NEURAL CIRCUITRY OF CONDITIONED AND UNCONDITIONED FEAR: EFFECTS OF LESIONS OF THE BED NUCLEUS OF THE STRIA TERMINALIS

by

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ABSTRACT

The bed nucleus of the stria terminalis (BNST) is suggested to play a role in certain types of fear, such as unconditioned fear and conditioned fear to long duration stimuli, but not others such as short duration conditioned fear stimuli. The main goal of the present research is to better understand the role the BNST plays in conditioned fear and unconditioned fear behavior. To study conditioned fear, a conditioned fear-potentiated startle paradigm was used where a light previously paired with a shock potentiates the acoustic startle reflex. For unconditioned fear, rats were exposed to a predator odor, trimethylthiazoline, to elicit unconditioned freezing behavior. Adult male Sprague-Dawley rats were first conditioned to a light. The rats were then subjected to neurotoxic lesions of the posterior division of the BNST. Following a week of recovery, the rats were tested for fear-potentiated startle. Several days later, they were tested for unconditioned freezing to TMT. Results demonstrate that lesions of the posterior division of the BNST significantly reduce cue-specific fear-potentiated startle, without affecting startle when the light was not present. This indicates that the BNST lesions interfered with expression of learned fear, but not the ability to startle, nor background anxiety, which is an increase in "baseline" startle after fear conditioning compared to pre-fear conditioned startle. Freezing to TMT was also reduced slightly in BNST lesioned animals, but this reduction was not statistically significant. Our data is contrary to previous research focused on the anterior nuclei of BNST, and suggests that the BNST has a heterogeneous collection of nuclei or cells that can affect both conditioned and unconditioned fear.

Chapter 1

INTRODUCTION

1.1 On Fear, Anxiety, and Behavior

Fear is critical to survival. Fear initiates several species-specific behaviors that allow animals to assess and respond to potential dangers. The expression of fear in mammals can be seen as immobility/freezing, fleeing, fighting, or avoidance behaviors (Schulkin, Morgan, & Rosen, 2005). However, the response is dependent on the perception of danger, the situational ability to express one response over the other, and the variation of responses expressed by different species (Blanchard & Blanchard, 1990).

A clear distinction is made between phasic and sustained fear in Davis et al. (2010). In the article, fear (phasic) is defined as an adaptive state of apprehension to an impending threat. When time is taken into consideration, the onset of fear is rapid but then dissipates quickly once the threat is no longer present. On the other hand, anxiety (sustained fear) is evoked by threats that are both less specific and less predictable and are generally physically or psychologically distant. Considering time, anxiety is defined as a future-oriented mood state that is activated by distal or potential threats that expresses arousal and vigilance. Anxiety is thus a long-lasting state of apprehension. It is these emotional states of fear and anxiety that have led behavioral

neuroscientists to explore the neuroanatomical and neurophysiological correlates of fear, anxiety, and ultimately mood related disorders (LeDoux, 1996; Price, 1999; Rosen & Schulkin, 1998).

1.2 Neural Circuitry of Fear

There are two major brain regions that are particularly important for the integration and initiation of fear and anxiety behavior. These regions are the amygdala and the bed nucleus of the stria terminalis (BNST). Both brain regions have similar outputs to areas such as the periaqueductal gray (PAG), the nucleus reticularis pontis caudalis (RPC), and the hypothalamus. Some other important auxiliary brain regions that help to mediate fear are the medial prefrontal cortex (mPFC), which plays an active role in the inhibition and extinction of fear (Morgan et al., 1993, 2003), and the hippocampus (Sanders et al. 2003), which may play a role in contextual fear conditioning. When these three structures act in a parallel manner, a proper fear response can be generated. However, several studies have shown differentiation between the basic functionalities of the amygdala and the BNST.

1.3 The Amygdala

Although the amygdala is composed of several different anatomical nuclei, the mechanisms underlying the fear-learning process have been associated specifically within the central (CeA) and the basolateral (BLA) nuclei of the amygdala. Animal

models have shown that a majority of sensory information is received by the BLA via direct projections from the thalamus, the associated posterior intralaminar nucleus (PIN), and indirectly from other areas such as the auditory association cortex, and the polymodial association cortex (Li, Stutzmann, & LeDoux, 1996; Kandel, Schwartz, & Jessell, 2000). The BLA then passes the information to the CeA, which contains a majority of the output sites to the brain stem (e.g. PAG and RPC) and the hypothalamus (Davis & Shi, 2000; Kapp, Supple, & Whalen, 1994; Davis, 1992). This connectivity between the BLA and the CeA was recently explored in Amano et al. (2011), which also implicated that the BLA and basomedial (BM) are involved in the same role of information transmission from BLA to CeA. Because of the amygdala's diverse interconnectivity with other brain regions, including the BNST, major research focus has been directed at these associated regions and their implications in fear.

1.4 The Amygdala and Phasic Fear

The amygdala is essential to implicit associative learning, especially when a neutral sensory stimulus has been paired with an aversive one (Hitchcock & Davis, 1987, 1991; Campeau & Davis, 1995; LeDoux, 2000). In earlier studies of the amygdala, the neutral non-aversive stimulus, also called the conditioned stimulus (CS), was typically only presented for a short duration of time (about 3-4 seconds) and co-terminating with a 0.5-1 second aversive unconditioned stimulus (UCS) (Hitchcock & Davis, 1991). Thus, the amygdala has traditionally been considered to be the

neurobiological substrate critical for classic Pavlovian fear conditioning. The Pavlovian paradigm of conditioning has shown that fear can be generated towards a non-harmful CS, such as a light or a tone, when it is paired with an aversive UCS, such as a foot shock. When the CS is presented, the conditioned response (CR) will occur. This is seen when a light or tone initiates freezing behavior after a subject has been trained to fear the CS. This is a very important theory concerning the acquisition of phobias to non-aversive stimuli and in certain scenarios that influence panic disorders, thus making the amygdala the region of interest in fear-learning and clinical anxiety disorders (Waddell, Morris & Bouton, 2006).

1.5 The Bed Nucleus of the Stria Terminalis

The BNST and the amygdala are strongly interconnected through reciprocal connections via the fiber bundle called the stria terminalis, thus making the BNST a part of the "extended amygdala". Three specific regions in the posterior division of the BNST called the inter-fascicular nucleus (if), the principal nucleus (pr), and the transverse nucleus (tr), all have reciprocal connections with the CeA, ventral PAG, and the several divisions of the hypothalamus (Krettek & Price, 1978; Sun & Cassell, 1993; Dong & Swanson, 2006). The projections to the lateral hypothalamus help to facilitate the activation of the sympathetic nervous system that is seen during fear (LeDoux, et al. 1988). Additionally, projections to the brain stem areas such as the dorsal motor nucleus of the vagus, nucleus of the solitary tract, and the ventrolateral

medulla have been implicated in heart rate and blood pressure modulation (Schwaber et al. 1982). This implies that both the amygdala and the BNST play an interactive and potentially complementary roles in the expression of fear related behaviors (Davis & Shi, 1999; Dong, Petrovich, & Swanson, 2001).

1.6 The BNST and Sustained Fear

In regards to fear behavior, the BNST has been implicated in the acquisition and maintenance of sustained fear behaviors (Davis & Lee, 1998; Walker & Davis, 1997). This anatomical dissociation was first defined in Hitchcock and Davis (1991) who reported that electrolytic lesions to the BNST did not affect fear-potentiated startle. Walker and Davis (1997) proposed that the BNST (but not the amygdala) is important in the expression of unconditioned fear using a light-enhanced startle paradigm that reflected an unconditioned response to anxiety producing stimulus. The evidence suggested an anatomical dissociation between the fear behaviors expressed by the amygdala and the BNST with the amygdala expressing acute fear behavior and the BNST the anxious, persistent fear phenotype.

However, the BNST was also shown to be involved in fear conditioning when the CS is long in duration and also during the reinstatement of extinguished fear, which differs from the function of the amygdala (Waddell, Morris, Bouton, 2006). Both short duration CS and long duration CS elicited a similar CR indicative by freezing of the increase in startle reflex response. However, the difference can be seen

in which neural circuits are responsible for either phasic or sustained fear CR (Wagner & Brandon, 1989). Therefore, when a CS is presented that is temporally persistent it may evoke a state of anxiety that subsequently prepares the organism for a distant, aversive event (Waddell, Morris, Bouton, 2006).

1.7 The BNST and Unconditioned Fear

Because the Walker and Davis study (1997) implicated the BNST and not the amygdala in unconditioned fear behaviors, their findings served as major branch point in the study of innate fear. A model to study unlearned fear was developed by taking advantage of the freezing behavior expressed upon exposure to a predator odor (Wallace & Rosen, 2000). Wallace and Rosen (2001) showed that neurotoxic lesions to the amygdala block long-term learning of contextually conditioned fear and did not affect fox-odor trimethylthiazoline (TMT) induced unconditioned freezing. Subsequently, Fendt, Thomas, and Apfelbach (2003) temporarily inactivated the BNST with muscimol, a $GABA_A$ agonist, and observed that it completely blocked the unconditioned freezing response to TMT. Conversely, the same study showed that temporary inactivation to the amygdala (specifically the CeA and BLA) yielded no effect on TMT freezing responses. These studies offered yet another functional neural circuit between the olfactory system, the medial nuclei of the amygdala, and the BNST for the expression of unconditioned fear to a predator odor.

Furthermore, researchers discovered that TMT exposure can influences c-fos mRNA production within the BNST and the ventral olfactory bulb (Janitzky et al., 2009; Kobayakawa et al., 2007). The study also showed no significant TMT influence within the dorsal olfactory bulb or in the amygdala. The production of immediate-early gene (IEG) c-fos is important because it codes for the Fos transcriptional factor protein that is usually used as a marker for activity within the neuron (Herrera & Robertson, 1996). Transcription of c-fos mRNA is made within minutes of exposure to an external stimulus and usually at maximum concentrations between 30 and 45 minutes after the exposure.

1.8 The BNST and Contextual Fear

More recently, contextual fear conditioning has also been associated with the BNST. In Duvarci, Bauer and Pare's (2009) experiment, they examined how lesions to the BNST affect the magnitude of conditioned fear response to a CS+ or a CS-. It was determined that the BNST is not necessary for CS+ response but that it is important in fear expression to the CS-. Lesions thus decreased the inappropriate fear responding to the CS-, suggesting that the BNST is not required for the expression of conditioned fear but it does help determine the selectivity of conditioned fear responses.

Similarly, Poulos et al. (2010) also implicated the anteromedial division of the BNST as playing a compensatory role in the acquisition of contextual fear conditioning after extensive training and in the absence of the BLA. This was seen

with an increase in c-fos in the BNST following lesions to BLA during the recall of fear memory after extensive fear conditioning. These results imply a protein synthesis-dependent plasticity in the BNST. The connection between contextual fear learning and the BNST was further supported by Cullinan, Herman, and Watson (1993) who found that the BNST receives inputs from the hippocampal formation (HPF) and sends outputs to the vPAG. Furthermore, Dong, Petrovich, and Swanson (2001) who show that the posterior BLA is the only BLA nuclei that projects to BNST and facilitates the expression of contextual fear conditioning.

1.9 The Current Study

The BNST thus seems to be essential for long-duration fear conditioned stimuli, unconditioned fear response to predator odors, and contextual fear conditioning. However, a majority of the studies listed examined the role of the anterior division of the BNST (adBNST). As stated earlier, Dong and Swanson (2006) implicated several posterior division BNST (pdBNST) nuclei in the expression of defensive behaviors. Therefore, we wished to observe if the pdBNST is involved in both short-duration conditioned and sustained fear and unconditioned fear. If the pdBNST plays a similar role in both conditioned and unconditioned fear, excitotoxic lesions to the pdBNST should then significantly reduce or even block freezing behavior towards the predator odor TMT. On the other hand, in a fear-potentiated startle paradigm that assess increases in startle to noise trials subsequent to fear

conditioning (background anxiety), and fear potentiation to the CS, elimination of the pdBNST should have no effect on short-duration conditioned fear stimuli and it should diminish background anxiety because it is because it hypothesized to be an unlearned phenomenon.

Thus, it is hypothesized that damage to the pdBNST will (1) reduce freezing to predator odor TMT, (2) reduce background anxiety in a fear-potentiated startle test, and (3) not affect short duration fear-signaled potentiation of startle. By increasing our understanding of the neural circuitry and the role that the pdBNST plays in it, we hope to also gain an increased understanding of conditioned and unconditioned fear. This can translate into future research that is aimed at the development of pharmacological agents that will alleviate certain psychological disorders that are associated with the over-expression of sustained fear such as Post-Traumatic Stress Disorder (PTSD) or Panic Disorder (PD).

Chapter 2

METHODS AND MATERIALS

2.1 Subjects

Forty male Sprague-Dawley rats were received from Charles River Laboratories International, Inc. The rats were kept on a 12:12-hr light-dark cycle and housed in pairs prior to surgery. Twenty-six animals underwent surgery and fourteen animals were used as controls. Food and water were available ad libitum during all stages of the experiments. During fear conditioning, the rats weighed between 250-275 g. However, after surgery, they had grown to approximately 290-470 g.

2.2 Fear-Potentiated Startle Paradigm

Eight SR Lab startle chambers with clear Plexiglas cylinder subject holders (San Diego Instruments, San Diego, CA) were used for matching, training, and testing. The conditioned stimulus (CS) was presented using three parallel LED lights mounted onto one wall of the chamber. The unconditioned stimulus (UCS) was presented using a floor of ten parallel 4-mm diameter stainless steel tubes each separated by 4-mm distance. The floor delivers approximately a 0.5 mA footshock during training. Below the cylinder holder, an accelerometer with a piezoelectric crystal assesses changes in

pressure (startle response from subjects) that can generate a voltage and create a startle score.

The experiment began with three days of acclimation/matching sessions that were followed by one day of classical fear conditioning (training). Lesions were given approximately three days after fear conditioning and subjects were allowed seven days to recover prior to testing. After recuperation, the subjects were tested using the fear-potentiated startle protocol.

During the three days of startle acclimation/matching, each session began with a 5-minute acclimation period followed by 30 trials of startle stimuli. Each trial presented a noise burst of either 95, 105, or 115 decibels. Each noise level was presented a total of 10 times in a predetermined pseudorandom order delayed by 15-second intervals.

After the third day of matching, the subjects startle amplitude was averaged to obtain a mean startle score that could be used to sort them into two groups (sham and lesion) with similar levels of startle. On the fourth day, the rats were placed into the startle chambers and given a 5-minute acclimation period. After acclimation, 10 pairings of 3 seconds of light (CS) ended with a 500 ms 0.6 mA footshock (UCS). The pairings presentation intervals ranged between 60 to 180 seconds.

Three to four days following fear conditioning, rates were given excitotoxic lesions. The surgery and injections are described below in the Surgery section. After infusion of the neurotoxic lesions and a seven day recuperation interval, the subjects were tested for background anxiety and fear-potentiated startle. The testing sessions

consisted of 5-minutes of acclimation followed by 70 startle trials with 15-second intervals. However, the first 10 trials consisting of 95 dB noise bursts were not used in the analysis because the trials are used as a habituation-acclimation process to allow for habituation to the startle stimuli. The following trials were divided into two types of trial presentations: noise alone or noise and light. In the noise alone trials, a noise burst of 95, 105, or 115 dB was presented in the absence of a light (CS). However, in the noise and light trials, a noise burst of 95, 105, or 115dB was presented with a light stimulus (CS). This design allowed for each noise burst level to be presented 10 times per trial type (noise alone v. noise and light), totaling 60 trials.

2.3 Trimethylthiazoline Exposure Paradigm

Four custom-made ventilated Plexiglas freezing chambers were used for both acclimation and testing sessions. The chamber floor was made of ten parallel 4mm diameter stainless steel tubes separated by a 4 mm distance. The floor had the capability to deliver a footshock but no shock was delivered during experimentation. In order to record the freezing behavior of the subjects, they were recorded using a video camera linked to a computer running Freeze Frame.

For two days prior to TMT testing and after recovery from neurotoxic lesions and FPS testing, subjects were acclimated to the freezing chambers for 10 minutes. After the two acclimation sessions, animals were exposed to approximately 38.8 μL

(19.4 μL per side of the chamber) of TMT over a 10-minute period to assess percentage of freezing behavior expressed.

2.4 Surgery

Subjects were anesthetized with an isoflurane/oxygen mixture and placed into a Kopf non-puncture stereotaxic apparatus. Neurotoxic lesions of the BNST were created by infusion a mixture of 5.0 μg ibotenic acid, 5.0 μg N-methyl-D-aspartate, and 5.0 μL 1.0M phosphate buffer solution. Sham rats received the same treatment except no neurotoxic mixture was infused into the BNST. A 1.0 μL Hamilton chromatography microliter syringe was mounted to the stereotaxic and connected to a Harvard brand infusion pump. The needle was lowered bilaterally, at the following locations: AP -0.8, ML -1.5, DV -6.7 (Paxinos & Watson, 2007). 0.2 μL was infused bilaterally over 2 minutes. After each infusion, the needle was kept in place for 5 min before being removed. Post surgery, the subjects were monitored with the body temperature maintained with a heating pad on low heat until the subjects began to be active. The rats recovered for seven days prior to the testing session (See Experimental Design above).

2.5 Tissue Collection

At the conclusion of the behavioral experiments, the subjects were given an overdose of ketamine mixed with xylazine (87 mg/13mg/kg:IP) and perfused

transcardially with 0.1M phosphate buffered saline solution (PBS), followed by 4% paraformaldehyde solution in PBS at a pH of 7.3. The brains were then removed and stored in 4% paraformaldehyde for one day, followed by storage in 30% sucrose in 4% paraformaldehyde solution until slicing. Coronal cross-section tissue samples of the entire BNST region were taken at a thickness of 40 μm. The sections were stored in microfuge tubes filled with cyroprotectant solution made of glycerol and ethylene glycol in Tris-buffered solution (TBS) and stored at -20°C.

2.6 Immunohistochemistry

NeuN immunohistochemical staining was used as a qualitative measure of the accuracy of lesion placement. Selected sections were washed in TBS and then incubated in primary antibodies solution (1:500 monoclonal anti-NeuN antibody; Chemicon international Temecula CA) diluted in 0.4% Triton-X/TBS for 48 hours at 5°C. After the incubation period, the tissue was washed in TBS and placed into secondary antibody solution (1:600, anti-mouse IgG made in horse antibody; Vector Labratories Inc., Burlingame CA) for one hour at 25°C. The tissue was once again washed in TBS and incubated for 30 minutes in avidin-biotin-peroxidase complex solution (ABC Elite Kit; Vector Laboratories Inc., Burlingame CA) at 25°C. Following ABC exposure, the tissue was washed in TBS and then incubated for 10 minutes in diaminobenzedine (DAB)-nickel solution. Following the reaction, the

tissue was wet-mounted onto gelatin-covered slides, dehydrated for one night, and then coverslipped the following day with Permamount solution.

2.7 Data Analysis

For the fear-potentiated startle paradigm, three startle scores were used for statistical analyses: Pre-Fear, Noise-alone, and Light + Noise. The pre-fear startle scores were obtained from each rat from the third acclimation session by averaging all of the trials (30 total/animal). The same was done to the Noise-alone and Light+Noise trials to obtain respective Noise-alone and Light+Noise startle scores.

The effect of lesions to the posterior BNST in the fear-potentiated startle test was analyzed by a mixed model ANOVA with a between-subjects measure of the lesion and within-subjects measure of fear-potentiated startle (Light+Noise vs. Noise-alone). Cue-specific fear was analyzed in two ways. The first was by using absolute fear-potentiated startle scores that is calculated by subtracting the average noise startle amplitude from the average light + noise startle amplitudes for each rat. The second way was by using a proportional fear-potentiated startle score for each rat that is computed by dividing the absolute fear-potentiated startle score by the average noise startle amplitude score and making it into a percent.

Additionally, a measure of change in startle amplitude after fear conditioning, or what is being called “background anxiety” was calculated by comparing Pre-Fear

startle score to Noise-alone startle scores. The analysis was done using a mixed model ANOVA with a between-subject measure of lesions and within-subject measure of background anxiety (Pre-Fear vs. Noise-alone). An absolute background startle score was computed by subtracting the Pre-Fear startle scores the Noise-alone startle scores.

For the TMT exposure paradigm, two sets of freezing scores were used for statistical analyses: Acclimation scores and TMT exposure scores. The analysis was done using a mixed model ANOVA. Additionally, the overall trial time (10 minutes) was divided into two segments of 5 minutes (initial and final) and similarly analyzed using a mixed model ANOVA.

Chapter 3

RESULTS

3.1 Histology

Histology was used as a qualitative measure to determine that the lesions had been made in the appropriate location. The sites and extent of the lesions to the pdBNST are shown in Figure 1. There were a total of 9 animals with confirmed bilateral lesions. However, one subject's data was not used for statistical analysis because the subject had excessively high startle data. Additionally, the data from 17 animals was not used for statistical analysis because the subjects either only had unilateral lesions (n=6) or the lesion missed the target area completely (n=11). The NeuN stains revealed cellular loss in the pdBNST for lesioned animals while sham animals showed no cellular loss (Figure 2).

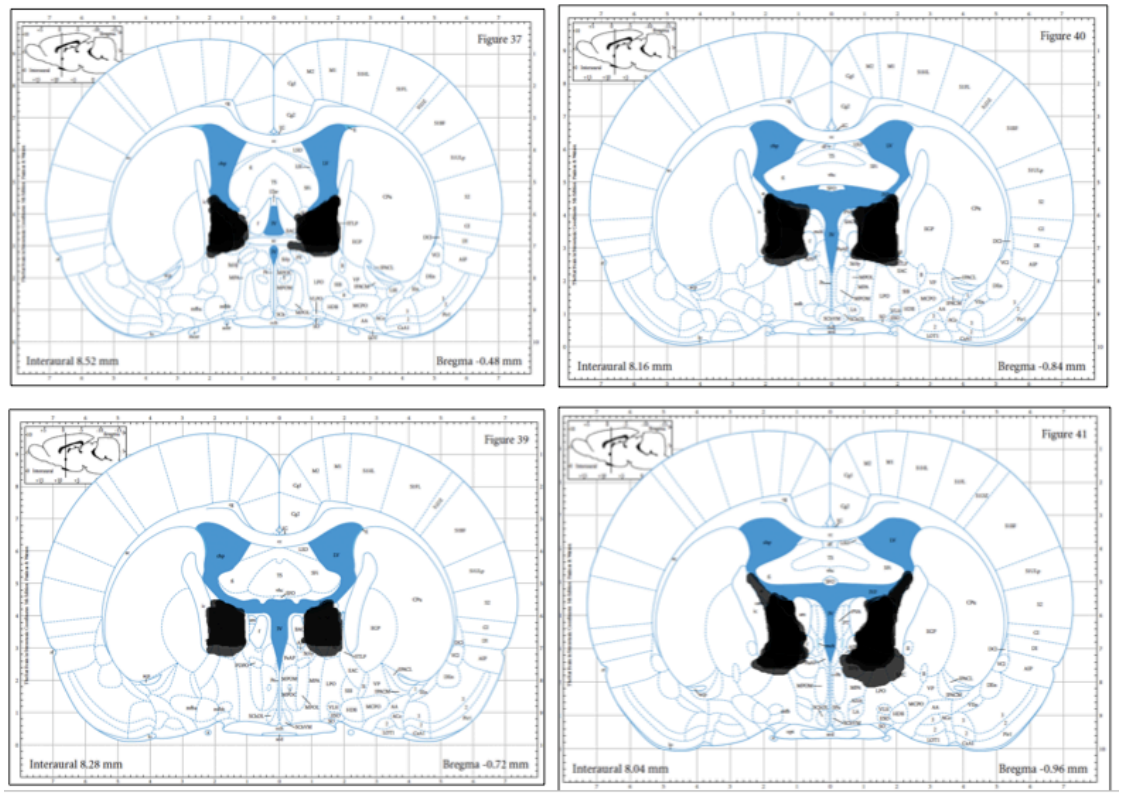
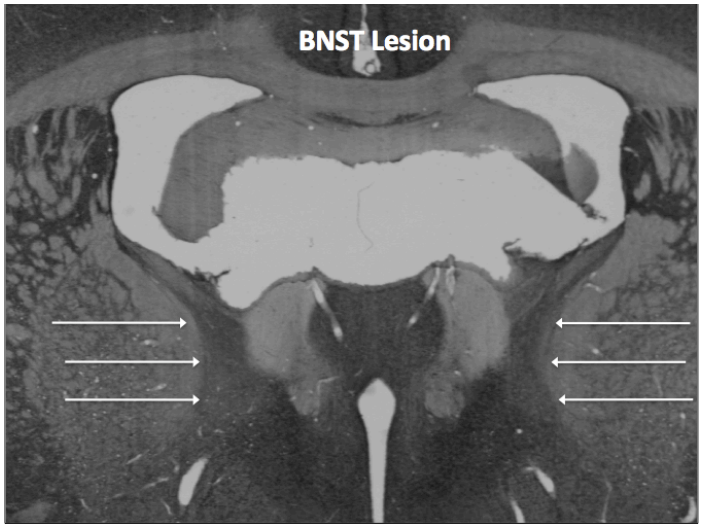


Figure 1. Histological reconstruction of injection sites NMDA/ibotenic acid into the posterior division of the bed nucleus of the stria terminalis (from -0.48mm to -0.96mm from bregma). Coronal sections were taken from the atlas of Paxinos and Watson (2007). Gray areas represent the minimum extent of the lesions while black areas represent the maximum extent of the lesion per coronal area.

A.



B.

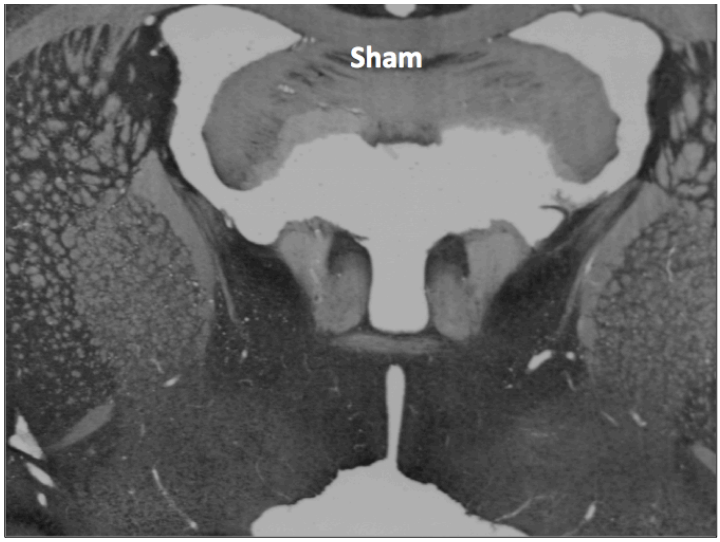


Figure 2. NeuN immunoreactivity in coronal section of posterior division of the BNST. (A) Cellular loss in posterior division of the BNST indicated with arrows. (B) Sham comparison displays no cellular loss in posterior division of the BNST.

3.2 Fear-Potentiated Startle

In comparing Noise-alone startle to Pre-Fear startle scores, that is background anxiety, a mixed model ANOVA showed that there was no main group effect for sham vs. lesion ($F_{1,20}$=0.129, p< 0.7232). There was a significant within main effect of an increase in startle in Noise-alone trials compared with Pre-Fear trials ($F_{1,20}$=20.148, p<0.0002). Moreover, there was no interaction effect. This indicates that bilateral lesions to the BNST did not affect background anxiety using absolute background anxiety scores. ($F_{1,20}$=0.156, p<0.697, Figure 3).

Similarly, in comparing Light+Noise startle to Noise-alone startle scores, that is cue-specific fear potentiated startle, a mixed model ANOVA revealed that there was no main effect for sham vs. lesion ($F_{1,20}$=1.734, p<0.2028). However, the data showed a significant within main effect between the Light+Noise and Noise-alone trials ($F_{1,20}$=13.688, p<0.0014). Additionally, there was an interaction effect indicating that lesions to the BNST did affect absolute cue-specific fear-potentiated startle scores ($F_{1,20}$=14.263, p<0.0012, Figure 4). This data is supported using proportional fear-potentiated startle scores (Figure 5). The overall data indicates that bilateral lesions to the pdBNST after classical conditioning had no specific effect on background anxiety, but it did significantly reduce the expression of cue-specific fear-potentiated startle (Figure 6).

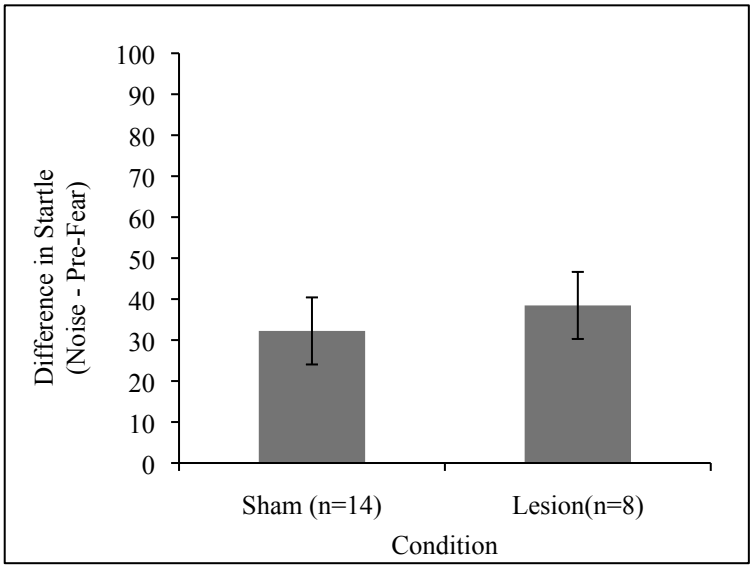


Figure 3. Absolute background anxiety data. Differences between average noise and average pre-fear startle scores between groups.

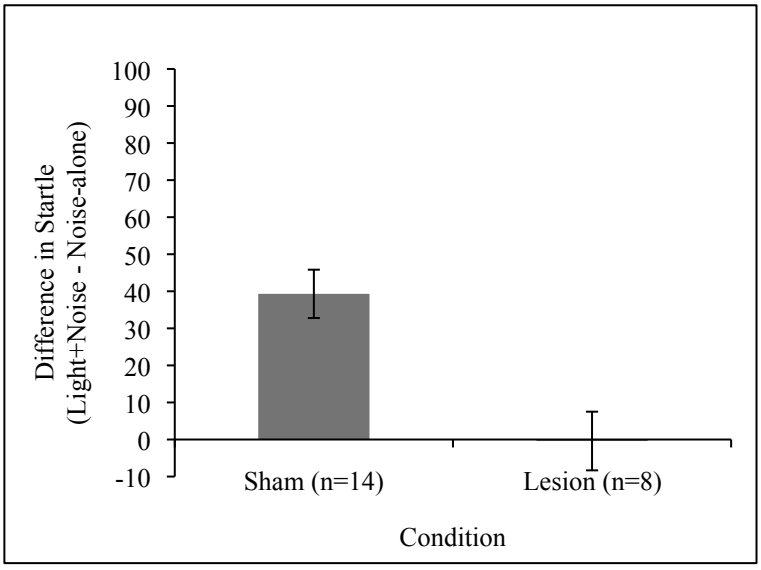


Figure 4. Absolute fear-potentiated startle scores between groups. Difference between average light + noise and average noise alone trials.

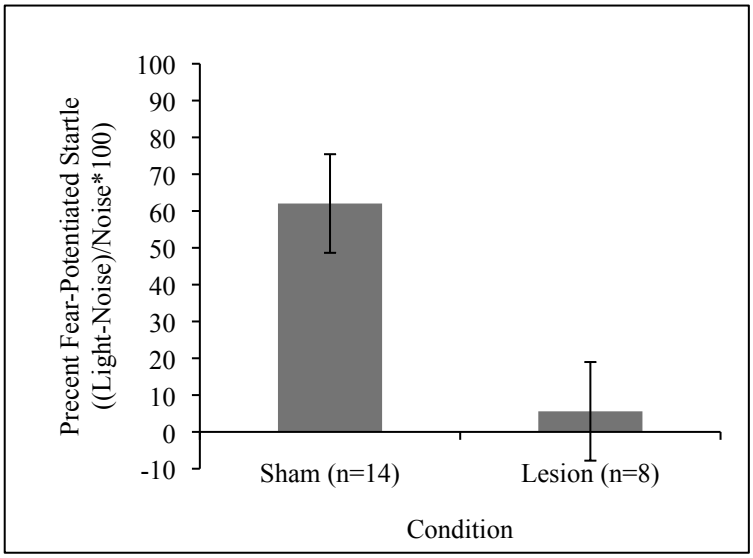


Figure 5. Proportional fear-potentiated startle scores between groups. Difference between average light + noise and average noise divided by average noise startle scores.

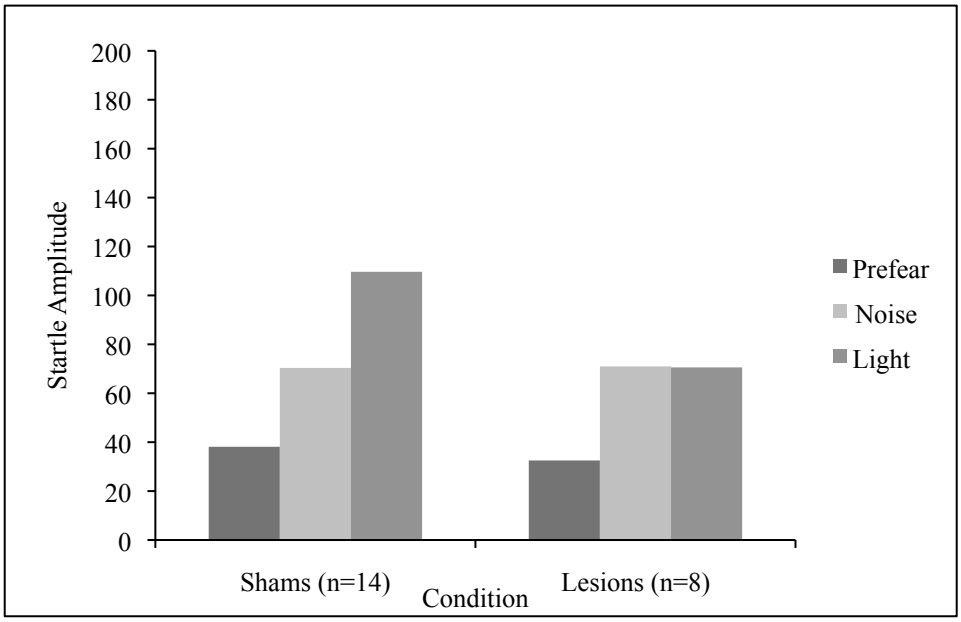


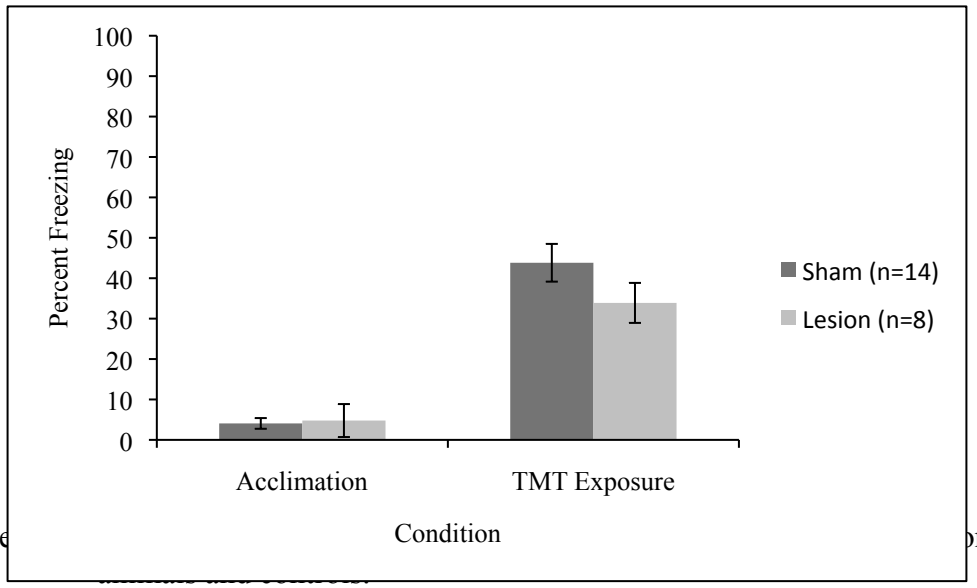
Figure 6. Fear-potentiated startle scores for control and histologically confirmed bilateral lesions.

3.3 TMT Exposure

The comparison of the overall TMT exposure and Acclimation sessions (10 minutes each) was performed with a mixed model ANOVA. The analysis revealed that there was no main between-group effect for sham vs. lesion ($F_{1,19}$=1.164, p<0.294). However, the within-main effect between the Acclimation and the TMT exposure session was statistically significant ($F_{1,19}$=85.877, p<0.001). There was no significant interaction effect however ($F_{1,19}$=2.204, p<0.154, Figure 7).

The data was further divided into two 5-minute intervals (initial and final) to achieve a better analysis of lesion effects to the BNST. For the initial 5-minutes, a mixed model ANOVA revealed no main between measure effect ($F_{1,19}$=0.19, p<0.668), a significant within main effect ($F_{1,19}$=17.204, p<0.0005), and no significant interaction effect ($F_{1,19}$=0.216, p<0.648, Figure 8).

However, analysis of the final 5-minute through a mixed model ANOVA showed that there was no main between-group effect ($F_{1,19}$=1.729, p<0.204) but there was a significant within main effect indicating a difference between the freezing scores between acclimation and TMT exposure sessions ($F_{1,19}$=135.63, p<0.0001). Additionally, there was a trend towards an interaction effect indicating that lesions to the BNST facilitates attenuation of freezing behavior when exposed to TMT ($F_{1,19}$=4.211, p<0.0542, Figure 9). The overall data indicates that bilateral lesions to the pdBNST have a marginal effect on the expression of freezing behavior to predator odor (TMT) and it is more easily seen during the final 5-minutes of exposure.



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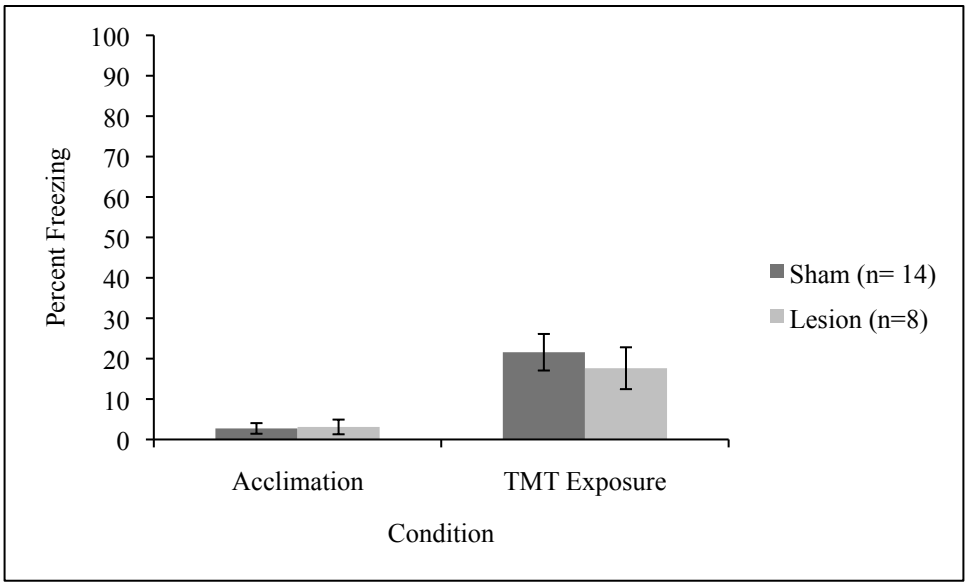

Figure 8. Initial 5 minutes of acclimation and TMT exposure.

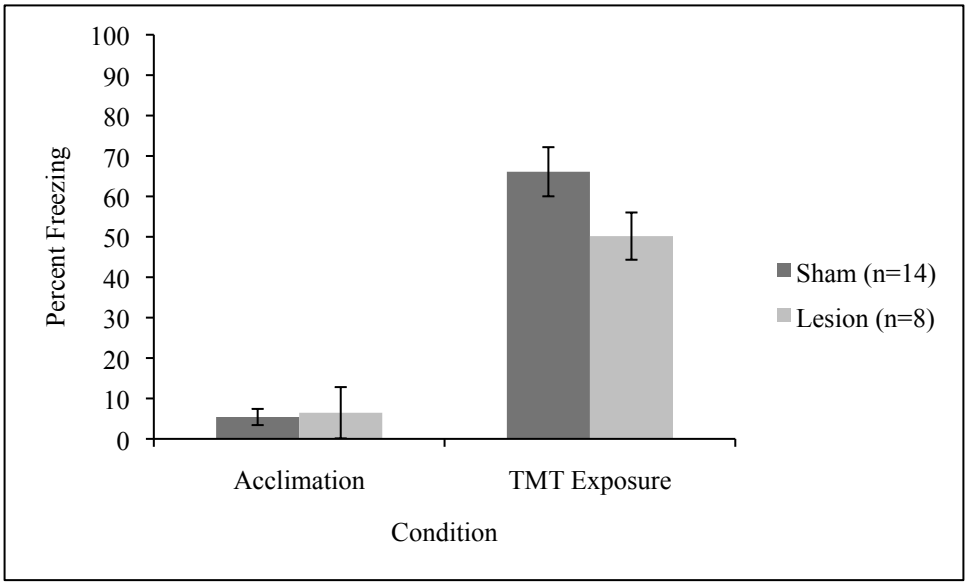

Figure 9. Final 5 minutes of acclimation and TMT exposure.

Chapter 4

DISCUSSION

4.1 The Role of the pdBNST in Phasic and Sustained Fear

This study found that lesions to the posterior division of the bed nucleus of the stria terminalis (pdBNST) significantly reduced cue-specific fear-potentiated startle, but had no affect on startle in the absence of the light (i.e. background anxiety). Additionally, freezing to TMT was marginally reduced in animals that had lesions to the pdBNST. The amalgamation of this data indicates that lesion to the pdBNST interferes with both the expression of learned fear (not including ability to startle or background anxiety) and unconditioned fear.

When comparing our results to prior research, it stands as a stark opposition to the conventional neuroanatomical dissociation between the BNST and the amygdala regarding conditioned fear learning. The traditional notion postulates that the expression of phasic fear is mainly dependent on the amygdala (Hitchcock & Davis, 1987, 1991; Campeau & Davis, 1995; LeDoux, 2000). Moreover, Hitchock and Davis (1991) showed that the BNST had no effect on phasic fear after electrolytic lesions to the area. Rather, the BNST was thought to have been involved in the acquisition and expression of sustained fear (Davis & Lee, 1998; Walker & Davis, 1997; Waddell, Morris, Bouton, 2006). However, in a recent study performed by Ravinder et al. (2013), a single injection of the SSRI fluoxetine (which can exacerbate symptoms of

anxiety) into the BNST induced an increase in Arc expression (a molecular marker of activity and plasticity) and subsequently enhanced conditioned fear memory and Arc expression in the CeA. These results indicate that the BNST and the CeA communicate and thus are not completely autonomous from each other during conditioned fear learning.

An important difference between these previous studies and ours was the location subjected to lesions or inactivations. Previous studies focused on the anterior division of the BNST (adBNST) while our interest was in the posterior division of the BNST (pdBNST). Just as the subnuclei within certain other regions of the brain (hippocampus, amygdala) have specific functional roles in the expression of behavior, there may also be a difference between the behavioral output of the adBNST and the pdBNST.

Differences in behavioral expression have been studied in the division of the BNST. For example, the adBNST seems to be involved in the maintenance of homeostasis (Dong et al., 2000; Dong and Swanson, 2003, 2004). On the other hand, Dong and Swanson (2006) used an anterograde tracer called *Phaseolus vulgaris*-leucoagglutinin (PHAL) to determine the projections of the three major subnuclei in the pdBNST, which are the interfascicular (BNSTif) nuclei, the transverse nuclei (BNSTtr), and the principal nuclei (BNSTpr). Although research by multiple lab groups (Segovia & Guillamon, 1993; Newman et al., 1997; Wood, 1998; Newman, 1999; Simerly, 2002) have already determined that the BNSTpr is integral in the control of the neuroendocrine system and reproductive behavior, little is known about

the function of the BNSTtr and BNSTif except that it may be involved in the expression of defensive behaviors such as fight or flight (Shaikh et al., 1986; Kollack-Walker & Newman, 1995; Newman, 1999; Canteras et al., 2001).

The topographical work performed by Dong and Swanson (2006) demonstrated that the BNSTif and BNSTtr have complementary innervation projection patterns to the upper brainstem, lateral septal, and hypothalamic regions that are in control of defensive and reproductive behaviors. Specifically, there are strong projections from the BNSTtr to the medial amygdala and to the ventralPAG (freezing). The BNSTif projects to the MEApv and MEAd. Similarly, the BNSTtr also terminates onto the substantia innominata, medial and capsular parts of the CeA, and MEad. This data is supportive of the results obtained from our present study because it shows that the pdBNST is important in the expression of fear behavior. Therefore, while the adBNST is associated with sustained fear, unconditioned fear, and contextual fear conditioning, the pdBNST may have an integral role in the expression of conditioned fear, a proposition supported by our fear-potentiated startle data. Therefore, this provides a way to integrate the function of the pdBNST into the previous literature.

Although animals that received lesions to the pdBNST did not show a significant reduction in the amount of freezing behavior to a predator odor in comparison to control animals, this does necessarily mean that the area is not important in the expression of unconditioned fear. As seen in studies performed by Fendt, Thomas, and Apfelbach (2003), inactivation of the adBNST causes a

significant reduction in freezing to TMT. This was supported by research done by Shipley et al. (1995) that the BNST is highly connected to the olfactory system. Interestingly, inactivation of the adBNST did not bring freezing down to pre-TMT exposure levels. Dong and Swanson's (2006) research showed that both the transverse and interfascicular nuclei have projections to the adBNST. This interconnection may suggest that the two divisions work together in the expression of freezing behavior. This is not unreasonable considering that the transvere nucleus has dense projections to the vPAG that is integral in expressing freezing behavior to an unconditioned stimulus (Fanselow, 1991; Fanselow et al., 1995).

4.2 Limitations

Yet, there are some underlying limitations with our experimental design. For example, animals assigned to the control group did not receive a vehicle injection (i.e. saline solution or artificial cerebral spinal fluid (aCSF)). Additionally, data obtained from unilaterally lesioned animals (not shown or discussed in this paper) showed a significant decrease in cue-specific fear-potentiated startle and no decrease in background anxiety, which only adds to the necessity of having control animals undergo the process of a needle insertion and vehicle infusion into the pdBNST to remove the possibility that the effect obtained was a result of the surgery itself.

4.3 Conclusion

This main objective of this study was to understand the role of the posterior division of the BNST in both conditioned and unconditioned fear. Lesions to the pdBNST after fear conditioning was not expected to effect cue-specific fear-potentiated startle but it was expected to attenuate background anxiety in the fear-potentiated startle paradigm. Yet, the opposite effect was found in both cases. Furthermore, although it was expected that lesions would attenuate the unconditioned fear response to a predator odor, only a marginal effect was found.

Future research will have the control subjects undergo all steps of the surgery, minus the actual infusion of the NMDA/ibotenic acid. This will serve as a better control during comparison of fear behavior across groups. Also, selective inhibition of different neurons within the pdBNST using optigenetics would provide increased anatomical accuracy and it may even elucidate the function of the individual nuclei in the pdBNST.

In conclusion, the information gathered from this current study is important in facilitating a better understanding of the neural circuitry for fear. A greater elucidation of the fear circuit is highly important because it can be used to developed novel pharmacological agents that are aimed at treating certain psychological disorders associated with pathological anxiety such as Panic Disorder (PD), Generalized Anxiety Disorder (GAD), or Post-Traumatic Stress Disorder (PTSD).

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