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5-Ht1a Antagonism within the Bed Nucleus of the Stria Terminalis Modulates Anxiety-Like Behaviors in Rats

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5-HT1A ANTAGONISM WITHIN THE BED NUCLEUS OF THE STRIA TERMINALIS MODULATES ANXIETY-LIKE BEHAVIORS IN RATS

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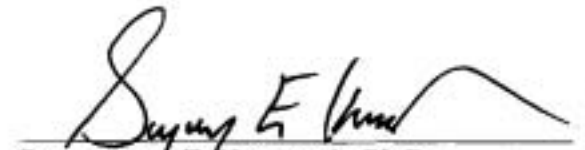

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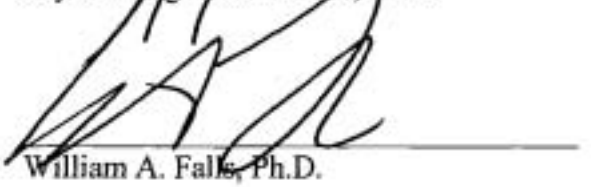

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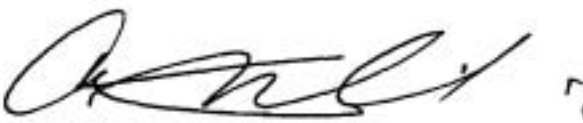

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Abstract

Substantial evidence suggests that serotonin (5-HT) activation within the brain modulates anxiety-like behavior. The bed nucleus of the stria terminalis (BNST) has been argued to mediate anxiety-like behavioral responding, and the activation of 5-HT systems may modulate anxiety-like behavior via the release of 5-HT within the BNST. Prior studies have suggested that the 5-HT1, 7 agonist 5-carboxyamidotrytamine (5-CT) is anxiolytic, which is consistent with a reduction in BNST activity via the activation of postsynaptic 5-HT1A receptors. However the anxiolytic effects of 5-CT could also have been mediated by 5-HT7 receptor activation. Hence, to isolate the effects of 5-HT1A on anxiety-like behavior, we infused the 5-HT1A antagonist WAY-100635 (0, 0.04, 0.4, and 4.0 µg/µl in saline vehicle) into the BNST of rats immediately before social interaction or acoustic startle testing. For social interaction testing pairs of rats were administered two 5-sec 1-mA footshocks immediately after infusion, removed from the chamber and measured for social interaction in a separate testing apparatus. For acoustic startle testing, rats were placed in boxes and measured for the percentage increase in test (post-infusion) startle from baseline (pre-infusion) startle. Anxiety levels were operationalized as the amount of social interaction per line cross and the percentage increase in startle following drug infusion. WAY-100635 dose dependently decreased social interaction, indicative of an anxiogenic effect. Interestingly, 0.4µg/µl of WAY-100635 decreased startle, indicative of an anxiolytic effect. These data suggest that activation of the 5-HT systems modulates anxiety-like behavior by altering activity within the BNST.

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Introduction

Anxiety is the most prevalent of all psychological disorders in America, affecting over 40 million people annually (DuPont et al., 1998). Anxiety disorders include panic disorder, obsessive compulsive disorder, generalized anxiety disorder, and phobic disorder. While the symptoms that underlie these clinical disorders are well characterized, the brain mechanisms that produce anxiety in humans and animals are less clear. Anxiety has been described as a fear response that persists over an extended period of time even if a threat is not immediately present (Walker, Toufexis, & Davis, 2003). For example, a normal adaptive fear response includes the set of behaviors activated in the face of a threat, as in the case of a person walking through the woods who encounters a bear. An individual with an anxiety disorder will exhibit this same fear response too intensely or at an inappropriate time. Due to the time course and lack of specificity of anxiety, disorders involving anxiety represent a maladaptive response that interferes with the everyday functioning of an individual's life. Although anxiety disorders afflict human populations, many experimental manipulations needed to understand the mechanisms that underlie anxiety-like behaviors cannot be performed on human subjects. By using animal models, these questions can be explored through the ability to control and utilize various manipulations that may influence anxiety-like behavior.

Behavioral Paradigms

The neurobiology of anxiety has been extensively studied, however, is still not well understood. The mechanisms argued to mediate anxiety-like behavioral states are numerous and involve various neurochemicals and brain areas. Researchers that study animal models of anxiety have designed a multitude of behavioral paradigms that have

been argued to quantify varying anxiety levels. The acoustic startle response is a common behavior utilized to measure the emotional state of an animal through measurement of the natural reflexive action to jump (startle) in response to a loud noise burst. An underlying anxiety-like state has been argued to mediate some increases in startle responding; although, other manipulations can increase startle without affecting anxiety. For example, serotonin injected onto the spinal cord increases the amplitude of the startle reflex and without having any influence on affect (Davis, Astrachan, Gendelman, & Gendelman, 1980). In order to determine whether an experimental manipulation, such as a pharmacological treatment or stressor exposure modulates anxiety, comparisons are often made between the startle amplitude exhibited prior to the manipulation (baseline) and those exhibited after the manipulation. An anxiogenic (anxiety producing) response is assumed if the rat demonstrates elevated startle levels after the manipulation when compared to baseline; whereas lower startle levels in comparison to baseline are indicative of an anxiolytic (anxiety reducing) effect (for review, see Davis, 1989). To ensure that these changes in startle amplitude are reflective of changes in affect rather than changes in motor activity, multiple behaviors are often measured.

The acoustic startle response is a reflexive response that can be modulated in anxiety-provoking situations, however, animals can also be tested for varying levels of anxiety through placement into an approach-avoidance conflict situation (Handley, 1995), such as social interaction testing. Social interaction in rodents is an ecological behavior used in the study of anxiety and has been demonstrated in natural settings (File & Pallab, 2003). Using the social interaction behavioral paradigm developed by File

(1978), an experimenter pairs rodents that are naïve to each other, and measures the amount of time the pair interacts; including sniffing, following, boxing, fighting or grooming. An anxiolytic response to an experimental manipulation would be suggested if rats spend longer amounts of time in social interaction in comparison to control treated rats (File & Hyde, 1978). While acoustic startle and social interaction are used in many experiments examining anxiety, anxiety is often characterized by a coordinated complex set of behavioral responses that are not limited to those just described.

Brain and Behavioral Correlates

An expansive area of research has been devoted to examining the specific brain mechanisms associated with changes in anxiety behaviors in humans and anxiety-like behaviors in animal species. Most of the circuitry associated with the modulation of maladaptive anxiety-like behaviors is the same as those that are important in modulation of behaviors associated with an adaptive fear response. For example, fear conditioning procedures, in which a neutral stimulus (conditioned stimulus) is paired with an aversive or noxious stimulus (unconditioned stimulus) so that the conditioned stimulus (CS) comes to elicit a fear response (Ledoux, 1998), have implicated subregions of the amygdala in the acquisition and expression of these responses.

The lateral and basolateral amygdala (LA/BLA) are brain regions where sensory information is assigned an affective valence, and relayed to the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST), which coordinate behavioral responding (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; LeDoux, Farb, & Ruggiero, 1990; Davis, 1992; Walker et al., 2003; Shammah-Lagnado, Alheid, & Heimer, 2001). The CeA and BNST share a similar developmental history, which leads to similar

morphology, chemoarchitecture and physiology (Alheid, de Olmos, & Beltramino, 1995). The CeA and BNST also both project to areas that are responsible for coordination of specific responses to stress such as those previously described (Walker et al., 2003). For example, the BNST and CeA both coordinate similar anxiety/fear-like behavioral responses such as increased startle and freezing through projections to the nucleus reticularis pontis caudalis (Davis, 1989) and periaquiductal grey as well as decreases in social interaction via the central grey (for review, see Walker et al., 2003). This suggests that the CeA and BNST might play a major role in the expression and acquisition of conditioned fear (Davis, 1992).

Outcomes from behavioral studies have led Davis and colleagues (2003) to suggest that there may be a differentiation between the role of the CeA and BNST in mediating adaptive fear responding versus the maladaptive responding that characterizes anxiety disorders in humans. Extensive research has examined the role of the BNST within anxiety-like behavior in animal models through lesion, pharmacological and immunohistochemical techniques (Walker & Davis, 2002; Davis, 1998; Duncan, Knapp, & Breese, 1996; Hammack, Richey, Watkins, & Maier, 2004). For rats, presentation of a startle eliciting noise burst in the context of bright light causes elevations in startle in comparison to noise burst presentation in dimly lit arenas, which has been called light enhanced startle and is BNST-mediated (Walker & Davis, 2002). Lesions made to the BNST with the glutamate antagonist 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinozaline-2, 3-dione (NBQX) block the expression of light enhanced startle; however, lesions of the central nucleus of the amygdala have no effect on startle (Walker & Davis, 1997). Interestingly, light-enhanced startle in rodents is analogous to dark-

enhanced startle observed in humans; a person that is placed within a dark room will show elevated startle to a noise burst in comparison to a startle eliciting noise burst within a brightly lit room (Grillon, Pellowski, Merikangas, & Davis, 1997).

Several other anxiety-like behaviors are mediated by the BNST. For example, the BNST also mediates fear-like responding to long-duration conditioned stimuli (Waddell, Morris, & Bouton, 2006). In this, a 10-minute tone or 1-minute tone was paired with a foot-shock and rats were measured for the amount of suppressed bar presses which had been previously paired with receiving food. Lesions made to the BNST blocked the expression of conditioned suppression to the 10-minute tone but not to the 1-minute tone (Waddell et al., 2006), and previous studies have shown that the CeA mediates fear conditioning to short duration conditioned stimuli (Fendt & Fanselow, 1999)

During a paradigm called learned helplessness, the lack of control over stress can produce a pathological anxiety-like state which has been argued to model anxiety disorders in humans (Maier & Watkins, 2005). Helpless animals exhibit exaggerated fear conditioning and decreases in social interaction, (Maier et al., 1993; Short & Maier, 1993; Short, Patel, Lee, Talarico, 2000). BNST lesions made prior to learned helplessness treatment blocked the anxiogenic behavioral consequences normally produced by exposure to uncontrollable stress (Hammack et al., 2004). The behaviors associated with learned helplessness are mediated by increases in serotonin (5-HT) activity, and reducing 5-HT activity attenuates learned helplessness behaviors (Maier et al., 1993). Hence these studies suggest that increased serotonergic release within the BNST may mediate behaviors associated with learned helplessness.

The BNST contains some of the highest levels of extrahypothalamic corticotropin-releasing hormone (CRF) found in the central nervous system (CNS). Increases in CNS CRF activity is anxiogenic, and has been shown to elevate startle levels. Lee and Davis (1997) found that neurotoxic lesions of the BNST but not the amygdala blocked the enhanced startle responding observed after intracerebroventricular (ICV) CRF. Moreover, rats administered local infusions of CRF into the BNST demonstrated enhanced startle whereas rats administered local CRF infusions into the amygdala did not (Lee & Davis, 1997). Local CRF BNST infusions also decreased the time spent in open arms in the elevated plus-maze task, consistent with an anxiogenic effect (Sahuque et al., 2006). This suggests that CRF receptor activation within the BNST mediates anxiety-like responses through promoting an anxiogenic response.

Studies of neural activation have also provided support for the role of the BNST in anxiety-like behaviors. Fos, a protein product of the immediate early gene c-Fos, is expressed when a neuron is excited, and is often used as a marker of neural activation (Duncan et al., 1996). Systemic administration of anxiogenic pharmacological agents such as the benzodiazepine inverse agonist FG-7142, the 5-HT2A receptor agonist mCPP, the alpha2 adrenergic receptor antagonist yohimbine, or caffeine has led to increases in Fos activation in the BNST and the CeA (Singewald, Salchner, & Sharp, 2003). This study suggests that both areas may be involved in fear and anxiety circuitry. However, other studies have demonstrated the activation of the BNST but not the CeA when animals were exposed to an anxiogenic behavioral treatment. For example, in the social defeat paradigm, two male rodents are placed within the same cage and one is allowed to defeat the other. The defeated animal responds with defensive and anxiety-

like behaviors in the presence of any other conspecific (Martinez, Phillips, & Herbert, 1998). Social defeat activated the BNST as compared to Fos-levels in control animals (Martinez et al., 1998). A similar study demonstrated that repeated exposure to social defeat within rats resulted in increased Fos expression in the BNST but not in the CeA thus supporting the hypothesis that the activation of BNST neurons mediates anxiety-related behaviors (Chung, Martinez, & Herbert, 1999). Finally, using functional magnetic resonance imaging (fMRI) techniques, Straube et al. (2007) found that humans who have phobias of spiders exhibited increased activation in the BNST in comparison to individuals without this phobia while anticipating the presentation of spider-associated stimuli. (Straube, Mentzel & Miltner, 2007).

Based on these collective data, Davis and colleagues (2003) have proposed that the CeA and BNST mediate anxiety-like responding to two distinct types of stimuli. The CeA modulates behaviors to stimuli that are specific and produces behaviors that are quick in onset and terminate shortly following the removal of the stimuli. However the BNST modulates behaviors that are "sluggish" to initiate and persist long after the behavioral eliciting stimuli has been terminated. The stimuli that provoke anxiety- like behavioral responses are diffuse, non-specific and longer in duration. Although the distinction between these two areas in their involvement in fear and anxiety-like behavior is still unclear, the results of previous experiments suggest that malfunctioning of the BNST is likely to mediate the behavioral expression of some anxiety disorders in humans.

Neurochemistry

The specific brain areas that have been argued to modulate anxiety-like and fear related behavior can be modulated by various neurochemicals. Gamma-aminobutyric acid (GABA), the major neurotransmitter that mediates inhibition in the brain, is widely distributed and manipulations of GABAergic systems have been demonstrated to influence the anxiety/fear- related behavior. For example, benzodiazepines are common GABA(A) receptor allosteric modulators that increase the efficacy of GABA in opening GABA(A)-coupled chloride ion channels, and result in enhanced inhibition of neurons by GABA (Clement & Chapouthier, 1998; Nutt & Malizia, 2001). Benzodiazepine agonists are anxiolytic, and inhibit brain regions associated with the fear circuitry, such as the amygdala (Clement & Chapouthier, 1998; Nutt & Malizia, 2001). Additionally, systemic administration of a GABA receptor agonist in rats resulted in an anxiolytic effect as measured by the increases in time spent in the open arms of an elevated-plus maze (Rodgers & Dalvi, 1997). The infusion of benzodiazepines into the BLA resulted in an anxiolytic effect of increased time spent in social interaction within rats, suggesting that the BLA may be a site of therapeutic action for this class of drugs (Gonzalez, Andrews, & File, 1996). The role of GABA within the BNST is discussed below.

Norepinephrine manipulations have also been shown to modulate anxiety-like behaviors in response to stress (Connor & Davidson, 1998; Morilak et al., 2005). Norepinephrine antagonists for both the α1- and β- receptors injected into the lateral septum attenuated defensive burying of a shock probe placed within an animal's cage (for review, see Morilak et al., 2005). Moreover, blockade of the α1 receptors in the CeA attenuated anxiogenic effects within social interaction while α1- and β- receptor blockade

in the lateral BNST attenuated anxiogenic effects in the elevated-plus maze (Cecchi, Khoshbouei, & Morilak, 2002; Cecchi, Khoshbouei, Javors, & Morilak, 2002). Connor and colleagues (1998) argue that reduced norepinephrine receptor sensensitivity found within clinical populations with anxiety disorders is due to the chronic high concentrations of circulating norepinephrine within this population.

As previously discussed, CRF has also been shown to have both direct and indirect effects in modulating anxiety-like behaviors, including those associated with learned helplessness Administration of a large dose of intracerebroventricular CRF increased behaviors associated with learned helplessness when the animals were tested 24 hours later (Ronan, Kramer, Kram, & Petty, 2000). Moreover, the administration of CRF to rats directly into the serotonergic dorsal raphe nucleus (DRN), a brain area associated with learned helplessness, produced learned helplessness-like behaviors 24 hours later (Hammack et al., 2002). The administration of CRF antagonists into the DRN prior to administration of inescapable shock attenuated the behavioral expression of anxiety-like behavior (Hammack et al., 2003). Administration of CRF and CRF agonists to rats has also been found to increase anxiety levels as measured indicated through potentiated startle (Lee & Davis, 1997). Although systemic and intra-DRN administration of CRF, and CRF agonists and antagonists have been found to modulate the behavioral expression of learned helplessness and acoustic startle, these effects might be indirect through excitation of serotonergic neurons within the DRN (Hammack et al., 2003; Kirby, Rice, & Valentino, 2000).

While pharmacotherapies have been developed to modulate some of the previously described neurotransmitters involved in anxiety (i.e. benzodiazepines),

currently, the most widely prescribed medication for anxiety disorders are selective serotonin reuptake inhibitors (SSRIs). SSRIs block the reuptake of endogenously released 5-HT, causing it to be maintained within the synaptic space for longer periods of time. The efficacy in this pharmacological agent in the treatment of anxiety disorders suggests that 5-HT likely modulates anxiety. While some mechanisms through which 5-HT activation modulates anxiety-like behavior have been explored, its exact role is still unclear. Intrinsic 5-HT release (endogenous) and pharmacological manipulations (exogenous) which can mimic 5-HT, or increase or decrease 5-HT within the brain have different effects on anxiety depending on the area of neuronal activation and activation of specific 5-HT receptor subtypes. Furthermore, pharmacological manipulations of serotonergic effects can also differentially affect anxiety depending on the length of treatment (Handley, 1995).

Although SSRIs have been shown to be beneficial in the treatment of anxiety, clinical evidence has demonstrated that patients will often feel more anxious within the first week of treatment before feeling less anxious following longer treatment. For example, it has recently been documented that a single dose of 20mg of citalopram, an SSRI, potentiates the expression of fear and anxiety in the presence of threatening or aversive stimuli in healthy human participants (Grillon, Levenson, & Pine, 2007). Within rats, acute treatment with SSRI fluoxetine results in an anxiogenic effect evidenced through decreased social interaction and increased number of escapes from the aversive qualities of an airjet (Salchner & Singewald, 2002). The acute versus chronic SSRI treatment effects have also been demonstrated within animals, with acute treatment with SSRI citalopram administered systemically to rats enhancing auditory fear

conditioning in rats whereas rats treated for 22 days with citalopram demonstrating decreased freezing when placed in the context previously associated with the receiving shock (Burghardt, Sullivan, McEwen, Gorman, & LeDoux, 2004).

5-HT release can also have different effects on anxiety behavior depending on brain region. Disruption of 5-HT release within the amygdala through administration of 5,7-dihydroxytryptamine (5,7-DHT) resulted in an anxiolytic effect on the punished drinking test, but had no effect on behavior in the elevated-plus maze (Sommer et al., 2001). These inconsistent results suggest that the two behaviors may be differentially sensitive to serotonergic activity within the amygdala. An anxiolytic effect was found after 5,7-DHT lesions made to the median raphe nucleus through increasing time spent in open arms in the elevated plus maze task if animals had be previously put through a stressor. This lesion and previous exposure to stress also increased time spent in the anxiogenic context of a bright compartment when animals were tested in the light-dark box task in comparison to animals that did not receive the neurotoxic lesion (Andrade & Graeff, 2001). Other anxiogenic effects have been found following disruption of 5-HT activity within the septum through increased performance of defensive aggression, escape behavior and enhanced startle responses (for review see Handley, 1995).

As described above, the activation of 5-HT systems can produce different effects on anxiety like behavioral responding depending on the length/dosage of serotonergic administration as well as the brain region in which 5-HT is modulated. However, confusion regarding the effects of 5-HT activation on behavioral responding is most often explained by the large number of receptor subtypes to which 5-HT binds. There are over eighteen different receptor subtypes that bind 5-HT and these receptors can mediate very

different effects on neuronal activity, including acute inhibition (hyperpolarization) and/or excitation (depolarization), and a variety of long term responses (Uphouse, 1997). That multiple serotonergic receptor subtypes can be located within the same brain area and on the same cell adds complexity to an already complex system.

5-HT has a similar high affinity for both receptors within the 5-HT1 and 5-HT7 families, and preferentially binds to these receptor subtypes when endogenous 5-HT levels are low (Palacios, Raurich, Mengod, Hurt, & Cortés, 1996). The 5-HT1 family is a G-protein coupled receptor that mediates its activity through reducing adenylyl cyclase activity and/or opening inwardly rectifying K+ (GIRK) channels resulting in a hyperpolarizing response which decreases serotonergic release and neuronal firing (Uphouse, 1997; Lanfumey & Hamon, 2004; Gross, Santarelli, Brunner, Zhuang, & Hen, 2000). The 5-HT2 and 5-HT4 receptors are G-protein coupled but lead to excitatory responses through a slow membrane depolarization through increasing phospholipase C and adenylate cyclase, respectively (Uphouse, 1997). The 5-HT3 receptor is the only receptor that is linked to a ligand-gated cation channel and mediates a fast excitatory response, but desensitizes rapidly. 5-HT5, 5-HT6 and 5-HT7 receptors are also G-protein excitatory receptors that are poorly understood in mechanism of action but add to complexity of understanding the mechanism through which 5-HT modulates behaviors (Uphouse, 1997).

Long term serotonergic pharmacological treatments, such as SSRIs, most likely lead to different behavioral effects due to changes in receptor sensitivity following chronic activation of these receptor subtypes. These adaptations include, but are not limited to, receptor downregulation, upregulation, and changes in protein cascades

(Uphouse, 1997). These adaptations may be one reason behind the change in anxiety observed across time during SSRI treatment. The previously discussed results suggest that 5-HT, BNST, and the receptors to which 5-HT binds play some role in the modulation of anxiety, and the therapeutic effect of SSRI treatment. Therefore, the goal of the current set of experiments is to further elucidate the role that 5-HT might be have within the BNST in anxiety.

Anatomy

The BNST is a complex heterogeneous structure with groups of cells that have different morphology, projection patters and neurochemistry, leading some to divide the structure into over 30 distinct subregions (Ju & Swanson, 1989; Ju, Swanson, & Simerly, 1989). The anterolateral group, including the oval nucleus, has been most implicated in anxiety-like responding, in part, due to its afferent and efferent projections and its neurochemistry. The oval nucleus of the BNST contains two distinct regions: the shell, which is composed of layers of interneurons, and the core, containing both interneurons and projection neurons (Larriva-Sahd, 2006). Relatively short projections originating from the oval nucleus innervate the anterolateral and anterodorsal areas of the BNST (Dong, Petrovich, Watts & Swanson, 2001a). The oval nucleus region of the BNST is highly connected with the medial and lateral CeA, receives sparse projections from the BLA, and also projects to areas that are responsible for coordination or motor movements such as the paraventricular hypothalamic nucleus (Dong, Petrovich & Swanson, 2001b, Dong et al., 2001a). The oval nucleus may serve as a connection between the limbic system (extended amygdala) and motor responses (Larriva-Sahd, 2006). Similarly the anterolateral BNST is highly connected with the medial, lateral and ventral capsular CeA

and also highly interconnected with the serotonergic and anxiety-related caudal DRN (Dong & Swanson, 2004; Vienante, Stoeckel, & Freund-Mercier, 1997; Commons, Connolley, & Valentino, 2003; Dong et al., 2001a; Cassell, Freedman, & Shi, 1999). Additionally, electrical stimulation of the lateral BNST alters cardiovascular responses and has been suggested to play a role in coordination of responses to aversive stimuli (Dunn & Williams, 1995; Alheid, 2003; Commons et al., 2003).

The anterolateral group of the BNST, which includes the oval nucleus and anterolateral subregions, contains dense populations of neurons that can co-express GABA, CRF, enkephalin or neurotensin (Ju et al., 1989; Sun & Cassell, 1993; Vienante et al., 1997; Phelix, Liposits, & Paul, 1992; Peto, Arias, Vale, & Sawchenko, 1999; Day, Curran, Watson, & Akil, 1999). However anatomical data suggest that these neurochemicals may be distributed in different populations of BNST neurons. For example, there is a consistent lack of co-expression between enkephalin and CRF within the anterolateral BNST, suggesting that these neuropeptides are expressed by different BNST cell types. Furthermore, electrophysiological and pharmacological data suggest that 5-HT receptor subtypes may be differentially distributed on neurons, with co-localization of 5-HT1A, 2A, and 7 receptors on one population of BNST neurons, and separate distinct populations of BNST neurons that express only the 5-HT1A or 5-HT7 receptor subtypes. Lastly, projections from this BNST region contain neuropeptides whose release can have either inhibitory or excitatory effects in terminal regions. This suggests that the circuitry associated with modulation of activity within the BNST is highly complex. Interestingly, this region, which has been associated with anxiety-like responding, is targeted by 5-HT projections from the caudal DRN (Commons et al.,

2003), which has also been associated with anxiety-like responding (Hammack et al., 2002).

Physiology

The literature reviewed above suggests that 5-HT may modulate anxiety by modulating BNST activity. Electrophysiological and immunohistochemical studies have begun to examine how 5-HT affects neuronal activation within the BNST. As described earlier, 5-HT can modulate neurons within the BNST as demonstrated by Fos activation within the BNST following treatments with 5-HT agonists such as mCPP (Singewald et al., 2003). An earlier study found that systemic injection of 5-HT1A receptor subtype agonist, flesinoxan, increased Fos within the BNST. Although a 5-HT1A agonist would be expected to reduce BNST activity, the BNST is densely populated with GABAergic neurons, therefore interneurons within the BNST normally under inhibition via GABA would now become active due to disinbitition. Flesinoxan may also attenuate DRN activity, releasing the BNST from normal serotonin-mediated inhibition. Regardless of mechanism, the increased Fos activation following flesinoxan suggests that 5-HT can modulate activation of neurons within the BNST (Compaan, Groenink, van der Gugten, Maes & Oliver, 1996).

Direct modulation of BNST neuronal activity by 5-HT application has also been examined. The neuronal responses to various neurotransmitters can be determined using electrophysiological techniques that measure the intrinsic properties of individual neurons *in vitro*. Using whole cell patch clamp techniques, Rainnie (1999a) isolated specific neurons in the dorsal portion of both the lateral and medial BNST and measured the response to 5-HT bath application. Within these areas, BNST neurons exhibited

multiple responses to 5-HT, such that single neurons could respond to 5-HT with depolarization, hyperpolarization, hyperpolarization followed by depolarization or no change in membrane potential (Rainnie, 1999a). As previously discussed, the anterolateral group of the BNST has been hypothesized to have a critical role in modulation of responses to stressful stimuli (Alheid, 2003; Commons et al., 2003). Therefore Levita and colleagues (2004) sought to examine the response of neurons within this BNST subregion to 5-HT application, extending the findings of Rainnie (1999a), in a larger sample of neurons. When a 50μM concentration of 5-HT was applied to the cells, 11% of these cells responded with pure hyperpolarization, 25% responded with depolarization and 45% of these cells had a mixed response of hyperpolarization followed by depolarization (Levita, Hammack, Mania, Li, Davis & Rainnie, 2004).

The various responses to 5-HT within the BNST are mediated by at least four different 5-HT receptor subtypes present within the BNST, including 5-HT1A, 2A, C and 7 receptors (Levita et al., 2004; Hammack, Haensly & Rainnie, 2005). The 5-HT1A receptor mediates all direct neuronal inhibition by 5-HT within the BNST (Levita & Hammack, 2004) while the 5-HT2A, C and 7 receptors mediate excitation (Hammack, Mania, & Rainnie, 2005). Because BNST 5-HT1A receptors mediate BNST inhibition, activation of the 5-HT1A receptors within the BNST should be anxiolytic. The aim of the current set of studies is to examine the role of this receptor subtype in its modulation of anxiety-like behaviors.

5-HT1A Receptor Subtype

The activation of the 5-HT1A receptor has been argued to mediate the anxiolytic effects of serotonin. Mice that lack the 5-HT1A receptor have been shown to avoid

stressful situations, which is indicative of higher levels of anxiety (Lanfumey & Hamon, 2004). When 5-HT1A knock-out (KO) mice were exposed to foot-shock, they demonstrated enhanced freezing and increased heart rate in comparison to wild-type controls (Gross et al., 2000). Studies have also demonstrated increased levels of anxiety in 5-HT1A KO mice through less time spent in open-arms of the elevated plus maze and less time spent in the center during the open-field task (Heisler et al., 1998; Parks, Robinson, Sibille, Shenk & Toth, 1998; Overstreet et al., 2003).

Pharmacological agents that target the 5-HT1A receptor modulate fear/anxiety-like behavior. Administration of the 5-HT1A partial agonist buspirone to rats following training in fear-potentiated startle was sufficient in attenuation of fear-potentiated startle, although it did not affect baseline startle (Risbrough, Brodkin, & Geyer, 2003). Rats that were administered 5-HT1A receptor agonist 8-OH-DPAT thirty minutes prior to shock demonstrated a dose dependent reduction in ultrasonic vocalizations in comparison to animals that did not receive treatment, again consistent with an anxiolytic behavioral effect (De Vry, Schreiber, Melon, Dalmus & Jentzsch, 2004). Interestingly, marmoset monkeys administered systemic injections of the 5-HT1A antagonist WAY-100635 demonstrated decreased anxiety-like behavior when placed in a maze where escape was only possible through close proximity to a predator, indicative of an anxiogenic action of 5-HT1A activation (Barros et al., 2003). These mixed findings are most likely due to the fact that systemic drug injection produces non-specific drug effect in multiple regions, and suggesting that different drugs may have different sites of action.

Aim of Current Study

While studies are beginning to examine the effects of the 5-HT1A receptor subtype microinjected into specific areas of the brain, only a few pharmacological manipulations have examined the role BNST 5-HT1A modulation in the modulation of anxiety-like behavior. Levita et al. (2004) administered 5-carboxamindotryptamine (5CT), an agonist that has high affinity for the 5-HT1A, 1B, 1D, 5 and 7 receptor subtypes, onto BNST slices found that this agonist produced a predominantly inhibitory response profile using whole-cell patch-clamp electrophysiological techniques. Consistent with BNST inhibition, intra-BNST injection of 5CT decreased baseline acoustic startle amplitude in comparison to animals that received vehicle treatment. These results suggest that activation of the 5-HT1A receptor subtype within the BNST is anxiolytic (Levita et al., 2004). Although the previous results suggest that activation of the 5-HT1A mediates anxiolytic responses within the BNST, 5CT could also have its anxiolytic effects through activation of the 5-HT7 receptor within the BNST. Preliminary experiments within our lab sought to block this behavioral effect of 5-CT on baseline startle through concomitant administration of selective 5-HT1A antagonist, WAY-100635. However, results indicated that the antagonist was having an effect without concomitant administration of an agonist which suggested that there was endogenous tonic serotonin release. The current set of experiments sought to better explore this finding through measurement of social interaction and baseline acoustic startle in response to various doses of WAY-100635. Adult male Sprague-Dawley rats received bilateral BNST cannulations, allowed to rest, assigned to drug groups and were measured for anxiety levels utilizing the above behaviors. Due to the anxiolytic effects

of agonists at the 5-HT1A receptor subtype within the baseline acoustic startle paradigm, it is expected that blockade of this receptor subtype within the BNST will promote anxiogenic effects within both behavioral paradigms.

Methods

Animals

Male Sprague-Dawley rats (200-275 g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were singly housed and kept on a 12 hr light/dark cycle (lights on at 7 AM) with food and water available *ad libitum*. Animals were given one week of rest upon arrival to the facility prior to behavioral testing or surgery. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont.

Apparatus

Footshock. A conditioning chamber (Med Associates, St Albans, VT) was used to administer shocks prior to social interaction testing. The 30 x 25 x 35 cm chamber was constructed out of aluminum and clear polycarbonate and contained a grid floor made up of twenty stainless steel bars (4.8 mm diameter) that were spaced 16.0 mm apart. The testing chamber was contained within a larger (65 x 50 x 55 cm) sound attenuating chamber (Med Associates, St. Albans, VT). Scrambled footshocks were delivered through the grid floor using a SG500power source (Med Associates, St. Albans, VT) controlled by Med PC IV software installed on an OptipleX GX240 computer (Dell Computer Corporation, Round Rock, TX).

Social Interaction. Social interaction testing was conducted in a room with a brightness of 128 lux within a 55 x 55 cm opaque white plastic box with 50 cm high walls (United States Plastics Corp., Lima, OH). In order to measure locomotor activity, the box was divided into smaller squares with horizontal and vertical separations every 11 cm using red tape. An analogue video camera (Panasonic MiniDV, PV-GS35,

Secaucus, NJ) mounted vertically above the test arena was used to record social interaction and locomotor activity.

Startle. The four stabilimeter chambers used for startle testing were 15 x 15 x 8 cm wire mesh and Plexiglas boxes that contained a grid floor of four stainless steel bars (6.0mm in diameter) spaced 18.0mm apart (Med Associates, St. Albans, VT). Each chamber hung from four springs and rested on a rubber stopper within a 90 x 70 x 70 cm sound-attenuating chamber. A high frequency speaker (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located approximately 5 cm away from the back of the startle chamber delivered the 50-ms white noise burst startle stimuli (5ms rise-decay) created by a computer sound file (0-22 kHz) and amplified by a Radio Shack Amplifier (100 Watt; Model MPA-200; Tandy, Fort Worth, TX). Background noise of 60dB was generated through a random noise generator (General Radio Company; Concord, MA).

The startle response elicited through each noise burst was measured through the displacement of the accelerometer located at the bottom of the cage. The voltage produced was proportional to the velocity of displacement, with startle amplitude defined as the maximal peak-to-peak voltage (displacement) during the first 200-ms after stimulus onset. The accelerometer output was amplified (PCB Piezotronics, Model 483B21) and digitized by an InstruNET converter (GW Instruments, Model 100B; Somerville, MA) interfaced to a Macintosh G3 computer.

Surgery

BNST Cannulation. On the day of surgery, rats were weighed and brought into the surgical suite. Isoflorane was vaporized into oxygen through an Isotec vaporizer (Fraser Harlake, Orchard Park, NY) which flowed into a plastic chamber in which the

animal was initially anesthetized. Once anesthetized, the animal was removed from the chamber and the surgical area was shaved. The subject was then fitted into the stereotaxic instrument (KOPF Instruments; Tujunda, CA) using blunt ear bars and the bite bar set to a height of -3.5 mm. Isoflorane was delivered through a nose cone secured to the bite bar. A heating blanket set at the lowest setting was placed under the animal to encourage proper circulation during surgery.

Proper aseptic preparation was completed with cleaning of the surgical area with Bentadine scrub (Purdue Product L.P., Stamford, CT) 3 times followed by a rinse of 70% ethyl alcohol. A single incision was made on the dorsal surface of the skull with a scalpel. The skull was exposed and thoroughly cleaned with cotton-tipped applicators and gauze. Occasionally 0.3 mL hydrogen peroxide (3%) was used in order to clear the area of blood. Four hemostats were used to pull away the skin at four corners of the incision area to expose the surface of the skull. Using a 395 Variable Speed MultiPro Rotary Tool Kit (Dremel) 4 screw holes were through the skull at the outermost corners of the surgical site. Screws (Small Parts, Miami, FL & PlasticsOne, Roanoke, VA) were then screwed halfway into the drilled holes in order to stabilize the skull cap.

Both stereotaxic arms were each fitted with a 22 gauge stainless steel guide cannula that were 2 cm long (PlasticsOne, Roanoke, VA) and attached to the stereotaxic frame using specially designed holders (PlasticsOne, Roanoke, VA). Guide cannulae were aimed at the BNST at an angle of 20º in order to avoid placement in the lateral ventricle. For each side, the tip of the guide cannulae were centered on bregma and aimed at a site just dorsal to the BNST based on the coordinates of the Paxinos and Watson brain atlas (1998) (26mm lateral, 3.82 mm posterior to bregma). Once it was

determined where each guide cannula would enter the skull, a hole was drilled and the surface of the dura was exposed. Once holes were drilled, guide cannulae were lowered until the tip touched the dura. Both guide cannulae were aimed 5.3mm ventral to the dura surface.

Following implantation, guide cannulae were secured to the skull using "cold cure" denture material and cross linking methyl methacrylate liquid compound (Co-Oral-ite, Diamond Springs, CA). Dummy cannulae were made from stainless steel wire (dia.014 in., Small Parts, Miami, FL) and placed into each guide cannula extending approximately 1 mm beyond cannula tip to prevent clogging. The dummy cannulae were secured to the skull cap with denture material. After the denture material dried, the subject was removed from the stereotaxic instrument and administered one subcutaneous injection of 0.3 ml (.3mg/ml:5 ml sal) of buprenorphine (Hospira, Lake Forest, IL) and one subcutaneous injection of 1 ml Lactated Ringers Solution. Approximately .1 ml of Marcaine (Hospira Inc., Lake Forest, IL) was administered dropwise around the incision site as a topical anesthetic. The subject was allowed to recuperate under a heat lamp and brought back to colony room upon revival. Two post-operative .3 ml subcutaneous buprenorphine injections were administered the day following surgery, and post-operative checks were maintained for seven days during the subject's recuperation as per IACUC protocol. Rats were allowed to recuperate for one week after surgery before brain infusion and behavioral testing. During this week, rats were handled every day in order to perform post operative checks and habituate them to experimental procedures.

Guide Cannulae Placement Verification. On the day of sacrifice the rat was weighed, brought into the surgical area within their home cage and received an injection

of 150 mg/kg sodium pentobarbital in 7.8% isopropyl alcohol (Fort Dodge Animal Health, Fort Dodge, IL). Under deep anesthesia, the rat was removed from its cage and placed onto a wire mesh covering which rested over a catch bucket.

An incision across the abdomen and below the xiphoid process was made. The rat received an injection of .2-.3 ml Heparin (Abraxis, Shaumburg, IL) directly through the diaphragm into the heart to reduce blood coagulation. Cuts were made up both sides of the lungs and across the diaphragm to allow to access to the heart. One incision was made to the left ventricle to allow a blunted 14- gauge needle attached to a systolic pump (Manostat, Barrington, IL) to be inserted into the aorta and another incision was made in the right atrium. Saline was then pumped through the body. After approximately 30 seconds, 4% Paraformaldehyde or Formalin 10% was pumped through the same system in order to fix brain tissue. Once sufficiently fixed, the brain was removed and placed in a vial containing either Paraformaldehyde 4% or Formalin 10% for postfixing for at least 24 hours.

Brains were sectioned (60-90 μm) on either a freezing microtome or cryostat at -30°C. Slices were stored in phosphate buffered solution and kept refrigerated until staining. Brain sections were mounted onto chromium aluminum subbed slides and stained with cresyl violet. Slides were treated with a series of dehydrating washes, cresyl violet incubation and washing. Following cresyl violet staining, slides were coverslipped using mounting medium (Richard Allen Scientific; Kalamazoo, MI).

Guide cannulae placement was verified under a light microscope and notes were kept as to which area the guide cannulae tracts extended.

Drug Administration

Three doses of WAY-100635 and one vehicle control treatment were used during the behavioral studies described below. Drug aliquots containing 10.0 µl of 8.0 µg WAY-100635 per 0.5 µl de-ionized water were frozen until needed. On the day of testing, the aliquots were allowed to thaw and further diluted with 10.0µL 1.8% Saline so that the drug mixture contained 8.0 µg WAY-100635 per 1.0 µl .9% Saline. Aliquots were further diluted to achieve appropriate drug concentrations (8.0µg/µl, 0.8µg/µl and 0.08µg/µl). Vehicle treatments consisted of equivolume 0.9% Saline. Rats were assigned to one of the four treatments prior to behavioral testing and received 0.5µl of solution per side. The resulting drug amounts infused into the BNST were 4.0µg/0.5µl WAY, 0.4µg/0.5µl WAY, 0.04µg/0.5µl WAY or 0.5µl vehicle.

For drug infusions, subjects were removed from their cages and handheld in a towel. The dummy cannulae were removed from guide cannulae and the rats were injected by hand, one side at a time, through the guide cannulae aimed at the BNST. The entire infusion process took an average of 5 minutes per rat. Infusions were made through 28-gauge stainless steel tubing (Plastics One, Roanoke, VA) connected to 10.0µL Hamilton syringe through PE-50 tubing (Plastics One, Roanoke, VA). The injector extended 1.0mm beyond the tip of the guide cannula, into the BNST. The injector was left within the guide cannula for approximately 1 minute to allow for drug diffusion.

Specific Procedures

Experiment 1: WAY-100635 Dose Response within Social Interaction. 64 male Sprague-Dawley rats were given bilateral cannulations of the BNST as described above. After being allowed to rest for one week, during which time rats were handled to

habituate them to injection procedures, pairs of animals were randomly assigned to one of four drug treatment groups (4.0µg/0.5µl WAY, 0.4 µg/0.5µl WAY, 0.04 µg/0.5µl WAY, or vehicle). On the test day, two animals were removed from colony room and transported within their home cage to the test room and allowed to rest for 5 minutes. Subjects were removed from their cages and administered assigned treatment as described above.

Following injections, animals were left to rest in their "displaced" home cage for 5 minutes and then transferred to the conditioning chamber for an acclimation period of 5 minutes. Both subjects then received two 1 mA shocks for 5 seconds each separated by 1 minute and left for a period of 15 minutes within the conditioning chamber. The parameters for this shock procedure is based off previous work by Amat and colleagues (1998a, 1998b) who demonstrated that administration of two consecutive shock increases 5-HT release in projection regions of the dorsal raphe nucleus over a 120 minute period of time.

Rats were then removed from conditioning chamber and transferred to the social interaction box. Subjects were placed on opposite corners and the remaining 10 minutes of testing were recorded with a camera. Following the interaction testing, the animals were removed from the social interaction box and returned to their home cages. Perfusions and histological verification of cannulae placements were subsequently conducted as described above to determine whether proper placement was achieved.

Videos were scored by a blinded rater for the amount of time that animals spent in active social interaction and locomotor activity. Social interaction and locomotor activity were operationalized through including following, sniffing, boxing, and grooming (see

File et al., 1978) and total number of line crosses, respectively. Based on a previous study, anxiety levels were defined as the amount of social interaction per line cross, with higher ratios indicative of lower levels of anxiety. Pairs of animals were treated as an n=1 so as to eliminate inflation. Prior to data analysis, data was cleaned and screened for outliers. A data point was considered an outlier and eliminated from analysis if it was more than two-standard deviations from the mean. Pairs of animals that contained a rat that had a histologically verified off-placed cannula and had received the highest dose of WAY-100635 were compared through planned contrasts against vehicle treated pairs to check for drug effects at areas surrounding the BNST. A one-way ANOVA was conducted for locomotor activity, total amount of social interaction and social interaction controlling for locomotor activity with planned contrasts performed to analyze differences between pairs that received vehicle treatment versus those that received varying doses of WAY-100635. A Person's correlational analysis was also conducted in order to determine the relationship between total social interaction and line crosses.

Experiment 2: WAY-100635 Dose Response within Baseline Startle. 38 male Sprague-Dawley rats were given bilateral cannulations of the BNST as described above. After being allowed to rest for one week, animals were assigned one of four treatment groups (4.0μg/0.5μl WAY, 0.4 μg/0.5μl WAY, 0.04 μg/0.5μl WAY, vehicle) and tested using the acoustic startle paradigm. The experiment was conducted over a three day period for each rat. The first two days each consisted of a single 20 minute run of startle testing. Two days of startle testing were performed before drug treatments in order to acclimate the rats to the test procedure. As will be discussed later, the second day of startle testing was used to ensure that startle amplitudes did not differ between treatment

groups. On the third day each animal was run through another single 20 minute run of startle testing (baseline), administered their designated drug treatment and then run through a single 45 minute run of startle testing (test). Rats were then placed back into their home cages and returned to their colony suite. Perfusions and histological verification of cannulae placements were subsequently conducted as described above to determine whether proper placement was achieved.

For each acoustic startle test, rats were placed in the startle chambers in the dark, and a 60 dB background noise was presented continuously in order to eliminate any noise competition. During the 5 minute acclimation period, activity levels in the absence of acoustic startle stimulus presentation were measured every 30 seconds through the same accelerometer device used to measure startle amplitudes. After the 5 minute acclimation period, rats were measured for their response to acoustic startle stimuli every 30 sec for either 15 or 40 minutes (depending on test). In order to avoid habituation, rats were presented with startle stimuli of 3 different intensities (95 db, 100 db, and 105 db) that were presented in a pseudorandom order such that all three intensities were presented within each block of three trials. Upon termination of the program, rats were removed from the boxes and returned to their home cages and startle boxes were cleaned with soap and water between sessions to avoid odor transfer between animals.

In order to ensure that drug treatment groups did not systematically differ in startle levels prior to drug administration; rats were matched into groups. For group matching, the last 3 minutes of startle amplitudes recorded during the second day of baseline startle testing were averaged for each rat. Subjects were then assigned into

treatment groups so that the mean startle amplitudes were approximately the same between groups.

Acoustic startle amplitude was utilized as an index of anxiety levels. Rats were screened out that had startle amplitudes that were at the maximum measurement unit greater than 50% of the time. This was done because "clipping" of data exemplifies rats that had startle amplitudes that were beyond the range of measurement, therefore it would be impossible to gain an accurate average of these rats' startle amplitude.

For each rat, a percent increase in startle amplitude produced by drug infusion was determined by dividing averages of startle amplitudes obtained for each 3-minute increment of time during test (after WAY-100635 infusion) by the average startle amplitude during the last 3-minutes of baseline startle (immediately prior to WAY-100635 infusion). A repeated-measures general linear model was used to compare treatment groups for differences within treatment groups across time, between treatment groups in average percent increase in startle amplitude produced by drug infusion and the interaction between treatment and time. A one-way ANOVA and planned contrasts were used to compare differences between the different WAY-100635 treated and vehicle treated rats.

For each 3-minute increment of time, there were two trials conducted at each noise burst intensity (95dB, 100dB, 105dB). A percent increase in startle amplitude for each intensity produced by drug infusion was determined by dividing startle amplitudes for each 3-minute increment of time during test (after WAY-100635 infusion) by the average startle amplitude during the last two trials from baseline (immediately prior to WAY-100635 infusion) for each noise burst intensity. This data was then analyzed

through use of a repeated-measures general linear model for each noise burst intensity in order to compare treatment groups for differences within treatment groups across time for, between group differences in average percent increases in startle amplitude produced by drug infusion and the interaction between treatment and time. A one-way ANOVA and planned contrasts were used to compare differences between the different WAY-100635 treatment groups and the vehicle treated group.

To ensure that drug effects were due to changes in anxiety and not changes in overall activity (locomotor changes), activity levels were also analyzed for each treatment group. Similar to previous analyses, changes in activity levels were determined through taking the average of the last three minutes of activity during baseline and dividing it out of averaged 3-minute activity test segments, resulting in a percentage increase in activity due to treatment. A repeated measures general linear model was run to compare groups for differences within treatment groups across time, between treatment groups for average percentage increase from baseline and the interaction between treatment and time. A one-way ANOVA and planned contrasts were used to compare differences between the different WAY-100635 treatment groups and the vehicle treated group. A Person's correlational analysis was also conducted in order to determine the relationship between activity levels and startle amplitudes. Further analysis was also conducted through use of a one-way ANOVA and ANCOVA in order to ensure that changes in startle were not due to differences in startle box location.

Results

Histology

Once rats had been perfused and brains were postfixed in Formalin 10% or Paraformaldehyde 4% and representative slices of cannulations were kept for Cresyl Violet staining and verification. The area of interest for the current studies was the dorsal lateral BNST. Cannulations were included in analysis if they were between 0.20 mm and 0.92 mm behind Bregma and fell within the borders of medial, lateral and dorsal portions of the BNST and the ventral border of the parastrial nucleus. Figure 1 and Figure 3 are representative illustration of those cannulations that were considered hits for social interaction and startle respectively.

Because pairs of animals were treated as one score in social interaction testing, a missed cannulation in one of the animals in the pair resulted in the pair being eliminated from analysis. A total of five rats (five pairs) were determined to be misses during histological verification. Two pairs of animals were eliminated from analysis, however three pairs that were deemed misses and had also received an infusion of 4.0μg/0.5μL were analyzed as another treatment group in order to examine whether drug effects were due to spread to surrounding areas. None of the rats tested within the startle testing were considered to be misses.

Social Interaction Testing

As described above, the effect of drug treatment on locomotor activity, social interaction and social interaction controlling for locomotor activity were determined. One pair of animals was removed as an outlier (more than two-standard deviations from the mean) and, as previously stated, two pairs were eliminated that were determined to be

missed BNST cannulations and had not received 4.0µg/0.5µl. An additional 4 pairs were eliminated from analysis due to errors made while conducting the experiment procedure. The resulting group numbers can be found in Table 1.

Locomotor activity tended to increase dose dependently, however this difference did not achieve significance $F(4,25)= 1.011$, $p=.424$ (Figure 2b). Conversely social interaction tended to decrease dose dependently, however these differences were also not significant between treatment groups $F(4, 25)= 2.124$, $p=.114$ (Figure 2c). Although there was no significant difference in activity levels or total social interaction between groups, Pearson's correlational analysis showed that these factors were significantly related to each other such that increases in locomotor activity were related to increases in social interaction $r(21)= .444$, $p<.05$.

Because social interaction correlated with locomotor activity, and based on previous studies examining social interaction (Short & Maier, 1993), social interaction was determined by examining the amount of social interaction per line cross. A significant dose-dependent decrease is social interaction scores was achieved $F(4,25)= 3.156$, $p<.05$ (Figure 2a). A significant decrease in social interaction per unit activity was found for rats treated with 4.0µg/0.5µl in comparison to vehicle treated rats $t(21)=2.269$, $p<.05$; demonstrative of an anxiogenic effect of intra-BNST 5-HT1A antagonism.

Due to the efficacy of the highest dose (4.0µg/0.5µl) of the antagonist in increasing anxiety levels as measured through social interaction, analyses were conducted simultaneously to evaluate drug effects of this dose on rats whose cannulae were implanted outside of the BNST (Figure 3). There was no significant differences found in planned contrasts between this group of animals and vehicle treated animals on any of the

behavioral indices. Therefore these results suggest that the anxiogenic effect found following treatment with 4.0µg/0.5µl dose of WAY-100635 was not due to effects at an area neighboring the BNST.

Acoustic Startle Test

The effect of 5-HT1A antagonism on startle was determined by examining the percent increase in startle from baseline to test across time, increase in startle to the different noise burst intensities from baseline to test, as well as the relation between startle amplitude and activity. One rat was removed from analysis due to clipping (startle amplitudes beyond the range of measurement) of data for more than 50% of the test trials. The resulting group numbers can be found on Table 1. Due to programming errors, the last 10 minutes of the 40 minute run contained blocks of three trials that did not contain all three intensities and were presented with intertrial intervals of 10 or 20 sec. This error in programming resulted in exclusion of this time period from further analysis and use of the first 30 minutes of startle responses to noise bursts.

Activity levels, determined by pre-noise burst deflections of the accelerometer, were analyzed in order to ensure that changes in startle amplitudes were not due to changes in activity due to drug effects. Activity averages and percent changes for each treatment group were compared against startle effects during the respective time interval. A repeated-measures analysis demonstrated that there was an effect of drug treatment on activity levels $F(3,33)= 12.637$, $p<.05$. A planned contrast indicated that the lowest dose of WAY-100635 (0.04 µg/0.5µl) increased activity levels in comparison to vehicle treated animals $t(33)=.227$, $p<.05$ (Figure 4). These results suggest that the drug is having an effect on activity; these activity changes may be influencing subsequent startle

levels. A Pearson's correlational analysis demonstrated that there was no significant correlation between activity and startle amplitude or the change in activity levels and change in startle amplitude from baseline to test. The lack of relationship between activity and startle amplitudes leads to the conclusion that the effect of drug treatment on activity levels is independent of any effect on startle amplitude. Therefore, no additional analyses were conducted examining the role that the drug had on activity in affecting startle amplitude.

Startle changes were calculated as a percentage increase from baseline (immediately prior to infusion) to test (immediately following infusion) and were analyzed through examining differences within treatment group effects of time, between treatment groups and the interaction of treatment and time. A repeated-measures ANOVA demonstrated that there was a significant within subjects effect of time $F(9, 297)= 2.497$, $p<.05$. However there was no significant between group effect of drug treatment or interaction effect of drug treatment across time (Figure 5b). Therefore these results indicate that blockade of the 5-HT1A receptor within the BNST had no effect on anxiety as measured by startle.

In order to examine if startle differed for the groups at particular noise burst intensities, change in startle from baseline to test was examined for each intensity. There were no significant differences across time, between drug treatment groups or for the interaction between treatment group and time for startle in response to a 95 dB or 100dB noise burst (Figure 6a & 6b). There was a significant effect across time for change in startle in response to the 105dB noise burst $F(9, 297)= 3.904$, $p<.05$, but similar to the other noise intensities there was no significant difference between drug groups or

between groups across time on change in startle amplitude in response to the 105dB noise burst (Figure 6c). An analysis of covariance (ANCOVA) was also performed in order to determine if location of the startle boxes were influencing any differences in startle between the treatment groups. There was no effect of box placement on change in startle amplitude, further demonstrating that effects were not due to equipment effects. Finally, an analysis was conducted in order to verify that there were no significant differences in baseline (prior to infusion) startle between treatment groups. A one-way ANOVA demonstrated that there were no significant differences between treatment groups in their average baseline startle. These results further support that blockade of the 5-HT1A receptor within the BNST has no effect on anxiety as measured through startle.

Discussion

The current set of experiments found that blockade of the 5-HT1A receptor within the BNST modulated anxiety-like behavior. WAY-100635 dose-dependently decreased social interaction, which was indicative of an anxiogenic effect. Similar doses of WAY-100635 did not effect on baseline startle. These data suggest that the modulation of BNST 5-HT1A activity modulates anxiety-like behavior, although some differences between these two paradigms (social interaction and acoustic startle responding) are apparent.

Anxiety is the most prevalent of all psychological disorders and has been extensively studied; however the neurobiological underpinnings are still poorly understood. As mentioned above, previous evidence suggests that the BNST serves as an interface between some anxiogenic stimuli and behavioral responses. The BNST receives input from the caudal DRN (Commons et al., 2003), BLA (Dong et al., 2001a; Dong et al., 2001b) and CeA (Dong et al., 2001a; Dong et al., 2001b), which all have been shown to mediate fear and anxiety-like behaviors. Consistent with an anxiogenic role of BNST activation, the BNST projects to areas that mediate specific anxiety-associated behavioral responses such as the nucleus reticularis pontis caudalis and central grey, which mediate startle responding and social interaction, respectively (for review, see Walker et al., 2003). Changes in BNST activity modulates many anxiety-like behaviors including those associated with learned helplessness (Hammack et al., 2004), light-enhanced startle (Grillon et al., 1997), social defeat (Martinez et al., 1998; Chung et al., 1999) and long-duration conditioned fear-like responding (Waddell et al., 2006).

As noted above, the activation of 5-HT systems has been shown to increase and decrease anxiety-like behavior depending on whether activation is chronic or acute (Handley, 1995; Grillon et al., 2007), the brain region in which 5-HT is modulated (Sommer et al., 2001; Andrade et al., 2001) and the 5-HT receptor subtype targeted (Uphouse, 1997; Lanfumey, 2004). The 5-HT1A receptor has been the most widely studied in mediating the effects of serotonin activation on anxiety-like behavior. Despite this attention, few studies have examined the role of the 5-HT1A receptor activation within the BNST in mediating anxiety-like behavior. One report demonstrated that the activation of this 5-HT receptor subtype within the BNST might promote an anxiolytic response within the acoustic startle paradigm (Levita et al., 2004); however, the agonist used in this study, 5-carboxyamidotryptomine, also binds with similar affinity to 5-HT7 receptors, which are also found in the BNST. The 5-HT7 receptors are G-protein linked excitatory receptors; activation of which would promote an opposing effect to the 5-HT1A receptor (Uphouse, 1997). By blocking the 5-HT1A receptor with antagonist WAY-100635, which is much more selective to 5-HT1A receptors than 5-CT, the current studies sought determine if the anxiolytic effect found in the Levita et al., 2004 study was driven through activation of the 5-HT1A or 5-HT7 receptors within the BNST. An anxiogenic effect of WAY-100635 in social interaction testing was consistent with prior data suggesting that activation of the 5-HT1A receptor mediates an anxiolytic response.

The Effect of BNST 5-HT1A Antagonism on Social Interaction

WAY-100635 infusion into the BNST produced a dose dependent decrease in social interaction per line cross, with the highest dose (4.0µg/0.5µl) of WAY-100635 promoting the greatest anxiogenic effect. The dose-dependency of WAY-100635 is

consistent with an action at 5-HT1A receptors. Within this behavioral paradigm, locomotor activity (line crosses) and total amount of social interaction (following, sniffing, grooming, etc.) were significantly correlated to each other such that higher amounts of activity were associated with higher amounts of social interaction, although the antagonist did not significantly alter either measure independently. The lack of a significant difference between treatment groups on activity levels suggests that the anxiogenic effect of 4.0µg/0.5µl WAY-100635 on social interaction was not mediated by drug induced changes in activity. It is important that social interaction scores from rats with cannulae accidentally implanted outside of the BNST and treated with the highest WAY-100635 dose were not different from control rats because it supports that the site of action for WAY-100635 was the BNST and not a neighboring area.

As a 5-HT1A antagonist, WAY-100635 would not alter neuronal excitability unless an agonist, such as endogenous 5-HT, is activating the 5-HT1A receptor. Hence, following WAY-100635 infusion, rats were administered two consecutive shocks because previous studies showed that this treatment was sufficient to induce serotonergic release in anxiety-related brain areas (Amat et al., 1998a; Amat et al., 1998b). The anxiogenic effect of 5-HT1A blockade within the BNST following shock suggests that activation of the 5-HT1A receptor would promote an anxiolytic effect; supporting the previous results by Levita and colleagues (2004). The dose dependent anxiogenic effect of WAY-100635 suggests that the highest dose is required in order to achieve maximal antagonism of the 5-HT1A receptors within the BNST. The reduced efficacy of the lower doses may have occurred due to lack of maximal 5-HT1A receptor binding; allowing some serotonin to bind to 5-HT1A receptors.

The Effect of BNST 5-HT1A Antagonism on Acoustic Startle

In contrast to the effects found within social interaction, WAY 100635 did not alter acoustic startle responding. Similar to the social interaction analyses, a significant effect of drug on activity could suggest that changes in startle amplitude were due to effects on motor systems. A significant increase in activity was found for the lowest WAY-100635 dose (0.04µg/0.5µl). A correlation was performed to see if activity levels were related to startle amplitudes; however, no significant relation was found. The lack of correlation between these two behavioral indices indicates that the startle amplitudes performed by the animals following drug infusion were likely not due to drug-induced changes in activity.

Baseline (pre-infusion) startle differences between groups were eliminated by matching rats into treatment groups based on their second day of startle testing so that the average of the last 3 minutes of startle amplitudes did not significantly differ. This matching procedure was conducted prior to drug infusions in order to ensure that there were no differences between treatment groups that would mask or potentiate the drug effects of WAY-100635. A one-way ANOVA, further demonstrated that there were no significant differences between treatment groups in their baseline (pre-infusion) startle amplitudes, which may have otherwise influenced test (post-infusion) startle amplitudes.

An analysis of covariance (ANCOVA) was performed in order to determine if the changes observed in startle from baseline (pre-infusion) to test (post-infusion) varied across the different test chambers. This analysis was conducted to ensure that the experimental contexts did not differ and that differences in equipment did not affect the results. No significant difference was found for an effect of testing chambers on changes

in startle response. Separate analyses were also performed to examine if changes in startle amplitude were different for each of the three different noise intensities. Startle amplitudes in response to the highest noise burst intensity (105dB) decreased slightly across time, but there were no differences between treatment groups in startle amplitudes at any noise burst intensity as evidenced by a lack of a significant interaction between time and treatment group. Hence, the lack of effect of WAY-100635 was not influenced by drug induced changes in activity levels, particular noise intensity or equipment confound.

The Role of 5-HT1A Receptors in Anxiety

Most studies suggest that pharmacological activation of 5-HT1A receptors using agonists such as 8-OH-DPAT, buspirone, ipsapirone and gepirone, have anxiolytic behavioral effects (Dekeyne, Brocco, Adhumeau, Gobert & Millan, 2000; Stanhope & Dourish, 1996; Heiser & Wilcox, 1998), which has also been observed in rodents and humans, and drugs such as Buspirone are prescribed for the treatment of anxiety disorders (for review, see Heiser et al., 1998). Treatment with 8-OH-DPAT, a commonly used 5-HT1A agonist, has been shown to have anxiolytic properties in rat. For example 8-OH-DPAT increased punished responding within the Vogel conflict paradigm when administered systemically (Dekeyne et al., 2000). Conditioned suppression of lever pressing within the conditioned emotional response test was also decreased in rats after systemic treatment with the 5-HT1A agonists ipsapirone and gepirone (Stanhope et al., 1996). Moreover, the anxiolytic effects on the conditioned emotional response test and Vogel conflict paradigm were blocked through pretreatment with WAY-100635, the 5-HT1A antagonist used in the present studies (Dekeyne et al., 2000; Stanhope et al.,

1996). The anxiogenic effect of WAY-100635 on social interaction reported here is consistent with the literature reporting anxiolytic responses to systemic injections of 5-HT1A agonists, although the lack of effect of WAY-100635 on startle is not consistent with this literature. Possible reasons explaining the difference in anxiety effects of WAY-100635 on social interaction and startle will be examined later in discussion.

Although several studies have reported effects of 5-HT1A manipulations on social interaction in rodents (see below), fewer have investigated the effects of serotonergic manipulation on acoustic startle. Systemic injection of 8-OH-DPAT or the 5-HT2 receptor agonist, mescaline, has been shown to increase startle, indicative of an anxiogenic effect (Nanry & Tilson, 1988; Davis, 1987). However, the site of action of these compounds in the modulation of anxiety-like behavior is not known. For example, systemic administration of serotonergic drugs may modulate spinal serotonin receptors altering the startle response directly, rather than changing an underlying anxiety-like state (Davis et al., 1980). Importantly, in the current study, WAY-100635 was injected directly into the BNST and hence would be unable to affect serotonin receptors at distal sites such as the spinal cord. Moreover, no relation was observed between changes in activity and changes in startle, and, no changes in activity were found in the social interaction experiment. Hence, it is likely that any effect of WAY-100635 on startle would have been indicative of modulation an underlying anxiety state, rather than motor systems.

The Role of 5-HT1A Receptor in Social Interaction

Previous studies have examined the role of 5-HT1A receptor activation/inactivation in the modulation of social interaction using both systemic

injections and brain infusions of 5-HT1A agonists and antagonists. Systemic administration of 5-HT1A agonists such as 8-OH-DPAT usually increased social interaction, which is indicative of an anxiolytic effect (Dekeyne et al., 2000; Picazo, Lopez-Rubalcava, Fernandez-Guasti, 1995), and typically these effects were blocked by the pretreatment of WAY-100635 (Dekeyne et al., 2000). These findings are consistent with the anxiogenic effect observed after administration of the 5-HT1A antagonist within social interaction in the current study. As mentioned above, the brain region mediating the effects of systemically administered serotonergic drugs on anxiety is unknown, although our current studies suggest that the BNST may be a critical site of action for these effects.

Other studies in which 5-HT1A agents were injected into discrete brain regions, found different effects on social interaction depending on the region targeted. Intra-median raphe nucleus administration of 5-HT1A agonist 8-OH-DPAT has led to increases in social interaction due to activation of MRN 5-HT1A receptors and inhibition of MRN activity (Andrews, Hogg, Gonzalez & File, 1994; File, Gonzalez & Andrews, 1996). Lesions made to the DRN serotonergic neurons blocked the anxiolytic effects of systemic 5-HT1A agonistic effects within social interaction (Picazo et al., 1995). Interestingly, 5-HT1A agonist treatments aimed at projection regions of the MRN and DRN, such as the basolateral amygdala and the hippocampus, have found anxiogenic effects on social interaction (Andrews et al., 1994; File et al., 1996; Gonzalez, Andrews & File, 1996). Although not addressed in the current set of experiments, the basolateral amygdala and hippocampus are other brain regions that receive 5-HT input from the

DRN and MRN and hence may be other sites by which 5-HT might modulate fear/anxiety-like behavior.

Few studies have examined the effect of 5-HT1A drugs within the BNST. 5-HT1A receptors can be located either a pre-or post-synaptically. For example, activation of somatodendritic presynaptic 5-HT1A autoreceptors within the DRN produces a well known reduction in 5-HT production and 5-HT release from terminals. In addition, post-synaptic 5-HT1A receptor activation also leads to an inhibitory effect on neuronal firing in projection regions of the raphe nuclei (Uphouse, 1997). Systemically administered 5-HT1A agonists and antagonists likely act at both pre- and/or postsynaptic 5-HT1A receptors; hence, it is difficult to determine the site of action for behavioral changes produced by drugs administered in this manner. The previously described results suggest that activation of presynaptic receptors within the MRN and DRN is anxiolytic. Interestingly, 5-HT1A-induced decreased DRN serotonin activity may still modulate anxiety-like behavior via action at postsynaptic 5-HT1A receptors in the BNST. Whereas high levels of BNST serotonin likely bind to several 5-HT receptor subtypes that increase and decrease BNST activity, lower levels of circulating serotonin within the BNST may preferentially act at postsynaptic 5-HT1A receptors, which inhibit BNST neuronal activity to produce less anxiety. The anxiogenic effects of WAY-100635 on social interaction are consistent with an anxiolytic role for the activation of postsynaptic BNST 5-HT1A receptors.

Different Effects of BNST 5-HT1A Antagonism on Social Interaction & Acoustic Startle

These studies found differential effects of BNST 5-HT1A antagonism on social interaction and the baseline acoustic startle response. While the acoustic startle response

is a reflexive measurement of the current emotional state, social interaction measures changes in emotional state through use of an approach/avoidant paradigm which incorporates uncertainty about the novel environment and also introduces a social component (Handley, 1995; File et al., 1978). One goal of this study was to determine if the 5-HT1A receptor had the same functional properties across these two different of anxiety measures. The difference in findings between these two studies may be due to a different role of 5-HT or the BNST in modulating these behaviors. However, Levita et al. (2004) demonstrated an anxiolytic effect of BNST 5-HT manipulation on the startle paradigm, which was consistent with the anxiogenic effect on social interaction by blockade of the 5-HT1A receptor.

Another explanation for the different effects observed between these two experiments is that two difference procedures were employed. For the social interaction test, two-consecutive shocks were given after administration of the 5-HT1A antagonist, which were not administered before testing the startle response. Shock was not administered during startle testing because pilot data demonstrated that WAY-100635 modulated startle in the absence of shock, which suggested that the BNST contained endogenous circulating 5-HT in this testing paradigm.

As previously discussed, both the 5-HT2 and 5-HT7 receptors are also located within the BNST. 5-HT has the highest affinity for the 5-HT1A and 5-HT7 receptors, followed by the 5-HT2 (Palacios et al., 1996). Because WAY-100635, as an antagonist, does not have any action other than blocking 5-HT1A receptors, it is likely that the anxiogenic effect of WAY-100635 on social interaction was achieved due to the activation of both the 5-HT2 and 5-HT7 receptors by high levels of endogenous

circulating 5-HT following administration of shock. Therefore, social interaction testing was most likely influenced by the activation of both the 5-HT7 and 5-HT2 receptors due to high levels of endogenous 5-HT release. The lack of shock administration prior to startle testing and the habituation procedure that was employed the two days prior to drug infusion testing most likely resulted in a lower amount of endogenous 5-HT release. Therefore, the low levels of endogenous 5-HT may have activated the 5-HT7 and/or 5-HT2 receptors at such a low amount that the behavioral effect could not be detected. While activation of both 5-HT2 and 5-HT7 receptors is excitatory, they could be differentially located on interneurons and/or projection neurons and/or result in the release of various neuropeptides such as GABA, CRF, neuropeptide Y, enkephalin or neurotensin (Ju et al., 1989; Sun & Cassell, 1993; Vienante et al., 1997; Phelix et al., 1992; Peto et al., 1999; Day et al., 1999). Therefore, the postsynaptic location of the 5-HT2 or 5-HT7 receptors could result in release of excitatory or inhibitory neuropeptides. The behavioral response is ultimately dependent on the net result of integration of these different excitatory and inhibitory inputs and projections.

Recent evidence has suggested that WAY-100635 may be an agonist at the dopamine 2, 3 and 4 (D2, D3, D4) receptor subtypes (Chemel, Roth, Arbruster, Watts & Nichols, 2006). Initially, Forster et al. (1995) reported that WAY-100635 was 100 times more selective for the 5-HT1A receptor subtype than for the D2 and D4 receptor subtypes. However this has been refuted by Chemel and colleagues (2006) who found that WAY-100635 was only 10 times more selective for the 5-HT1A receptor subtype than the D4 receptor, but more than 100 times more selective for the D2 and D3 receptor subtypes. Postmortem in situ hybridization studies in humans show that the area of the

BNST highly expresses D1 and D2 mRNA (Hurd, Suzuki & Sedvall, 2001). However, autoradiography studies have failed to find evidence for D4 receptors to be localized within the BNST within rats (Primus, Thurkauf, Xu, Yevich, McInerney, Shaw, Tallman & Gallagher, 1997). Because of the low affinity of binding to the D2 receptor within the BNST and the lack of D4 receptor localization within this area, it is unlikely that the effects of WAY-100635 observed in the current studies were mediated by DA receptor binding.

Limitations and Conclusions

While the preceding results suggest a role for 5-HT1A receptors within the BNST in modulation of anxiety, there are some limitations. The lack of consistency between paradigms raises some concerns. It is unclear if the different effects found within the two studies were due to differences in endogenous circulating serotonin, differences in the type of anxiety being measured, a combination of both, or some other variable. Additional studies are ongoing in order to investigate if 5-HT1A antagonism would be anxiogenic if rats were administered two consecutive shocks prior to being tested for acoustic startle, although these studies are beyond the scope of this paper.

The target area within the current studies was the anterolateral region of the BNST due to its importance in modulating anxiety-like behavior as demonstrated through stimulation, neurochemical and anatomical data (Casada & Daphne, 1991; Phelix et al., 1992; Alheid et al., 1995). While the majority of neurons within this area are GABAergic (Cullinan, Herman, & Watson, 1993; Erlander, Tillakartne, Feldblum, Patel, & Tobin, 1991), these neurons may also release other neuropeptides such as CRF, enkephalin, CCK, neurotensin, neuropeptide Y and others that may or may not inhibit

postsynaptic sites. Therefore, activation of 5-HT1A receptors within the BNST may inhibit the firing of neurons that carry excitatory and inhibitory receptors adding complexity to the mechanism through which the BNST modulates anxiety-like behavior. While this complexity exists, the current results and the effects found by Levita and colleagues (2004) suggest that activation of this anterolateral region of the BNST seems to increase anxiety-like behavior.

The current results suggest an anxiolytic action of the 5-HT1A receptor activation within the BNST, however there are at least three other functional serotonin receptors within this area (Singewald et al., 2003; Levita et al., 2004; Fox, Hammack, & Falls, in press). As previously discussed, these receptors may be located on various types of neurons and the ratios of these activated receptors may have differed between the current behavioral paradigms. Because of the lack of knowledge for the role of the activation of other 5-HT receptor subtypes within the BNST on anxiety-like behavior, it is unknown which receptor/s mediated the behavioral effects observed following BNST 5-HT1A antagonism. Future studies focusing on the role of these receptors and the interaction between the various receptors within the BNST will help to better understand the role that serotonin in the BNST plays in anxiety.

While the two current studies yielded different results, the known serotonergic release during the social interaction experiment and the previous results found by Levita et al. (2004) suggest that inhibition within the BNST via the activation of the 5-HT1A receptor is effective in reducing anxiety states. These studies provide insight to possible targets of future pharmacotherapies and how those that target the 5-HT1A receptor subtype may be beneficial in reducing anxiety.

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Figure & Table Captions

Table 1. Group numbers for social interaction and startle testing, including animals not used for statistical analysis.

Figure 1. A schematic representation of BNST guide cannulae placement for social interaction testing. Points represent the injector tip for animals receiving varying doses of WAY-100635. X's represent those animals that were considered misses and also included in analysis. Coronal sections are shown from -0.20 through -0.92mm relative to bregma.

Figure 2. Figure 2a- Treatment with WAY-100635 dose dependently decreased the amount of social interaction per unit of activity with 4.0µg/0.5µl promoting an anxiogenic effect. Figure 2b- Locomotor activity was defined in terms of line crosses made during a 10 minute period by the animals paired during social interaction; no significant differences were found. Figure 2c- Total amount of social interaction (sniffing, grooming, boxing, etc.) measured over a 10 minute period. 5-HT1A receptor antagonism within the BNST or neighboring brain areas did not affect locomotor activity. WAY-100635 doses: 4.0µg/0.5µl, 0.4 µg/0.5µl, 0.04 µg/0.5µl, vehicle.
*$p<0.05$ with respect to vehicle treated group.

Figure 3. A schematic representation of BNST guide cannulae placement for startle testing. Points represent the injector tip for animals receiving varying doses of WAY-100635. Coronal sections are shown from -0.20 through -0.80mm relative to bregma.

Figure 4. Intra-BNST blockade of 5-HT1A receptors increased activity during acoustic startle testing. Rats treated with 0.4 µg/0.5µl WAY-100635 demonstrated an increase in

activity in comparison to vehicle treated rats. *p<0.05 with respect to vehicle treated group.

Figure 5. Figure 5a- No significant effect was found for treatment groups on percentage increase in startle from pre-infusion to post-infusion testing. Figure 5b- Average percentage increase across time in startle amplitudes from pre-infusion to post-infusion testing. The percentage increase in startle amplitude diminished over time. WAY-100635 doses: 4.0µg/0.5µl, 0.4 µg/0.5µl, 0.04 µg/0.5µl, vehicle

Figure 6. Figure 6a- No significant effect was found for treatment groups on percentage increase in startle amplitude from pre-infusion to post-infusion testing in response to 95dB noise bursts. Figure 6b- No significant effect was found for treatment groups on percentage increase in startle amplitude from pre-infusion to post-infusion testing in response to 100dB noise bursts. Figure 6c- A significant effect was found across time such that there was a decrease in percent change as time progressed. WAY-100635 doses: 4.0µg/0.5µl, 0.4 µg/0.5µl, 0.04 µg/0.5µl, vehicle

Table 1

	VEHICLE	0.04µg/0.5µl	0.4µg/0.5µl	4.0µg/0.5µl	Miss 4.0µg/0.5µl	Excluded From Analysis
Social Interaction N= 26	6	6	5	6	3	7
Baseline Acoustic Startle N= 27	10	8	10	9	N/A	1

Figure 1

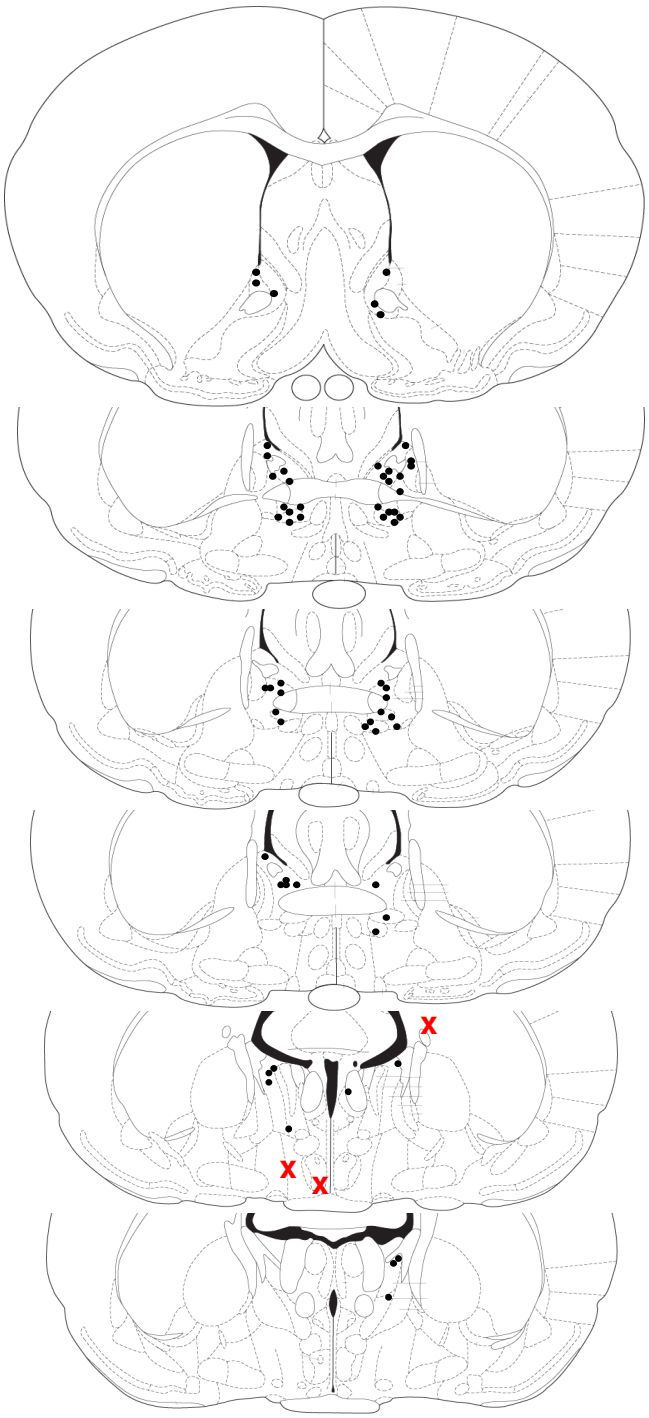
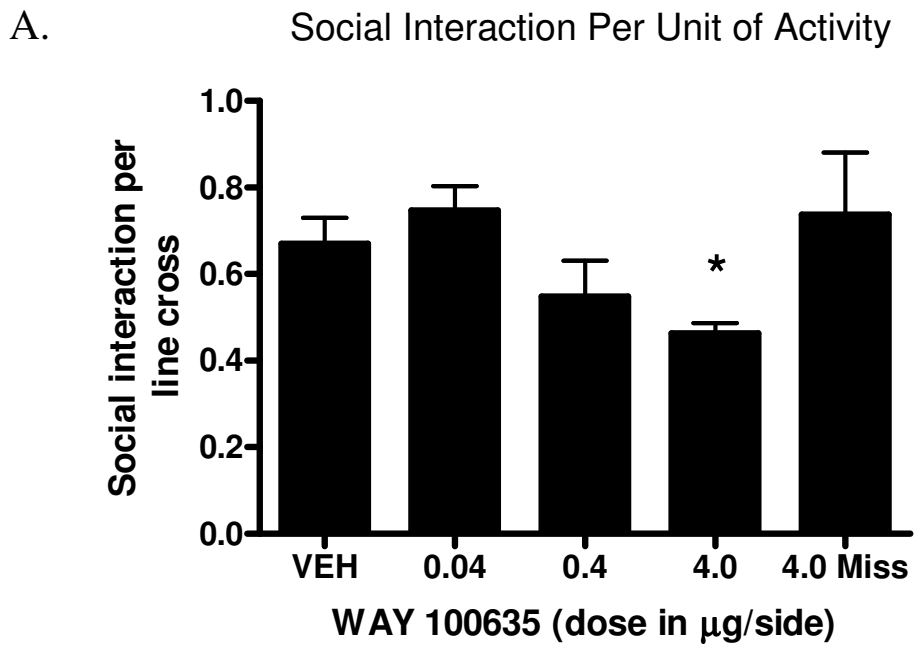

bregma -0.22 mm
bregma -0.26 mm
bregma -0.30 mm
bregma -0.40 mm
bregma -0.80 mm
bregma -0.92 mm

Figure 2


A.
Social Interaction Per Unit of Activity
Social interaction per line cross
1.0
0.8
0.6
0.4
0.2
0.0
*
VEH
0.04
0.4
4.0
4.0 Miss
WAY 100635 (dose in µg/side)

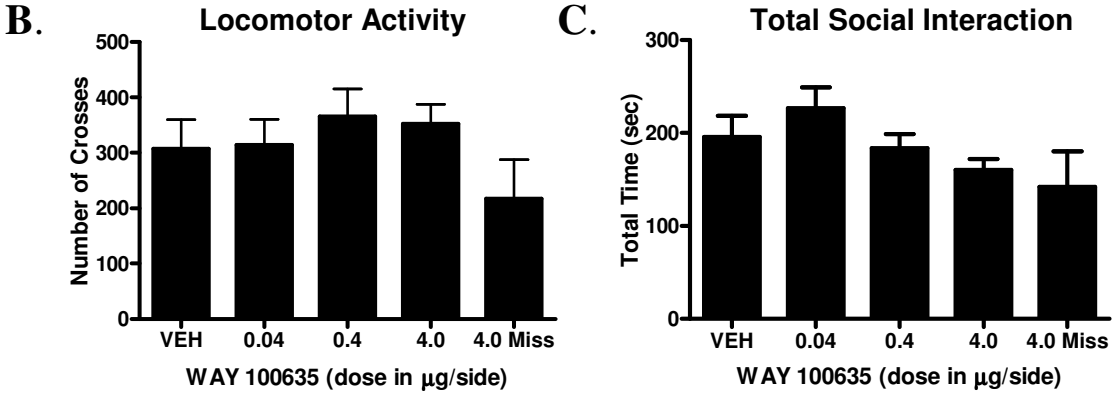

B.
Locomotor Activity
Number of Crosses
500
400
300
200
100
0
VEH
0.04
0.4
4.0
4.0 Miss
WAY 100635 (dose in µg/side)
C.
Total Social Interaction
Total Time (sec)
300
200
100
0
VEH
0.04
0.4
4.0
4.0 Miss
WAY 100635 (dose in µg/side)

Figure 3

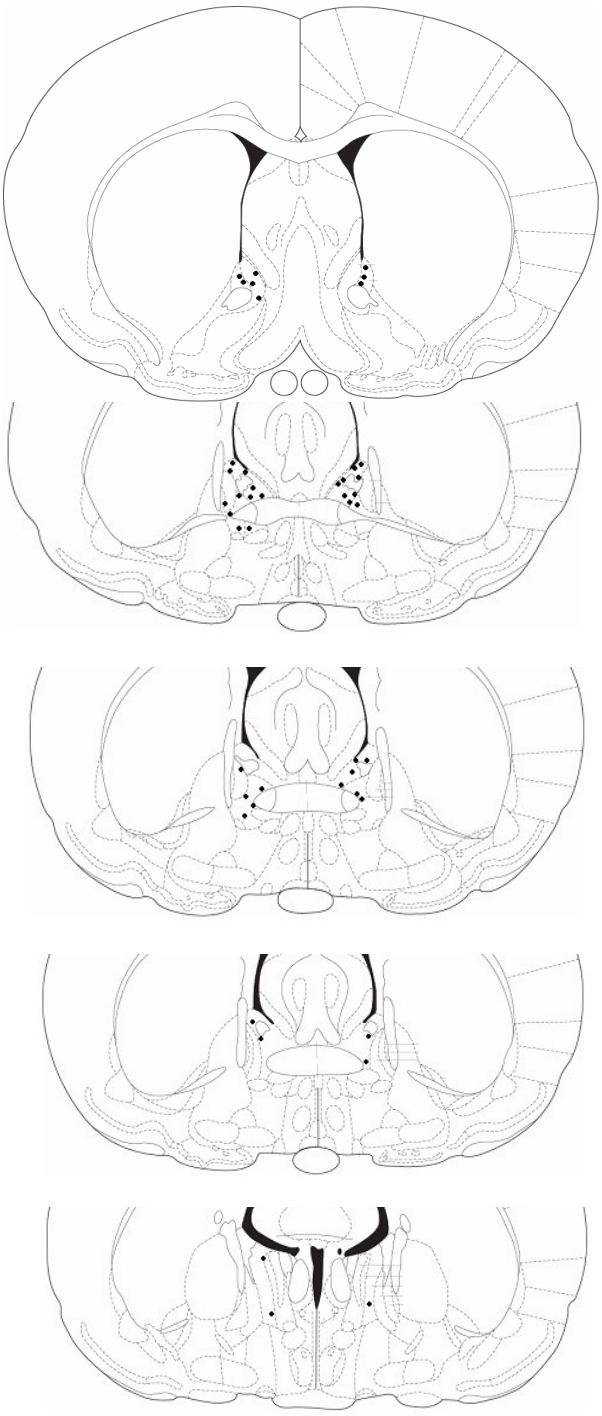


Figure 4

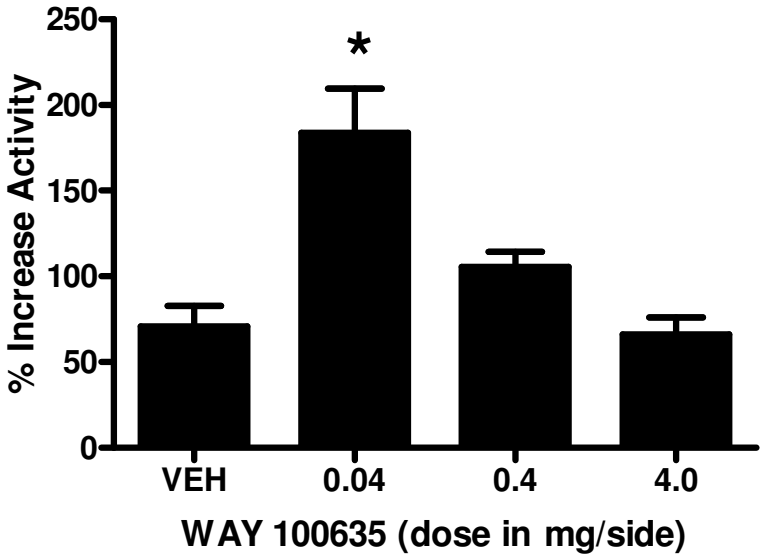
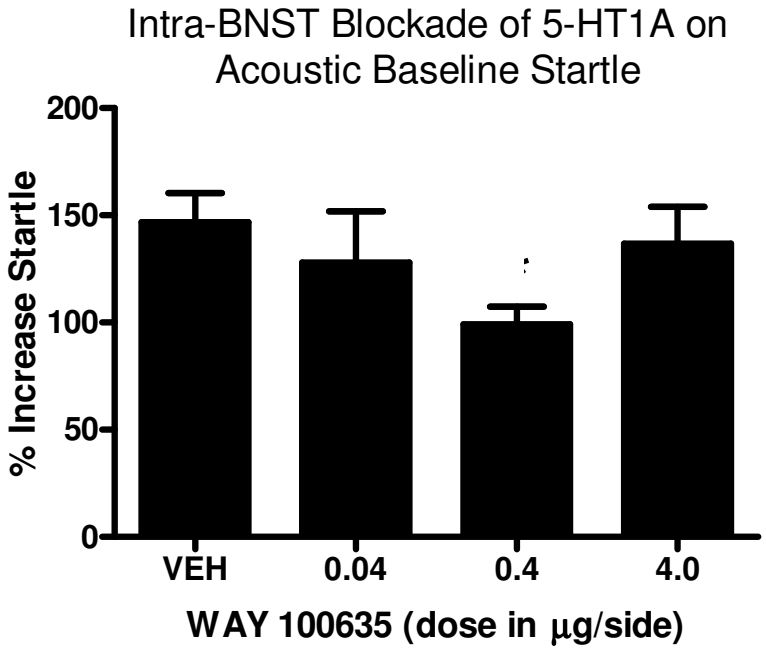

250
200
150
100
50
0
% Increase Activity
*
VEH
0.04
0.4
4.0
WAY 100635 (dose in mg/side)

Figure 5

A.


Intra-BNST Blockade of 5-HT1A on Acoustic Baseline Startle
% Increase Startle
200
150
100
50
0
VEH
0.04
0.4
4.0
WAY 100635 (dose in µg/side)

B.

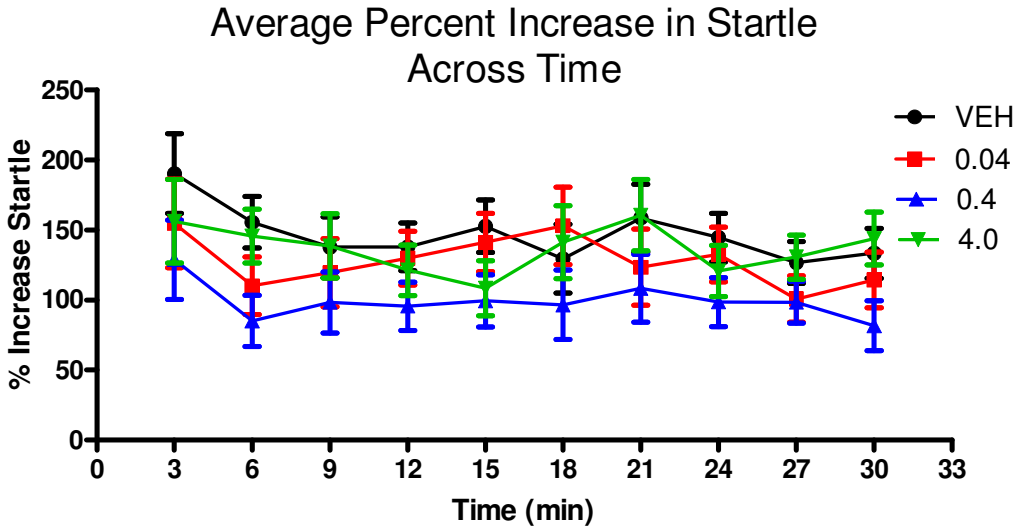
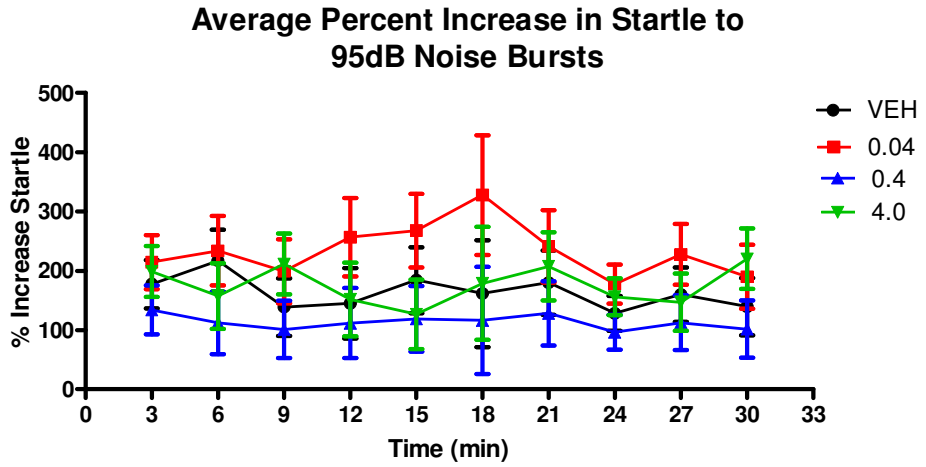
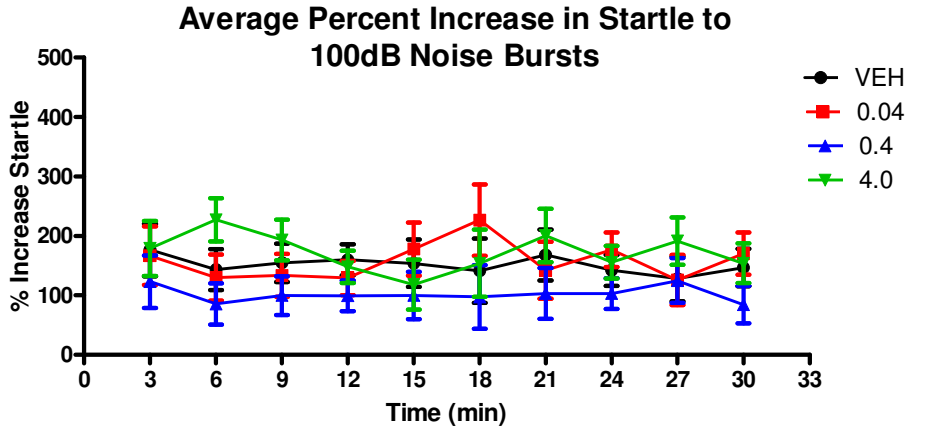

Average Percent Increase in Startle Across Time
% Increase Startle
250
200
150
100
50
0
0
3
6
9
12
15
18
21
24
27
30
33
Time (min)
VEH
0.04
0.4
4.0

Figure 6

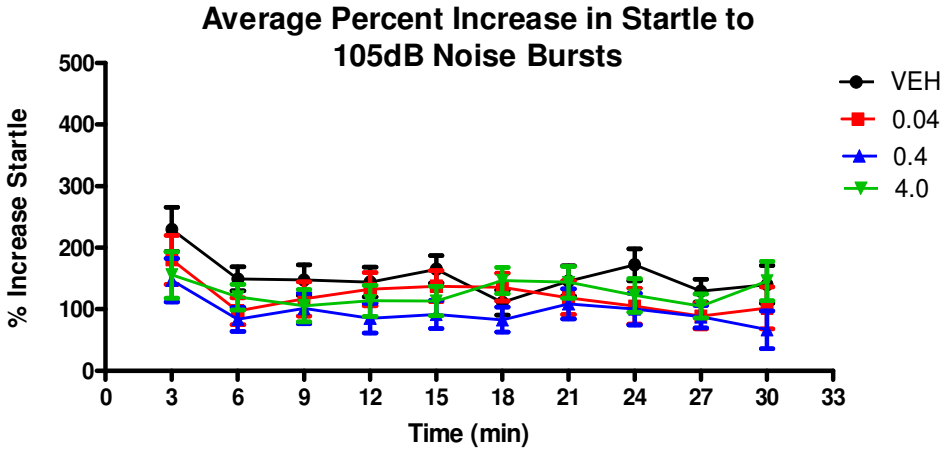
A.


Average Percent Increase in Startle to
95dB Noise Bursts
% Increase Startle
Time (min)
VEH
0.04
0.4
4.0

B.


Average Percent Increase in Startle to
100dB Noise Bursts
% Increase Startle
Time (min)
VEH
0.04
0.4
4.0

C.


Average Percent Increase in Startle to
105dB Noise Bursts
% Increase Startle
Time (min)
VEH
0.04
0.4
4.0