Drew University

College of Liberal Arts

Bed Nucleus of the Stria Terminalis: Potential Odor Responsivity and Cannabidiol Modulation

A Thesis in Neuroscience

by

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Abstract:

Anxiety disorders exist at a high prevalence in the United States. Current research examining anxiety-related brain regions, as well as studies on drugs that alter the activity in these regions, contribute to improvements of therapies for the disorder. Recently, studies looking characterizing the activity of the bed nucleus of the stria terminalis (BNST) have determined that this region plays a key role in sustained anxiety and contextual fear, as opposed to the well defined amygdala, which is primarily involved in phasic fear. Further, presentation of predator odors have induced anxiety behaviors in animal models, likely because of the non-volatile processing projections from the vomeronasal organ (VNO) to accessory olfactory bulb (AOB), medial amygdala (MeA), and finally, the BNST. In the present study, electrophysiological methods were used to search for baseline and odor-elicited activity in the BNST to further confirm this region's role in anxiety, as well as test the potential for this methodology to be an efficient tool in studying anxiety. It was hypothesized that the BNST would present with baseline activity in the anesthetized rat and have non-volatile odor (2-heptanone) elicited activity, but not volatile odor-elicited activity. Ultimately, there was sufficient baseline activity but no odor-elicited activity to either odor types. Previously obtained data recorded in the olfactory tubercle (OT) were also analyzed to provide a comparison for baseline and odor-elicited activity. The OT had a similar baseline frequency but, in contrast, were responsive to isoamyl acetate, 2-heptanone, propyl butyrate, and 1,7-octadiene. Additionally, intracerebroventricular doses of cannabidiol (CBD; 1, 2, and 3 μL) were presented to the rats while being presented with odors to test the if drug's potential therapeutic effects may be enacted through altering the activity in the BNST. It was hypothesized that CBD would change the activity of both baseline and odor-elicited activity; however, since there was no odor-elicited activity, only the effect on baseline activity could be observed. CBD did not consistently change baseline activity. One of three cells presented with 1 μL of CBD demonstrated a change in activity that occurred at an appropriate time relative to drug administration. Overall, the potential for using this model and drug likely remains in question due to the potential areas of improvement including altering the mechanism of odor or drug introduction as well as fine tuning existing methods.

Contents:

Introduction:

Anxiety Disorders

Anxiety disorders, including generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), and posttraumatic stress disorder (PTSD), are among the most prominent group of mental illnesses in the United States (National Institute of Mental Health, 2017). Recent statistics suggest that 31.1% of adults in the country suffer from one of many anxiety disorder subtypes at some point during their life. While many might experience phasic feelings of anxiety during stressful situations, having prolonged and frequent feelings of anxiety, along with a number of physical abnormalities, which interfere with daily activities, constitute anxiety disorders (National Institute of Mental Health, 2018). Whether the anxiety disorder be GAD, PTSD, or OCD, symptoms typically include mild to severe feelings of nervousness, a lack of control, shortness of breath, increased heart rate/ blood pressure, or worriedness. The etiology of this spectrum disorder remains debatable, with irregularities or patterns stemming from neuroanatomical differences, neurotransmitter/neuroendocrine imbalances, as well as genetic or environmental influences (Martin et al. 2009). Due to the large prevalence of those affected by the disorder, many resources have been devoted to characterizing anxiety and its origins in order to ameliorate symptoms.

In general, anxiety disorders are associated with abnormal function in a series of brain structures that show evidence of processing emotion (Martin et al. 2009). This series of structures, particularly involving the limbic system and frontal cognitive areas, works to evaluate situations utilizing brain regions associated with memory, affect, cognitive state, and sensory input from the internal and external environment. Several key structures include the

hippocampus, the amygdala, and the orbitofrontal cortex (OFC). The OFC, for instance, is involved in integrating and associating sensory stimuli in order to make decisions based on previous stimulus-reinforcement (Rolls, 2004). The region incorporates both primary reinforcers, like taste or touch, as well as relate abstract information, such as money, to support understanding of rewards and punishment and ultimately lead to the expression of appropriate behaviors and emotions. Patients or animal models with damage to this region exhibit difficulty in making stimulus-reward or stimulus-punishment associations, making it challenging to react appropriately to stimuli on both a social and emotional level.

Additionally, the hippocampus aids in stimulus-association modulation through managing and facilitating the creation of positive, negative, or neutral memory of stimuli (Martin et al. 2009). As the survival of species is largely dependent on avoidance of threats and sustaining of life promoting factors, it makes sense that the hippocampus be recruited to create memory of previously encountered stimuli. In aiming to address how the dysfunction of this region might contribute to anxiety disorders, Schumacher et al. (2018) inactivated various groups of cells in the hippocampi of rats and placed them in an environment where there were aversive, appetitive, and neutral stimuli. They found that ventral CA3 inactivation led to more approach to the conflict cues while inactivation of the ventral CA1 fibers lead to increased avoidance behavior to the conflict cues. These results further solidify the hippocampus' role in beneficial or survival promoting anxiety that leads to the appropriate level of avoidance behavior, as well as the specific cell groups that work in opposition to respond appropriately.

Another structure, the amygdala, is highly associated with fear-related activity, memory, aggression, defense, as well as species specific behavior (Martin et al. 2009). To further support

its involvement in fear and anxiety, the central nucleus of the amygdala (CeA) has many projections to regions such as the lateral hypothalamus to control heart rate and blood pressure, the parabrachial nucleus to influence respiration, the basal forebrain to impact attention and arousal, as well as the central gray area that is associated with freezing behavior and social interaction. The amygdala has been noted to be involved in the initiation of many of these behaviors in quick response. Dysfunction of activity in the amygdala can lead to the expression of anxiety disorder-like symptoms via all these subsequent regions. Evidence from fear conditioning and fMRI studies have shown how plasticity in the lateral amygdala (LA) is critical for fear responses via the basolateral amygdala (BL) (Lee et al., 2018). Previous studies where the BL was inactivated also found an extinction of fear-responses to stimuli while optogenetic excitation of the regions that project to the hippocampus was able to induce anxiety states in subjects.

Other studies using fMRIs look to identify potential biomarkers of anxiety disorders (Blair et al. 2016). For instance, fMRI was conducted while participants demonstrated various types of social reference learning. Patients that were diagnosed with social anxiety disorder, for example, displayed a higher level of activity in the amygdala during the social recognition learning and especially during times when being presented with other people's negative reactions. The authors claim that the higher level of activity is representative of faulty observational learning. In contrast, a recent case study of a female patient demonstrated how bilateral calcifications of the amygdala lead to a total extinction of fear (Lilienfeld et al. 2018). These results are consistent with the idea that anxiety disorders and heightened fear response involves an upregulation of amygdala activity.

These regions, particularly the hippocampus and amygdala, also go beyond making associations and initiating behaviors, but also cause a physiological response via their projections through hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1**; Martin et al. 2009) The HPA axis translates central nervous system activity to endocrine activity through the cascade of events that follow from the hypothalamus, to the pituitary, and then adrenal cortex (Smith & Vale, 2006). Activation of the hypothalamus will lead to the release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN), which leads to the release of of adrenocorticotropic hormone (ACTH) through cyclic adenosine monophosphate (cAMP) in the pituitary gland. The release of ACTH will bind to receptors of the adrenal cortex and promote the synthesis and release of glucocorticoids. Glucocorticoids will both regulate the activity of the HPA axis and lead to the physiological changes throughout the body that accompany stressors.

Again, the amygdala and hippocampus will influence the HPA axis according to exposure to stimuli. The hippocampus, for instance, will project to the hypothalamus to provide inhibitory input causing a decreased release of corticotropin releasing factor (CRF) by the hypothalamus, adrenocorticotropic hormone (ACTH) by the anterior pituitary, and further, glucocorticoid, such as cortisol, by the adrenal cortex (Vashist & Schneider, 2014; **Figure 1**). This is dependent on the level of stress being experienced. For instance, mild to moderate stressors will lead to increased activation of the hippocampus to allow for synaptic plasticity and rearrangement, while strong to severe will lead to inactivation of the hippocampus (Kim et al., 2015).

The opposite can occur when there is positive input from the amygdala. With increased activation of the amygdala in response to fear, there will be excitatory signal projecting to

hypothalamus, as well as inhibitory signal projecting to the hippocampus to disinhibit its own projections to the hypothalamus (**Figure 1**). This leads increased release of the stress hormone, cortisol, which is typically dependent on both circadian regulation and physical or emotional stressors. Cortisol is also associated with adrenal exhaustion resulting in increased sensitivity to stressors when exposed to chronic stress, providing further relevance to anxiety. In general, cortisol regulates the stress response by increasing glucose availability for muscles and the brain while decreasing the actions of non-essential systems including the digestive and immune system (Mayo Clinic, 2016). Chronic stressors have been seen to lead to dysregulation of the systems modulated, leading to physical and mental illness.

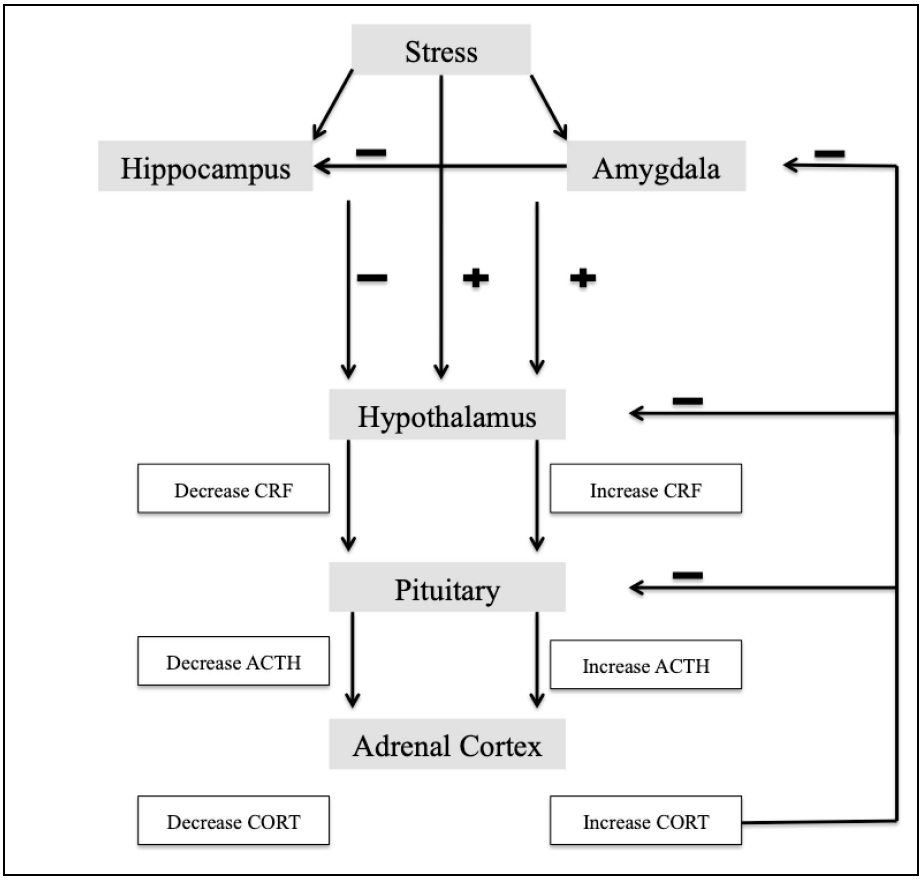


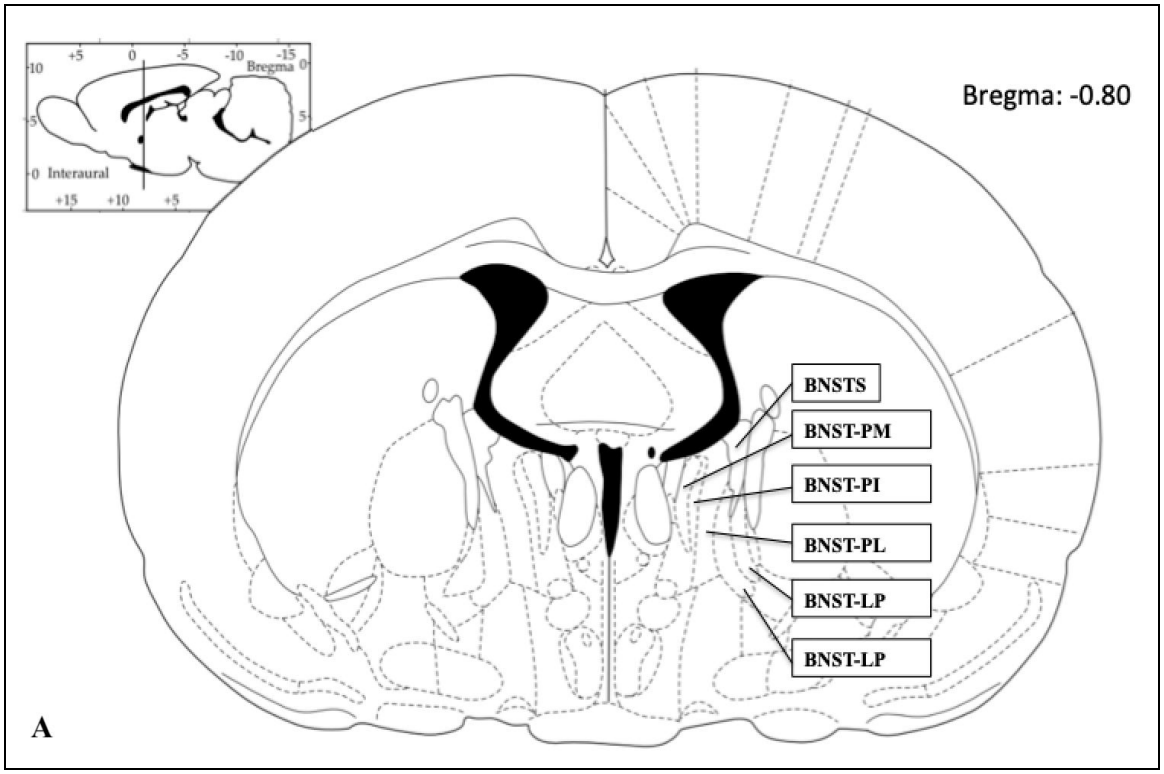
Figure 1: The flow chart describes how stressors or feedback from the hippocampus or the amygdala can influence the HPA axis (Vashist & Schneidel, 2014). Positive feedback is depicted by a plus sign and negative feedback is depicted by a negative sign. The boxes on the

sides indicate the secretions of each structure and how they are impacted by input.

BNST

The bed nucleus of the stria terminalis (BNST) is another brain region, comparable to the amygdala, that has also been found to play an integral role in anxiety and stress response (Lebow & Chen, 2016). The BNST is rostral to the amygdala, ventral to the lateral septum and dorsal to the hypothalamic preoptic area (**Figure 2b**; Crestani et al., 2013). Also known as the "extended amygdala", the BNST sub regions appear to be a continuation of amygdalar structures connected through the stria terminalis and ventral pathway. For instance, the central amygdaloid nucleus (CeA) is mirrored by the lateral BNST and the medial amygdaloid nucleus (MeA) is mirrored by the medial division of the BNST. In addition to their projections between each other, they also project to effector centers, such as the hippocampus, insula, and mPFC, and hypothalamus, in parallel (Gorka et al., 2017; Sulliven et al., 2014). As a result, both the amygdala and BNST are involved in the production and modulation of fear states and responses through behaviors and physiological changes (Lebow & Chen, 2016). Differences between these two regions, however, include how the amygdala is more involved in a phasic fear response, while the BNST is more active in the sustained fear response and social facilitation (Rodriguez-Sierra et al., 2016). Given its role in sustained/contextual fear response, it is no surprise that the BNST has connections with the hippocampal-hypothalamic circuit (Lebow & Chen, 2016). More specifically, the dorsomedial nucleus of the BNST receives input from the hippocampus, as well as dopaminergic inputs from the ventral tegmental area (VTA; part of the reward system pathway) and serotonergic inputs from the raphe nuclei, then projects to the paraventricular nucleus (PVN) of the hypothalamus in order to acquire learned contexts and respond according to previous

exposure. There is a great deal of interconnectivity and common targets with the CeA and BNST; however, the BNST is historically less studied.



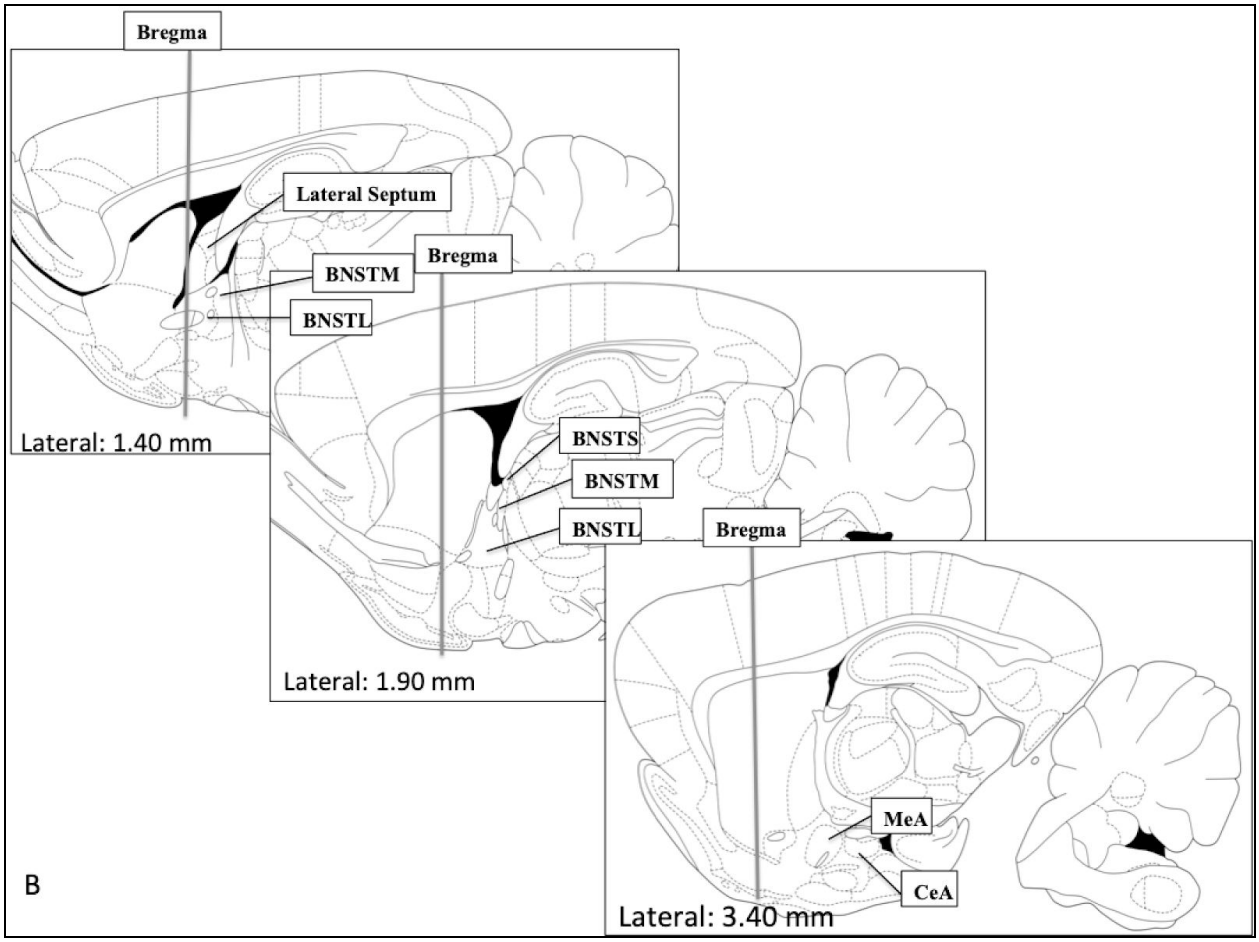


Figure 2: A- The coronal slice, taken -0.80mm behind bregma, indicates the location of four posterior BNST subregions, including the BNSTS (supracapsular part), BNST-PM (posteromedial), BNST-PI (posterointermediate), and BNST-LP (posterolateral). **B-** The three atlasas (Paxinos, 2007) depict where the BNST is in relation to other regions and in relation to bregma (gray line). Panels in the back are more medial and panels in the front are more lateral. In the panel farthest back is the lateral septum, which is lateral to the BNST but less lateral to the midline. In the middle panel are three regions of the BNST (BNSTS; MPM: medial division, posteromedial part; BNSTL: lateral division). In the third and closest panel are the medial and central amygdala, which are more posterior (in relation to bregma) and more lateral than the BNST.

Existing research on this brain region determined that there are ultimately between 12-18 cell groups of the BNST within the anterior and posterior regions, 12 of which are described in **Table 1** below (Lebow & Chen 2012). These cell groups differ in the neuronal receptors, neurotransmitters released, as well as transporters and proteins integrated. Though there is

integration and cross talk between the two regions, the posterior region (BNSTp) is involved with reproductive and defensive behaviors, while the anterior region (BNSTa) is more involved with anxiety regulation and contextual fear (Davis et al. 2010). For instance, optogenetic excitation of the neurons in the oval nucleus, having postsynaptic dopamine (DA) receptors that receive input from the VTA, resulted in anxiety-like responses. Connections such as with the fusiform and rhomboid nucleus to the periaqueductal grey and VTA play into the pain, risk, and reward systems that contribute to whether past or previous stimuli should invoke anxiety. Other nuclei are also involved in the physical response through hypothalamic and brainstem connections. Interestingly, studies have found that noradrenaline is released from the ventral BNST in response to synthetic fox odor (2,3,5- Trimethyl-3-thiazoline; inducing an innate fear response), unconditioned stimuli, and conditioned stimuli without the presentation of the unconditioned stimuli.

The posterior division, comprised of the principal, interfascicular, and transverse nuclei, receives inhibitory input from different regions of the amygdala (CeA and MeA), and excitatory input from the paraventricular thalamus (emotional behavior and valence), and hippocampus (Lebow & Chen, 2012) . As this region leans towards a more social function, it is a sexually dimorphic collection of nuclei with varying levels of estrogen and androgen receptors. While the posterior division primarily supports defensive behaviors, social recognition, and physical responses (mediated by the HPA axis) through direct projections, it also has dense interconnectivity with the anterior division of the BNST to incorporate multiple inputs and mediate appropriate stress responses. This summation of data provides reason and explanation for the BNST's role in anxiety and valence across numerous regions of the BNST; however,

primarily in the anterior division. It is important to consider that researchers who have looked at these brain regions in detail have mostly done so using rodent models. Post-mortem and fMRI studies (revealing increased activation when anticipating anxiety-provoking situations) show that the BNST is composed of lateral, medial, central, and ventral divisions, where the ventral composes the majority of the posterior region (Lebow & Chen, 2012). Current research has indicated that both rat and human BNSTs are sexualy dimorphic and have similar posterior pathways with connections with the MeA and hypothalamus.

Table 1: Description of eleven anterior and posterior subregions of the BNST

Region	**Function**	**Features and Connectivity**
Anterior Division		
Oval Nucleus	Integrates mood and negative valence; promotes anxiety behavior	Inhibits the CeA, VTA, and hypothalamus through GABAergic projections. High levels of corticotropin-releasing factor (CRF) neurons. Receives input from VTA via DA receptor neurons.
Anterolateral BNST	Involved in valence assessment prior to stimulus presentation. Contributes to the regulation of drinking and fluid homeostasis as part of HPA response.	Inhibits neuroendocrine centers of the paraventricular (PVN) hypothalamus and has steroid hormone receptors for oxytocin, for example. Receives input from ventral subiculum and dorsal raphe serotonergic inputs. Projections to vagus nerve.
Fusiform nucleus	The fusiform, juxtacapsular, and rhomboid nuclei are	Projects to CeA, PVN, nucleus accumbens, periaqueductal gray (pain), and reticular nuclei (attention).

	less studied but have overlapping involvement in anxiety, pain, and reward systems.	High levels of corticotropin-releasing factor (CRF) neurons.
Juxtacapsular nucleus		Projects to CeA, basolateral amygdala, substantia nigra, and dorsal raphe
Rhomboid nucleus		Projects to CeA, VTA, substantia nigra, nucleus accumbens, and globus pallidus (reward); PVN, reticular nucleus, periaqueductal gray (pain). High expression of GABA, Glutamate, dynorphin (opioid system), and dopamine receptors.
Ventral BNST	Involved in assessing the appropriateness of and readiness for the response to sensory input	Has the largest density of noradrenergic fibers in the brain (sympathetic nervous system involvement). Receives input from the medulla and nucleus of the solitary tract of the brainstem (relating to cardiovascular response).
Anteromedial BNST	Contributes to the regulation of drinking and fluid homeostasis as part of HPA response.	Projections to Barrington's nucleus (fluid homeostasis), PVN, and CeA.
Dorsomedial BNST	Integrating social information, initiation of fight or flight behaviors, and valence or reward response modulation. Hypothesized to be a region where projections to the PVN leading to chronic CRF lead to dysregulation of the autonomic systems.	Has the most projections to the hypothalamus of the BNST subnuclei. Projects to AVP, oxytocin neurons of the magnocellular PVN (GABAergic; producing thyrotropin-releasing hormone, CRF, somatostatin, and gonadotropin-releasing hormone (likely suppressing latter two during flight or flight response as growth and reproduction are non-essential) Receives input from MeA, CeA, hippocampus, insula, prefrontal cortex, dopaminergic projections from VTA, serotonergic projections from the raphe nuclei, and noradrenergic

		neurons from the locus coeruleus.
Posterior Division		
Principal nucleus	Inhibits some medial BNST subnuclei that activate the HPA axis according to social and valence input.	Projects to hypothalamic areas that regulate reproduction and defensive behaviors such as the pre-mammillary bodies and PVN. Has a levels of GAD65, an enzyme that will synthesize inhibitory GABA neurotransmitter.
Transverse nucleus	Involved in social recognition and stress response.	Both project to the VTA, substantia nigra, and other mesolimbic brain regions. Both receive input from the anterior olfactory bulb and MeA. The transverse nucleus has inhibitory projections to the PVN (stress regulation)
Interfascicular nucleus		

BNST and Anxiety

Early studies on the BNST were able to explain how there is a difference between fear and anxiety and the process by which it is modulated. For instance, Sullivan et al. (2004) explain how the amygdala, specifically the central nucleus (CeA), has direct connections to the PVN of the hypothalamus (leading to increased CRF release with stress). In this way, it directly influences the release of the glucocorticoid hormone, corticosterone (CORT). The amygdala, based on situational assessments, signals for the release of CORT in the PVN of the hypothalamus as part of the HPA axis, which contributes to the ability to adapt and respond to physical and psychological stressors. Building upon this direct connection, the CeA has direct connections to the BNST, which also has connections to the PVN to mediate the HPA response. This insinuates that the BNST can modulate activity that passes from the CeA to the PVN and also project its own signal incorporating other valence and socially driven input. To further

examine this, Sullivan et al. (2004) created lesions in the CeA or the BNST to test what specific role each structure has in modulating HPA response. Before creating the lesions, the authors presented the rats with either a cue or a context and either a paired shock or no shock. The cue stimulus (presenting a tone) served to induce phasic fear, while the context stimulus (putting the rat in one of two chambers) served to induce sustained anxiety. The authors gave the rats one of two lesions and measured the subsequent freezing behavior. Sullivan et al found that the CeA lesioned rats experienced a decrease both cue and contextual induced freezing behavior, while the BNST lesioned rats experienced only decreased freezing behavior to contextual stimuli. Additionally, CORT release was found to be less following BNST lesion. These results suggest that the CeA is more involved in modulating fear responses, and the BNST is more involved in modulating anxiety driven responses. Still, it is important to note that the BNST lesions were made in a series of 3 and histological analysis revealed that medial and lateral regions were compromised. The author's results may not have ruled out that other preserved BNST regions could be involved in phasic fear.

More recently, studies comparing the CeA and BNST continue to differentiate between fear and anxiety pathways at a more precise level. For instance, Haufler et al. (2013) explain how previously conducted studies, using methods like drug infusions or lesions, suggest that the BNST is more involved with contextual anxiety and cue induced fear. These studies, however, still fail to address the specific path of fear response that is hidden by dense interconnectivity. To explore this subject further, Haufler et al. conducted their own study where tetrodes were placed in the anterolateral (BNST-AL) and anteromedial (BNST-AM) BNST of rats before conditioning them to a specific context (the cage they were placed in plus a foot shock). Results demonstrated

that the two regions were mostly active when presented the conditioned stimulus. Additionally, the BNST-AM had a higher level of activity when the behavioral response was freezing as opposed to continued movement. This further confirmed that there is functional variation between regions of the BNST, and suggests that one region might be more involved in anxiety than the other.

Moreso, the most common types of cells in the BNST are GABAergic (having cells that produce and release inhibitory neurotransmitter), suggesting the BNST has an inhibitory in function (Gungor & Pare, 2014). This is in line with previous studies that suggest that the BNST is responsible for the constant evaluation of context or threat, and is likely a complex structure with many reciprocal connections. On the other hand, some studies have shown how intra-BNST injections of calcitonin gene-related peptide (CGRP), which is involved in pain processing, leads to the excitation of BNST targets. To understand if the BNST is primarily inhibitory or excitatory when regulating anxiety states, Gungor and Pare recorded BNST-AL neurons *in vitro* using patch clamp and measured the effect of CGPR. The authors found no effect of CGPR on the firing pattern of the BNST-AL cells and no difference in amplitude in the excitatory postsynaptic potentials (EPSPs). Instead, they found an increase in inhibitory postsynaptic potential (IPSP) amplitude. This difference, however, did not correlate with the addition of CGRP. Overall, the data indicated that CGRPs are acting postsynaptically, and that the BNST is primarily inhibitory in nature. From this data, we can consider how the BNST becomes dysfunctional or how one can use medication to alter its activity. For instance, the BNST might be activated by a threatening context, which in turn inhibits the CeA so that there is less excitation of the HPA axis.

Using predator odors to probe anxiety related regions

In the past, presenting odors to rodents and measuring the subsequent behavior has been a model for examining the nature of anxiety responses. In general, triggering olfactory processing can occur through a simple pathway from olfactory receptor to olfactory bulb to olfactory cortex (piriform cortex, anterior olfactory cortex and olfactory tubercle (OT)), or to and from non-olfactory regions (Wesson & Wilson, 2011). Stimulation of these olfactory and non-olfactory regions depend on the secondary olfactory bulb neuron type. Tufted cells projecting from the olfactory bulb respond better to lower concentrations and have broader receptive field in comparison to the other secondary olfactory cell type, mitral cells.

Previous studies have employed biological volatile odorants to stimulate the olfactory and non-olfactory regions. Some have looked at multimolecular natural odors (e.g. from foods/plants) while others have looked at the importance of their monomolecular components (e.g. limonene or propyl butyrate) that are important for pattern recognition and association (Barnes et al., 2008). Others have investigated how monomolecular predator odor components have influenced behavior and brain activity. It is important to acknowledge the distinction between these two because of the different pathways that volatile and non-volatile (pheromone derivatives) information takes in the brain (Bergan et al., 2014). Volatile odors like limonene or propyl butyrate will be detected in the main olfactory epithelium (MOE) which will then project to the main olfactory bulb (MOB) and then to the primary olfactory cortex or lateral amygdala. In comparison, non-volatile odors like 2-heptanone (pheromone derivative) will be detected in

the vomeronasal organ (VNO) and proceed to the accessory olfactory bulb (AOB) and then to the MeA, BNST (interfascicular division), and hypothalamus.

From a behavioral standpoint, previous studies have taken advantage of the latter pathway (originating in the VNO) to examine the differences between the anxiety and fear responses in the BNST and amygdala. For instance, in an early study by Fendt, Endres, and Apfelbach (2003) trimethylthiazoline (TMT, a molecule extracted from fox feces) was presented to rats that had either the amygdala or BNST inhibited by $GABA_A$ receptor agonist and measured subsequent freezing. The authors found that the inactivation of the BNST but not inactivation of the amygdala stopped freezing behavior, indicating that the BNST holds a more prominent responsibility to responding to innately frightening stimuli. This is interesting because it makes sense given the pathway (from the VNO) that it activates and the need for prey to quickly or innate identify predators to ensure survival; however, the data contrast other sources that claim that the BNST is more involved with contextual fear (anxiety) and the amygdala is more involved with phasic fear. Still, other behavioral studies studies have found that lesions in the posterior division of the BNST impair male rodent opposite-sex odor investigation to eliminate pheromone odor preference, for example (Been & Aras, 2010). This further solidifies at least the posterior division's role in innate fear relating to pheromone odor components, social interaction, and survival behavior.

To look at the precise involvement of the regions and subregions involved in anxiety, odors can also be presented to anesthetized rodents to measure subsequent activity and gain a better understanding of anxiety. Using electrophysiological techniques can serve to identify distinct nuclei within the amygdala, for example, that are involved in this fear response. For

instance, when Govic and Paolini (2015) presented freely breathing rats with cat urine, or distilled water as a control, they found that the MeA had the greatest selective response in comparison to the basolateral nuclei, which had the second greatest selective response, and the CeA having the third. There are no apparent electrophysiological studies similar to this looking at the BNST; however, one might be able to infer that if the MeA is having the most selective response to the alarm odor (stemming from the VNO), than the regions that it projects to, including the interfascicular region of the posterior BNST, then the BNST might also experience a secondary selective response.

Evidence supporting that the BNST would have a selective response to non-volatile odors, much like the MeA did *in vivo*, has been previously seen *in vitro*. For example, one study by Rodriguez-Sierra et al. (2016) aimed to examine the nature of the BNST in anxiety disorders by creating a PTSD model. They did so by exposing a group of rats to predator odor (non-monomolecular: soiled cat litter containing feces, urine, and hair) and then tested for the PTSD-like expression through performance in the Elevated Plus Maze, where rats who spent time in the open arms were considered resilient and rats that spent the duration of the test in the secure closed arms were put in the PTSD-like group. Following their classification, respective brains were removed and sliced to test activity and responsivity in the amygdala and BNST by using patch clamp. The authors found variations among the subregions of each structure; however, found that in cells, such as the BNST-AM (anteromedial) cells, there was higher responsivity in the PTSD rat than the non-PTSD/resilient rats in particular. The data suggest that odors can invoke sustained neuronal changes in these regions, particularly the BNST-AM, that are relevant to anxiety disorders. It is interesting, however, that they chose to only look at the

anterior BNST and not the posterior BNST. They did so because of its more prominent role in sustained anxiety; however, it might have been useful to see what types of synaptic changes occured in the posterior division given the model that they used employing non-volatile odors.

Pharmacotherapies for Anxiety

Anxiety disorders are disruptive in one's daily life and are often treated with psychotherapy, pharmacotherapy, or both. Pharmacotherapy approaches target different neurochemicals and receptors to manipulate the activity in various brain regions, especially those that might play a role in anxiety. As there is a high prevalence of psychiatric disorders, it is reported that 1 in 6 Americans take psychiatric drugs whereas 1 in 50 did thirty years ago (ADAA, 2019). Broadly, there are four major classes of drugs used to treat anxiety disorders: selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), benzodiazepines, and tricyclic antidepressants (TCAs).

SSRIs and SNRIs are typically the first class of drug to be prescribed and exhibit anxiolytic effects after taking the drug for 2-6 weeks. SSRIs, SNRIs, and TCAs block the reuptake of serotonin or norepinephrine to increase the concentration of serotonin in the synapse so that postsynaptic ion channels have a greater probability of opening to create dramaticized postsynaptic responses, as well as trigger a signaling cascade of other neurotransmitters, second messengers, and gene transcription to enact long term cellular changes (Farach et al. 2012; ADAA, 2019). Benzodiazepines are used for short-term management and produce anxiolytic effects quickly by binding to GABA-A receptor complex. This allows for chloride ions to enter the cell to bring the cell farther from its threshold making it less likely to fire an action potential. There are a number of other drug types beyond these four classes, such as calcium modulators

for example, yet all produce an array of side effects (ADAA, 2019). For instance, the reuptake inhibitors may cause jitters, insomnia, weight gain, or digestional issues. Benzodiazepines may lead to depression, fatigue, decreased motor skills or cognitive functions, and are associated with dependency that can lead to withdrawal symptoms (Bandelow, Michaelis, & Wedekind, 2017). Negative side effects, as well as the high prevalence of these drugs not producing sufficient anxiolytic effects in patients, has led to the search for more pharmacotherapies.

Cannabidiol

Cannabidiol (CBD) is the most abundant compound in the *Cannabis sativa* plant. In fact, the plant's extract can be composed of up to 40% CBD (Campos et al., 2012). CBD is not as well known as the plant's hallucinogenic compound, **Δ**-9-tetrahydrocannabinol (THC) (Norris *et al*, 2016). Instead of having a cyclic ring with an ether like THC, CBD has a hydroxyl group where one of the adjacent methyl groups forms a double bond with the extra electron density after decarboxylation from its precursor, cannabidiolic acid (Rudd, 2018; **Figure 3**). These structural differences lead to CBD not being psychoactive and having a number of potential therapeutic uses (Klein et al., 2017). Previous findings suggest that the drug has antidepressant, anxiolytic, anticonvulsant, and anti-inflammatory effects through a number of mechanisms involving the cannabinoid CB1 and CB2 receptors, GPR55 receptors, TRPV1 cation channels, and the 5-HT receptors. (Campos et al., 2012).

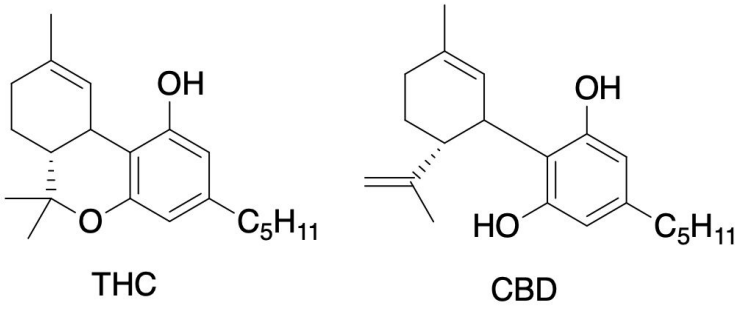


Figure 3: The structure of THC in comparison to CBD.

Endocannabinoid receptor system

With regards to the cannabinoid receptor system, CBD has been previously found to have low levels of affinity for CB1 and CB2 receptors and was at one point considered to act as an inverse agonist at low concentrations (Pertwee, 2008). CB2 receptors are found abundantly in the peripheral nervous system and are involved modulating the inflammatory response through altering cytokine release, for example. On the other hand, CB1 receptors are found mainly in the CNS and modulate neurotransmitter release (acetylcholine, noradrenaline, dopamine, serotonin, glutamate, etc.) and further, neuronal activity. When CB1 receptors are acted upon by endogenous antagonists, there is a decrease in adenylyl cyclase that results in a decrease in neuronal excitability and transmission (Mackie, 2008). In the absence of agonists, CB1 receptors exist at an equilibrium of active and inactive states to produce a basal activity level. A neutral antagonist would prevent binding of an agonist, which would have no effect on basal activity. In contrast, an inverse agonist binds selectively to the inactive-state, decreasing the amount of active-state receptors and lowering the basal activity.

More recently, it has been reported that CBD more accurately acts as a negative allosteric modulator (NAM) of CB1 receptors (Laprairie et al., 2015). In an *in vitro* study using HEK 293A cells to represent receptor signalling and the high CB1 expressing ST*Hdh*$^{Q7/Q7}$ cells from

the striatal motor pathway, Laprairie et al. tested CBD's NAM effect. The authors measured parameters such as ligand cooperativity, efficacy and orthosteric/allosteric ligand affinity (K_A and K_B) while using both agonists (endogenous 2-AG and exogenous THC) and an antagonist (O-2050). Ultimately, they found that CBD gave rise to negative cooperativity and decreased ability for the ligand to perform its normal cell response. Additionally, CBD decreased GPCR signalling and arrestin signalling, consequently preventing alternative signalling pathways that arrestin introduces. The results were not found to be dependent on the agonists and antagonist presence. Importantly, the authors note that the ST*Hdh*$^{Q7/Q7}$ cell type also express several other receptor types in high quantities, including GPR55 and $5HT_{1A}$ receptors. This potentially implies that the NAM effects may be occuring as a result of interactions with other receptor types.

GPCRs

While CBD and other cannabinoids have been found to act as ligands to CB1 and CB2, these interactions fail to account for the wide range of effects this class of molecule seem to have within the body (Sharir & Abood, 2011). Other receptor types are now being considered cannabinoid receptor subtypes, including GPR55. As the CB receptors are mediated by g-protein coupled receptors (GPCRs), it has been previously suggested that the CBD mechanism is potentially claiming all G protein that GPCRs need for signalling because the proteins have a higher affinity for the inactive-state receptor (Pertwee, 2008). The potential actions of CBD through the GPR55 receptor have been most recognized through studies on epilepsy, as CBD's anticonvulsant properties is one that is most studied. For instance, Kaplan et al. (2017) looked at a model of Dravet syndrome, where Scn1a$^{+/-}$ mutant mice expressed the phenotypic repeated seizures. CBD doses of at least 100 mg/kg were found to be protective against seizure

convulsing, frequency, and duration. Additionally, when recording action potentials in the hippocampal dentate gyrus, a major area of seizure propagation in connection with the limbic system (Hosseinzadeh et al. 2016), they found reduced excitability. For one thing, they found increased GABAergic activity in the dentate gyrus, which produced overall inhibition (Kaplan et al., 2017). The authors also attributed this finding to CBD's interaction with the GPR55 receptor, based on their ability to mimic these effects with a GPR55 receptor antagonist. Mechanisms explaining these studies would include CBD blocking GTPγS from binding to GPR55 to diminish function during *in vitro* studies (Ryberg et al., 2007; Sharir & Abood, 2010). Further, this process is likely mediated by the cannabinoid ligand CP55940, which proceeds to activate or inhibit GPR55. Additionally, blocking activity by CBD has been found to be independent of any CB1 or CB2 activity (Ryberg et al., 2007).

Finding similar results, Klein et al. (2017) used several rat models of epilepsy, where seizures were induced. Rats were administered CBD intraperitoneally at doses ranging from 50 to 250 mg/kg and the level of protection from seizures it provided was characterized by the lack of convulsing behavior exhibited. The authors found that there was dose dependent protection, where doses around 50 to 150 mg/kg were most effective. Klein et al. point to several possible molecular explanations for their findings including CBD modulating excitability through interactions with GPR55 as well as blocking voltage gated sodium channels.

TRPV1

Other channels involved in particular may be the TRPV1 channels, which are responsible for the transduction of chemical and mechanical stimuli into electrical stimuli to be sent to the brain through its non-selective cation permeability (Ianotti et al. 2014). While TRPV1 channels

are found mostly in the peripheral nervous system, transducing and modulating ascending nociceptive signals, some hypothesize that TRPV1 receptors also have a presence in the central nervous system. In a study looking at the effects of CBD on TRPV1 channels, HEK293 cells expressing the channel were exposed to CBD and capsaicin, a TRPV1 ligand (Ianotti et al. 2014). Using patch clamp methodology, results indicated that CBD decreased activity at the TRPV1 receptors through desensitizing it and other members of this receptor family (TRPV2 and TRPA1). The authors suggest that CBD is preventing the phosphorylation of TRPV1 (as seen in the Western blot analysis of CBD and non-CBD *in vitro* cell cultures), which has previously been seen as a hallmark of TRPV1 sensitization. Additionally, the authors used immunofluorescence on rat hippocampal brain slices to reveal that there is a high association of TRPV1 with glutamatergic synapses and not GABAergic synapses. These are significant findings not only because they suggest that CBD can modulate nociceptive systems in periphery, but also potentially cognitive processes through decreasing excitatory activity in the hippocampus.

5-HTRs

Cognitive processes are also likely to be modulated by CBD's effects on serotonin or 5-HT receptors. Russo et al. (2005), who, unveiled how the drug acts as an agonists at 5-HT1a receptors, claims that this receptor is responsible for many of CBD's therapeutic actions, including its strong anxiolytic effects. The authors use an *in vitro* setup where they monitored the activity of radioligands on the 5-HT_{1a} receptors while in the presence of varying concentrations of CBD. Results revealed how CBD dose dependently displaced a known 5-HT agonist ([3H]8-OH-DPAT), indicating that cell function was a result of CBD acting as the agonist at

these receptors. Additionally, CBD acted in a similar way that 5-HT itself does through increasing GTP binding (as seen through GTPγS binding) which decreased cAMP concentrations due to negative coupling. These results suggest that CBD would lead to decreases in activity *in vivo*, much like its actions on the other receptor types would.

In general, the 5-HT system modulates circadian rhythms, motor activity, cognition, and emotions (Bocchio et al. 2016). CBD might have the ability to modulate these processes to some degree through the 5-HT receptor, especially regarding emotions and stress responses. More recent studies have looked at freezing behavior in response to stressors when given injections of CBD in regions such as the prelimbic/infralimbic regions of the prefrontal cortex (Fogaca et al., 2014; Marinho et al., 2015). CBD is able to decrease freezing behavior through its actions on the 5-HT receptors, but is further proved to be an agonist when the protective effects are significantly reversed by the addition of 5-HT_{1A} antagonist, WAY100635. Additionally, electrophysiological studies have also conveyed that CBD reducing firing activity in the ventral tegmental area (in dopaminergic neurons) is dependent on the functioning of 5-HT_{1A} receptors in the nucleus accumbens by using receptor antagonist NAD 299 (Norris et al., 2016).

All together, these various receptors are spread throughout the nervous system, allowing CBD to have its range effects. Within the central nervous system, the CB1 receptors are found to be in high density in the amygdala and hippocampus (Bitencourt et al. 2008). If CBD is acting as an inverse agonist, preventing the active state in these brain regions, or decreasing the affinity of the CB1/CB2 endogenous ligands, then it might ultimately be modulating anxiety and fear conditioning (Laprairie et al., 2015). Alternatively, CBD may be increasing GTP binding in negatively coupled GPR55 to prevent changes in channel conformation in cells of the frontal

cortex, striatum, and hippocampus(Sharir and Abood, 2011). More so, the drug may be having major inhibitory effects in areas which have high expression of 5-HT_{1A} receptors, such as the prefrontal cortex, amygdala and hippocampus (Garcia-Garcia et al., 2014).

CBD in Previous studies

As there is a consistent association of receptors that CBD interacts with in regions such as the amygdala and hippocampus, further research into its anxiolytic effects would be justified. During the process of recognizing stressful stimuli that induces phasic fear, there is an initial increase in 5-HT release, causing higher activity the dorsal raphe nuclei (DRN) (Bocchio et al. 2016). Further downstream, there is a similar relationship between increased 5-HT release and fear in the basolateral amygdala (BSA). Previous studies have looked into the activation of the amygdala when using 5-HT agonists such as SSRIs, which CBD seems to mimic (Arrant et al., 2013). Findings such as decreased c-fos fluorescence indicating less activity in the presence of SSRIs suggest that 5-HT agonists may modulate and lower anxiety levels. Given previous findings that CBD has several mechanistic similarities to 5-HT agonists, CBD should have similar anxiolytic effects.

Further, behavioral studies employing fear conditioning while administering CBD at different time points have demonstrated the drug's large scale ability to decrease fear learning, likely through serotonergic pathways with the hippocampus and amygdala. For instance, CBD reduced firing activity in the ventral tegmental area, as long as 5-HT receptors were not antagonized, in Norris et al.'s study (2016). There was also reduced freezing behavior and more rapid fear extinction in rats receiving CBD. Multiple studies seem to suggest that CBD is

preventing fear memory consolidation and retrieval in general (Jukus et al., 2016). Overall, there seems to be a gap of missing research between CBD's effect on brain regions with high expression of its prefered receptors and the behavioral implications. While Norris et al. (2016), for example, are one of few to focus the electrophysiological effects of CBD following fear conditioning, there are limited studies on CBD using electrophysiology in general. Additionally, these studies do not have a focus on activity in the amygdala or related regions.

CBD and the BNST

CBD has been found to decrease freezing behavior in response to contextual/learned situations, as noted by the above examples. The BNST is involved with this type of fear response, which has been seen through studies that did not use CBD and is also inferred by its dense interconnectivity (Arrant et al., 2013; Lebow & Chen, 2016). CBD was seen to act on regions that project to the relevant subnuclei of the BNST, such as the dorsal raphe nuclei and basolateral amygdala. It is possible that either the mechanisms of CBD can alter the regions that project to the BNST, impacting the BNST's subsequent activity, and/or the CBD can manipulate the activity in the BNST directly.

As CBD has been found to act similarly to an SSRI/ 5-HT agonist, one could infer that if SSRIs play a role in anxiety and fear learning facilitated in the BNST, then CBD should also modulate BNST activity. Studies by Gomes et al. (2011), for example, have looked at CBDs ability to modulate the BNST. The authors injected CBD or vehicle systemically into rats and then observed that the rats receiving CBD exhibited attenuated fear responses to foot shocks. After using the 5-HT_{1A} receptor antagonist WAY100635, there was a decrease in attenuated fear response to the contextual fear stimulus. This observation suggests that CBD is attenuating

contextual fear behavior and responses, but also that the drug is at least partially doing so through the 5-HT_{1A} receptor. In any case, the BNST has been reported to have high densities of CB1 receptors in the anterolateral division of the BNST, TRPV1 receptors, and 5-HT receptors. These are all possible targets for CBD to modulate activity in the BNST.

Current study

The connectivity and subsequent function of this lesser known brain region, the BNST, has led to research centered around better characterizing what subregions are involved in contextual fear and how this information can be used to ameliorate conditions such as GAD or PTSD (Rodriguez-Sierra et al., 2016). Previous studies have identified how inactivating different regions of the BNST result in varying behavioral or molecular responses in contextual fear models. Still, many individual BNST regions are not clearly characterized, where some regions seem to play a larger role in contextual fear than others. Up to this point, there has been little electrophysiological research on the BNST in comparison to other methods. While behavioral studies often employ predator odors to measure subsequent freezing activity when the BNST is either lesioned or intact, there is little data on neuronal firing differences to these odors. This study examined the possibility of presenting odors, including 2-heptanone as a predator odor component, to anesthetized rats to examine differences in BNST firing from baseline activity using electrophysiology. The posterior division was targeted during recording sessions, as it is most involved in social behaviors that might be responsive to predator odor, mating, defensive, and aggressive behaviors (Lebow & Chen, 2016). Moreso, within the posterior division is the interfascicular region of the BNST, which has direct connections to the olfactory bulb, increasing the possibility of recording odor-tuned cells. In turn, this region has connectivity to the oval

nucleus of the anterior BNST, which integrates contextual information with positive and negative valence to direct mood. In light of the interconnectivity and its role in both social and fear response facilitation, this study aims tested the hypothesis that the BNST will be odor-responsive, where it demonstrates change in firing rate in response to pheromone odor (2-heptanone) but not other non-predator/non-species odors.

Previously recorded data from recordings done in the olfactory tubercle (OT) were also analyzed. The OT receives direct input from the olfactory bulb and is a part of the olfactory cortex (Wesson & Wilson, 2011). It receives input delivering a wide range of odor-elicited activity. It continues to propagate this signal to several different non-olfactory targets that include certain divisions of the amygdala, for example. These previously recorded data will serve as a comparison for both the baseline and odor-elicited activity of the BNST. It is more likely that while the BNST will have odor-elicited activity to the pheromone odor, the cells of the OT will be responsive to both pheromone and non-pheromone odors.

Additionally, the effect of cannabidiol (CBD) on both baseline or possible odor-elicited activity was tested because of its many interactions with receptors including the cannabinoids (CB1 and CB2 receptors), GPR55 receptors, TRPV1 cation channels, and the 5-HT receptors (Campos et al., 2012). Previous studies using CBD have found that the drug significantly reduces conditioned freezing behavior, which is analogous to results found when inactivating the BNST (Norris et al., 2016; Sullivan et al., 2004). CBD was also given at doses of 1, 2, and 3μL. While previous studies have not used CBD intracerebroventricularly with the same methodology and purpose, other studies have found 2 μL has been an effective dose for modulating sleep (Murillo-Rodriguez et al. 2006). As the drug has yet to be utilized with these methods, the three

doses were administered to allow for possible dose curve responses. It is also plausible that CBD alters BNST activity, particularly to that triggered by predator odor, because of the BNST's concentration of both 5-HT and cannabinoid receptors. In light of these previous data, this study also tested the hypothesis that CBD both changes BNST baseline activity and odor-elicited activity.

Methods:

Experimental subjects. Six male and female Long-Evans rats were used as subjects for *in vivo* electrophysiology. Four of the rats were administered CBD during the recordings at doses ranging from 1-3 µL (a higher and lower dose from what Murillo-Rodriguez et al. (2006) determined to be effective when CBD is administered intracerebroventricularly), while two were not administered drug. Rats were kept in individual housing with a 12 hour light/dark schedule, with food and water available *ad libitum*. All experiments were performed during the light phase. Experiments were conducted in accordance with the guidelines of all pro IACUC and are in agreement with NIH guidelines for ethical use of animal subjects and research.

Surgery. Rats were fully anesthetized using ketamine and acepromazine (1g/mL; 3g/kg) with isoflurane to inhale as needed. A stereotaxic frame, including ear bars that were inserted into the posterior aspects of the orbital sockets, was used to stabilize the skull during the surgical procedure. After exposing the dorsal skull using a #10 blade scalpel, clearing the fascia, stopping bleeding using a Bovie High-Temp Cautery, and clearing the skull with hydrogen peroxide, two bilateral rostral holes (1.5 mm) were drilled for stoelting stainless steel screw placement. An aluminum post was secured between the screws using dental cement to stabilize the skull during recording times. Next, two bilateral 1.5 mm holes were drilled according to appropriate atlas

coordinates from bregma over the right intrafascicular region of the BNST (AP -0.8 mm, ML, 1.5 mm) for the electrode to pick up activity in the desired region, having connections with the olfactory bulb (Lebow & Chen, 2016), and over the left ventricle (AP -0.8 mm, and -1.5 mm) to later inject the dose CBD for four of the rats.

Electrophysiology. The rats were transferred onto an anti-vibration table within a Faraday cage, blocking any electrostatic noise, and stabilized by connecting the head post to a attached to the table. The rats were grounded to Faraday cage and two connected tungsten electrodes were lowered into the hole over the BNST to collect differential recordings. The electrodes were lowered until reaching a depth of -5 mm below surface. Action potentials that were seen as both positive and negative-going waveforms were picked up and monitored using the A-M systems Inc Audio Monitor (Model 3300) oscilloscope, as well as the Microelectrode Amplifier (Model 3600), and recorded using Spike2 software.

Stimulus Presentation. Once the electrode was lowered to a cell(s) with action potentials consistent in amplitude, form, and direction, seven odors (1,7- octadiene, propyl butyrate, 2-heptanone, isoamyl acetate, heptanal, limonene, and mineral oil as a control (**Figure 2**); odors previously found to alter the activity of brain regions that project into the BNST; Rennaker et al., 2007)) were presented using an air dilution olfactometer at 1L /min flow with nitrogen (Wesson & Wilson, 2010). The seven odors were presented at 30 s intervals at random orders for 18,000 s and activity was recorded using Spike2 software.

Drug Administration. After recording baseline activity in response to the eight odors, cannabidiol was administered (1-3μL; 1μg/mL) by lowering a 5μL Hamilton microsyringe with a beveled tip into the drilled hole over the left ventricle (AP -0.8 mm, and -1.5 mm).

Immediately following CBD or saline injections, the seven odors were again presented to the anesthetized rat for 18,000s at 30 second intervals. Recordings were continuous throughout. Brains were removed, coronally sliced at 50 µm using a microtome, and mounted on glass slides where they were they were Nissl stained using cresyl violet or neutral red?(Wesson & Wilson, 2010). Slides were examined under a dissecting microscope to confirm electrode position in the BNST.

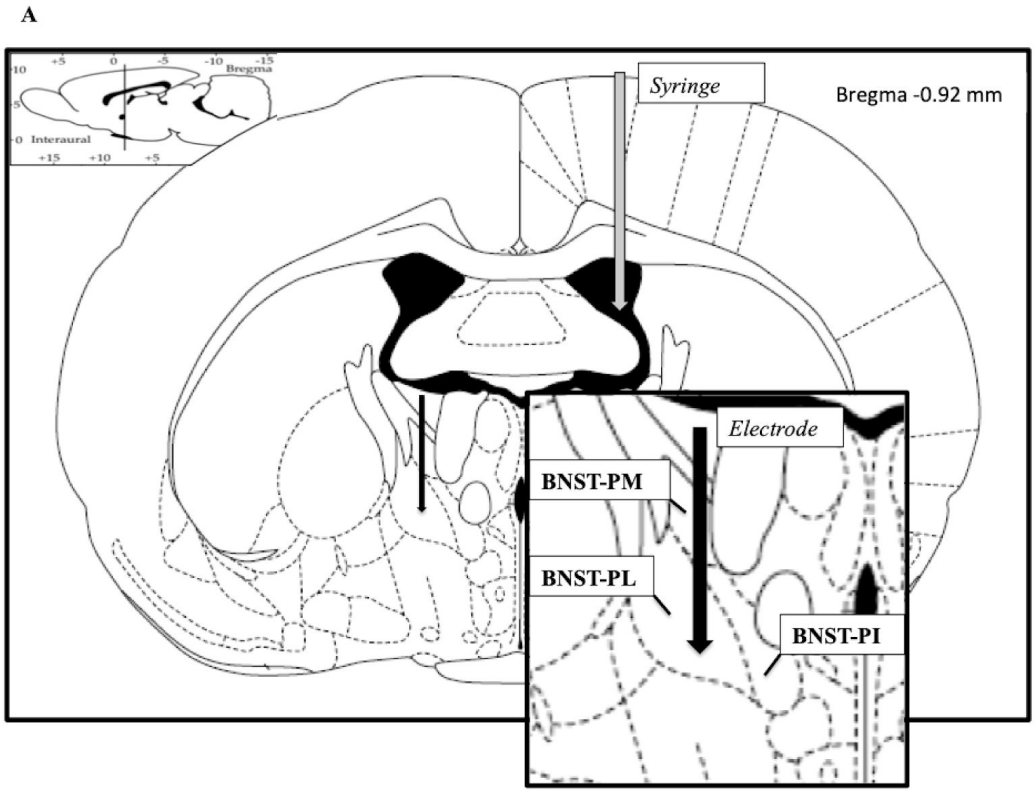
Data analysis. Spike2 data files containing electrophysiological activity that occurred during the recording were uploaded to Offline Sorter where cells differentiated from one another based on wave from. Data was further analyzed in NeuroExplorer using perievent rasters and rate histograms to identify odor responsivity and changes in firing/cell presence over time or in relation to drug administration

Olfactory Tubercle. Previously collected data from recordings in the olfactory tubercle, which were not exposed to CBD, were sorted in Offline Sorter and analyzed in NeuroExplorer using only odors used in the current experiment. In total, 25 cells from the olfactory tubercle were analyzed to compare to the 25 cells found in the BNST after having not found odor-elicited activity in the cells of the BNST.

Results:

Histology: Stained slices of the rat brains determined that the recording in rat 4 was in the BNST. The locations of the recordings from other brains were not determinable as a result of an error when placing slices on slides. **Figure 4** depicts that the electrode's final AP coordinates in rat 4 were slightly more posterior than the original target but still had ML and DV coordinates that placed it in the BNST medial posterior division, which receives input from locations that

process non-volatile odors. While this division does include the intrafascicular region of the BNST, we were not able to determine if it was within that exact target nucleus. It is likely that the electrode was somewhere within the posteromedial, posterolateral, or posterointermediate BNST. While exact placement of each electrode could be not determined, **Figure 5** shows the range of coordinates that recordings occurred in. In total, 10 of the 14 recording depths could be in the BNST, where two are verified using histology. These recordings fell within the range of 4 to 8 mm in ventral depth, making them likely to be in the posterior BNST, and were recorded in all but rat 2. Recordings with a depth less than 4 were likely in the medial, supracapsular BNST or another region. Recordings that were done 7 mm or below were likely in the LPO, MPO or another region. Recordings that were not assumed to be in the posterior region were grouped as "other".



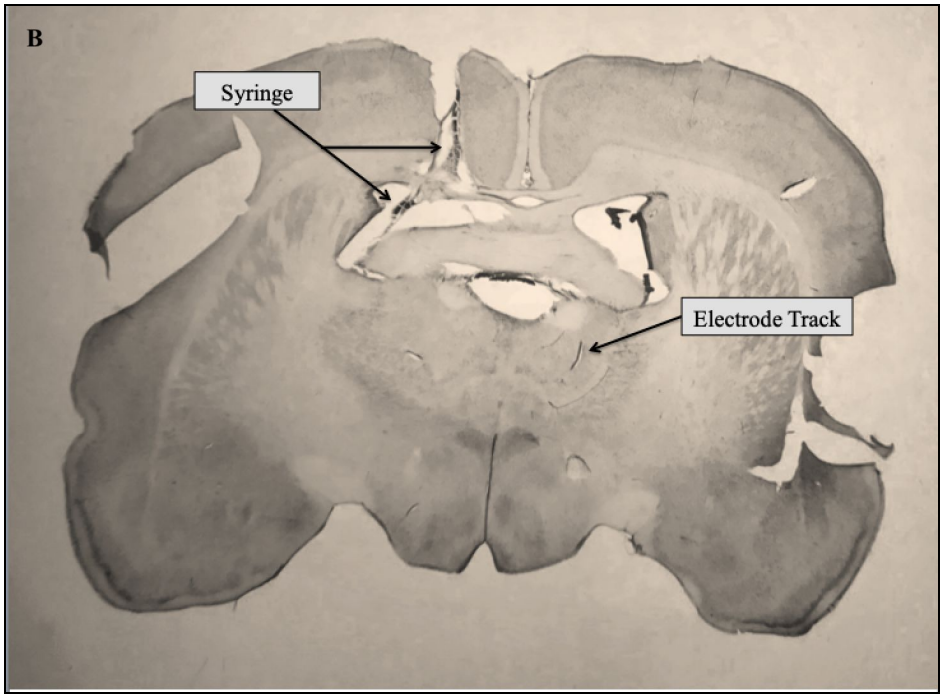


Figure 4: The rat brain atlas (Paxinos, 2007) in panel **A** depicts histological evidence of electrode tract, as shown by the black arrow, for rat 4. The tracks appear to most prevalent and ventral in more posterior sections (-0.92 mm from bregma) than the original target (-0.80 mm from bregma). This likely left our recordings to be in the posteromedial, posterolateral, or posterointermediate regions of the BNST. It also confirms the placement of the syringe into the left ventricle. These findings came from the slice pictured in panel **B.**

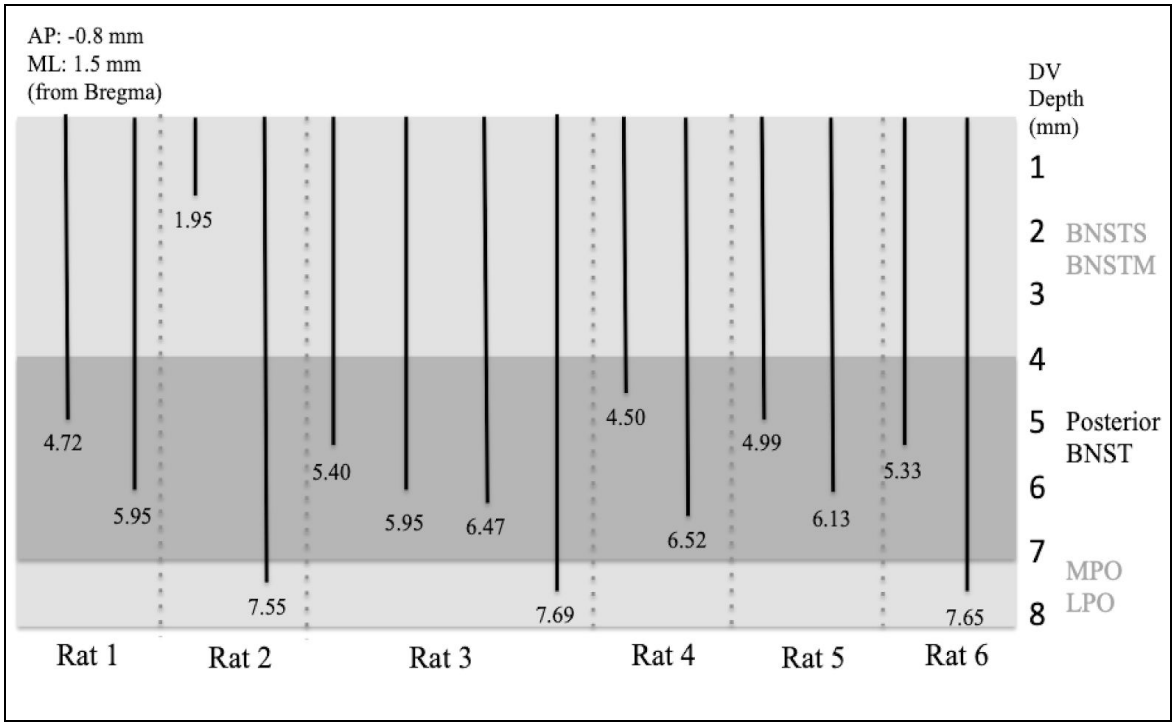


Figure 5: The figure displays the ventral depth at which the electrodes were placed across each of the 6 rats at the same targeted posterolateral stereotaxic coordinates. Electrode depths falling in the dark gray region is estimated to be in the posterior division of the BNST. If above, it was likely located in the BNSTS, BNSTM, or other region. If below, it was likely that the electrodes were located in other brain regions including the medial preoptic nucleus (MPO) or lateral preoptic area (LPO). There were 14 recordings total, where 4 of fell outside of the region predicted to be the BNST and were categorized as "other".

Baseline Activity. Active cells were found in the BNST indicating that electrodes and other equipment were working properly. Baseline activity varied from cell to cell. The previevent rasters show how there is no difference in firing to any of the odors (volatile or alarm odors) for any of the cells (**Figures 7 and 8)**, suggesting that this activity is representative of their baseline activity. In the OT, varying basal activity was seen between cells during the time before odor presentation. **Table 2** notably shows that there was a similar average frequency (4.46 Hz for the BNST and 5.05 Hz for the OT). The four recordings that picked up on 5 cells in non-BNST regions had a lower mean frequency and number of spikes picked up. The BNST was as noisy as a region that definitively responds to odors such as the ones used in this study (OT),

indicating that any negative findings will not be a result of recording in a brain region that is quiet in an anesthetized rat. This is further seen by the distribution of the frequencies in the histogram in **Figure 6**, where most cells fired under 1 imp/sec, but cells from all three categories had frequencies up to 31 Hz.

Table 2: Cells, where they were recorded, and their activity

	BNST	Other	OT
Number of Cells	20	5	26
Mean Frequency (Hz)	4.67	1.16	5.05
Standard Deviation	6.21	1.34	8.28

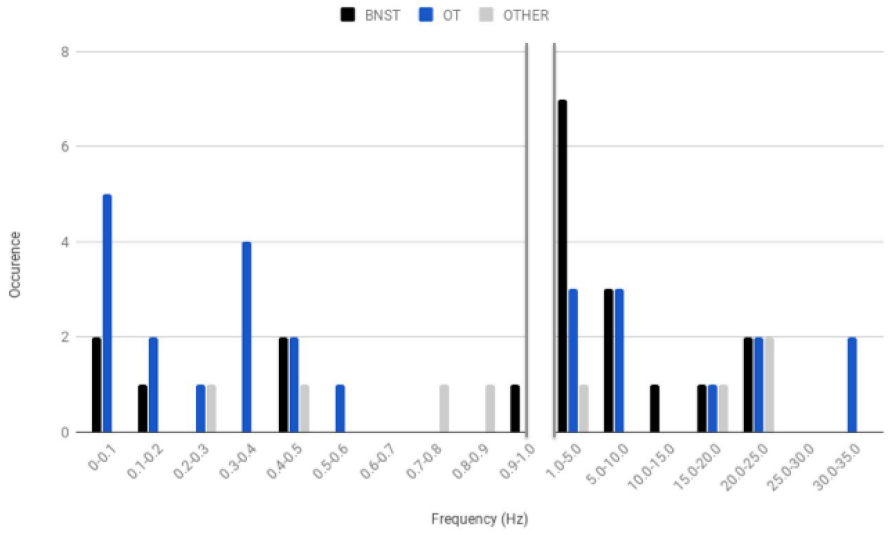
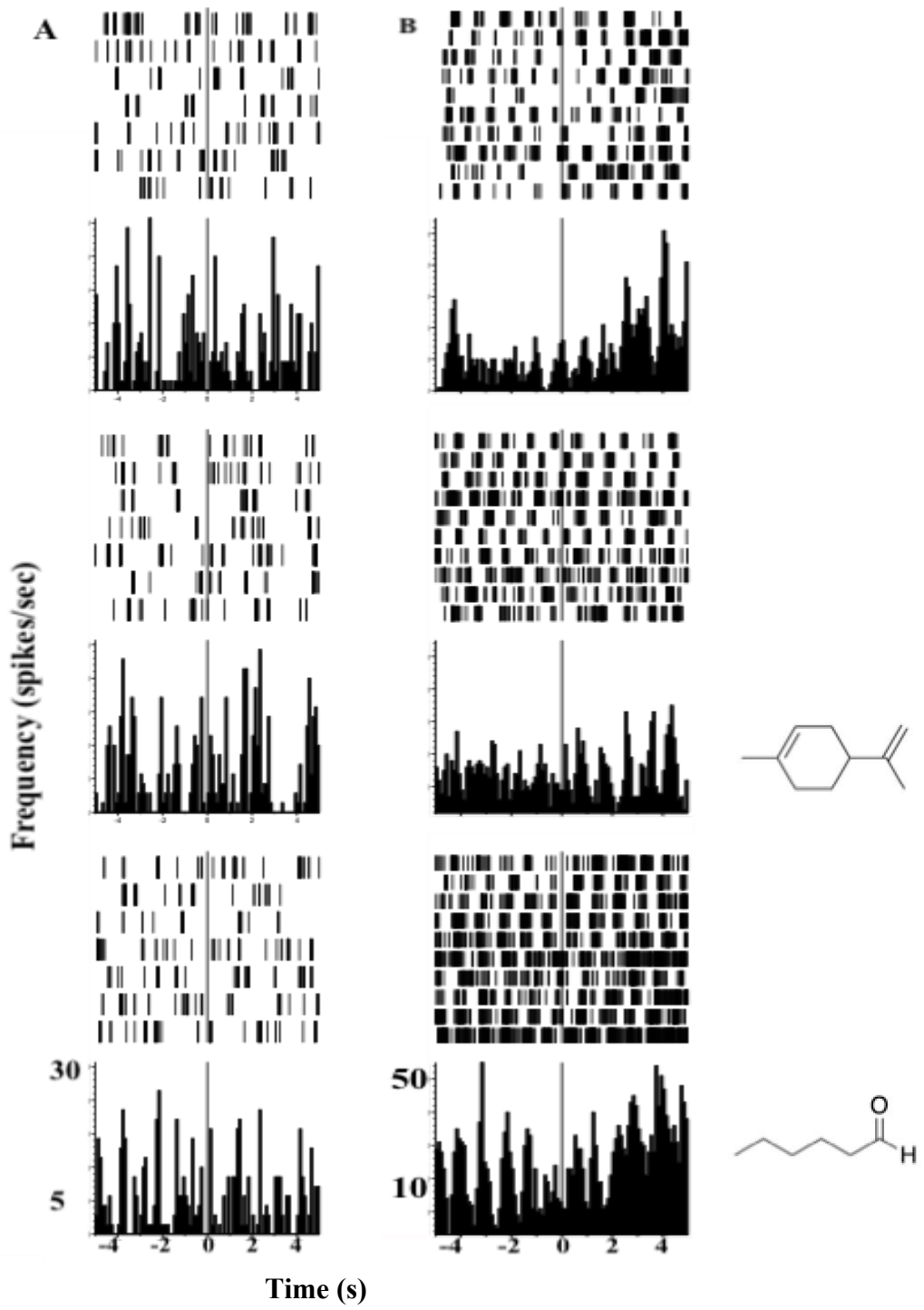


Figure 6: Occurrence of frequencies for the BNST, the OT, and cells where the ventral depth was not within the target region ("other"). As the majority of frequencies fell below 1 Hz, the break in the table indicates where the scale changes to increments of 5 Hz. Most frequencies

were below 1.0 Hz, however there were a few cells across all three brain region categories that reached between 1 and 35 Hz.

Odor Response. Cells in the BNST did not display any odor-elicited at the time of or after odor presentation. There was no variation when the rat was presented with mineral oil, limonene, heptanal, isoamyl acetate, 2-heptanone, propyl butyrate, or 1,7- octadiene (**Figure 7A**). There was no BNST odor response recorded across all six rats, including recordings where CBD was administered in doses ranging from 1-3 μL (**Figure 8**). Firing activity was consistent across all prevent rasters (within cells) and did not show any variation around or after "time 0", at which the odor was present. In comparison, OT cells that were presented with the same odors did show signs of tuning. Specifically, OT cells increased their firing rate when presented isoamyl acetate, 2-heptanone, propyl butyrate, or 1,7- octadiene (**Figure 7b**). These findings were fairly consistent across most cells throughout the different recordings (**Table 3**).


A
B
Frequency (spikes/sec)
30
5
50
10
-4
-2
0
2
4
Time (s)
O
H

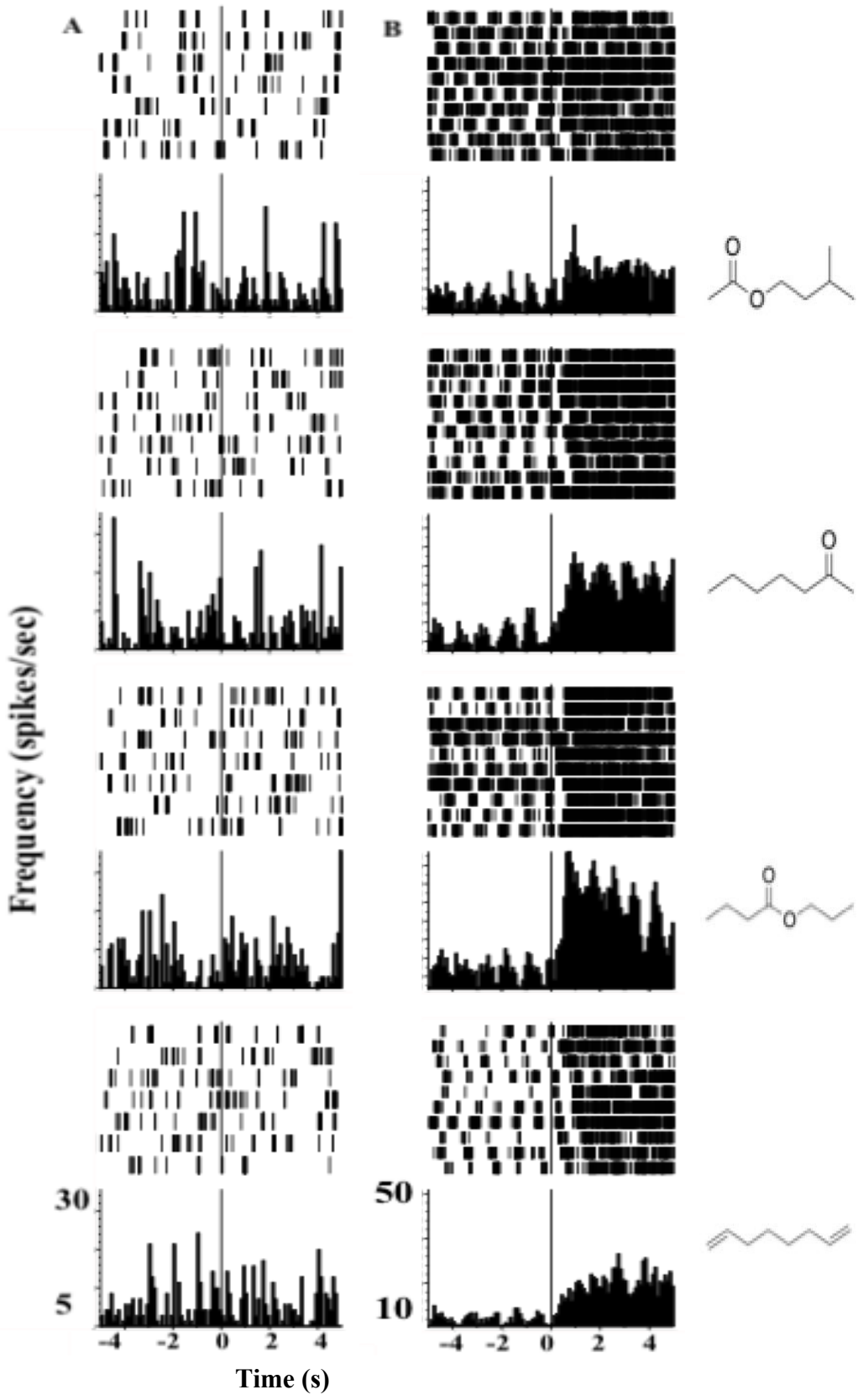

A
B
Frequency (spikes/sec)
30
5
50
10
-4
-2
0
2
4
Time (s)

Figure 7: The above perievent rasters display recordings from the BNST (**A**; rat 4; ventral depth of 4.51 mm) and the OT (**B**; rat A8F, cell **A**; ventral depth of 25.35 mm). The rasters above the histograms display rows of activity that represent the progressing presentations (7-10) of one odor, where each row of dashes represent the spikes occurring at and around the time of odor presentation. Below, the histograms display the total activity (summed from the above raster) at each time point across all presentations (before and after odor presentation at time 0 s, which lasts for the following 2 s). From top to bottom, the odors are mineral oil, limonene, heptanal, isoamyl acetate, 2-heptanone, propyl butyrate, and 1,7-octadiene. The cell in the BNST does not show any odor-elicited activity. In contrast, the cell in the OT is most responsive to 2-heptanone, propyl butyrate, and 1,7-octadiene, where activity increases within 1 s of odor presentation and persists beyond 5 s.

Table 3: Number of cells that responded to each odor in the BNST or OT (of 26).

	BNST	OT
mineral oil	0	1
limonene	0	8
heptanal	0	22
isoamyl acetate	0	19
2-heptanone	0	22
propyl butyrate	0	23
1,7-octadiene	0	16 (of 17)

CBD Activity. As there were no odor- elicited activity in the cells of the BNST, CBD did not alter any odor response or induce any odor response across the six rats. This can be seen when comparing non-odor responsive CBD-treated cells in the BNST to non-CBD treated odor responsive cells in the OT (**Figure 8**). With regards to baseline activity, CBD inconsistently and infrequently altered firing frequency. Two of the six rats received 1 µL of intraventricular CBD. **Figure 9** shows rate histograms for one cell recorded in rat 3. While one cell (3A) did change waveform over the course of the trial, its timing or behavior did not coincide with CBD

administration, which occurred about 600 s apart. The one cell displayed in the figure (3B), however, starts firing in less than 100 s from the time of CBD administration. These results suggest that CBD may be altering this cell's firing rates at 1 μL ICV doses. On the other hand, rat 4, who also received 1 μL of CBD, did not show any administration dependent change. Cells receiving 1 μL of CBD varied in percent change throughout the trial, with Cell A from rat 3 being most dramatic but not at the time of administration (**Figure 10**). Rat 4 (1 μL) and other rats that received 1-3 μL of CBD all exhibited a general flux in activity that was consistent throughout the recording. The cells recorded in those rats did not appear to change their activity in response to CBD, including any induction of odor-elicited activity.

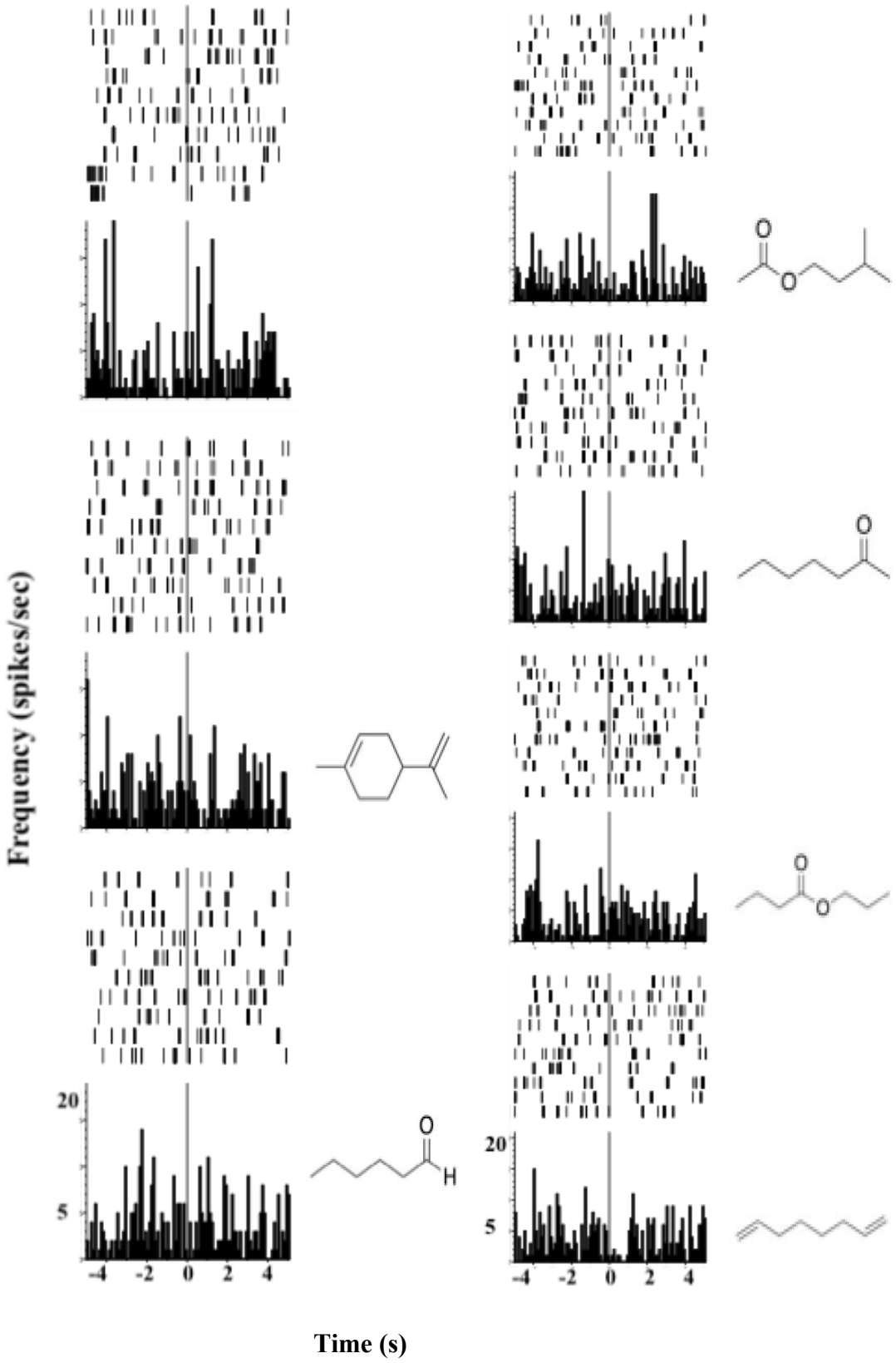


Figure 8: The above perievent rasters display recordings from the BNST(**A**; rat 4; ventral depth

of 6.52 mm) after 1 uL CBD administration. The rasters display rows of spikes that occur during the progressing presentations (10) of one odor, which are summed in the histogram below. As There is no observable change in the firing of these cells at or after the time of odor exposure (0 s) From top to bottom, the odors are mineral oil, limonene, heptanal, isoamyl acetate, 2-heptanone, propyl butyrate, and 1,7-octadiene. The cell in the BNST does not show any odor elicited activity in the presence of CBD.

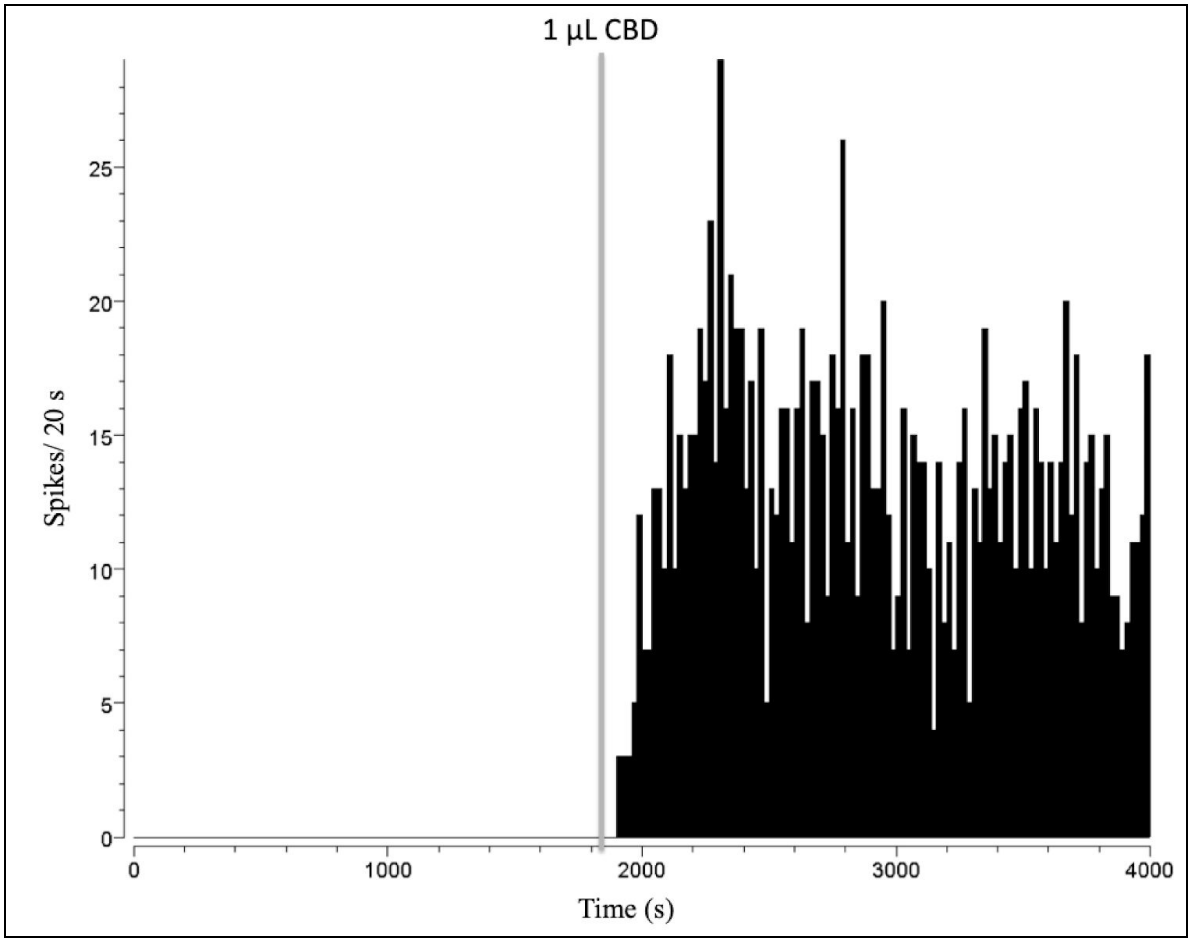


Figure 9: The total activity of one cell from rat 3 (ventral depth 5.95 mm) across a full recording is displayed in the above rate histogram. The activity does not include odor-elicited activity but occurred during a recording session while odors were being presented. The grey line indicates the time that 1 uL of CBD was administered ICV (1830 s). The cell starts firing within 100 s from when CBD was administered, suggesting a possible interaction of the drug with this particular BNST cell.

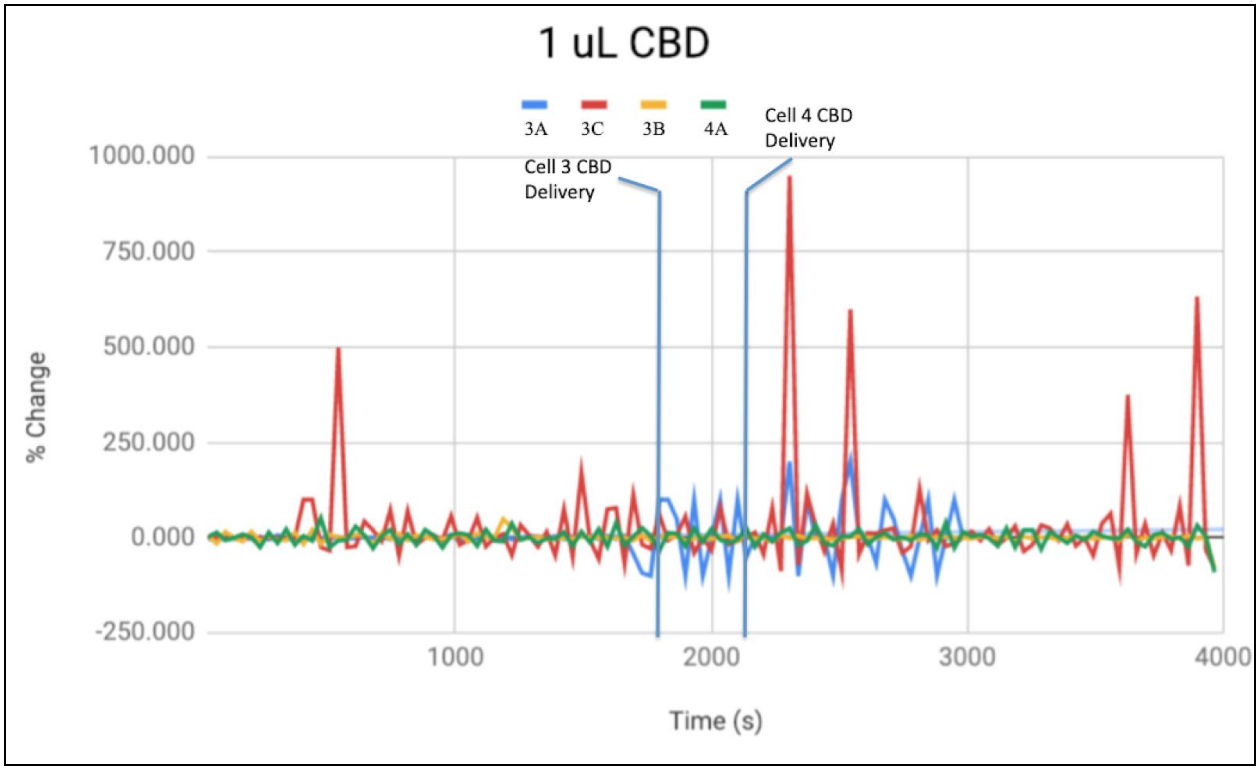


Figure 10: The percent change for all cells recorded when administering 1 uL are shown above. Percent changes for all cells reach 100% on and off throughout the recording. Around the time of CBD administration, for 3A and 3C especially, there is a co-occurring positive percent change of the largest magnitude.

Discussion:

Basal Activity and Odor response

The Bed Nucleus of the Stria Terminalis (BNST) is a structure analogous to the amygdala, however more specialized to facilitate the contextual fear response and anxiety instead of the phasic fear response (Lebow & Chen, 2016). The structure is also important for social recognition, especially to cues from their own species or from their predators, and thus uses its anxiogenic function to drive survival promoting behavior. Previous studies have examined the regions involvement in anxiety using behavioral models, employing odors to invoke conditioned fear, as well as *in vitro* electrophysiological models. In this study, *in vivo* electrophysiological methods were used to better characterize the activity in this region.

Additionally, odors (volatile and non-volatile) were presented to examine if this region is odor responsive in anesthetized rats to explore the the possibility of using odor presentation to continue our understanding of the BNST and its role in anxiety. Ultimately, there were no odor responsive cells found in the BNST.

It is important to first note that only electrode placement in the posterior division of the BNST of rat 4 could be confirmed using histological analysis. Looking at slices of other brains suggest that there might be close to accurate placement; however, the exact location of the recording was not determined. Instead, the depth of each recording was noted and then used to classify which cells were recorded in the BNST and which cells were recorded in other regions (**Figure 5**). To help ameliorate this issue in locating electrode placement in the future, it might be valuable to employ the use of dyes, such as Fluorogold, to better identify where electrode placement was (Bergan, Ben-Shaul, & Dulac, 2014). The electrode is dipped in the dye before lowered into the brain and identified after other histology. While electrode placement accuracy likely varied between brains, and depth of recording was fairly dependent on where recordable potentials were found, histology confirmed that placement was in the posterior division for at least four cells. This result is critical to the hypothesis that the targeted interfascicular (posterior) BNST would be responsive to 2-heptanone only because of its interconnectivity with the MeA, AOB, and VNO where the odor is detected (Bergan, Ben-Shaul, & Dulac, 2014).

During the recordings across this study, active cells were found in the BNST. It was not necessarily a quiet region in the anesthetized rats, which becomes more obvious when compared to another brain region. Recordings from a previous study looking at odor-elicited activity in the OT were analyzed and compared to the data collected in the BNST. While this allowed for a

comparison to a definitively odor responsive brain region, it also provided a comparison for overall activity. Ultimately, the mean frequencies and total spikes across all the data in the BNST was quite similar to that of the OT (**Table 2**). This can further solidify that the BNST's baseline activity is not particularly low under anesthesia, considering its cells were as active as a region increasing its firing to odors, and also that the frequency would likely be high enough to detect changes in response to odor presentation.

Previous studies report that the BNST has a broad range of activity, where cells might have a firing rate between zero and 32 Hz (Haufler, Nagy, & Pare, 2013). This is comparable to the data from the current study, where frequency in the BNST ranged from 0.117 Hz to 23.517 Hz. Additionally, previous data suggest that the BNST-AL and -AM have low average firing rates less than 4 Hz. Posterior division frequencies are not characterized in the study reporting these data. Still, this firing rate is similar to the average firing rate determined in the current study of 4.67 Hz. While it is important to note that the lack of certainty about electrode placement combined with these data might suggest that electrodes were recording in the anterior division, frequencies from the rat that was determined to be in the posterior division ranged from 0.117 to 5.61 Hz. It is also important to mention that the overall activity found in this brain region, as well as other regions using similar methods of electrophysiology, are biased towards active cells. When lowering the electrode into the the brains to record data, the electrode is stopped when placed

The hypothesis that there would be 2-heptanone odor-elicited response in the BNST may also not have been supported because of the location of the recording. Again, even if recordings were definitely in the interfascicular region, we still may not have been in the exact nuclei that is

innervated by the accessory olfactory bulb or medial amygdala, which will also project non-olfactory related information. Conversely, because the region of the BNST that has connections to olfaction and other sensory input that is socially driven, the odors we used may not be appropriate for signal propagation to the BNST. In any case, there is still a gap in previous data when it comes to the posterior division's role in anxiety. Odors, such as trimethylthiazoline have been able to invoke contextual anxiety responses in the past (e.g. lesions within the BNST blocks fear behavior in response to the fox odor component, trimethylthiazoline, which also induces noradrenaline in this region; Fendt, Siegl, & Steiniger-Brach, 2005). Further research needs to be done to address how the interconnectivity between the anterior and posterior BNST allow for the communication of predator odors to cross paths and invoke anxiety.

Though it was surprising that there was no response to the predator odor component 2-heptanone, it might be reasonable to include within-species/opposite sex pheromone components in the future. This could be useful in evaluating the sexually dimorphic component of this brain region, which could also be another source of error given that we used both male and female rats (Lebow & Chen, 2016). Still, previous studies have further confirmed the posterior division's role in both odor detection and anxiety. For instance, lesions in the posterior division of the BNST were made and the rats were placed back into their environment containing opposite sex odors (Been & Aras, 2010). Lesions in male rodents eliminated opposite-sex odor investigation because of the lack of pheromone odor preference. This finding demonstrates that the posterior division is at least a point at which odor information passes through on its way to being further processed and perceived as within sex or opposite sex odors. The rats were still able to identify that there was an odor, however did not show its normal preference to

opposite-sex odors. This speaks to the interesting role that the posterior division plays in social interaction.

Moreso, the direct effect of 2-heptanone has been observed in another model using physically stressed rats (Gutierrez-Garcia et al., 2007). The authors review how non-volatile pheromone odors are transmitted to the BNST through the AOB and MeA to set off a physical response by projecting to the PVN, periaqueductal grey, and locus coeruleus, for example. This is similar to how rats smelling urine from other physically stressed rats can lead to physical stress in the rats smelling the urine. In this study, 2-Heptanone is used as the alarm odor. Interestingly, it is found in non-stressed mouse urine and in humans. It was presented to receptor partner rats through the urine of physically stressed rats. To test the long term anxiety of both the physically stressed and receptor partner rats, they used the forced swim test where immobility and giving up on finding a way out was classified as despair or anxiety. The authors found that both the stressed rats and the receptor partner, who had previous exposure to 2-heptanone, experienced the longest durations of immobility in comparison to non-stressed/non-2-heptanone smelling rats. These effects were interestingly ameliorated by the tricyclic antidepressant, imipramine. Again, the authors hypothesize that it was 2-heptanone's pathway to the BNST that invoked the long term changes in the receptor partner rats.

In the current study, there was also no tuned response to any of the volatile odors. If recordings were truly done in the posterior division of the BNST, this would be in support of the current hypothesis and in line with previous findings stating that volatile odors are picked up in the MOE and projected to other olfactory cortices or the lateral amygdala (Lebow & Chen, 2016). In contrast, the OT respond consistently to all the volatile odors with the exception of

limonene. Again, the OT receives direct monosynaptic input from the olfactory bulb and is a part of the olfactory cortex (Wesson & Wilson, 2011). Interestingly, the medial division of the OT is in line with the BNST. In any case, the OT is mostly innervated by tufted cells. Tufted and mitral cells are second order neurons, where tufted cells are more sensitive to odors at low concentrations and have a wider receptive field in comparison to the mitral cells that innervate the piriform cortex in a more precise manner. This likely explains how most of the cells recorded had similarly tuned responses to hepatanal, isoamyl acetate, 2-heptanone, propyl butyrate, and 1,7-octadiene. In contrast, the MeA has been found to be innervated by both tufted and mitral cells of the AOB (Salazar & Brennan, 2001). Given that these two regions both project to the BNST, it is likely that both second order cells are involved with odor signaling to the BNST. As an additional note, studies previously done in the same lab looking at the amygdala also found few odor responsive cells within the group that they recorded. It might be that with every structure or transmission that activity conveying information about odors pass through, there is a degradation or loss of signal. Perhaps it takes much more significant input to be conducted through to the BNST.

A possible reason that there was no response to 2-heptanone, despite its plausibility, deals with the overlooked fact that presentation of this odor needs to be directly and actively pumped to be delivered to the VNO (Ben-shaul et al. 2009). This would naturally require exploratory behavior of the rodent that is not possible in the anesthetized rat. While some have found ways to incorporate methods that allow for the pumping of stimulus in the anesthetized rat during electrophysiological recordings, the current study did not employ the appropriate methods to deliver stimulus to the VNO to activate the AOB, MeA, and consequently, the BNST. Altering

the methods to allow for active uptake of the odors would likely increase the odds of the hypotheses being supported, given the stimulus would follow a more appropriate path of activation. While additional research likely needs to be done to see where the second order cells specifically project to, this information leaves room for the possibility that recordings from the current study took place in region that responds to the specificity of mitral cell input.

CBD

CBD was injected intracerebroventricularly (1, 2, and 3 uL) to examine any change in BNST odor-elicited or baseline activity. Previous studies have found that CBD reduces contextual fear, a prominent function of the BNST, and that the drug can interact with many receptors in the CNS, including 5-HT and cannabinoid receptors that are abundant in the BNST (Norris et al., 2016; Campos et al., 2016). Given previous findings, this study aimed to address the hypothesis that CBD would proportionally change both basal and odor elicited activity in the BNST. As there were no odor responsive cells in the BNST, no evaluations could be made on whether CBD manipulated that activity; however, the lack of odor response indicated that the activity recorded was representative of the BNST's basal activity. This made it a bit more simple to evaluate CBD's effect on basal activity. Still, any changes to the basal activity were not found consistently.

While rat 3, who received 1 uL CBD, demonstrated an appropriately timed change in frequency in one cell within 100 s of drug administration (**Figure 10**), I would hesitate to describe this phenomenon as a dose-dependent response due to the lack of change across the two cells recorded in rat 4 and the one other cell from rat 3 (who also received 1 uL). Cell A from rat 3 did decrease in change waveform after 600 s, which was presumed to not be related to drug

administration. Still, Cell B starts firing in less than 100 s from when CBD was administered. These data suggest that CBD might be modulating the activity of this one cell; however, one of twenty six cells changing its firing within a reasonable time of drug administration is not convincing enough to make generalizations. While other studies using ICV injection do not necessarily address the same topics as the current study, they do share that CBD is rapidly absorbed and that they began their relevant recordings directly after drug administration (Murillo-Rodriguez et al., 2006). Additionally, CBD has a half life of around 9 hours. This further confirms that any change in activity would have happened close to the time of administration, which only Cell B from rat 3 did.

Cells from rats 3 and 4 that received 1 µL of CBD (1 µg/µL did fluctuate its activity at least by 100% throughout the recording and at the time of drug administration. At time of CBD administration, for 3B especially, there is a co-occurring positive percent change of a large magnitude. Other cells did not exhibit this difference, but instead the fluctuate constantly (**Figure 10**) It might be that this dose was too low for consistent changes. Murillo-Rodriguez et al. (2016) describe how, in previous work, 2 µg of CBD (this study used 1-3 µg) was sufficient to reduce anxiety behavior/time spent in closed arms through the elevated plus maze. The authors also were interested in how CBD can modulate sleep through similar IVC injections to later measure the c-fos expression as a measure of activity in waking-related brain regions (such as the hypothalamus and dorsal raphe nucleus). They found also found that 2 µg (1µL of CBD [10 µg/5 uL]) was sufficient to invoke these changes.

While previous studies have confirmed that 2 µg of CBD, twice as much as the amount that we were able to observe any activity change in, was able to rapidly diffuse to relevant brain

regions and alter activity. There was still little variation in activity after 2-3 μL of CBD (2-3 μg) were injected. Rate histograms for these cells did not reveal any changes in activity that were specific to the time of injection. All variations in activity throughout the recording, which were dramatic in some cells, were consistently repetitive. Again, the posterior division is said to be more involved in social and threat valuation and it is the anterior division that is more involved in contextual fear (Lebow & Chen, 2016; Daniel & Rainnie, 2016). It is possible that whatever region we were recording in was not densely populated by receptors that CBD might interact with, such as the 5-HT or CB1 receptors. In this case, the cells recorded could not have their activity manipulated by the CBD if there was no venue for the drug to act through.

Behavioral studies, however, would suggest that it is more likely that recording in various locations of the BNST would be impacted by CBD. Gomes, Resstel, and Guimaraes (2011), for example, looked into the anxiolytic effects of CBD through the 5-HT1A receptors. They performed intra-BNST injections on rats using a cannula, and then tested their performance in an elevated plus maze. Rats that received the intra-BNST injections of CBD demonstrated less anxiety in the maze by spending more time in the open arms. This anxiolytic effect was reversed by the 5HT receptor antagonist WAY100635 to further confirm that CBD's anxiolytic effects are primarily facilitated through the 5HT1A receptor, much like an SSRI. In this light, there should probably have been some sort of change in recorded activity, although we still cannot rule out the location aspect. Future studies similar to the current one may want to use intra-BNST injections to gain more precession. Additionally, lower doses can be used (Gomes, Resstel and Guimaraes used 15, 30, and 60 nmol) while still having the assurance that enough drug is reaching the targeted area. While this technique is more time consuming and requires more

equipment and precision, the method is economically favorable with regards to purchase of the drug that can be fairly costly.

Another technique that might be useful in the future would be to use optogenetics to activate the receptors in the BNST. Previous studies have looked at how serotonin 2c receptors in the dorsal raphe nucleus, for instance, were activated through a melanopsin-based optogenetic probe through Gq signaling pathway that the receptors are coupled through as GPCRs (Spoida et al., 2014). Spoida et al. found that the Gq signaling pathway revealed that the serotonergic neurons were sending negative feedback onto subsequent serotonergic cells to modulate anxiety-like behavior in the mice. While this study does not look at the 5-HT_{1A} cells, it does reveal that anxiety disorder related behaviors can be modulated through the optogenetic activation of g-proteins coupled to specific receptors in specific brain regions. Using this technique could give information about the receptor and cellular activity in the BNST that contributes to anxiety, as well as help us examine the simulated effects of CBD on these BNST receptors without the limitation of the 9 hour half life.

Even with the dense interconnectivity, it may not be possible to test contextual fear modulation by CBD in the BNST through odor presentation. Pheromone presentation has been both considered an innate trigger but also has been used to instill contextual anxiety in rodents. It remains unclear whether measuring the activity in the BNST in response to pheromones picked up in the VNO will tell us about the nature of contextual anxiety or if it is possible. Future studies altering the method of active uptake of non-volatile odors to the VNO will help to clarify this. While we did not find odor response across any of the cells of the BNST, we were able to see that it has a similar firing frequency to another structure that has been seen as

odor-responsive, the OT. This methodology should be progressed further to determine if it is possible to record odor-elicited activity in the BNST with greater certainty. If it is possible, it would be a significant advance to better characterizing the nature of anxiety and the brain structures involved. The methods are a faster and more efficient window into the direct responses of these brain regions in comparison to the abundant behavioral studies. The ability to characterize the activity or dysfunction of the BNST, using these methods, may improve the ease of research on finding therapies for psychiatric disorders.

In addition, the hypothesis regarding CBD modulation on basal activity was not supported and we were not able to address any CBD modulation of odor-elicited activity. It may be that CBD does not impact basilar activity in any of the regions that our electrode was placed, but it may modulate fear inducing odor activity that was not produced in this study as a result of there not being active pumping of non-volatile odors by the VNO. Again, future studies might consider employing intra-BNST injections or trying different doses of CBD. Previous studies on CBD's influence on behavior, on what receptors it interacts with, and on what receptors exist in the BNST all support the reasoning behind why CBD might have an effect on BNST activity and its role in anxiety in general. Further research will be necessary to address all the unknowns about the drug in order to confirm its benefits over other psychoactive and side-effect rich therapies commonly prescribed today.

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