

Going with the flow: the potential relationship between cerebral blood flow in the bed nucleus of the stria terminalis and anxiety-like behavior

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Abstract

Much is known about the role of Bed Nucleus of the Stria Terminalis (BNST) in behavioral anxiety, as are the mechanisms of cerebral blood flow. However, a direct connection between the two is yet to be investigated. The objective of the present study was to examine if pharmacological manipulation of cerebral blood flow in the BNST could produce an anxiogenic phenotype in animal test subjects. Subjects were bilaterally cannulated in their BNST, and the L-type calcium channel antagonist *diltiazem* was infused directly into the region. Then subjects were tested for behavioral anxiety on an elevated plus maze. Results were nonsignificant as experimental subjects did not show increases in anxiety-like behavior compared to control subjects. However, future studies utilizing this paradigm could enhance researcher's and clinician's understanding of the neurological system behind behavioral anxiety, and potentially lead to new effective therapies for anxiety disorders.

Introduction

The complexities of the nervous system, especially the brain, are astounding. Interest in the brain dates back as early as the 2nd century, and since then research and knowledge of its anatomy and physiology has grown extraordinarily. Despite its spectacular role in maintaining overall function within the body, the brain has been implicated in a wide range of somatic and psychological disorders. Understanding how the brain works in respect to the study of mental functions and behaviors is crucial for understanding the development and maintenance of psychological disorders like anxiety.

When evaluating any pathology or behavioral response such as anxiety, the interplay between biological and environmental influences should be considered. Nevertheless, it is the purpose of this project to attempt to isolate one neurobiological mechanism that may help scientists, researchers and clinicians to better understand the connection between the brain and

anxiety disorders. Specifically, my work will focus on the relationship between cerebral blood flow in the bed nucleus of the stria terminalis (BNST), an area known to play a critical role in the modulation of stress and anxiety, and anxiety-related behaviors. This study investigates whether or not manipulation of blood flow in this region can directly influence anxiety-like behavior. The following sections will outline the literature regarding stress/ anxiety in relation to the BNST, neurovascular coupling (NVC) and cerebral blood flow, and a study conducted by myself and colleagues that was designed to measure the potential relationship between NVC in the BNST and anxiety-like behavior.

Stress and anxiety

The physiology and psychology of stress and anxiety

Stress is often a generic term used to describe any deviation of the body from homeostasis. The stress response is the body's reaction to any stressor, good or bad, presented in the environment. Our bodies first respond to stressors via activation of the sympathetic branch of the autonomic nervous system. Also known as the "fight or flight" response, the sympathetic nervous system is responsible for mobilizing the body's energy resources to deal with the threatening situation. The consequential signaling cascade begins in the brain, is transmitted through the spinal cord and is conducted to virtually every tissue throughout the body. The body's coordinated behavioral, physiological and hormonal reaction to activation of the sympathetic nervous system in response to an environmental stressor is defined as the stress response. This response includes, but is not limited to, increased heart and respiratory rates, increased blood pressure, pupil dilation, inhibition of digestive functions, increased blood glucose levels, secretion of norepinephrine and epinephrine, and decreased sensitivity to painful

stimuli (Kandel, Schwartz, Jessel, Siegelbaum & Hudspeth, 2013; Martini, 2006). Normally these symptoms subside as the threat of the stressor passes, but when the system is dysfunctional, it can lead to debilitating disorders of anxiety. Researchers are still trying to achieve a more comprehensive explanation of the neurobiological mechanisms that underlie behavioral anxiety disorders; the present study attempts to address this topic.

Anxiety is a cognitive and physiological state that causes an individual uneasiness or worry, and can sometimes be induced by stress. It is a common response that nearly every human will experience at one point in his or her life. Symptoms of anxiety often include accelerated heart rate, shortness of breath, dizziness, chest pain, fear of losing control or “going crazy”, intrusive and persistent memories of the anxiety-inducing experience, difficulty concentrating, avoidance behavior, panic and more (American Psychiatric Association, 2000). In the case of anxiety disorders, these symptoms persist for extended periods of time, and often in inappropriate situations. They cause the individual large amounts of distress, and interfere with normal functioning (American Psychiatric Association, 2000).

The National Institute of Mental Health cites that anxiety disorders affect about 40 million Americans (18%) age 18 and older in a given year, and are often co-morbid with other types of mental disorders such as depression and substance abuse (National Institute of Mental Health, 2009). The Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR) notes the prevalence of some specific, and particularly devastating anxiety disorders. According to the DSM-IV-TR, the lifetime prevalence rate of Post Traumatic Stress Disorder (PTSD) is 8%, 5% for generalized anxiety disorder, 3-13% for social phobia, and up to 3.5% for panic disorder. Because anxiety disorders are abundant in society, and can be so devastating to those that suffer from them, it is important that researchers and clinicians gain

insight into the causes of a disorder's onset and symptoms. In doing so, hopefully means to prevent initial onset and combat the symptoms of a progressive pathology can be advanced to help those suffering to improve their quality of life.

Various brain regions have been associated with anxiety and anxiety disorders including the bed nucleus of the stria terminalis (BNST), the amygdala, the prefrontal cortex, the hippocampus and the brainstem (Ventura-Silva et al., 2012). Much research, including the present study, has focused on the role of the BNST in the maintenance and expression of anxiety.

The Bed Nucleus of the Stria Terminalis (BNST)

There has been extensive research conducted on animal models that suggests that chronic stress is a precursor to general anxiety and an enhanced state of fear. Many studies point to the BNST as a critical site for the regulation of anxiety and fear responses.

The BNST is a small limbic forebrain structure that is thought to be the key mediator of slow-onset and persistent stress responses to sustained threats (Walker, Toufexis, & Davis, 2002; Radke, 2009). BNST activation is also believed to be largely responsible for the learned association between fear and a specific context. Accordingly, research has suggested that the prolonged maintenance of anxiety, often in non-fearful contexts, is modulated principally by the BNST (Radke, 2009).

Clinical anxiety disorders are often explained as an inability to appropriately inhibit anxiety in non-stressful situations; many anxiety-stricken humans tend to express a general sense of fear and anxiety in safe scenarios (Radke, 2009; Duvarci, Buaer & Pare, 2009). For example, a major symptom of PTSD is a state of anxiety induced by a specific trauma that persists even after the trauma-related cues are gone (Radke, 2009; Christianson et al., 2011). Individuals

suffering from PTSD are often overwhelmed with intrusive thoughts and explicit memories of the trauma long after the trauma has occurred. Because the BNST is thought to be responsible for the modulation of persistent anxiety, it is hypothesized that many anxiety disorders reflect a dysfunction of the normal neural systems associated with the BNST.

The anatomy and physiology of the BNST

The BNST is composed of anteromedial, anterolateral and posterior divisions (Ventura-Silva et al., 2012). It receives excitatory glutamatergic projections from the basolateral amygdala (BLA), the prefrontal cortex (PFC), and the hippocampus, and sends projections to hypothalamic and brainstem regions (Walker et al., 2002; Radke, 2009, Ventura-Silva et. al., 2012).¹ It is likely that upon stimulation of the BLA, PFC and/or hippocampus, the BNST subsequently becomes activated and leads to the activation of the hypothalamic and brainstem regions that mediate the physiological and behavioral responses to stressful and often fearful stimuli. Thus, when the BNST and/or related structures are active, the organism should be exhibiting anxiety.

Ventura-Silva et al. (2009) conducted a study that aimed to compare the pattern of activation of neurons in the BNST under basal conditions and after exposure to an anxiogenic stimulus in control and chronically stressed rats. They examined cFOS expression in both groups before and after subjects were exposed to a startle stimulus. cFOS is a protein, and transcription of cFOS is upregulated in response to cellular activity. Researchers found that cFOS was indeed upregulated in the BNST of chronically stressed rats as compared to control rats following exposure to an anxiogenic stimulus. They concluded that chronic stress shifts the pattern of BNST activation to reflect an excited, anxiogenic mode (Ventura-Silva et al., 2012). Researchers

¹ See figure 1

also used an elevated plus maze (EPM) to test whether or not these animals expressed an anxiogenic phenotype. An EPM is a measure of behavioral anxiety that will be defined in more detail in a later section. Essentially, they found that stress did increase anxiety-like behavior on the EPM, and also induced BNST activity. Researchers suggest that activation of the BNST via environmental stress was responsible for causing the observed anxiogenic phenotype (Ventura-Silva et al., 2012).

Christianson et al. (2011) also used cFOS expression to analyze BNST activity after exposure to a stressor. Researchers exposed rats to inescapable tail shocks either with or without a safety signal. The safety signal was a stimulus presented to the animals that indicated that a stress-free period would soon follow. They tested cFOS expression and used social exploration as a measure of behavioral anxiety in both groups. Researchers found that cFOS expression was diminished in the BNST of animals that received a safety signal immediately after receiving a stressor, and that rats exposed to the safety signal exhibited greater social exploration. This finding suggests that the BNST may become less active in animals that are supposedly reassured that the fearful stimulus has passed, and consequently exhibit more social exploration indicative of reduced anxiety (Duvarci et al., 2009). Additionally, Christianson et al. (2011) found that after chemical lesions of the BNST with the sodium channel antagonist tetrodotoxin (TTX), animals showed decreased freezing behavior, indicative of decreased anxiety, during later exposure to stress. Again, this suggests that the BNST is necessary for the exhibition of behavioral anxiety, and that inactivation of the BNST may help diminish anxiety-like behavior. It is probable that in individuals with anxiety disorders, the mechanism of BNST inactivation is somehow compromised.

The BNST and the amygdala: anxiety versus fear

The amygdala is a limbic structure associated with the emotion of fear and fear expression. Fear and anxiety elicit similar physiological and behavioral responses in an animal, and as a result it is sometimes difficult to distinguish between the two. Additionally, both the amygdala and the BNST play a role in the stress-activated anxiety response, and have numerous overlapping projections (Duvarci et al., 2009). The following research lends support to the notion that fear and anxiety are actually quite different: fear is argued to represent a short-duration response to an imminent threat, whereas anxiety is argued to represent a long-duration response to a future threat (Walker et al., 2002). Research suggests that the BNST is crucial for the expression of anxiety rather than fear.

Various studies have utilized designs that compare the BNST and the amygdala to isolate the differences between fear responding and anxiety responding. Duvarci et al. (2009) used fear conditioning to simultaneously assess the influence of the amygdala and the BNST on behavior. Fear conditioning is a type of learning that is often modulated by the amygdala and occurs when a neutral stimulus becomes associated with a fearful stimulus. After repeated associations, the neutral stimulus can elicit feelings of fear in the absence of the fearful stimulus or imminent threat (LeDoux, 1996). Duvarci et al. (2009) conditioned rats to fear a tone by pairing it with a foot shock. They then analyzed freezing behavior in response to the tone and used an elevated plus maze to test anxiety 5 days later. They found that sham rats showed a normal acquisition of conditioned fear responses, demonstrated by increased freezing behavior. These rats also exhibited anxiety-like behavior on the elevated plus maze 5 days after training. Interestingly, they found that BNST-lesioned rats also showed normal fear acquisition as evidenced by initial freezing behavior but demonstrated *less* anxiety-like behavior on the elevated plus maze 5 days

later. This led the researchers to conclude that BNST lesions do not disrupt the pathways from the CeA to the hypothalamus and brainstem, structures that modulate the magnitude of initial fear acquisition and responding. However, BNST lesions did disrupt the exhibition of behavioral anxiety days later. Duvarci and colleagues also found that although BNST lesions did not affect fear responding to the initial conditioned stimulus, they did dramatically reduce inappropriate fear responding to a conditioned stimulus that was not originally paired with a foot shock. Together, these findings indicated that the BNST is crucial for both the maintenance of anxiety, and the selectivity of fear responding.

In a similar study, Walker et al. (2002) found that disrupting BLA function reduced and could even abolish autonomic responses to conditioned fear and fear-potentiated startle, but that lesions to the BNST did not block fear potentiated startle. Findings of both studies support the claim that perhaps two interconnected but distinct pathways exist: one for fear and one for anxiety. The fear pathway is mediated by the amygdala and governs a short-term response to a specific threat cue. The anxiety pathway is mediated by the BNST and is characterized as a slow-response system that once activated, can influence behavior long after the fearful stimulus has been terminated (Walker et al., 2002).

In respect to human behavior and PTSD, it is consistently observed that traumatic stress exaggerates fear and anxiety even after the trauma has subsided. Christianson et al. (2011) suggest that it is possible that the intense fear that occurs during a trauma changes the way fearful emotions are expressed after the trauma and causes the individual to generalize this fear to other non-fear related cues. It is possible that there are cellular changes that take place in the neurons of the BNST that can account for this inappropriate and prolonged state of anxiety. Perhaps chronic stress or fear sensitizes neurons in BNST pathways (Walker et al., 2002). If so,

upon any type of stressful stimulation, the BNST would elicit an anxiety-like response even if the stimulatory cue were not a fearful one. It is also possible that neurons in the BNST are highly plastic, and that the cellular changes that occur in response to chronic stress or fear explain the long-duration response characteristic of BNST elicited anxiety (Christianson et al., 2011). Finally, it is our prediction that changes in blood flow in the BNST may also modulate activity of neurons in this area, and contribute to sensitization of these responses.

CRH and the BNST

The mechanisms by which corticotrophin releasing hormone (CRH) influences the anxiety response are also critical to the understanding of BNST physiology and anxiety-like behavior. CRH is often cited as a vital component of the hypothalamic- pituitary- adrenal axis, or HPA- axis. In the HPA-axis, the hypothalamus is activated in response to stress and releases CRH, which binds to receptors in the anterior pituitary gland. The anterior pituitary gland then releases adrenocorticotrophic hormone (ACTH), which binds to receptors on the adrenal cortex of the adrenal medulla. The adrenal cortex then releases glucocorticoid hormones (like cortisol), which then act on neuronal receptors at various body tissues to induce a physiological stress response. The HPA-axis functions as a negative feedback loop such that when levels of cortisol get too high, cortisol binds to receptors on the hypothalamus and anterior pituitary to signal the cessation of its own release.

Both exposure to chronic stress and the application of corticosterone (the rat glucocorticoid) induce anxiety in animals. Roman et al., (2011) found that repeated central injections of CRH produce anorexia in a rodent model. Anorexia is a common symptom of

anxiety in both humans and animals, and researchers suggest that it is likely mediated by CRH-containing neurons in the BNST.

Ventura-Silva et al. (2012) found CRH type₁ and type₂ receptors to exist in the BNST of rat brains, and also point out that the BNST has projections both from the PFC and to the hypothalamus. Researchers propose that upon activation of excitatory CRH₁ receptors in the anteromedial nucleus of the BNST, glutamatergic projections from the BNST to the hypothalamus are activated, and anxiety-like behaviors result via excitation of the HPA-axis. Conversely, CRH₂ receptors in the posterior nucleus of the BNST receive glutamatergic input from the PFC and are responsible for the termination of CRH release and the reduction of anxiety. Researchers propose that chronic anxiety may reflect a disturbance in the PFC-BNST CRH₂ receptor pathway such that the negative feedback mechanism of the HPA-axis is compromised. If so, this is possibly be the mechanism by which long-term anxiety is maintained.

Research by Walker et al. (2002) supports this allegation as well. They found that infusions of CRH into the ventricles dramatically increased the startle response in control animals, indicating that CRH can indeed elicit anxiety. However, they also found that lesions to the BNST blocked CRH-enhanced startle, that CRH infused directly into the BNST increased startle amplitude, and that infusions of a CRH receptor antagonist into the BNST blocked the effects of intracerebroventricular CRH infusions (Walker et al., 2002). These results were not consistent when the tests were applied to other areas like the hippocampus and amygdala. This evidence further supports the hypothesis that the BNST is essential for the expression of behavioral anxiety. It is possible that chronic stress can lead the BNST to become overactive and lead to persistent activation of excitatory CRH pathways. This over excitation may also

contribute to impairments in the HPA-axis negative feedback mechanism and lead to a state of long-duration anxiety (Ventura-Silva et al., 2012).

Implications

Taken together, results of studies conducted thus far lead to the conclusion that activation of BNST is absolutely critical for the modulation of anxiety and the expression of an anxiogenic phenotype. Specifically, BNST activity is responsible for the production of long-term behavioral anxiety after exposure to repeated stress (Duvarci et al., 2009; Roman et al., 2011; Radke, 2009; Ventura-Silva et al., 2012; Walker et al., 2002).

As Duvarci et al. (2009) suggests, it is possible that BNST neurons have a higher baseline and/or sensory driven level of excitability in subjects that express an anxious phenotype compared to subjects that do not. Additionally, Roman et al., (2011) propose that chronic stress (like that which can cause anxiety disorders) cause morphological changes in BNST neurons like increased dendritic length, and an overall increase of BNST volume. It is possible that pharmacological interventions that reduce BNST excitability may prove useful for the treatment of anxiety disorders. The present study utilized a pharmacological intervention into the BNST to mimic the effects of chronic stress. The following section aims to describe the mechanisms of neurovascular coupling and the pharmacological system of cerebral blood flow in respect to activation of the BNST. To date, no studies have been conducted that propose a link between blood flow in the BNST and the production of anxiety-like behavior.

Cerebral Blood Flow and Neurovascular Coupling

Cerebral Blood Flow

Although the brain only contributes to 2% of overall body weight, it receives 15% of the body's cardiac output and uses approximately 20% of the body's total oxygen supply (Kandel et al., 2013). The 750-1000 mL of blood the brain receives every minute function to deliver oxygen, glucose and other nutrients to active cells, while simultaneously removing carbon dioxide, lactic acid and other metabolic waste products (Kandel et al., 2013). Blood vessels and arterioles in the brain are highly specialized to detect local changes in oxygen and carbon dioxide concentrations, and dilate or constrict to increase or decrease blood flow respectively. Oxygen, glucose and carbon dioxide concentrations in the blood must be strictly regulated to maintain a proper pH within brain cells, and oxygen is necessary for cellular mitochondria to produce ATP for energy (Koehler, Roman & Harder, 2009). Changes in the concentration of any of these substances can impair the health and functionality of cells and lead to a dangerous cardiovascular accident or stroke. Anoxia, or a lack of oxygen, is particularly detrimental as it can cause irreversible damage within minutes (Kandel et al., 2013). Accordingly, the brain has developed very specialized mechanisms to regulate and maintain precise blood flow and nutrient delivery.

The mechanisms of normal neurovascular coupling

When a region of the brain becomes active, it needs an increased supply of oxygen and glucose to support the increase in local neuronal activity. The process by which the brain detects increases in local neuronal metabolic activity and responds by increasing blood flow to the active region is termed neurovascular coupling (NVC) or functional hyperemia (Filosa et al., 2006).

The most noteworthy players in NVC include active neurons, astrocytes, and myocytes (smooth muscle cells).² Astrocytes are supporting cells that exist to provide a variety of functions

² See figure 2

to support the activity of neurons. Astrocytic endfoot processes interact with the smooth muscle cells of cerebral blood vessels and are also in close contact with local neuronal synapses.

Basically, under normal circumstances, astrocytes are able to detect the degree of local activity through interaction with local synapses, and signal to the smooth muscle cells of cerebral blood vessels to dilate in response to increased activity (Longden et al., 2011). Vasodilation allows more blood flow and consequently more oxygen and glucose delivery to the area to facilitate energy usage in the active cells. Changes in intracellular calcium and subsequently changes in extracellular potassium are mechanisms by which increased neuronal activity can be coupled to vascular activity.

More specifically, when a neuron is stimulated, it releases glutamate, which activates metabotropic glutamate receptors (mGluR) on astrocyte endfoot processes. Activation of mGluRs initiates a cascade of events in which various signaling molecules within the astrocyte cause an increase of local calcium within the astrocytic endfoot (Koehler et al., 2009). When the concentration of calcium within the endfoot process increases, large-conductance voltage - dependent potassium channels (BK channels) on the endfoot open and potassium is released into the extracellular space between the astrocyte and the smooth muscle cells. This local increase in potassium activates inward rectifier potassium channels (K_{ir}) on the smooth muscle cell, and causes a potassium efflux from the myocyte. As potassium levels within the myocyte decrease, the cell becomes hyperpolarized, and voltage- dependent calcium channels on the myocyte decrease activity, and vasodilation ensues (Filosa et al., 2006; Longden et al., 2011; Girourard et al., 2009; Koehler et al., 2009). ³

³ see figure 3

L-type calcium channels and smooth muscle cells

In this project, we attempted to bypass the entire neuron- astrocytic circuit described above and manipulate blood flow via smooth muscle cells only. We wanted to see if inducing an increase in blood flow in the BNST could affect behavior, and we wanted to avoid any possible non-vascular effects that may contribute to behavioral modifications. A brief explanation of isolated smooth muscle cell anatomy and physiology is essential to understand the premise of the present study.

Various types of calcium channels exist in copious amounts throughout different body tissues. Pertinent to this study is a type of voltage-gated calcium channel called an L-type calcium channel. It is our hypothesis that, L-type calcium channels exist most abundantly on vascular smooth muscle cells in the brain, and not on astrocytes or neurons in the BNST. On vascular smooth muscle cells, L-type calcium channels are important for cytosolic control of calcium and muscle contractility. When the myocyte becomes depolarized, L-type calcium channels are activated, and there is a flux of cytosolic calcium from the extracellular space into the smooth muscle cell. This influx causes contraction of the myocyte, and subsequent vasoconstriction. L-type calcium channel action can be decreased by potassium channels on the myocyte, which upon potassium efflux, become hyperpolarized and initiate vasodilation (Berridge, 2008).

Neurovascular coupling and stress

In some pathologies, including chronic stress, the normal mechanisms of NVC seem to be impaired. Thomas Longden and the Hammack Laboratory at the University of Vermont investigated whether or not NVC could be altered by chronic stress. They found that in

chronically stressed animals, NVC in the amygdala was in fact compromised. In particular, Longden found smaller peak dilation, and a shorter duration of dilation in the blood vessels of stressed animals as compared to the vessels of non-stressed animals (Longden, 2011). He also found that the spread of calcium signaling to smooth muscle cells in the amygdala was compromised in chronically stressed animals as compared to non-stressed controls (Longden, 2011). This led to the hypothesis that chronic stress can impair NVC in the amygdala. This led to the question of whether or not this theory also works in the BNST, and if it works in the opposite direction, i.e. whether or not manipulation of cerebral blood flow can cause a change in neuronal activity, and consequently elicit a particular behavioral response.

The goal of this study was to examine one potential connection between neurological activity in the BNST and the expression of anxiety. We used a pharmacological agent to manipulate blood flow in the BNST and considered whether or not it had an effect on neuronal activity in the region. We then examined whether or not it had the potential to mimic the effects of chronic stress and produce an observable anxiogenic behavioral response. If results are significant, we expect to have insight into a potential cellular mechanism that underlies the modulation of behavioral anxiety. To the best of our knowledge, this is the only study that has been conducted that investigated this specific relationship.

EXPERIMENTAL STUDY

Hypothesis

As stated above, the central question in this project is whether or not manipulation of blood flow in the BNST can heighten neuronal activity and consequently facilitate anxiety-like behavior. In this study, we assumed that upon stimulation such as stress, neurons in the BNST

become active and the normal mechanisms of NVC ensue. We aimed to manipulate the normal mechanism of NVC in the BNST using the L-type calcium channel antagonist *diltiazem*. We hypothesized that blocking L-type calcium channels on the smooth muscle cells of blood vessels in the BNST would lead to vasodilation and a subsequent increase in blood flow in the region (Berridge, 2008; Lipscome, Helton & Xu, 2004). We then investigated whether or not inducing an increase in blood flow in the BNST could mimic the effects of chronic stress and create an angiogenic phenotype in our test subjects. Because L-type calcium channels are most abundant on smooth muscle cells, we anticipate that *diltiazem* will not have significant non-vascular effects, and that results of the study will be mostly due to the isolated manipulation of blood flow. We used an elevated plus maze as a measure of behavioral anxiety. We hypothesized that *diltiazem*-treated rats would spend less time on the open arms of the EPM compared to vehicle-treated rats and that this would be indicative of increased anxiety-like behavior.

Diltiazem

The molecular formula for *diltiazem* is $C_{22}H_{26}N_2O_4S$, and it is a well-known L-type calcium channel antagonist that works to specifically block voltage-gated calcium channels on muscle cells (Lipscome et al., 2004). Because of its antagonistic properties at the level of the myocyte, it is often used to treat hypertension and other cardiovascular disorders (Rogalsky & Todorov, 2009). In this project, we infused *diltiazem* directly into the BNST with the hypothesis that it would cause vasodilation and increase blood flow to the area.

Methods and Materials

Subjects

In the present study, adult male Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). The animals were housed individually, maintained on a 12-hour light/dark cycle and allowed food and water *ad libitum*. All procedures were conducted in concordance with the Office of Animal Care and Management and the Institutional Animal Care and Use Committee at the University of Vermont. Steps were taken to minimize pain and suffering to all animals. Rats were chosen as test subjects because of the known similarities between the neurocircuitry of humans and rats, and the observed similarities in their anxiety response to stress. It is predicted that many of the principles involved in rat neurocircuitry may be similar to those of humans. It is possible that results may generate information that could potentially be generalized to benefit humans in the future.

Surgical Procedure

For intra-BNST injections, rats were anesthetized with a mixture of oxygen (1%) and isoflurane vapor (2.0-5.0%) and secured in a stereotaxic device with blunt ear bars. Reflexes were checked to ensure complete anesthetization and monitored along with respiration rate throughout the duration of the procedure. After the head was shaved, a rounded scalpel was used to make an initial incision to expose the skull and bregma. The skull was cleaned, and four holes were gently drilled in the corners of the exposed area using a round point drill bit. Screws were then inserted into the holes using a hemostat to form the basis of the skull -cap. Arms were added to the stereotaxic device and a stainless steel cannula was placed into the holder at the end of the arm. A cannula was lowered onto bregma and the following BNST coordinates were taken: anterior-posterior = (-2.8 mm), medial-lateral = (+5.0 mm), and dorsal-ventral = (-8.0mm). Small holes were made in the skull at these locations and cannulae were inserted bilaterally into the

BNST. A dental cement solution was mixed and applied to the skull covering the bottom of the screws and cannulae to form a skull- cap. Another layer of cement was applied and left to dry. The rat was removed from the stereotaxic device and returned to its home cage for a week-long post- operative recovery period.

Infusions

Rats were infused using a piece of plastic tubing and a 1.0 microliter infusion tube. The infusion tube was attached to the plastic tubing and a small internal cannula was placed at the end. Rats were constrained with cannulae exposed, and the internal cannula was gently inserted into each cannula. Control rats were infused with 0.2 microliters of artificial cerebrospinal fluid (aCSF) solution and experimental rats were infused with 0.2 microliters of 50 micromolar *diltiazem*. The internal was allowed to remain inside the BNST cannula for one minute following infusion. After each infusion, rats were placed in the testing room for a five- minute acclimation period.

EPM testing and analysis

An elevated plus maze (EPM) is an apparatus used to measure anxiety. It consists of a roof-less plus-shaped structure with two open and two closed arms. The entire apparatus is elevated off the ground. The model is based on a rat's preference to dark, closed spaces, and its aversion to open, elevated spaces. In theory, a more anxious rat will spend more time on the closed arm where it is more comfortable, and less time on the open arm where it is exposed to more light and in danger of falling off the maze.

The room containing the maze was dimly lit by a red light and testing was conducted with minimal noise. Rats cannot see the red light, thus testing essentially occurred in the dark. One by one, animals from each group were placed in a closed arm of the maze and allowed to roam freely for five minutes while a video recording was taken. Measures of total time spent on open versus closed arms were taken for each subject and evaluated. Times were recorded using EthoVision XT computer software. Average time for open arm and closed arm exploration were taken for each group, and the groups were compared using an unpaired t-test.

Perfusions and histology

After testing, each animal was anesthetized with pentobarbital sodium and transcardially perfused with 9% saline solution followed by 4% paraformaldehyde solution. The brains were removed, post-fixed in paraformaldehyde, and refrigerated until sliced. Each brain was sliced using a cryostat and mounted on slides for cannula verification.

Results

Unfortunately histology could not be obtained for this report. However, if available, anatomic position of the BNST in our tissue slices would have been determined using a rat brain atlas. Location of right and left cannula placements would have been determined for each brain. If the cannulae were indeed placed in the BNST, it could be concluded that our drug reached the desired region of interest. Data of these animals' EPM times would be included in the analysis. If cannulae were judged to be in any area other than the BNST, data from these animals' EPM times would not be included in analysis.

Open and closed arm times were measured for both control (n= 16) and *diltiazem* (n = 16) treated groups. Unpaired t-tests with Welch's correction were used to compare average time spent on the open arms and average time spent on the closed arms between groups. The average total time spent on the open arms was not significantly greater in control rats (n=15) as compared to *diltiazem*-treated rats (n=14) (two-sample unpaired t-test, $t=0.6953$, $p < 0.05$). The average total time spent on the closed arms was also not significantly greater between the *diltiazem*-treated rats (n=16) compared to control rats (n=15) (two-sample unpaired t-test, $t= 0.05263$, $p<0.05$).⁴

Discussion

Pharmacological intervention did not significantly produce anxiety-like behavior in test subjects. Results could be interpreted to support or refute our hypothesis. *Diltiazem*-treated rats did spend less time on the open arms of the EPM compared to vehicle-treated rats, which may demonstrate an increase in anxiety-like behavior. On the other hand, it is also possible that vasodilation in the BNST does not increase anxiety-like behavior at all. Perhaps NVC works exclusively such that cerebral blood flow is only altered in response to neuronal activity, but that manipulation of cerebral blood flow cannot influence neuronal activity. Thus, even if *diltiazem* did induce vasodilation in the BNST, it would not cause activation of neurons in the region. Accordingly, anxiety-like behavior would not be observed. As a result, it is reasonable to believe that a direct link between cerebral blood flow and behavior does not exist at all.

Future Directions

⁴ see figure 4

Because no studies have yet investigated the relationship between blood flow and behavior, it is impossible to suggest whether or not these results support existing theories. Nevertheless, because results of this pilot study do show a trend, it lays a foundation for future studies in this field. Future research that addresses this hypothesis should utilize larger groups of animals to increase statistical power. Additionally, if results of a future study using a different type of L-type calcium-channel antagonist were to show a similar trend, it would serve to corroborate the existing data. Perhaps a future research design could incorporate the use of an L-type calcium channel agonist as well. In respect to the current hypothesis, infusions of an L-type channel agonist into the BNST should produce results opposite from those seen here: an anxiolytic phenotype in animals. It may also be beneficial to utilize various different types of anxiety measurements in addition to an elevated plus maze. Measures of anxiety could be taken using a social exploration test, for example.

This study was designed to simply examine whether or not manipulation of blood flow in the BNST could mimic the effects of chronic stress and produce an anxiogenic phenotype. Expanding the study to include a group of animals that have undergone a chronic variate stress treatment may produce interesting results. Because stress has been shown to impair the normal calcium signaling and vessel dilation in neurovascular coupling mechanisms, (Longden, 2011) it is possible that *diltiazem* could remedy these dysfunctional processes by improving impaired vessel dilation. If this were true, and impaired NVC as a result of chronic stress is a cause of anxiety, *diltiazem* treatment could potentially alleviate the effects of chronic stress on anxiety-like behavior. On the other hand, it is possible that treating chronically stressed animals with *diltiazem* may serve to exacerbate behavioral anxiety. That is, if impairments in NVC are not caused by chronic stress, but an animal is exhibiting an anxiogenic phenotype, vessel dilation in

the BNST could intensify existing anxiety. Results of Longden's work suggest the former, whereas results of this study suggest the latter. Thus, a study combining the premise of each experiment may provide a more comprehensive explanation.

Limitations

Although results of this study did demonstrate a trend to support our hypothesis, no conclusions can be drawn from the data. The absence of the cannulae verifications makes the data unreliable. Many tissue slices were damaged and clear verification could not be obtained, and all others were inaccessible. For concrete support for the hypothesis, histological verification that cannulae were indeed placed in the BNST is a critical piece of data missing from this study. This data is necessary to determine whether or not the experimental drug reached the region of interest, and consequently if observed effects were a result of BNST manipulation.

It is possible that there were also uncontrollable extraneous factors that contributed to variability in the results. For example, noise during the time of testing could have influenced anxiety in the test subjects. It is possible that the stress of surgery and drug infusions contributed to an overall increase in activity in test subjects as well. Furthermore, variability in the effectiveness of drug delivery during infusions could have impacted results. Finally, it is possible that *diltiazem* did have effects on non-vascular responses. Tests to verify the exclusivity of *diltiazem*'s actions on myocytes should be considered.

The purpose of this study was to examine the relationship between cerebral blood flow and behavior, but it would have been valuable to make use of techniques that could examine the calcium spread in blood vessels, and the activity of neurons in the BNST both before and after drug infusions. These strategies may include whole cell patch clamping of neurons in the BNST,

vessel dilation imaging, or fluorescent tissue staining. For example, if whole cell patch clamping revealed that *diltiazem* did not in fact have vascular effects, and imaging techniques demonstrated increase vessel dilation in response to *diltiazem* treatment, results would be more reliable.

Results of this study support existing literature that claims that the BNST is a site crucial for the modulation of behavioral anxiety. Additionally, it suggests that pharmacological interventions designed to decrease cerebral blood flow in the BNST may be able to decrease anxiety-like behavior. If future research supports the results in this study, it would be the first time cerebral blood flow was linked directly to behavior. This notion potentially applies to numerous other types of neurological disorders as well, and the claims made here are likely worth further investigation.

Conclusions

Disorders of the nervous system are complicated and fascinating. Ranging from instances of extraordinary autobiographical memory, to devastating and puzzling conditions such as Alzheimer's, Disease the assortment of neurological abnormalities is tremendous. Anxiety disorders characterize only a small subset of psychological and neurological diseases, yet to those that suffer from chronic anxiety, it can be a wholly exhaustive and disruptive part of daily life. Panic disorders, post-traumatic stress disorder, social phobias, and obsessive-compulsive disorder are just a few examples of anxiety pathologies that can devastate lives. An anonymous source from the National Institution of Mental Health (2009) reports of panic disorder, "In between attacks, there is this dread and anxiety that it's going to happen again. I'm afraid to go back to places where I've had an attack. Unless I get help, there soon won't be anyplace where I

can go and feel safe from panic”. As illustrated, this person’s anxiety got so bad that he or she developed an intense fear of going anywhere at all. Another person’s report of General Anxiety Disorder exemplifies how significantly anxiety can disrupt normal function: “When my problems were at their worst, I’d miss work and just feel terrible about it. Then I worried that I’d lose my job. My life was miserable until I got treatment” (National Institute of Mental Health, 2009). Intense anxiety can impair a person’s ability to leave the house, go to work, interact with other people, and lead a normal life. Often these things cause even more anxiety, and the disease spirals out of control. It is crucial that those people who suffer from symptoms like those described above get help, and almost always, victims of anxiety long for a cure.

Treatments for anxiety often include both pharmacological and therapeutic intervention styles. Correcting chemical imbalances, or dysfunctional neurobiological mechanisms can have a great impact on the effectiveness of behavioral therapy. It is the hope that advancements in neurobiology, pharmacology and psychology can lead to a deeper understanding of the pathology of anxiety in general. Hopefully, new, more effective and collaborative techniques may be developed to erase anxiety as a disorder, and ease the suffering of thousands.

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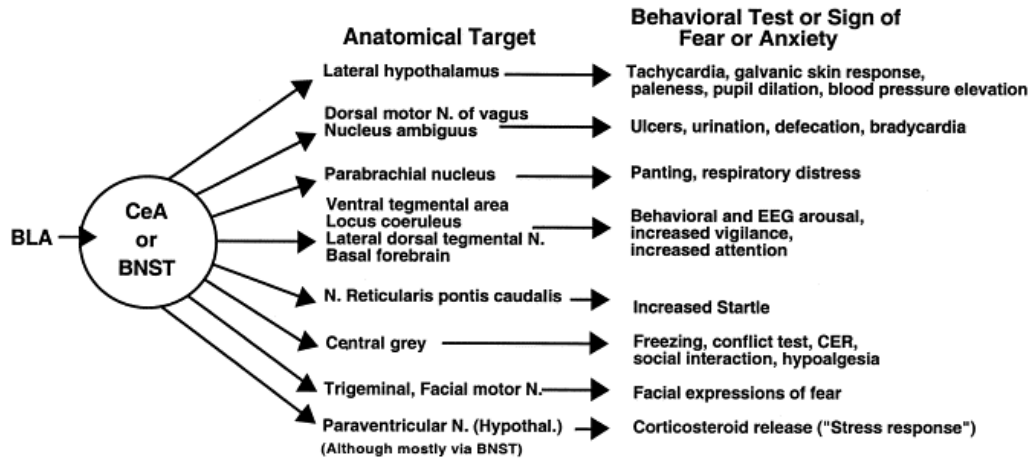
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Appendix

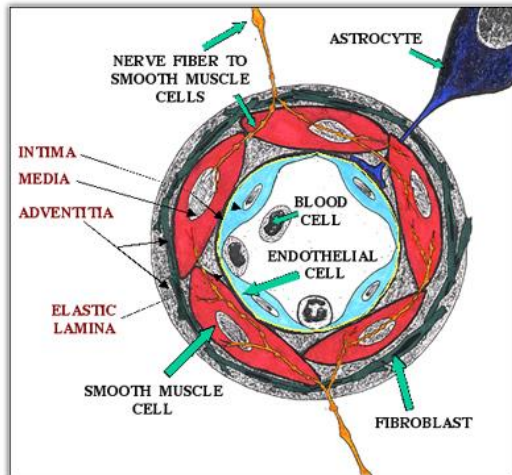
Figures

1.



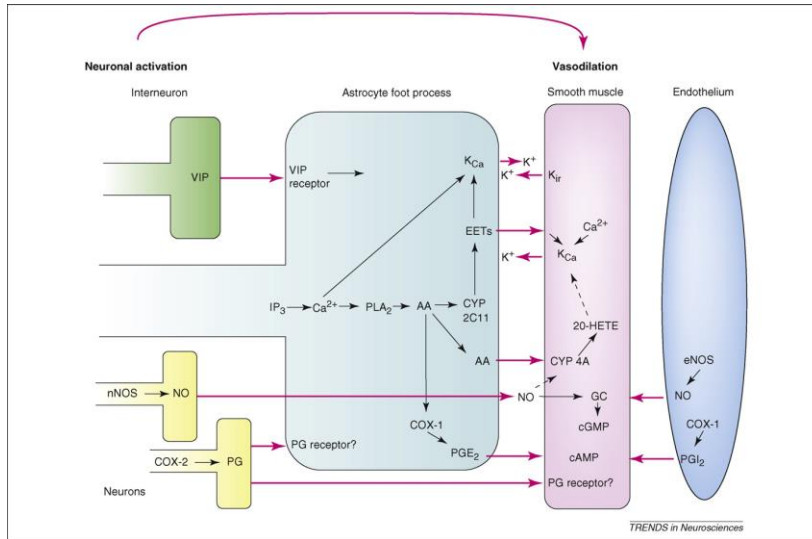
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2.



(figure taken from: <http://www.brain-aneurysm.com/about.html>)

3.



(figure taken from:

Koehler, R.C., Roman, R.J., Harder, D.R. (2009). Astrocytes and the regulation of cerebral blood flow.

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