infusions) and were subjected to inescapable swim stress. The animals were placed into a plastic bucket about 12 inches in diameter filled with room temperature water (21-23˚) at a height of 11 inches for 15 minutes. After the stress exposure, the animals were thoroughly dried with a towel before being returned to their respective home cages. Animals that were in the unstressed groups were returned to their home cages after the infusions.

Classical conditioning

All rats were trained with delay eyeblink conditioning 24h following the stress procedure. The animals were run with a total of 400 trials, 100 trials per day for 4 consecutive days. In this delay conditioning paradigm, the female rats were exposed to an 80-dB, 850ms white noise conditioned stimulus (CS) that coterminated with a 100ms, 0.5mA periorbital eyelid stimulation unconditioned stimulus (US). Eyeblinks were measured by significant changes in the magnitude of the electromyographic (EMG) activity recorded from the eyelid muscles. In order to be considered a conditioned eyeblink response (CR), EMG activity had to have a duration of 10 ms and exceed 0.3mV and 3 SD of the baseline activity recorded during a 250ms period before the onset of the CS. The eyeblink conditioned responses that were counted to assess learning in this study also had to be fairly well-timed and occur within 250ms before the onset of the US (Figure 6). Animals with poor EMG signals were excluded from the study.

Statistical analysis

In both of the experiments, the percentage of CRs emitted was analyzed with a 2 x 2 x 4 (muscimol/vehicle aCSF by stress/no stress across four sessions of training) mixed factor

analysis of variance (ANOVA). The first 100 trials of the first session were also averaged in blocks of 20 trials. A 2 x 2 x 5 (muscimol/aCSF vs. stress/no stress across five 20-trial blocks on the first day of training) mixed factor ANOVA was used to analyze the percentage of conditioned responses. These analyses were followed by Newman-Keuls post-hoc comparisons.

Histology

Rats were injected intraperitoneally with a lethal dose of sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.9% saline solution for exsanguination. This was followed by 10% buffered formalin. Prior to brain extraction, obturators were replaced with injectors connected to 10µl Hamilton syringes to infuse 0.5µl Evans blue dye (1mg/ml) to mark the site of infusion. Brains were then dissected out and post-fixed in formalin for at least 24h. The brains were transferred from the 10% buffered formalin to a 30% sucrose-formalin solution for at least 3 d for cryoprotection. When the brains were fully saturated, they were frozen and sectioned into 40-µm-thick coronal sections using a cryostat. Every third slice was mounted onto pre-gelled slides and stained with 0.1% neutral red to verify the accuracy of cannula placements. A rater blind to group assignments in the behavioral data assessed cannula tip locations. If the tip of the injection cannula, which protruded 0.5 mm beyond the guide cannula, was within the dorsal boundary of the prelimbic cortex, then it was considered to be in the correct location prelimbic infusions. For infralimbic infusions, the cannula tip sites needed to be within the infralimbic region, leaving the prelimbic area intact. Placements within the mPFC were between +3.20 and +2.70 mm relative to bregma. The sites of drug infusion were assessed by track markings of the infusion cannula. Rats were excluded from analysis if placements were not within either the prelimbic or infralimbic areas, or if the mPFC was excessively damaged by the cannula or the infusions.

Experiment 1. Neuronal activity in the prelimbic cortex is necessary to suppress learning after stress in females.

Introduction

The role of the medial prefrontal cortex in learning and mnemonic processes has been well characterized (Fuster, 1973; Goldman-Rakic, 1996; Eichenbaum et al., 1983; Kronforst-Collins & Disterhoft, 1998; Morgan et al., 1993; Miller & Cohen, 2001). Stress can affect mPFC-dependent learning, often disrupting these behaviors (Arnsten, 2009; Lupien & Lepage, 2001). It has been previously reported that the medial prefrontal cortex is engaged during stress to affect subsequent learning only in females and not males (Maeng et al., 2010). This effect of stress on eyeblink conditioning is not only observed with a specific type of uncontrollable stressor but occurs with a variety of stressors such as tail shock, foot shock, and swim stress (Shors et al., 1992; Shors, 2001; Leuner et al., 2004). The finding in this brain region is important in that it identifies a neural substrate that is specific to females that may explain why women are more negatively affected by stress and thus are more vulnerable to stress-related disorders than men. There is also compelling evidence in PTSD and depressed patients pointing to structural and functional abnormalities within the mPFC and its connectivity to the amygdala (Steele et al., 2007). Functionally distinct roles have been described for subregions within the mPFC; relevant to the present study are the prelimbic and infralimbic cortices. Therefore, investigating the PL and IL separately may provide valuable information on a more specific involvement or mechanism in how stress modifies learning in females. Interestingly, compared to the infralimbic region, the prelimbic area has dense projections to and from the basolateral amygdala (Buchanan et al., 1994; Vertes, 2004). Because the mPFC-BLA connection is also

necessary in the stress-induced suppression of eyeblink conditioning in females, this has strong implications for a critical role for the PL in the female stress effect.

Most of the literature investigating the role of the prelimbic area in processes related to stress, anxiety, and fear have demonstrated that PL neural activity drives the expression of anxiety and conditioned fear (Blanco et al., 2009; Jinks & McGregor, 1997; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2009). Because of its connectivity to the amygdala and its role in fear, it was hypothesized that prelimbic inactivation during swim stress exposure would prevent the suppression of learning in females 24h later. Many studies have demonstrated that lesions of the prelimbic mPFC impair trace eyeblink conditioning but do not affect delay eyeblink conditioning (Weible et al., 2000; Takehara et al., 2003). However, it has been reported that the PL may also be necessary for delay eyeblink conditioning (Gilmartin & McEchron, 2005). In order to avoid disruption of eyeblink conditioning performance due to damage of this region, we used delay eyeblink conditioning to assess learning. Furthermore, transient inactivation of the PL was only during the stressor exposure which occurred 24h before and not during training so that PL inactivation would not affect learning itself. The sex differences in the effects of stress (enhanced learning in males and impairment in females) are also observed in delay eyeblink conditioning as well. In this experiment, we wanted to examine whether activity of the prelimbic subregion is specifically required for deficits in learning after stress. Therefore, this experiment was conducted only in females, and the PL was temporarily inactivated- neural activity was inhibited only during the stressor and not during delay training.

Methods

To determine whether the prelimbic subregion of the medial prefrontal cortex is necessary to induce the stress effect on learning in females, the PL of adult female rats were bilaterally infused with either muscimol or aCSF vehicle in a different context from training or the stress procedure. Immediately following infusions, animals were taken into another room and were either stressed or unstressed. This yielded four different groups: vehicle aCSF and no stress (n=7), vehicle aCSF and stress (n=6), muscimol and no stress (n=10), and muscimol and stress (n=8). Because the rats were stressed and trained in two different contexts, it is unlikely that there were contextual effects on conditioned responding observed during training (Shors et al., 1997; Wood et al., 2001). Furthermore, it can be noted that the effects of stress on learning in females are not due to the altered pain sensitivity or changes in the unconditioned response after the stressor (Wood & Shors, 1998; Bangasser & Shors, 2004). The amplitude of the UR during training is not affected by stressor exposure 24h later; therefore, the possibility that females respond differently after stress due to alterations in the perceived intensity of the US can be eliminated (Bangasser & Shors, 2004). 24h after the stressor exposure, all rats were trained with delay eyeblink conditioning for four consecutive days.

Results

In order to dissociate the roles of the prelimbic and infralimbic subregions of the mPFC in the stress effect on learning, they were examined separately. Experiment 1 was conducted to determine whether neuronal activity within only the prelimbic area of the medial prefrontal cortex was necessary for the stress-induced impairment of eyeblink conditioning in females. Animals were implanted with bilateral cannula with tip placement aimed at the prelimbic area of

the mPFC for drug infusions (Figure 7). A 2 x 2 (independent measures: no stress/stress x vehicle/muscimol) repeated measures analysis of variance (ANOVA) across four days of training revealed a main effect of drug [$F(1,27)=8.65$; $p<0.01$] and stress [$F(1,27)=7.77$; $p<0.01$]. There was also an effect of session on the percentage of conditioned responding across the four days of delay conditioning [$F(3,87)=6.68$; $p<0.01$], which indicated that learning had occurred. A one-way repeated measures ANOVA indicated that vehicle-treated stressed females exhibited minimal or no evidence of learning as sessions of training progressed [$F(3,15)=0.16$; $p>0.05$]. The analysis also revealed a significant interaction of drug and stress [$F(1,27)=13.12$; $p<0.01$] and a significant three-way interaction between drug, stress, and session [$F(3,81)=2.75$; $p<0.05$]. A Newman-Keuls *post hoc* test revealed that the females that received bilateral aCSF injections into the PL before the stressor expressed fewer conditioned responses than those that were not stressed ($p<0.01$). Interestingly, muscimol-injected stressed females also emitted more CRs than those in the vehicle/stress group ($p<0.01$). As illustrated in figure 9, unstressed rats injected with muscimol 24h before training performed similarly to the control animals ($p>0.05$). Therefore, muscimol alone did not affect conditioned responding 24h later. It should also be noted that although there appears to be a significant amount of cortical damage caused by the cannulation, it did not disrupt performance as evidenced by the control (aCSF/no stress) animals that were able to learn across the sessions of training.

The first session of 100 trials was analyzed with a 2 x 2 repeated measures ANOVA as five 20-trial blocks to consider effects of muscimol and stress in the prelimbic cortex on early acquisition. This was assessed to account for any differences of effects on early learning that may affect later learning; animals may not be able to learn the task or may differ in the rate of acquisition that may influence the later sessions of training. The analysis revealed an effect of

drug [$F(1,27)=4.42$; $p<0.05$] and block [$F(4,108)=20.90$; $p<0.01$]. Again, females infused with vehicle aCSF and stressed did not learn well across the first 20-trial blocks of delay eyeblink conditioning [$F(4,20)=2.76$; $p>0.05$].

Learning criterion for animals that learned well was 60% conditioned responding in at least one session of training. To illustrate how well the rats learned after stress and how it was affected by inactivation of the prelimbic cortex, the percentage of animals that reached the learning criterion of 60% conditioned responding was calculated and are represented in Figure 11. As previously observed, all or most of the animals that were not exposed to swim stress learn well, whereas all of the stressed females with intact prelimbic areas did not. Interestingly, most (~87%) of the stressed females whose prelimbic cortices were inactivated were able to learn well. Therefore, bilateral infusions of muscimol into the PL prevented the effect of stress on conditioning, suggesting that activity in the PL is necessary to impair learning after stress in female rats.

Discussion

The purpose of Experiment 1 was to determine whether the prelimbic subregion of the medial prefrontal cortex, separate from the infralimbic area, is necessary to suppress learning after stress in females. Because of its dense projections to the BLA and excitatory role in the production of fear responses, it was hypothesized that muscimol inactivation of the PL would prevent the stress effect on learning in females. Consistent with the hypothesis, vehicle-treated females exposed to the stressor did not learn well and emitted fewer conditioned responses than those females that were not stressed. However, females whose prelimbic areas were inactivated during the stressor exposure learned well and performed similarly to rats that were unstressed,

emitting more conditioned responses than the stressed controls. These results indicate that the prelimbic area is critically involved in the stress-induced impairment in associative learning in females.

The mPFC-BLA pathway is critical specifically in females to suppress learning after stress. Disconnecting communication between the structures prevents stress-induced decremented responding (Maeng et al., 2010). Because the prelimbic cortex projects heavily to the basolateral amygdala, it is possible that the PL-BLA circuit is engaged during stress to suppress eyeblink conditioning in females. Fear conditioning studies posit that the prelimbic area is involved in the expression or production of fear responses; PL inactivation prevents the conditioned freezing behavior typically observed at the start of extinction training, and activation of the PL impairs extinction learning and increases conditioned freezing to the tone (Corcoran & Quirk, 2007; Blum et al., 2006; Vidal-Gonzalez et al., 2006; Sierra-Mercado et al., 2011). Multichannel unit recordings of firing activity of PL neurons revealed that pattern of PL activity mirrored that of conditioned freezing behavior such that increases in neuronal firing during the CS tone presentation correlated with increases in freezing behavior (Burgos-Robles et al., 2009). If neuronal activity within the PL enhances the fear and stress response, inhibiting this activity within the prelimbic region may attenuate the effects of stress on learning and explain why these females were able to learn well with PL inactivation during stress. It should be noted that during the inactivation of the PL, IL activity and structural responses to the stressor is maintained throughout the experiment and may be producing the attenuation of the female stress effect. The infralimbic mPFC has been implicated in the inhibition of amygdalar activity and thus, through actions of this region alone, may be reducing the response to stress.

The mechanism underlying the stress-induced learning suppression in the present results may involve prelimbic communication with the basolateral amygdala. The prelimbic cortex receives inputs from the BLA, and in turn, sends excitatory inputs to the BLA to influence stress and fear responses (Brinley-Reed et al. 1995; Likhtik et al., 2005; McDonald et al., 1996; Rosenkranz & Grace, 2002; Vertes, 2004). Similar to the PL, inactivation of the basolateral amygdala reduces conditioned fear expression (Muller et al., 1997; Sierra-Mercado et al., 2011). Interestingly, just as in the mPFC, transient inactivation of the basolateral amygdala with muscimol during exposure to stress prevents the impairment of eyeblink conditioning in females (Waddell et al., 2008). Together these data indicate that both the prelimbic cortex and basolateral amygdala (and the connections between them) are necessary for the expression of fear responses and are implicated in the stress effect on eyeblink conditioning in females as well. Therefore, in this experiment, inactivation of the prelimbic area during the stressor may have disrupted excitatory input or activation of neurons in the BLA necessary to elicit the stress response and thus prevented the stress effect. Additionally, exposure to acute stress on an elevated platform in a brightly lit room disrupts the long-term potentiation (LTP) in the prelimbic mPFC normally induced by theta burst stimulation to the basolateral amygdala in male rats (Maroun & Richter-Levin, 2003). LTP is also suppressed in the hippocampus after stress, and this impairment in synaptic plasticity has been associated with deficits in hippocampal-dependent learning and memory processes (Garcia et al., 1997; Xu et al., 1997, 1998; Akirav and Richter-Levin, 1999; McEwen & Sapolsky, 1995). Therefore, it is possible that in the present study, swim stress may alter LTP in the PL-BLA pathway to impair learning in females, but due to inactivation of the prelimbic cortex during the stressor, the deleterious effects on learning may

have been prevented. Future studies targeting just the connection between the prelimbic cortex and the basolateral amygdala will be able to address these possibilities.

Experiment 2. Activation of the infralimbic cortex during the stressor is not necessary for the stress effect on learning in females.

Introduction

In contrast to the prelimbic cortex, the infralimbic region of the medial prefrontal cortex does not have robust projections to the basolateral amygdala. Instead, it projects more to the central amygdala. Specifically, the IL projects to GABAergic intercalated (ITC) cells in the amygdala that inhibits the centromedial amygdala to reduce fear output (Vertes, 2004; Royer et al., 1999; Paré et al., 1995; Cassell & Wright, 1986; Sesack et al., 1989; Hurley et al., 1991; McDonald et al., 1996; Quirk et al., 2003; Freedman et al., 2000). Thus, the infralimbic mPFC has been extensively studied for its role in the inhibition of fear, particularly in extinction learning (Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006; Milad & Quirk, 2002). Inactivation of the infralimbic mPFC impairs extinction learning, and more conditioned freezing to a tone that had been previously paired with foot shock is observed in these animals compared to controls (Sierra-Mercado et al., 2011). One and three exposures to uncontrollable swim stress induces morphological alterations (dendritic retraction) in infralimbic but not prelimbic neurons of the mPFC and concomitantly impairs extinction learning (Izquierdo et al., 2006). Furthermore, sex differences in the effects of chronic stress on BLA-projecting IL mPFC neurons were observed; there was a stress-induced increase in dendritic arborization in ovariectomized females treated with estrogen, suggesting increased synaptic activity (Shansky & Morrison, 2010). As noted previously, communication between the mPFC and BLA is necessary to impair learning after stress in females. However, the subregions of the mPFC were not differentiated in this

effect, though evidence suggests that stress alters IL structure and function and can influence BLA activity to inhibit the effects of stress on learning.

Based on findings indicating that the infralimbic cortex is sensitive to stress and exerts an inhibitory influence over the BLA, which is critically involved in the stress-induced learning suppression in females, experiment 2 was conducted to determine whether the IL specifically modulates this stress effect. To test this hypothesis, the infralimbic region was temporarily inactivated with muscimol only during acute stressor exposure and not during training in female rats. 24h later, the animals were trained with delay eyeblink conditioning.

Methods

Experiment 1 revealed that the prelimbic neural activation is necessary to induce the stress effect in females. To examine whether the infralimbic area of the medial prefrontal cortex is similarly or differentially involved to impair learning after stress in females, the IL was bilaterally inactivated with muscimol infusions restricted to this region during the swim stressor, and learning was assessed 24h later in experiment 2. There were four groups. One group of females received bilateral microinfusions of vehicle aCSF and were not stressed (n=8). The second group received vehicle aCSF and were exposed to swim stress (n=7). The remaining two groups consisted of female rats that were infused with muscimol and were swim stressed (n=7) and were not stressed but returned to their home cages following infusions (n=7). Conditioned responding across four days of training (100 trials/day) was assessed.

Results

Experiment 2 focused on the role of the infralimbic subregion of the mPFC in the stress-induced suppression of eyeblink conditioning in female rats (Figure 10). These animals were bilaterally cannulated with targets within the infralimbic mPFC for drug infusions (Figure 8). A 2 x 2 x 4 analysis of variance for stress (no stress/stress) x drug (aCSF/muscimol) x training session (session 1 -4) revealed no effect of drug [$F(1,24)=0.81$; $p>0.05$], but a main effect of stress [$F(1,24)=17.49$; $p<0.01$]. In this analysis, there was also an effect of session [$F(3,72)=6.69$; $p<0.01$], confirming that learning had occurred across the four days of eyeblink conditioning. A one-way repeated measures ANOVA, however, revealed that stressed females of both the muscimol [$F(3,18)=0.68$; $p>0.05$] and vehicle [$F(3,18)=0.91$; $p>0.05$] treatment groups did not learn well as training sessions progressed.

A repeated measures ANOVA assessing early acquisition across the first five 20-trial blocks on the first day of training revealed an effect of stress [$F(1,24)=10.06$; $p<0.01$] and blocks [$F(4,96)=9.47$; $p<0.01$]. There was also a significant drug x block interaction [$F(4,96)=2.69$; $p<0.05$]. A Newman-Keuls *post hoc* analysis confirmed that the unstressed females that received either bilateral vehicle aCSF or muscimol injections into the IL emitted more conditioned responses than the vehicle-treated females that were stressed ($p<0.05$). It should also be noted that there seems to be a considerable amount of cortical damage caused by the cannulation of the IL; however, eyeblink performance was not disrupted as the control (aCSF/no stress) females were able to learn across the four sessions of training. Additionally, aCSF-treated and stressed females did not increase conditioned responding in the five 20-trial blocks of the first training session [$F(4,24)=1.11$; $p>0.05$], indicating that they were not able to learn to perform the task. In contrast, muscimol-treated females that were stressed did show evidence of learning during

early acquisition [$F(4,24)=3.02$; $p<0.05$], but this was not maintained throughout the later sessions of training as described above.

To further characterize the effects of drug and/or stress on associative learning, a learning criterion of 60% of conditioned responding was used to identify the animals that learned in each treatment. In both of the unstressed groups, all or most of the animals learned well, reaching at least 60% conditioned responses on at least one session of training. In contrast, only about 28% of the females that were infused with aCSF and stressed reached this learning criterion (Figure 12). This was an expected result for the stress vehicle-treated animals; decremented responding is characteristic of learning in stressed females. Interestingly, like the vehicle/stress females, most of the animals in the IL muscimol/stress group were also not able to learn well with only about 14% of the rats reaching criterion.

Discussion

Experiment 2 was conducted to determine whether the inhibition of infralimbic mPFC neuronal activity during swim stress would attenuate the effect of stress on learning in females. The structural and functional effects of stress (acute and chronic) have been described within this region as well as its inhibitory control of the amygdala in terms of extinction learning. It was important to identify the role of the infralimbic cortex in this paradigm in addition to the prelimbic area as fear conditioning studies have demonstrated that these two adjacent subregions play different and sometimes opposite roles. In the present study, stressed female rats that received vehicle infusions during the stressful experience emitted fewer conditioned responses than those females that were not stressed. In other words, stressor exposure suppressed associative learning in this group of female rats. It was expected that if the infralimbic area is

necessary to elicit the stress-induced deficit in eyeblink conditioning, then transient inactivation of this region during the stressful experience would prevent this stress effect. Conversely, if the IL is not a necessary structure in the circuitry modulating learning after stress, then inhibiting neural activity within this area during stress with muscimol would not alter the effect of stress on learning, and decremented conditioned responding would occur in these females. The results of this experiment illustrated the latter.

The animals that received muscimol inactivation of the infralimbic cortex and were swim stressed performed similarly to the vehicle-treated stressed females, emitting fewer conditioned responses than their unstressed counterparts. It is important to note the performance of the muscimol-treated stressed females across the 5 20-trial blocks of the first session of training. It appears as though they learned similarly to the unstressed females, expressing more conditioned responses than the vehicle-treated stressed females, during early acquisition. However, the impairing effect of stress on learning in the IL-inactivated stressed group appears in the later sessions of training. This might be explained by a possible difference in the latency of the stress effect. Differences in neuronal activity patterns between the PL and IL have been reported in a different type of learning (Burgos-Robles et al., 2013). Therefore, the effects of stress may take longer to appear in the presence of prelimbic activity during the stressor and in the absence of IL activity. Future studies could examine the onset and duration of activity within these regions in the female stress effect on learning. Nonetheless, these data suggest that neuronal activity within the infralimbic subregion during a stressful experience is not critically involved in the learning impairment after stress in females.

The anatomical data of projections to and from the infralimbic cortex support an inhibitory role for IL mPFC on amygdalar function. This region does not project to the

basolateral amygdala but instead projects to GABAergic intercalated cells of the amygdala that inhibit activity of the centromedial amygdala; the output of the centromedial amygdala is the response to fear and stress. Therefore, it is possible that by inhibiting neural activity within the IL with muscimol infusions during the stressful event, the inhibitory control of the amygdala by this brain area is no longer present, and the suppression of learning induced by stress is unaltered. This may not be the case as the IL-inactivated stressed females did not learn worse than the vehicle-treated stressed animals as a greater response to stress or suppression might be expected due to the loss of inhibitory control. Furthermore, although the IL was inactivated during stressor, prelimbic mPFC activity remained intact and as the previous fear conditioning studies suggest, may alone be driving the response to stress and impairing eyeblink conditioning in these females. Another possibility is that the IL may not be acting through GABAergic mechanisms and thus have no effect with activation of $GABA_A$ receptors in this study. However, findings that demonstrate impairments in extinction have been made with infusions into the infralimbic cortex, using sodium channel blocker tetrodotoxin (TTX; Sierra-Mercado et al., 2006) as well as N-methyl D-aspartate (NMDA) and β-adrenergic receptor antagonists (Burgo-Robles et al., 2007; Mueller et al., 2008) and protein synthesis inhibitors (Santini et al., 2004). Future studies using other agents to block activity within this region will elaborate on this possibility.

GENERAL DISCUSSION

There is a nearly two-fold higher prevalence of stress- and anxiety-related mental illness in women compared to men (Tolin & Foa, 2006; Breslau et al., 1997; Kessler et al., 2005; Nolen-Hoeksema & Girgus, 1994; Foa & Street, 2001). Although sex differences in humans and nonhuman animals have been characterized in the neurobiology underlying these psychopathologies, the mechanisms that produce these differences or make women so much more vulnerable to stress than men is not well understood (Goldstein et al., 2010; Lebron-Milad et al., 2012). The hippocampus, amygdala, and prefrontal cortex have been strongly implicated and extensively studied in the neurocircuitry of anxiety, fear, and stress disorders (for review, see Shin & Liberzon, 2010). These brain regions are sexually dimorphic and interconnected to each other. The critical involvement of each in the response to stress and modulation of learning are of particular importance because individuals who suffer from these types of mental illness often describe difficulty in executing certain cognitive tasks and have symptoms that can be exacerbated by life stress. Structural differences within the hippocampus, amygdala, and prefrontal cortex have also been reported in depressed and PTSD patients compared to healthy individuals.

In our laboratory, we have demonstrated that the effects of stress (of various types and duration) on eyeblink conditioning differ between the sexes as do the neural substrates engaged by the stressful experience that modify subsequent learning. Exposure to acute inescapable stress facilitates eyeblink conditioning in male rats, whereas in females, exposure to the same stressor suppresses learning (Wood & Shors, 1998; Beylin et al., 1998; Wood et al., 2001; Bangasser et al., 2005; Waddell et al., 2008; Maeng et al., 2010). The hippocampus and basolateral amygdala are critically involved in the modification of learning by stress in both

males and females (Bangasser & Shors, 2007; Waddell et al., 2008). Though these brain structures are shared in the circuitry mediating stress modification of eyeblink conditioning, there are other brain regions in which males and females diverge. One brain region that is necessary for the stress-induced enhancement of conditioned eyeblink responding in males but not for the learning deficit in females is the bed nucleus of the stria terminalis (Bangasser et al., 2005). In females, activity in the medial prefrontal cortex during stress exposure is critical to impair learning; however, this is not the case in males, and the mPFC is not necessary for the male stress effect (Maeng et al., 2010). This finding is important because it suggests that the mPFC is a site that mediates the negative effects of stress on learning only in females and may help explain the greater risk for disorders associated with stress in women. It is also consistent with other reports indicating that the mPFC is involved in the modulatory effects of stress on learning (Shansky et al., 2004; Arnsten & Goldman-Rakic, 1998; Cerqueira et al., 2007; Murphy et al., 1996; Birnbaum et al., 1999; Radley et al., 2006). Stress of various types, intensities, and duration can induce alterations in structure and function within the medial prefrontal cortex, affecting its communication with other neural structures and its influence on certain behaviors. Anatomically, the medial prefrontal cortex is interconnected with the hippocampus and basolateral amygdala (Vertes, 2004; Hoover & Vertes, 2007). Therefore, the involvement of the mPFC in the stress-induced suppression of learning may lie in its anatomical connections to these critical regions in the neurocircuits that mediate the modulation of learning by stress. The mPFC-BLA pathway seems especially important to study because it specifically has been identified to be an essential component in the circuitry for the female stress effect on eyeblink conditioning (Maeng et al., 2010).

Aside from these connections, the medial prefrontal cortex may impair eyeblink conditioning after stressful experience in females through effects of estrogen. For instance, estrogen influences genomic processes in the prefrontal cortex that can alter transcription factor gene expression in the prefrontal cortex (Wang et al., 2004). Estrogen receptors are abundantly expressed in this region, especially the estrogen receptor beta, which has been associated with affect and responses to stress (Weiser et al., 2008; Rissman, 2008; Wang et al., 2004; Shughrue et al., 1997; Shughrue & Merchenthaler, 2001; Zhang et al., 2002). Figueiredo et al. (2002) demonstrated that immediate early gene c-fos activity (common marker for neuronal activation) induced by restraint stress exposure was reduced in proestrus females compared to females in other stages of estrous. Similarly, the debilitating effect of stress on eyeblink conditioning in females is only observed when the stressful event is experienced during a particular phase of the estrus cycle when estrogen levels are low and on the rise (Shors et al., 1998). Therefore, neuronal activity within the mPFC and behavioral responses to stress may change depending on the phase of the estrous cycle and thus are influenced by fluctuating levels of circulating estrogen and other female gonadal hormones. In light of these data, it is possible that the medial prefrontal cortex may be modulating the female stress effect on learning via an estrogen-mediated mechanism. The interaction between stress and estrogen within the mPFC will be discussed.

As mentioned, it was previously determined that an acute stressful event disrupts classical eyeblink conditioning in female rats and does so via a mechanism that critically involves the medial prefrontal cortex (Wood et al., 2001; Maeng et al., 2010). However, the mPFC is heterogenous in structure, and different roles have been described for its various subregions. Many studies examining the medial prefrontal cortex use this term to refer to

different subregions collectively in this general area of the brain. Here, we discuss the mPFC as the prelimbic and infralimbic areas. The findings in males describe inherent differences in the distributions of projections and functions between the prelimbic and infralimbic subregions of the mPFC and also in their responses to stress and fear. Therefore, dissociating these two subregions may be critical to further elucidate the circuitry underlying the role of the mPFC in the modulation of learning by stress in females.

Distinctive roles for the prelimbic (PL) and infralimbic (IL) regions of the medial prefrontal cortex have been described. This is not very surprising because these areas project to and from different brain regions and thus implies that there may be different functions for both. For instance, the infralimbic area been noted to be involved in more visceral or autonomic functioning, whereas the prelimbic area has been more closely associated with limbic and cognitive functioning (Vertes, 2004). We previously reported that the mPFC (both PL and IL), BLA, and communication between these two structures are essential for the learning suppression after stress in females (Maeng et al., 2010; Waddell et al., 2008). Due to differences between the IL and PL both in terms of their projections and function, it is possible that these specific areas may also be differentially mediating the stress effect on learning in females. For this reason, it is important to identify potential differences. If these differences do exist, they may also be communicating differently to the BLA. Activation of the mPFC alone is not sufficient to induce the female stress effect and appears to involve interaction with the BLA.

In the present set of experiments, we explored the possibility of subregional differences in the role of the mPFC to suppress learning after an acute stressful experience in female rats. To do so, either the prelimbic or the infralimbic cortex was bilaterally inactivated during an episode of inescapable swim stress. Here we demonstrate that muscimol inactivation of the

prelimbic area of the mPFC during swim stress prevents the suppression of learning after stress that we typically observe in females; in contrast, inactivation of the infralimbic subregion did not. Animals without neural activity within the prelimbic cortex during the stressor performed similarly to the unstressed females regardless of drug treatment. Female rats with inhibited neural activity within the infralimbic cortex behaved similarly to the stressed females with intact infralimbic activity during stress exposure, showing little evidence of learning. Together, the present data suggest that the subdivisions of the mPFC differentially contribute to the stress-induced suppression of learning in females; the prelimbic area, but not the infralimbic area, is necessary to elicit this effect. Due to the time course of inactivation during stress and training in the absence of drug, it can be concluded that the prelimbic cortex is critically engaged during the stressful experience and is not necessary for learning.

The findings here may be specific to the learning paradigm used in these experiments; however, there are not many studies that distinguish between prelimbic from infralimbic cortices for direct comparison. It is of particular clinical significance because we use an experimental paradigm that demonstrates a detrimental effect of stress on learning that is specific to females. This may be related to the higher vulnerability to stress disorders in women than men. The potential interactions among the prelimbic cortex, basolateral amygdala, and estrogen to suppress learning after a stressful experience in females will be discussed. Finally, combining the current and previous data, a female-specific neurocircuit that is engaged by stress to modify learning in eyeblink conditioning will be proposed.

Stress-induced suppression of classical eyeblink conditioning: Prelimbic cortex vs. Infralimbic cortex

The discrimination between the PL and IL was not a surprising outcome because anatomical and functional differences have been described previously (Vertes, 2004; Burgos-Robles et al., 2013; Hoover & Vertes, 2007; Vidal-Gonzalez et al., 2006; Ball & Slane, 2012; Sierra-Mercado et al., 2011; Tavares et al., 2009; Izquierdo et al., 2006; Radley et al., 2006). For example, multi-channel unit recordings of the prelimbic and infralimbic neurons during an appetitive operant conditioning task revealed different firing patterns in male rats; PL neurons displayed a rapid, but transient response to the reward, whereas IL neurons exhibited a slower and longer response (Burgos-Robles et al., 2013). This result may be important to take into account because it suggests a different time course of action between the IL and PL during the execution of the same behavior. Thus, it is possible that the activity in the PL and IL may be necessary at different times during the stressor exposure and throughout eyeblink conditioning in our study. Bland et al. (2005) not only illustrated sex differences in the responses of the mPFC to tailshock stress with greater c-fos mRNA expression overall in males than females, but they also demonstrated differences between the prelimbic and infralimbic areas in the females that were not observed in males. Stress-induced c-fos activation increased compared to unstressed controls in female rats in both PL and IL areas at 0 and 60 minutes after the stressor; however, the level of c-fos mRNA expression in the PL was significantly greater at 60 minutes than 0 minutes after the stressor (Bland et al., 2005). These data suggest that differentiating between the PL and IL response to stress may be especially critical in females.

Here, we have demonstrated that neuronal activity in the prelimbic but not the infralimbic mPFC during the swim stressor is necessary and may modulate the learning impairment that

occurs in females. This is consistent with studies that report that PL activity is involved in the expression of conditioned fear (Choi et al., 2010, 2012; Sierra-Mercado et al., 2011). Moreover, prelimbic neuronal firing activity is greater in animals that fail to recall extinction, learning that requires the inhibition of conditioned fear responses; these animals exhibit enhanced expression of conditioned fear (Burgos-Robles et al., 2009). It is interesting to note that in contrast to the IL, there are very few connections between the prelimbic cortex and the BNST, a structure demonstrated to be critical for the male and not female stress effect (Vertes, 2004; Bangasser et al., 2005). This provides anatomical support for the exclusion of a role for the BNST in the stress-induced learning suppression in females. Although we cannot eliminate the possibility that the PL and IL could be modulating each other, these data provide evidence that neural activity within the prelimbic mPFC and not in the IL is critical to impair learning after stress in females. The circuit described for the role of the PL in expression of fear involves excitatory input from the prelimbic mPFC to the BLA, which activates the central amygdala for enhanced fear expression (Sotres-Bayon et al. 2004; Likhtik et al. 2005). Thus, stress exposure may activate the prelimbic area and induce morphological alterations (i.e. dendritic remodeling) that affect BLA activity, making subsequent learning more sensitive to modification by stress in females.

The infralimbic mPFC, on the other hand, is most often evaluated for its role in fear extinction learning. Stimulation of neurons within this subdivision of the mPFC inhibits conditioned fear (Sierra-Mercado et al., 2011). The IL projects to and activates inhibitory intercalated cells in the amygdala. These cells connect the BLA to and inhibit the central amygdala, reducing fear output (Berretta et al., 2005; Sotres-Bayon et al. 2004; Likhtik et al. 2005). It has been demonstrated that IL neurons that do not project to the BLA exhibit no stress-

induced morphological changes. However, in stressed females treated with estrogen, IL neurons that do project to the BLA displayed increased dendritic length, suggesting increased synaptic connectivity (Shansky & Morrison, 2010). This finding suggests that our brief swim stress exposure may induce morphological changes in the IL such as dendritic hypertrophy, which could overexcite neurons and make them vulnerable to excitotoxicity. Stress-induced neuronal death in the infralimbic cortex could lead to dysfunction of the region, exciting instead of inhibiting amygdalar activity, rendering the females in our study to be more sensitive to stress as indicated by their deficits in eyeblink conditioning. Because the IL appears to exert its influence via GABAergic mechanisms, it may be necessary to use other forms of inactivating pharmacological agents during stress such as sodium channel blocker TTX or lidocaine.

The presence of connections between cortical subregions allows for the possibility that mPFC subdivisions modulate each other. These connections may contribute to their functional differences. The IL receives afferent inputs mainly from the PL within the mPFC, whereas the PL receives more overall inputs from cognitive/limbic structures (Hoover & Vertes, 2007). Thus, the prelimbic and infralimbic areas may be interacting with each other to regulate the response to stress. Inactivation of both the PL and IL without subregional discrimination during the stressor prevented the stress-induced impairment on learning in females. This was also observed with inactivation of only the PL and not the IL. Together, these findings could suggest that the prelimbic area modulates the infralimbic area, perhaps because the PL responds faster to the stressor and before the IL to influence learning. Infralimbic inactivation did not prevent the stress-induced learning deficit in the females as prelimbic inactivation did. Thus, it seems unlikely that communication between the two structures during the stressor is necessary for this

effect of stress. Instead, the suppression of learning in response to stress in females appears to rely more on PL activity.

As mentioned previously, Burgos-Robles et al. (2009) assessed activity of prelimbic neurons in response to the tone during fear conditioning. It was demonstrated that the conditioned tone responses in the PL correlated with freezing behavior during habituation, conditioning, and extinction. Failure to extinguish conditioned fear responding was associated with increased neuronal firing of prelimbic neurons and deficient infralimbic neuronal activity. Stimulation of either the IL or PL and simultaneous electrophysiological recordings of neuronal activity could expand on the activity profiles of the PL and IL during stressor exposure and training. Differences in the firing patterns of the PL and IL neurons may result in differential modulation of BLA activity, which can also modify the female stress effect on learning.

Stress-induced suppression of classical eyeblink conditioning: Prelimbic cortex-basolateral amygdala pathway

As suggested, it is highly unlikely that one neural area is responsible for suppressing learning after stress in females. Other structures are involved, as demonstrated by the finding that activation of the mPFC alone in the absence of stress is not sufficient to elicit the learning deficit induced by stress in females. Studies in humans comparing neural correlates of PTSD and other stress and anxiety disorders have revealed overlapping circuitry in the hippocampus, amygdala, and prefrontal cortex (Shin et al., 2005; for review, see Shin & Liberzon, 2010). We have also previously demonstrated that activity within the hippocampus and basolateral amygdala is necessary to impair learning after stress exposure in females. Additionally, the mPFC-BLA connection was specifically examined and found to be critical in females. It is well

established that the amygdala regulates the emotional response to stressful, fear-inducing, and negative stimuli (LeDoux, 2000; Hendler et al. 2003; Wright et al. 2003). Interestingly, women have greater abnormalities in prefrontal cortico-amygdalar circuits, whereas men have abnormalities in the prefrontal cortico-striatal pathways, supporting the idea that different neural regions are affected by mental illness between the sexes (Kong et al., 2013). These data suggest that in females, the prelimbic cortex, hippocampus, and basolateral amygdala may be communicating during stress within a complex integrated network that contributes to the modulation of learning by stress.

Stress- and anxiety-related psychiatric disorders can be linked to a dysregulation of emotion. Abnormalities in the structure and function of brain regions that are integral to stress and emotion regulation are crucial to investigate. As described above, the prefrontal cortex and amygdala are of particular interest (Wang et al., 2013; Tang et al., 2012; Koenigs & Grafman, 2009). Although studies have investigated the connection between the medial prefrontal cortex and the amygdala across various paradigms, it is important to note that many primarily target the prelimbic area and its connections to the amygdala. Furthermore, the PL mPFC projects primarily to the BLA, whereas the IL has a wide distribution of projections throughout the amygdala, the least of which are to the basolateral amygdala (Vertes, 2004; Hurley et al., 1991). Maroun and Richter-Levin (2003) demonstrated that inescapable stress (30 minutes on a brightly lit elevated platform) prevents long-term potentiation in the medial prefrontal cortex induced by theta burst stimulation of the basolateral amygdala in rats. However, their recording electrodes were confined to only the prelimbic subregion, and thus, this study reported an effect specific to the BLA-PL pathway. In another study, stress-induced palatable food-seeking in female rats was examined (Calu et al., 2013). This experiment was conducted to explore the mechanisms

underlying the relapse to unhealthy eating during dieting typically provoked by stress in women. A pharmacological stressor, yohimbine, elicited palatable food-seeking behavior in female rats; this stress effect on behavior was dependent on the dorsal medial prefrontal cortex, which also included the prelimbic area but not the infralimbic region (Calu et al., 2013). In women with PTSD, increased activation of the amygdala and decreased activation of the dorsolateral prefrontal cortex (a possible homolog of the rodent prelimbic mPFC) were observed as the subjects anticipated presentations of negative images during an emotional anticipation task. In this study, increased prefrontal cortex activation correlated with less severe PTSD and better performance on another cognitive task that assessed inhibition and attention shifting ability (Aupperle et al., 2012). These findings suggest that the prefrontal cortex (prelimbic) and amygdala are responding differently to emotionally negative stimuli in women suffering from PTSD than they do in healthy women. These abnormalities may represent dysregulated affective processing and cognitive control systems. Depressed women are more likely to engage in ruminative behavior, or constant self-focus, which can worsen negative affective states and symptoms of depression (Tamres et al., 2002; Lopez et al., 2009; Nolen-Hoeksema & Aldao, 2011). Interestingly, women with major depressive disorder exhibited increased activation of the dorsolateral prefrontal cortex, medial prefrontal cortex, and amygdala during rumination compared to control subjects (Cooney et al., 2010). These findings highlight a central role of the prelimbic mPFC and amygdala that is specific to women with stress-related illness.

In laboratory animals, the literature indicates that the prelimbic cortex does not play a role in conditioned fear inhibition but instead facilitates conditioned fear expression. Activation of the PL may also enhance the response to stress as the critical brain regions in neurocircuitry mediating these responses overlap. It has been posited that activation of the prelimbic region of

the mPFC activates the basolateral amygdala, which in turn activates the centromedial amygdala (CMA) to elicit a fear response (Figure 13; Quirk et al., 2003; Sierra-Mercado et al., 2011; Correll et al., 2005). Therefore, in our experiment, it is possible that acute exposure to swim stress activates a similar circuitry, inducing activity within the prelimbic mPFC, BLA, and centromedial amygdala. CMA activation may then act on eyeblink conditioning circuitry to elicit the response to stress that impairs eyeblink conditioning in the females. Shansky and Morrison (2010) demonstrated that BLA-projecting neurons in the infralimbic mPFC of ovariectomized female rats treated with estrogen increased dendritic branching and spine density after chronic stress exposure. This dendritic remodeling may be associated with increased connectivity or function of the IL and BLA. Although a similar chronic stress-induced and estrogen-mediated increase in dendritic branching and length was reported in the female PL, it is unknown whether PL-BLA interactions in response to stress may be similar to this finding in the IL-BLA pathway (Garrett & Wellman, 2009). It seems likely that BLA-projecting neurons of the prelimbic cortex may undergo stress-induced morphological alterations that influence functional activity to mediate stress effects on learning in females. Some studies suggest dopaminergic modulation; stress exposure may stimulate dopaminergic pathways that impair mPFC function and strengthen amygdalar activity (Rosenkranz & Grace, 2002, 2003; Arnsten, 2009). Future studies are necessary to identify specific mechanisms underlying the prelimbic mPFC communication with the basolateral amygdala and the effects of stress on this pathway.

The opioid system is most often associated with pain and related sensory mechanisms that are mediated by its activity in the periphery. However, opioids can also convey information about the environment to the central nervous system for cognitive processing. Endogenous opioids fall into three major classes: enkephalins, endorphins, and dynorphins, with

corresponding receptor types, delta, mu, and kappa, respectively. Although they tend to induce similar analgesic effects when bound to their specific peripheral receptors, their central functions differ. For instance, mu and delta opioid receptor activation induces elevations in mood (Broom et al., 2002; Rubinstein et al., 1996), whereas kappa opioid receptor activation tends to induce states of dysphoria or depression (Bruchas et al., 2010). These differences in action in turn may mediate different responses to stressful life events. For instance, mu-opioid receptor knockout (MOP-KO) mice display a reduced expression of stress-induced emotional responses in behavioral tests for anxiety and depression. Animals lacking the mu-opioid receptor entered more open arms in the elevated plus maze and spent more time in them compared to wild-type animals. MOP-KO mice were also more mobile in the tail-suspension and forced swim tests (Ide et al., 2010). Interestingly, increases in corticosterone levels in response to stress were reduced in MOP-KO animals, suggesting an interaction between endogenous opioids and stress-induced corticosterone release. In women with major depressive disorder and women that do not respond to antidepressant treatment, a positron emission tomography (PET) scan measuring mu-opioid receptor binding activity revealed differences compared to healthy controls (Kennedy et al., 2006). This study demonstrated that brain areas involved in emotion regulation, such as the mPFC and amygdala, exhibited increased mu-opioid neurotransmission during a sustained sadness state in women suffering from depression. This was in contrast with their matched controls, who exhibited reduced mu-opioid neurotransmission (Kennedy et al., 2006). Furthermore, Yim et al. (2010) examined 370 euthymic (non-depressed, healthy) women from which blood samples were taken several times during 15-37 weeks of pregnancy and then 9 weeks postpartum. This study reported that women who had high levels of beta-endorphins during pregnancy had a 3-fold risk of developing postpartum depression. These findings suggest

that endogenous opioids may act in circuitry that regulates responses to stress and enhance sensitivity to stress in women.

In our laboratory, systemic injections of a nonselective opioid receptor antagonist, naltrexone, prevented the stress-induced enhancement of eyeblink conditioning in males, suggesting that stress may be modulating subsequent learning via an opioidergic mechanism (Maeng & Shors, unpublished). This has yet to be explored in the female stress effect but may also be involved as studies have reported a critical role for endogenous opioids in responses to stress (Mellon & Bayer, 1998; Amir, 1982; Shors et al., 1990; Ide et al., 2010) and in women with major depressive disorder and postpartum depression (Kennedy et al., 2006; Yim et al., 2010). Together, these data suggest possible mechanisms through which the PL-BLA pathway may mediate the negative effects of stress on learning in females. Infusions of naltrexone, centrally administered to the prelimbic mPFC during the stressor, could be used to investigate opioidergic modulation of stress-induced learning suppression in females.

Stress-induced suppression of classical eyeblink conditioning: ovarian hormones

Women are especially vulnerable to stress and disturbances in mood during drastic hormonal fluctuations (Brummelte & Galea, 2010). Moreover, the higher prevalence of depression and stress-related mental illness in women than men appears during puberty until menopause (Katiala-Heino et al., 2003; Ruiz et al., 2000; Nolen-Hoeksema & Girgus, 1994; Kessler et al., 1993; Hyde et al., 2008; Sonnenberg et al., 2000; Silberg et al., 1999; Marcotte et al., 2002; Piccinelli & Wilkinson, 2000). Stress exposure during pregnancy, a time of profound hormonal changes, can lead to postpartum depression (Brummelte & Galea, 2010). Therefore, ovarian hormones such as estrogen have been implicated in the etiology of depression in women.

Likewise, in laboratory animals, the effect of stress on learning in females appears during puberty and dissipates when they become aged and are no longer cycling (Hodes & Shors, 2005, 2007). Stress modifies learning differently during pregnancy and postpartum in rodents as well, which illustrates the influences of changing hormonal profiles during each reproductive stage (Leuner & Shors, 2006; Lemaire et al., 2006; Maeng & Shors, 2012). Exposure to inescapable stress increases the release of the rodent stress hormone corticosterone, which enhances eyeblink conditioning in the males (Beylin & Shors, 2003). Although corticosterone levels are also increased after the stressor in females, removing the adrenal glands that secrete corticosterone did not prevent the female stress effect (Shors et al., 1998; Wood et al., 2001). Because the suppression of learning due to stress in females is not dependent on the presence of corticosterone, these effects could be attributed to the activational influences of estrogen (Wood et al., 2001; Wood & Shors, 1998; Beylin et al., 1998; Shors et al., 1998). For instance, administration of an estrogen receptor antagonist and ovariectomy prevents the suppression of learning after stress (Wood et al., 2001; Wood & Shors, 1998). Furthermore, the most profound stress-induced impairment in learning in females is observed when the animals are exposed to the stressor in the diestrus 2 phase of the estrous cycle when estrogen levels are low and are increasing towards its peak in proestrus when they are trained (Shors et al., 1998; Wood et al., 2001; Wood & Shors, 1998). Thus, the decremented conditioned responding after stressor exposure in females is not regulated by corticosterone as it is in males but by ovarian hormones.

Although there are some reports of the protective effect of estrogen on mPFC-mediated learning, others demonstrate an enhanced response to stress in the mPFC in the presence of estrogen. Menopausal women and nonhuman animals that were ovariectomized perform better on working memory and mPFC-dependent tasks following estrogen treatment (Duff & Hampson,

2000; Sinopoli et al., 2006). The human and nonhuman primate dorsolateral prefrontal cortex, which may be homologous with the rodent prelimbic cortex, is important for cognition and seems to be an important target for these effects of estrogen (Goldman-Rakic, 1988). Estrogen concentration levels in this brain region are reportedly higher than in other cortical areas (Bixo et al., 1995). Furthermore, fMRI and PET studies indicate that the dorsolateral prefrontal cortex may be selectively activated by estrogen (Berman et al., 1997; Shaywitz et al., 1999; Ohkura et al., 1994). Similar to the effects of stress on mPFC morphology, increases in dendritic spine density have been reported in the dorsolateral prefrontal cortex of female rhesus monkeys treated with estrogen (Hao et al., 2006; Tang et al., 2004).

Estrogen can also enhance sensitivity to stress. As described previously, restraint stress impairs performance on an mPFC-mediated learning task in females with high estrogen levels but not in males or low estrogen females (Shansky et al., 2006). In addition, chronic restraint stress induces increases in dendritic branching only in infralimbic neurons that project to the BLA and only in females that were treated with estrogen (Shansky & Morrison, 2010). This finding indicates that the presence of estrogen induces stress sensitivity in the IL-BLA pathway. We present data here suggesting that the prelimbic but not the infralimbic cortex is necessary for the stress-induced impairment of conditioning in females. It would therefore be useful to know whether the PL-BLA pathway is similarly modulated by stress and estrogen. Perhaps exposure to stress during the diestrus 2 phase when estrogen levels are low alters mPFC morphology and activity. These alterations could be further influenced or maintained by the presence of estrogen at the start of training, when concentrations are high in proestrus. Therefore, the suppression of learning after stress observed in the present study may be occurring via an interaction between stress and estrogen within the prelimbic cortex.

In the current study, we did not assess levels of corticosterone because the stress-induced learning deficit in females is not influenced by the stress hormone. Instead, the female stress effect depends on the presence and levels of estrogen at the time of the stressor. Therefore, it may be necessary to determine whether the inactivation techniques used in this study affect ovarian hormone levels. To further pursue this estrogen-mediated mechanism, future studies infusing estrogen antagonists into the prelimbic cortex could be conducted. The amygdala also contains estrogen receptors (Jasnow et al., 2006; Shughrue et al., 1998), and estrogen infusion influences amygdalar function in fear and emotional responses (Frye & Walf, 2004). Thus, stress and estrogen may be interacting not only in the PL but may also affect the PL-BLA circuit. Based on the reviewed evidence, it would be expected that blocking estrogen receptors during the stressful experience in the prelimbic area would attenuate the stress-induced impairment in eyeblink conditioning.

Putting it all together: female-specific circuit

The facilitation of eyeblink conditioning in males and impairment in conditioning in females after stress are mediated by neural structures and circuitry that also differ between the sexes (Wood & Shors, 1998; Wood et al., 2001; Waddell et al., 2008; Bangasser et al., 2005; Hodes & Shors, 2005; Maeng et al., 2010; Bangasser & Shors, 2010). The hippocampus and the basolateral amygdala are necessary for both the stress-induced facilitation of learning in male rats and the suppression of learning after stress in female rats (Bangasser & Shors, 2007; Waddell et al., 2008). The circuitries diverge, however, at the level of the bed nucleus of the stria terminalis and the medial prefrontal cortex (Bangasser et al., 2005; Maeng et al., 2010). The bed nucleus of the stria terminalis is necessary to enhance conditioning in males after stress

but is not necessary to suppress learning in females. Conversely, neuronal activity within the mPFC is necessary during the stressor to impair learning in females but not to facilitate learning in males. In light of these present data, the prelimbic subregion of the mPFC specifically mediates the stress effect in females and may do so via communication with the basolateral amygdala.

As discussed, the prelimbic cortex projects heavily to the basolateral amygdala which can in turn act on neurons in the central amygdala for fear expression (Sotres-Bayon et al. 2004; Likhtik et al. 2005; Sierra-Mercado et al., 2011; Quirk et al., 2003). This connection between the prelimbic mPFC and BLA may also modulate the emotional response to stress as there is an overlap in critical brain regions in the neurocircuitry that mediate conditioned fear expression and the stress effect on eyeblink conditioning. In our paradigm, the central amygdala may connect to components in eyeblink conditioning circuitry, which has been well characterized (Medina et al., 2002; Christian & Thompson, 2003; Lee & Kim, 2004). Specifically, the central amygdala may influence the conditioned eyeblink response via its projections to the pontine nuclei of the CS pathway and the trigeminal nucleus of the US pathway (Figure 14). These CS and US pathways converge in the interpositus nucleus of the cerebellum, which activates downstream cerebellar targets that produce an eyeblink conditioned response (Price & Amaral, 1981; Weisz et al., 1992; Canli & Brown, 1996; Whalen & Kapp, 1991). For instance, lesions of the amygdala can prevent the conditioned enhancement of the eyeblink response (Weisz et al., 1992). Furthermore, electrical stimulation of the central nucleus of the amygdala can modulate the speed of the eyeblink reflex as well as its amplitude via the direct projection from the central amygdala to eyeblink reflex circuitry (Canli & Brown, 1996; Whalen & Kapp, 1991).

Although there are no direct projections from the mPFC to the hippocampus, massive hippocampal projections to the PL have been reported (Hoover & Vertes, 2007; Swanson, 1981; Jay & Witter, 1991; Verwer et al., 1997; Moser & Moser, 1998; Risold & Swanson, 1996). The hippocampus is dense with glucocorticoid receptors and also undergoes drastic alterations in structure and function following stress exposure (Shors et al., 2001; McEwen & Sapolsky, 1995). These morphological changes can also differ between the sexes (Galea et al., 1997). Furthermore, the hippocampal-prefrontal pathway is important for learning and memory functions and is also sensitive to stress (Laroche et al., 2000; Floresco et al., 1997; Bannerman et al., 2004; Moser et al., 1993; Czerniawski et al., 2009; Henke, 1990). For instance, male rats that were subjected to acute inescapable stress for 30 min on an elevated platform exhibited impaired long-term potentiation (LTP) in the prefrontal cortex following high frequency stimulation of the hippocampus (Rocher et al., 2004). As mentioned, in addition to the BLA, bilateral lesions of the hippocampus prevented the effect of stress on eyeblink conditioning in both males and females (Bangasser & Shors, 2005). Interestingly, as in the mPFC, differential roles for the dorsal and ventral hippocampus have been described (Czerniawski et al., 2009; Moser et al., 1993; Fanselow & Dong, 2010). The dorsal region of the hippocampus has been implicated in spatial memory processes (Moser et al., 1993). In contrast, the ventral hippocampus is involved in emotion and responses to stress (Henke, 1990). Therefore, the ventral region of the hippocampus could also be communicating with the PL mPFC to impair learning in stressed females. A disconnection technique could determine whether in addition to the mPFC-BLA pathway, communication between the mPFC and hippocampus, specifically the PL and ventral hippocampus respectively, is also necessary to impair learning after a stressful event in females.

The hippocampus, amygdala, and prefrontal cortex constitute a highly interconnected network involved in learning and stress processes (Ishikawa & Nakamura, 2003; Floresco et al., 1997; Orsini et al., 2011; Thierry et al., 2000; Sotres-Bayon et al., 2012). A circuit for fear modulation via inputs of the hippocampus and amygdala to the prelimbic cortex has also been described. Inactivation of the basolateral amygdala reduced PL activity and reduced conditioned fear responding, whereas inactivation of the ventral hippocampus increased conditioned fear responding and prelimbic activity in animals that had been extinguished (Sotres-Bayon et al., 2012). Thus, it is likely that the underlying circuitry mediating how the exposure to an acute stressful event modulates subsequent learning involves communication between these three neural substrates. Stress-induced alterations in structure and function of the ventral hippocampus could modulate activity of the prelimbic cortex and interact with the basolateral amygdala to modify eyeblink conditioned responding in females. There is some evidence to suggest that this circuitry may be mediated by estrogen, but further study is needed to confirm this idea.

Conclusions

Profound sex differences in the incidence of stress- and anxiety-related disorders are observed but are not fully understood. Women are twice as likely as men to develop these psychopathologies, suggesting that they are more disadvantaged by stressful experience than men. In laboratory animals, acute inescapable stress exposure enhances eyeblink conditioning in males, whereas learning is drastically impaired in females. These opposing effects of stress between the sexes demonstrate a greater sensitivity to stress in females than males. Here we determined that the medial prefrontal cortex, and now more specifically the prelimbic area of the

mPFC, is a critical brain region that is involved in the stress-induced suppression of learning in females but not the enhancement of conditioning in males.

It has previously been demonstrated that the medial prefrontal cortex and its communication with the basolateral amygdala are critically involved in the stress-induced suppression of learning in females. The mPFC is a complex structure comprised of several other subregions in addition to those discussed in this study that also have specific functions and influences on stress and learning behaviors. The ventromedial PFC (PL and IL subdivisions) has been strongly and consistently associated with responses to stress as well as mental illness and affective disorders. However, due to growing evidence that prelimbic and infralimbic subregions of the mPFC function differently and may differentially respond to stress, it was important to explore the possibility that they could have distinctive roles in the stress-induced suppression of learning.

The present data suggest that the prelimbic cortex is critically engaged by stress to impair learning in females, whereas the infralimbic cortex is not. This finding has important clinical implications because the rodent prelimbic area may be homologous to the human and nonhuman primate dorsolateral prefrontal cortex, in which many studies have reported abnormalities in patients with depression and PTSD. Additionally, a hyperactive amygdala and decreased activation of the mPFC have been associated with PTSD and depression (Drevets, 2003; Milad et al., 2006; Rauch et al., 2006; Liberzon et al., 2003; Shin & Liberzon, 2010). The inverse relationship between these two regions suggests that stressful life experiences that disrupt mPFC structure and function can also lead to emotional dysregulation due to a loss of prefrontal control of the amygdala. This mechanism may be mediating ruminative behaviors and recursive negative mood and thoughts, which predominantly occur in women.

Studies in humans find alterations in the mPFC and amygdala associated with stress-related psychopathologies in both men and women albeit in different ways. Therefore, understanding the sex differences in the incidence of these disorders may rely on identifying more specific subregional differences. Dissociating the function of the PL and IL and determining how they respond to and are affected by stress may be crucial to our understanding of why women respond to stress differently than men. Many women are resilient to and are able to cope with stress without developing mental illness, whereas some are not. It is possible then that individual differences in PL and IL function may predict vulnerability to stress-related disorders. Here we provide data to suggest that communication between the prelimbic cortex and basolateral amygdala enhances vulnerability to stress specifically in females. Therefore, the circuit including the prelimbic cortex (or dorsolateral prefrontal cortex) and amygdala may be especially responsive in women that are more susceptible to depression and other stress-related psychiatric disorders after experiencing a stressful life event. If it is, this may be a female-specific target for therapy and treatment for depression, PTSD, and other mental illness that can be precipitated by stress.

LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMY	Amygdala
ANOVA	Analysis of variance
AP	Anterior posterior
BLA	Basolateral nucleus of the amygdala
BNST	Bed nucleus of the stria terminalis
CMA	Centromedial amygdala
CR	Conditioned response
CS	Conditioned stimulus
DLPFC	Dorsal prefrontal cortex
DV	Dorsal ventral
EMG	Electromyographic
GABA	γ-aminobutyric acid
HPC	Hippocampus
HPA	Hypothalamic-Pituitary-Adrenal axis
IL	Infralimbic
IPN	Interpositus nucleus
ITC	Intercalated cells
ML	Medial lateral
MPFC	Medial prefrontal cortex
NMDA	*N*-methyl-D-aspartate
PL	Prelimbic
PON	Pontine nucleus
PTSD	Post-traumatic stress disorder
TGN	Trigeminal nucleus
UR	Unconditioned response
US	Unconditioned stimulus
VMPFC	Ventromedial prefrontal cortex

Figure 1

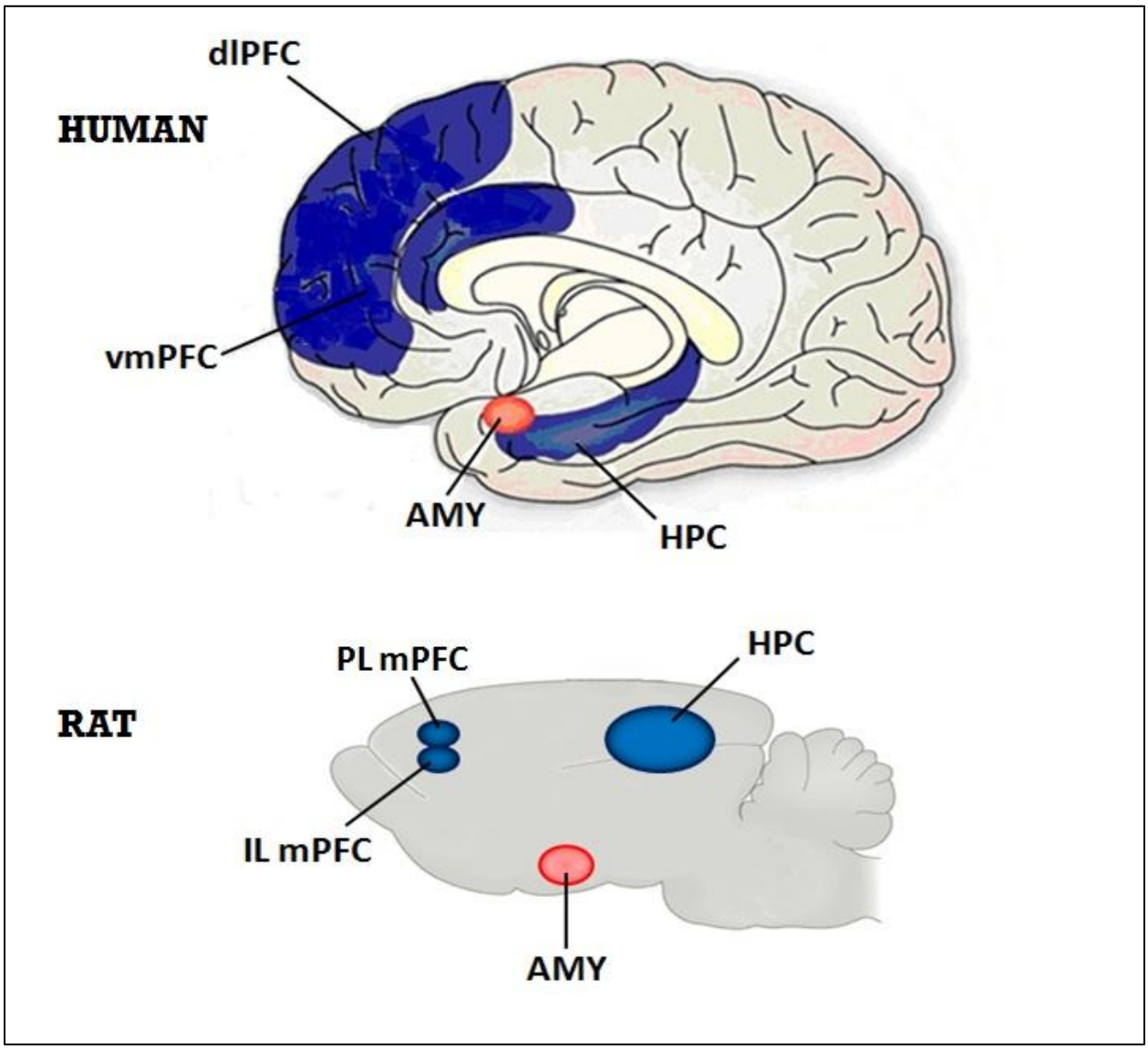

Figure 2

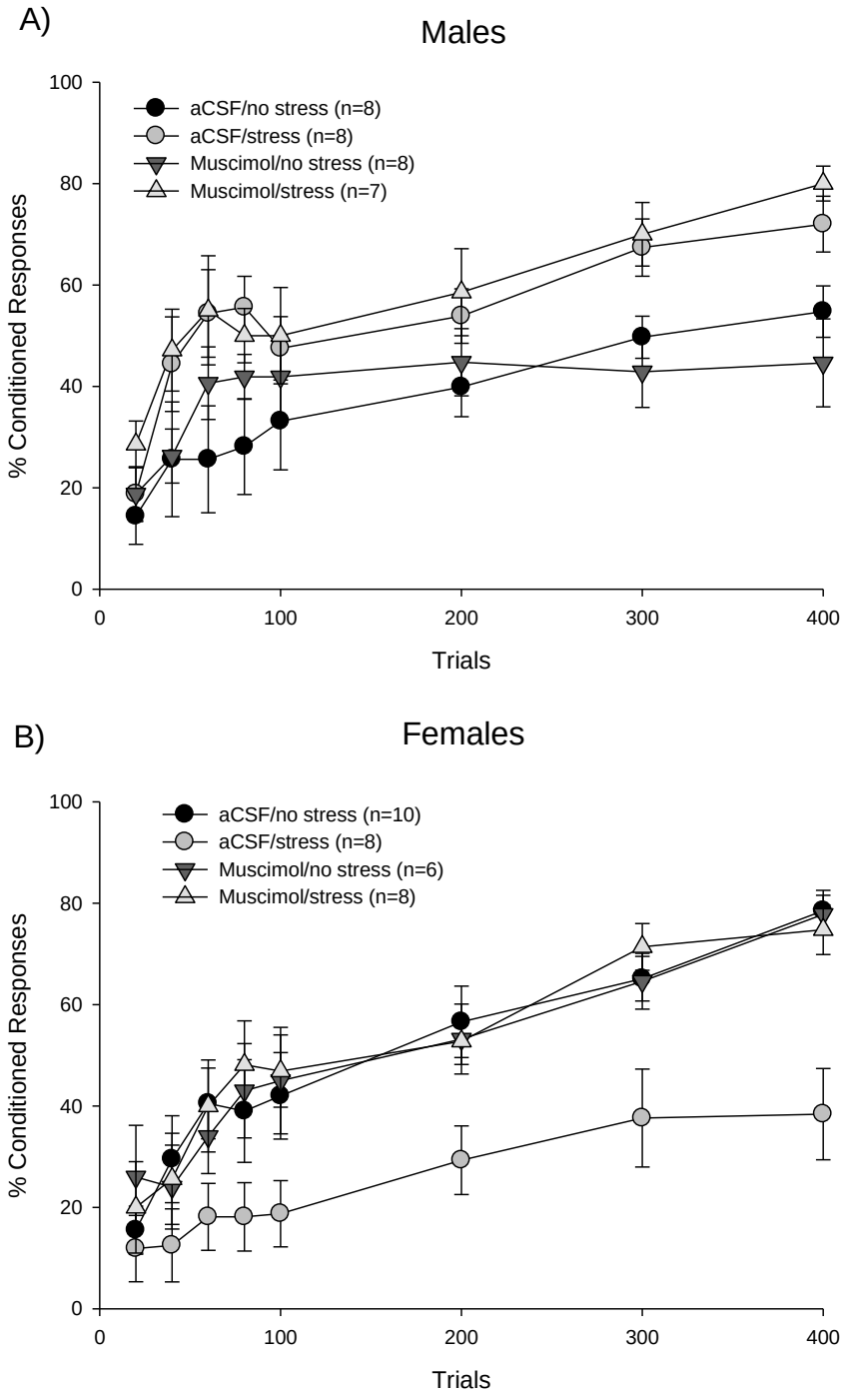
A)
Males
aCSF/no stress (n=8)
aCSF/stress (n=8)
Muscimol/no stress (n=8)
Muscimol/stress (n=7)
% Conditioned Responses
Trials
B)
Females
aCSF/no stress (n=10)
aCSF/stress (n=8)
Muscimol/no stress (n=6)
Muscimol/stress (n=8)
% Conditioned Responses
Trials

Figure 3

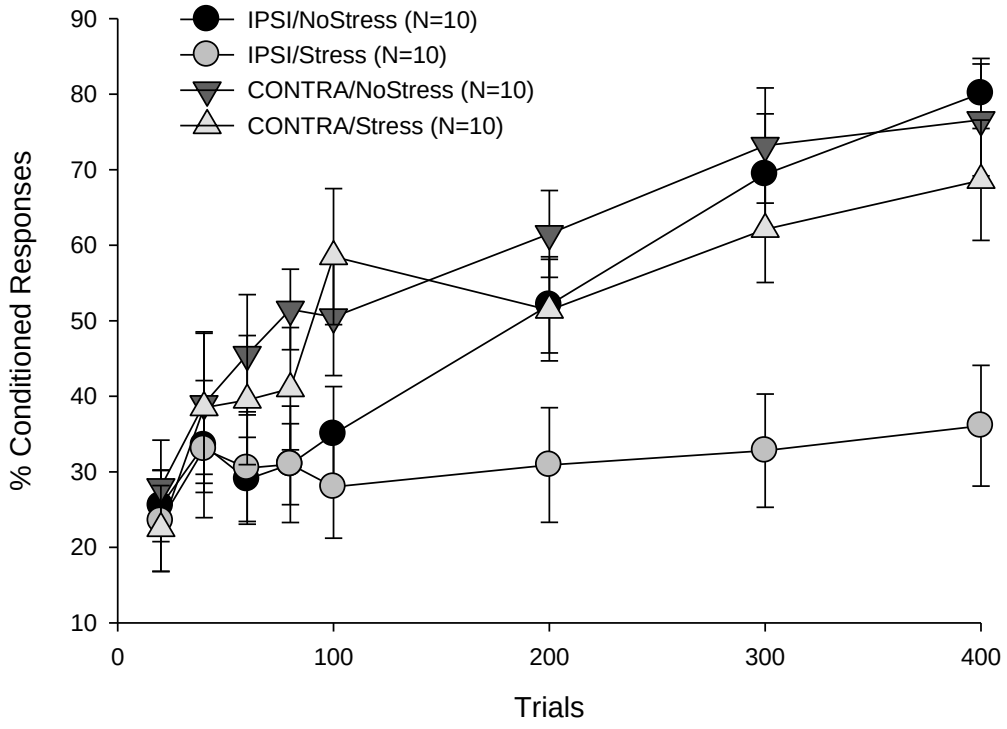
IPSI/NoStress (N=10)
IPSI/Stress (N=10)
CONTRA/NoStress (N=10)
CONTRA/Stress (N=10)
% Conditioned Responses
Trials
90
80
70
60
50
40
30
20
10
0
100
200
300
400

Figure 4

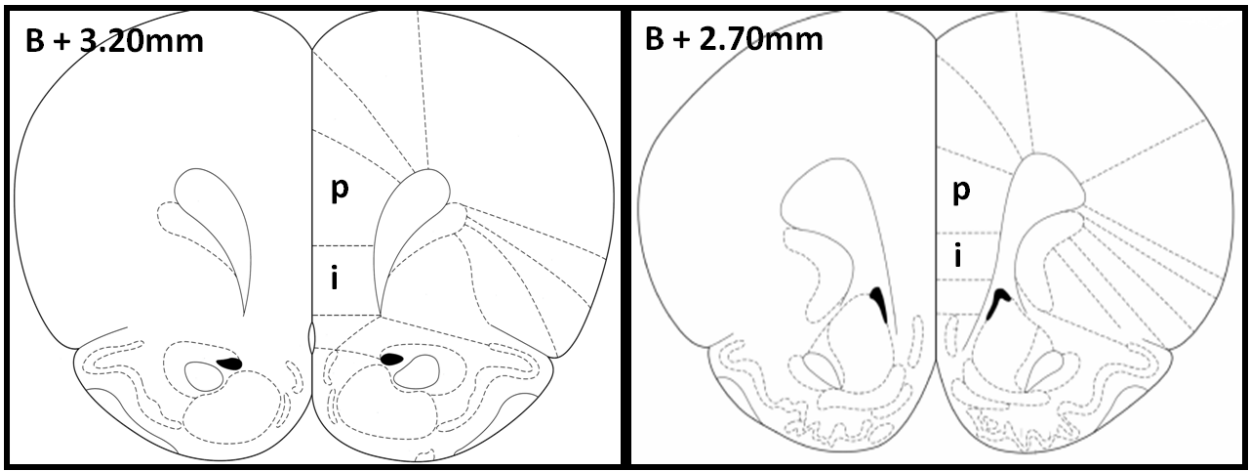

Figure 5

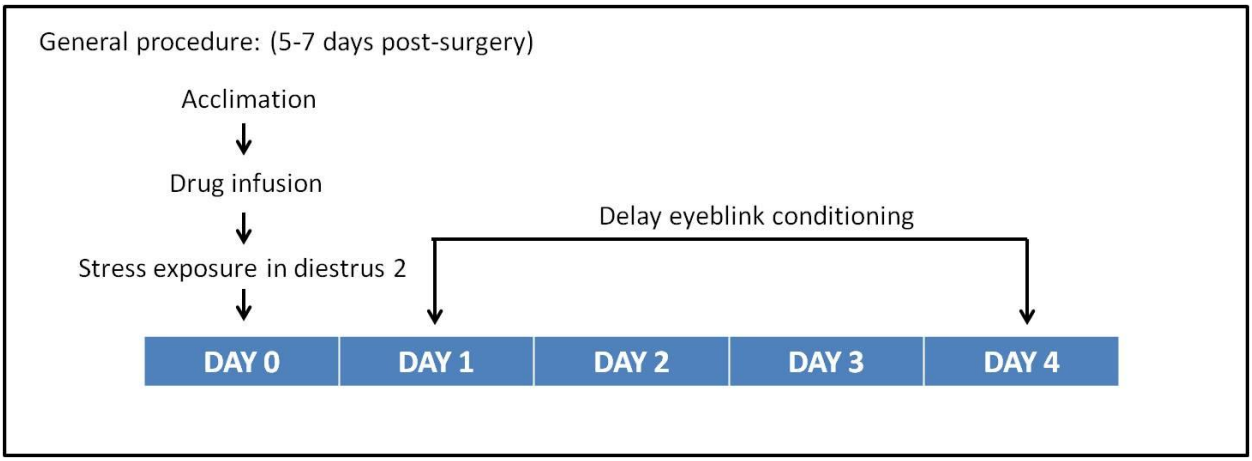
General procedure: (5-7 days post-surgery)
Acclimation
Drug infusion
Stress exposure in diestrus 2
Delay eyeblink conditioning
DAY 0
DAY 1
DAY 2
DAY 3
DAY 4

Figure 6

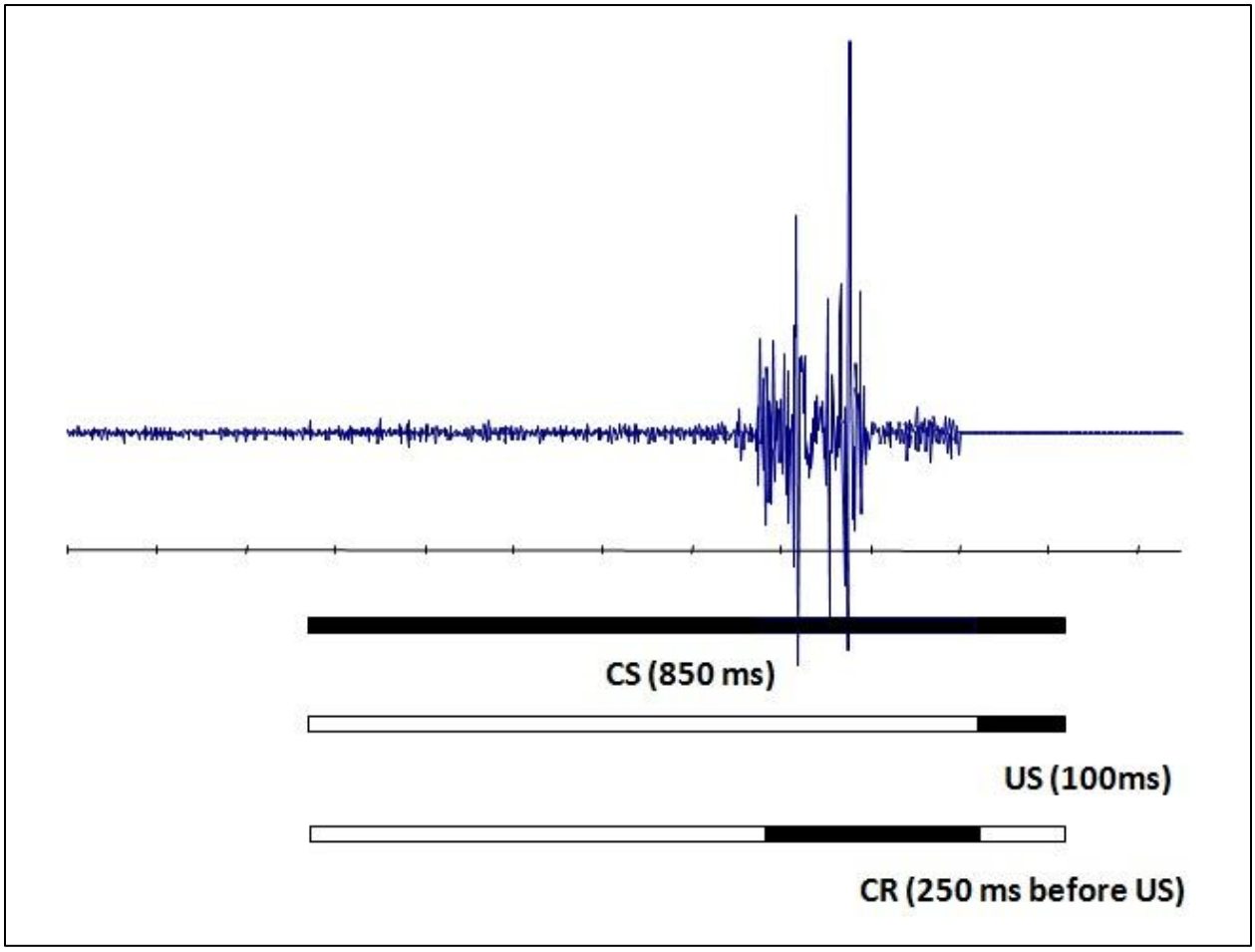

Figure 7

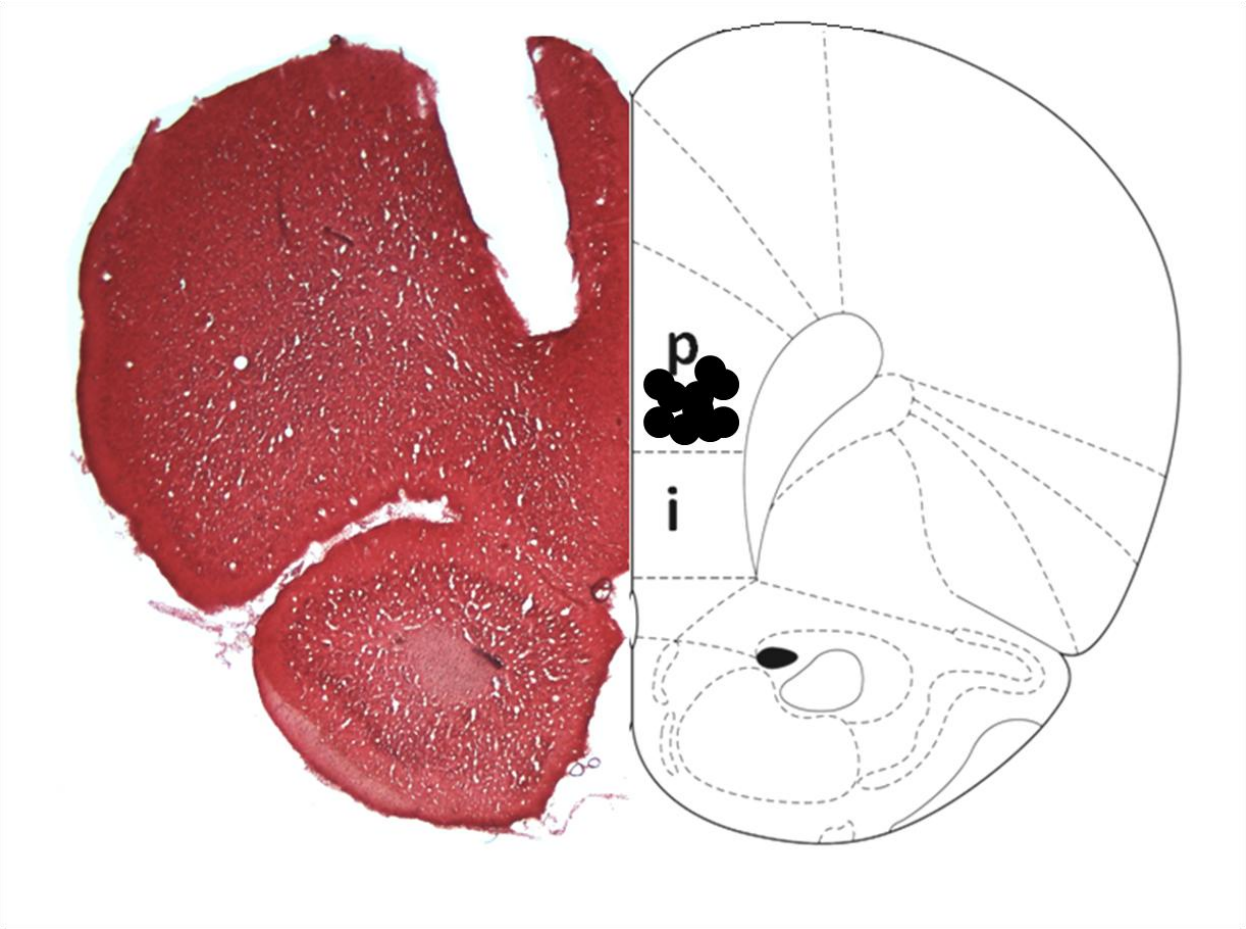

Figure 8

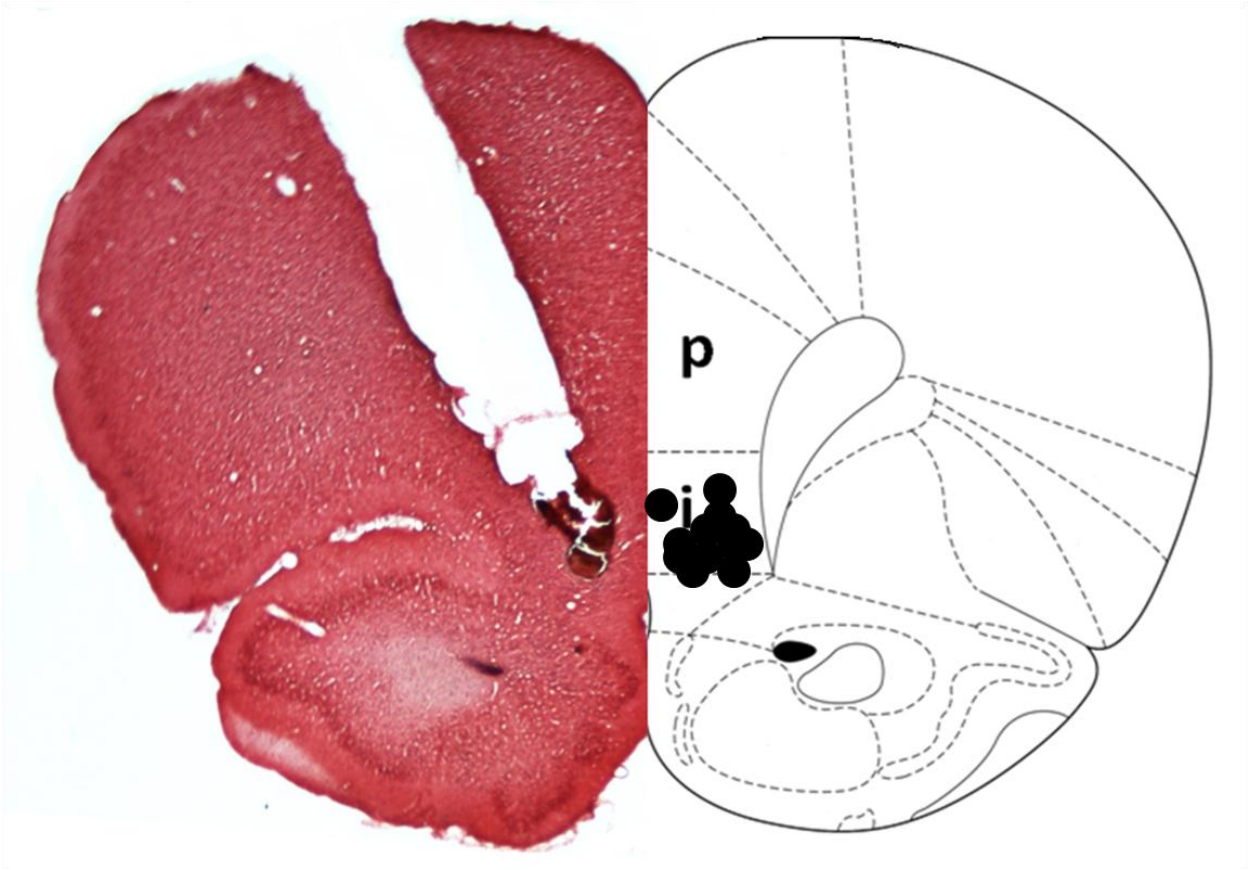

Figure 9

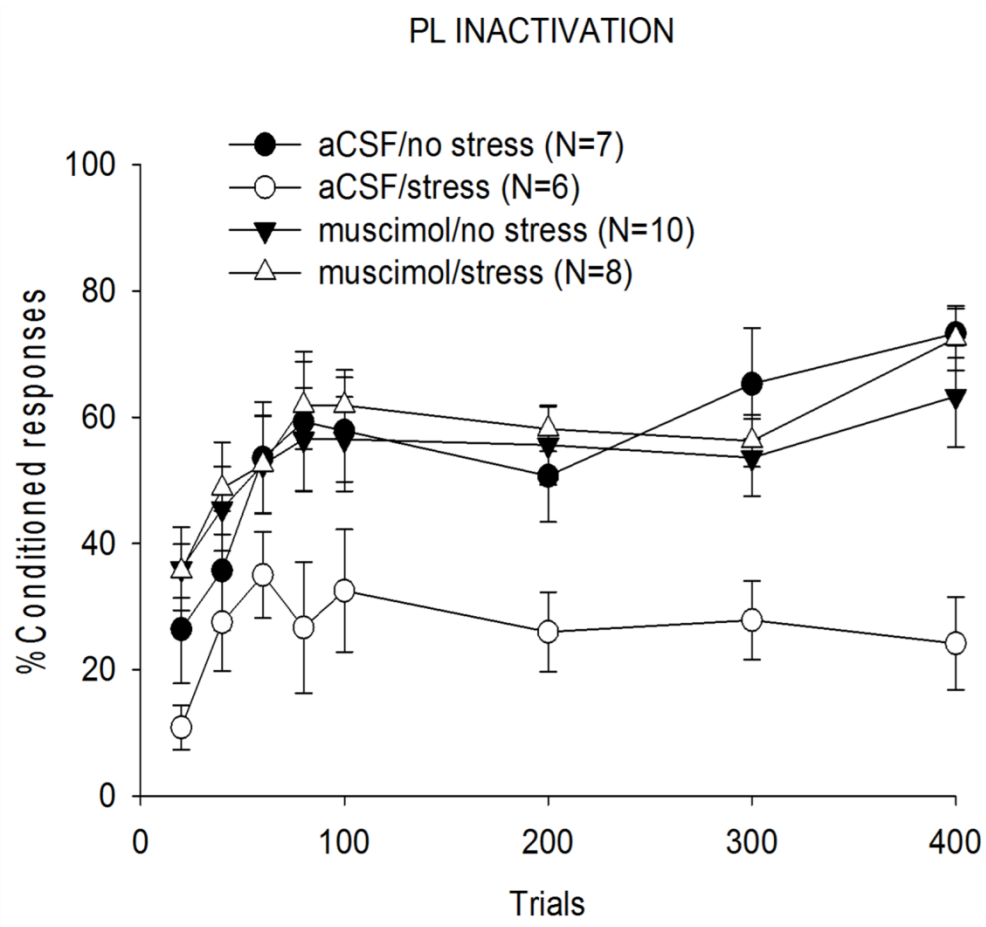
PL INACTIVATION
aCSF/no stress (N=7)
aCSF/stress (N=6)
muscimol/no stress (N=10)
muscimol/stress (N=8)
% Conditioned responses
100
80
60
40
20
0
0
100
200
300
400
Trials

Figure 10

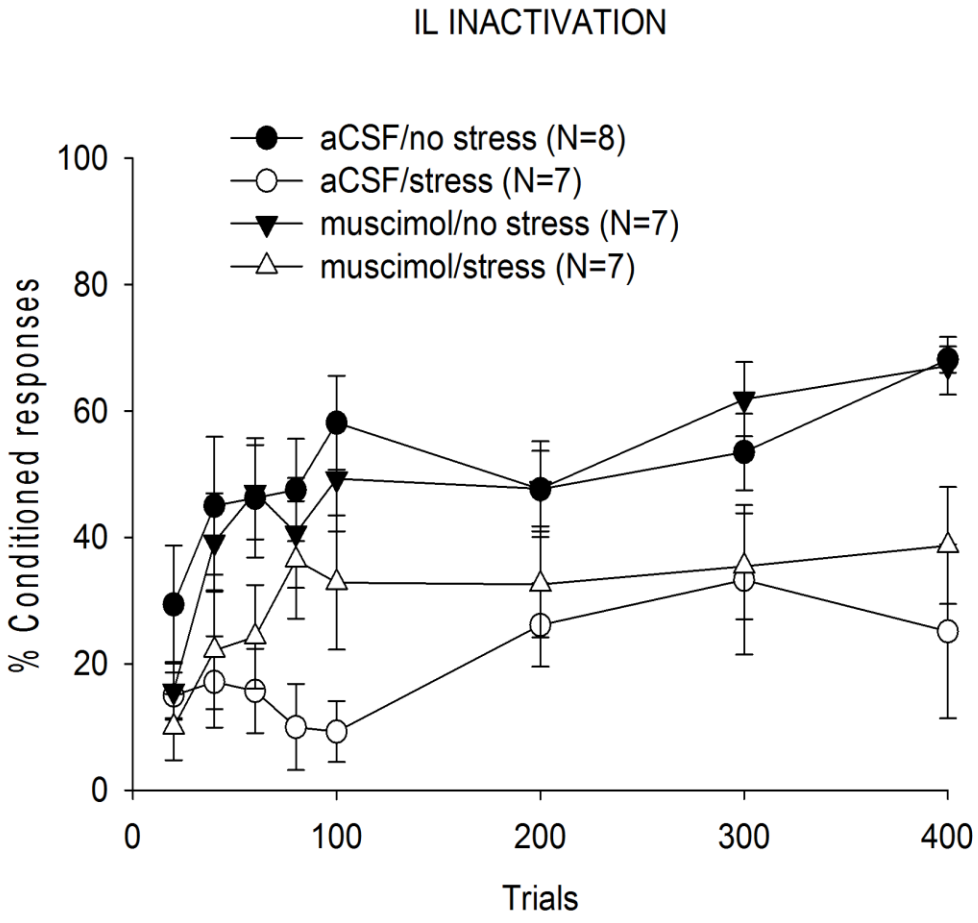
IL INACTIVATION
aCSF/no stress (N=8)
aCSF/stress (N=7)
muscimol/no stress (N=7)
muscimol/stress (N=7)
% Conditioned responses
100
80
60
40
20
0
0
100
200
300
400
Trials

Figure 11

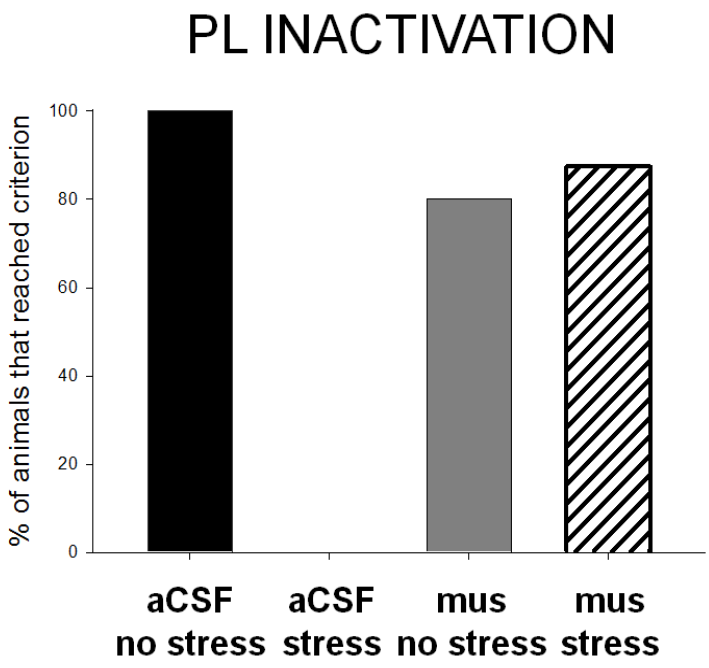
PL INACTIVATION
% of animals that reached criterion
100
80
60
40
20
0
aCSF no stress
aCSF stress
mus no stress
mus stress

Figure 12

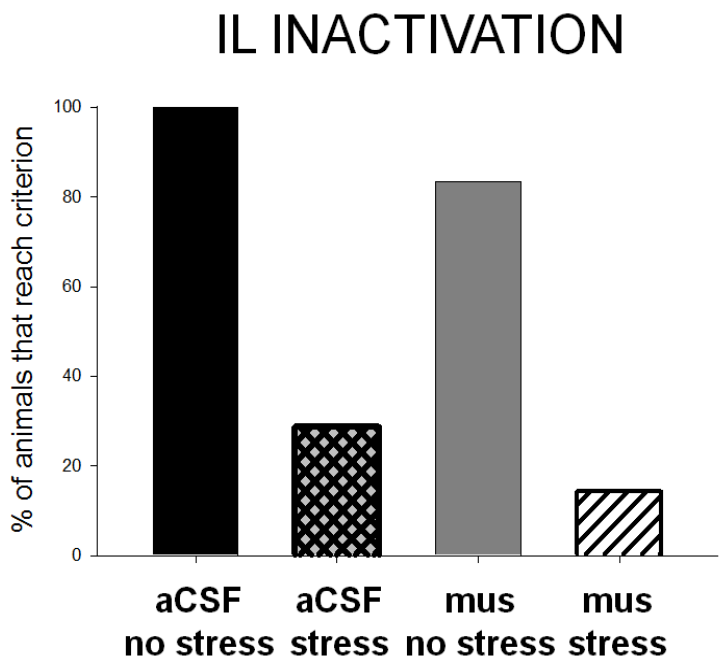
IL INACTIVATION
% of animals that reach criterion
100
80
60
40
20
0
aCSF no stress
aCSF stress
mus no stress
mus stress

Figure 13

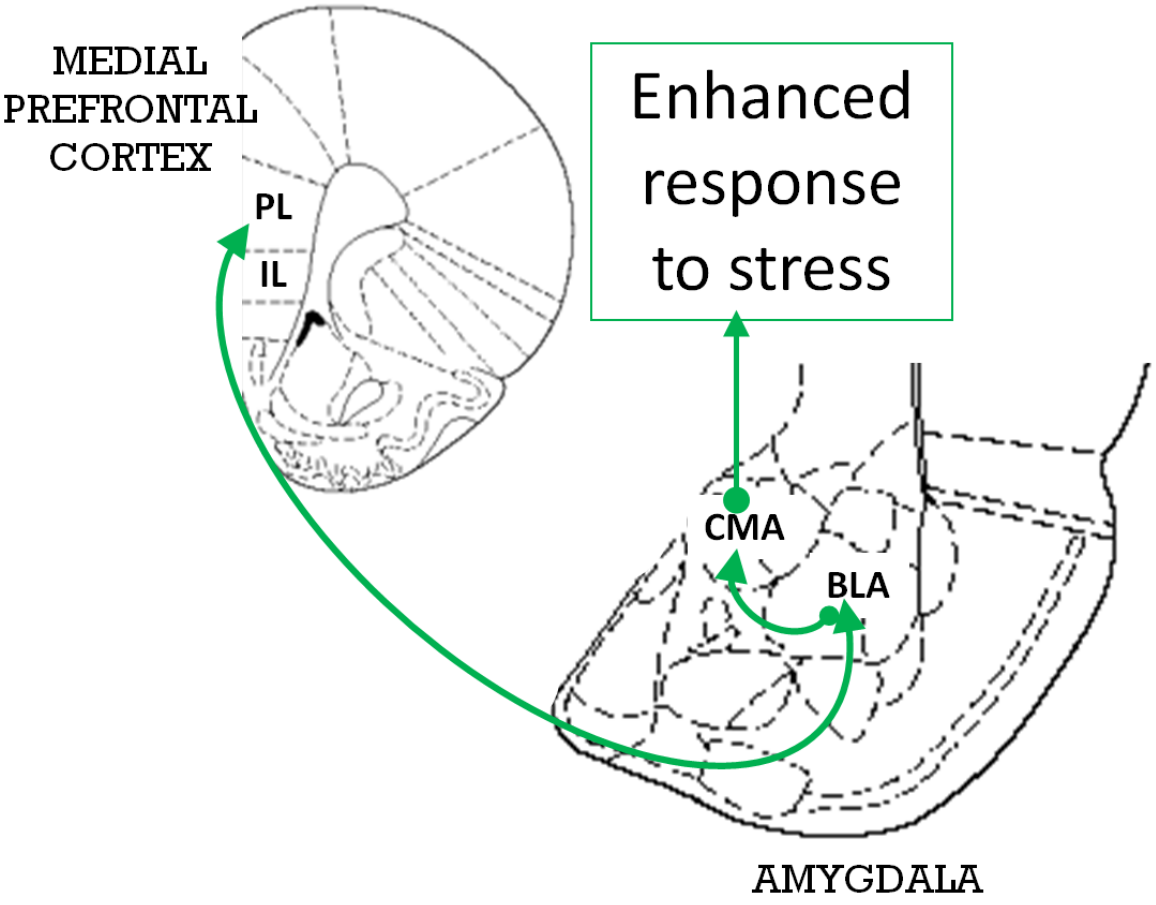
MEDIAL
PREFRONTAL
CORTEX
PL
IL
Enhanced
response
to stress
CMA
BLA
AMYGDALA

Figure 14

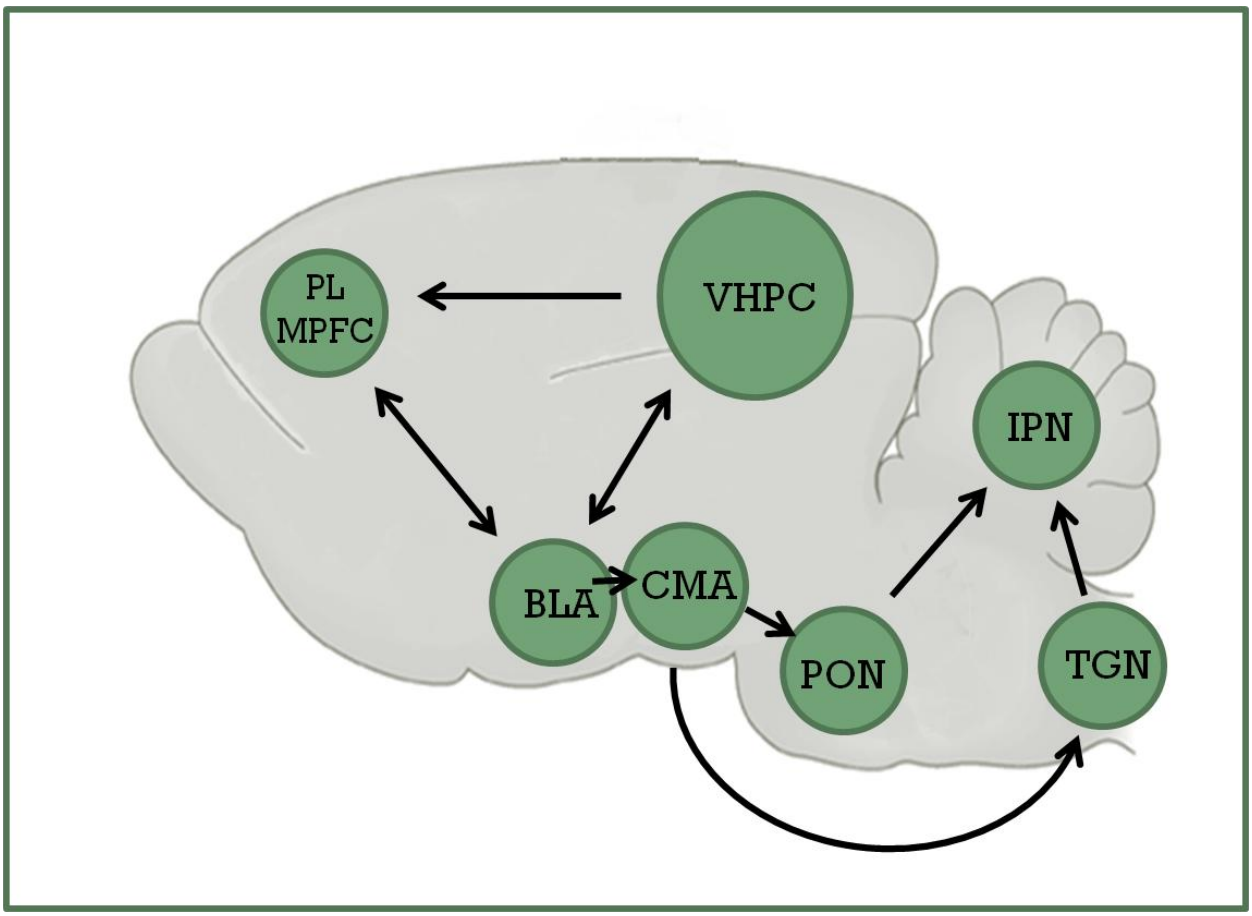

FIGURE LEGENDS

Figure 1. Critical brain regions of the emotion regulation circuitry in humans and rats. dlPFC: dorsolateral prefrontal cortex; vmPFC: ventromedial prefrontal cortex; AMY: amygdala; HPC: hippocampus; PL mPFC: prelimbic medial prefrontal cortex; IL mPFC: infralimbic prefrontal cortex.

Figure 2. Sex differences in mPFC inactivation. In a previous study, the mPFC was inactivated with muscimol or infused with aCSF during the stressor. One day later, animals were trained with delay conditioning. A, Inactivation of the mPFC did not prevent the stress-induced facilitation of learning in males. B, In contrast, mPFC inactivation in females prevented the suppression in learning after stress (Maeng et al., 2010).

Figure 3. mPFC-BLA disconnection. Acute stress exposure disrupted learning in females with ipsilateral mPFC-BLA lesions. In contrast, conditioned responding of animals with contralateral lesions was not impaired by stress and was similar to the performance of the unstressed females of both types of lesions. Thus, communication between the mPFC and BLA is necessary to impair learning after a stressful event in females (Maeng et al., 2010).

Figure 4. Diagram of the prelimbic (p) and infralimbic (i) subregions of the medial prefrontal cortex. The prelimbic subregion is located more dorsally and the infralimbic area more ventrally within the mPFC. (Image adapted from Paxinos & Watson, 1997).

Figure 5. Experimental timeline for Experiment 1 and 2. 5-7 days following cannulation and headstage surgery, animals were lavaged to monitor estrous cycle. When in diestrus 2, all female rats were acclimated to the conditioning chambers, infused with either vehicle aCSF or $GABA_A$ agonist muscimol. Immediately following infusions, the animals were either exposed to 15

minutes of swim stress or were returned to their home cages. 24h later, all rats were trained with four days of delay eyeblink conditioning.

Figure 6. Illustration of a conditioned eyeblink response. In the delay conditioning paradigm employed in this study, the 850ms white noise conditioned stimulus coterminated with a 100ms periorbital shock unconditioned stimulus. An eyeblink that occurred 250ms before the onset of the unconditioned stimulus was considered a conditioned response and was counted to assess learning.

Figure 7. Cannulation of the prelimbic cortex. This 0.1% neutral red-stained section and diagram at Bregma + 3.20mm illustrate the location of the bilateral cannula tip placement within this region. The cannula tips were implanted at an angle of 15˚to avoid damage to the sinus. Animals whose infusion sites were not in the prelimbic area were excluded from the study. (Image adapted from Paxinos & Watson, 1997).

Figure 8. Cannulation of the infralimbic cortex. This section at Bregma + 3.20mm (stained with 0.1% neutral red) and diagram illustrate the location of the cannula tip placement within this region. Cannula tips were angled at 30˚ to avoid damage to the overlying prelimbic cortex. Animals whose infusion sites were not in the infralimbic area were excluded from the study. (Image adapted from Paxinos & Watson, 1997).

Figure 9. Prelimbic medial prefrontal cortex inactivation. The prelimbic cortex is necessary for the stress effect on eyeblink conditioning in female rats. Vehicle-treated females that were swim stressed 24h before training showed little evidence of learning and emitted significantly fewer conditioned responses than those that were not stressed ($p<0.05$). The stressed females that received muscimol inactivations of the prelimbic mPFC were able to learn and learned well,

expressing more conditioned responses than the stressed controls ($p<0.05$). These data suggest that neural activity within the prelimbic subregion of the mPFC is critically involved in the stress-induced suppression of learning in females.

Figure 10. Infralimbic medial prefrontal cortex inactivation. The infralimbic medial prefrontal cortex is not necessary for the effect of stress on eyeblink conditioning in female rats. Females treated with aCSF vehicle that were trained 24h after swim stress exposure did not learn well and emitted significantly fewer conditioned responses than those that were not stressed ($p<0.05$). The females that received muscimol inactivations of the infralimbic mPFC during the stressor were also unable to learn like the stressed vehicle-treated females ($p>0.05$), expressing fewer conditioned responses than the unstressed animals ($p<0.05$). These data suggest that neural activity within the infralimbic subregion of the mPFC during the stressful event is not critical to impair conditioning in females.

Figure 11. The percentage of animals that reached a learning criterion of 60% conditioned responding in the prelimbic mPFC inactivation experiment. Animals that emitted at least 60% conditioned responses in one or more sessions of delay conditioning were considered to have learned well. No females in the aCSF/stress group met this criterion and learned well compared to the animals in the other three groups. Most of the muscimol-treated stressed animals did learn well just as the unstressed females.

Figure 12. The percentage of animals that reached a learning criterion of 60% conditioned responding in the infralimbic medial prefrontal cortex inactivation experiment. Animals that emitted at least 60% conditioned responses in one or more sessions of delay conditioning were considered to have learned well. Very few females in the aCSF/stress group met this criterion

and learned well in contrast to the percentage of animals that did learn well in the unstressed groups of either treatment. Like the stressed vehicle-treated animals, most of the stressed animals that received muscimol inactivation of the IL mPFC during stress did not learn well and failed to reach 60% conditioned responding during training.

Figure 13. mPFC-BLA circuits in stress modification of learning. In fear conditioning, this circuitry has been described for the expression of fear and the inhibition of fear responses. Assuming there is an overlap of circuitry with that mediating the responses to stress, we propose similar mPFC-BLA interactions that might modulate the stress-induced learning suppression in females. Prelimbic activity sends excitatory input (green) to the basolateral amygdala, which stimulates the central amygdala to elicit the response to stress and impair conditioning. Infralimbic activity excites intercalated cells, which sends inhibitory input (red) to the central amygdala to reduce the response to stress. PL: prelimbic mPFC; IL: infralimbic mPFC; CMA: centromedial amygdala; BLA: basolateral amygdala; ITC: intercalated cells of the centrolateral amygdala.

Figure 14. Potential circuitry mediating the stress-induced suppression of eyeblink conditioning in females. Stress exposure may alter activity and/or structure of and the connections between the ventral hippocampus, basolateral amygdala, and prelimbic medial prefrontal cortex to modify eyeblink conditioning in females. PL MPFC: prelimbic medial prefrontal cortex; VHPC: ventral hippocampus; BLA: basolateral amygdala; CMA: centromedial amygdala; IPN: interpositus nucleus; TGN: trigeminal nucleus; PON: pontine nuclei.

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