CONTRIBUTIONS OF THE BED NUCLEUS OF THE STRIA TERMINALIS TO TEMPORALLY UNCERTAIN THREAT

An Undergraduate Research Scholars Thesis

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ABSTRACT

Contributions Of The Bed Nucleus Of The Stria Terminalis To Temporally Uncertain Threat

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Anxiety is a major public health concern. The bed nucleus of the stria terminalis (BNST) is a highly conserved brain region that has been implicated in anxious behaviors. However, the precise mechanisms of the BNST's involvement in anxiogenesis are not well known. Fear conditioning is an important and clinically relevant model through which we can probe the role of the BNST in anxiety-like behaviors. One possibility is that the BNST is recruited to anxiety when the aversive outcome is temporally uncertain. That is, the BNST appears to be involved in aversive learning and memory when animals expect a negative stimulus but are unable to determine *when* that stimulus will occur. The following experiments will directly test this possibility by inhibiting BNST activity using the N-methyl-D-aspartate receptor antagonist, APV, to during learning to temporally predictable or unpredictable threats. It was found that the BNST is involved in the learning of unpredictable threat, where timing of an aversive event is unknown to the subject. These data are important for the implications of BNST's involvement in fear and anxiety formation as well as future brain therapies regarding treatment of anxiety-related disorders.

DEDICATION

I dedicate this research to my father, Cody French. Thank you for your constant support of my college career and daily reminders to rise above failure.

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I would like to thank Travis Goode for being the best mentor in my research experience. For the past two years, he has provided me with priceless knowledge, support, and guidance in my experience in the research lab. Thank you for inspiring me to become a better student and well-rounded young lady.

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NOMENCLATURE

BNST	Bed Nucleus of the Stria Terminalis
PTSD	Post Traumatic Stress Disorder
LTP	Long Term Potentiation
ANOVA	Analysis of variance
TR	Trial
BL	Baseline
CS	Conditioned stimulus
US	Unconditioned stimulus
APV	Amino-5-phosphonovaleric acid

CHAPTER I

INTRODUCTION

Anxiety and fear

Pathological anxiety is one of the most common and debilitating forms of human mental illness (Kessler et al. 2005). Regrettably, current treatments are imperfect, include side effects, and are difficult to maintain in the long-term (Vervliet et al. 2013; Goode and Maren 2014). As such, it is increasingly important that we probe the brain for novel therapeutic targets. Moreover, increased understanding of the circumstances under which particular brain regions are active will grow our insight in to the psychological phenomena governing learning and memory.

Bed nucleus of the stria terminalis and temporally uncertain threat

The bed nucleus of the stria terminalis (BNST), a collection of neurons buried deep within the brain, has been identified as an important mediator of anxiety (Goode and Maren 2017). Humans, rats, and other mammals all share similarities in brain structures governing responses to threats, including the BNST. As such, we can use this animal model to probe BNST function during anxiety. Indeed, the BNST has been an area of increased study, however, the factors that determined BNST's contributions to fear and anxiety are not well known. Studies that have lesioned the BNST have found effects on fear and anxiety only if the animals are trained without clear signals of *when* aversive stimuli (e.g., footshock) will occur (Sullivan et al. 2004; Hammack et al. 2015). However, the role of temporally uncertain threat has not been systematically explored with regards to BNST function. Thus, the primary research question of this thesis asks whether the BNST is required for learning to anticipate uncertain threats, which is may be associated with the development of anxiety.

To accomplish this goal, we utilized DL-2-amino-5-phosphonovaleric acid (APV), a drug that blocks the NMDA-subtype of glutamate receptors in the brain and blocks learning. We made intracranial infusions of APV the BNST of rats to determine whether inhibiting synaptic plasticity in the BNST that prevents learning new information about threats and their consequences (i.e., disrupting the BNST's "neural plasticity") (Kim et al. 1992). We performed this manipulation during acquisition of Pavlovian fear conditioning in rats using auditory tones that are temporally predictive of shock or not. The cues predictive of shock were auditory stimuli (10 second 80 decibel, 2 kHz tones) that immediately preceded an aversive footshock (2sec, 1mA). However, for the unpredictable circumstances, the order of the tone-shock pair was simply reversed – where the shock occurred prior to the start of the tone. Rats were then tested to the cues in the absence of drugs to determine whether learning-induced plasticity within the BNST is essential for exhibiting fear to a temporally uncertain threat. This work will generate an important contribution to our understanding of the role of the BNST in anxiety, as it is not yet known whether plasticity within the BNST is essential for fear to temporally uncertain and diffuse threats (Davis et al. 2010; Goode and Maren 2017).

CHAPTER II

METHODS

Subjects

Subjects consisted of thirty-two adult males (n=16) and females (n=16) Long-Evans (Blue Spruce) rats (obtained from Harlan). Rats were individually housed in a climate-controlled vivarium, with free access to standard rodent chow and water. Cages were changed once a week with fresh bedding. All handling, surgical, and behavioral procedures were approved by the Texas A&M University Animal Care and Use Committee.

Handling

Prior to the beginning of the surgical and experimental process, all animals were handled in order to get them familiar with human interaction. Animals were handled for 1-2 min each day for one week.

Surgery

Prepatory Stages

To begin surgery, an animal was retrieved form the vivarium and placed into a tank receiving a simultaneous supply of isoflurane and oxygen in a closed and concealed space for about five to six minutes. Once the animal was anesthetized, they were immediately removed from the chamber and situated into a surgical stereotaxic frame that was delivered a constant supply of oxygen with mediated levels of gaseous isoflurane (5%).

Once the animal was situated in the stereotaxic frame, the isoflurane was brought down to a level of four, and the animals head was carefully buzzed with the electric razor. Once the head was buzzed, isoflurane levels were further decreased to 2-1% and iodine tablets were

spread upon the hairless area, rotating the tablet front to back and reversed in order to prevent bacterial infection. Betadine was applied to the skin and the lubricant and the tear lubricant as added to the eyes. Next, a small incision was made on the tissue above the skull. The skull was exposed following the incision, and the skull was then leveled with bregma and lambda on an even plane. Once all bleeding was mostly ceased, hemostats were used to clamp down four corners of the incision in order to get a clear view of the skull. The coordinates in which we used to guide the surgical process is as follows: Anterior/Posterior: -.2 mm, Medial/Lateral: +/- 2.65 mm, and Dorsal/Ventral: -6.5 mm (coordinates are relative to bregma) (cannula were angled at ten degrees with tips aimed towards the midline). Small holes were drilled to secure smaller jeweler's screws (for securing the head cap) and additional small holes were made for the passage of the guide cannulas. Once cannulas were set into place according to the coordinate position, a head cap was created with the use of dental cement. All screws were covered completely, but the cannula was not completely covered in order to allow dummy cannula to be inserted. Neosporin was then administered around the skull area and head cap and animals were provided a Rymadil-containing bacon-flavored tablet to ease transition from surgery. Animals were monitored during a period of post-surgery recovery and then returned to their homecages for 1 week before the onset of behavioral training.

Intracranial microinfusions

To acclimate animals to the process of intracranial infusions, animals had their dummy cannulas changed out (twice) during the week of recovery prior to behavioral training. Immediately prior to the beginnings of behavioral training and testing, animals were infused with the NMDA receptor antagonist APV ("APV-BNST") or vehicle ("Vehicle"; sterile saline) into the BNST. NMDA stands for N-methyl-D-aspartate, in which the main function of the receptor

includes the activity of the amino acid glutamate (Glu), which plays a central role in both the normal and abnormal functioning of the central nervous system (CNS). Glu is recognized to be the main excitatory neurotransmitter in the CNS. For the intracranial micro infusions, the process involved transporting the animals from the vivarium to a distinct room in the laboratory and gently removing the stainless-steel obturators from the guide cannulas. Drug or vehicle-filled injectors were inserted into the guides and drug or vehicle was infused into the BNST over the course of 1 min. After an additional minute to allow for diffusion of the solution, the injectors were removed, and clean obturators were inserted into the guides. Animals were then immediately transported to the training chambers.

Training and testing

All behavioral training and testing occurred within distinct rooms and chambers in the laboratory. These chambers are calibrated to detect movement, and therefore record the percentage of freezing animals across the trials. The conditioned stimulus (CS) for the experiment was a 10-sec, 2 kHz, 80 dB auditory tone. The unconditioned stimulus (US) was a 2-sec, 1 mA footshock. Accordingly, animals were submitted to fear conditioning procedures using either forward (“Forw”; CS-then-US) or backward (“Back”; US-then-CS) training.

Specifically, animals (in squads of eight rats) were transported to the testing chambers (the chambers were scented with a distinct odor to generate a unique context; Context A). After five minutes of acclimation to the context, animals experienced twelve forwards or backwards trials. Each trial was separated by 1 min. After the final trial, animals were returned to their homecages for 48 hours. Subsequently, animals were tested to the CS (five trials; 3-min baseline) in a novel context. 24 hours later, animals were returned to the original training context for a 20 min test. 24 hours after the first round of tests, animals were retrained to the forwards or

backwards stimulus (identical to before) but without administration of drug or vehicle. Animals were then submitted to the CS and context tests once more.

Histology

Thirty-two rats received intracranial microinfusions into the BNST. One subject was excluded due to off-target cannula placements (data shown in figures represent the final group totals, with all rats receiving bilateral infusions into the BNST), resulting in the following group numbers: Forw-APV-BNST (n=7); Forw-Vehicle (n=8); Back-APV-BNST (n=8); Back-Vehicle (n=8).

Statistics

All data were submitted to analysis of variance (ANOVA). Fisher's protected least significant difference (PLSD) test was used for post-hoc analyses following a significant omnibus *F* ratio in the ANOVA. Alpha was set to 0.05. All data are expressed as mean (+/- SEM) unless stated otherwise.

CHAPTER III

RESULTS

Behavior

Immediately after animals were infused with APV ("APV-BNST") or vehicle ("Vehicle") they were conditioned to the forward or backward CS (Figure 1). Across the training session, a main effect of conditioning trial was detected ($F_{1,27} = 123.907$, $p < 0.0001$); no other main effects or interactions were detected in the ANOVA. These data indicated that freezing increased across the training session, and that drug administration did not affect the ability of animals to engage in freezing during training.

Figure 1

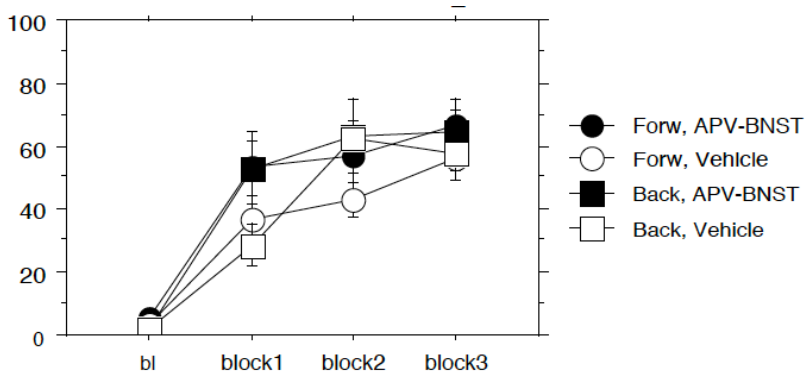

Figure 1. Conditioning to a forward or backward stimulus following infusion of APV or vehicle into the BNST. Y-axis depicts freezing in percentages (BL corresponds to the baseline period; block1-3 represents mean freezing at across 4-trial conditioning blocks).

Forty-eight hours later, rats were tested to the CS (in the absence of the US) in a novel context (Figure 2). Repeated measures ANOVA of testing trials revealed a significant main effect of trial ($F_{1,27} = 27.326$, $p < 0.0001$), and drug assignment ($F_{1,27} = 10.712$, $p < 0.005$). Split by training assignment ANOVA indicated a main effect of drug for Back animals ($F_{1,14} = 10.099$,

$p < 0.01$). These data indicate that APV administration into the BNST during training affects fear expression to the temporally uncertain CS selectively.

Figure 2

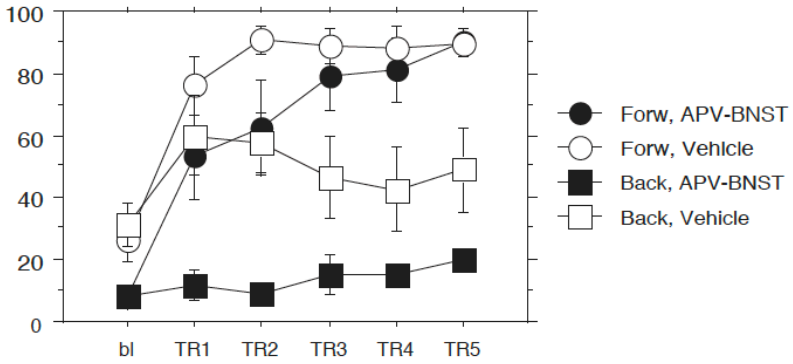

Figure 2. Drug-free test to the conditioned stimulus (in the absence of the unconditioned stimulus) in a novel context. Y-axis depicts freezing in percentages (BL corresponds to the baseline period; TR1-5 represents mean freezing at each test trial).

Twenty-four hours after the CS test, animals were returned to the original training context in the absence of the CS or US (Figure 3). A significant main effect of drug administration was detected across the session ($F_{1,28} = 11.806$, $p < 0.005$), but with no other significant main effect or interactions detected. These data indicate that NMDA receptors in the BNST also mediate contextual fear, likely a property of the temporal uncertainty of training context.

Figure 3

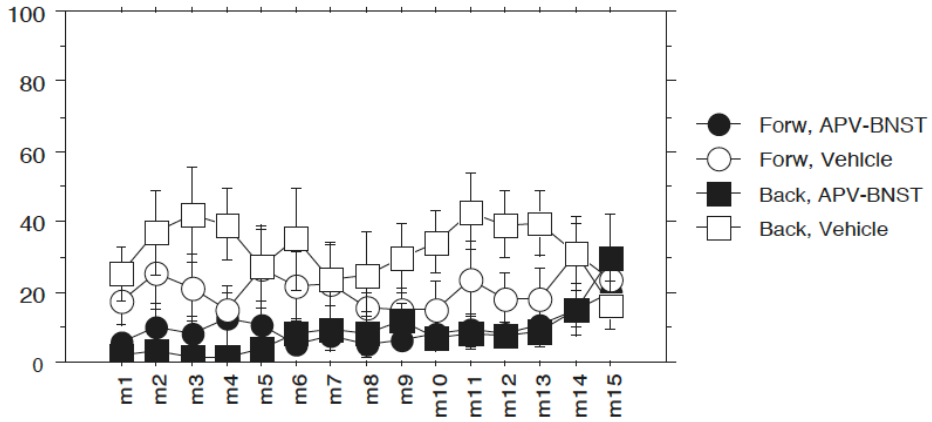

Figure 3. Drug-free test to the conditioning context in the absence of the CS or US. Y-axis depicts freezing in percentages; m1-15 depict mean freezing at each minute of the test.

CHAPTER IV

CONCLUSION

The current study reveals that the BNST plays a specific role in the plasticity-related learning processes that occur during fear conditioning to a temporally uncertain threat. The results revealed that NMDA receptors in the BNST is essential for learning about temporally uncertain stimuli, but not when the cues are highly predictive. Although our study mainly focuses on the timing of uncertain threats, other research has also explored the region of the BNST and how it can contribute to the formation of fear-related anxiety disorders.

Emerging evidence suggests that the BNST is a critical node in the stress response neurocircuitry and may play a significant role in anxiety, a highly debilitating disorder. There lies a unique role for the BNST in contextual fear as well as sustained, anxiety-like responses in rodents (Avery et al. 2016). This particular paper identifies the distinction between fear and anxiety, just as our research attempts to define. It is understood that anxiety is a future-oriented state elicited by threats that are physically distant, psychologically distant, or unpredictable. Fear, however, is a phasic state of heightened arousal and orienting towards an immediate and identifiable danger. A similar contrast can be seen in rodents, where anxiety-like behaviors are elicited by physically distant threats, or diffuse contextual threats such as location previously paired with footshock like that designed in our experiment. In contrast, fear-like behavior is elicited by more physically proximal or imminent threats (Avery et al. 2016).

This distinction between fear and anxiety provides a useful perspective for designing and interpreting human threat studies. Avery assesses BNST function during anticipation of threat in humans in order to translate the findings to engagement of the BNST in humans. In their

findings, it is seen that BNST showed the greatest response in humans when an unpredictable threat was introduced, as opposed to predictable or no threat contexts. These findings correlate with our results in that we see the unpredictability of events recruits BNST. Extending to the human model, the data provided strong evidence that the BNST tracks anxiety elicited by threat in humans. Human structural connects mirror that of rodent structural components, although the vast expansion and elaboration of structures in the human mandates further research of the precise spatial mapping for connection in the human brain. Furthermore, this paper reflects the similarity of the data in our research, although we have focused more on the role of the BNST in the anticipation of uncertain threats.

Other research on the BNST also focuses in the function of the BNST as the extended amygdala. To reiterate, the distinction made between fear and anxiety is addressed in our research, where predictable and unpredictable shock was evaluated. Our experimental design focused on the BNST activity associated with unpredictability. Rather than being associated with a discrete cue, the aversive event was associated with contextual cues (Davis et al. 2010). Conditioning to context was found to be unpredictable for all cohorts of rats, whether they were trained in the FW or BW condition. This further emphasizes the importance in timing in recruiting the BNST to predict an aversive event in a context associated with training. In the human model, context conditioning studies evaluated by assessing startle levels in the absence of light (Davis et al. 2010). In these experiments, data showed that human response was consistent with animal data, where context conditioning was found to be greater in the unpredictable context, compared with that in the no shock or predictable context. This further suggests that relation between data among animals and humans is quite similar. Understanding that animal

data found in research can be highly correlated to implicating human response demonstrates the effectiveness of the BNST in both animals and humans.

Acknowledging that fear and anxiety are similar but nor identical, laboratories are attempting to develop a more operational definition of fear and anxiety, which have been termed 'phasic' and 'sustained' fear. With the attempt to define the distinction between the two, it is also important to include the research that suggests the amygdala in itself also interacts with the BNST in responses to unknown threat. Although it appears definite that BNST is not required for the genesis of defensive behaviors triggered by discrete threatening cues, evidence suggests that it can modulate the processing of such cues (Gunger and Paré 2016). It is seen that in other research such as Gunger's, that the BNST's role is not limited to the generation of aversive responses to diffuse threats but that it also shapes the impact of discrete threatening stimuli. Therefore, it is important to note that BNST is implied to have a close interaction with central amygdala, where in threatening conditions, interactions between the two locations likely determine the intensity and specificity of aversive responses. Another area of our data that is similar to other research can be found in the experimental design in evaluating the extended amygdala (Walker and Davis 2008). It was found that measuring fear-potentiated startle tests using conditioned stimuli that vary in *length* suggested that the central nucleus of the amygdala and the lateral division of the BNST were involved in short-term versus long-term fear responses (i.e., phasic and sustained fear, respectively) (Walker and Davis 2008).

In contrast to the previous studies discussed, other BNST research has involved lesions, which was not a method used in our experiment. The only data we intend to present in relation to lesions, is that our findings suggest the same effect of freezing can be found in the BNST without the need for removing that section of the animal's brain. Our data found that inactivation of the

NMDA receptors in the BNST was more than sufficient in representing the effectiveness of context and APV alone. However, for the purpose of examining the effect of BNST activity in other research with complete extraction of the BNST, behavioral responses from these data will be examined. It was found that BNST lesions elicited higher levels of movement, and lower levels of freezing. This further suggests that the BNST plays an especially important role in unconditioned fear and anxiety, given that the lesion contributed to inactivity of the BNST altogether and possibly recruited other areas surrounding the lesioned site. To better explain these particular results in an example, BNST lesions disrupt a rat's spontaneous preference for a dark over a light chamber, a response generally believed not to involve condition (Walker and Davis, 1997; Sullivan et al. 2004). Therefore, BNST is involved in contextual processing of threats, as well as certain aspects of unconditioned fear (Sullivan et al, 2004). Just like our results, these data show similarity in that the BNST is mainly involved in fear responses to contextual stimuli, including both behavioral and neurological elicited by learned and unlearned situations.

A final study also involving the lesions of the BNST is that of Hammack and his colleagues – where the rats received lesions, followed by condition trials in exposure to a context paired with footshock. Results indicated that BNST lesions caused a deficit to learning to the aversive situation. This further provided strong evidence that the BNST is involved in the *timing* of onset events, where the BNST is mainly recruited in aversive conditioning to long-duration, and not just contextual, conditional stimuli. Although less consistent with the view that BNST becomes activated *after* prolonged fear, it is hypothesized that BNST is involved when the onset of a cue has a remote temporal relation to shock. This is consistent with our research, only in that the timing is further suggested to be involved in the activity of the BNST in learning of the cues

associated with the anticipated aversive event. Results showed that the BNST lesions reduced freezing in the longer duration conditioning rather than the short-term conditioning situation. This suggests that the freezing to a contextual CS is *not* affected by a BNST lesion unless it is longer than the 1-minute duration period (shorter duration). The results suggest that the status of a CS as a contextual stimulus does not necessarily guarantee a role for BNST activity (Hammack et al. 2015). Hammack's inclusion of testing the *timing* of shock intervals brings the results down to how BNST is recruited *after* lesion (therefore inactiving BNST altogether and permanently) in correlation to aversive condition and their respective behavioral responses.

Summary

Overall, our results contribute to understanding the BNST's role in learning about threat. This, in turn, may help to improve potential behavior and brain therapies by targeting the BNST in human subjects. These finding have important implications for future anxiolytic therapies of fear- and anxiety-related disorders (including posttraumatic stress disorder, PTSD) that target the BNST.

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