THE PREFRONTAL CORTEX COMMUNICATES WITH THE AMYGDALA TO IMPAIR LEARNING AFTER AN ACUTE STRESSFUL EXPERIENCE IN FEMALES

by

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Psychology

written under the direction of

Dr. Tracey J. Shors, PhD

and approved by

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New Brunswick, New Jersey

May 2010

ABSTRACT OF THE THESIS

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Exposure to an acute stressful event enhances classical eyeblink conditioning in male rats, whereas exposure to the same event dramatically impairs performance in females (Wood & Shors, 1998; Wood et al., 2001). We hypothesized that stress affects learning differently in males and females because different brain regions and circuits are being activated. In the first experiment, we determined that neuronal activity within the medial prefrontal cortex (mPFC) during the stressful event is necessary to disrupt learning in females. In both males and females, the mPFC was bilaterally inactivated with GABA agonist muscimol prior to the stressor. Inactivation only prevented the impaired performance in females; it had no consequence for performance in males. Previous studies indicate that neuronal activity within the basolateral amygdala (BLA) during the stressful event is necessary for the impaired performance in females (Waddell et al., 2008). In the second experiment, we hypothesized that the mPFC communicates with the BLA to disrupt learning in females after the stressor. To test this hypothesis, these structures were disconnected from each other with unilateral excitotoxic (NMDA)

lesions on either the same or opposite sides of the brain. Females with contralateral lesions, which disrupt the connections on both sides of the brain, were able to learn after the stressful event, whereas those with ipsilateral lesions, which disrupt only one connection, did not learn after the stressor. Together, these data indicate that the mPFC is preferentially engaged in females during stress to impair subsequent learning and does so via communication with the amygdala.

Acknowledgements

I would like to thank my advisor, Dr. Tracey J. Shors, for her support and scholarly guidance throughout every phase of this project. I would also like to thank Dr. Jaylyn Waddell for her contributions to these studies and for her mentorship in training me with the techniques used to conduct these experiments. Last but not least, I would like to thank Drs. Tim Otto and Mark West for serving on this committee and for their valuable advice and suggestions.

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Introduction

Traumatic life experiences tend to be more debilitating in women, rendering them twice as likely as men to develop stress and anxiety-related disorders (10.4% versus 5.0%; Kessler et al., 1995; Carter-Snell & Hegadoren, 2003; Foa & Street, 2001; Tolin & Foa, 2006). This vulnerability in women may relate to sex differences in the stress response, which have been reported in laboratory animals. For example, stressors such as inescapable swim stress or brief stimulations to the tail enhance a type of associative learning, classical eyeblink conditioning, in male rats and mice, whereas the same stressors elicit profound learning deficits in females (Wood & Shors, 1998; Wood et al., 2001).

These behavioral differences in response to stress are mediated by sex differences in neural and hormonal processes within specific brain regions. For example, the hippocampus and basolateral amygdala (BLA) are critically engaged in both males and females to modify learning after stress (Bangasser & Shors, 2007; Waddell et al., 2008); however, the bed nucleus of the stria terminalis (BNST) is necessary only in males (Bangasser et al., 2005; Bangasser & Shors, 2008). One brain region that has not been evaluated in this context is the medial prefrontal cortex (mPFC). This brain region is a likely participant because it is activated during the stress response and interconnects with the hippocampus, BNST, and BLA (Vertes, 2004, 2006; Cerqueira et al., 2008; Diorio et al., 1993). Furthermore, stress-related disorders are associated with differences in both structure and function of the mPFC (Bremner et al., 1999; Drevets, 2000; Rajkowska, 2000; Luine, 2002). One study finds that mPFC-mediated learning is more sensitive to stress in females than in males (Shansky et al., 2006). Therefore, in the first experiment,

we hypothesized that the mPFC would be critically engaged in females, but less so in males, to modify learning after a stressful experience. To test this hypothesis, the mPFC was inactivated during the stressor. One day later, both sexes were trained to learn the classically conditioned eyeblink response. Overall, we found that the mPFC is critically engaged during the stressor to reduce learning in females but is not to enhance learning in males.

The mPFC and amygdala interconnect to affect emotional responses to stress, presumably via anatomical connections between them (Heidbreder & Groenewegen, 2003; Vertes, 2004). For example, lesions to the prefrontal cortex reduce the extinction of a fear response, which depends on the amygdala to learn (Morgan & LeDoux, 1995). Based on these interactions, we hypothesized that the mPFC and amygdala communicate with each other to reduce learning after stress, specifically in females. To test our hypothesis, both structures were excitotoxically lesioned on either the same (ipsilateral) or opposite (contralateral) sides of the brain. Those with ipsilateral lesions would have one intact connection, whereas those with contralateral lesions of the brain would have neither connection intact. In support of our hypothesis, females with contralateral lesions performed as if they had not experienced the stressful event, indicating that mPFC and BLA communication is critical for the stress-induced learning impairment.

Materials and Methods

Experiment 1: mPFC Inactivation

Subjects. Male and cycling female Sprague-Dawley rats between 90-120 days of age were used and obtained from a breeding facility at Rutgers University. Rats were housed in groups of 3-4 until surgery. Following surgery, rats were housed alone in standard plastic "shoebox" cages (44.5 cm long, 21.59 cm wide, and 23.32 cm high). Rats were maintained on ad lib access to rat chow and water on a 12 hr light-dark cycle. All experiments were conducted with full compliance to the rules and regulations specified by the PHS Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Vaginal Cytology. To monitor the 4 phases of the estrus cycle, samples of loose vaginal cells were taken with cotton-tipped swabs soaked in sterile saline and rolled onto slides (lavage). The slides were then stained with 1% toluidine blue, rinsed and dehydrated with 95% ETOH for estrus phase assessment under a light microscope. Proestrus is characterized by purple staining of epithelial cell nuclei, estrus by masses of aggregated dark blue cornified cells, diestrus 1 by dark leukocytes and scattered epithelial cells, and diestrus 2 by similar cell types but more sparse. It has been determined that the stress effect was most pronounced in females when they are stressed in diestrus 2 and trained 24 hrs later in proestrus (Shors et al., 1998). Therefore, females used in this study were lavaged daily after a 1-week recovery period following surgery, stressed in diestrus, and trained in proestrus, when estrogen concentrations are increasing (Shors et al., 1998;

Wood et al., 2001). Animals that failed to exhibit a normal estrus cycle were eliminated from the study.

Surgery. All rats were anesthetized with sodium pentobarbital (50 mg/kg for males and 40 mg/kg for females). After being placed in the stereotaxic instrument, the scalp was cleaned with Betadine, and an incision was made. Guide cannulas (23 gauge, Plastics One, Roanoake, VA) were implanted into the mPFC, bilaterally aimed at the junction of the prelimbic and infralimbic cortex. Cannulas were implanted at a 15º angle at the following coordinates relative to bregma: (AP: +3.1 mm; ML: ±1.6 mm; DV: -3.3 mm from dura). Following cannulation, both cannulas and headstages were fitted onto the skull with dental cement and anchored by skull screws. The headstages were attached to 4 electrodes; two delivered the unconditioned stimulus (US) of periorbital stimulation, and two recorded electromyographic (EMG) activity as a measure of blinks. The electrodes (insulated stainless steel wire with a diameter of 0.005 in.) were implanted through the upper eyelid muscle. The insulation was removed from a section of each electrode in order to make contact with the muscle. Each of the electrode wires was coiled securely in place.

Muscimol Infusions. During infusions, stylets were replaced with infusion cannulas protruding 1 mm past the guide cannula. Infusion cannulas were attached to a microinfusion pump via polyethylene tubes connected to 10 µl Hamilton syringes. The syringe and tubes were filled with water, and a small air bubble separated the water from artificial cerebral spinal fluid (aCSF) or muscimol solution. Rats were infused with 0.5

µg muscimol at a dose of 1 µg/µl into each hemisphere. The drug was infused at a rate of 0.125 µl/min over 4 min for a total of 0.5 µl.

Stress Procedure. At least 7 days after the surgery, rats were acclimated to the conditioning chamber (60 min) and spontaneous blinks were recorded. They were then transported to a separate context and infused with aCSF or muscimol. Half of this group was transferred into a different context and placed in a dark soundproof chamber. They were loosely restrained and exposed to 30 low intensity (1 mA, 60 Hz, 1 sec) stimulations to the tail. This is the minimum amount of stress necessary to induce the opposite effects of stress on classical eyeblink conditioning (Shors & Servatius, 1997; Shors, 2004).

Classical Conditioning. 24 hrs later, rats were returned to the conditioning chamber and exposed to ten white noise stimuli alone (250 ms, 80 dB, ITI 25 $\pm$ 5 s) before the first session. This procedure is used to assess potential effects of the stressor or infusion on sensitized responses to the CS (blinks during first 100 ms of the CS) (Servatius & Shors, 1994). The rats were then trained with 400 trials (100 trials/day) of paired stimuli using a 80-dB, 850 ms burst of white noise CS overlapping with a 100 ms, 0.5 mA periorbital stimulation of the eyelid (US). These studies were conducted using a delay conditioning procedure in which the CS and the US overlap in time and co-terminate. Eyeblinks were assessed by significant changes in the magnitude of the EMG response recorded from the eyelid muscles. Activity that exceeded 10 ms, 0.3 mV (and > 4 standard deviations) when compared to activity within the 250 ms pre-CS baseline recording period (Figure 1) were considered indicative of an eyeblink.

Histology. After behavioral testing, rats were given a lethal dose of sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.9% saline solution followed by 10% buffered formalin. Brains were extracted and post-fixed in formalin for at least 24 hrs. The brains were then cryoprotected in a 30% sucrose-formalin solution for at least 3 days, after which the brains were frozen and sectioned into 40 µm-thick coronal sections using a cryostat. Every third section was mounted onto gelled slides and stained with cresyl violet to verify accuracy of cannula placements. A rater, blind to behavioral data, assessed cannula placements. The locations were considered accurate 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 and at least 1 mm above the ventral boundary of the infralimbic cortex. Placements within the mPFC were between +3.20 and +2.70 mm relative to bregma. A reconstruction of placements that were considered accurate is shown in Figure 2. Site of drug infusion was assessed by track markings of the infusion cannula. Rats were excluded from analysis if placements were not within the mPFC or if the mPFC was excessively damaged by the cannula or the infusion. The final number of animals in each group was as follows: Males/Vehicle/No Stress: n=8; Males/Vehicle/Stress: n=8; Males/Muscimol/Stress: n=7; Females/ Vehicle/No Stress: n=10; Females/Vehicle/Stress: n=8; Females/Muscimol/Stress: n=8.

Experiment 2: mPFC ↔ BLA Disconnection

Subjects. Cycling female Sprague-Dawley rats between 90-120 days of age were obtained from a breeding facility at Rutgers University. Rats were housed in groups of 3-4 until surgery. Following surgery, rats were housed alone in standard plastic "shoebox"

cages (44.5 cm long, 21.59 cm wide, and 23.32 cm high). Rats had ad lib access to rat chow and water and were maintained on a 12 hr light-dark cycle. All experiments were conducted with full compliance to the rules and regulations specified by the PHS Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Vaginal Cytology. As in the first experiment, phases of the estrus cycle were monitored via lavage and those without normal cycles were eliminated from the study.

Surgery. Female rats were anesthetized with sodium pentobarbital (50 mg/kg). After being placed in the stereotaxic instrument, the scalp was cleaned with Betadine, and an incision was made. Excitotoxic lesion sites were infused with NMDA via a 10 µl Hamilton syringe attached to a microinfusion pump. For lesions of the medial prefrontal cortex, the syringe tip was aimed at the prelimbic/infralimbic junction (AP: +3.0/+2.5 mm; ML: ±0.7 mm; DV: -4.5 mm from skull), and 0.1 µl of 10 mg/ml NMDA was infused at a rate of 0.1 µl/min. Coordinates for lesions of the basolateral amygdala were as follows: AP: -2.8 mm; ML: ±4.8 mm; DV: -8.5/-8.3 mm from skull (20 mg/ml NMDA; volume: 0.25 µl/ 0.15 µl; rate: 0.1 µl/min). All contralateral and ipsilateral lesions were counterbalanced and assessed subsequently for possible lateralization effects. Following infusions of excitotoxin, headstages were fitted onto the skull with dental cement and anchored by skull screws. The headstages were attached to 4 electrodes; two delivered the unconditioned stimulus (US) of periorbital stimulation, and

two recorded electromyographic (EMG) activity as a measure of blinks. The electrodes were implanted as described in Experiment 1.

Stress Procedure. At least 7 days were allowed for recovery time after surgery. Cycling rats in diestrus 2 were placed into the conditioning chamber for an acclimation period. Rats to be stressed were then transferred to a separate room into an enclosed soundproof box and underwent brief stress exposure as described in Experiment 1.

Classical Conditioning. Prior to either stress exposure or none, the rats were placed into the conditioning boxes for a habituation period in which they acclimated for 1 h while spontaneous blinks were recorded and then 24 hrs later, were returned to the chamber. As in Experiment 1, rats were observed for a sensitization period then began training with delay eyeblink conditioning.

Histology. After behavioral testing, rats were administered a lethal dose of sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.9% saline solution followed by 10% buffered formalin. Brains were extracted and post-fixed in formalin for at least 24 hrs. The brains were then cryoprotected in a 30% sucrose-formalin solution for at least 3 days, after which the brains were frozen and sectioned into 50 μm-thick coronal sections using a cryostat. Every third section was mounted onto gelled slides and stained with the cresyl violet to verify lesion size. A rater, blind to behavioral data, assessed lesion placements. Rats were excluded from the study if lesions were misplaced or incomplete. Lesions were identified by the location of the needle track, absence of nerve

cell bodies, and gliosis, or the presence of darkly stained astrocytes (Bangasser et al., 2005). The extent of the smallest and largest lesion is presented in Figure 3. The final number of animals in each group was as follows: Ipsilateral/no stress: n=10; Ipsilateral/stress: n=10; Contralateral/no stress: n=10; Contralateral/stress: n=10.

Results

Experiment 1: mPFC Activity in Males vs. Females

In the first experiment, the mPFC was inactivated during the stressor in males and females. All animals were trained with delay conditioning 24 hrs later. Anticipatory conditioned responses (CRs) prior to the US were counted and averaged across sessions of 100 trials (Figure 4). Males were analyzed separately from females. Three groups of males were trained: one group whose mPFC was inactivated during the stressor, another stressed and injected with saline and a third saline group that was not stressed. To conserve animals, an unstressed group injected with muscimol 24 hrs before training was not tested. The independent measures were stress versus no stress and inactivation with muscimol versus saline vehicle infusion.

To assess acquisition of the CR across trials of training in males, a repeated measures ANOVA across the 4 sessions of trials was conducted on each group. All three groups increased the number of CRs as training progressed [$F(3, 60)=22.33$; $p<0.01$]. There was no interaction between the groups and responding across the sessions [$F(6, 60)=0.13$; $p>0.05$)]. The percentage of responses differed among groups when the CRs were collapsed across the four sessions of training trials [$F(2, 20)=7.81$; $p<0.01$]. A Tukey HSD *post hoc* analysis confirmed that performance during training of the unstressed group injected with aCSF before the stressor emitted fewer CRs than the stressed group injected with either muscimol ($p<0.01$) or aCSF ($p<0.05$). In males, those that were exposed to the stressor emitted more CRs than those that were not exposed to the stressor ($p<0.05$) (Figure 4A). The increase in responding occurred regardless of whether or not the mPFC was inactivated with muscimol during the stressor. Thus,

muscimol infusion prior to stress exposure did not abolish the subsequent facilitation of eyeblink conditioning elicited by stress in males.

The first 5 blocks of twenty trials were analyzed with a repeated measures ANOVA. There was no effect of group [$F(2, 20)=3.24$; $p>0.05$] or block x group interaction [$F(8, 80)=0.79$; $p>0.05$]. However, there was a main effect of blocks of trials [$F(4, 80)=8.81$; $p<0.01$], as males increased their conditioned responding across trial blocks. Thus, there was no effect of drug treatment or stress on early learning but an effect on conditioned responding in the later sessions.

As in males, three groups of females were trained: one group whose mPFC was inactivated during the stressor, another stressed and injected with saline and a third saline group not stressed. To assess acquisition across training in females, a repeated measures ANOVA across the four sessions of trials was conducted on each group. The analysis revealed an effect of session, as conditioned responding increased across training days [$F(3, 69)=29.09$; $p<0.01$]. There was no interaction among the groups (stress with and without muscimol or no stress) and performance across trials of training [$F(7, 161)=1.18$; $p>0.05$]. Using the percentage of CRs across trials of training as the dependent measure, there was a main effect of group [$F(2, 23)=11.65$; $p < 0.01$]. A Tukey HSD *post hoc* test confirmed that the females that were injected with a vehicle in the mPFC before the stressor emitted fewer responses than those that were not stressed ($p<0.01$). However, those that were stressed while their mPFC was inactivated learned well, emitting more CRs than those that were stressed in the presence of the vehicle ($p<0.01$) (Figure 4B). These data indicate that neuronal activity within the mPFC during a stressor is necessary to impair performance of the CR in females.

To assess the effects of stress or muscimol infusion on early acquisition, a repeated measures ANOVA was run on five 20-trial blocks for the first 100 trials. There was no effect of group [$F(2, 23)=2.60$; $p>0.05$] or group x trial interaction [$F(8, 92)=1.23$; $p>0.05$]. However, there was an effect of blocks [$F(4, 92)=9.94$; $p<0.01$], as the animals learned and increased responding as blocks of trials proceeded. Therefore, stress or drug infusion did not differentially influence early responding but rather altered performance during training in the later sessions.

Experiment 2: Communication Between the mPFC and the Amygdala in Females

Experiment 2 assessed whether communication between the mPFC and BLA was necessary to impair learning in females after acute stress exposure. To do so, females were stressed either with ipsilateral or contralateral lesions to the mPFC and BLA. The behavioral results are presented in Figure 5. A repeated measures ANOVA across the four sessions of 100 trials of training revealed an effect of session [$F(3, 108)=32.99$, $p<0.01$], indicating that animals increased their conditioned responding as training progressed. The main effect of group was also significant [$F(3, 36)=5.83$, $p<0.01$]. Planned comparisons confirmed that females that were stressed with lesions on the same side of the brain responded with fewer CRs than those that were not stressed with the same type of lesions [$F(1, 36)=10.21$, $p<0.01$]. Furthermore, females in this group (stressed with ipsilateral lesions) also emitted fewer CRs than those in the other three groups [planned comparison: $F(1, 36)=16.47$, $p<0.01$]. However, those that were stressed with lesions on opposite sides of the brain performed no differently than their unstressed counterparts [planned comparison: $F(1, 36)=0.99$, $p>0.05$]. Importantly, the lesions did

not alter performance itself. Females that were not stressed but had lesions on the same side performed similarly to those that were not stressed and had lesions on the opposite sides [planned comparison: $F(1, 36)=0.46$, $p>0.05$].

To assess learning in the early trials of training, a repeated measures ANOVA was conducted on the first 100 trials, which are presented in Figure 5 in blocks of 20 trials. There was no main effect of group [$F(3, 36)=1.14$, $p>0.05$]. However, there was an effect of blocks of trials [$F(4, 144)=10.13$, $p<0.01$] and an interaction between group and blocks of trials [$F(4,144)=1.98$, $p<0.05$]. Tukey HSD *post hoc* comparisons revealed that although exposure to the stressor or type of lesion did not influence responding in the first four 20-trial blocks of training, the stressed females with ipsilateral lesions emitted fewer CRs than the stressed females with lesions on opposite sides of the brain by the last block of 20 trials ($p<0.05$). Moreover, there was no difference in early responding between the unstressed females with lesions on the same side and the stressed and unstressed females with lesions on opposite sides ($p>0.05$). Thus, the effects of lesion and stress on learning were apparent during the first session of training and persisted throughout the remaining 3 sessions as asymptotic performance was reached.

Rats were considered to have learned the response if they emitted at least 60% CRs during 2 consecutive sessions of training. Using this criterion, we determined that all the groups learned except those that were stressed with unilateral and ipsilateral lesions to the mPFC and the BLA (Figure 6). Thus, stressed females whose mPFC and amygdala were still in communication did not learn, whereas those that had disrupted communication on each side learned the CR as well as those that were not exposed to the stressor. These data indicate that communication between the mPFC and BLA is

necessary to impair associative eyeblink conditioning in females after an acute stressful event.

Discussion

Exposure to an acute stressful event enhances classical eyeblink conditioning in males but profoundly disrupts this type of learning in females (Wood & Shors, 1998). In fact, most females that are exposed to the stressor do not show much evidence of learning, even after hundreds of trials of training (Leuner & Shors, 2006). Here we report that the mPFC is critically engaged during the stressor to induce this impairment. This was determined by inactivating the brain region with muscimol during the stressful event and training the animals one day later in the absence of the drug. When the mPFC was functionally inactivated, stressed females were able to learn. Given the time course, we conclude that the mPFC is critically engaged during the stressful event and is not necessary during the process of learning. Interestingly enough, inactivation of the medial prefrontal cortex (mPFC) during the stressful event did not prevent the enhanced conditioning in males. Thus, neural activity within the mPFC is necessary to disrupt learning in females but is not necessary to enhance performance in males after stress. These results are novel because they indicate that activity within the mPFC during a stressful event is necessary to impair future learning in females.

It is somewhat surprising that neuronal activity within the mPFC was *not* necessary to enhance learning in males after the stressor. The mPFC is involved in numerous processes related to associative learning, including eyeblink conditioning (Kronforst-Collins & Disterhoft, 1998; Fuster, 2001; Takehara et al., 2002; Goldman-Rakic, 1995). Furthermore, the mPFC is activated during stress (Cerqueira et al., 2008), is densely populated with glucocorticoid receptors (Lupien & Lepage, 2001), and also plays a role in the hypothalamo-pituitary-adrenal response to stress (Diorio et al., 1993;

Figueiredo et al., 2003; Radley et al., 2006). In humans, stress-related disorders have been associated with differences in both the structure and function of the mPFC (Bremner et al., 1999; Drevets, 2000; Rajkowska, 2000). Similarly, stress exposure in rodents induces dendritic remodeling within the mPFC (Brown et al., 2005; Shansky & Morrison, 2009; Garrett & Wellman, 2009). The female mPFC seems especially sensitive to stress (Shansky et al., 2006; Garrett & Wellman, 2009; Ter Horst et al., 2009), as well as fluctuating estrogen concentrations (Gerrits et al., 2006). Others find that the mPFC modulates the effects of controllability on processes of learning. Specifically, Maier and colleagues found that inactivation of the mPFC during the stressor prevented the protective effects of "controllability" on helplessness behavior and fear conditioning (Amat et al., 2005). In other words, animals that established control over the stress were still helpless if the mPFC was not functioning while they did so. Moreover, these animals expressed more fear during conditioning, even though they had established control over the stressor (Amat et al., 2005). In previous studies, we found that the effects of stress on eyeblink conditioning are mediated by controllability (Leuner et al., 2004), although we did not manipulate controllability here. One would expect that males with control would express more eyeblink conditioning when the PFC was inactivated. To our knowledge, the role of the mPFC in the detection of controllability has not been examined in females.

In the second experiment, we further determined that the mPFC communicates with the basolateral nucleus of the amygdala (BLA) to impair learning after stress. Females that had lesions on opposite sides of the brain, i.e. those in which the connections between the mPFC and the amygdala were disrupted in both hemispheres,

were able to learn well after the stressor. In contrast, stressed animals that received unilateral lesions on the same side of the brain did not learn. It is presumed that this learning deficit was maintained by the one intact connection between the mPFC and BLA. The neurophysiological process by which this occurs is unknown. There are many reciprocal anatomical and physiological interactions between the mPFC and the amygdala (Krettek & Price, 1977; Porrino et al., 1981; Quirk et al., 2003; Hoover & Vertes, 2007). Notably, there are direct projections from the mPFC to the amygdala and extended amygdala (McDonald et al., 1999; McDonald, 1991). Interestingly, most studies indicate that the mPFC suppresses amygdalar activity though most corticoamygdalar projections are excitatory (Rosenkranz & Grace, 2001; Sotres-Bayon et al., 2004). This may occur via excitation of GABAergic BLA interneurons that decrease excitatory input to the central nucleus of the amygdala (Grace & Rosenkranz, 2002). There are also projections from the basolateral amygdala to the frontal cortex (Kita & Kitai, 1990), especially to the dendritic spine heads of mPFC layers II and V (Bacon et al., 1996). Stimulation of the BLA also modifies neuronal responses in the mPFC. Based on latency, it appears that some connections are monosynaptic while others are polysynaptic (Perez-Jaranay & Vives, 1991). However, projection neurons from the BLA to the mPFC are immunoreactive for glutamate and/or aspartate, supporting direct monosynaptic excitatory input to the mPFC from the BLA (McDonald et al., 1989).

The functional significance of the reciprocal connectivity demonstrated between the BLA and mPFC has been extensively explored, especially as they interact with stress and learning. As noted, the mPFC can suppress activity within the BLA when it is activated first, suggesting a distinct modulatory role for the mPFC in its communication

with the amygdala (Likhtik et al., 2005; Rosenkranz & Grace, 2001; Sotres-Bayon et al., 2004). Stimulation of the mPFC also can reduce physiological responses in the central nucleus of the amygdala, suggesting that it may modulate activity between the BLA and the central nucleus (Quirk et al., 2003), perhaps via inhibitory intercalated cells (Paré, 2003; Quirk et al., 2003). The deficit in learning examined here may be mediated by activity within the mPFC, which then modulates the expression of fear as it is expressed via output from the amygdala. Indeed, many studies report inhibitory control of the amygdala by the mPFC during emotional learning, such as during the extinction of fear (Morgan et al., 1993; Morgan & LeDoux, 1995; Quirk et al., 2000; Sotres-Bayon et al., 2004).

Alternatively, the amygdala may modulate activity in the mPFC to impair learning. Acute stress exposure prevents the induction of LTP *in vivo* when recording from cells within the mPFC in response to stimulation of the BLA-mPFC pathway (Maroun & Richter-Levin, 2003). Others find that BLA stimulation modulates neuronal activity in the mPFC (Perez-Jaranay & Vives, 1991). Furthermore, fear conditioning, which relies on the amygdala, can inhibit activity of prefrontal cortical neurons (Garcia et al., 1999), again pointing to amygdalar regulation of the mPFC. Alternatively, it could be that concurrent activity within the BLA and the mPFC is necessary to impair learning after stress. Both structures are involved in learning simple associations, and their activity can modulate performance of the conditioned eyeblink response (Kronforst-Collins & Disterhoft, 1998; Powell et al., 1996; Lee & Kim, 2004). They project not just to each other but to brain structures involved in the limbic-hypothalamic-pituitary-adrenal (LHPA) stress circuit (López et al., 1999). In previous studies, we found that the

hippocampus is involved in these effects of stress on learning (Bangasser et al., 2007). Thus, activity within the mPFC may communicate with the amygdala by way of the hippocampus to disrupt learning after stress.

The vast majority of studies about the mPFC and BLA have been conducted exclusively in males. However, some studies have examined and find sex-specific effects. For example, stress and estrogen treatment induce more dendritic arborization in neurons that project from the BLA to the mPFC when compared to the same measures in males or ovariectomized females (Shansky & Morrison, 2009). Because the deficit in learning expressed by stressed females reported here is dependent on the presence of estrogen (Wood & Shors, 1998), it is likely that estrogen is acting within one and/or the other structure to modulate learning. In summary, the present data suggest that the mPFC and the amygdala interact with each other to impair associative learning specifically in females. Minimally, these findings indicate that males and females are using different brain regions and circuits to modify learning after stress. More generally, they may provide clues as to why women are so much more vulnerable than men are to stress-related mental illness, such as post-traumatic stress disorder (PTSD) and depression.

Figure Captions

Figure 1. mPFC-BLA disconnection procedure. Animals with contralateral excitotoxic lesions received a unilateral lesion to the mPFC and a unilateral lesion to the BLA in opposite hemispheres. The contralateral lesion disrupted communication between the mPFC and BLA across both hemispheres. Animals with ipsilateral lesions received a unilateral lesion of the mPFC and a unilateral lesion of the BLA within the same hemisphere. Thus, the connection between the two structures was preserved in one hemisphere in these rats. If the concurrent activation of both the mPFC and BLA is necessary for the learning deficit after stress, then it would be expected that this impairment would be prevented in the contralateral females but maintained in the ipsilateral females.

Figure 2. Histology of mPFC inactivation. Cannula tip placements within the mPFC were between +3.20 and +2.70 mm relative to bregma as shown in these coronal sections (Paxinos & Watson, 1998). Animals were included if tips of the injection cannula were within the dorsal boundary of the prelimbic cortex and at least 1 mm above the ventral boundary of the infralimbic cortex. (p: prelimbic region of the medial prefrontal cortex; i: infralimbic region of the medial prefrontal cortex.)

Figure 3. Histology of mPFC-BLA disconnection. ***A***, mPFC lesions. ***B***, BLA lesions. Largest lesions (in gray) and smallest lesions (in black) of rats included in this study are depicted here on coronal sections (Paxinos & Watson, 1998). The unilateral images are representative of lesions in both hemispheres. Brain sections of the mPFC and BLA were

stained with 0.1% cresyl violet to verify sites of excitotoxic damage (marked by arrowheads). Note the darkly stained astrocytes and absence of cell bodies in lesioned tissue (4X). ***C***, Intact mPFC. ***D***, Lesioned mPFC. ***E***, Lesioned BLA. ***F***, Intact BLA.

Figure 4. mPFC inactivation. The mPFC was inactivated with muscimol or infused with saline during the stressor. One day later, animals were trained with delay conditioning. ***A,*** Muscimol-treated stressed males emitted more CRs than the saline-treated unstressed males, but performed similarly to those that were stressed and infused with saline. Thus, inactivating the mPFC did not prevent the stress-induced facilitation of learning in males. ***B,*** Stressed females infused with saline expressed fewer CRs than their unstressed counterparts. However, when females were infused with muscimol during the stressor, they increased responding and performed similarly to those that were unstressed. Thus, in contrast to males, mPFC inactivation in females eliminated the decremented conditioned responding following stress exposure.

Figure 5. mPFC-BLA disconnection. Acute stressful experience 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 for the stress-induced learning deficit in females.

Figure 6. The percentage of female rats in each lesion and stress condition that met learning criterion. Animals that learned emitted at least 60% CRs in at least two (of the four) consecutive sessions of 100 trials.

Figure 1

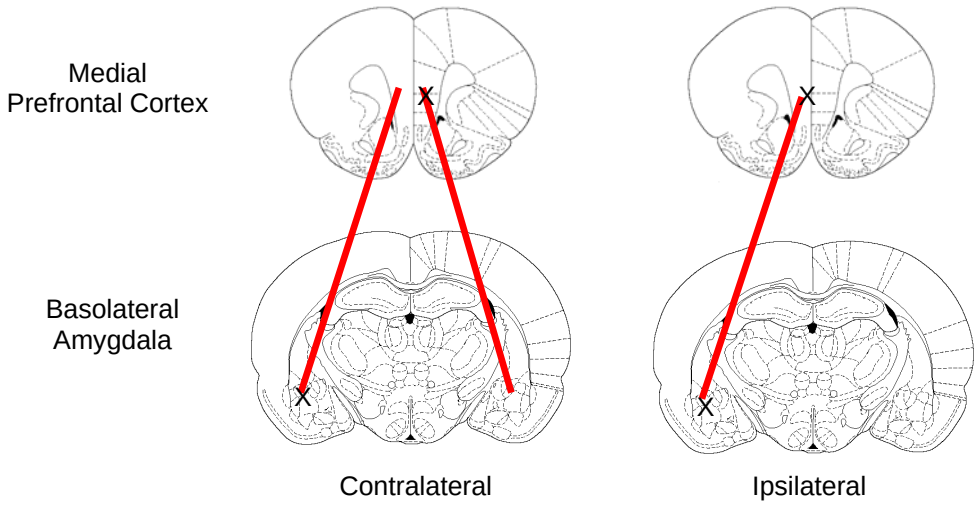

Medial
Prefrontal Cortex
Basolateral
Amygdala
Contralateral
Ipsilateral

Figure 2

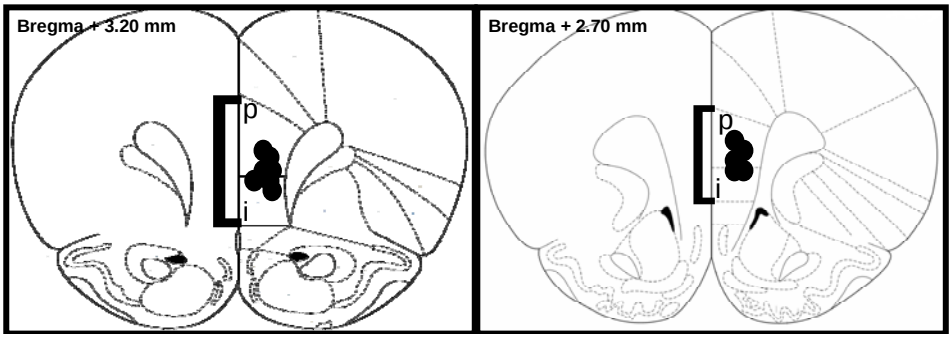


Figure 3

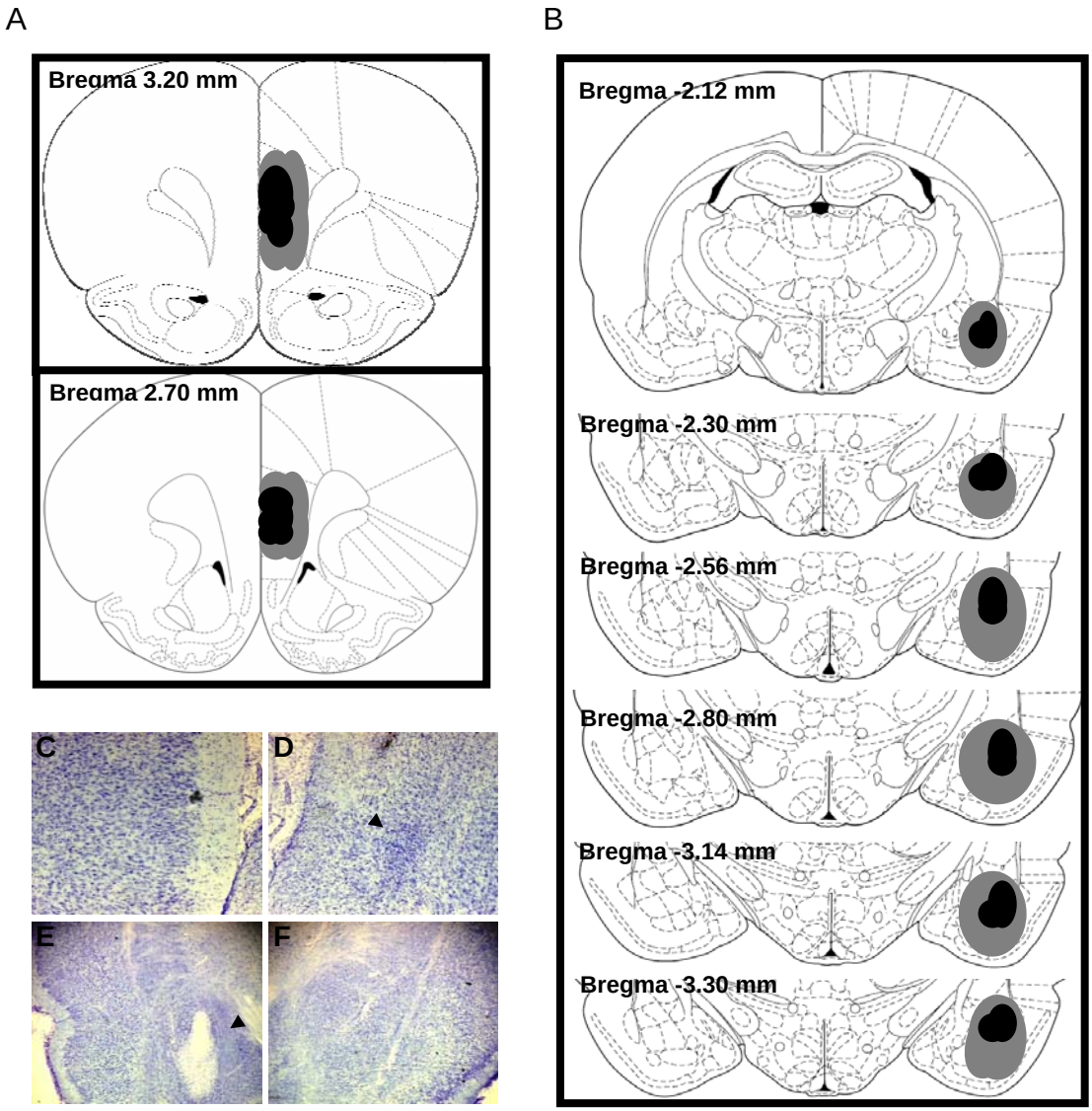

A
B
Bregma 3.20 mm
Bregma 2.70 mm
C
D
E
F
Bregma -2.12 mm
Bregma -2.30 mm
Bregma -2.56 mm
Bregma -2.80 mm
Bregma -3.14 mm
Bregma -3.30 mm

Figure 4

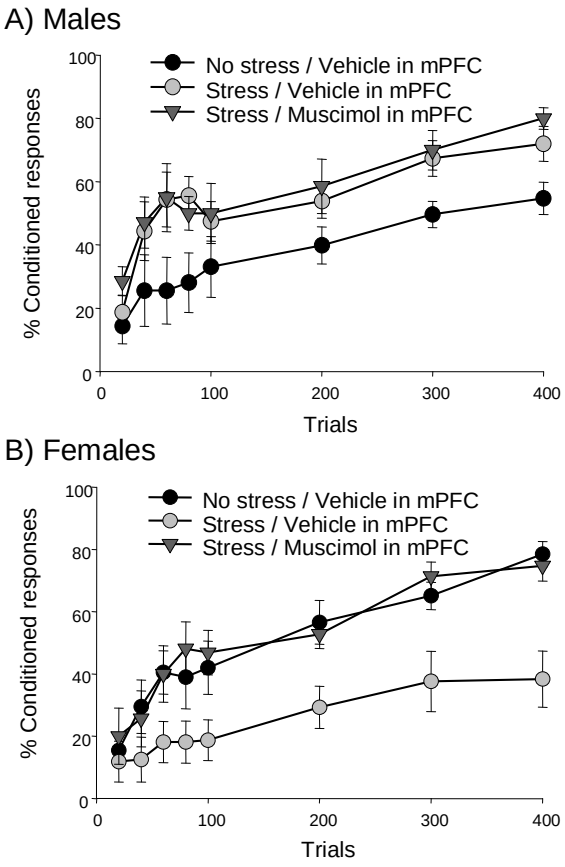

A) Males
No stress / Vehicle in mPFC
Stress / Vehicle in mPFC
Stress / Muscimol in mPFC
% Conditioned responses
Trials
B) Females
No stress / Vehicle in mPFC
Stress / Vehicle in mPFC
Stress / Muscimol in mPFC
% Conditioned responses
Trials

Figure 5

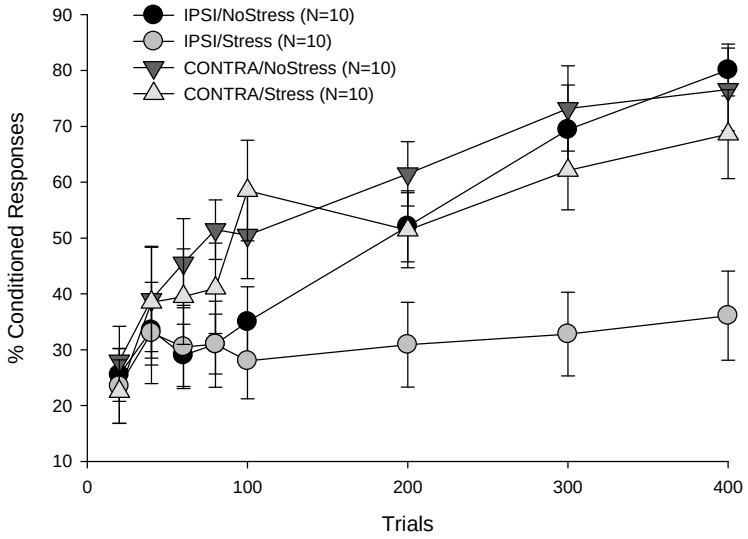
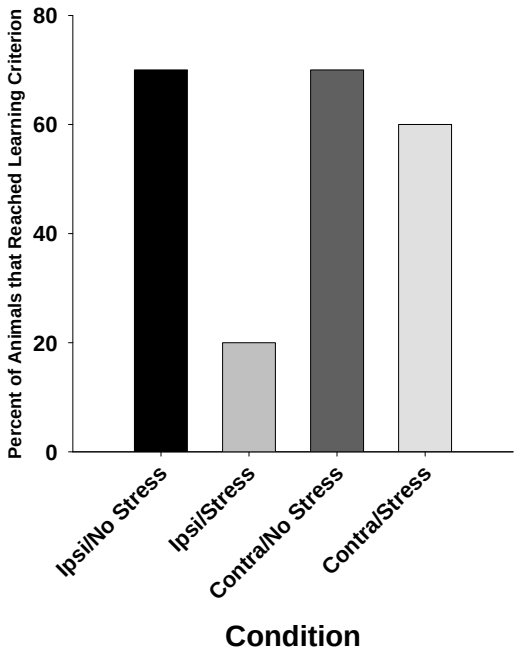

IPSI/NoStress (N=10)
IPSI/Stress (N=10)
CONTRA/NoStress (N=10)
CONTRA/Stress (N=10)
% Conditioned Responses
Trials
90
80
70
60
50
40
30
20
10
0
100
200
300
400

Figure 6


Percent of Animals that Reached Learning Criterion
80
60
40
20
0
Ipsi/No Stress
Ipsi/Stress
Contra/No Stress
Contra/Stress
Condition

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