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Metergoline, the basolateral amygdala and the ventral pallidum: Implications for panic disorder

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METERGOLINE, THE BASOLATERAL AMYGDALA AND THE VENTRAL PALLIDUM: IMPLICATIONS FOR PANIC DISORDER

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Panic disorder (PD) is a common mental illness characterized by recurring spontaneous panic attacks. Scientific investigation into PD has been accelerated by the development of rat models of PD. These models can be validated by responses to intravenous sodium lactate (NaLac), including tachycardia, that are similar to PD patient responses. Previous work on established PD models has suggested that antagonism of serotonin (5-HT) receptors in the basolateral amygdala (BLA) may be sufficient to model PD. To test this hypothesis metergoline (MET), a 5-HT receptor antagonist, or vehicle was microinjected into the BLA of anesthetized rats. Following the microinjection, NaLac or d-mannitol was infused intravenously. Heart and respiratory rates were recorded and analyzed for evidence of panic-like reactions.

Previous work suggests that 5-HT signaling in the BLA may influence the activity of neurons in the ventral pallidum (VP). Additionally, the VP may play a role in restraining anxiety-related behaviors. Concurrent with the previously described experiment, extra-cellular single unit recordings were made from the VP. The data were analyzed for changes in firing rate frequency as a result of the treatments.

The results indicated that only the rats treated with MET and NaLac exhibited tachycardia, confirming the utility of these treatments for modeling panic. Hypoventilation was observed in all d-mannitol treated rats, and hyperventilation was observed in all NaLac treated rats. This suggests respiratory rate is not a sufficient indicator of panic. Decreases in VP activity were observed in the rats treated with the vehicle and d-mannitol, and no other significant changes were observed. This demonstrates that the MET microinjection disinhibits the VP. Additionally, NaLac disinhibits the VP. This suggests that the VP plays a role in the chemosensory response to NaLac.

METERGOLINE, THE BASOLATERAL AMYGDALA AND THE VENTRAL PALLIDUM: IMPLICATIONS FOR PANIC DISORDER

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CHAPTER I

THE PROBLEM AND ITS BACKGROUND

Introduction to the Problem

Panic disorder (PD) is a disease that affects nearly 5% of the population (Kessler et al., 2006). PD involves "[r]ecurrent unexpected panic attacks" (American Psychiatric Association, 2013, p. 208). Alterations in serotonin (5-hydroxytryptamine, 5-HT) signaling have been implicated in PD (Maron & Shlik, 2006). The unexpected nature of the panic attacks complicates the experimental study of PD (Cowley & Arana, 1990). Progress has been made investigating PD patients by using various panicogenic treatments to elicit panic attacks (Charney, Heninger, & Breier, 1984; Charney, Heninger, & Jatlow, 1985; Schruers, Van De Mortel, Overbeek, & Griez, 2004).

I.V. infusion of sodium lactate (NaLac) is a commonly used panicogenic treatment (Cowley & Arana, 1990). PD patients report that NaLac-induced panic is similar to naturally occurring panic attacks (Goetz, Klein, & Gorman, 1996). The physiological response to NaLac-induced panic includes tachycardia and hyperventilation (Cowley & Arana, 1990; Gorman et al., 1988).

Another strategy to overcome the limitations in studying PD is to generate animal models of the disorder. The NaLac-induced physiological response can be used to verify

these models (Sajdyk & Shekhar, 2000; Shehkar, Sajdyk, Keim, Yoder, & Sanders, 1999). The use of PD models allows for invasive study of the underlying neurobiology.

A role for the basolateral amygdala (BLA) in PD is well established. Pharmacological manipulations that chronically disinhibit the BLA successfully model PD as evidenced by NaLac-induced tachycardia (Sajdyk & Shekhar, 2000; Sanders, Morzorati, & Shekhar, 1995). The BLA is synaptically arranged such that 5-HT exerts an overall inhibitory influence on the projection neurons, and it has been suggested that release of 5-HT in the BLA may restrain anxiety-related behaviors (Hale et al., 2010; Rainnie, 1999). This suggests that antagonism of 5-HT receptors (5-HTRs) in the BLA should lead to a failure to restrain anxiety-related behaviors; hence, it should result in susceptibility to NaLac-induced panic.

The ventral pallidum (VP) is the output structure for the limbic loop, a network of neural structures theorized to regulate responses to positive affective states (Groenewegen, 2003; Maurice, Deniau, Menetrey, Glowinski, & Thierry, 1997). A role for the VP in processing panic hasn't been investigated; however, a few studies suggest the VP may function to restrain anxiety-related behaviors (Cromwell & Berridge, 1993; Duncan, Knapp, & Breese, 1996; Nikolaus, Huston, & Hasenöhrl, 2000). This suggests the VP may also have a role in restraining the response to panicogenic treatments.

Additionally, the VP, and the entire limbic loop, is functionally and anatomically interconnected with the BLA (Maslowski-Cobuzzi & Napier, 1994; McDonald, 1991; Mogenson & Yim, 1983; Woolf & Butcher, 1982). Projection neurons in the BLA produce spontaneous action potentials and are overall inhibited by 5-HT (Rainnie, 1999). BLA neurons project to the VP and contain an excitatory neurotransmitter (McDonald,

1991; Woolf & Butcher, 1982). This suggests that antagonism of 5-HTRs in the BLA should disinhibit the projection neurons. The projection neurons, in turn, should excite neurons in the VP.

There are three general aims of this study. First, determine if 5-HTR antagonism in the BLA is sufficient to model PD as evidenced by a NaLac-induced response. Second, determine the influence of tonic 5-HT signaling in the BLA on the firing rates of spontaneously active VP neurons. Third, determine if the VP has a role in the response to panicogenic treatments.

Hypotheses

Pharmacological manipulations that disinhibit the BLA successfully model PD as evidenced by NaLac-induced tachycardia (Sajdyk & Shekhar, 2000; Sanders, Morzorati, & Shekhar, 1995). The BLA is synaptically arranged such that 5-HT exerts an overall inhibitory influence on the projection neurons, and it has been suggested that release of 5-HT in the BLA may restrain anxiety-related behaviors (Hale et al., 2010; Rainnie, 1999). Therefore, antagonism of the 5-HTRs in the BLA should disinhibit it and prevent the ability of 5-HT release to restrain anxiety-related behaviors. Hence, the first hypothesis is that microinjection of metergoline (MET), a 5-HTR antagonist, into the BLA followed by i.v. NaLac will result in tachycardia. Support for this hypothesis would indicate successful development of a PD model.

NaLac-induced hyperventilation is observed in PD patients, but respiratory activity is not usually assessed in rat models of PD (Gorman et al., 1988). Therefore, if respiratory activity is assessed in a model of PD it should mirror the changes in

cardiovascular activity, similar to PD patients. Hence, hyperventilation is also an indicator of a panic-like reaction and the second hypothesis is that the combination of MET and NaLac will result in hyperventilation. Support for this hypothesis would also indicate the successful development of a PD model.

Projection neurons in the BLA produce spontaneous action potentials and are overall inhibited by 5-HT (Rainnie, 1999). BLA neurons project to the VP and contain an excitatory neurotransmitter (McDonald, 1991; Woolf & Butcher, 1982). Therefore, antagonism of 5-HTRs in the BLA should disinhibit the projection neurons causing increased excitation of the VP neurons. Hence, the third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons. Support for this hypothesis would indicate that tonic 5-HT in the BLA exerts an inhibitory influence on the spontaneous firing rate of VP neurons.

A role for the BLA in the response to anxiogenic and panicogenic agents, including NaLac, is well established (Hale et al., 2010; Hodges, Green, & Glenn, 1987; Sajdyk & Shekhar, 2000). The BLA and the limbic loop, including the VP, are anatomically and functionally interconnected (Maslowski-Cobuzzi & Napier, 1994; McDonald, 1991; Mogenson & Yim, 1983; Woolf & Butcher, 1982). A role for the VP in restraining anxiety-related behaviors is emerging (Cromwell & Berridge, 1993; Duncan, Knapp, & Breese, 1996; Nikolaus, Huston, & Hasenöhrl, 2000). The suggested role of the VP and its connectivity with the NaLac-responsive BLA suggests the VP will exhibit a response to NaLac also. Therefore, the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. Support for this hypothesis would establish a role for the VP in the response to panicogenic treatments.

Procedures

Rats were anesthetized with urethane. A wound clip was attached to the skin over the heart to serve as an EKG electrode. Stereotaxic surgery was performed; a guide cannula was lowered into the BLA and a glass electrode, filled with pontamine sky blue dye in 2M saline (PSB), was lowered into the VP. An i.v. catheter was inserted into one femoral vein.

The signals from the electrodes were amplified, filtered and displayed on oscilloscopes. Window discriminators were used to isolate the desired signals and transmit them to a computer-interface device. An additional data channel was used to record the manual push of a button. Pushing the button at each observed respiration allowed for the recording of the respiratory data by the same computer program.

To isolate a single neuron, the electrode was advanced ventrally with a micromanipulator until action potentials were observed with at least a 3:1 signal to noise ratio. If all the action potentials exhibited a consistent amplitude and shape, and the firing pattern remained consistent, then the signal was presumed to be from a single unit. The window discriminator was set to allow a single peak from each action potential waveform to fall within the trigger window.

An injection cannula was backfilled with MET or the vehicle solution (VEH), and it was inserted into the BLA through the guide cannula after obtaining at least 5min of baseline activity from a single neuron. Respiratory activity was monitored for 1min. 0.2µl of MET or VEH were microinjected into the BLA over the course of 30s. Seven minutes after the microinjection, respiratory activity was monitored for 1min. Ten minutes after the microinjection, 10ml/kg of NaLac or d-mannitol (MAN) was infused through the i.v.

catheter over the course of 15min. Respiratory activity was then monitored for 1min every 5min after the i.v. infusion began and continued until 15min after the i.v. infusion ended.

To mark the injection site for histological verification, 15min after the i.v. infusion ended the injection cannula was removed from the guide cannula and backfilled with PSB. It was reinserted and 0.2µl PSB was microinjected into the BLA over 30s. To mark the recording site, a current was passed through the electrode for 20min to expel the PSB.

To obtain the brain, the electrode was slowly extracted followed by the guide cannula. The rat was sacrificed with an overdose of urethane through the i.v. catheter, and then it was transcardially perfused with 0.9% saline followed by 4% para-formaldehyde in 0.1M phosphate buffer. The brain was removed, stored overnight in the fixative solution and then placed in a 20% sucrose solution.

To verify the electrode and cannula placements, coronal sections of the brain were cut with a cryostat. The sections were washed with phosphate-buffered saline then mounted on microscope slides in dilute phosphate buffer. The slides were stained with neutral red, cover-slipped and examined with a light microscope to verify deposition of PSB in the targeted structures.

Data Analyses

The design of this experiment technically conforms to a three-way mixed ANOVA with microinjection and i.v. infusion as between subject factors and time as a within subjects factor. However, the hypothesized result is a three-way interaction, and

three-way interactions can be difficult to interpret in the context of this type of design. Therefore, initial analyses will focus on between-subject differences, and follow-up analyses will focus on within-subject differences.

The first hypothesis is that microinjection of MET into the BLA followed by i.v. NaLac will result in tachycardia. To determine if heart rate changes differed among the experimental groups, a difference score for each subject was calculated as the percent change of the mean heart rate during the last 5min of the i.v. injection compared to mean heart rate for 5min prior to the microinjection. The heart rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

The results of the analysis indicated that the MET/NaLac group had heart rate changes that were significantly different than the other rats; therefore, I tested if the MET/NaLac combination produced heart rates significantly greater than baseline. The EKG data from 5min prior to the microinjection through the end of the i.v. infusion were grouped into 60s bins. The binned heart rate data from the MET/NaLac group were subjected to a repeated measure ANOVA with *post hoc* Dunnett corrected pairwise comparisons.

The second hypothesis is that microinjection of MET into the BLA followed by i.v. NaLac will result in hyperventilation. To determine if respiratory rate changes differed among the experimental groups, a respiratory rate difference score for each subject was calculated as the percent change of the mean respiratory rate during the last 5min of the i.v. injection compared to mean respiratory rate for 5min prior to the microinjection. The respiratory rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

The results indicated that the groups treated with NaLac had respiratory rate changes significantly different than the groups treated with MAN regardless of the microinjection they received; therefore, I tested if the i.v. infusions caused respiratory rates significantly different from baseline. A 30s bin was generated from the respiratory data collected at baseline and during the i.v. infusion. The data were subjected to a two-way mixed ANOVA with i.v. infusion as the between subjects factor and time as the within subjects factor. *Post hoc* Bonferroni corrected pairwise comparisons were used to examine the effect of time within each group. *Post hoc* Sidak corrected pairwise comparisons were also used to examine the effect of group at each time point.

The third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons; the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. The factorial design of this experiment allows simultaneous testing of these two hypotheses. To determine if the firing rate changes differed between treatment groups, a firing rate difference score for each subject was calculated as the percent change of the mean firing rate during the last 5min of the i.v. injection compared to the mean firing rate for 5min prior to the microinjection. The firing rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

The VEH/MAN group had firing rate changes that were significantly different than the VEH/NaLac group; therefore, I tested if these groups exhibited significant changes in firing rates at the end of the i.v. infusion compared to baseline. The firing rate data from 5min prior to the microinjection through the end of the i.v. infusion were grouped into 60s bins. The binned data were subjected to a two-way mixed ANOVA with time as a within subjects factor and group as a between subjects factor. *Post hoc*

Bonferroni corrected pairwise comparisons were used to examine the effect of time within each group. *Post hoc* Sidak corrected pairwise comparisons were also used to examine the effect of group at each time point.

To determine if there were any differences in the distributions of types of response between the groups, the difference scores for each neuron were classified as exhibiting an increased firing rate, decreased firing rate, or no change in firing rate. An increased firing rate was defined as a firing rate difference score greater than the mean plus one standard deviation, a decreased firing rate was defined as a firing rate difference score less than the mean minus one standard deviation and the remaining difference scores were classified as exhibiting no change. The total numbers of neurons exhibiting decreased firing rate, no change, or increased firing rate were tabulated for each group. The distributions of responses for the groups were compared using a Pearson χ^2 test.

CHAPTER II

REVIEW OF RELATED LITERATURE

Panic Attacks and Panic Disorder

The lifetime prevalence for panic disorder (PD) with or without agoraphobia is estimated to be as high as 4.8%, and the lifetime prevalence for experiencing a panic attack, without PD, is estimated to be 23.5% (Kessler et al., 2006). Currently, panic attack is defined by the DSM-5 (American Psychiatric Association, 2013, p. 208) as:

> [A]n abrupt surge of intense fear or intense discomfort that reaches a peak within minutes, and during which time four (or more) of the following symptoms occur: **Note:** The abrupt surge can occur from a calm sate or an anxious state.
>
> (1) Palpitations, pounding heart, or accelerated heart rate.
> (2) Sweating.
> (3) Trembling or shaking.
> (4) Sensations of shortness of breath or smothering.
> (5) Felings of choking.
> (6) Chest pain or discomfort.
> (7) Nausea or abdominal distress.
> (8) Feeling dizzy, unsteady, lightheaded, or faint.
> (9) Chills or heat sensations.
> (10) Paresthesias (numbness or tingling sensations).
> (11) Derealization (feelings of unreality) or depersonalization (being detached from one-self).
> (12) Fear of losing control or "going crazy."
> (13) Fear of dying.

PD is defined as "[r]ecurrent unexpected panic attacks" (American Psychiatric Association, 2013, p. 208). The panic attacks must lead to significant distress or dysfunction, must not be caused by a drug or medical condition, and must not be better

explained by another diagnosis (American Psychiatric Association, 2013). Evidence for two subtypes of PD has been reported recently (Briggs, Stretch, & Brandon, 1993; Roberson-Nay & Kendler, 2011). The two subtypes can be distinguished by the presence of respiratory symptoms during panic attacks, items 4-6, 13 and, to a lesser extent, item 10 of the diagnostic criteria.

Recommended primary treatments for PD include cognitive behavioral therapy (CBT), selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors, tricyclic antidepressants (TCAs) and benzodiazepines (BDZs) or combinations thereof. No single treatment method has demonstrated superior outcomes, but the reuptake inhibitors are preferred due to their ease of use and safety as compared to CBT and other drug therapies, respectively. Combinations of these treatments are recommended when patients fail to respond adequately to a single treatment (American Psychiatric Association, 2009). Patients with the respiratory subtype of PD experienced better outcomes with eight weeks of imipramine, a TCA, while those with the non-respiratory subtype responded better to alprazolam, a BDZ (Briggs et al., 1993).

The high lifetime prevalence for experiencing a panic attack and their essential nature to PD highlight the importance of understanding the etiology of panic attacks; however, the spontaneity of panic attacks makes experimental study difficult (Cowley & Arana, 1990). Intravenous infusion of a sodium lactate solution (NaLac) has been shown to be a useful method for eliciting panic attacks with greater frequency in PD patients compared to control subjects (Liebowitz et al., 1984). A number of other pharmacological challenge tests differentiate PD patient groups from control groups based on susceptibility to their panicogenic or anxiogenic properties. These treatments

include, among others, inhalation of carbon dioxide (CO_2) enriched air and systemic administration of caffeine or yohimbine (Charney, Heninger, & Breier, 1984; Charney, Heninger, & Jatlow, 1985; Schruers, Van De Mortel, Overbeek, & Griez, 2004). PD patients with the respiratory subtype are more likely than those with the non-respiratory subtype to panic following CO_2 inhalation, hyperventilation, breath-holding or caffeine challenge tests (Freire, Perna, & Nardi, 2010).

NaLac-induced panic in humans is assessed by a variety of subjective methods, which has likely contributed to the variability in reports of the intensity of the evoked response and the degree to which it is specific to PD. The subjective experience caused by NaLac in PD patients is reported as being very similar to spontaneous panic attacks (Goetz, Klein, & Gorman, 1996). Objective assessment is a possibility as NaLac causes a number of physiological changes, including tachycardia, in all subjects; subjects who panic due to NaLac exhibit greater increases in heart rate than those who don't (Cowley & Arana, 1990). There is also evidence for hyperventilation in PD patients but not controls due to NaLac (Gorman et al., 1988).

Similar to humans, rat models of PD exhibit tachycardia in response to NaLac; interestingly, unlike humans, control rats do not also show increased heart rates (Shehkar, Sajdyk, Keim, Yoder, & Sanders, 1999). Behavioral evidence for a panic-like state in the rat includes decreased social interaction time, increased ambulatory and non-ambulatory motor behaviors, as well as conditioned avoidance (Hale et al., 2010; Sajdyk & Shekhar, 2000; Shekhar & Keim, 1997). Together, these findings support the appropriateness of using rat models of PD in order to understand the neurobiology of panic attacks.

One criticism of the NaLac-induced panic literature is a lack of appropriate control conditions; thus, it remains to be determined if the panic is induced by the infusion of a high-osmolarity solution, the presence of lactate in the infusate, the presence of sodium in the infusate or resulting changes in other blood-chemistry parameters (Cowley & Arana, 1990). Studies using hypertonic solutions of various sugars as controls have demonstrated that infusions of high-osmolarity solutions are not sufficient for eliciting panic attacks in either humans or rats (Molosh et al., 2010; Pitts & McClure, 1967).

Studies using hypertonic sodium chloride (NaCl) solutions as a control suggest that this may be sufficient to induce panic in both humans and rats (Molosh et al., 2010; Peskind et al., 1998). No respiratory measures were collected with hypertonic NaCl infusion in rats. Compared to NaLac infusion in humans, however, hypertonic NaCl infusion does not produce the same changes in blood parameters indicative of hyperventilation. Furthermore, some of the patients reported experiencing different intensities of dyspnea in response to the two infusions. This has important implications when considering the recent evidence for a respiratory subtype of PD, and it suggests that while hypertonic NaCl infusion may be sufficient to induce symptoms reported by all PD patients, it may do so without inducing symptoms typical for PD patients exhibiting the respiratory subtype.

Neurobiology of Serotonin

5-Hydroxytryptamine (5-HT, serotonin) is a monoamine neurotransmitter biosynthesized in two steps from the dietary amino acid tryptophan (TRP). TRP is

converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. 5-HTP is converted to 5-HT by aromatic amino decarboxylase. In mammals, the majority of 5-HT is found in the digestive system; it can also be isolated from the blood as it is stored in platelets. In the body, 5-HT causes contraction of smooth muscles and constriction of blood vessels. Within the brain, 5-HT is synthesized in axon terminals, packaged into vesicles and released in an action potential dependent manner, mainly by neurons with cell bodies in the raphe nuclei. 5-HT is cleared from the extracellular fluid and deposited into the cytosol by the serotonin transporter (SERT). 5-HT in the cytosol is either repackaged into vesicles or degraded by monoamine oxidase to 5-hydroxyindole acetic acid (Nichols & Nichols, 2008). Packaging into vesicles occurs via vesicular monoamine transporter type 2 (Liu & Edwards, 1997).

The raphe nuclei form six distinct nuclei near the midline throughout the brainstem and comprise two groups. The rostral group has projections that ascend to the forebrain and includes the caudal linear nucleus, the dorsal raphe nucleus (DRN), and the median raphe nucleus. The caudal group has projections that descend to areas in the brainstem and to the spinal cord. The caudal group includes the raphe magnus, the raphe obscurus nucleus, and the raphe pallidus nucleus. There are also serotonergic neurons in the lateral reticular formation that are considered part of the caudal group (Hornung, 2003).

After being released, 5-HT diffuses through the extracellular fluid where it can bind to and activate 5-HT receptors (5-HTRs) present in cell membranes. Presently, there are fourteen known 5-HTRs. They are classified into seven families, 5-HT_1 through 5-HT_7. The 5-HT_3 receptor is a ligand-gated, non-selective cation channel; the thirteen

other 5-HTRs are all G-protein coupled receptors, which can be classified more broadly based on the preferentially coupled G-proteins. The 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} subtypes are $G_{q/11}$ coupled, the 5-HT_4, 5-HT_6 and 5-HT_7 subtypes are G_s coupled, and the 5-HT_{1A}, 5-$HT_{1B,}$ 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{5A} and 5-HT_{5B} subtypes are $G_{i/o}$ coupled (Nichols & Nichols, 2008). Humans do not express 5-HT_{5B} and rats do not express 5-HT_{1E} (Gloriam, Fredriksson, & Schiöth, 2007).

The effectiveness of SSRI treatments, which inhibit the SERTs from transporting 5-HT out of the synapse, for PD is just one example of the evidence implicating the serotonergic system in PD. In addition, SSRI treatment reduces the response to a variety of panicogenic challenges in PD patients. Reducing central 5-HT levels by diets free of TRP increases the response to CO_2 challenge, and augmenting 5-HT levels by 5-HTP supplementation decreases the response. Imaging studies have revealed reduced 5-HT_{1A} density in un-medicated but not remitted PD patients. Similarly, reduced SERT density has also been observed in several brain regions; remitted PD patients exhibit normal density in most but not all areas examined (Maron & Shlik, 2006).

Forebrain Neuronal Circuitry Relevant to Panic Attacks

The Ventral Pallidum

The limbic loop is one of several cortico-striato-pallido-thalamo-cortical loops in the brain and it is thought to be critical for generating appropriate behavioral responses to rewarding stimuli. The prefrontal cortex (PFC), nucleus accumbens (NAC), ventral pallidum (VP) and mediodorsal thalamus (MD) are the brain structures that form the

major components of the loop (see Figure 1. Groenewegen, 2003; Maurice, Deniau, Menetrey, Glowinski, & Thierry, 1997).

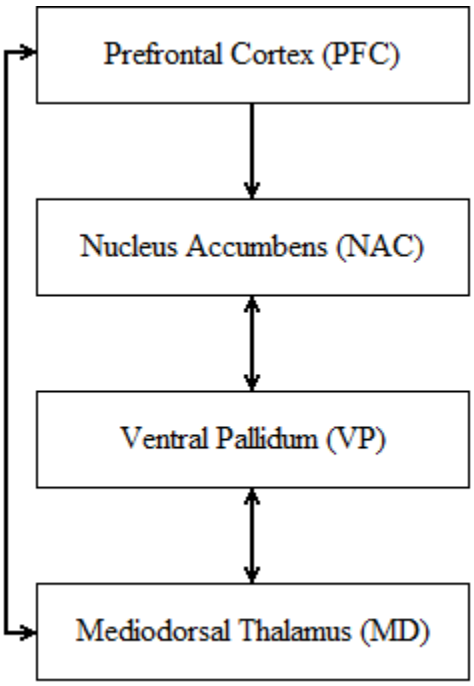

Figure 1. The Arrangement of the Major Structures in the Limbic Loop.
Arrows represent projections from one structure to another; not all known projections are illustrated.

The VP is a subcortical structure defined as the ventral extension of the globus pallidus that extends rostrally beneath the anterior commissure. It can be differentiated from the globus pallidus because the VP is densely innervated with substance P containing axons. Several distinct types of neurons have been identified in the VP. Large projection neurons containing acetylcholine (ACH) that are observed in the VP are considered to be part the nucleus basalis (NB), a group of ACH containing neurons found distributed throughout the basal forebrain. It is these neurons which project to the cortex and amygdaloid nuclei. Projection neurons containing GABA have also been identified;

some of these neurons are characterized as NB neurons based on shared target areas while those projecting to other areas are considered to originate from the VP proper. Recent work has indicated that GLU containing projection neurons are also present in the NB, and this has led to suspicions that this type of neuron also exists within the VP. In addition to the projection neurons, the VP also appears to contain GABAergic interneurons (Bengston & Osborne, 2000; Gritti et al., 2006; Haber et al., 1985; McDonald, Muller, & Mascagni, 2011; Pang, Tepper, & Záborszky, 1998).

As the output structure for the limbic loop, the VP should receive ventral striatal input, and it should provide output to the thalamus. The VP meets both these criteria. It has the necessary afferent, as well as efferent, connections with many striatal areas in the ventral portion of the brain including the NAC, ventromedial caudate-putamen, olfactory tubercle and fundus striatum. The VP also has the necessary efferent, as well as afferent, connections with the MD (Fuller, Russchen, & Price, 1987; Groenewegen, Berendse, & Haber, 1993; Haber, Groenewegen, Grove, & Nauta, 1985).

In addition to serving as a direct output pathway for the limbic loop, good evidence has accumulated suggesting that it also serves as the first structure of an indirect pathway. As a structure it can be subdivided into the lateral VP (VPl) and the medial VP (VPm). The VPm has a higher density of axons containing neurotensin. It is the predominant source of projections to the ventral tegmental area and MD and thus comprises the direct pathway. The VPl has a higher density of calbindin. It is the predominant source of projections to the subthalamic nucleus and substantia nigra reticulata. Based on organizational similarities to the motor loop, the VPl is thought to be

part of an indirect pathway (Groenewegen et al., 1993; Maurice et al., 1997; Zahm, 1989).

The VP also has afferent and efferent connections to the amygdala, the neurons of the horizontal diagonal band, PFC, parafascicular nucleus, lateral hypothalamus, parabrachial nucleus and periaqueductal gray. Additional efferent connections from the VP include the septum and entorhinal cortex. Amygdaloid efferents to the VP appear to come from all areas except the lateral nucleus. VP efferents to the amygdala target primarily the basolateral nucleus but also the lateral nucleus, basomedial nucleus, central nucleus and periamygdaloid cortex. The VP efferents to the BLA are comprised of the NB neurons located within the region, and the afferent connections from the BLA have been observed to form synapses with the NB neurons in the VP. These afferents also come in contact with other neuron types, but no clear synapses were observed (Fuller et al., 1987; Groenewegen et al., 1993; Vertes, 2004; Woolf & Butcher, 1982; Záborszky, Léránth, & Heimer, 1984).

Input to the VP from neuromodulatory brainstem areas include the serotonergic DRN and linear raphe nucleus, the dopaminergic ventral tegmental area, the histaminergic mammillary nuclei and the noradrenergic locus coeruleus. For all of these areas, excepting the linear raphe nucleus, reciprocal outputs from the VP have been demonstrated. The VP has been demonstrated to project to more brainstem areas than those that innervate it; these include the substantia nigra compacta, substantia nigra reticulata, retrorubral area, median raphe nucleus and raphe magnus (Fuller et al., 1987; Groenewegen et al., 1993; Panula, Pirvola, Auvinen, & Airaksinen, 1989).

A role of the VP in panic attacks has not been investigated in the literature; however, there are some data to suggest it may have such a role. Microinjections of substance P into the VP are anxiolytic in the elevated plus maze (Nikolaus, Huston, & Hasenöhrl, 2000). Lesions of the VP cause rats to exhibit an aversive reaction to sucrose (Cromwell & Berridge, 1993). Increased c-fos expression is observed in the VP after a shuttle box foot shock avoidance paradigm (Duncan, Knapp, & Breese, 1996). These studies suggest that the VP may play a role in restraining anxiety-related behaviors. Further support for this idea comes from the interconnections between the VP and the amygdala, a structure long thought to be critical to states of fear and anxiety.

The Amygdala

The amygdala is a subcortical region in the temporal lobe comprised of distinct nuclei. The basolateral nucleus, or basal nucleus by alternate convention, of the amygdala (BLA) lends its name to the basolateral complex (BLC), which also includes the basomedial nucleus, or accessory basal nucleus, (BMA) and the lateral nucleus (LA). Theories about the functions of the nuclei in the BLC are diverse; however there is some general agreement that the complex receives and integrates information from the sensory thalamus and secondary sensory cortices along with information about the hedonic or aversive value of stimuli. Traditional theories suggested the BLC controlled the central amygdaloid nucleus (CEA) via projections from the BLA, but it has recently been suggested that the two structures operate in parallel (Balleine & Killcross, 2006; Davis, Rainnie, & Cassel, 1994; Pape & Pare, 2010). Reciprocal connections that have been demonstrated between the amygdaloid nuclei and the hippocampal formation appear to

be important for associating emotional values with contextual information (Phillips & LeDoux, 1992; Pitkänen, Pikkarainen, Nurminen, & Ylinen, 2000).

The BLA is comprised of two basic types of neurons: spiny pyramidal glutamatergic projection neurons and spine sparse non-pyramidal GABAergic interneurons (Davis et al., 1994); however, the majority of the neurons projecting to the MD are large non-pyramidal GABAergic neurons. In the BLA there are four families of interneurons that can be distinguished based on the presence of parvalbumin (PV), somatostation (SOM), vasoactive intestinal peptide (VIP) or cholecystokinin (CCK) in the absence of VIP; additionally, there are interneurons that do not contain any of those markers (Mascagni & McDonald, 2007). The activity of the pyramidal cells is kept under tonic inhibition by the interneurons (Rainnie, 1999).

PV positive cells are the major interneuron family representing approximately 45% of the GABAergic cells in the BLA (McDonald & Mascagni, 2001). The PV interneurons receive excitatory input from the pyramidal cells and reciprocate with inhibitory output; thus they are well positioned to provide feedback inhibition to BLA output neurons (Muller, Mascagni, & McDonald, 2006; McDonald, Mascagni, Mania, & Rainnie, 2005). The PV interneurons are themselves interconnected by synapses and gap junctions, and similar to PV interneurons in the cortex, this organization is proposed to be responsible for entrainment of neuron groups into rhythmic oscillatory activity (Muller, Mascagni, & McDonald, 2005).

The BLA sends outputs to all four of the major limbic loop structures and receives inputs from the PFC, VP and MD (see Figure 2). Additional projections to the BLA come from other areas containing NB neurons including the neurons of the horizontal diagonal

band, the neurons of the vertical diagonal band, substantia inominata and lateral preoptic area. Additional projections from areas that don't contain NB neurons include the septum, caudate-putamen, temporal cortex, paraventricular nucleus, parataenial nucleus, periaqueductal gray and bed nucleus of the stria terminalis. Reciprocal projections from the BLA to the bed nucleus of the stria terminalis and caudate-putamen have also been demonstrated (McDonald, 1991; Woolf & Butcher, 1982).

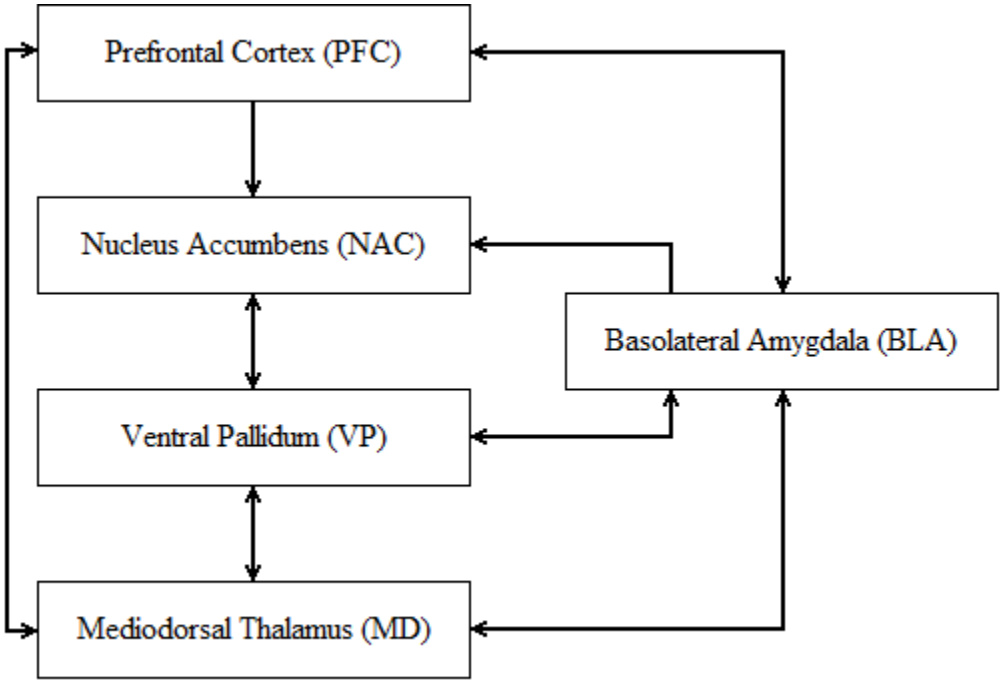

Figure 2. The Arrangement of the BLA with the Limbic Loop.
Arrows represent projections from one structure to another; not all known projections are illustrated.

The BLA is innervated by various brainstem neuromodulatory systems. It receives dopaminergic input from the ventral tegmental area, substantia nigra pars compacta, substantia nigra pars lateralis and the retrorubral area. Norepinephrine input comes primarily from the locus coeruleus but also from cells with somata located more

caudally in the brainstem (Fallon, Koziell, & Moore, 1978; Usunoff, Itzev, Rolfs, Schmitt, & Wree, 2006). 5-HT input is provided by the DRN, particularly from a region referred to as the shell of the dorsal part of the dorsal raphe nucleus (DRDSh) (Abrams et al., 2005; Ma, Yin, Ai, & Han, 1991; Muller, Mascagni, & McDonald, 2007). Histaminergic innervation has been demonstrated and appears to arise from the tuberomamillary nucleus (Köhler, Swanson, Haglund, & Wu, 1985; Panula et al., 1989). While projections from the pedunculopontine nucleus are suspected to contain ACH, ACH input arising from the NB is well recognized (Heckers & Mesulam, 1994; Woolf & Butcher, 1982).

Approximately 10% of DRN neurons that contain 5-HT project to the AMN, and nearly all of the DRN cells that project to the AMN contain 5-HT. The AMN also receives serotonergic input from the ventral periaqueductal grey (Ma et al., 1991). The major target of serotonergic DRN terminals within the BLA are the distal dendrites of pyramidal cells, but synapses also occur with non-pyramidal interneurons (see Figure 3. Muller et al., 2007). 5-HT concentration in the BLA increases following inescapable shock but not escapable shock (Amat, Matus-Amat, Watkins, & Maier, 1998). Injections of 5-HT into the BLA reduced punished responding (Hodges, Green, & Glenn, 1987). *In vitro* 5-HT has been demonstrated to modulate both excitatory and inhibitory transmission in a dose-dependent manner (Rainnie, 1999); it has also been demonstrated to cause a long-term depression (LTD) in response to LA stimulation during application followed by a long-term potentiation (LTP) after stimulation has ceased (Huang & Kandel, 2007).

There is evidence to suggest that the BLA may be involved in the etiology of panic attacks. Microinjections of bicuculline methiodide, a $GABA_A$ antagonist, into the BLA result in increased heart rate, blood pressure and locomotor activity, and microinjections of muscimol, a $GABA_A$ agonist, produces anxiolytic effects in the elevated plus maze (Bueno, Zangrossi, & Viana, 2005; Sanders & Shekhar, 1991). Microinjections of GLU receptor agonists also increase heart rate and blood pressure, while microinjections of GLU receptor antagonists attenuate the effects of bicuculline methiodide microinjections (Soltis, Cook, Gregg, & Sanders, 1997). Chronic microinjections of bicuculline methiodide, at sub-threshold doses, result in a model of PD characterized by sensitization to the cardiovascular and anxiogenic effects of bicuculline methiodide, as measured by social interaction and punished responding tests, as well as susceptibility to lactate-induced increases in cardiovascular activity and decreases in social interaction (Sajdyk & Shekhar, 2000; Sanders, Morzorati, & Shekhar, 1995). Chronic sub-threshold co-administration of bicuculline methiodide and GLU receptor antagonists prevents priming of the cardiovascular response and results in increased social interaction time (Sajdyk & Shekhar, 1997).

Systemic administration of various anxiogenic drugs, including the panicogenic agents caffeine and yohimbine, produced correlated increases in c-fos expression, a marker of neuronal activity, in BLA PV interneurons and serotonergic DRN neurons (Hale et al., 2010). NaLac, similar to other panicogenics, increased c-fos in the DRDSh of control rats, but the increase was not observed in rats made susceptible to NaLac-induced panic by chronic L-allylglycine, an inhibitor of GABA synthesis, microinjected into the dorsomedial hypothalamus (Johnson, Lowry, Truitt, & Shekhar, 2008). These

studies also suggest that deficient 5-HT signaling in the BLA may lead to a panic-prone state. In fact, it has been suggested that the release of 5-HT from DRN neurons onto BLA interneurons functions as a negative feedback loop to restrain anxiety-related behaviors (Hale et al., 2010).

Serotonin Receptors, the Amygdala and Anxiety-Related Behaviors

The BLA contains 5-HT_{1A} receptors and mRNA (Pompeiano, Palacios, & Mengod, 1992). Presynaptic 5-HT_{1A} receptors in the BLA inhibit the release of GABA and GLU (Cheng, Wang, & Gean, 1998; Koyama et al., 2002); however, this idea is disputed (Rainnie, 1999). There is also evidence indicating the presence of postsynaptic 5-HT_{1A} receptors on some neurons (Rainnie, 1999; Stein, Davidowa, & Albrecht, 2000). Antagonism of 5-HT_{1A} receptors reduces the long-term depression cause by 5-HT *in vitro* (Huang & Kandel, 2007). Injections of a 5-HT_{1A} agonist into the BLA resulted in anxiogenesis during a social interaction test but not during an elevated plus-maze test. The anxiogenic effect was reversed by a 5-HT_{1A} antagonist (Gonzalez, Andrews, & File, 1996). Interestingly, intra-BLA injections of a 5-HT_{1A} agonist increased punished responding indicating an anxiolytic effect (Hodges et al., 1987).

5-HT_{1B} receptors and mRNA are also present within the BLA (Bruinvels et al., 1994; Bruinvels, Palacios, & Hoyer, 1993). Increased expression of 5-HT_{1B} receptors by DRN neurons decreased fear-potentiated startle, an AMN dependent phenomenon, suggesting the receptors are located on DRN terminals within the AMN (Clark, Vincow, Sexton, & Neumaier, 2004). There is evidence that 5-HT_{1B} receptors function to presynaptically inhibit GLU release from BLA terminals in efferent regions, but not

within the BLA (Guo and Rainnie, 2010; Rainnie, 1999). It has been demonstrated that chronic passive observation of aggression increases the 5-HT_{1B} receptor density in the BLA, and chronic treatment with the antidepressant buspirone decreases the density (Sato, Skelin, & Diksic, 2010; Suzuki, Han, & Lucas, 2010).

5-HT_{1D} and 5-HT_{1F} receptors and mRNA are also present in the BLA, and 5-HT_{1E} mRNA is present in the primate AMN (Bruinvels et al., 1993; Bruinvels et al., 1994; Lucaites, Krushinski, Schaus, Audia, & Nelson, 2005); however, in regards to the BLA, that is the extent of the knowledge about those receptor subtypes.

5-HT_{2A} receptors are expressed in the BLA postsynaptically on pyramidal dendrites and on GABAergic dendrites and cell bodies, including large non-pyramidal GABA neurons that project to the MD. Approximately 75% of interneurons expressing 5-HT_{2A} receptors also express PV, and approximately 33% also express SOM (Jiang et al., 2009; McDonald & Mascagni, 2007). 5-HT_{2A} agonists increase the activity of BLA interneurons leading to an inhibition of projection neurons (Rainnie, 1999). Inescapable stress, in the form of physical restraint coupled with repeated tail shocks, reduces 5-HT_{2A} mRNA and receptor density, and chronic buspirone increases the density (Jiang et al., 2009; Sato et al., 2010).

5-HT_{2C} mRNA and receptors have also been demonstrated in the BLA (Pompeiano, Palacios, & Mengod, 1994; Rocha, Rigo, Di Scala, Sandner, & Hoyer, 1994). Systemic injections of a 5-HT_{2C} agonist were anxiogenic in an elevated plus maze and intra-BLA ketanserin, a 5-$HT_{2A/2C}$ antagonist prevented the effect (de Mello Cruz et al., 2005). Intra-BLA injections of 5-HT_{2C} agonists have produced an anxiogenic effect in open-field testing including novel object approach (Campbell & Merchant, 2003).

5-HT_3 mRNA has been detected in the amygdala (Tecott, Maricq, & Julius, 1993). The receptors are expressed by non-pyramidal and possibly pyramidal neurons in the BLA. The interneurons expressing the 5-HT_3 receptor do not express any of the typical marker proteins (Mascagni & McDonald, 2007). The receptors are located both pre- and postsynaptically in the amygdala (Miquel et al., 2002). In the LA 5-HT has been shown to cause a fast excitatory post-synaptic potential that desensitizes within 100ms; 5-HT_3 antagonists but not antagonists for fast excitatory post-synaptic receptors block the response (Sugita, Shen, & North, 1992). Consistent with a quickly desensitized response, 5-HT_3 agonists transiently increase GABA release in the BLA, unless the cell is pre-exposed to a 5-HT_{1A} agonist (Koyama et al., 2002). Injections of a 5-HT_3 antagonist into the BLA resulted in increased social interaction time in a brightly lit unfamiliar testing chamber, but the treatment failed to alter punished responding (Higgins, Jones, Oakley, & Tyers, 1991).

5-HT_4 receptors and mRNA can be detected in the BLA (Vilaró, Cortés, & Mengod, 2005). Antagonism of the 5-HT_4 receptors prevents the long-term potentiation (LTP) caused by 5-HT application *in vitro*, and a 5-HT_4 agonist is sufficient to induce LTP (Huang & Kandel, 2007).

5-HT_6 mRNA and receptors have been detected in the BLA (Roberts et al., 2002; Ward et al., 1995). Systemic injections of a 5-HT_6 agonist increase GABA levels in the AMN and other brain regions without affecting GLU levels (Schechter et al., 2008). Localization of this receptor in the BLA has yet to be investigated, but it is theorized to function as an excitatory receptor on GABA interneurons in the various brain regions that express it.

The presence of 5-HT_7 receptors and mRNA in the BLA has been demonstrated in the guinea pig (To, Bonhaus, Eglen, & Jakeman, 1995). In the rat BLA, however, neither were detected (Gustafson, Durkin, Bard, Zgombik, & Branchek, 1996). 5-HT_{2B} and 5-HT_{5A} receptors also are not detected in the BLA (Duxon et al., 1997; Oliver, Kinsey, Wainwright, & Sirinathsinghji, 2000).

The relationship between anxiety-related behaviors and 5-HT signaling in the BLA is complicated by the variety of receptor subtypes and testing procedures employed. It is clear, however, that 5-HT signaling in the BLA does indeed have important consequences for anxiety-like states in rats.

Interactions Between the Ventral Pallidum, the Amygdala and the Nucleus Accumbens

There are multiple pathways from the BLA to the VP (see Figure 3). Electrical stimulation in the BLA evoked short latency excitations and inhibitions with onset times indicative of monosynaptic effects. Long latency inhibitions and excitations were also evoked in the VP suggesting additional polysynaptic effects. Complex responses consisting of two or more of the evoked components were also observed (Maslowski-Cobuzzi & Napier, 1994).

The monosynaptic onset speed of the short latency excitations implicates a direct excitatory pathway from the BLA to the VP. Additional studies indicate that GLU release from BLA projection neurons onto VP neurons is responsible for the short latency excitations (Fuller et al., 1987; Mitrovic & Napier, 1998).

The monosynaptic onset speed of the short latency inhibitions implicates a direct inhibitory pathway from the BLA to the VP that is probably GABAergic, however,

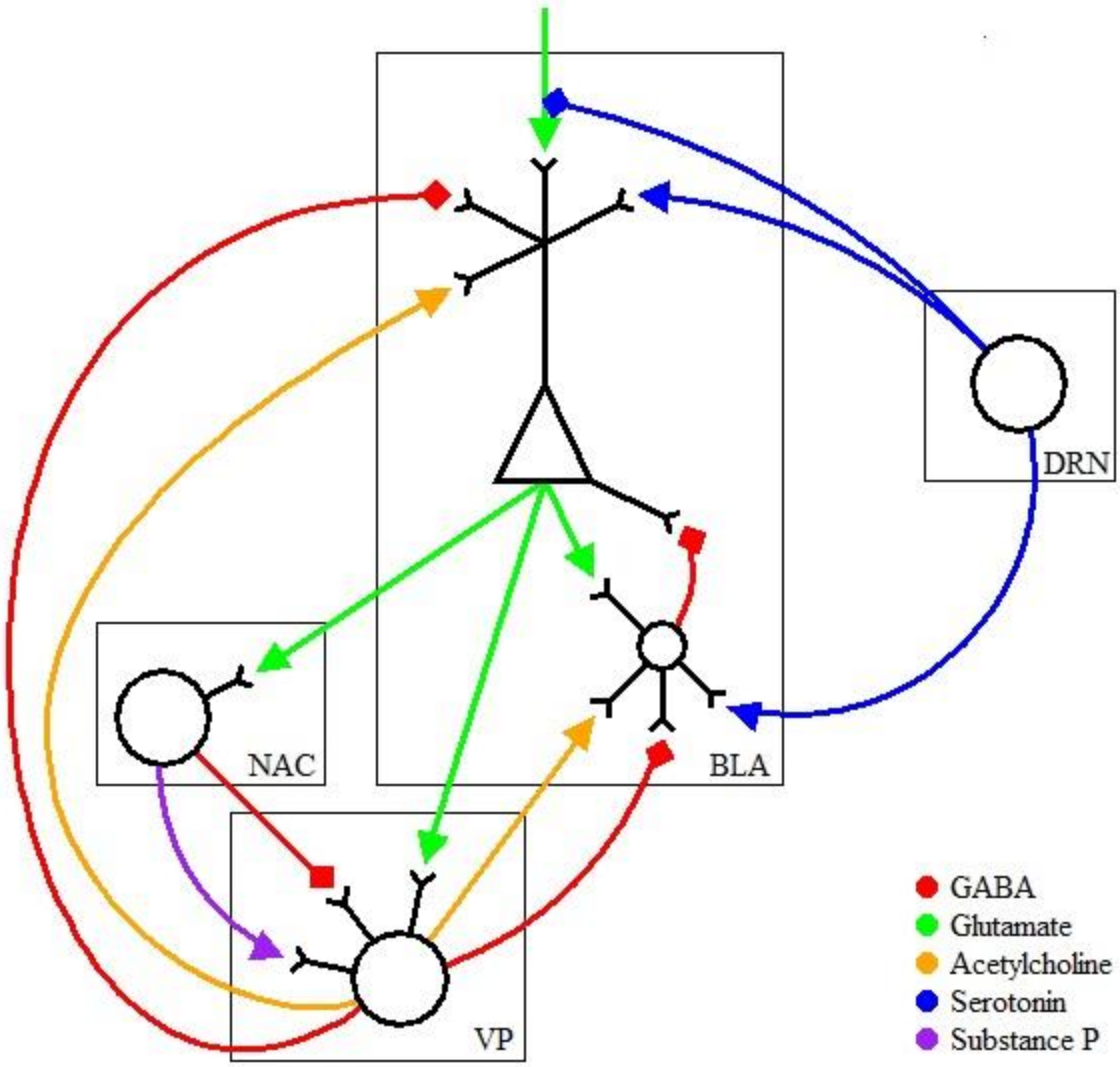

Figure 3. Diagram of the Pathways Between the BLA and VP, Including Pathways from the DRN to the BLA.

Arrows represent excitatory synapses, and diamonds represent inhibitory synapses; not all known synapses are illustrated.

GABAergic pyramidal neurons have not been observed in the BLA (Carlsen, 1988). The proximity of the intercalated cell masses to the BLA suggest that they may also be activated by electrical stimulation of the BLA (Mitrovic & Napier, 1998), and GABAergic projections from these cells to the VP have been demonstrated (Paré &

Smith, 1994). These experiments suggest that GABA release from intercalated cells onto VP neurons is responsible for the short latency inhibitions.

The polysynaptic onset speed of the long latency inhibitions allows for the possibility of an indirect inhibitory pathway. Inactivation of the NAC by procaine injection attenuated the long latency inhibitions in 54% of cells exhibiting this response (Mogenson & Yim, 1983). This fact suggests GLU excitation of NAC cells that send inhibitory efferents to the VP are involved in this response. Electrical stimulation of the NAC evokes short latency, monosynaptic inhibitions in the VP that can be attenuated by intra-VP GABA antagonists (Mitrovic & Napier, 1998). Intra-NAC injections of GLU agonists result in locomotor hyperactivity that can be attenuated by intra-VP injections of a GABA antagonist (Shreve & Uretsky, 1988). These experiments demonstrate a GLU activated GABAergic projection from the NAC to the VP. It is likely that at least some of the long latency inhibitions are the result of GLU release from BLA neurons onto NAC GABAergic neurons that project to the VP.

The polysynaptic onset speed of the long latency excitations allows for the possibility of an indirect excitatory pathway. Inactivation of the NAC by procaine injection did not affect the long latency inhibitions, however, only four cells exhibiting evoked excitations were tested and the proportion exhibiting long latency excitations was not reported (Mogenson & Yim, 1983). Thus, it is premature to conclude that an inhibitory pathway from the NAC is not involved in the polysynaptic excitations. Indeed, it has been suggested that GLU excitation of substance P containing neurons that project from the NAC to the VP could be responsible for polysynaptic excitations. Substance P is an agonist at metabotropic receptors whereas GABA is an agonist at faster acting

ionotropic receptors, which would explain why the polysynaptic excitations are observed following the polysynaptic inhibitions (Mitrovic & Napier, 1998).

The VP not only receives input from the BLA but also projects to it. Electrical stimulation of the cholinergic forebrain, including the VP, elicited primarily short latency, monosynaptic excitations followed by long latency inhibitions in BLA pyramidal cells; also observed were short latency, monosynaptic inhibitions followed by long latency excitations in pyramidal cells and short latency excitation in interneurons (Mello, Tan, & Finch, 1992; Washburn & Moises, 1992).

VP neurons containing ACH have been shown to project to the BLA (Woolf & Butcher, 1982). In the BLA, axons containing ACH primarily form synapses with the distal dendrites of pyramidal cells; however, synapses with the somata and dendrites of PV interneurons have also been verified, and it is likely that synapses are formed with additional interneuron types (Muller, Mascagni, & McDonald, 2011). ACH has excitatory actions on both GABAergic and glutamatergic neurons in the BLA; therefore, the excitation-inhibition sequence observed following VP stimulation can be explained by direct excitation of pyramidal neurons by ACH followed by indirect inhibition via the excitation of interneurons (Mello et al., 1992; Washburn & Moises, 1992).

Some of the neurons projecting from the VP to the BLA contain GABA (Mascagni & McDonald, 2009). These neurons primarily form synapses with the dendrites and somata of PV interneurons, but synapses are also observed with the dendrites and somata of pyramidal cells (McDonald et al., 2011). The inhibition-excitation sequence observed following VP stimulation can be explained by direct

inhibition of pyramidal neurons by GABA followed by disinhibition via the inhibition of interneurons (Mello et al., 1992).

CHAPTER III

HYPOTHESES AND EXPERIMENTAL DESIGN

Hypotheses

There are three general aims of this study. First, determine if 5-HTR antagonism in the BLA is sufficient to model PD as evidenced by a NaLac-induced response. Second, determine the influence of tonic 5-HT signaling in the BLA on the firing rates of spontaneously active VP neurons. Third, determine if the VP has a role in the response to panicogenic treatments. Consideration of the relevant literature lead to the development of four specific hypotheses to complete the three aims.

Pharmacological manipulations that disinhibit the BLA successfully model PD as evidenced by NaLac-induced tachycardia (Sajdyk & Shekhar, 2000; Sanders, Morzorati, & Shekhar, 1995). The BLA is synaptically arranged such that 5-HT exerts an overall inhibitory influence on the projection neurons, and it has been suggested that release of 5-HT in the BLA may restrain anxiety-related behaviors (Hale et al., 2010; Rainnie, 1999). Therefore, antagonism of the 5-HTRs in the BLA should disinhibit it and prevent the ability of 5-HT release to restrain anxiety-related behaviors. Hence, the first hypothesis is that microinjection of metergoline (MET), a 5-HTR antagonist, into the BLA followed by i.v. NaLac will result in tachycardia. Support for this hypothesis would indicate successful development of a PD model.

NaLac-induced hyperventilation is observed in PD patients, but respiratory activity is not usually assessed in rat models of PD (Gorman et al., 1988). Therefore, if respiratory activity is assessed in a model of PD it should mirror the changes in cardiovascular activity, similar to PD patients. Hence, hyperventilation is also an indicator of a panic-like reaction and the second hypothesis is that the combination of MET and NaLac will result in hyperventilation. Support for this hypothesis would also indicate the successful development of a PD model.

Projection neurons in the BLA produce spontaneous action potentials and are overall inhibited by 5-HT (Rainnie, 1999). BLA neurons project to the VP and contain an excitatory neurotransmitter (McDonald, 1991; Woolf & Butcher, 1982). Therefore, antagonism of 5-HTRs in the BLA should disinhibit the projection neurons causing increased excitation of the VP neurons. BLA neurons also project to the NAC, the limbic loop structure upstream from the VP (McDonald, 1991; Woolf & Butcher, 1982). Stimulation of the NAC often produces short-latency inhibition of the VP followed by long-latency excitation (Mitrovic & Napier, 1998). Therefore, antagonism of 5-HTRs in the BLA should increase excitation of the NAC neurons producing augmented short-latency inhibition and long-latency excitation in the VP. The expected effects of 5-HTR antagonism in the BLA are increased excitation in the VP via a direct pathway and an indirect pathway through the NAC. Hence, the third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons. Support for this hypothesis would indicate that tonic 5-HT in the BLA exerts an inhibitory influence on the spontaneous firing rate of VP neurons.

A role for the BLA in the response to anxiogenic and panicogenic agents, including NaLac, is well established (Hale et al., 2010; Hodges, Green, & Glenn, 1987; Sajdyk & Shekhar, 2000). The BLA and the limbic loop, including the VP, are anatomically and functionally interconnected (Maslowski-Cobuzzi & Napier, 1994; McDonald, 1991; Mogenson & Yim, 1983; Woolf & Butcher, 1982). A role for the VP in restraining anxiety-related behaviors is emerging (Cromwell & Berridge, 1993; Duncan, Knapp, & Breese, 1996; Nikolaus, Huston, & Hasenöhrl, 2000). The suggested role of the VP and its connectivity with the NaLac-responsive BLA suggests the VP will exhibit a response to NaLac also. Therefore, the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. Support for this hypothesis would establish a role for the VP in the response to panicogenic treatments.

Experimental Design

A factorial design will be used to ensure that any observed panic-like response is due to the effects of both MET and NaLac. One factor will be defined by the microinjection; either MET or the vehicle solution (VEH) used to deliver the MET. A second factor will be defined by the i.v. infusion; either NaLac or d-mannitol (MAN). MAN, in an equimolar amount, has been chosen as a control solution to eliminate detection of any effects due to differences in solution osmolarity. A third factor, time, is also present in this experiment and will be included in some of the analyses.

This experiment technically conforms to a three-way mixed design with microinjection and i.v. infusion as between subject factors and time as a within subjects factor. However, the hypothesized results for heart and respiratory rates are three-way

interactions, and three-way interactions can be difficult to interpret in the context of this type of design. Therefore, initial analyses will focus on between-subject differences, and follow-up analyses will focus on within-subject differences.

Difference scores will be calculated to examine between-subject effects collapsed across time. The difference scores will be calculated as the percent change during the last 5min of the i.v. infusion compared to a 5min baseline and subjected to two-way ANOVAs. Significant results will be followed-up with the indicated one-way, or two-way mixed, repeated-measure analysis and appropriate *post hoc* analyses.

This combination of analyses for examining within and between-subject effects is appropriate for the design for two interrelated reasons. First, this combination of analyses is able to more specifically test the hypotheses. Second, this type of combination of analyses is generally necessary for interpreting three-way interactions in mixed designs with more than a few repeated measures.

The responses of VP neurons to both systemic and direct drug applications are heterogeneous (Heidenreich, Mitrovic, Battaglia & Napier, 2004). Therefore, additional analyses may be necessary to examine if the distributions of types of response differs among groups. Each neuron will be classified as exhibiting an increased firing rate, no change, or a decreased firing rate.A Pearson χ^2 analyses will be used to compare the distributions.

CHAPTER IV

METHODS

Procedures

MET was dissolved in 1.3% glacial acetic acid then diluted with an equal amount of solution containing .05M pH 7.4 phosphate buffer and .04M sodium hydroxide. The final concentration was 10 nmol per 0.2 µl, and this is the volume that was administered in the experiment. VEH was prepared identically, except that MET was omitted. The solution was determined to have pH = 4.5; the desired concentration of MET was incompletely solvated in vehicle solutions of greater pH. NaLac was prepared as a 0.5M solution, and 0.5M MAN was prepared for an equimolar control; both solutions were administered at 10mg/kg.

To construct an electrode, a 1.2mm OD borosilicate glass capillary tube with inner filament was pulled to a point in a vertical gravity puller (David Kopf Instruments). The resulting electrode was filled with pontamine sky blue dye in 2M saline (PSB). The impedance of each electrode was determined and the electrodes used ranged from 3.8-6.8MΩ.

Male Sprague-Dawley rats (weighing 307-507g) were anesthetized with urethane (1.5mg/kg intraperitoneal), the top of their heads were shaved and they were secured in a

stereotaxic apparatus connected to ground. A metal wound clip was attached to the skin over the heart as an electrocardiogram (EKG) electrode. A longitudinal incision was made through the scalp, and the skull was cleared of tissue. To allow access to the BLA and the VP, two burr holes were drilled through the skull, and the underlying meninges were removed. A 27g stainless steel guide cannula was lowered into position dorsal to the BLA (2.8mm posterior, 4.5-4.8mm lateral, 6.0-6.2mm ventral to bregma). A glass electrode was lowered through the other burr hole into the VP (0.3mm posterior, 2.2 mm lateral to bregma 7.0mm ventral to the dorsal surface of the brain). An intravenous (i.v.) catheter was inserted into one femoral vein, and patency was maintained by injecting 0.9% saline as necessary.

To monitor neural activity, a silver wire was connected to the positive lead of an AC differential amplifier (World Precision Instruments), and the negative lead was connected to the stereotaxic apparatus. The silver wire was inserted into the electrode, and the signal was amplified with a 10k gain, high-pass filtered at 300Hz and low-pass filtered at 3kHz. The output from the amplifier was connected to a 60Hz notch filter (Mentor). The output from the notch filter was split and routed to a digital oscilloscope (Tektronix) and an analog oscilloscope (Hitachi) through a window discriminator (Mentor). The output from the window discriminator was routed to a data acquisition unit (Cambridge Electronic Design) connected to an IBM-compatible PC running Spike 2 software (Cambridge Electronic Design). After isolating a single unit, as described later, the software recorded the occurrence of each action potential.

To monitor heart rate, the positive lead of an AC/DC differential amplifier (AM Systems Inc.) was attached to the wound clip over the rat's heart, and the negative lead

was connected to the stereotaxic apparatus. The AC signal was amplified with a 1k gain, high-pass filtered at 10Hz and low-pass filtered at 100Hz. The output from the amplifier was connected to an analog oscilloscope (Tektronix) through a window discriminator (World Precision Instruments). The output from the window discriminator was routed to the same data acquisition unit as the signal from the electrode. The window discriminator was set to allow a single peak from each EKG waveform to fall within the trigger window; this resulted in the software recording the occurrence of each heartbeat.

To monitor respiration rate, an AC stimulator (Grass Electronics) was set to output a single 5v 10mW pulse upon manual press of a button. The output from the stimulator was routed to the same data acquisition unit as the signals from the electrode and wound clip. To acquire respiratory data, I pressed the stimulator button upon each visually observed inhalation by the rat; this resulted in the software recording the occurrence of each respiration.

To isolate a single neuron, the electrode was advanced ventrally with a micromanipulator until action potentials were observed with at least a 3:1 signal to noise ratio. If all the action potentials exhibited a consistent amplitude and shape, and the firing pattern remained consistent, then the signal was presumed to be from a single unit. The window discriminator was set to allow a single peak from each action potential waveform to fall within the trigger window; this resulted in the software recording the occurrence of each action potential. If the criteria were not met before the electrode reached 7.6mm ventral to the dorsal surface of the brain then the electrode was removed from the brain. The electrode was then reinserted after being repositioned 0.1mm posterior, repositioned 0.1mm medial or replaced then repositioned.

To test the hypotheses, a 36g stainless steel injection cannula, that was designed to protrude from the guide cannula 1mm, was attached to a 10µl Hamilton syringe via polyethylene tubing. It was backfilled with MET or VEH and inserted into the BLA through the guide cannula after obtaining at least 5min of baseline activity from a single neuron. Respiratory activity was monitored for 1min. 0.2µl of MET or VEH were microinjected into the BLA over the course of 30s using a syringe pump (KD Scientific). Seven minutes after the microinjection, respiratory activity was monitored for 1min. Ten minutes after the microinjection, 10ml/kg of NaLac or MAN was infused through the i.v. catheter over the course of 15min using the same syringe pump. Respiratory activity was then monitored for 1min every 5min after the i.v. infusion began and continued until 15min after the i.v. infusion ended.

To mark the injection site for histological verification, 15min after the i.v. infusion ended the injection cannula was removed from the guide cannula and backfilled with PSB. It was reinserted and 0.2µl PSB was microinjected into the BLA over 30s using the syringe pump. To mark the recording site, the rat and stereotaxic apparatus were electrically isolated from ground and all other equipment. A DC stimulator (Grass Electronics) and constant current unit (Grass Electronics) were used to pass a 150v 2mA current through the electrode for 20min.

To obtain the brain, the electrode was slowly extracted followed by the guide cannula. The rat was sacrificed with an overdose of urethane through the i.v. catheter, and then transcardially perfused with 0.9% saline followed by 4% para-formaldehyde in 0.1M phosphate buffer. The brain was removed, stored overnight in the fixative solution and then placed in a 20% sucrose solution.

To verify the electrode and cannula placements, coronal sections of the brain were cut with a cryostat. The sections were washed with phosphate-buffered saline then mounted on microscope slides in dilute phosphate buffer. The slides were stained with neutral red, cover-slipped and examined with a light microscope to verify deposition of PSB in the targeted structures.

Data Analyses

The design of this experiment technically conforms to a three-way mixed ANOVA with microinjection and i.v. infusion as between subject factors and time as a within subjects factor. However, the hypothesized result is a three-way interaction, and three-way interactions can be difficult to interpret in the context of this type of design. Therefore, initial analyses will focus on between-subject differences, and follow-up analyses will focus on within-subject differences.

The first hypothesis is that microinjection of MET into the BLA followed by i.v. NaLac will result in tachycardia. To determine if heart rate changes differed among the experimental groups, a difference score for each subject was calculated as the percent change of the mean heart rate during the last 5min of the i.v. injection compared to mean heart rate for 5min prior to the microinjection. Outliers were detected using the Studentized residual and removed from all heart rate analyses. Next, a baseline heart rate was calculated for each subject as the mean heart rate for 5min prior to the microinjection. To ensure the treatment groups did not differ initially, the baseline heart rate data were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

To determine if heart rate changes differed among the experimental groups the heart rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors. The results of the analysis indicated that the MET/NaLac group had heart rate changes that were significantly different than the other rats; therefore, I tested if the MET/NaLac combination produced heart rates significantly greater than baseline. The EKG data from 5min prior to the microinjection through the end of the i.v. infusion were grouped into 60s bins. The binned heart rate data from the MET/NaLac group were subjected to a repeated measure ANOVA with *post hoc* Dunnett corrected pairwise comparisons.

The second hypothesis is that microinjection of MET into the BLA followed by i.v. NaLac will result in hyperventilation. To determine if respiratory rate changes differed among the experimental groups, a respiratory rate difference score for each subject was calculated as the percent change of the mean respiratory rate during the last 5min of the i.v. injection compared to mean respiratory rate for 5min prior to the microinjection. Outliers were detected using the Studentized residual and removed from all respiration rate analyses. Next, a baseline respiratory rate was calculated for each subject as the mean respiratory rate for 5min prior to the microinjection. To ensure the treatment groups did not differ initially, the baseline respiratory rate data were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

To determine if respiratory rate changes differed among the experimental groups, the respiratory rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors. The results indicated that the groups treated with NaLac had respiratory rate changes significantly different than the groups treated

with MAN regardless of the microinjection they received; therefore, I tested if the i.v. infusions caused respiratory rates significantly different from baseline.

A 30s bin was generated from the respiratory data collected at baseline and during the i.v. infusion. The data were subjected to a two-way mixed ANOVA with i.v. infusion as the between subjects factor and time as the within subjects factor. *Post hoc* Bonferroni corrected pairwise comparisons were used to examine the effect of time within each group. *Post hoc* Sidak corrected pairwise comparisons were also used to examine the effect of group at each time point.

The third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons; the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. The factorial design of this experiment allows simultaneous testing of these two hypotheses. VP firing rates are expected to vary, therefore no statistical test for outliers was conducted; however, to ensure the treatment groups did not differ initially, a baseline firing rate was calculate for each subject as the average firing rate for 5 min prior to the microinjection. The baseline firing rate data were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors.

To determine if the firing rate changes differed between treatment groups, a firing rate difference score for each subject was calculated as the percent change of the mean firing rate during the last 5min of the i.v. injection compared to the mean firing rate for 5min prior to the microinjection. The firing rate difference scores were subjected to a two-way ANOVA with microinjection and i.v. infusion as factors. The VEH/MAN group had firing rate changes that were significantly different than the VEH/NaLac group;

therefore, I tested if these groups exhibited significant changes in firing rates at the end of the i.v. infusion compared to baseline.

The firing rate data from 5min prior to the microinjection through the end of the i.v. infusion were grouped into 60s bins. The binned data were subjected to a two-way mixed ANOVA with time as a within subjects factor and group as a between subjects factor. *Post hoc* Bonferroni corrected pairwise comparisons were used to examine the effect of time within each group. *Post hoc* Sidak corrected pairwise comparisons were also used to examine the effect of group at each time point.

To determine if there were any differences in the distributions of types of response between the groups, the difference scores for each neuron were classified as exhibiting an increased firing rate, decreased firing rate, or no change in firing rate. An increased firing rate was defined as a firing rate difference score greater than the mean plus one standard deviation, a decreased firing rate was defined as a firing rate difference score less than the mean minus one standard deviation and the remaining difference scores were classified as exhibiting no change. The total numbers of neurons exhibiting decreased firing rate, no change, or increased firing rate were tabulated for each group. The distributions of responses for the groups were compared using a Pearson χ^2 test.

CHAPTER V

RESULTS

The procedures were performed on 51 subjects. 29 subjects had cannula injections, verified by histology, into the BLA or just dorsal into the LA. 14 subjects were excluded from all analyses because histology demonstrated the cannula injections did not involve the BLA. Histology was inconclusive for one subject. After the stereotaxic coordinates were refined cannula placements were at least 90% accurate. Of the 51 total subjects, 27 had electrode placements, verified by histology, in the VP. 17 subjects were excluded from the firing rate analyses because histology demonstrated electrode placements outside the VP. Histology was inconclusive for two subjects. The electrode placements were at least 75% accurate, including subjects used to collect pilot data. Histology was not performed on seven subjects; these subjects were included in the analyses due to the demonstrated accuracy of the procedures.

Results from the Heart Rate Data Analyses

In order to test the first hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in tachycardia, several analyses were conducted. First, a difference score for each subject was calculated as the percent change of the mean heart rate during the last 5min of the i.v. injection compared to mean heart rate for 5min prior

to the microinjection. Four outliers were excluded from the analyses based on the Studentized residuals of the difference scores (see Figure 4). Two subjects were excluded from the VEH/LAC group, Studentized residuals = -3.07 and 3.27. One subject was excluded from the MET/MAN group, Studentized residual = -2.93. One subject was excluded from the MET/NaLac group, Studentized residual = 2.95. Two additional subjects were excluded due to incomplete EKG recordings.

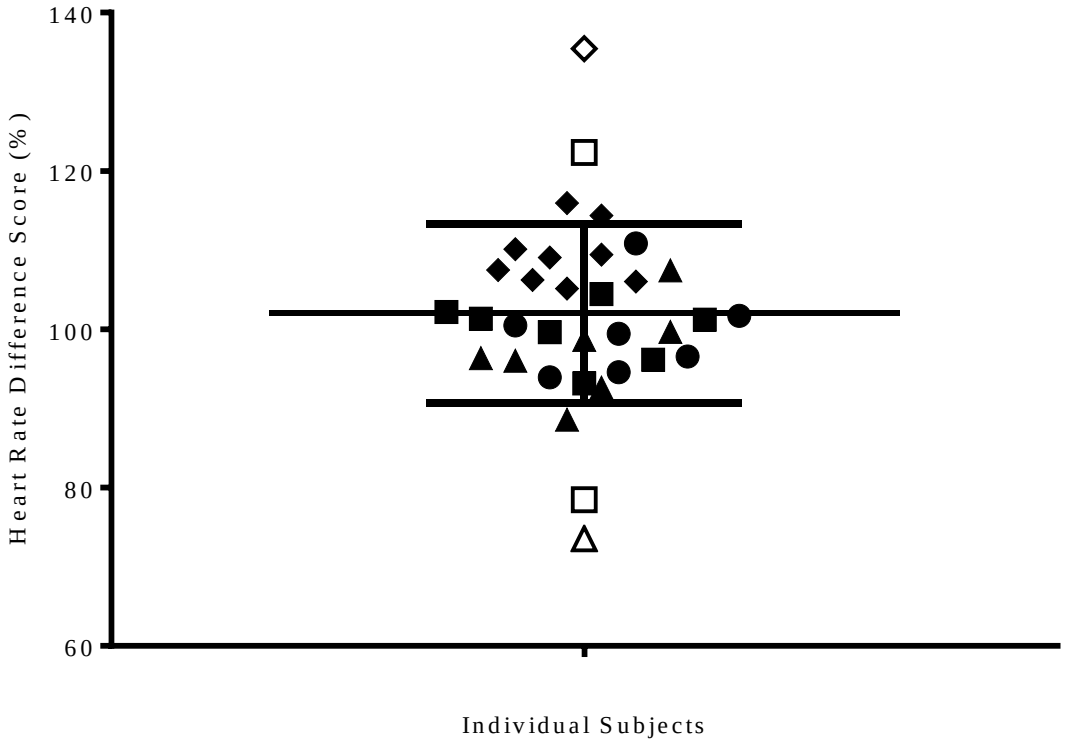

*Figure 4.*Individual Heart Rate Difference Scores.

Individual subjects from all treatment groups are spread over the x-axis for clarity. Circles represent the VEH/MAN group, squares represent the VEH/NaLac group, triangles represent the MET/MAN group and diamonds represent the MET/NaLac group. Open symbols represent the outliers that were excluded from the heart rate analyses. The central line represents the mean and the error bars represent the standard deviation.

Table 1. Descriptive Statistics for the Baseline Heart Rates (Hz)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	5.29	0.50	7	5.75	0.29	7
NaLac	5.42	0.60	7	5.48	0.30	9

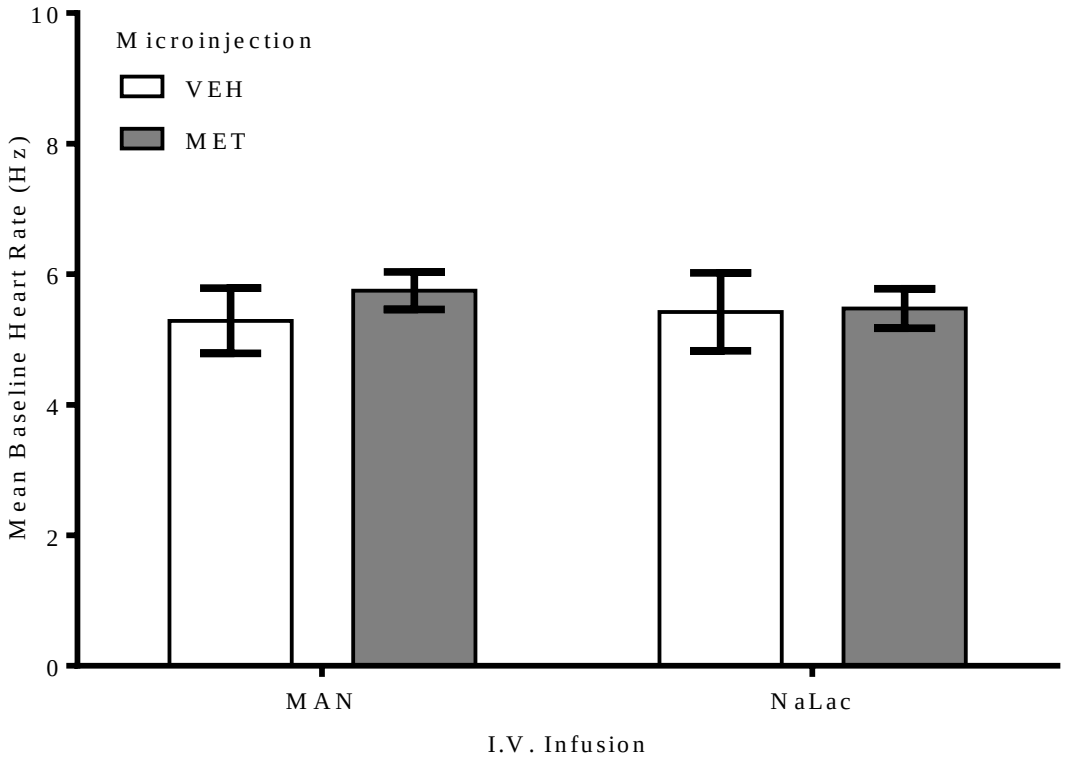

Figure 5. Baseline Heart Rate Data for Each Treatment Group.

Error bars represent the standard deviations.

Next, a baseline heart rate was calculated for each subject as the mean heart rate for 5min prior to the microinjection. A two-way ANOVA was conducted on the baseline heart rate data to ensure the treatment groups did not differ at baseline (see Table 1 for the descriptive statistics). The results of the two-way ANOVA on the baseline heart rate data indicate no significant effect of microinjection, $F(1, 26) = 2.61$, $p = .118$, no significant effect of i.v. infusion, $F(1, 26) = 0.19$, $p = .671$, and no significant interaction, $F(1, 26) = 1.68$, $p = .207$ (see Figure 5). There were no significant differences in the

baseline heart rates; therefore the heart rate difference scores are not confounded by the baseline heart rate.

Table 2. Descriptive Statistics for the Heart Rate Difference Scores (%)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	99.65	5.74	7	97.05	5.92	7
NaLac	99.74	3.85	7	109.31	3.73	9

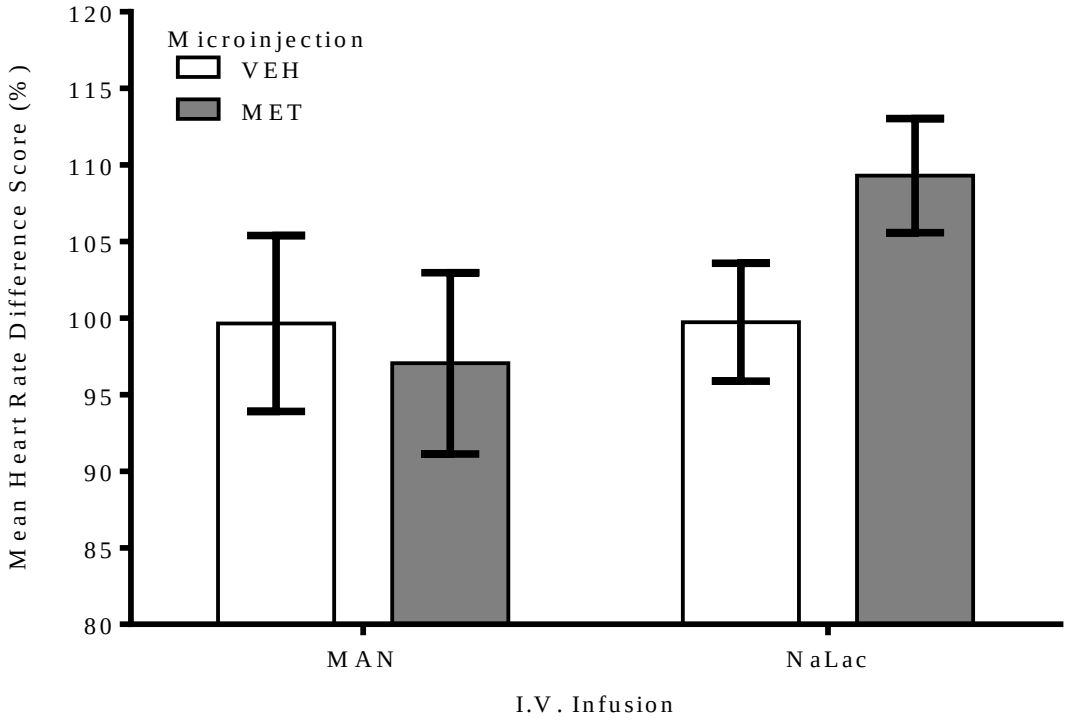

Figure 6. Mean Heart Rate Difference Scores for Each Treatment Group.

Error bars represent the standard deviations.

A two-way ANOVA was conducted on the heart rate difference scores (see Table 2 for the descriptive statistics). The results indicate a significant effect of i.v. infusion, $F(1, 26) = 12.08$, $p < .01$, and a significant interaction between microinjection and i.v. infusion, $F(1, 26) = 11.73$, $p < .01$. Microinjection itself was not a significant factor, $F(1, 26) = 3.86$, $p = .060$. Comparison of the group means reveals that the effect of i.v.

infusion is driven by the interaction, and that the MET/NaLac group had greater heart rate difference scores (see Figure 6).

Table 3. Descriptive Statistics for the MET/NaLac Group's Heart Rate (Hz) Over Time (min)

Time	Mean	*SD*	*n*
1	5.47	0.29	9
2	5.47	0.30	9
3	5.48	0.31	9
4	5.47	0.31	9
5	5.48	0.30	9
6	5.47	0.31	9
7	5.47	0.36	9
8	5.45	0.34	9
9	5.45	0.35	9
10	5.45	0.35	9
11	5.44	0.34	9
12	5.45	0.34	9
13	5.46	0.32	9
14	5.49	0.32	9
15	5.49	0.32	9
16	5.49	0.31	9
17	5.53	0.29	9
18	5.60	0.29	9
19	5.62	0.31	9
20	5.64	0.32	9
21	5.68	0.29	9
22	5.73	0.29	9
23	5.77	0.29	9
24	5.82	0.29	9
25	5.87	0.27	9
26	5.91	0.28	9
27	5.95	0.28	9
28	5.99	0.30	9
29	6.01	0.29	9
30	6.03	0.30	9

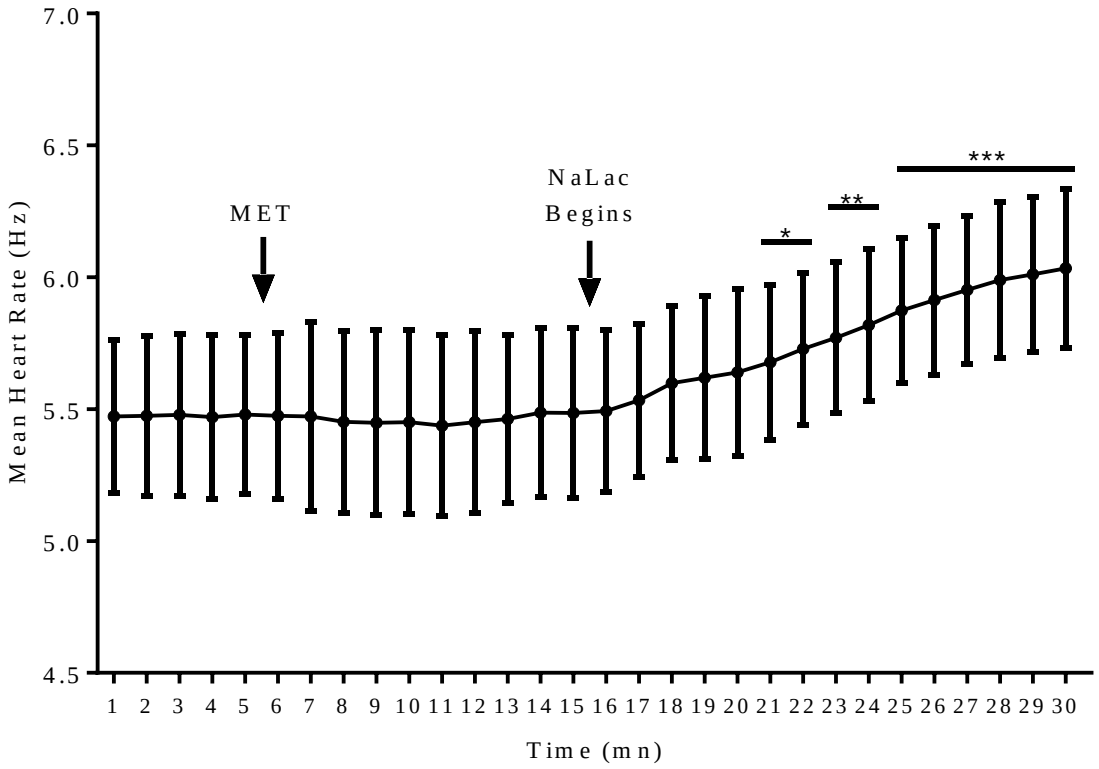

Figure 7. Mean Heart Rate Scores for the MET/NaLac Group Over Time.

*, $p < .05$, **, $p < .01$, and ***, $p < .001$ compared to minute 1. Error bars represent the standard deviations.

The MET/NaLac group had heart rate changes that were significantly different than the other groups; therefore, I tested if the MET/NaLac group had greater heart rates at the end of the i.v. infusion than at baseline. The heart rate data for each subject was averaged into 60s bins (see Table 3 for the descriptive statistics). The results of the repeated measures ANOVA on the binned heart rate data for the MET/NaLac group revealed a significant effect of time, $F(2.046, 16.37) = 37.10$, $p < .0001$ (see Figure 7). *Post hoc* Dunnett analysis revealed that heart rate was significantly increased, compared to minute 1, at minute 21-22 ($p < .05$), minutes 23-24 ($p < .01$), and minutes 25-30 ($p < .001$).

The first hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in tachycardia, is supported by the results of the heart rate analyses. The results indicate that the MET/NaLac group had significant increases in heart rate, and these changes in heart rate were significantly different than the other treatment groups' changes.

Results from the Respiratory Rate Data Analyses

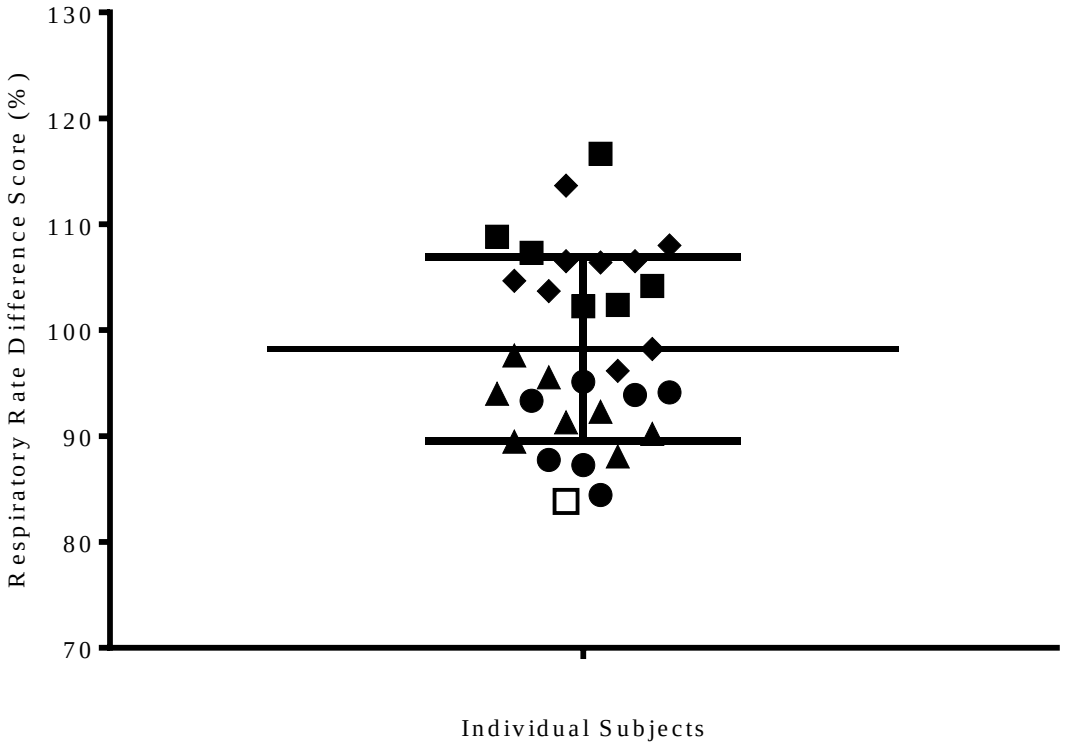

*Figure 8.*Individual Respiratory Rate Difference Scores.

Subjects from all treatment groups are spread over the x-axis for clarity. Circles represent the VEH/MAN group, squares represent the VEH/NaLac group, triangles represent the MET/MAN group and diamonds represent the MET/NaLac group. The

open square represents the outlier that was excluded from the heart rate analyses. The central line represents the mean and the error bars represent the standard deviation.

A set of analyses was conducted to test the second hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in hyperventilation. First, a respiratory rate difference score for each subject was calculated as the percent change of the mean respiratory rate during the last 5min of the i.v. injection compared to mean respiratory rate for 5min prior to the microinjection. One outlier was excluded from the VEH/NaLac group based on the Studentized residual of the difference scores, Studentized residual = -5.50 (see Figure 8). An additional five subjects were excluded due to incomplete respiratory recordings.

Table 4. Descriptive Statistics for the Baseline Respiratory Rates (Hz)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	1.60	0.15	7	1.53	0.17	8
NaLac	1.49	0.29	6	1.62	0.15	9

Next, a baseline respiratory rate was calculated for each subject as the mean respiratory rate for 5min prior to the microinjection. A two-way ANOVA was conducted on the baseline respiratory rate data to ensure the treatment groups did not differ at baseline (see Table 4 for the descriptive statistics). The results of the two-way ANOVA on the baseline respiratory rate data indicate no significant effect of microinjection, $F(1, 26) = 0.17$, $p = .686$, no significant effect of i.v. infusion, $F(1, 26) = 0.00$, $p = .980$, and no significant interaction, $F(1, 26) = 1.99$, $p = .171$ (see Figure 9). There were no significant differences in the baseline respiratory rates; therefore the respiratory rate difference scores are not confounded by the baseline respiratory rate.

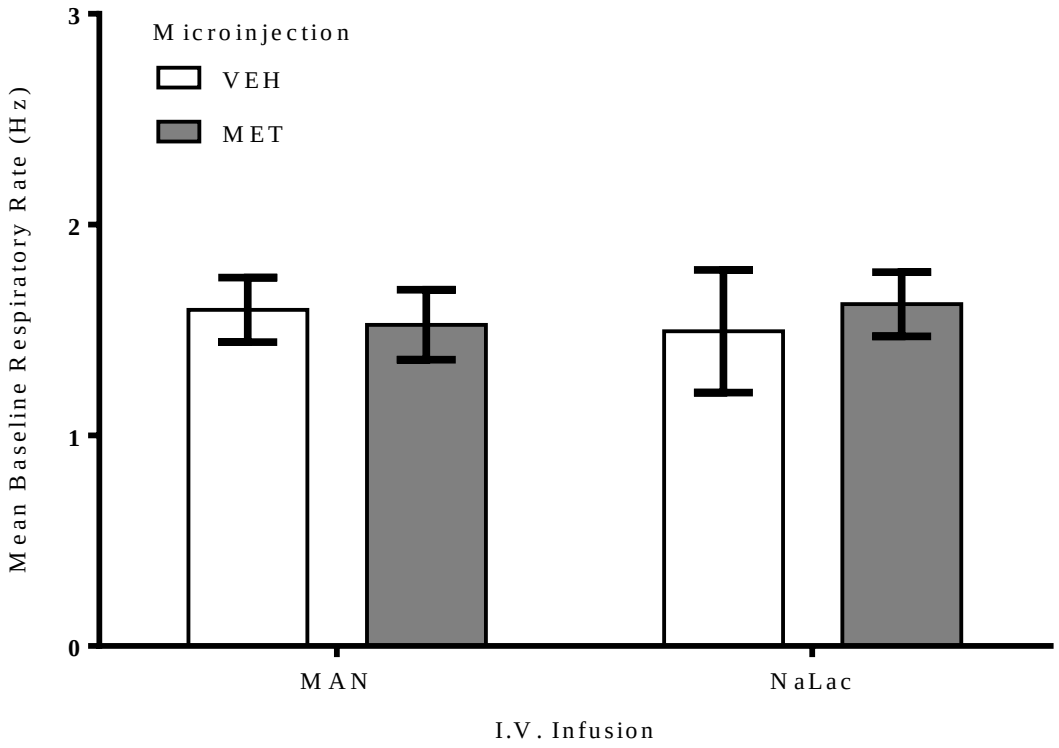

Figure 9. Baseline Respiratory Rate Data for Each Treatment Group.

Error bars represent the standard deviations.

A two-way ANOVA was conducted on the respiratory rate difference scores (see Table 5 for the descriptive statistics). The results indicate a significant effect of i.v. infusion, $F(1, 26) = 71.69$, $p < .0001$, but no significant effect of microinjection, $F(1, 26) = 0.03$, $p = .861$, and no significant interaction, $F(1, 26) = 1.10$, $p = .304$. Comparison of the group means reveals that the subjects treated with NaLac had greater respiratory rate difference scores than the subjects treated with MAN, regardless of the microinjection (see Figure 10).

Table 5. Descriptive Statistics for the Respiratory Rate Difference Scores (%)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	90.85	4.24	7	92.32	3.23	8
NaLac	106.94	5.45	6	104.87	5.20	9

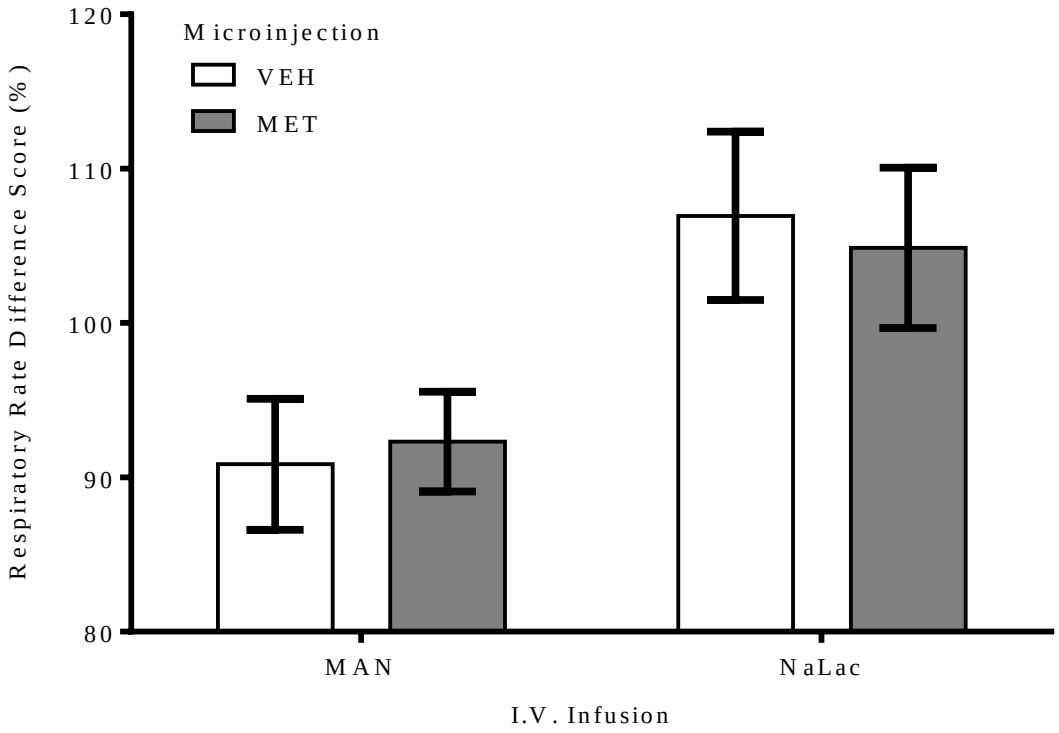

Figure 10. Mean Respiratory Rate Difference Scores for Each Treatment Group. Error bars represent the standard deviations.

The rats that were treated with NaLac had respiratory rate changes significantly different than the rats treated with MAN, regardless of the microinjection treatment they received; therefore I tested if the i.v. infusions resulted in increased respiratory rates at the end of the i.v. infusion compared to baseline. A 30s bin was generated from the respiratory rate data collected at baseline and during the i.v. infusion for each subject (see Table 6 for the descriptive statistics). The binned respiratory rate data were subjected to a two-way mixed ANOVA with i.v. infusion as a between subjects factor and time as a within subjects factor. The results of the two-way mixed ANOVA on the binned respiratory rate data indicate no significant effect of time $F(3, 84) = 1.18$, $p = .324$ and no significant effect of i.v. infusion $F(1, 28) = 3.60$, $p = .068$. The results do indicate a

significant interaction $F(3,84) = 23.75$, $p < .0001$ (see Figure 11). *Post hoc* Bonferonni within group analyses revealed a significant decrease from baseline 5min after the MAN i.v. infusion began ($p < .001$), as well as at 10min and 15min after the infusion began, $p < .0001$. Additionally, the anlayses indicated a significant increase from baseline 10min after the NaLac i.v. infusion began ($p < .01$) and 15 min after the infusion began, $p < .001$.

Table 6. Descriptive Statistics for the Respiratory Rates (Hz) Over Time by I.V. Infusion

		MAN			NaLac	
Time	Mean	*SD*	*n*	Mean	*SD*	*n*
Baseline	1.56	0.16	15	1.57	0.22	15
5min I.V.	1.47	0.15	15	1.61	0.31	15
10min I.V.	1.44	0.16	15	1.65	0.27	15
15min I.V.	1.43	0.15	15	1.66	0.25	15

The results of the two-way ANOVA on respiratory rate changes indicated a significant effect of i.v. infusion, however, the results of the two-way mixed ANOVA on respiratory rate only indicated a time by infusion interaction. Therefore, I conducted *post hoc* Sidak corrected pairwise comparisons to determine if the respiratory rate differed between the groups at any time point (see Figure 11). *Post hoc* between group analyses revealed a significant difference between groups 10min and 15min after the i.v. infusion began, $p < .05$.

The second hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in hyperventilation, is not supported by the results of the respiratory rate analyses. Rather, NaLac is sufficient to cause hyperventilation and MAN is sufficient to cause hypoventilation, regardless of the microinjection treatment.

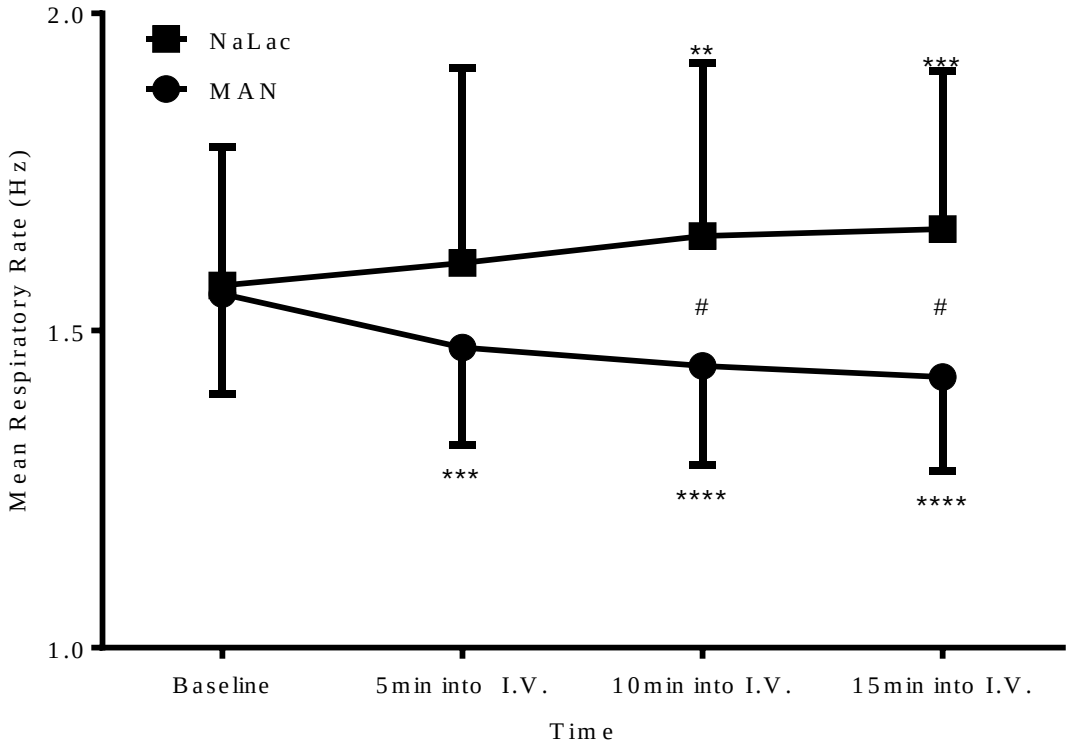

Figure 11. Mean Respiratory Rate for Each I.V. Infusion Group Over Time. **, $p < .01$, ***, $p < .001$, ****, $p < .0001$ compared to baseline. #, $p <..05$, between-groups comparison. Error bars represent the standard deviations.

Results from the Firing Rate Data Analyses

The third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons; the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. No test for outliers was conducted because changes in VP neural activity are expected to be heterogeneous (Heidenreich, et al., 2004). Of the 27 subjects with proper electrode placements, three were removed from the analyses due to improper cannula placement or incomplete single-unit activity recordings.

Table 7. Descriptive Statistics for the Baseline Firing Rates (Hz)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	6.08	4.43	7	6.17	5.58	6
NaLac	2.87	1.97	5	11.83	8.92	6

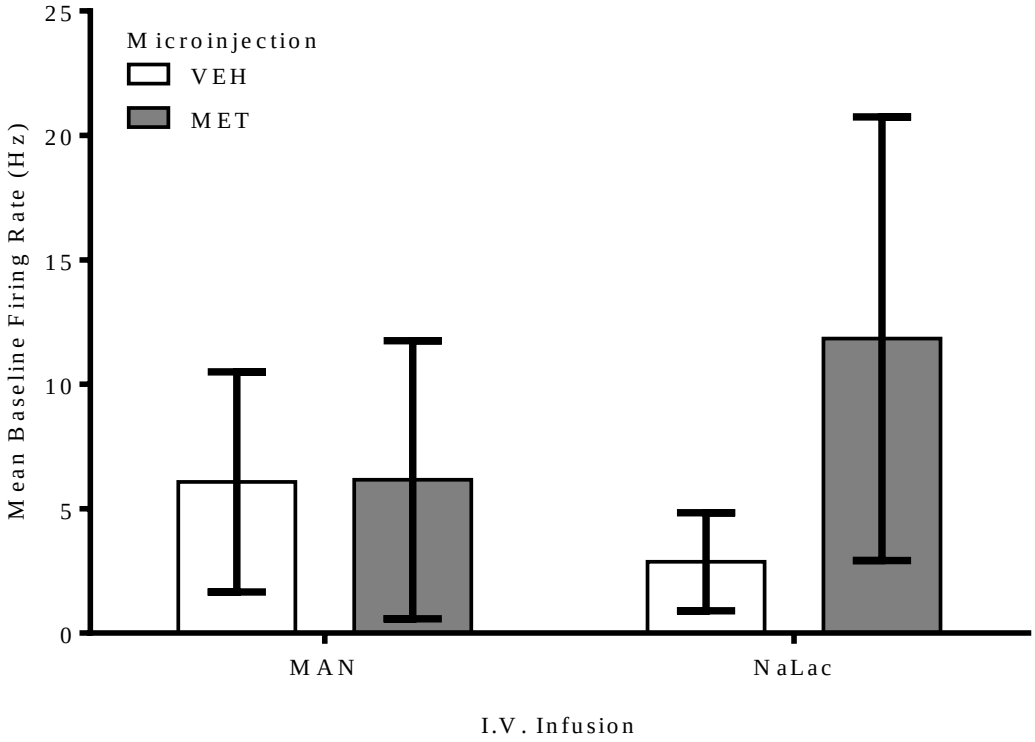

Figure 12. Baseline Firing Rate Data for Each Treatment Group.

Error bars represent the standard deviations.

To ensure the treatment groups did not differ initially, a baseline firing rate was calculated for each subject as the mean firing rate for 5min prior to the microinjection (see Table 7 for the descriptive statistics). The results of the two-way ANOVA on the baseline firing rate data indicate no significant effect of microinjection, $F(1, 20) = 3.52$, $p = .075$, no significant effect of i.v. infusion, $F(1, 20) = 0.26$, $p = 0.615$, and no significant interaction, $F(1, 20) = 3.39$, $p = 0.080$ (see Figure 12). There were no significant

differences in the baseline firing rates; therefore the firing rate difference scores are not confounded by the baseline firing rate.

Table 8. Descriptive Statistics for the Firing Rate Difference Scores (%)

	VEH			MET		
	Mean	*SD*	*n*	Mean	*SD*	*n*
MAN	39.86	31.21	7	90.14	57.01	6
NaLac	134.21	105.18	5	73.55	47.48	6

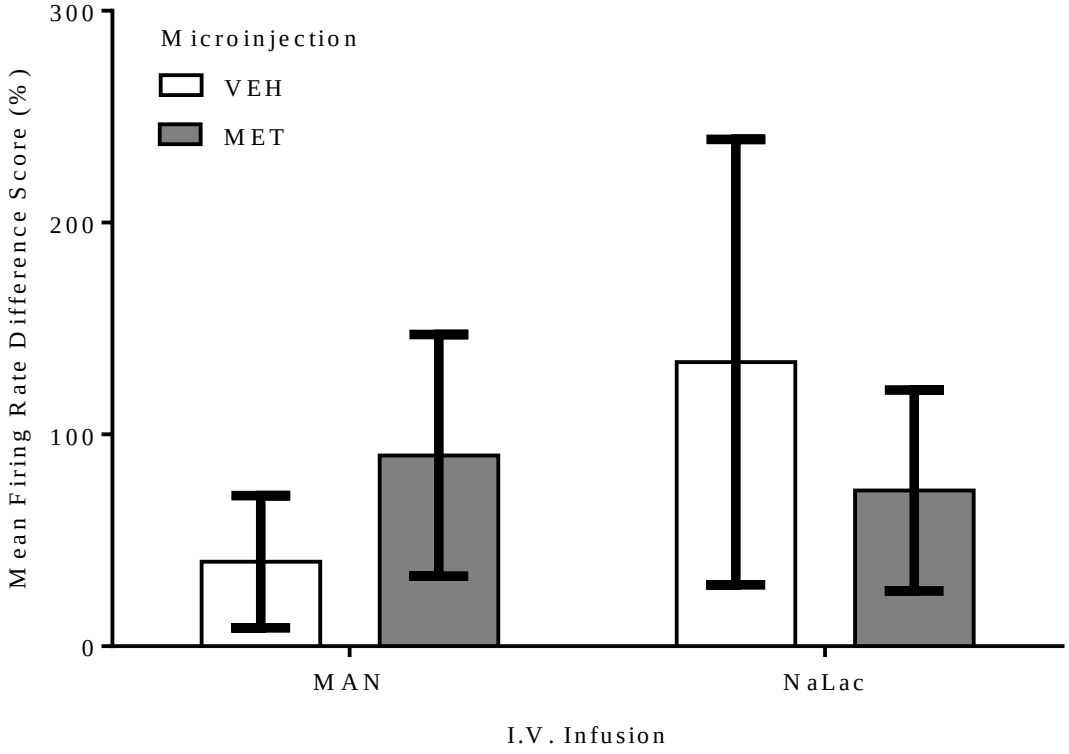

Figure 13. Mean Firing Rate Difference Scores for Each Treatment Group. Error bars represent the standard deviations.

To determine if the firing rate changes differed between treatment groups, a firing rate difference score for each subject was calculated as the percent change of the mean firing rate during the last 5min of the i.v. injection compared to mean firing rate for 5min prior to the microinjection (see Table 8 for the descriptive statistics). The results of the two-way ANOVA on the firing rate difference scores indicate a significant interaction,

$F(1, 20) = 4.69$, $p < .05$, but no significant effect of i.v. infusion, $F(1, 20) = 2.30$, $p = .145$, or microinjection, $F(1, 20) = 0.04$, $p = .841$. Comparison of the group means reveals that the i.v. infusion did not affect the MET treated subjects; however, for the VEH treated subjects, MAN resulted in decreased firing rate difference scores (see Figure 13).

Table 9. Descriptive Statistics for the VEH Treated Groups' Firing Rates (Hz) Over Time (min)

	VEH/MAN			VEH/NaLac		
Time	Mean	*SD*	*n*	Mean	*SD*	*n*
1	6.30	4.49	7	3.12	2.13	5
2	5.73	4.38	7	2.08	2.19	5
3	5.02	4.67	7	2.48	1.78	5
4	5.31	4.14	7	3.25	2.50	5
5	5.49	4.04	7	3.43	2.64	5
6	6.02	3.96	7	2.27	2.03	5
7	6.29	4.92	7	1.99	1.82	5
8	6.19	4.98	7	2.03	2.44	5
9	6.15	4.63	7	3.15	2.98	5
10	5.14	3.59	7	2.11	2.62	5
11	5.08	4.29	7	2.40	2.85	5
12	4.74	4.06	7	2.48	2.91	5
13	4.48	4.01	7	2.46	2.64	5
14	4.55	3.91	7	3.29	2.86	5
15	4.26	3.88	7	2.62	2.33	5
16	5.66	5.41	7	2.19	2.19	5
17	4.08	4.20	7	2.21	2.65	5
18	4.09	4.14	7	1.98	2.40	5
19	4.26	4.21	7	2.15	2.82	5
20	4.10	4.11	7	2.16	2.82	5
21	3.75	3.64	7	2.19	2.96	5
22	3.60	3.35	7	2.26	2.87	5
23	3.43	2.89	7	2.49	3.50	5
24	3.45	2.94	7	2.42	3.17	5
25	4.63	4.96	7	2.42	3.19	5
26	3.54	3.04	7	3.47	3.99	5
27	2.99	2.60	7	2.14	2.60	5
28	3.04	2.51	7	2.32	2.80	5
29	3.12	2.62	7	2.52	2.72	5

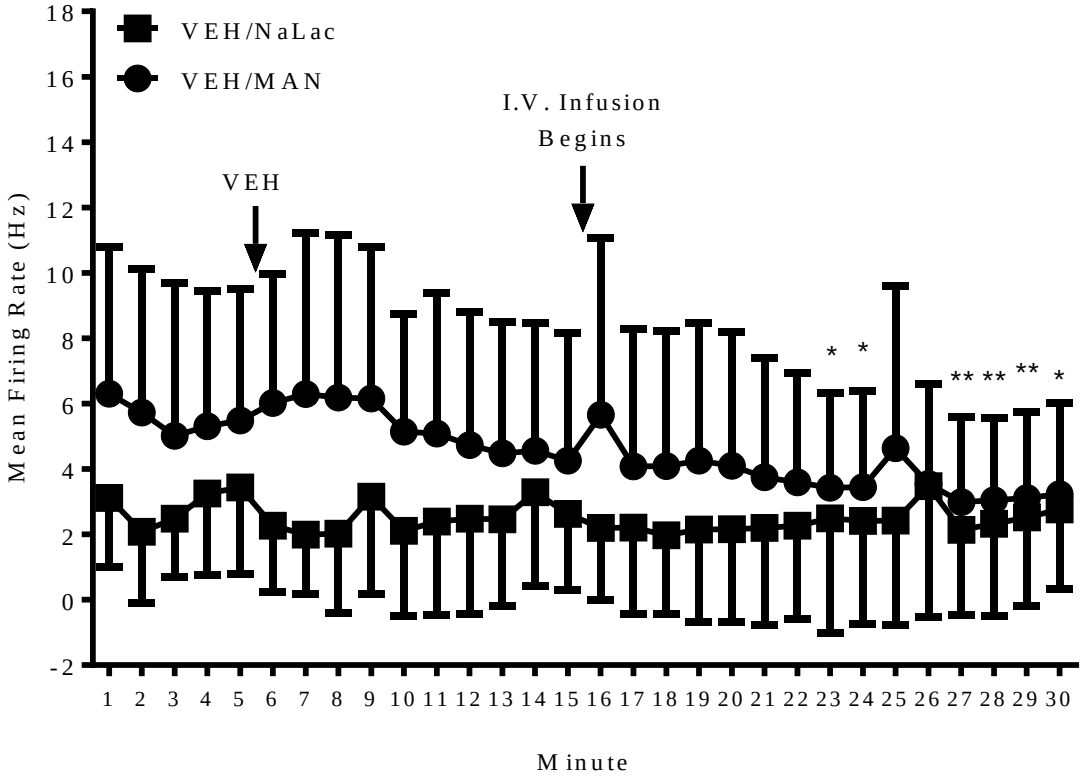

Figure 14. Mean Firing Rate for the VEH/MAN and VEH/NaLac Groups Over Time. *, $p < .05$ and **, $p < .01$ compared to minute 1. Error bars represent the standard deviations.

The VEH/MAN group had firing rate changes that were significantly different than the VEH/NaLac group; therefore, I tested if these groups exhibited significant changes in firing rates at the end of the i.v. infusion compared to baseline. The firing rate data for each subject was averaged into 60s bins (see Table 9 for the descriptive statistics) and subjected to a two-way mixed ANOVA with group as a between subjects factor and time as a within subjects factor. The results of the two-way mixed ANOVA on the binned firing rate data for the VEH/MAN and VEH/NaLac groups revealed a significant effect

of time, $F(29, 290) = 1.74$, $p < .05$, no significant effect of group, $F(1, 10) = 1.26$, $p = .288$, and a significant interaction, $F(29, 290) = 1.55$, $p < .05$. *Post hoc* Bonferonni within group analyses revealed significant decreases, compared to minute 1, in the firing rate of the VEH/MAN group at minutes 23-24 and 30, $p < .05$, as well as at minutes 27-29, $p < .01$. There were no significant changes in the firing rate of the VEH/NaLac group. Additionally, *post hoc* Sidak between group analyses revealed no significant differences between the groups at any time (see Figure 14). These results confirm that the source of the significant interaction in the two-way ANOVA on firing rate difference scores is a decreased firing rate in the VEH/MAN group.

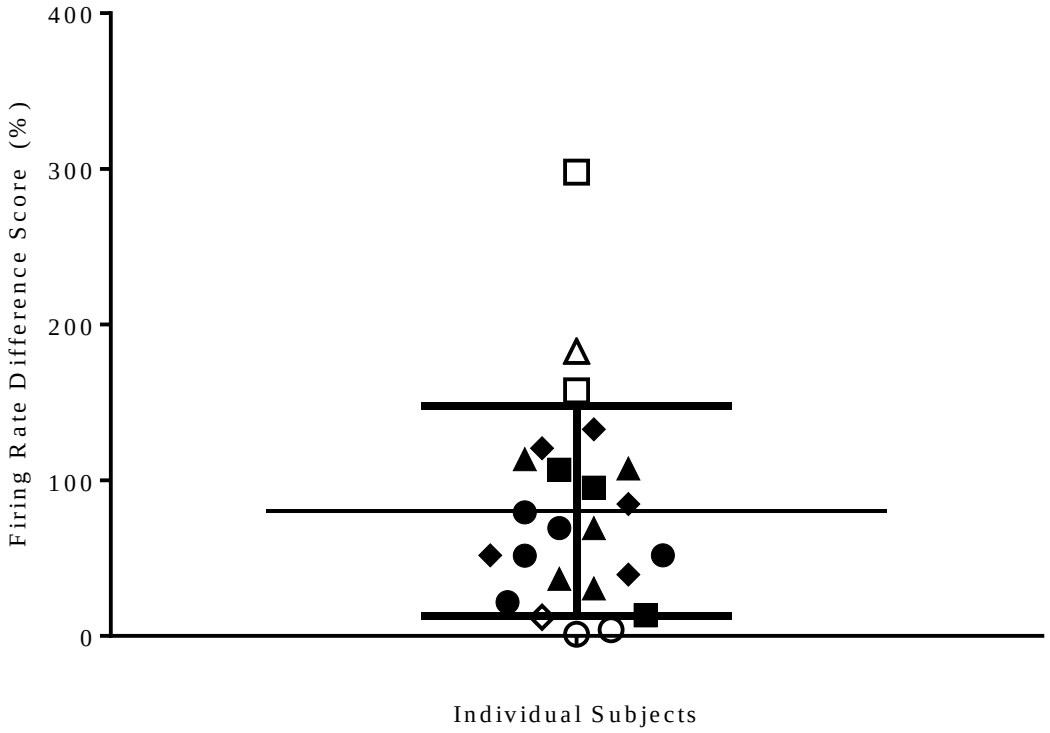

*Figure 15.*Individual Firing Rate Difference Scores.

Individual subjects from all groups are spread over the x-axis for clarity. Circles represent the VEH/MAN group, squares represent the VEH/NaLac group, triangles represent the

MET/MAN group and diamonds represent the MET/NaLac group. Open symbols represent the subjects that were considered to have exhibited changes in firing rate. The central line represents the mean and the error bars represent the standard deviation.

Table 10. Classification of Firing Rate Responses by Group

Microinjection	I.V. Infusion	Firing Rate Difference Score (%)	Response Category
VEH	MAN	51.64	No Change
		51.85	No Change
		1.05	Decrease
		79.43	No Change
		21.66	No Change
		69.41	No Change
		4.00	Decrease
	NaLac	157.53	Increase
		95.35	No Change
		13.38	No Change
		106.74	No Change
		298.04	Increase
MET	MAN	113.69	No Change
		182.73	Increase
		107.63	No Change
		69.25	No Change
		30.67	No Change
		36.84	No Change
	NaLac	39.38	No Change
		120.50	No Change
		132.81	No Change
		84.80	No Change
		12.03	Decrease
		51.76	No Change

To determine if there was any difference in the distribution of types of responses between the groups, the difference scores for each neuron were classified as exhibiting an increased firing rate, decreased firing rate, or no change in firing rate. An increased firing

rate was defined as a difference score greater than the mean plus one standard deviation, a decreased firing rate was defined as a difference score less than the mean minus one standard deviation and the remaining difference scores were classified as exhibiting no change (see Figure 15). The total numbers of neurons exhibiting decreased firing rate, no change, or increased firing rate were tabulated for each group (see Table 10). The distributions of responses for the groups were compared using a Pearson χ^2 test (see Figure 16). The results of the Pearson χ^2 test revealed no significant differences between the treatment groups, $\chi^2(6, N = 24) = 7.91$, $p = .245$. These results indicate that the significant change in firing rate detected by the previous analyses is not due to an increase in the proportion of neurons being inhibited.

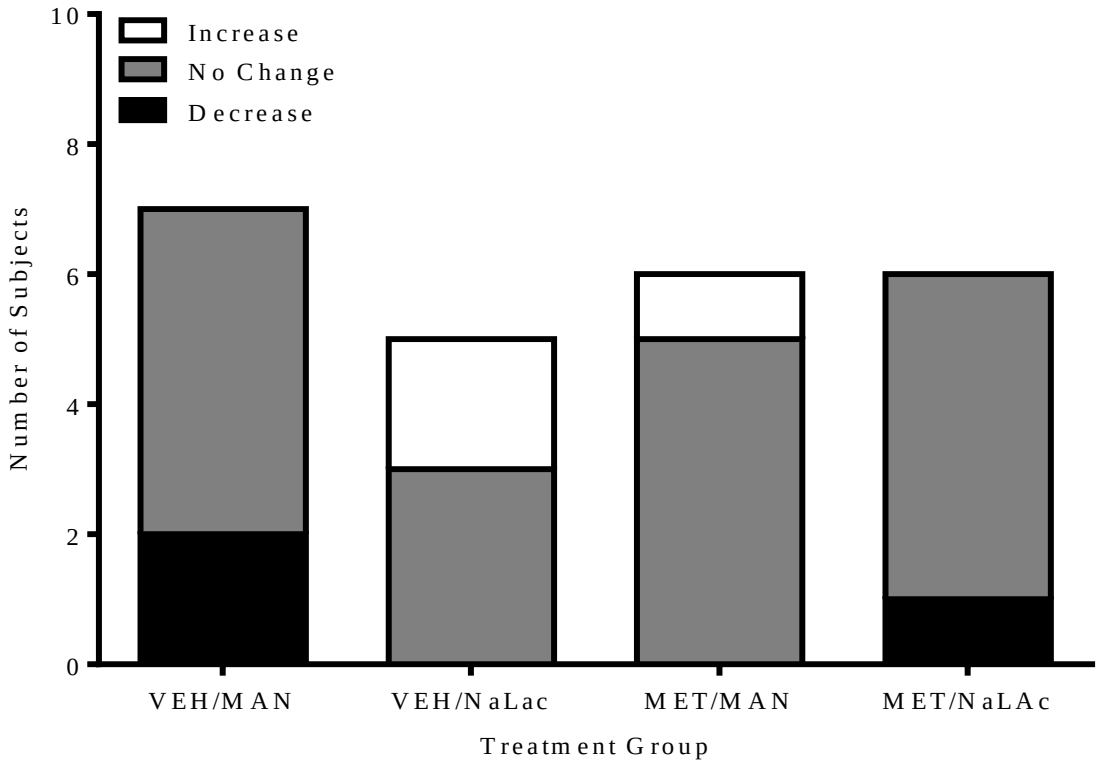

Figure 16. The Distribution of Firing Rate Responses for Each Treatment Group.

The third hypothesis, that MET will increase the firing rate of spontaneously active VP neurons, is not supported by the results of the firing rate analyses; neither is the fourth hypothesis, that NaLac will result in changes in the firing rate of VP neurons. Rather, the combination of VEH and MAN resulted in significantly decreased firing rates, and these changes in firing rate were significantly different than the other treatment groups' changes. Additionally, the changes in firing rate are not due to an increase in the proportion of neurons being inhibited.

CHAPTER VI

DISCUSSION, CONCLUSIONS AND IMPLICATIONS

Discussion

The first hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in tachycardia, is supported by the results of the study. The MET/NaLac group had significantly greater heart rates at the end of the i.v. infusion compared to baseline and the change in heart rate was greater than the change in heart rate exhibited by the other groups. Therefore, 5-HTR antagonism in the BLA is sufficient to model PD as evidenced by NaLac-induced tachycardia.

Activation of 5-HT_{2A} receptors in the BLA has been previously hypothesized to function to restrain anxiety-related behaviors (Hale, et al., 2010); however, to my knowledge this is the first study to demonstrate, via direct manipulation, that the 5-HT receptors in the BLA function to restrain panic. Inactivation of the receptors with the microinjection of MET was sufficient to cause susceptibility to NaLac-induced tachycardia. This indicates a panic-like reaction. Consistent with their hypothesized function, inactivation of 5-HT receptors in the BLA resulted in a failure to restrain the panic-like reaction to NaLac. It is important to note that MET is an antagonist at a variety

of 5-HT receptors, including 5-HT_{2A}; therefore, it cannot be concluded that inactivation of 5-HT_{2A} receptors is solely responsible for the observed effects.

Disrupted 5-HT signaling has been implicated in the etiology of PD by a number of studies; importantly, SSRIs are the preferred treatment option for PD. Studying how disrupted 5-HT signaling leads to NaLac sensitivity could improve PD treatment. Additionally, the finding that only an acute MET treatment was necessary to cause NaLac sensitivity suggests that non-pathological inhibition of 5-HT signaling in the BLA, possibly via unintentional tryptophan depletion, may be relevant to the panic attacks experienced by the general population.

The second hypothesis, microinjection of MET into the BLA followed by i.v. NaLac will result in hyperventilation, is not supported by the results of this study, because MET is not necessary for the effect. The groups that received NaLac had significantly greater respiratory rates at the end of the i.v. infusion compared to baseline. The groups that received MAN had significantly smaller respiratory rates at the end of the i.v. infusion compared to baseline. The NaLac groups had a significantly greater respiratory rate than the MAN groups at the end of the i.v. infusion.

The dissociation of the respiratory effects from the cardiovascular effects was unexpected. The observation that hyperventilation wasn't greater in the MET/NaLac group may be due to the use of respiratory rate to assess respiratory function. Evidence for hyperventilation in human PD patients has usually relied on measurements of pCO_2. One study compared various measures of respiratory function in response to NaLac and concluded that increased respiratory volume contributed to the hyperventilation response far more than increased respiratory rate did (Gorman, et al., 1988).

More pronounced, and even more surprising, than the NaLac-induced hyperventilation is the MAN-induced hypoventilation. One possible explanation for this effect is the increased plasma-osmolality produced by the infusion. With chronic manipulation in dogs, plasma-osmolality is negatively correlated with respiratory rate; and acute hypo-osmolality induces hyperventilation (Andersen & Jennings, 1988; Andersen, Sarda & Jennings, 1990). However, acute hypo-osmolality in the rat does not induce hyperventilation (O'Connor & Jennings, 2001). To my knowledge, the effects of hyper-osmolality on respiration have not been examined in the rat. It is more likely that the hypoventilation is due to the urethane anesthetic or other surgical procedures. Regardless of the cause of the hypoventilation, the modest hyperventilation produced by NaLac should be considered along with a reversal of the hypoventilation.

Two aspects of the presently reported model of PD combine to offer high throughput for future studies. The first aspect is the use of heart rate as the dependent variable indicating a panic-like reaction. Similar to established models of PD, under the control conditions the variability in heart rate is small; therefore, panic-like responses can be detected using a small number of subjects. The second aspect is the use of an acute microinjection in the presently reported model. Established models of PD, in contrast, employ chronic microinjections administered over several days. The acute microinjection allows the entire procedure to be completed on a single day while the subject is maintained under anesthesia; this also greatly simplifies the necessary surgical procedures.

The primary limitation of the presently reported model of PD, the lack of behavioral validity, is a trade-off with the high throughput design. It was observed that

the respiratory, neural and cardiovascular responses are all dissociated; therefore, it is probable that the affective responses, and consequently behavioral responses, are also dissociable from the other responses. Established models of PD describe procedures for behavioral testing of awake, behaving animals, and there is no reason the MET administration can't be employed in the described procedures. Therefore, to overcome the lack of behavioral validity, the effects of MET microinjection into the BLA followed by i.v. NaLac should be determined in behavioral paradigms that reveal the negative affective state induced by established models of PD.

The third hypothesis is that MET will increase the firing rate of spontaneously active VP neurons; the fourth hypothesis is that NaLac will result in changes in the firing rate of VP neurons. The results indicate that the VEH/MAN group had significantly decreased firing rates, and these changes in firing rate were significantly different than the other treatment groups' changes. Additionally, the changes in firing rate are not due to an increase in the proportion of neurons being inhibited; therefore the treatment must result in an enhanced inhibitory response compared to the other treatment groups. These results, though significant and interesting, do not support the hypotheses.

Changes in neural activity in the VP due to the VEH microinjections in the BLA are not entirely unprecedented. The VEH solution was determined to have pH = 4.5, and BLA neurons express acid-sensing ion channels (ASICs) which function to excite neurons when the extracellular pH is reduced (Ziemann, et al., 2009). Thus, one possible mechanism for the reduced firing rate in the VP is that the VEH microinjection excited inhibitory interneurons; the interneurons then inhibited the excitatory projection neurons from the BLA to the VP. Therefore, the excitatory drive in the VP was reduced and the

firing rate decreased. It remains to be determined how a presumably transient decrease in pH can lead to a change in neural activity 20min later; however, ASICs are permeable to Ca^{++} which can trigger intracellular cascades. Intracellular Ca^{++} signaling can also lead to genomic effects, which take some time to manifest.

The proposed reduced excitatory drive is also consistent with the observation that the proportion of neurons exhibiting a decreased firing rate did not differ between the groups. An alternative mechanism for the decreased firing rate involves recruitment of inhibitory neurons in the NAC that project to the VP. This mechanism cannot be excluded as a potential contributor to the observed effect. However, given that the axon from a single neuron generally makes synaptic contact with multiple other neurons, one would expect that this alternative mechanism would affect the distribution of responses.

The same VEH solution was used for all groups; however, only the VEH/MAN group exhibited decreased firing rates. The previously suggested explanation is compatible with the observed lack of effect in the remaining treatment groups. MET microinjections are expected to inhibit the interneurons; thus, including MET in the VEH solution results in opposing ASIC-mediated excitations and 5-HTR antagonist-induced inhibitions. The opposing effects could balance each other and result in the lack of observed changes in the VP firing rates.

The VEH/NaLac group did not exhibit decreased firing rates either. In mice, CO_2 inhalation has been demonstrated to reduce the pH of the BLA sufficiently to activate ASICs; therefore, respiratory activity, BLA pH and BLA neural activity are intimately associated (Ziemann, et al., 2009). The respiratory rates of the NaLac treated subjects increased significantly. Increasing respiratory rate is a well-known mechanism animals

use to increase blood pH. Thus, the increased respiratory rate in the VEH/NaLac could increase the pH of the BLA sufficiently to counteract the acidic microinjection and result in the lack of an observed effect on VP firing rates. In fact, NaLac infusions have been demonstrated to increase blood pH (Peskind, et al., 1998).

The proposed mechanisms for VP firing rate observations suggest a potential mechanism for the NaLac-induced panic in the MET treated group. MET microinjections into the BLA are expected to inhibit the interneurons in that structure. ASIC-mediated excitation has been observed in the BLA at pH 7.2, before any manipulation of pH was performed (Ziemann, et al., 2009). Therefore, the increased blood pH caused by a NaLac infusion could inhibit interneurons in the BLA by reducing the tonic ASIC-mediated excitation. The inhibition produced by the combination of these two treatments may be responsible for the initiation of the panic-like reaction. Changes in blood parameters due to NaLac are also detected in other brain structures (Molosh, et al., 2010). Therefore, it cannot be determined from this study alone if the effects of NaLac on the BLA are necessary or sufficient to precipitate the panic-like reaction following the MET microinjection.

The third hypothesis, that MET will increase the firing rate of spontaneously active VP neurons, was designed to determine the influence of tonic 5-HT signaling in the BLA on the firing rates of spontaneously active VP neurons. Neither of the groups treated with MET exhibited increased firing rates; however, the significant interaction in the two-way ANOVA on firing rate changes indicates that the VP firing rate is influenced by the MET microinjection. The VEH/MAN group exhibited significantly decreased firing rates; the firing rates of the MET/MAN group did not. As revealed by the reversal

of the VEH-induced inhibition, MET disinhibits VP neurons. The hypothesis is supported by the results of this study. However, the effect of MET is confounded with the effect of the VEH; therefore, the influence of tonic 5-HT signaling in the BLA on the firing rates of spontaneously active VP neurons could not be determined by this study.

The fourth hypothesis, that NaLac will result in changes in the firing rate of VP neurons, was designed to determine if the VP has a role in the response to panicogenic treatments. Neither of the groups treated with NaLac exhibited significant changes in firing rate; however, the significant interaction in the two-way ANOVA on firing rate changes indicates that the VP firing rate is influenced by the NaLac infusion. The VEH/MAN group exhibited significantly decreased firing rates; the firing rates of the VEH/NaLac and MET/NaLac groups did not. As revealed by the reversal of the VEH-induced inhibition, NaLac disinhibits VP neurons. The hypothesis is supported by the results of this study.

There were no significant differences observed in the firing rates of the VEH/NaLac group and the MET/NaLac group; therefore the VP is likely involved in the chemosensory response to NaLac infusion rather than in restraint or generation of the panic-like reaction. The response of the VP to the taste of NaCl is modulated by the sodium-load in the body (Tindell, Smith, Peciña, Berridge & Aldridge, 2006). The VP is sensitive to the sodium-load in the body and the NaLac infusion increases this load. Therefore, the proposed role for the VP in chemosensation of the NaLac infusion is consistent with previously demonstrated functions for the VP. If the role of the VP is in the chemosensation of the NaLac infusion, its function may be specific to that treatment

rather than panicogenic treatments in general. Therefore; it remains to be determined if the VP has a role in the response to panicogenic treatments.

Conclusions

This study demonstrates that acute antagonism of 5-HTRs in the BLA is sufficient to model PD, as evidenced by NaLac-induced tachycardia; respiratory rate does not appear to be a sufficient indicator of a panic-like response. It is true that the effects of the 5-HTR antagonist are confounded with the effects of the VEH solution; however, the effects of the VEH solution are reversed by the effects of the NaLac infusion; therefore, the antagonism of 5-HTRs is sufficient and the effect doesn't require activation of ASICs.

To my knowledge, this is the first study to demonstrate, via direct manipulation, that 5-HT receptors in the BLA function to restrain panic. Increased throughput is one advantage this model offers over established models of PD. Additionally, the acute treatments used in this model suggest it is relevant to non-pathological alterations in neural function that could underlie the panic attacks experienced by the general population.

Firing rates in the VP are decreased by microinjections of an acidic VEH solution into the BLA. This suggests that ASICs in the BLA are located on interneurons that inhibit the neurons projecting from the BLA to the VP. These firing rate changes were not observed in the group treated with MET, demonstrating that 5-HTR antagonism in the BLA disinhibits the VP. Antagonism of 5-HTRs prevented the VEH-induced changes. This suggests that 5-HTRs and ASICs exert similar influences over the projection neurons from the BLA to the VP. The effect of 5-HTR antagonism was confounded by

the VEH in this study; therefore, the influence of tonic 5-HT signaling in the BLA on the firing rates of spontaneously active VP neurons could not be determined.

VEH-induced decreased firing rates in the VP were reversed by the NaLac infusion; thus NaLac disinhibits VP neurons, and the VP has a role in the response to panicogenic treatments. VP neurons were disinhibited in both the VEH/NaLac group and the MET/NaLac group; therefore the VP is likely involved in the chemosensory response to NaLac infusion rather than in restraint or generation of the panic-like reaction. Its function may be specific to that treatment; therefore, it remains to be determined if the VP has a role in the response to panicogenic treatments.

Implications for Future Studies

The conclusions from this work generate several important implications for future studies. It was demonstrated that acute antagonism of 5-HTRs in the BLA is sufficient to model PD. Given the evidence implicating disrupted 5-HT signaling in PD patients, future experiments relating PD to alterations in neural signaling should consider using the presently reported model.

NaLac produced increased respiratory rates regardless of the microinjection treatment, indicating that respiratory rate is not an appropriate indicator of a panic-like reaction in rat models. In humans, hyperventilation is usually assessed by pCO_2 levels but can also be detected by respiratory volume. Given the recent discussion about a respiratory subtype of PD, it behooves researchers in the field to develop methods to properly assess hyperventilation in models of PD.

The VP is inhibited by acidic microinjections into the BLA. It is disinhibited by both 5-HTR antagonism in the BLA and i.v. NaLac infusions. Considering the anatomical arrangement of the limbic loop and the BLA, future research should determine if 5-HTR activation in the BLA functions as an inverse gain control for processing in the limbic loop. For example, microinjections of 5-HT into the BLA should inhibit VP neurons.

The VP is likely involved in the chemosensory response to NaLac infusion rather than in restraint or generation of the panic-like reaction. Given that evidence exists for a role of the VP in restraining anxiety-related responses, this conclusion does not negate that possibility; the VP may serve multiple functions. Future research on the role of the VP in PD should use other panicogenic agents to generate clear conclusions.

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