Differential Activation of Nitrergic Neurons in the Dorsal Raphe Nucleus of Acute Restraint Stressed Male Rats

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DIFFERENTIAL ACTIVATION OF NITRERGIC NEURONS IN THE DORSAL RAPHE NUCLEUS OF ACUTE RESTRAINT STRESSED MALE RATS

Committee Chair: Godwin Ananaba, Ph.D.

Dissertation dated December 2016

The Dorsal Raphe Nucleus (DRN) is a complex brain region that has been implicated in disorders such as anxiety and depression. The DRN is divided into subregions through its rostrocaudal and mediolateral axis. It has been reported that after a single restraint session there is differential spatial activation of nitric oxide synthase (NOS) across the DRN. The temporal profile of NOS activity during acute stress is not known but it is important because duration of acute stress is associated with different general responses. In this report rats were restrained for 1, 3, or 6 hours and nicotinamide adenine phosphate diaphorase (NADPH-d) was stained as an index to NOS activity to determine the spatial-temporal profile of NOS throughout a 6 hour restraint. Astrocyte reactivity was also measured to determine whether NOS activation correlated with GFAP expression since astrocytes react to neural activity and store and release l-arginine, the precursor for nitric oxide production. The results showed that the DRN had a dynamic response to acute restraint stress, most notably in the caudal lateral wings where activation increased after 3 hours of restraint (p = > 0.001) but neuron count decreased
after 6 hours (p = 0.040). Astrocytes did not correlate with NOS activation but they showed spatial-temporal differences as well whereas they were more active in the rostral half of the DRN. In conclusion, the present study suggests that NOS produced in the DRN may have a role in prolonged exposure to acute stress and that subregions show differential NOS activation.
DIFFERENTIAL ACTIVATION OF NITRERGIC NEURONS IN THE DORSAL RAPHE NUCLEUS OF ACUTE RESTRAINT STRESSED MALE RATS

A DISSERTATION

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

INDIA S. NICHOLS

DEPARTMENT OF BIOLOGICAL SCIENCES

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CHAPTER I
INTRODUCTION

Virtually every person has had to deal with some form of stress in their lifetime. Stress is a physiological or physical alteration which results in an interaction between neuronal and endocrine systems and ultimately results in the production of glucocorticoids from the adrenal glands. The process for this is termed “flight or fight” response. “Flight or fight” is a mechanism our bodies use to cope with situations that are deemed life threatening. The fight or flight response is triggered when a stressful stimulus activates the hypothalamic-pituitary-adrenal (HPA) axis, in particular, the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. Corticotropin releasing factor (CRF), the primary regulator of the HPA axis is released from the PVN. CRF reaches the anterior pituitary and triggers the release of adrenocorticotropic hormone (ACTH). ACTH then targets the adrenal cortex where the production of glucocorticoids, cortisol in humans and corticosterone in rodents, is stimulated and ultimately released. Constant stimulation of the fight or flight response is often associated with depression.¹

Depression from overstimulation of the HPA axis stems from disruptions in neurotransmitters involved in mood regulation. For example CRF links stress and depression through regulation of serotonergic cells in the dorsal raphe nucleus (DRN).²
The DRN is the major source of 5HT in the brain and it is highly implicated in anxiety disorders and depression.\(^3\text{-}^6\) However, at least 50\% of the DRN is non-serotonergic with a large portion of NO producing neurons which have a unique topographical distribution and colocalization pattern across the DRN.\(^7\)

The most common class of antidepressants is serotonin specific reuptake inhibitors (SSRI) such as Paxil, Prozac, and Zoloft. SSRI’s function by blocking receptors called reuptake receptors on the presynaptic neuron and astrocytes that would take up the neurotransmitter after a signal has been transmitted. Blocking 5-HT reuptake receptor maintains 5-HT in the synaptic cleft, and it continues binding to the postsynaptic receptor, transmitting a signal. Some classes of serotonin and norepinephrine reuptake inhibitors, such as Effexor, suppress nitric oxide as secondary mode of action. Studies show that the presence of nitric oxide synthase (NOS) inhibitors have antidepressant effects.\(^8\) The problems with current antidepressants are: (1) less than 50\% of patients show full remission, (2) there is possible increase of suicidal tendencies, and (3) it has no effect in about 40\% of patients.\(^9\) So it is necessary to develop new drug targets and understanding the role of NO in the pathogenesis of acute stress may help identify NO as a potential therapy.

1.2 Background

The dorsal raphe nucleus (DRN) is a topographically unique brain region that is functionally organized into distinct subregions based on the localization of serotonin (5-HT) neurons. The subregions are divided into rostrocaudal and mediolateral dimensions. The DRN has been implicated in both depression and anxiety disorders.\(^6\) Although the
nucleus contains the densest population of 5HT neurons in the brain there is a large non-5HT domain which includes NOS neurons. Similar to 5HT, NOS neurons maintain a unique topographical distribution and colocalization pattern across the DRN.

Nitric Oxide synthase (NOS) neurons have been found to colocalize with tryptophan hydroxylase (TPH), the enzyme that produces 5HT, across the midline of the DRN in approximately 75% of cells. In contrast, NOS does not colocalize with TPH in the lateral wings of the DRN, but there is evidence of 5HT receptors colocalizing with NOS. Also, recently it has been revealed that the NOS cells in the most caudal lateral wings colocalize with cholinergic cells of the lateral dorsal tegmental. NOS neurons in the caudal lateral wings are highly implicated in the stress response. Acute restraint stress shows differential NOS activation across subregions of the DRN where the rostral ventromedial region and caudal lateral wings were the most activated. Meanwhile, NOS produced in the DRN plays a role in antidepressant-like and anxiolytic-like effects.

These studies combined with the present study provide evidence that the caudal lateral wings are an important component in how the DRN processes stress information. Here we will add that there is a differential temporal response especially in the lateral wings which highlight the importance of the lateral wings in the stress response.

1.3 Rationale
Restraint stress increases NOS activation in several brain areas including the DRN. Previous studies showed a significant increase of NOS activity in the DRN following a single restraint. The increase was particularly seen in caudal lateral wing
regions where NOS is independently localized from serotonin. Other areas of the brain that show differences in NOS activation are the paraventricular nucleus of the hypothalamus and the supraoptic nucleus of the hypothalamus. Both display a significant increase of NOS activation at 3 hours then a decrease at 6 hours. There is also evidence that NO produced in the DRN is involved in antidepressant-like behavior.\textsuperscript{16}

Duration and modality of restraint has varying effects on the rat brain and behavior. Rats that are exposed to single episode of an hour restraint may show signs of anxiety or depression up to 24 hours later.

The objective of this experiment is to reveal NOS activation patterns in the DRN in a spatial-temporal manner. Astrocyte density is analyzed because it is a possible source for NOS to obtain arginine; therefore, increased reactivity of astrocytes may correlate to NOS activation.

The hypothesis of this study is that NOS activation and GFAP expression will be differentiated throughout the DRN as stress is processed over a 6 hour length of restraint stress.

\textbf{1.4 Research Questions}

1. What is the spatial-temporal profile of NADPH-D in the DRN in response to acute stress?
2. Does astrocyte density and CAT expression correlate with NOS activation during stress?
1.5 Hypotheses

1. Nitric Oxide synthase activation is differentiated in a spatial-temporal manner in the dorsal raphe nucleus.

2. Astrocytic density will be a reflection of NOS activation.

1.6 Specific Aims

*Objective 1*: Demonstrate that NOS activation is differentiated in a spatial-temporal manner.

Specific Aim 1: **Determine the pattern of NOS activity in the dorsal raphe nucleus in response to increasing lengths of restraint stress.** Using NADPH-d to determine NOS activation within the topography of the DRN will reveal the pattern of NOS activity in each subregion at different lengths of restraint.

Specific Aim 2: **Determine differences in the spacing of swellings on nitrergic axons of the DRN following different lengths of restraint stress.** Nitric oxide diffuses out of the cells as soon as it is produced therefore it uses nonsynaptic release. However, nonsynaptic release through varicosities allows for a summation of NO. An increase in intervaricosity density may provide evidence that there increased NO being released from fibers projecting to the DRN. This aim will provide evidence on how varicosity axons of the DRN change in response to stress by measuring inter-varicosity distance for each region and compare the mean spacing. Studying the fibers will show whether a particular region has more varicosity fiber innervation compared to other regions and if
there is a difference in intervaricosity space in stressed rats compared to non-stressed rats.

*Objective 2:* Demonstrate the pattern of astrocyte activity in the DRN following different durations of restraint stress.

*Specific Aim 3:* **Determine whether astrocytes respond to acute stress in the DRN and if there are differences in expression throughout the DRN**

Since astrocytes have been shown to be reactive in stress signaling, here we will use antiGFAP to stain astrocytes and observe/measure differences in astrocytic reactivity during restraint stress.
CHAPTER II
LITERATURE REVIEW

2.1 Dorsal Raphe Nucleus

The dorsal raphe nucleus (DRN) is a heterogeneous brain structure containing axons that modulate all levels of the brain and therefore is implicated in a variety of functions. It extends from the nucleus of Edinger Westphal, through the pons, and into the rostral portion of the medulla at the level of the tegmental nuclei. In the horizontal plane it is between the cerebral aqueduct and fourth ventricle and extends ventrally between the trochlear nuclei and medial longitudinal fasciculi (Figure 1). The nucleus has a role in sleep, antidepressant, anxiolytic effects, and transmission of nociception. It is a neurochemically complex structure that accounts for about 40-50% of serotonin (5-hydroxytryptamine, 5-HT) production in the brain. The DRN is anatomically defined by clusters of 5-HT-containing cells which run in a rostral-caudal manner. These clusters create subdivisions, which are: dorsomedial (DRD), ventromedial (DRV), and bilateral (lateral wings) sub-regions (DRL) (Figure 2). Given that the DRN is responsible for most of the brain’s serotonin and it innervates all levels of the brain, the main function of this region was once thought to release 5-HT to all levels of the brain at the same time. However, there is a large non-5HT domain of the DRN, which includes dopamine, GABA, and/or NO.
**Figure 1.** Paxino and Watson Adaptation of Dorsal Raphe Nucleus. The dorsal raphe nucleus (images adapted from Paxinos and Watson rat brain atlas) Coronal sections: (a) Bregma -6.3mm, (b) Bregma -8.3mm, (c) Bregma -9.3mm. Horizontal section with DRN between aqueduct and 4\textsuperscript{th} ventricle. The dorsal raphe nucleus is distinguished by the aqueduct (red box). As you move towards more caudal regions the aqueduct becomes larger.
These neurochemicals may interact with serotonin by co-localizing in the same neuron or may act independently of serotonin.\textsuperscript{15,20} As a matter of fact at least 70\% of neurons in the midline co-express NOS and 5-HT but nitrergic neurons in the caudal lateral wing are completely independent of 5-HT (Figure 3).\textsuperscript{7,15} Furthermore, subregions of the DRN are differentially activated in response to stimuli and produce different responses depending on the region activated. For example mid-caudal parts of the DRN are involved in anxiety processing while the DRL subregion is responsive to panic-evoking situations.\textsuperscript{17}
Figure 3. Serotonin and nNOS in the DRN. Okere and Waterhouse\textsuperscript{15} showed serotonin and nNOS localization in the DRN. Serotonin (red) co-localizes with nNOS (green) in the rostral midline but the caudal DRN shows complete independent localization of nNOS and serotonin.

Also in response to acute stress, studies report that the caudal-DRL exhibits more nitrergic neural activity than the rostral-DRL while the rostral DRD and DRV show more nitrergic neural activity than their respective caudal regions.\textsuperscript{15} These findings may be due to the fact that the subdivisions have different paths for their axonal projections.

DRN divisions are important because each subregion sends axons to different parts of the brain. The rostrally located DRD and DRV regions project largely to cortical structures while the lateral wings project only to subcortical structures.\textsuperscript{7,22} These subcortical structures include the thalamus, hypothalamus and amygdala, which are involved with sleep and attention, stress regulation, and emotions and fears, respectively. The hippocampus region receives moderately dense projections from the caudal DRN.
and virtually none from the rostral DRN. However, neocortical regions, like the temporal lobe, receive significantly denser projections from the rostral DRN than the caudal DRN. Dorsal raphe cells that give rise to cortical projections are clustered in three distinct rostral to caudal groupings, such that neurons projecting to motor, somatosensory, and visual cortices are concentrated in the dorsomedial, suprafascicular ventromedial, and interfascicular ventromedial subregions of the DRN, respectively.

Generally, the rostral midline DRN is associated with motor functions and has specific connections to the caudate–putamen, basal ganglia, striatum, and motor cortex whereas the caudal midline regions are implicated in stress and anxiety responses.

Lastly, the DRN is important in mood regulation and psychological disorders. Its size and 5-HT component are different among several mental health disorders. In major depressive disorder the area of the DRN is decreased, bipolar disorder is characterized by a decreased size serotonergic neurons, and suicide is characterized by an increased DRN area and higher density but decreased size of serotonergic neurons.

2.2 Physiological Nitric Oxide

Nitric Oxide (NO) is a diffusible gas synthesized from L-arginine by nitric oxide synthase (NOS) (Figure 4). There are three isoforms of NOS; neuronal NOS (NOSI or nNOS), endothelial NOS (eNOS, or NOSIII), and inducible NOS (iNOS or NOSII). The amino acid sequence homology between the subtypes is less than 59% in the same species and higher than 80% for the same subtype between species. iNOS is normally found in the cytosol of cells in the immune system, like macrophages.
Figure 4. Synthesis of NO from Arginine. Synthesis of NO from l-arginine by NOS schematic by Aurelio et al., 2009. Following an increase in calcium concentrations, calmodulin binds to NOS and activates it. L-arginine is oxidized to NO and citrulline in the presence of 4 other co-factors including NADPH which is also oxidized to NADP+. Once NO is produced it diffuses out of the cell and binds to guanylyl cyclase (GC) where cGMP is ultimately produced.

Unlike nNOS and eNOS, iNOS tightly binds calmodulin and its activity is calcium independent. It is stimulated in response to cytokines or microbial agents and plays a role in the inflammatory response. Inducible NOS is also involved in stress induced depression. In fact, after 4 weeks of an unpredictable chronic mild stress there is increase in iNOS expression in the cortex.

eNOS is constitutively expressed and generally found in the endothelium of blood vessels. It produces NO that is responsible for blood vessel dilation via binding to the heme group of guanylyl cyclase and increasing cGMP production.

nNOS is generally localized in neurons of the central and peripheral nervous system, it is also constitutively expressed, binds guanylyl cyclase, and increases cGMP production. nNOS activation is induced by increases in intracellular calcium levels, glucocorticoids at the mRNA level, and transcription factors. nNOS uses a PDZ
motif to bind to NMDA receptor.\textsuperscript{31} When glutamate binds to NMDA receptor it subsequently activates nNOS by increasing intracellular calcium.\textsuperscript{31}

Unlike other neurotransmitters, NO diffuses out of a neuron upon its synthesis and binds directly to its target cell. Therefore, it can use both synaptic and nonsynaptic transmission. It also acts retrogradely on a presynaptic cell from a postsynaptic cell while most neurotransmitters cannot.\textsuperscript{35}

Detecting NO is difficult because it is highly reactive and has a short half-life. Therefore, the localization and activation of NOS is widely used in studies. NADPH-diaphorase histochemical technique is used based on the fact that all NOS enzymes contain a binding site for NADPH-d and display diaphoretic activity (Figure 5).\textsuperscript{31} So NADPH-d is a widely accepted marker for NOS activity. In fact, early studies show that in both brain and peripheral tissue NOS and NADPH-d are identical.\textsuperscript{36} Nitroblue tetrazolium is the substrate that binds to NADPH-d which reduces tetrazolium to a blue-black formazan in the presence of NADPH. Therefore, the more active enzymes in a neuron the more formazan will be produced and the more intense the blue-black stain. Other techniques to detect NOS are immunohistochemistry antibodies raised against each isoform, cDNA or cRNA probes to determine expression of their respective mRNAs, and/or assays that measure L-citrulline, cGMP, nitrites, and nitrates.\textsuperscript{31}

As a neurotransmitter, Nitric Oxide (NO) acts a modulator and has been linked to memory, learning, and antidepressant affects.\textsuperscript{16, 37} It may act as a reuptake inhibitor for other neurotransmitters and also aids in synaptic plasticity.\textsuperscript{38}
Neurotransmitter uptake by nerve endings is a mechanism of inactivation for many released neurochemicals, such as dopamine, glutamate, and serotonin, hence the reason most antidepressants block the uptake of 5-HT.\textsuperscript{38} Nitric Oxide also potentiates the role of neurotransmitters. It does this for GABA in the medulla oblongata by acting on the presynaptic neuron.\textsuperscript{39} By NO increasing GABA activity it has an inhibitory role because GABA is an inhibitory neurotransmitter.

Nitric Oxide is able to shut down both NOS activation and expression through a negative feedback regulation.\textsuperscript{40} A heme-iron bond on NOS may either be reduced.

\textbf{Figure 5.} Various types of varicose fibers in caudal DRL. Red arrows indicate varicosities on axons.
(ferrous) or oxidized (ferric) and both ferric- and ferrous-nitrosyl complexes exist with NOS, which leads to inhibition.\textsuperscript{41} nNOS largely employs inhibition by ferrous-nitrosyl complex regardless of the NO concentration (70-90\%).\textsuperscript{42} For all NOS, a loss of activity appears when heme binds NO that accumulates in a solution as a consequence of chemical equilibrium.\textsuperscript{43} NO is able to inhibit NOS expression for both eNOS and iNOS.\textsuperscript{44}

2.3 Nitrergic Neurons and Nitric Oxide Activity

NO plays a regulatory role in the stress response. It is highly expressed in the HPA axis where it modulates the release of ACTH and corticosterone.\textsuperscript{31} Furthermore NOS activation is increased in the HPA axis following stressful stimuli.\textsuperscript{31} In depression NO production increases and inhibition of NO production has antidepressant like effects.\textsuperscript{45-46}

Previous studies show that nitrergic neurons co-localize with 5-HT in the rostral dorsal and rostral ventral regions of the DRN but are independent of 5-HT in the caudal lateral wings (Figure 3).\textsuperscript{15} Acute restraint stress increases NOS activation in all subregions of the DRN but most significantly in the caudal DRL.\textsuperscript{15} It was also shown that NO produced in the DRN has antidepressant-like and anxiolytic-like effects.\textsuperscript{16} Spiacci Jr. showed that when L-Arg was injected into the DRN, it significantly increased anxiolytic-like behavior and an L-arginine inhibitor produced anxiety-like and depressant-like behaviors. In general though, it has been shown that inhibitors of nNOS induce antidepressant-like effects in animal models and a 2012 study concluded that stress induced NO from iNOS may contribute to depressive-like behaviors in mice.
models.\textsuperscript{8, 33, 47-48} All together this shows that NO produced in the brain has different effects depending on where it is produced and that NO produced specifically in the DRN lateral wings may play a role in antidepressant and anxiolytic behaviors.

Besides depression and anxiety behaviors, NO has been implicated in schizophrenia and bipolar disorder.\textsuperscript{49-50} Postmortem brain sections show an increase in NOS concentrations in the cerebellum of schizophrenic patients and overall NOS activity was higher in platelets of drug naïve schizophrenic patients compared to healthy individuals and drug-treated schizophrenic patients.\textsuperscript{51-52} Patients with bipolar disorder have higher levels of nitrites in their plasma compared to healthy patients.\textsuperscript{47}

NO is involved with sleep regulation so there is no surprise that its concentration is on a circadian cycle. In various brain regions NOS activity was highest during the dark period and lowest in the light period. More specifically, the hypothalamus had highest NOS activity between 3:00 pm and 3:00 am.\textsuperscript{53}

Nitrogic efferents of the DRN are a topic that needs further exploration, but with at least 70\% of midline cells co-expressing both 5HT and NOS it is likely that 5HT projections and NOS projections of this region are the same. This is especially evident in the spinal cord and forebrain where 80\% of 5-HT neurons also contain NOS. In contrast, in descending spinal cord regions 5-HT neurons do not contain NOS.\textsuperscript{7} Nitrogic only neurons have been reported to project to the barrel field cortex of the trigeminal pathway, 70-80\% of those neurons coming from the midline, and 30-40\% of DRL neurons project to the ipsilateral ventral posteromedial nucleus of the thalamus (VPM), and the contralateral principal nucleus of V (PrV).\textsuperscript{7, 22} This is further supporting evidence that
lateral wing projections are solely to subcortical structures and there are functional topographical differences of the DRN.

Other brain regions besides the DRN where NOS is present include the hypothalamus. In the hypothalamus NO regulates the release of several hormones including CRH. It is involved in the hypothalamic-pituitary response to cytokines. Cytokines are induced by lipopolysaccharides and viral products. Interleukin-2 activates nNOS and NO induces the release CRH. The presence of NOS and the role of NO in the hypothalamus is important because this is a region where the DRN projects axons and previous studies have also showed that restraint stress alters NOS activity in the hypothalamus.

2.4 Dorsal Raphe and Varicose Fibers

Just as the DRN projects to all levels of the brain it also receives input from all levels of the brain. The DRV and DRL both receive input from the same brain regions however the lateral wings received some inputs from brain regions that the DRV does not. Some of the regions that project to the DRV and DRL are: anterior perifornical nucleus, ventral part of the nucleus of the stria terminalis, lateral preoptic area, paraventricular nucleus of the hypothalamus, lateral and posterior hypothalamus, periventricular region of the third ventricle, ventral tegmental area, dorsolateral periaqueductal gray, pedunculopontine tegmental nucleus, laterodorsal tegmental nucleus, medial parabrachial nucleus, dorsomedial and ventrolateral tegmental areas in pons, median raphe and raphe magnus nuclei, gigantocellular nucleus, lateral paragigantocellular nucleus, prepositus nucleus, within the paramedian myelinated bundles in medulla oblongata and, finally,
nucleus of the solitary tract. The DRL also has a unique set of projection fibers which are
the diagonal band of Broca, rostral DRN, lateral parabrachial nucleus, caudal most
pontine gray medial to the genu of the 7th nerve and medial vestibular nucleus. 7 The
paraventricular nucleus of the hypothalamus is noteworthy because it is the main
component of the hypothalamus involved with the stress response and other autonomic
functions and it projects to both the DRV and DRL.

The characteristics of incoming fibers are important because they give
information like relative signal transmission speed and how neurotransmitters are
released. Thick axon fibers tend to increase conductance and therefore offer faster
transmission over longer distances than thin fibers.54 Some axons have fibers that have
the appearance of beads on a string; those beads are called varicosities or axon boutons
(Figure 5).

Varicosities allow for neurotransmitters to be released before they reach the
synapse, therefore release is nonsynaptic. These varicosities are up to 2 µm in diameter
and about 3 µm in length and similar to traditional synapses, are packed with vesicles and
mitochondria.55 Varicosities contain neurofilaments and the distance between them
called intervaricosity distance can be as little as 0.2 µm.55 The inter-varicosity distance is
important for several reasons. The mean spacing between varicosities is a reflection of
synaptic density and varicosity density along the axon can be an index of localized
neuronal interactions.56 Information about varicosities is important for drug discovery
because they allow for release of transmitters outside of a synapse and therefore allow for
binding to high affinity extra synaptic receptors.57 The midbrain periaqueductal gray
(PAG) which mediates the emotional coping response to different stressful paradigms
displays an increase in varicose axons as well as a decrease in distance between varicosities on an axon.\textsuperscript{58}

NO, as mentioned earlier, uses both nonsynaptic and synaptic forms of transmission. Varicosities are beneficial for NO release because it provides multiple sources for NO to be released. When NO is released from multiple sources with a distance of \(<200 \, \mu\text{m}\) from each other it summates and diffuses at a further distance than it would from a single source.\textsuperscript{59}

\textbf{2.5 Stress}

There are several types of psychological stress including; acute stress and chronic stress. Chronic stress is a debilitating type of stress that may stem from childhood trauma. It is a daily occurrence and people who experience it often cannot see a way out of their situation which they view as miserable.\textsuperscript{60} Acute stress is momentary and comes from past or future events. Some symptoms of acute stress are: emotional distress, muscular problems, heartburn and other stomach problems.\textsuperscript{60} An example of acute stress would be getting in a car accident or planning for a big event. Acute stress may also transition to chronic stress. For instance an individual being laid off at work is an acute stress situation, but when the job search is prolonged it could lead to chronic stress. Also other more serious types of acute stressors such as experiencing death could trigger emotions such as depression, irritability, and anxiety. All of these examples of stressful events trigger the HPA axis response.

Stress may also help worsen or increase risk of conditions such as obesity, diabetes, and/or cancer.\textsuperscript{61-62, 63} Affective disorders, such as depression, are associated
with stress which may be triggered by either chronic or acute stressors. When a person is overly stressed it leads to over activity of the HPA axis, reduced serotonin, and increased NO production amongst a host of other differences. An overactive HPA axis leads to increased levels of cortisol and impaired feedback regulation. Receptors for cortisol become desensitized which leads disturbances in neurotransmission.

Restraint stress is an uncontrollable stress situation that influences changes in behavioral and autonomic responses, like mean arterial pressure and heart rate increase. Restraint stress induces anxiety and depressive like behaviors in rats. Animals exposed to restraint stress showed decreased exploratory activity in an open field, increased immobility in a forced swimming test, and reduced exploration of the open arms of an elevated plus maze. The psychological and physical changes associated with restraint come from distress and aversive nature of having to remain immobile.

Some papers use immobilization while others use restraint as techniques to induce stress. The difference between restraint and immobilization is that immobilization limits range of locomotion without inhibiting movement of specific limbs. An example of immobilization is putting an animal in a restraint device, leaving room for their head and tail, the limbs are free but the area is too small for the animal to move about. An example of restraint is taping an animal’s limbs to a board or pad, so that movement restriction is placed specifically on the limbs.

A single episode of restraint is considered a model for acute stress. Chronic stress is modeled by consecutive days of being restrained, so a 1 hour restraint for 7 days is an example. The length of time an animal is in restraints corresponds to different behaviors, chemical release, and neuronal activation. For example one hour of restraint shows an
increase in ACTH levels, increase in corticosteroid levels, and an increase in anxiety behavior. 2-5 hours of restraint correspond to an increase in ACTH response, decreased level of activity and exploration, decrease in spatial memory, and increase in hippocampal NO, while 6 or more hours shows dendritic retraction, decreased spatial memory, and decreased reward related motivation. Although just a 15 minute restraint is long enough to induce ACTH and corticosterone secretion, decrease food intake, increase CRF, and increase neuron activity.

Restraint stress effects NOS activation in other brain regions. In the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, NOS activation is significantly increased after a 3 hour restraint but after 6 hours of restraint there is significant decrease. The periaqueductal gray showed significant increases in the number of varicosities on axons and NOS activation following a 3 hour restraint.

2.6 Astrocytes

Astrocytes are a type of glia that plays many roles in the brain. They are the most abundant type of cell found in the central nervous system. The various roles of astrocytes include assisting with neural signaling, reuptake and breakdown of neurotransmitters from the synaptic cleft, maintain ionic homeostasis in microenvironment, and provide neurons with nutrients. Astrocytes were originally organized into two distinct morphological categories, fibrous astrocytes or protoplasmic. Fibrous astrocytes have many extended processes and are located primarily in white matter. Protoplasmic astrocytes have many short branched processes and are found mainly in gray matter. However, other morphological types have been found to be significant in different brain
regions. For example in the olfactory bulb layers, which are anatomically and functionally distinct, there is a differential distribution of astrocyte morphology subtypes. More specifically, there were 6 subtypes identified: unipolar, irregular, wedge-shape, circular, semicircular, and elongate. These subtypes are characterized by the shapes of their processes, the extension of their processes, and the overall astrocyte shape. The presence of different morphological types in functional distinct layers of the olfactory bulb suggests that astrocyte morphology is important and may have functional relevance.

Astrocytes are important in neural signaling in that they participate in neurotransmitter reuptake and breakdown and may provide transmitters to both postsynaptic and presynaptic cells. Reuptake receptors on the astrocyte are an important function of synaptic regulation. When a neuron is fired into the synaptic cleft reuptake receptors take up neurotransmitters that do not bind to postsynaptic receptor, which effectively stops the signal from transmitting any further.

Astrocytes have structures on their processes called endfeet, which surround blood vessels in the brain. Endothelial cells of the blood brain barrier form very tight junctions, which only small molecules such as O₂ and CO₂ can cross. Larger molecules are regulated through various transport systems. Glucose passes through on GLUT transporters and neutral amino acid on L1 amino acid transport system.

2.7 Astrocyte Plasticity

Astrocytes express glial fibrillary acidic protein (GFAP) which is an intermediate filament cytoskeletal protein; it is also a marker for reactive astrocytes. Astrocytes often become reactive as a response to neural injury and neurodegenerative diseases reflecting
an increase in the number and size of cells expressing GFAP.\textsuperscript{78} GFAP may be necessary for the formation of stable glial processes in response to neuronal signals. Changes that occur in reactive astrocytes may also be a reflection of increase in astrocyte metabolism and protein synthesis as a response to changes in the microenvironment.\textsuperscript{79} Evidence shows that the transition of astrocytes from resting to activation is associated with the expression of new molecules not normally detected in resting astrocytes and upregulation of molecules normally present in low levels. For example after facial motor neuron injury astrocytes express NADPH-d and there is increased NO production.\textsuperscript{80} Increase in the cytoskeleton proteins, GFAP may assist with wound repair by either helping to strengthen the tissue surrounding injury or glial scarring may help form a wall to prevent neuronal injury from spreading or help form a barrier to hinder regenerating neurites from growing out.\textsuperscript{79}

Changes in GFAP expression have been associated with chronic stress and depression. Animals exposed to activity stress for 6 consecutive days showed an increase in GFAP expression in the hippocampus.\textsuperscript{81} While in human and rat’s depression is marked by a decrease of GFAP expression in the limbic region.

### 2.7 Arginine Transport

The brain gets arginine from 4 major sources which are: (1) From the blood transported to brain tissue through the blood brain barrier (BBB); (2) It may come from the cerebral spinal fluid transported through the choroid plexus ependymal cells; (3) It may derive from protein degradation; and (4) It may be recycled from citrulline via argininosuccinate.\textsuperscript{82,83,84}
Arginine is recycled from citrulline in many cells. There are two enzymes that must be present in order for recycling to take place. Citrulline and aspartate are used by argininosuccinate synthetase (ASS) to form argininosuccinate. Argininosuccinate is then cleaved by argininosuccinate lyase (ASL) to form arginine.\textsuperscript{84} Immunohistochemical experiments showed differential expression of ASS and nNOS neurons and many NOS neurons did not contain both, like NOS neurons of the periaqueductal gray. There was some colocalization for example neurons in the dorsolateral tegmental area contained both nNOS and ASS.\textsuperscript{85} In situ hybridization showed co-localization of both ASS and ASL in all nNOS neurons.\textsuperscript{84}

Arginine from the BBB is obtained by astrocytes via a family of transporters that facilitate the transport of cationic amino acids called y+ transport system. The y+ transport family consists of three types of cationic amino acid transporters (CAT). The transporters are responsible for the transport of arginine, lysine, and orthinine across the BBB.\textsuperscript{82} The 4 CA transporters are: CAT-1, CAT-2A, CAT-2B, and CAT-3 each differently expressed in rat brain cells.\textsuperscript{86,87} CAT-1 is expressed in neurons, astrocytes, oligodendrocytes, and endothelial cells, CAT2B is in neurons and oligodendrocytes, and CAT-3 is detected in neurons only.\textsuperscript{88}

Astrocytes act as a site of storage and mediator between arginine and neurons.\textsuperscript{89-90} In fact, nitrergic fibers and endothelial cells are often localized adjacent to arginine positive astrocytes.\textsuperscript{91}

Given that arginine is stored and released from astrocytes where nitrergic containing cells are in close proximity the proposed model for glia-neuron interaction concerning arginine is: Arginine is released from astrocytes after stimulation by non-
NMDA glutamate receptors, after it is released it is transported to NOS containing neurons via y+ transport system where it is utilized by NOS to produce NO (Figure 6).92

**Figure 6.** NOS-Glia interaction. Arginine transport by y+ transport system following astrocyte stimulation by glutamate.
CHAPTER III
MATERIALS AND METHODS

Ethical Considerations

The animals were cared for per the Morehouse School of Medicine Internal Animal Care and Use Committee (IACUC). They were housed in a climate controlled environment. The restraints used were specifically designed to accomplish research goals that are unmanageable or impractical to accomplish by any other means. Provisions were made for observation of the animals at appropriate intervals. The animals had access to food and water except when restrained.

3.1 Animal Care Statement

Male Long Evans rats were maintained in the local animal facility under standard environmental conditions and had unlimited access to rodent chow pellets and water. Experimental and control rats were age- and weight- matched because of age dependent variables in endogenous NOS activity. The experiments conformed to the local and NIH regulations guiding the use of experimental animals for scientific investigation.

3.2 Study Design

Twenty four (24) Long Evans rats were used for this study. Their weights were between 170-200 grams which is associated with age 45-50 days. The animals were grouped into one of four groups which are unrestrained, restrained for: 1 hour, 3 hours, or...
6 hours. The unrestrained rats serve as the control. The restraints were conducted between 9:00 am and 3:00 pm when nitric oxide synthase activity is at a low point during its circadian rhythm.

3.3 Description of the setting

Rats were housed in a climate controlled environment and acclimated to their new environment for a week prior to restraints. A regular 12/12 light cycle was used and food and water were provided ad libitum.

3.3.1 Transcardial Perfusion

After restraints rats were anesthetized with isoflurane and perfused with 4% paraformaldehyde (PFA) to preserve the brain tissue. Toe pinch reflex was used to determine when the anesthetic has worked. While the rat was being anesthetized the perfusion apparatus was set up. The infusion pump was prepared with two 1 liter glass beakers. One beaker contained phosphate-buffered saline (PBS) and the other contained 4% PFA fixative in 0.1 M PBS. Controlled release of a valve allowed PBS to flow through a tube into an attached hypodermic needle that was connected to the infusion pump. The needle was placed in the left ventricle of the rat’s heart and the right atrium was punctured to allow blood to escape. The animals were then decapitated and the brain was collected and post fixed overnight at 4°C followed by immersion in 30% sucrose until ready to be sectioned.
3.3.2 Brain Sectioning

Each brain was mounted on a removable cryostat platform. Tissue-Tek® Optimum cutting temperature (O.C.T.) formulation embedding medium (Sakura Finetec USA, Inc., Torrance, CA 90501 USA) for frozen tissue specimen was used to ensure most favorable sectioning environment, to add additional firmness, and to preserve the structure and shape of the brain tissue to facilitate the cutting process. At room temperature, a small amount of Tissue-Tek ® was placed on the cryostat (Micron International, HM 500 OM) platform. The brain/Tissue-Tek ® complex was placed in an auxiliary holding corridor inside the cryostat and allowed to freeze to a temperature of about -20°C. Once this temperature was reached, the platform with the brain attached was placed on the main cryostat corridor. The brains were cut in the coronal plane in an anterior or posterior direction into sections 20 microns thick. The sections were collected in 12 1ml microtubes with cryoprotectant.

3.4 Tissue Staining

3.4.1 NADPH-d for NOS Activation

Tissues are prepared by washing 3x with PBS in a 24 well plate, floating sections were stained with NADPH-d (incubated in0.1 M PBS, pH 7.4, containing 0.3% Triton-X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml NADPH) overnight in a 37°C incubator. Following incubation the sections were washed 3x at 5 minutes each in PBS and mounted on gelatin-subbed microscope slides using free-floating tissue mounting method. The sections were dried overnight in a slide drawer. Then with gradients of
alcohol and xylene the sections were cleared and dehydrated. Permount mounting medium was used to cover slip the slides.

3.4.2 Astrocyte IHC

Using an ABC staining kit from vector labs sections were incubated for 45 minutes with diluted normal blocking serum and excess was blotted from sections. They were then incubated overnight with anti-GFAP antibody diluted in PBS (1/100). Washed in buffer for 5x2 minutes then incubated for an hour with diluted biotinylated secondary antibody. Then washed again for 5x2 minutes in PBS and incubated with ABC reagent for 30 minutes then rinsed for 5x2 minutes in PBS. Finally, the tissues were incubated for 30 minutes in diluted AMCA for blue fluorescent staining.

3.4.3 Image Acquisition

The DRN was divided into 6 subregions for immunohistochemical analysis. In the rostrocaudal dimension: rostral (-7.3 to -7.73 mm Bregma and caudal (-8.45 to -9.26 mm Bregma). For each dimension every 4th coronal section was obtained and stained.

A Zeiss microscope fitted for either AxioCamMR3 for RGB or AxioCAMMR3 for black and white was used for all images. Axio imaging software was used to capture all images. Images were taken at 10-fold objective and 20-fold objective. The exposure time for all fluorescence imaging was 906.0 ms at 100%

3.4.4 Image Processing

All image processing was done with ImageJ. Three (3) sections for each rostral-caudal subregion were used to obtain neuron counts, optical density measurements, and
intervaricose spacing for 5 rats and the mean for each rat for each experimental group and brain region was obtained. Neuron counts were done manually on 10-fold objective images by placing a ticker while ImageJ kept count. Optical density (OD) was calculated from the mean gray value measured by ImageJ. Mean gray value is the sum of the gray values of all the pixels in the selection divided by the number of pixels. As mentioned previously NADPH-d staining intensity is an indicator of how active a neuron is. An intense stain in a neuron is indicated by the dark coloring produced by the conversion of tetrazolium to formazan. So the optical density is a relative indicator of how active the NOS in a neuron is. The higher the OD the more active the NOS is in a given neuron compared to neurons with low ODs. Optical density measurements were taken on 20-fold objective images and the mean is of a collection of single neurons in the each subregion.

The minimum criterion to be included in the assessment of varicosity spacing was for a nitrergic process to have at least three sequential varicosities at least 5 varicosities were measured per region. Images for varicosities were intentionally taken in areas where varicose fibers were the highest.

GFAP expression was measured by an automated ImageJ count of the number of astrocytes and fibers expressed; 20-fold objective images were converted from RGB to 16-bit, then a threshold was set to cover all the astrocytes and fibers. Only particles of an area of >150 pixels and a circularity of 0.00-1.00 were counted.

NOS expression was counted using automated ImageJ counting on control and 6 hour restraint 10-fold objective images. Particles that were >65 pixels and .5-1.00
circularity were counted. The pixel sizes for GFAP and NOS counts were selected by trial and error until the appropriate size counted all neurons or astrocytes.
CHAPTER IV
RESULTS AND DATA

There were varying levels of activated neurons which is denoted by the intensity of NADPH-d staining. The more active the neuron the darker the NADPH-d stain (Figure 7 and Graph 1).

Figure 7. Various types of NADPH-d staining intensity. Staining intensities in the caudal DRN show various levels of NOS activity; (a) represents low activity in the DRD, (b) represents intermediate activity in the DRV, and (c) represents high activity in the DRL.
**Graph 1.** NADPH-d intensity measured by optical density of pixels. (a) Neurons from the dorsal region, (b) neurons from the ventral region, and (c) neurons from the lateral wings.

As well-known, NOS neurons in the DRN localize mostly to the rostral dorsal, rostral ventral, and caudal lateral wings (Figure 8). NOS activity in the lateral wings increases as the dorsal raphe nucleus becomes more caudal (Graph 2). However the opposite is evident for the dorsal and ventral regions where activity and expression decreases as the DRN becomes more caudal (Graph 2). The caudal lateral wings have the most NOS activation compared to any region of the DRN (Graph 2).
**Figure 8.** NOS activity in the (a) Rostral and (b) Caudal sub-regions. Notice the Caudal lateral wings have more intense staining which indicates more activity.
Graph 2. NOS activity in each DRN sub-region. Caudal lateral wing has the most NOS activation compared to all other sub-regions.

4.1 Lateral Wings

The lateral wings showed no significance in the number of activated neurons between control (rostral mean = 8, SEM = 1.50, caudal mean = 66, SEM = 2.77), 1 hour (rostral mean = 12, SEM = 1.78, p-value = .097, caudal mean = 68, SEM = 7.03, p-value = .660), and 3 hour (rostral mean =11 SEM = 1.84, p-value = .081, caudal mean = 69, SEM = 4.25, p-value = .962) restraint for either rostral or caudal. There is significance at 6 hour caudal (caudal mean = 52, SEM = 4.73, p-value = 0.040, rostral mean = 5, SEM = 1.26, p = 0.098) restraint where there is decrease in the number of activated neurons compared to the control (Graph 3).
Graph 3. Caudal DRL show 21% decrease in the number of active neurons.

Although NOS activity decreased in neurons the expression did not change between control (mean = 69, SEM = 5.08) and hours (mean = 65, SEM = 3.67, p = 0.211) (Figure 9 and Graph 4).
Figure 9. NOS expression does not change in control and 6 hour restraint.
Graph 4. NOS expression did not change between control and 6 hour restraint.

Optical density measurements showed differences in NOS activation. A 1 hour restraint displayed a 57% increase in the OD of neurons in the rostral DRL but there was no difference in the caudal DRL (rostral mean = 1.44, SEM = 0.098, p = -.019, caudal mean = 2.06, SEM = 0.192, p = 0.198) compared to the control (rostral mean = .912 SEM = 0.100). A 3 hour restraint in the rostral DRL did not show any differences (mean= 1.16 SEM = 0.176, p = 0.877) but neurons in the caudal DRL showed a 28% increase in OD compared to the control (mean = 2.58 SEM = .013, p = > 0.001). Finally, 6 hours of restraint showed no differences in either rostral (mean = 1.13 SEM=0.177 p = .285) or caudal (mean = 2.21 SEM 0.114 p = .407) regions compared to control rats (Graph 5).
Graph 5. Optical Density of the DRL. In (a), rostral show a 57% increase following 1 hour of restraint; in (b) caudal, there is 28% increase in NOS activity.
Immunofluorescence as well as NADPH-d staining displayed more varicose fibers in the caudal lateral wings than any other region (Figure 10).

![Figure 10](image)

Figure 10. Varicosity fibers in the DRN 40-fold objective stained for NADPH-d, varicosity fibers in rostral vs caudal subregions. NADPH-d stained fibers. The caudal lateral wing (f) has the most varicose fibers than other regions.

A 1 hour restraint did not show any differences in the rostral DRL (mean = 7.80 µm SEM = 1.147, p = .249) compared to the control (mean = 7.59 µm SEM = 1.747) of the same region. The caudal DRL showed a significant decrease in intervaricosity.
spacing (mean = 7.23 µm SEM = 0.517, p < 0.001). Restraint for 3 hours did not change the intervaricosity distance in the rostral DRL (mean = 8.60 µm SEM = 1.373, p = 0.401). However, intervaricosity distance decreased in the caudal DRL of 3 hour restraint (mean = 6.61 µm SEM = 0.798 p < 0.001); 6 hour restraint did not show any differences in intervaricosity distances for either region (rostral mean = 7.93 SEM = 1.749, p = 0.740, caudal mean = 9.41 µm SEM = 1.33, p = 0.381) (Graph 6).

**Graph 6.** Inter-varicosity distance measurement for fibers in the lateral wings. This graph compares each time point with the corresponding region in the control.

The restraint rats also showed more thin fibers than those of the control in the caudal DRL (Figure 11).
Figure 11. 20 fold objective images of varicosity axons in the caudal DRL. Restraint animals showed thinner varicose types compared to the control animals.

4.2 Ventrals

The ventral region did not have any significance in mean number of cells at any length of restraint (control: rostral mean = 56, SEM = 9.93, caudal mean = 16, SEM = 4.04, 1 hour: rostral mean = 58, SEM = 8.91, p = .538, caudal mean = 19, SEM = 2.56, p = 0.589, 3 hour: rostral mean = 64, SEM = 6.82, p = .747, caudal mean = 18, SEM = 4.10, p = 0.397, 6 hour: rostral mean = 66, SEM = 10.75, p = 0.562, caudal mean = 20, SEM = 4.54, p = 0.292).
NOS activity did not show any changes in rats restrained for 1 hour (rostral mean = 1.48, SEM = .082, p = 0.681, caudal mean = 1.08, SEM = 0.154, p = 0.384) compared to controls (rostral mean = 1.44, SEM = 0.185, caudal mean = 1.01, SEM= 0.131). A 3 hour restraint increased activity in the rostral (mean = 2.15, SEM = 0.127, p = 0.024) but not the caudal region (mean = 1.34, SEM = .245, p = 0.286). Finally, 6 hours of restraint showed similar differences as 3 hours of restraint, there was increase in the rostral region (mean = 2.01, SEM = .122, p = .029) but not the caudal region (mean = 1.47, SEM = .204, p = .072) (Figure 12 and Graph 7).

**Figure 12.** NOS activation in the Rostral Ventromedial.
Graph 7. NOS Activity in the DRV. NADPH-d staining revealed increased staining intensity following 3 hour and 6 hour in the rostral DRV.

By observation this region had the least amount of varicose fibers in both rostral and caudal end, but there were differences in the spacing on the fibers present (Figure 12). The rostral and caudal regions showed differences in the intervaricosity distance at 1 hour (rostral mean = 4.18 µm, SEM = 2.00, p = .042, caudal mean = 4.49 µm, SEM = 1.98, p = .020). A 3 hour restraint also showed decreased varicose spacing in both rostral and caudal regions (rostral mean = 4.19 µm, SEM = 0.771, p = 0.001, caudal mean = 4.49 µm, SEM = .984, p<.001). There was also significant decrease in varicose spacing 6 hour caudal region (caudal mean = 4.21 µm, SEM = 0.329, p<.001) but not the 6 hour rostral region (rostral mean = 4.95 µm, SEM = 1.20, p = .154 (Figure 13 and Graph 8).
Figure 13. Astrocytes in the rostral DRN. 20-fold objective of astrocytes in the rostral DRN AntiGFAP Immunofluorescence with AMCA revealed a 50% increase of GFAP expression in restraint rats.
Graph 8. There were differences in varicosity spacing at all lengths of restraint. In the rostral region (a) there was 31% decrease at 1 hour and 3 hour restraint but no changes in 6 hour restraint. The Caudal region showed 37% decrease for 1 and 3 hour restraint and 40% decrease for 6 hour restraint.
4.3 Dorsal

The mean number of cells in the dorsal region did not change between 1 hour (rostral mean = 16 +/- 4.407, p = .418, caudal mean = 6 +/- 2.64, p = .197) and 3 hour (rostral mean = 6.1 +/- .989, p-value = 0.301, caudal mean = 16 +/- 1.557, p = .261) restraint compared to the control (mean = 15 +/- 2.52). Restraint following 6 hours displayed a significant increase in the number of active neurons (rostral mean = 22 +/- 3.02, p = 0.043, caudal mean = 2.88).

Optical density measurements showed a 28% decrease in NOS activity in the rostral DRD of rats restrained for 1 hour (mean = 1.17 +/- 0.073, p = 0.005) compared to the control (mean = 1.568 +/- 0.128) but no changes in the caudal DRD (mean = 1.13 +/- 0.144, p = 0.119). Restraint following 3 hours did not show any significance in either rostral (mean = 1.85 +/-0.138, p= 0.169) or caudal (mean = .846 +/- 0.066, p = 410). A 6 hour restraint also did not change the OD in the rostral (mean = 1.73 +/- 0.162, p = 0.686) or caudal region (mean = 1.09, SEM = 0.162, p = 0.410).

Varicose measurements were not changed in the dorsal region for following 1 hour of restraint in either region. However varicosity spacing decreased in the caudal DRL following 3 hours and 6 hours of restraint (1 hour: rostral mean = 7.49 µm, SEM= 1.77, p = .145, caudal mean = 8.04 µm , SEM= 1.21, p = .589, 3 hour: rostral mean = 5.65 µm, SEM = 1.43, p = .747, caudal mean = 7.37 µm, SEM = 1.21, p = .040, 6 hour: rostral mean = 6.63, SEM = 1.28, p = .372, caudal mean = 7.27, SEM = 1.50, p = .033) compared to the control (rostral mean = 6.09 µm, SEM = 0.868, caudal mean = 9.13 µm, SEM = 1.51).
4.4 Astrocytes

There was no correlation between GFAP expression and NOS activity in either the rostral, caudal region or their respective sub regions (total rostral region: \( r = 0.02, p > 0.05 \), total caudal region = \( r = 0.108, p > 0.05 \)).

Restraint increased the number of GFAP astrocytes in the total DRN at all levels (control mean = 50, SEM = 13.38, 1 hour mean = 66, SEM = 15.24, \( p = 0.007 \), 3 hour mean = 63, SEM = 5.51, \( p = 0.011 \), 6 hour mean = 72, SEM = 17.58 \( p < 0.001 \)). The most significant differences were in the rostral DRN because all lengths of restraint showed increases in astrocytic density in that region (control mean = 47, SEM = 13.37, 1 hour mean = 75, SEM = 15.96, \( p > 0.001 \), 3 hour mean = 67, SEM = 16.97, \( p = 0.006 \), 6 hour mean = 69, SEM = 18.71, \( p = 0.005 \)) (Graph 8). The caudal region did not display differences until 6 hours of restraint (control mean = 50, SEM = 12.04, 1 hour mean = 50, SEM = 15.24, \( p = 0.391 \), 3 hour mean = 55, SEM = 10.83, \( p = 0.340 \), 6 hour mean = 75, SEM = 16.33), \( p = 0.005 \)) (Figure 14 and Graph 9).
Figure 14. Astrocytes in the caudal DRN. GFAP antibody staining with AMCA reveals increased GFAP expression following 6 hours of restraint (p=0.008) compared to the control.
Graph 9. Astrocyte reactivity in the rostral and caudal DR.
CHAPTER V
DISCUSSION

5.1 Lateral Wings

The lateral wings are the region where NOS neurons localize independently of 5-HT neurons and NOS activation is the most significant. Restraint stress did not have an effect on the mean number of neurons in the lateral wings in rats that were restrained for up to 3 hours. However, there was a decrease in the number of NADPH-d positive cells following 6 hours of restraint. The decrease is an indicator of decrease in NOS activation but not expression because measuring NOS neurons following NOS-1 antibody did not show changes in NOS expression. This suggests that there is some mechanism that is causing NOS activation to shut down following 6 hours of restraint.

Optical density which is an indicator of relative NOS activation was significantly increased in the caudal and rostral lateral wings. Specifically a 1 hour restraint increased rostral DRL activity and 3 hour restraint increased caudal DRL activity. 6 hour restraint did not show any differences in either rostral or caudal DRL activity besides a decrease in number of activated neurons.

The DRL only projects to subcortical structures. Since the caudal lateral wings are most activated at 3 hours of restraint one may assume that the DRN is highly involved with the modulation of subcortical structures through its NO domain following 3 hours of
restraint. Some of the subcortical regions that could be receiving nitrergic neurons from the DRN are the hypothalamus and amygdala. More specifically evidence shows that non-5HT neurons project to the basal forebrain and amygdala. There is also evidence that shows approximately 30-40% of neurons from the DRL that project to the trigeminal system are nitrergic. Further experiments need to be done to show an extensive projection path of DRL nitrergic cells.

By observation the caudal region displayed the most significant number of nitrergic fibers and varicose fibers than any other subregion. So fibers that project to the DRN had the most significant differences in the caudal region. Restraint decreased the spacing between varicosities in the caudal region but not the rostral region. 1 hour and 3 hour displayed decreased spacing between varicosities. 6 hours did not display any differences in varicosity spacing compared to the control. All levels of restraint displayed thinner varicose fibers than those in the control rats. That is suggestive that the fibers activated during restraint are different from those in nonrestraint rats.

Afferent fibers of the DRN include extensive input from various regions of the hypothalamus, medullary regions, and medial prefrontal cortex. The highest density of these projections are in the DRV and DRL subregions. In the total DRL region there were no significant differences in the number of GFAP expressing cells until 6 hours of restraint.

5.2 Ventral
The ventral region contains the most NOS neurons compared to all of other regions. NOS neurons of this region colocalize with 5-HT. Although there were no
differences in the number of NOS activated cells in the ventral region, NOS activity increased in the caudal DRV at 3 hours and 6 hours of restraint.

By observation the DRV had the least amount of varicose fibers than the DRL and DRD but displayed significant differences in mean spacing between varicosities in all lengths of restraint compared to the control. The rostral region showed differences up to 3 hours of restraint but no significant differences at 6 hours of restraint. The caudal region showed differences at all lengths of restraint.

Along with the DRL, the DRV receives dense fibers from the hypothalamus; whether these fibers are exactly nitrergic is not clear. However it is a possibility given that the pattern of NOS activation in the hypothalamus is consistent with a lack of differences in the varicose spacing of nitrergic neurons during 6 hours of restraint also the thin fibers of the ventromedial were similar to the fibers seen in the lateral wings.

Even though the ventral region displayed the least amount of GFAP expression than the other subregions, the total ventral region showed increased GFAP at all lengths of restraint in each rostro-caudal region.

### 5.3 Dorsal

The dorsal region contains the least amount of NOS neurons compared to all other regions. Stress by restraint increased the number of NADPH-d positive cells at 6 hours of restraint.

Following 1 hour of restraint there was a decrease in the OD of NADPH-d compared to the control. There were no differences in any other regions. Intervaricosity
distances did not show any differences between controls and restraint either. GFAP expression was significant at 6 hours of restraint in the total DRD.

5.4 Temporal Changes in the DRN

This study supports evidence that as the DRN processes stress it is using different populations of nitrergic neurons as evident by the differential activation of subregions and differences in the fiber types in each subregion. The temporal changes of NOS in the DRN have never been reported. Here it is shown that the DRN nitrergic neurons are differentially activated in each region at different time points throughout a 6 hour restraint. It also supports the notion that the DRN is involved in processing a prolonged exposure to stress, rather than the quick response as seen in fight or flight.

It has been shown that rats restrained for a single hour display behavior that indicates depression for up to 24 hours, this study showed the least changes in the DRN compared to the control following 1 hour of restraint (Figure 15). There were changes in intervaricosity distance in the lateral wings which continued through 3 hours of restraint, which may suggest the activation of a different set of neurons projecting to the DRN in response to acute restraint stress.
Three (3) hours of restraint showed the most activity of NOS being that both the caudal DRL and rostral DRV were significantly activated which are the regions with the most expressed NOS neurons compared to the dorsal region. Three (3) hours of restraint also decreased mean spacing between varicosities and showed. This is a crucial time point during the stress response for NOS activation in the DRN. It is also consistent with unpublished data that NOS activation is significantly increased in other brain regions following a 3 hour restraint compared to control, 1 hour, and 6 hour restraint. The mechanism of activation is not well understood but an increase in glutamatergic input
could be involved with the increase in activation, as well as an increase in regulation by neuronal localized CAT-3 but not CAT-1.

The most interesting finding is the decrease in NOS activation in the caudal DRL following 6 hours of restraint. The changes from 3 hours to 6 hours suggest a change in the response to stress, even though the stressor has not changed. The decrease here is also consistent with decreases seen in the hypothalamus PVN and SON.\textsuperscript{72} The PVN projects fibers to the DRL which may be a reason there is not significant differences in inter-varicosity distances after 6 hours of restraint compared to the control. It would also be interesting to see the activation of GABAergic fibers in the DRN following 6 hours of restraint. If there is an increase it may also explain the reason NOS is so lowly activated here.

\textbf{5.5 DRN Fibers}

Fibers of the DRN provide another source of NO. NO from these fibers may interact with the nitrergic neurons in the region but they may also interact with other types of neurons in the region such as the 5-HT neurons or cholinergic neurons. Since NO can act retrogradely on a presynaptic cell this NO may be interacting with other fibers that synapse in the DRN and influence the release of those other neurotransmitters.

GABAergic and Glutamatergic fibers also innervate the DRN which provide inhibitory and excitatory activity, respectively. NO is known to block the reuptake of glutamate which is how it is involved with LTP. It interacts with GABAergic cells. If NO is interacting with either one of these fiber types then it could be modulating its own activation as well as the activation of other neurons in the area.
NO diffusion from varicosities is an advantage because it allows for a big release of NO from one region. NO has a short half-life so in order for a signal to constantly be transmitted by NO, a cell has to constantly make NO until there is no longer a need for the signal. Varicosities have the potential to release a lot more NO than a straight axon so that the amount of NO meets the need to transmit a signal.

5.6 Differential GFAP Expression in the DRN

Increased GFAP is an indicator of astrocyte reactivity. It usually occurs as a response to neural injury and is found in neurodegenerative diseases. However, here there is increase in GFAP expression in neurons of animals that had a single episode of restraint. It has been reported that increased GFAP may be a reflection of an increase in metabolism and protein synthesis as a response to changes in the environment. Increased activity of the DRN may be the reason for increased GFAP expression during stress.

Although the differences of the DRN did not correlate with NOS activation, the differences in GFAP expression is more evidence of the differential response the DRN has in response to stress and provide evidence that astrocyte reactivity occurs more so in the rostral region than the caudal region despite the fact that the caudal region is more involved in the stress response. We also show that length of restraint is significant as 6 hours of restraint increased GFAP density in both rostral and caudal regions.

5.7 Future Directions

This study added a temporal component to which showed that the length of restraint makes a difference in NOS activation. While a 6 hour length of restraint may seem very intense it is still considered an acute restraint. Therefore, the differences in
response reported here are for varying levels of acute stress. The fact that 6 hours of restraint showed decrease of NOS activation in the DRL needs to be further explored. It may have to do with some feedback from other NOS neurons or from a different transmitter altogether. Studying the fibers that innervate the region as well as the fibers projecting to other regions in response to stress would give a more complete picture on the role of NO because it is a modulatory molecule for other neurotransmitters.

The role of NOS in the DRN following chronic stress, which would be modeled by several consecutive days of acute restraint, would be an important next step to elucidate the role NO plays in a more debilitating type of stress. Also, NO is involved with decreasing the response by the sympathetic nervous system (SNS) and since the DRN is involved largely with modulating the autonomic nervous system it would be interesting to see if NO from the DRN is directly involved with the modulation of the SNS.

Also these experiments measured the response immediately after stress has been applied. It would be interesting to see the NOS response in the DRN following a delay after varying levels of acute or chronic restraint. Following a week of survival, if the NOS activation is changed because of behavioral differences, this would be considered a model for post traumatic stress disorder.

The projections of NOS neurons from the DRN are not well known, especially to structures involved in the stress paradigm. Future studies that show the projection path of NOS neurons to the hypothalamus would be very beneficial to help complete the picture of the role NOS has during the stress response.
Lastly, the mechanism of increased activation of NOS in the DRN during stress is not known. Arginine may either come from blood vessels via CATs or it may be recycled from citrulline. Since reports show that increasing l-arginine in the DRN results in anti-depressant like behaviors it is likely that there is involvement with arginine transport through CATs. Although we did not conclude that from these experiments, it would be worth it to explore the possibilities of CAT3 or CAT2 with this involvement.
CHAPTER VI

CONCLUSION

These experiments together support already substantial evidence that subregions of the DRN are differentially involved in the process of stress signaling. It also shows that the temporal profile of the DRN is changed over a 6 hour period in regards to both astrocytes and NOS activation. Using NADPH-d as a marker for NOS activation we were able to show that DRN NOS activation in cell bodies and fibers is not only spatially differentiated across the DRN but there are differences in the temporal response of NOS activation following acute stress as well.

Immunohistochemistry using GFAP revealed that astrocytes in the rostral DRN were more active in response to stress than those of the caudal DRN. It also showed that 6 hours of restraint stimulate astrocyte activation in both rostral and caudal DRN. These experiments further support that DRN is a highly complex structure and its topography is important in how it processes stress.

These findings are important and should be taken into consideration when therapies for stress are developed. The DRN may be a candidate for drug target therapy since studies show that NO produced there has antidepressant like effects, but a timed release would also be beneficial as evidence here shows NOS is differentially released over time.
REFERENCES


