PHENOTYPICALLY DIFFERENT CELLS IN THE NUCLEUS OF THE SOLITARY TRACT: EXPRESSION OF GROUP I METABOTROPIC GLUTAMATE RECEPTORS AND ACTIVATION BY BAROREFLEXES

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

Phenotypically Different Cells in the Nucleus of the Solitary Tract: Expression of Group I Metabotropic Glutamate Receptors and Activation by Baroreflexes

Presented by James R. Austgen

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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For Kati and Roger, the highlights of my day, the loves of my life…

AND

To Darren Good, PhD, of the Dept. of Biology, Augustana College, Rock Island, IL…he taught me that teaching and research could be a good thing, and started me on this road to a PhD

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Abbreviations:

ACPD: aminocyclopentane -1S,3R-dicarboxylic acid

ADN: aortic depressor nerve

AP: area postrema

cGMP: cyclic guanosine monophosphate

CO: Carbon Monoxide

CS: calamus scriptorius

CSN: carotid sinus nerve

CVLM: caudal ventrolateral medulla

DHPG: 3,5-dihydroxyphenylglycine

Diaz: diazoxide

DMNV: dorsal motor nucleus of the vagus

FG: Fluoro-Gold

GABA: γ-aminobutyric acid

GAD65: glutamic acid decarboxylase isoform 65

GAD67: glutamic acid decarboxylase isoform 67

Glu: L-glutamate

HR: heart rate

iGluRs: ionotropic glutamate receptors

IR: immunoreactive

IX/X: Ninth and Tenth Cranial nerves

KYN: kynurenic acid

MAP: mean arterial pressure

mGluRs: metabotropic glutamate receptors

NA: nucleus ambiguus

NDS: normal donkey serum

NGS: normal goat serum

nNOS: neuronal nitric oxide synthase

NO: Nitric Oxide

NTS: nucleus of the solitary tract

PBS: phosphate buffered saline

PE: phenylephrine

PFA: paraformaldehyde

PVN: paraventricular nucleus of the hypothalamus

RM: repeated measures

RVLM: rostral ventrolateral medulla

SNA: sympathetic nerve activity

SPN: sympathetic premotor neuron

TH: tyrosine hydroxylase

TR-PBS: phosphate buffered saline with Triton X-100

VII: Facial nucleus

Phenotypically Different Cells in the Nucleus of the Solitary Tract: Expression of Group I Metabotropic Glutamate Receptors and Activation by Baroreflexes

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Abstract

Modulation and integration of visceral and central afferent information occurs in the nucleus of the solitary tract (NTS). The NTS is a heterogeneous nuclear region that contains afferent terminals, interneurons, and multiple synaptic receptors that process information before exiting the NTS to multiple nuclear regions within the brain. One such efferent pathway is to the caudal ventrolateral medulla (CVLM), which serves as the second central nuclear region of the arterial baroreflex. The exact nature of the modulation of the arterial baroreflex at the level of the NTS is still unknown. The studies presented here further clarify the role of Group I metabotropic glutamate receptors (mGluRs) on GABAergic, nitroxidergic and catecholaminergic neurons and neurons the project from the NTS to the CVLM. Additionally, we examine GABAergic, nitroxidergic and catecholaminergic neurons and neurons the project from the NTS to the CVLM activated in the NTS through acute hypertension and hypotension. Finally, we proposed a study to examine the expression of Group I mGluRs on neurons activated by hypertension and hypotension and neurons that project the NTS from the CVLM. These studies suggest that Group I mGluRs are preferentially expressed on CVLM projecting neurons, and that hypertension and hypotension activate separate populations of neuron in the NTS.

Chapter 1: Introduction

The autonomic nervous system serves to control a host of bodily functions without conscious thought. It is composed of two primary subdivisions, the parasympathetic and the sympathetic nervous system. These subdivisions work together to regulate important processes such as gastrointestinal function, pupillary diameter, urinary bladder emptying, body temperature and cardiovascular function. The parasympathetic nervous system contributes primarily to vegetative processes, causing pupillary constriction, increasing gastrointestinal motility and secretion, and slowing heart rate. In contrast, the sympathetic nervous system is important in the "fight or flight" response, dilating the pupils, decreasing gastrointestinal function, stimulating the heart and constricting blood vessels. Normal autonomic function, which is dependent on both the parasympathetic and sympathetic nervous system, is critical to the maintenance of homeostasis.

Impaired autonomic regulation, leading to cardiovascular dysfunction, contributes to a variety of disease states, including heart failure, hypertension, obesity, and diabetes (3). Specifically, the cardiovascular dysfunction in most of these cases includes increased sympathetic nervous system activity. Epidemiologic studies indicate a strong correlation between patient mortality and elevated levels of plasma norepinephrine, norepinephrine spillover, and muscle sympathetic nerve activity, which are markers for the level of sympathetic activation (14; 45). Thus, understanding the factors that influence autonomic regulation and result in autonomic dysfunction in disease is critical to the development of therapies for some of these disease states.

Many studies indicate that cardiovascular dysfunction is associated with a reduced capacity of the arterial baroreflex to respond to and regulate changes in arterial pressure (3; 84). This impairment of baroreflex function may involve either resetting of the reflex to regulate the circulation at a different arterial pressure and sympathetic nerve activity, or a reduction in the central gain or sensitivity of the reflex. In either case, baroreflex dysfunction likely contributes to both the pathophysiology of the disease and the inability to reverse cardiovascular dysfunction.

Although peripheral abnormalities clearly play an important role in the cardiovascular dysfunction associated with these disease states, a substantial component involves the central nervous system (3; 84). Mechanisms within the brain contribute significantly to modulation of autonomic function under normal conditions. Furthermore, central nervous system mechanisms are associated with both augmented sympathetic nervous system activity and baroreflex dysfunction in disease (3; 84). Studies outlined in this document focus on a critical region of the central component of the arterial baroreflex, the nucleus of the solitary tract. Specifically, we evaluate the distribution of a specific modulatory receptor, different cellular phenotypes, and neurons that project from the nucleus of the solitary tract to the caudal ventrolateral medulla, the next critical brain region in baroreflex control of the sympathetic nervous system. We also examine neuronal activation in response to increases and decreases in arterial pressure. These results provide important information relative to normal autonomic function, which can then be compared to results in animals with cardiovascular dysfunction. Comparisons of normal and

altered cardiovascular states may show that alterations in the factors listed above contribute to enhanced sympathetic nervous system activity and to the reduction in arterial baroreceptor function.

Arterial Baroreflex:

The arterial baroreflex is an autonomic reflex and buffering system that is responsible for the regulation of arterial blood pressure on a beat-by-beat basis (7; 9; 36; 56; 79; 92; 128). Arterial baroreceptors are mechanoreceptors with nerve endings imbedded in the walls of the aortic arch and carotid sinuses. The central nuclei of the baroreflex within the medulla oblongata receive afferent signals related to arterial blood pressure from the aortic depressor nerve (ADN) and carotid sinus nerve (CSN) (7; 9; 92; 128). The ADN, located in the vagus nerve bundle, has nerve endings that are found in the aortic arch. The CSN, which joins the glossopharyngeal nerve, has nerve endings in the region of the carotid sinus (7; 9; 128). The ADN and CSN have their cell bodies located in the nodose and petrosal ganglia, respectively, (9) and are made up of both A-type, myelinated and C-type, unmyelinated fibers. These populations of fibers are thought to signal different aspects of arterial pressure (7; 9; 128).

The baroreceptors are tonically active with their level of activity dependent on the basal arterial pressure. During periods of increased or decreased arterial pressure, expansion of the vessel wall is increased or reduced and baroreceptor activity increases or decreases, respectively (1; 128; 130). Thus, changes in arterial pressure influence baroreceptor afferent activity to the brain. The brain then

integrates this information and alters sympathetic and parasympathetic nervous system activity in response to those changes (7; 9; 36; 56; 92; 128), returning arterial pressure toward normal.

Central Nuclei of the Arterial Baroreflex:

Baroreceptor afferent information is relayed to the central nuclei of the medulla oblongata through an excitatory synapse. The afferents terminate in a dorsal strip of cells in the commissural nucleus of the solitary tract (NTS) and in the dorsomedial aspects of the medial NTS (24; 27; 31; 98; 138). These afferents activate second order neurons (neurons that receive primary afferent input) within the NTS by an excitatory amino acid. NTS neurons project to the dorsal motor nucleus of the vagus (DMNV) or the nucleus ambiguus (NA) to influence parasympathetic nervous system activity. The DMNV or NA is excited by the NTS, increasing vagal efferent activity to the parasympathetic ganglia on the heart, resulting in reflex bradycardia. In the sympathetic nervous system limb of the baroreflex (Fig. 1), the NTS activates a population of neurons in the rostral portion of the caudal ventrolateral medulla (CVLM) (33; 73; 131; 132). Rostral CVLM neurons subsequently project to the rostral ventrolateral medulla (RVLM) (33; 73; 131; 132). The RVLM is a tonically active nucleus that is a major source of sympathetic tone. The baroreflex-related projection from the CVLM to the RVLM is inhibitory, using the major inhibitory neurotransmitter γ- aminobutyric acid (GABA). As the RVLM is tonically active, the inhibitory projection from the CVLM decreases RVLM neuronal activity and sympathetic nervous system activity, reducing arterial blood

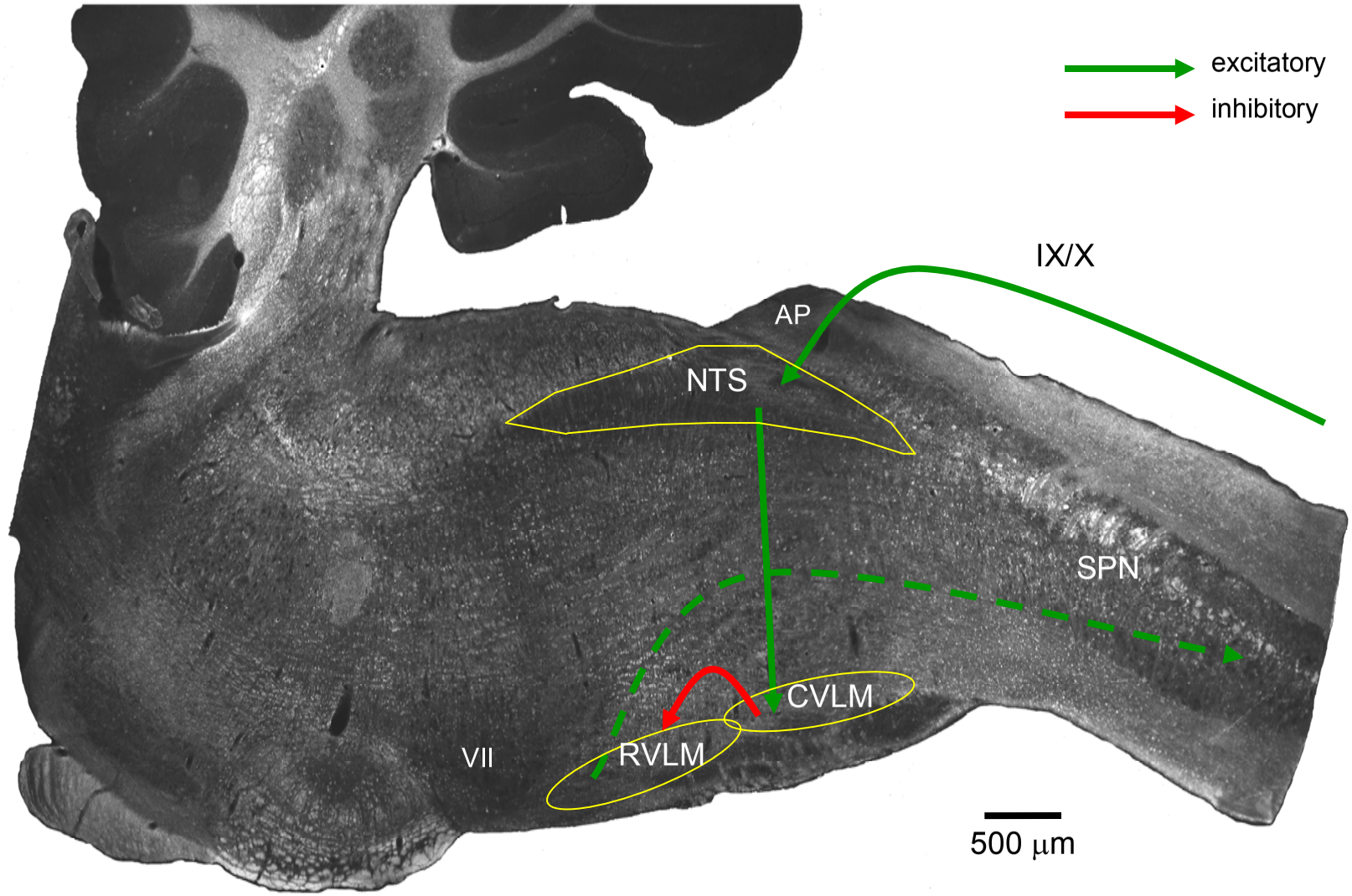

Figure 1**: Simplified model depicting the sympathetic nervous system branch of the arterial baroreflex in the rat.** Mosaic of sagittal photomicrographs of the rat hindbrain. AP: area postrema; NTS: nucleus of the solitary tract; CVLM: caudal ventrolateral medulla; RVLM: rostral ventrolateral medulla; SPN: sympathetic premotor neuron (that projects to the intermediolateral cell column of the spinal cord); IX/X: Glossopharyngeal and Vagus nerves; VII: Facial Nucleus.

pressure (5; 7; 9; 24; 31; 36; 59; 98; 138).

The arterial baroreflex normally functions to maintain arterial pressure near a set point (Fig 2A; star) by adjusting the level of sympathetic nerve activity (SNA). Increased baroreceptor stimulation decreases sympathetic nerve activity and increases parasympathetic nerve activity, blunting the increase in arterial pressure and returning arterial pressure to the set point. In response to decreases in blood pressure, baroreceptor activity is diminished. RVLM neuronal activity is disinhibited by removal of $GABA_A$-mediated inhibition from the CVLM, which increases SNA and subsequently blunts the decrease in arterial pressure, again returning pressure to the set point (7; 9; 36; 98; 138).

Arterial Baroreflex Plasticity:

The arterial baroreflex can be modulated both acutely and chronically, by a myriad of stimuli. For example, baroreflex control of sympathetic nerve activity or heart rate can shift along the horizontal (pressure) axis so that pressure is regulated at a higher or lower level (Fig 2B). This type of shift occurs in response to both acute (10-15 minutes) changes in blood pressure and long-term hypertension (in which there may also be a reduction in slope, Fig 2C) to maintain a new level of arterial pressure (42). In addition, the gain of the reflex can be altered. For example, a decreased gain would be associated with a decrease in the slope of the baroreflex curve and a reduced ability to compensate for changes in pressure (Fig 2C). The change in slope may also be accompanied by an alteration in the range of baroreflex regulation of sympathetic nerve activity or heart rate. Changes in gain

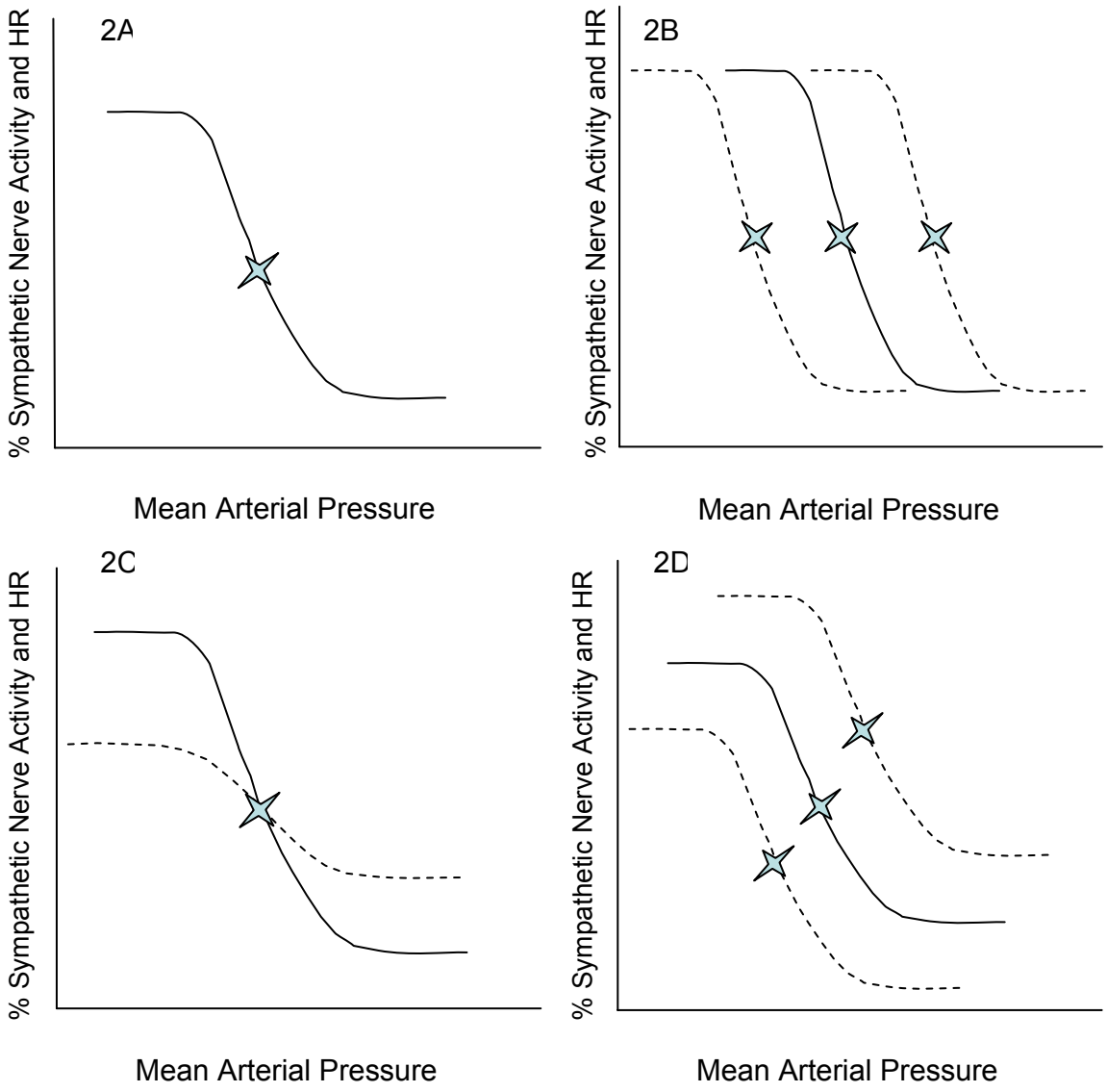

Figure 2: **Examples of arterial baroreceptor plasticity.** A. Normal curve. B. Shift along Pressure (horizontal) axis. C. Reduction in Gain. D. Shift along Output (vertical) axis and pressure axis. Dotted lines represent the possible alterations to the baroreflex curve. Star represents the set point of arterial pressure.

have been described after acute volume expansion or activation of the cardiopulmonary reflex (42) or in response to increases in circulating vasopressin (61), and chronically after cardiovascular deconditioning or during pregnancy (62; 67). Finally, the curve can shift up or down the vertical (sympathetic or heart rate) axis and left or right along the pressure axis, so that the output is regulated differently at a given pressure (Fig 2D). This occurs during acute exercise, when the curve shifts up and to the right, then shifts to the left and downward after exercise (post-exercise hypotension) before returning to normal levels (42).

The ability to modulate baroreflex function is critical to the regulation of arterial pressure under a variety of physiological and pathophysiological circumstances (64; 66; 66; 83; 83; 84; 121; 149; 149). For example, the shift in baroreflex regulation during exercise serves at least two functions. First, the upward shift in set point allows for an increase in sympathetic nerve activity and heart rate concomitant with an increase in blood pressure, unopposed by the baroreflex. Secondly, it allows for the individual to respond to other changes in blood pressure that may occur around the new set point. At least a portion of the modulation of baroreflex function occurs within the central nervous system (3; 84).

Nucleus of the Solitary Tract:

The NTS, located on the dorsal surface of the medulla oblongata, is a highly integrative region and receives inputs from a variety of visceral receptors and other brain regions. These inputs, as well as interneurons within the NTS, result in a collection of an estimated 42,000 cells and a million synapses (9). These multiple

inputs and interneurons, combined with the presence of numerous neurotransmitters and neuromodulators, provide the anatomic substrate for substantial integration of baroreceptor afferent activity within the NTS (7; 9; 84; 98; 138). Thus, the NTS is not a simple relay for afferent information. Rather, the ultimate output from the NTS is the culmination of the input signal and its modulation within the nucleus.

Afferent Projections to the NTS:

Among the multiple visceral afferents that terminate within the NTS are afferents from arterial baroreceptors, arterial chemoreceptors, cardiopulmonary receptors, lung inflation receptors, gastrointestinal and gustatory receptors (7; 9; 84; 98; 138). Although the NTS is a heterogeneous structure, there is a general viscerotopic organization to the termination of specific afferents within the NTS, allowing for subregions to be defined. Synaptic interactions between the afferents and the second order neurons are very specific, with most second order neurons receiving information from only one type of afferent (7; 9; 84; 98; 138). As stated previously, the arterial baroreceptor afferents terminate primarily in the commissural and dorsomedial subregion of the medial NTS. Chemoreceptor afferents terminate predominantly in the subregions lateral and ventrolateral to the solitary tract at the level of the fourth ventricle and in the medial and commissural subregions at the level of, and caudal to, the area postrema. Gustatory receptor afferents generally terminate in the medial subregion at the level of the fourth ventricle. Though there is a general localization of the second order neurons receiving these separate

afferents, there is considerable overlap of the regional distribution of those second order neurons within the whole of the NTS (9; 98).

Along with visceral or sensory afferents that terminate within the NTS, there are a number of medullary and supramedullary regions that send projections to the NTS. These include the area postrema (AP), the A5 cell group, the rostral ventrolateral medulla (RVLM), the periaqueductal gray, the paraventricular nucleus (PVN), and the lateral nucleus of the hypothalamus (7; 9; 36; 59). These regions all have a role in the regulation of cardiovascular activity, either through direct effects on sympathetic tone or hormone release. Information from these nuclear regions is in addition to information received from the visceral afferents. Therefore, the NTS is an important site for integration of information from both central and peripheral sources.

Synaptic Modulation in the NTS:

Signals that are received within the NTS undergo extensive integration. The NTS contains a large network of interneurons (neurons that do not project out of the region in which the soma are located) that influences synaptic activity of the second order or higher order NTS neurons (7-9; 36; 98). There are also numerous neurotransmitters and both pre- and postsynaptic receptors, that can positively or negatively influence the activity of NTS neurons (43; 59; 87; 106; 138; 144; 150; 160). Finally, gaseous neuromodulators, such as nitric oxide or carbon monoxide, may be released by afferent stimuli and subsequently modulate activity of the second order, higher order or interneurons (40; 41; 81; 82; 89; 97).

With regard to the baroreceptor afferent neurons, information is propagated across the synapse by an excitatory amino acid, shown in numerous studies to be glutamate (52-54; 146). Glutamate activates ionotropic glutamate receptors on the second order neuron, and ionotropic glutamate receptors in the NTS are required for baroreflex function (7-9; 56; 114; 144). In the sympathetic pathway of the arterial baroreflex (Fig. 1), second order neurons either project directly to the CVLM (13), or synapse with an additional neuron or neurons within the NTS, that then may project to the CVLM (7-9; 36; 55; 69; 98; 138). Therefore, arterial baroreceptor information is propagated from the mechanoreceptors to the CVLM through the NTS, likely crossing one or more synapses within the NTS. This suggests that arterial baroreflex information may be modulated by interneurons or other peripheral or central afferents projecting to the NTS.

Efferent Projections of the NTS:

In addition to the output to the CVLM, the NTS sends efferents to both medullary and supramedullary nuclei. Projections are sent to the RVLM, pre-autonomic areas of the PVN, the A5 cell group or to the intermediolateral cell column of the spinal cord, areas that are involved with sympathetic regulation (7-9; 13; 24; 36; 69; 75; 76; 98; 138). The NTS also has efferent projections to the DMNV or the NA, for control of parasympathetic tone. Further, the efferent projections to the neuroendocrine and magnocellular regions of the PVN influence vasopressin and pre-pituitary hormone release (9; 98; 138). Finally, the NTS has efferent projections to the central nucleus of the amygdala, which controls responses to stress (9; 98;

138), to the raphe nuclei, which influence multiple autonomic functions (2) and the respiratory centers of the ventrolateral medulla (9; 98; 138). Importantly, a majority of the efferent projections from the NTS are reciprocal. That is, the NTS both receives information from and sends information to a variety of brain regions involved in autonomic regulation.

Taken together, the evidence indicates that the NTS is a heterogeneous region, with many properties consistent with extensive modulation and integration. Understanding the effects of fine neuronal integration in this nucleus requires not only the knowledge of which cells are activated by a given stimulus, but also the phenotype of that cell, as well as the receptors that are present on that cell or the synaptic terminals surrounding it. The studies presented here use techniques to identify cell phenotypes, receptor expression, and a specific set of output neurons to further clarify neuronal activation and integration within the NTS. In the following sections, we will examine a type of receptor that can modulate synaptic activity and neuronal phenotypes that have been shown to have a role in regulation of cardiovascular function.

Pharmacology of Glutamate Receptors:

Propagation of baroreceptor information to second order neurons in the NTS requires an excitatory amino acid. L-Glutamate is considered the primary excitatory amino acid within the central nervous system. During fast excitatory transmission, glutamate activates ligand gated ionotropic glutamate receptors (iGluRs). These receptors allow the nonspecific movement of cations, which lead to cellular

depolarization (9). In the NTS, these iGluRs are essential for baroreflex function (7; 9; 56; 146), as stated above.

An additional group of receptors activated by glutamate is the metabotropic glutamate receptor family (mGluRs), a member of the G-protein receptor family (10; 32; 52; 114; 117; 118). Eight different genes for mGluRs have been sequenced. mGluRs are found throughout the CNS, though mGluR 6 is primarily localized to the retina (32; 117; 118; 128). The mGluRs traditionally are divided into three different subgroups, based on amino acid sequence homology and second messenger activity. Amino acid homology within a group is ~ 70%, and is generally less than 45% among groups. Group I mGluRs include receptor types 1 and 5, and activate phospholipase C, which induces phosphoinositide hydrolysis and increases intracellular Ca^{2+} concentrations. Group II includes receptor types 2 and 3, and are negatively coupled to adenylate cyclase, inhibiting the production of cAMP. Group III includes receptor types 4, 6, 7, and 8 and are also negatively coupled to adenylate cyclase. Although all groups can be found both pre- and postsynaptically (10; 32; 117; 118; 128), Group I mGluRs are generally postsynaptic and excitatory. In contrast, Group II and Group III mGluRs are generally found presynaptically and either increase or decrease neurotransmitter release.

Effects of mGluRs on Synaptic Activity:

mGluRs have been suggested to play a modulatory role in synaptic transmission. Long-term potentiation of cellular activity in the hippocampal CA1 and CA3 regions, the dentate gyrus, and the dorsolateral septal nucleus is initiated by

iGluRs, but its maintenance is dependent on mGluRs. Conversely, long-term depression of cellular activity can be induced in cerebellar Purkinje cells, hippocampal pyramidal neurons, striatal neurons and the visual cortex by mGluRs (10; 32; 117; 118; 128). These effects appear to be due to mGluRs inhibiting or potentiating the activity of various potassium and calcium channels (10). mGluRs are also expressed on cells that subsequently can influence synaptic activity. For example, Group I mGluR activation can induce the production of carbon monoxide (CO). Because CO is a highly diffusible gas and reacts with selected proteins, it in turn can influence the excitability of the affected cell or surrounding cells and terminals (89; 96). Also, Group I mGluRs have been shown to reduce release of GABA from interneurons by activation of the endocannabinoid system on Purkinje cells (19; 51). It is apparent that mGluRs are able to modulate synaptic activity on both a long and short term basis.

Role of mGluRs in the NTS:

It is likely that mGluRs modulate synaptic transmission within the NTS, subsequently influencing autonomic regulation. The anatomic distribution of mGluRs has been examined in the NTS. Immunohistochemical data indicate Group I, II, and III mGluRs are expressed throughout the NTS, and are located both on cell bodies and presynaptic terminals (63; 112). Studies using RT-PCR indicate the presence of Group I and II mRNA from NTS samples, consistent with the presence of Group I and II mGluRs in the NTS. The same study found mRNA for mGluRs 4, 7, and 8 within the nodose ganglion, which contains the cell bodies of visceral

afferents (72) that terminate in the NTS. Taken together, these data suggest that Group III mGluRs may be found in presynaptic terminals of the ADN or other visceral afferents entering the NTS, while Group I and Group II mGluRs are produced by cells located within the NTS boundaries.

The physiological effects of mGluRs have also been examined in the NTS. Although baroreflex function is abolished by blockade of iGluRs in the NTS, the decrease in arterial pressure and sympathetic nervous system activity in response to exogenous glutamate administration requires both iGluRs and mGluRs (48). Further, electrophysiological experiments found that activation of mGluRs in the NTS with the broad spectrum mGluR agonist 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid (ACPD) resulted in an excitatory effect in NTS neurons by increasing intracellular calcium levels due to postsynaptic activation of mGluRs, and a reduction in inhibitory currents, due to a presynaptic activation of mGluRs (52-54). Experiments using NTS slice preparations have shown that specific Group I mGluR agonists increase the number of action potentials to a given stimulus, due to activation of a voltage independent Na^+/Ca^{2+} exchanger and inhibition of outward K^+ currents on cells innervated by the ADN (134). These data suggest that Group I mGluRs are able to enhance cellular activity in NTS neurons that receive baroreceptor input, via a postsynaptic mechanism. There is, however, evidence suggesting that Group I mGluRs may facilitate the release of GABA from presynaptic terminals in the NTS (74), while other conflicting data suggest that only Group II mGluRs are able to affect GABA release (29). In dissociated NTS cells Group I mGluRs have been shown to facilitate or inhibit the activity of voltage gated calcium channels, dependent on the

specific type of calcium channel within the patch pipette (44). Thus, while Group I mGluRs may have some presynaptic effects, the majority of their action is mediated postsynaptically.

mGluRs in the NTS influence autonomic regulation. Microinjection of the Group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) into the NTS causes a reduction in mean arterial pressure (MAP), heart rate (HR) (104) and SNA (48; 49). These results are consistent with the concept that Group I mGluRs are able to activate neurons in the NTS that influence sympathoinhibition. Overall, Group I mGluR are located within the NTS and can increase synaptic excitability of cells that are involved with cardiovascular regulation. Therefore, our studies will focus on the expression of Group I mGluRs in the NTS.

Studies have examined the anatomic location of Group I mGluRs in the NTS and the effects of those receptors on NTS neuronal activity. However, little is known about the cell type on which those receptors are expressed. The studies presented in this project examined multiple cell phenotypes that are located within the NTS and, when activated, are able to affect cardiovascular function. The ability of Group I mGluRs to directly affect NTS neurons that project to baroreflex regions of the CVLM has not been examined previously. This project will examine both neurons in the NTS that project to the CVLM and the expression of Group I mGluRs on those CVLM projecting cells. Information from this project can then be compared to the expression of Group I mGluRs in the NTS in altered physiological or pathophysiological conditions.

Neuronal Subtypes within the NTS:

As stated above, the NTS is a complex network of afferent terminals, efferent cell bodies, and interneurons. Though the sympathetic arterial baroreflex is traditionally depicted as a three nuclear, two projection system within the hindbrain (Fig 1), those nuclear regions, especially the NTS, include numerous neurons that modulate synaptic transmission within the reflex. These studies examined three phenotypes that are well-documented cell populations within the NTS and that can, when activated, modulate synaptic activity and alter cardiovascular function.

GABA:

γ-amino butyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system (18; 55). Within the NTS, GABAergic neurons are believed to play a prominent role in influencing autonomic function (21; 55; 120; 142; 162). Bilateral microinjection of muscimol, a $GABA_A$ receptor agonist, or baclofen, a $GABA_B$ receptor agonist, into the NTS results in an increase in arterial pressure and sympathetic nerve activity (106; 144) and can reduce the gain of baroreflex mediated bradycardia (21). Unilateral microinjection of the $GABA_A$ receptor antagonist bicuculline into the NTS decreases baseline arterial pressure and sympathetic nerve activity (142). Also, bilateral microinjection of bicuculline, or phaclofen, a $GABA_B$ receptor antagonist, augments the excitatory response of NTS neurons to increasing arterial pressure (106; 142). These studies suggest a tonic inhibitory action of GABA on NTS neurons involved in arterial baroreflex function.

The distribution of GABAergic neurons in the NTS has been examined previously using immunohistochemical techniques or in situ hybridization for glutamic acid decarboxylase (GAD) in either its 65 kDa (GAD65) or 67 kDa (GAD67) isoform (27; 50; 140; 157). This enzyme synthesizes GABA from glutamate, and is commonly used as a marker for GABAergic neurons. These studies found GAD65 or GAD67 throughout the rostrocaudal extent of the NTS, in both cell bodies and terminals. The staining is heterogeneous within the subnuclear regions of the NTS (27; 50; 140; 157).

Though traditionally thought to be interneurons only, GABAergic neurons in the NTS have a varied and important function. NTS GABAergic neurons project to the respiratory regions of the ventral medulla and the cardiovascular regions of the PVN (12). Bailey et al (12) labeled GABAergic neurons with green fluorescent protein, which was coupled to the GAD promoter. GABAergic neurons were found to be second order neurons that receive direct excitatory innervation from the solitary tract, but also may receive inhibitory input from higher order GABAergic neurons in the NTS (12). Also, some GABAergic neurons were not directly activated by solitary tract stimulation, suggesting that those GABAergic neurons may be higher order interneurons in the NTS or not involved in baroreflex function. Thus, GABAergic neurons likely play an important role in modulation of NTS activity and may directly affect other cardiovascular and respiratory regions of the brain.

GABAergic neurons are critical in determining the baseline activity within the NTS. In addition, alterations of GABA release or GABA receptors can modulate NTS activity. This work ascertains the anatomic location of GABAergic neurons in

the NTS in relation to the Group I mGluRs, CVLM-projecting neurons, and neurons activated by increases or decreases in arterial pressure.

Nitric Oxide:

Nitric oxide (NO) is a gaseous substance that has a modulatory role throughout the body. Peripheral administration of the NO precursor L-arginine or NO donors such as sodium nitroprusside can induce hypotension through vascular dilation (82). Within the central nervous system, NO is a potent neuromodulator (41; 81; 82; 147) and, as a gas, easily diffuses through plasma membranes to influence cellular activity. Neuronal nitric oxide synthase (nNOS), or NOS type 1, is one of three enzymes responsible for the production of NO. nNOS expression has been shown to be present throughout the central nervous system. In the central nervous system, nNOS can be expressed in neurons or glia cells. However, within the NTS, nNOS is localized exclusively to neurons, in both cell bodies and vagal afferent terminals (22; 46; 47; 90-94). Therefore nNOS is located both presynaptically and postsynaptically. nNOS is activated by increases in intracellular Ca^{2+} concentrations, primarily through activation of ionotropic glutamate receptors (46; 82; 154) to produce NO from the amino acid L-arginine. NO acts as an autocrine and paracrine factor, as it may modulate the activity of the cell that produced it or other surrounding cells or terminals within ~200 μm (82; 154). NO activates soluble guanylate cyclase, which in turn increases intracellular levels of cyclic guanine monophosphate (cGMP). cGMP can activate receptors within the target cell, activate other intracellular second messengers, or directly activate membrane

channels (28; 82; 154). NO has also been shown to increase neuronal activation and induce gene transcription (47). Microinjection of NO precursors or donors into the NTS induces sympathoinhibition and decreases in arterial pressure and heart rate while administration of NOS inhibitors into the NTS increases SNA, arterial pressure, and results in tachycardia (60).

As nNOS is tonically active, NO production and release is a component of reflex modulation within the NTS. Microinjection of NOS inhibitors reduces the depressor response and bradycardia associated with activation of the arterial baroreflex and the cardiopulmonary reflex (57). nNOS inhibition also results in a greater sympathoexcitation during activation of the exercise pressor reflex (60). Finally, nNOS has been suggested to have a role in the development of hypertension. In the spontaneous hypertensive rat, the level of nNOS protein in the NTS decreases as the animal ages (and becomes hypertensive) in comparison to normal rats, thus suggesting nNOS and NO release aid in preventing hypertension (119).

NO plays a pivotal role in the NTS in maintaining normal body homeostasis. Hypertensive stimuli result in an activation of nitroxidergic neurons (27). However, it is unknown if periods of hypotension may also activate nitroxidergic neurons within the NTS. It is also unknown if the depressor response elicited by Group I mGluR activation is due to direct activation of nNOS neurons and if nitroxidergic neurons also project to the CVLM. This project will begin to address those questions.

Catecholamines:

Catecholaminergic neurons in the NTS receive input from, and project to, various medullary and supramedullary nuclear regions, including those that influence sympathoexcitation (69; 75; 76; 123; 143). Tyrosine hydroxylase (TH) is the initial and rate limiting enzyme in the production of catecholamines. TH catalyses the reaction that converts the amino acid tyrosine into L-DOPA (75; 76). This enzyme is located throughout the CNS and has been used as a marker for catecholaminergic neurons (75; 76). Catecholaminergic NTS neurons have been shown to project to the PVN, RVLM, and the amygdala (69; 75; 76; 123). Catecholaminergic cells, when activated, can influence the sympathetic nervous system through activation of neurons in the RVLM or pre-autonomic neurons of the PVN, hormone production through the neuroendocrine neurons of the PVN, or alter activity of the limbic system by stimulating neurons in the amygdala. Previous studies have shown that few catecholaminergic cells are activated by acute bouts of hypertension (27). In contrast , mRNA for TH has been shown to colabel with Fos-immunoreactivity (IR) after periods of hemorrhage (25). Other studies have examined the expression of dopamine-β-hydroxylase, an additional enzyme in the catecholamine cascade, in response to hypotension and also indicate that these neurons are activated (38).

The current studies examine catecholaminergic neurons in the NTS to determine if they express Group I mGluRs and whether those TH containing neurons are activated by hypertension or hypotension. These studies also confirm that catecholaminergic neurons of the NTS and CVLM projecting neurons are separate populations.

Fos as a Marker of Neuronal Activation:

Identification of neurons activated in response to hypertension or hypotension has been achieved using Fos immunohistochemistry. Fos is a protein that is transcribed from the *c-fos* gene. The proto-oncogene *c-fos* is a member of the immediate early gene family (37-39; 68; 124). *c-fos* mRNA is transcribed after periods of increased intracellular calcium and second messenger activity due to synaptic activity. The protein product Fos forms a heterodimer with the protein Jun, which then attaches to a target gene and induces transcription (37-39; 68; 124).

Production of Fos requires the cell to be activated beyond threshold. If a specific stimulus is paired with appropriate control conditions, it is possible to use Fos as a marker of synaptically evoked cellular activity within the central nervous system. Fos protein translation peaks at 90-120 minutes after initiation of the stimulus (37-39; 68; 124). Because of this, Fos protein derived from *c-fos* is used mainly to demonstrate an acute increase in synaptically evoked neural activity. Fos immunohistochemistry has been used extensively to map brain regions activated by the arterial baroreflex. Neuronal activation, as indicated by Fos expression, is localized primarily to neurons in a strip of the dorsal subnucleus of the commissural NTS and the dorsal, medial, and interstitial subnuclei of the medial NTS (23; 24; 26; 27; 58; 107-109; 122; 135; 157).

Fos immunohistochemistry has a number of distinct advantages that make it ideal for the current studies. It allows for the use of conscious animals subjected to specific stimuli. This is important as anesthesia is well known to alter baseline and reflex control of cardiovascular function. Unlike electrophysiological techniques, Fos

immunohistochemistry allows for evaluation of populations of neurons activated by specific stimuli, and examination of the regional distribution of that population of neurons. When combined with tract-tracing techniques or immunohistochemistry for other markers, it is possible to evaluate afferent or efferent projections of activated neurons, receptors expressed by those neurons, and the biochemical phenotype of those neurons. Finally, the effects of varying stimuli can be compared in similar nuclear regions, as the expression of Fos is somewhat qualitative (37-39; 68; 124).

Certain caveats must be considered when using Fos to detect neural activation. The *c-fos* gene is not activated by cellular activation in some cells. It is possible, then, to have neural activation but no Fos present. Fos protein is not expressed in neurons that are inhibited by a given stimulus, even though the inhibition may be critical to the response to that stimulus (37-39; 68; 124). Further, in addition to the requirement for an adequate stimulus to be able to visualize Fos, the duration of that stimulus is also important. In these studies drug treatments were for 90 minutes, after which animals were sacrificed. This paradigm, which also contained a set of control animals and extended baseline period, would likely prevent neural activation from nonspecific stimuli.

Activation of NTS Neurons:

An important goal of these studies was to understand which neurons in the NTS are activated in response to increases and decreases in arterial pressure. Increases in arterial pressure would increase baroreceptor afferent activity and activate neurons in the NTS (Fig.1). This has been shown repeatedly (23; 24; 26;

27; 58; 107-109; 122; 135; 157). Furthermore, with a normally functioning arterial baroreflex, periods of hypotension would decrease baroreceptor afferent activity and might be expected to result in reduced activity in the NTS. However, this does not appear to be the case, as hypotensive stimuli actually produce activation of NTS neurons (26; 122). Sinoaortic denervation, which prevents Fos-immunoreactivity in the NTS after a period of hypertension (23; 34; 122), also eliminates Fos-immunoreactivity after periods of hypotension (122). These data are consistent with the concept that both hypertension and hypotension-induced NTS neuronal activation are baroreflex mediated. This is paradoxical, in that the reflex responses to these stimuli are directionally opposite. One possible explanation is that different populations of NTS neurons are activated by increases and decreases in arterial pressure.

To our knowledge, no studies have examined the expression of Fos in the NTS after periods of both hypertension and hypotension in concert with evaluation of specific cell phenotypes. By examining Fos-immunoreactivity associated with cell phenotypes that have been shown to affect cardiovascular function, in addition to cells that project from the NTS to the CVLM, these studies examine if there is a phenotypic difference in activated neurons in the NTS after acute hypertension or hypotension.

Retrograde Tracer:

To identify neurons that project form the NTS to the CVLM, these studies used a retrograde tracer. Hydroxystilbamidine (Fluoro-Gold; FG), is a commercially

available compound that can be used as a fluorescent retrograde tracer (6; 80; 127; 152; 156). FG injected into the central nervous system is taken up by surrounding axons, neural terminals or cell bodies by passive diffusion (6; 80; 127; 152; 156). Once in the neuron, FG, which is a weak base, diffuses into the lysosomes, where it is protonated. The positively charged molecule cannot pass through the hydrophobic membrane of the lysosome, and the FG remains trapped within the cell as the lysosomes migrate along the cellular cytoskeleton in retrograde fashion toward the cell body (6; 80; 127; 152; 156).

FG is advantageous to use in immunohistochemical protocols. Its fluorescent nature and stability over time in fixed tissue allows for usage in immunohistochemical experiments without the requirement of a primary/secondary antibody protocol to visualize it and without the risk of loss of positive signal due to immunohistochemical protocols to detect other antibodies (6; 80; 127; 152; 156). When used with pharmacological mapping techniques (in these experiments by glutamate microinjections into the region of the CVLM) discrete FG microinjections can result in labeling of NTS neurons that project to the CVLM, and few neurons in other nuclear regions.

The use of FG does have some limitations. As stated above, FG can be taken up by fibers of passage in the target area. This can lead to FG labeling in areas that do not have terminals within the FG microinjected region. Also, FG can be quickly quenched in tissue with prolonged exposure to ultraviolet light (80; 152).

Using FG in conjunction with immunohistochemistry to identify specific neural populations, it will be possible to further clarify the phenotype of output neurons from

the NTS to the CVLM. We also will determine if there is a variation in the phenotype of CVLM projecting neurons activated in response to hypertension or hypotension.

Summary:

The NTS is a key region of the hindbrain for autonomic function. Its ability to integrate and modulate signals and convey that information to other nuclear regions is a vital component of autonomic regulation. Understanding the expression of Group I mGluRs and NTS neurons activated by hypertension or hypotension in relation to a group of three phenotypically different cells will allow for a better understanding of how that integration and modulation takes place in the NTS. Using a retrograde tracer to label neurons that project from the NTS to the CVLM will extend our knowledge regarding the ultimate information sent from the NTS to the CVLM, which are critical to arterial baroreflex function.

The information from these studies can be used to begin to determine if altered baroreflex function in different physiological or pathophysiological conditions is associated with alteration of Group I mGluR expression in the NTS, or an alteration in the phenotype of neuron activated in response to hypertension or hypotension.

Specific Aims:

Study 1: To examine the expression of Group I mGluRs on phenotypically different neurons in the NTS

Hypothesis A: Group I mGluRs are expressed on CVLM projecting and nitroxidergic neurons to a greater extent than on GABAergic or catecholaminergic neurons within the NTS.

Hypothesis B: CVLM projecting neurons within the NTS colabel with nitroxidergic cells to a greater extent that GABAergic or catecholaminergic cells.

This study will use retrograde labeling (to identify neurons in the NTS that project to the CVLM) in concert with immunohistochemical techniques to identify neurons that express Group I mGluRs and are GABAergic, nitroxidergic or catecholaminergic.

Study 2: To compare the number of phenotypically different neurons within the NTS that are activated by acute hypertension or hypotension.

Hypothesis A: Hypertension and hypotension produce similar overall levels of neural activation in the NTS.

Hypothesis B: Hypertension activates CVLM projecting and nitroxidergic neurons to a greater extent than GABAergic or catecholaminergic neurons.

Hypothesis C: Hypotension activates GABAergic or catecholaminergic neurons to a greater extent than CVLM projecting and nitroxidergic neurons.

This study will use retrograde labeling to identify neurons that project from the NTS to the CVLM. Animals will be administered drugs to increase or decrease arterial blood pressure, with a control group. Immunohistochemical techniques will allow identification of neurons activated by the change in arterial pressure, along with their phenotype (GABAergic, nitroxidergic or catecholaminergic).

Study 3: To examine the expression of Group I mGluRs on neurons within the NTS activated by acute hypertension or hypotension and neurons that project to the CVLM.

Hypothesis A: Hypertension and hypotension result in generally similar levels of activation of neurons within the NTS.

Hypothesis B: Neurons activated by hypertension or hypotension colabel with neurons that express Group I mGluRs.

Hypothesis C: Neurons that are activated by hypertension and project to the CVLM also express Group I mGluRs.

This study will use retrograde labeling to identify neurons that project from the NTS to the CVLM. Animals will be subjected to pharmacologically induced increases or decreases in arterial blood pressure. Immunohistochemical techniques will identify neurons activated by the change in arterial pressure and neurons that express Group I mGluRs.

Chapter 2: Expression of Group I Metabotropic Glutamate Receptors on Phenotypically Different Cells within the Nucleus of the Solitary Tract in the Rat

Background:

Group I mGluRs in the NTS have been suggested to serve as an excitatory receptor. Anatomic data describe the presence of Group I mGluR mRNA and protein in the NTS. Furthermore, activation of Group I mGluRs in the NTS results in a depressor response and sympathoinhibition. There is, however, little information regarding the expression of Group I mGluRs on specific cell phenotypes. This study categorized the expression of Group I mGluRs on three phenotypes of neurons known to influence autonomic regulation within the NTS. GABAergic neurons, which produce the primary inhibitory neurotransmitter, GABA, modulate synaptic transmission pre- and postsynaptically. Nitroxidergic neurons produce the gaseous neuromodulator NO that diffuses readily and can influence surrounding neurons. Catecholaminergic neurons are a population of neurons that project from the NTS to multiple regions of the forebrain and hindbrain. In addition, we studied neurons that project from the NTS to the CVLM. As described in Figure 1, arterial baroreflex information is passed through the NTS to the CVLM, after any modulation of that information. Therefore, the information that is propagated by the efferent projection from the NTS is the integration of a given input and its modulation in the NTS. This study will utilize the retrograde tracer Fluoro-Gold, microinjected into the CVLM, to identify neurons in the NTS that project to the CVLM, in conjunction with

examination of the expression of Group I mGluRs and GABAergic, nitroxidergic, and catecholaminergic neurons. This was done to determine if Group I mGluRs may directly influence CVLM projecting neurons, and to further characterize the phenotypes of neurons that project to the CVLM.

Abstract:

Group I metabotropic glutamate receptors (mGluRs) are a G-coupled receptors that modulate synaptic activity. Previous studies have shown that Group I mGluRs are present in the nucleus of the solitary tract (NTS), a nuclear region in which various visceral reflex afferent terminate. Microinjection of selective Group I mGluRs results in a depressor response and decreases in sympathetic nerve activity. There is, however, little evidence detailing which phenotype of neuron that Group I mGluRs are expressed on. The retrograde label Fluoro-Gold was microinjected in the caudal ventrolateral medulla of the rat. After five days, to allow transport of FG to the NTS, animals were sacrificed and immunohistochemically treated for Group I mGluRs and either glutamic acid decarboxylase isoform 67 (GAD67), neuronal nitric oxide synthase (nNOS) or tyrosine hydroxylase (TH). Distribution of GAD67, FG, nNOS, and TH was similar to previous reports.

Introduction:

The nucleus of the solitary tract (NTS), located in the dorsal medulla, is the site of the first synapse of cardiovascular, respiratory, and visceral afferent terminals (23; 27; 138). This includes arterial baroreceptor afferents, which are critical for control of arterial blood pressure on a beat to beat basis (98). The NTS is a highly integrative nucleus, and baroreceptor input is believed to undergo substantial modulation within the NTS. Output neurons from the NTS then relay this information to the caudal ventrolateral medulla (CVLM) through an excitatory projection. Neurons within the CVLM in turn inhibit spinally projecting pre-sympathetic neurons in the rostral ventrolateral medulla (5; 9; 23; 27; 36; 59; 98). Thus, the activity of NTS neurons projecting to the CVLM is a critical determinant of NTS influence on autonomic function.

Within the NTS, baroreceptor afferent terminals are believed to release the excitatory amino acid glutamate (146). Fast-acting ionotropic glutamate receptors within the NTS are required for normal baroreflex function (9); (56; 146). Metabotropic glutamate receptors (mGluRs) also are present in the NTS (30; 63; 72) and may influence cardiovascular regulation (30; 48; 49; 95; 134). Blockade of the sympathoinhibition and depressor response due to microinjection of glutamate into the NTS requires antagonism of both ionotropic glutamate receptors and mGluRs (48; 49; 114). NTS microinjection of a broad spectrum mGluR agonist or a Group I selective agonist produces a depressor and sympathoinhibitory response (48; 49). While these effects could be due to activation of several autonomic circuits, they are consistent with activation of baroreflex output neurons to the CVLM. It is currently

unknown whether CVLM projecting output neurons from the NTS express Group I mGluRs.

The NTS contains a wide variety of neurotransmitters and neuromodulators that influence cardiovascular regulation (4; 27; 56; 87). Group I mGluRs could thus affect cardiovascular function by modulating their activity. However, the specific neuronal subtypes in the NTS that are influenced by Group I mGluRs are unknown. GABAergic neurons are located throughout the NTS (27; 50; 140; 157). In addition, previous studies have shown the presence of neurons within the NTS containing neuronal nitric oxide synthase (nNOS) (77; 90; 91; 93; 147) or tyrosine hydroxylase (TH) (13; 23; 75; 76), synthetic enzymes for nitric oxide (NO) and catecholamines, respectively. Furthermore, NTS neurons containing nNOS, GAD67, (a GABA synthesizing enzyme), and TH all are activated by changes in arterial pressure, likely due to changes in baroreceptor activity (23; 27; 107; 157). Therefore GABA, NO, and TH containing neurons within the NTS all have the potential to influence arterial pressure, sympathetic nerve activity, and baroreflex function. There is, however, little evidence delineating which cell phenotypes within the NTS express Group I mGluRs.

The overall goal of this study was to examine the expression of Group I mGluRs on specific neuronal subtypes within the NTS. The expression of Group I mGluRs on CVLM projecting output neurons from the NTS was examined using the retrograde tracer Fluoro-Gold (FG). In addition, we evaluated the expression of Group I mGluRs on specific neurochemical phenotypes using immunohistochemistry for synthetic enzymes for GABA, NO and catecholamines in the NTS. Lastly, we

examined the neurochemical phenotypes of the CVLM projecting neurons that express Group I mGluRs.

Methods:

Animals:

Experiments were performed according to the guidelines in the NIH "Guide for the Care and Use of Laboratory Animals." The University of Missouri Animal Care and Use Committee approved all procedures and protocols. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275-325 grams were used (n=14). Rats were housed within an in-house animal facility on a 12 hour day/night cycle. Food and water were available ad libitum. All rats were given at least seven days to acclimate to the surroundings prior to any experimental procedure.

Surgical and Microinjection Procedures:

All recovery surgeries were performed under aseptic conditions. Rats were anesthetized with Isoflurane (AErane, Baxter [5% in 100% O_2, 2L per minute for induction; maintenance at 2-2.5%]). A catheter (PE10 fused to PE50) was inserted into the aorta via the femoral artery, and connected to a pressure transducer. Arterial pressure was measured using a DC amplifier (ADInstruments, Colorado) and mean arterial pressure (MAP) and heart rate (HR) were determined using a PowerLab Data Acquisition System (ADInstruments) connected to a Pentium computer. The rat was placed in a Kopf stereotaxic device, and the dorsal surface

of the medulla was exposed via a limited occipital craniotomy. Calamus scriptorius (CS), defined as the caudal pole of the area postrema (AP), was visualized, and the head of the rat was deflected downward until CS was 2.4 mm posterior to the interaural zero line. This positioned the medulla in the horizontal plane (79; 110). After determining stereotaxic coordinates for CS, a glass pipette filled with appropriate solutions was positioned within the brainstem for microinjection of Fluoro-Gold or for functional verification of the CVLM.

Fluoro-Gold Microinjections:

Once stereotaxic coordinates for CS were determined, a double-barreled pipette (OD 20-30 μm) with one barrel filled with L-glutamate (Glu, 10 mM) and the second filled with Fluoro-Gold (FG) (2% in dH_2O) was placed at the initial target stereotaxic coordinates for the CVLM. With the medulla positioned horizontally and CS positioned as described above, these coordinates were 0.2 mm caudal to and 2.0-2.2 mm lateral from CS, and 2.0-2.6 mm ventral to the dorsal surface of the brain. The location of the CVLM was confirmed functionally by responses to microinjection of Glu (30 nL). Criteria for identification of the CVLM were set at a depressor response of ~20 mmHg with a moderate (~40 beat per minute) decrease in HR. These criteria were met within 2-5 microinjections of Glu. Once the CVLM was localized, FG (15 nL) was injected at the same CVLM site through the second barrel of the pipette. Microinjections were made using a custom built pressure injection system. Fluid in the pipette was visualized through a 150x microscope with

a calibrated eyepiece micrometer, which allowed quantification of the volume injected by measuring the movement of the meniscus within the pipette.

Following the FG microinjection the pipette remained in the medullary tissue for an additional five minutes to minimize movement of FG up the injection tract. After removal of the pipette, the arterial catheter was removed and the leg and neck areas were sutured closed. Animals were given post-operative injections of Penicillin-D (0.2 ml, i.m.) and Buprenex (0.6 mg/ml, i.m.) to prevent infection and for pain management, respectively, and returned to their cages.

Transcardial Perfusion and Tissue Preparation:

Five days after FG microinjection, rats were deeply anesthetized with an i.p. injection of 0.2 ml Sleepaway (Ft. Dodge, IA) and transcardially perfused with 100-125 ml Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) followed by 450-500 ml of 4% paraformaldehyde. Brains were additionally post-fixed in 4% paraformaldehyde for two hours, and stored in 0.1M PBS. Brains were then blocked approximately at the level of Bregma -7mm, stripped of the dura, and embedded into 2% agar gel. Thirty micron sections were cut on a vibrating microtome (Vibratome, St Louis, MO), separated into a 1 in 6 series and then stored in 0.1M PBS. One set of sections was mounted on gel-coated slides, air-dried, covered in non-hard set Vectashield (Vector, Burlingame, CA), and the coverslip sealed with clear nail polish. This set of sections was used to determine the center of the FG injection and to determine boundaries of the NTS. If immunohistochemistry was performed within

one week, sections were stored at 4°C in 0.1M PBS. All other sections were stored in cryoprotectant at -20°C.

Immunohistochemistry:

Sections were treated with a modified immunohistochemical protocol (50) to visualize Group I mGluR, GAD67, TH, and nNOS containing cells. The specific steps of the protocol depended on the protein visualized (see below). Preliminary experiments were performed with varying concentrations of primary and secondary antibodies to optimize concentrations that provided the best positive signal with the least background signal. Each immunohistochemical protocol was a pairing of primary antibodies for Group I mGluRs and either GAD67, nNOS or TH. In addition, FG, which autofluoresces, was present in all series treated. All incubations in the immunohistochemical protocols were performed at room temperature on a shaker in sterile 48 well plates (Vector).

Group I mGluRs and GAD67:

Sections were incubated in 0.3% H_2O_2 in 0.1M PBS for 30 minutes then rinsed in 0.1M PBS for 15 minutes followed by incubation in pre-blocking solution (10% Normal Goat Serum [NGS, Jackson, West Grove, PA] in 0.1M PBS) for 30 minutes. Sections were rinsed again for 15 minutes in 0.1M PBS, then incubated for two days in a cocktail of rabbit anti-mGluR 5/1α (1:500, Chemicon, Temecula, CA) and mouse anti-GAD67 (1:3000, Chemicon) with 3% NGS in 0.1M PBS at room temperature on a shaker. After the two days, sections were rinsed in 0.1M PBS for

30 minutes before incubation for 2 hours in a cocktail of goat anti-rabbit Cy3 (1:300, Jackson) and goat anti-mouse biotinylated IgG (1:200, Vector) in 3% NGS in 0.1M PBS. Sections were rinsed in 0.1M PBS for 30 minutes, and placed into Streptavidin-Horseradish Peroxidase (1:200, PerkinElmer, Boston, MA) with 3% NGS in 0.1M PBS for 60 minutes. Sections were rinsed for 30 minutes, then incubated with TSA-biotin (1:100, PerkinElmer) with 3% NGS in 0.1M PBS for 15 minutes. Sections were rinsed for 30 minutes, then incubated with Neutravidin/avidin Oregon Green (1:300, Molecular Probes, Carlsbad, CA) with 3% NGS in 0.1M PBS for 2 hours. Sections were rinsed for 30 minutes, mounted on gel-coated slides, air-dried, coverslipped with Vectashield (Vector), and sealed with clear nail polish. Immunohistochemistry involving GAD67 was always performed within one week of perfusion of the tissue.

Group I mGluRs and nNOS or TH:

Sections were rinsed for 15 minutes in 0.1M PBS, followed by a 10% pre-blocking solution (10% Normal Donkey Serum [NDS, Jackson] in 0.1M PBS) for 30 minutes. Sections were rinsed again for 15 minutes in 0.1M PBS, then incubated for 48 hours in a cocktail of rabbit anti-mGluR 5/1α (1:500, Chemicon) and either mouse anti-nNOS (1:2000, Santa Cruz, Santa Cruz, CA) or mouse anti-TH (1:500, Chemicon) with 3% NDS in 0.1M PBS. After primary incubation, the sections were rinsed in 0.1M PBS for 30 minutes. Sections were then incubated with a cocktail of donkey anti-rabbit Cy3 (1:300 Jackson) and donkey anti-mouse Cy2 (1:200, Jackson) with 3% NDS in 0.1M PBS for two hours. Sections were rinsed in 0.1M

PBS for 30 minutes. Sections then were mounted onto gel-coated slides, dried, coverslipped with Prolong Gold (Molecular Probes) and sealed with clear nail polish.

Negative Controls:

For each immunohistochemical protocol, primary antibodies were withheld on some sections, to serve as non-immune controls. Control sections were examined to verify lack of specific positive labeling.

Microscopy and Image Analysis:

After the immunohistochemical procedure, sections were examined using an Olympus microscope (BX51), equipped with a 3-axis motorized stage (Ludl Electronic Products Ltd, Hawthorne, NY) and with filter sets for Oregon Green 488 or Cy2 [ex. λ480 nm; em. λ510 nm], Cy3 [ex. λ550 nm; em. λ570 nm], and Fluoro-Gold [ex. λ330 nm; em. λ515 nm]. Images were captured using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Image analyses and cell localization were performed using the software package Neurolucida (ver. 7.5, MicroBrightField, Willisonton, VT).

For each immunohistochemical group examined, CS was defined as the section in which the caudal pole of the area postrema (AP) is present. Sections from 360 μm caudal - 1260 μm rostral to CS were counted. Sections were outlined and the injection site of FG in the CVLM was verified. The NTS, tractus solitarius, and adjacent nuclei ipsilateral to the FG injection site were outlined. The top and bottom of the section in the Z-axis was defined and a stacked image of photomicrographs

was taken, with a distance of 1 micron between each image, representing the full thickness of the section. Three stacks of images were taken, one for each filter set without altering the position of any of the 3 axes. Positively immunoreactive (IR) and FG containing cells in the NTS were counted. The following criteria were set for counting cells: GAD67, TH, and nNOS-IR cells exhibited complete cytosolic labeling with a blank nuclear region. FG containing cells had either punctate labeling or completely filled cells in which no nuclear region was visible. mGluR-IR cells exhibited labeling that surrounded the soma. Cells with labeling that did not meet these criteria were not counted. When positive signals that fit the criteria above were observed under different filter sets, and these occurred in the same focal plane with similar cell morphology, the cells were considered double-labeled. Cells that contained positive signal with all three filter sets were considered triple-labeled.

Data Analysis:

After completion of analysis of each brain, the data were exported to the software package NeuroExplorer (ver. 4.5, MicroBrightField), separated into single, double and triple labeled cells in each specific outlined section (see above), and exported into a spreadsheet (Excel 11.656, Microsoft, Redmond, WA). The data were expressed in individual sections based on distance from CS. In addition, total counts, counts in sections caudal to the AP (caudal NTS; ~Bregma -15.24 to -14.4 mm), at the level of AP (postremal NTS; ~Bregma -14.4 to -13.56) and rostral to AP (rostral NTS; ~Bregma -13.56 to -12.96) were evaluated. Furthermore, the percentage of double-labeled cells relative to the number of each of the single

labeled cells was determined. Calculation of the percent of colabeling was achieved by dividing the number of colabeled cells by the total number of each of the single labeled cells. In this manner, the extent of colabeling was expressed as a percent of the total number of cells with each individual label. The total number of triple labeled cells of each immunohistochemical grouping was used to determine the percent of double-labeled cells that were also triple labeled. Graphs of data were generated using SigmaPlot (8.0, SPSS, Chicago, IL). Distribution of cells was determined by examination of sections in NeuroExplorer. Locations of various subnuclear regions were determined using a standard brainstem atlas (115).

For representative digital images of individual and co-labeled neurons, single images were taken with each appropriate filter of selected sections. These images were acquired with Neurolucida (MicroBrightField) and then imported, pseudocolored, and merged using Photoshop (ver. 7.0, Adobe Systems, San Jose, CA). Only brightness and contrast were adjusted for clarity.

Functional Verification of CVLM:

Experiments were performed to further verify that the functional criteria used for our FG microinjections were within the cardiovascular regions of the CVLM which receive input from a depressor region of the NTS (5). A separate group (n=4) of Sprague-Dawley rats was anesthetized with Isoflurane (AErane, Baxter [5% in 100% O_2, 2L per minute; maintenance at 2-2.5%]). Rats were instrumented as above. A tracheotomy was performed, rats were ventilated and a catheter (PE10 fused to PE50) was inserted into the inferior vena cava via the femoral vein. Once the dorsal

medullary area was visualized, rats were gradually removed from Isoflurane while receiving bolus injections (0.05 ml) of Inactin (100 mg/ ml, i.v. for a total dose of 100 mg/kg over ~30 min). Microinjections were performed using a triple-barreled pipette (OD 30-40 μm), containing Glu (10 mM), the general ionotropic glutamate receptor antagonist kynurenic acid (KYN, 40 mM), and 2% Chicago Sky Blue Dye. Once the CVLM was located by a depressor response to Glu (30 nL) as described above the pipette was moved to the region of the NTS (0.5 mm rostral and 0.5 mm lateral to CS, and 0.5-0.7 mm ventral from the dorsal surface). Glu (30 nL) was microinjected to verify the region of the NTS producing a depressor response. Appropriate pipette placement was verified by a 15-20 mmHg reduction in MAP in response to Glu. The pipette was returned to the CVLM and a depressor response was reconfirmed with an additional application of Glu. KYN (40 mM, 15 nL) was then microinjected into the CVLM to block ionotropic glutamate receptors. The pipette was then returned to the coordinates of the NTS, and Glu microinjected as before. Glu injections were repeated every five minutes until the depressor response recovered. After completion of the experiment, NTS and CVLM microinjection sites were each labeled with 15 nL of 2% Chicago Sky Blue dye. Animals were euthanized with 0.2 ml Sleepaway i.v. Brains were removed and placed into formalin for subsequent histological analysis.

Statistics:

All data are presented as mean ± SE. Hemodynamic responses to Glu microinjection into the CVLM were analyzed by a Student's t-test. Absolute values

of MAP and HR in response to Glu microinjection into the NTS under control conditions, after KYN injection and during recovery were analyzed by repeated measures (RM) two-way ANOVA. The change in MAP and HR due to Glu microinjection into the NTS before, during, and after KYN injection, the regional distribution of counted labels and the percent of colabeling were analyzed with one-way RM ANOVA. The rostrocaudal distribution of Group I mGluRs and FG in the different immunohistochemical protocols was compared by two-way RM ANOVA. When appropriate, ANOVA's were followed by a Student-Neuman-Keuls post-hoc analysis. Statistical analyses were performed using Sigma Stat software and significance was accepted at $P < 0.05$.

Results:

Validation of Immunohistochemical and Fluoro-Gold protocols:

Representative photomicrographs within the NTS of each neurochemical phenotype evaluated are shown in Figure 3. Photomicrographs of sections treated with antibodies were taken (Fig. 3 A1-D1), and without changing camera exposure time, images of non-primary control sections were taken (Fig. 3 A2-D2). No positively labeled cells were observed in the absence of the primary antibody.

Group I mGluRs and FG were included in each of the immunohistochemical protocols. To verify that inclusion of different antibodies, immunohistochemical protocols and cyroprotection did not affect the labeling of Group I mGluRs or FG, we compared the amount and distribution of Group I and FG labeling among the different protocols. There were no significant differences in the number or

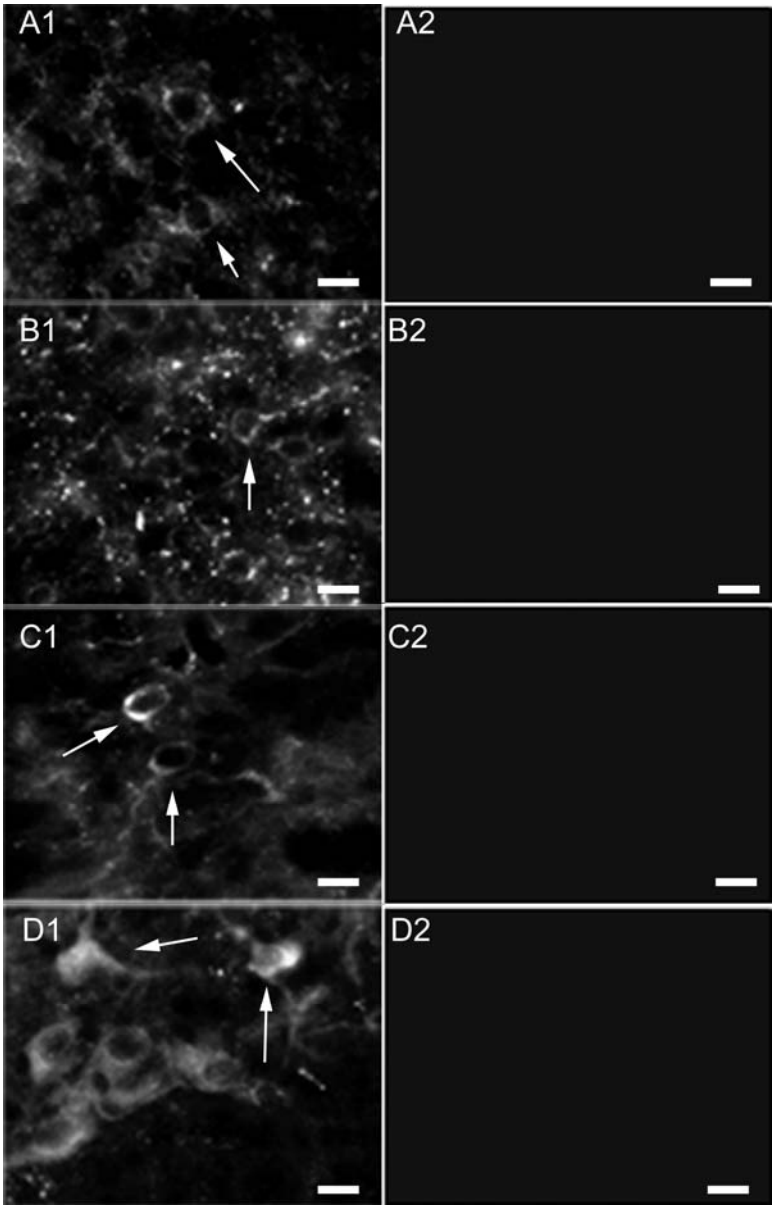

Figure 3: **Validation of immunohistochemical protocols.** Epifluorescent photomicrographs of immunopositive labeling for Group I mGluRs (A1), GAD67 (B1), nNOS (C1) and TH (D1). Controls (omission of primary antibody) are shown in A2-D2. Both positive and control images were taken from regions of the NTS that were examined in this study. Arrows in A-D denote positively labeled neurons. Scale Bar is 25 μm.

Control images were taken at the same exposure time as their positively labeled counterparts in adjacent sections and did not show any positive immunohistochemical labeling.

distribution of Group I mGluRs (Fig 4A) or FG labeled cells (Fig 4B) among any of the protocols. Mean values for the three protocols are represented by the solid lines.

Distribution of Immunolabeling within the NTS:

Cells that were immunoreactive (IR) for each marker were observed throughout the rostrocaudal distribution of the NTS. Figure 5 is a diagrammatical illustration showing the distribution of positive labeling for each marker at three rostrocaudal levels of the NTS in individual animals. Table 1 details the average number of positively labeled neurons per section within the three regions of the NTS examined. The description of positive labeling within the three regions of the NTS follows.

Group I mGluRs:

Group I mGluRs exhibited primarily punctate labeling that surrounded a large dark region, suggestive of labeling associated with the plasma membrane (Figs. 3A, 6A1-C1). Additional punctate labeling was exhibited throughout the NTS consistent with terminal labeling. Group I mGluRs were distributed extensively throughout the rostrocaudal axis of the NTS (Figs. 4A; 5A1-A3). The number per section of Group I mGluR-IR cells in the postremal and rostral regions of the NTS was significantly greater than in the caudal region (Table 1). Subnuclear distribution within the NTS was estimated by comparison to previous anatomical reports (115). Within the caudal NTS (Fig. 5A1), the Group I mGluR-IR cells were observed in the

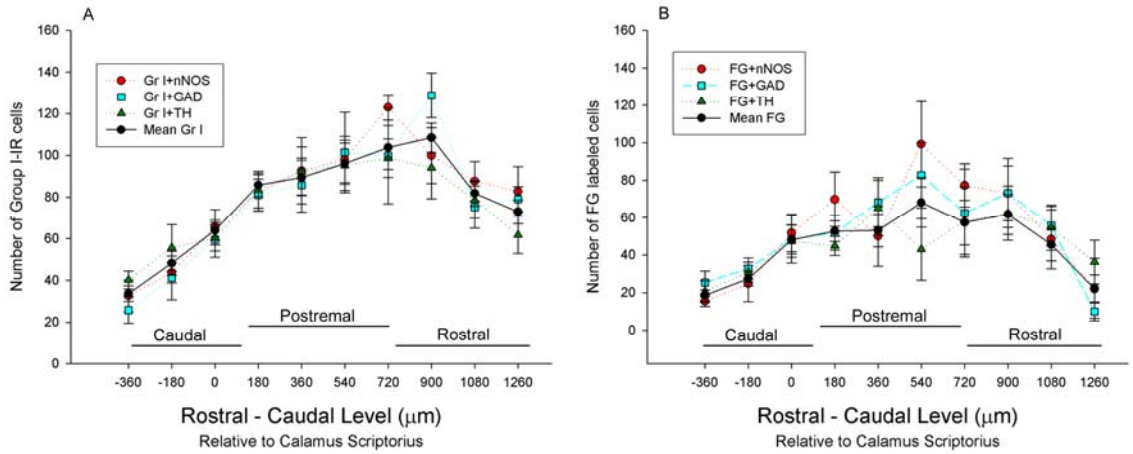

Figure 4: **Rostral-caudal distribution of Group I mGluRs and FG**. (A) Distribution of Group I mGluR immunoreactive neurons and (B) Fluoro-Gold (FG) containing neurons from separate protocols for immunohistochemical visualization of Group I mGluRs paired with GAD67, nNOS, or TH (n=5 each). The thick line depicts the mean of the three protocols for Group I mGluRs and FG.

There were no differences through the rostral-caudal distribution of the NTS in Group I mGluR expression or FG labeled neurons in the three immunohistochemical groups. These data indicate the reproducibility of our immunohistochemical protocols and imaging techniques.

Region	Gr I mGluR	FG	GAD67	nNOS	TH
Caudal NTS	45.9 ± 3.6 *	29.2 ± 4.2 *	30.7 ± 2.5 *	15.8 ± 2.8 *	16.9 ± 1.7 *
Postremal NTS	90.5 ± 5.4	57.7 ± 8.6	65.1 ± 7.5	49.3 ± 4.3 +	34.1 ± 4.2
Rostral NTS	91.3 ± 6.1 §	48.8 ± 9.8 §	82.1 ± 9.1 §	76.5 ± 8.1 §	26.5 ± 5.1
Totals	743.7 ± 37.6	433.9 ± 62.3	558.4 ± 42.6	441.2 ± 41.25	261.4 ± 18.58

*=p<0.05 Caudal vs. Postremal +=p<0.05 Postremal vs. Rostral §=p<0.05 Rostral vs. Caudal

Table 1: **Regional distribution of immunoreactive or CVLM retrogradely labeled cells in the nucleus tractus solitarius (NTS).** Number of positively labeled cells at each rostral-caudal level in the NTS for each phenotype: Group I mGluRs (Group I), Fluoro-Gold (FG), Glutamic Acid Decarboxylase 67 (GAD67), neuronal nitric oxide synthase (nNOS) and Tyrosine hydroxylase (TH). Numbers are mean ± SE. * P<0.05 postremal AP vs. caudal AP; + P<0.05 postremal AP vs. rostral AP; § P<0.05 rostral AP vs. caudal AP.

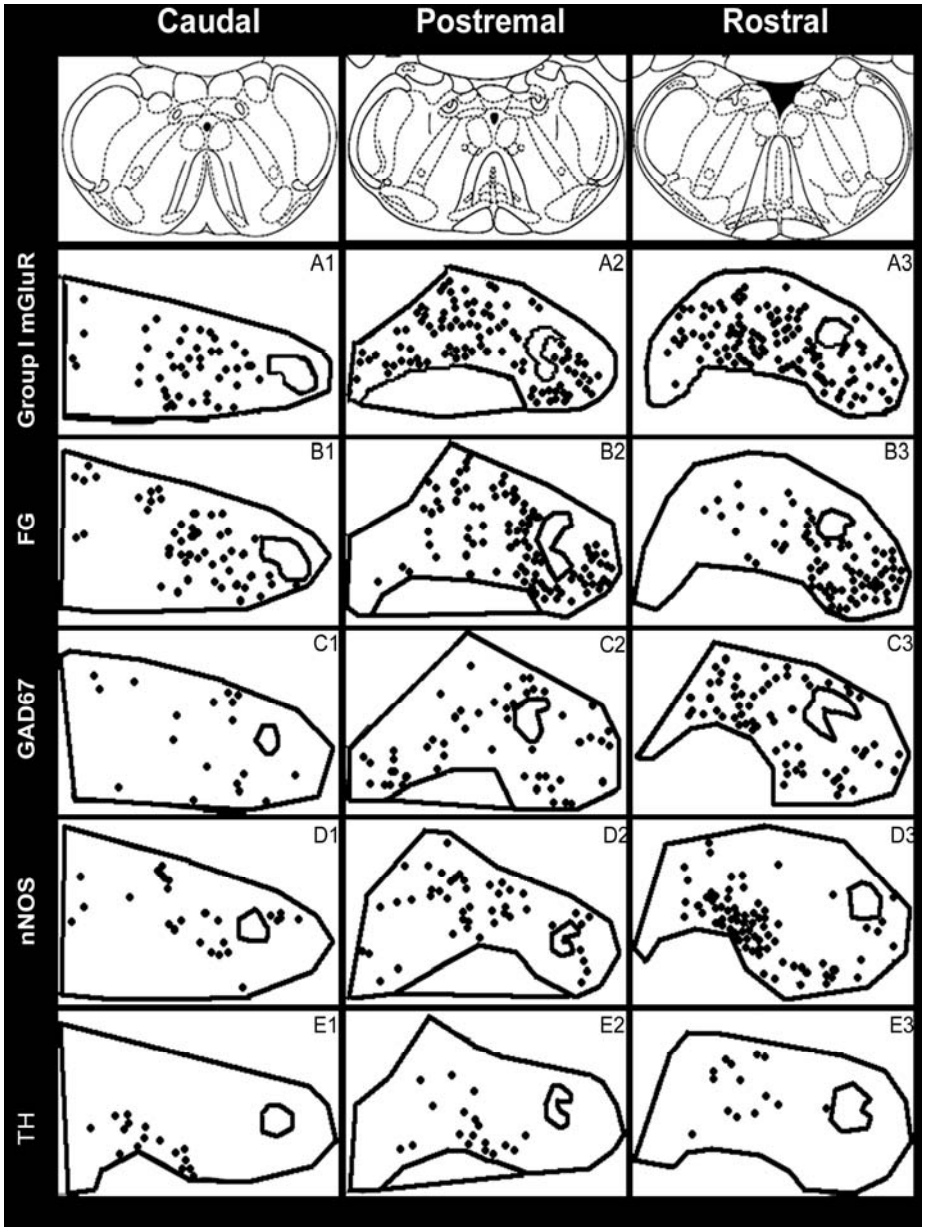

Figure 5: **Anatomic location of positive immunolabeling and CVLM projecting neurons in the NTS.** Representative diagrammatic images from the NTS of Group I mGluRs (A), FG (B), GAD67 (C), nNOS (D), and TH (E) labeling. Each point represents a labeled neuron. A1-E1: Representative sections of the NTS caudal to calamus scriptorius. A2-E2: Representative sections from the postremal NTS, at the level of the AP. A3-E3: Representative sections from the rostral NTS, through 540 μm rostral to the rostral margin of AP.

Group I mGluRs were widely expressed throughout the NTS. The distribution of the distinct phenotypes occurred in specific subregions (see text), as previously shown.

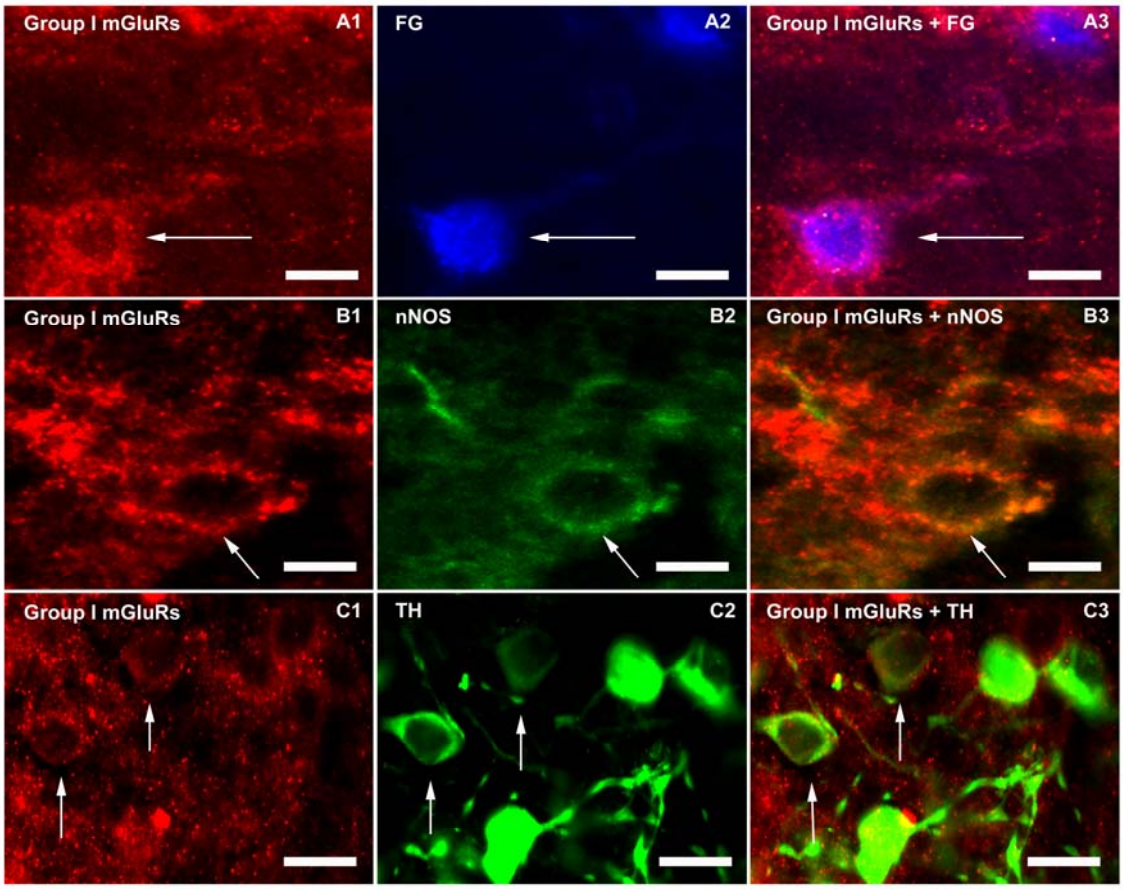

Figure 6: **Colabeling of Group I mGluRs with specific neuronal types**. Representative photomicrographs of Group I mGluR colabeling in the NTS. Pseudocolored photomicrographs of Group I mGluRs (panel 1) with FG (A), nNOS (B) and TH (C). Representative images were taken of cells dorsolateral to solitary tract (A, B) or in the medial subnucleus of the NTS (C). Representative photomicrographs of visualization of Group I mGluRs immunoreactivity (-IR, A1, B1, C1), labeling of FG (A2), nNOS-IR (B2) and TH-IR (C2), and merged images (A3-C3) to show cells positive for both signals. Cells that are colabeled are depicted by white arrows. Scale bar is 25 μm.

medial subnucleus, with some cells expressing Group I mGluRs in surrounding subnuclei. Group I mGluR-IR cells were located throughout the postremal region of the NTS (Fig. 5A2), with dense expression in the medial, intermediate, ventral, and ventrolateral subnuclei. The central subnucleus showed dense labeling in the rostral sections of the postremal NTS region. In the rostral NTS (Fig. 5A3), Group I mGluR-IR cells were present diffusely throughout the region, although a dense cluster was found in the medial subregion.

Retrogradely Labeled CVLM Projecting Cells:

Microinjection of FG into the CVLM resulted in neuronal labeling at all rostral-caudal levels of the NTS, primarily ipsilateral to the FG injection (Figs. 4B; 5B1-B3). All animals displayed a similar qualitative FG distribution in the NTS, for all the CVLM injection sites used in this study. Somal FG labeling was either punctate [presumed FG filled lysosomes; (156)] or appeared as completely filled cells. Some labeled soma also exhibited processes that were labeled (Figs. 6A2; 9A1-C1). Similar to Group I mGluR-IR, the number per section of FG labeled cells in the postremal and rostral NTS was significantly greater than in the caudal NTS (Table 1). Within the caudal region of the NTS (Fig. 5B1), labeling was observed primarily in the medial subregion, with some labeling in the dorsolateral, ventral, and ventrolateral subnuclei. In the postremal region of NTS (Fig. 5B2), the greatest amount of FG labeling was observed in areas that surrounded the solitary tract, including the lateral, ventrolateral, interstitial, intermediate, and posterior subnuclei. There was also a strip of labeling in the dorsolateral subnucleus. In the rostral NTS

(Fig. 5B3), FG containing cells exhibited heavy labeling in the lateral and ventrolateral subnuclei, with less labeling in the intermediate and interstitial subnuclei.

GAD67 Cells:

GAD67-IR cells exhibited staining with an unlabeled nuclear region, suggesting the labeling was cytosolic (Figs. 3B, 9A2). Also, punctate labeling was located throughout the NTS, consistent with presynaptic terminal labeling. There was a significantly greater number of positively labeled GAD67 neurons in the postremal and rostral regions compared to the caudal region (Table 1). However, within each level, GAD67-IR cells were widely distributed throughout the NTS (Fig. 5C1-C3).

nNOS Cells:

nNOS containing cells exhibited cytosolic labeling with a dark, unstained nuclear region (Figs. 3C, 6B2, 9B2). Most cells showed labeling in at least one process. Labeling on individual fibers was also visible. The number of nNOS-IR cells (Fig 5D1-D3) was significantly greater in the postremal and rostral regions than the caudal regions of the NTS, and there was significantly more labeling in rostral NTS than the postremal NTS (Table 1). Within the caudal NTS (Fig. 5D1), nNOS-IR cells were found in the medial and dorsolateral subnuclei, with less labeling lateral to the solitary tract. In the postremal region of the NTS (Fig. 5D2), nNOS-IR cells were located in the gelatinous, medial, and dorsolateral subnuclei, with some labeling in

the lateral subnuclei. In the rostral NTS region (Fig. 5D3), labeling was localized to the medial and central subnuclei.

TH Cells:

TH-IR cells were large, brightly labeled cells, with cytosolic labeling (Figs. 3D, 6C2, 9C2). Each cell exhibited labeling in at least one process, with some TH-IR cells having multiple labeled processes. Labeling on fibers surrounding the soma was also observed (Figs. 9C2). The average number of TH-IR cells per section was not different among the different NTS regions (Table 1). The overall distribution of TH-IR cells was discrete, with positive labeling found in specific subnuclei (Fig 5E1-E3). In the caudal NTS, TH positive cells were localized primarily to the medial and commissural subnuclei (Fig 5E1). In the postremal NTS region, groups of labeled cells were located in the medial and dorsolateral subnuclei (Fig 5E2). In the rostral NTS region the distribution of positively labeled TH cells was localized primarily in the medial subnucleus and the medial edge of the NTS along the floor of the fourth ventricle (Fig 5E3).

Colabeling of Group I mGluR Expressing Cells within the NTS:

The colabeling of Group I mGluRs with each of the other cell types was examined. Representative pseudocolored photomicrographs of Group I mGluR immunoreactivity on CVLM projecting, nNOS or TH containing cells within the NTS are shown in Figure 6. The number of dual labeled cells of each type as a percent of the number of Group I mGluR-IR cells are shown in Figure 7A. The percentage of

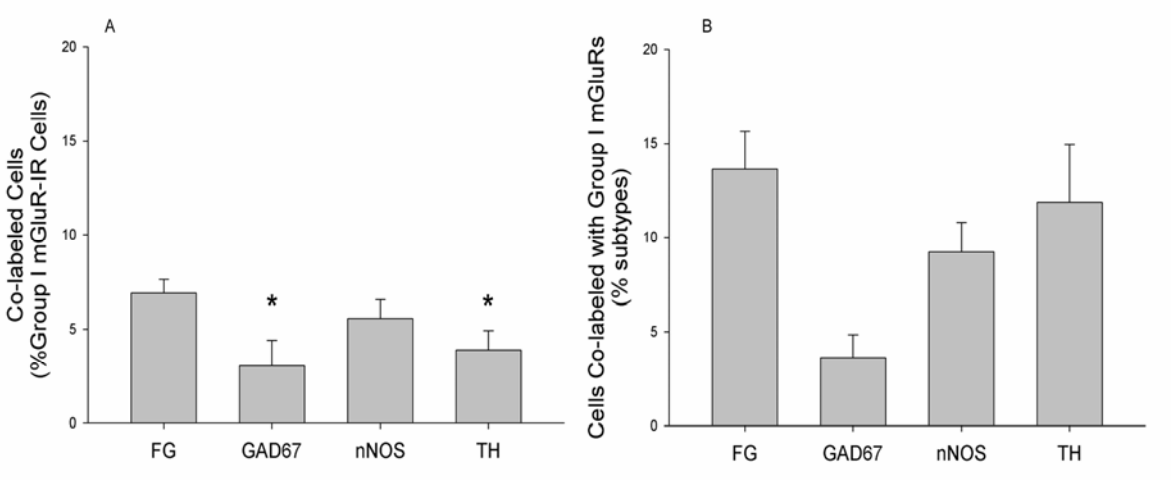

Figure 7: **Percent co-labeling of Group I mGluR positive cells in the NTS.** Group I mGluR-IR cells co-labeled with FG, GAD67, nNOS, or TH, expressed as (A) a percentage of the number of Group I mGluR-IR cells; (B) a percentage of the specific subtype of labeled cells. Bars are mean ± SE. * $P<0.05$ vs. FG.

These data suggest that Group I mGluRs are expressed on each of the phenotypes examined, but are preferentially expressed on CVLM projecting neurons.

Group I mGluR-IR cells that contained FG was greater than the percent of cells colabeled with GAD67 or TH. However, the percent of Group I mGluR-IR cells containing FG or nNOS was similar. Figure 7B presents the number of dual labeled cells as a percent of the number of cells of each individual specific subtype. There were no significant differences among the specific cell types in colabeling with Group I mGluR-IR cells, although there was a trend ($p=0.07$) for the percent of FG, nNOS, and TH containing cells that also express Group I mGluRs to be higher than the percent of GAD67-IR cells with Group I mGluR-IR. Representative examples of distribution of each colabeled pair in individual animals are displayed in Figure 8. Colabeling was not specific to particular subregions and occurred throughout regions containing single labels in the NTS.

Colabeling of CVLM Projecting Cells:

Colabeling of FG containing cells with GAD67, nNOS, and TH was also examined. Representative pseudocolored photomicrographs of colabeled cells within the NTS are shown in Figure 9. The relative amount of colabeling was determined as a percent of both the number of CVLM projection cells (containing FG) and of individual specific cell types, in the same fashion as Group I mGluRs (see above). A greater proportion of CVLM projecting cells (Fig. 10A) colabeled for Group I mGluRs compared to GAD67, nNOS, and TH colabeling. Relative to the number of cells of specific subtypes (Fig. 10B), the percentage of GAD67-IR cells that project to CVLM (FG) was significantly less than the percent of Group I mGluR-IR cells with FG. The percent of nNOS cells that contained FG was not significantly

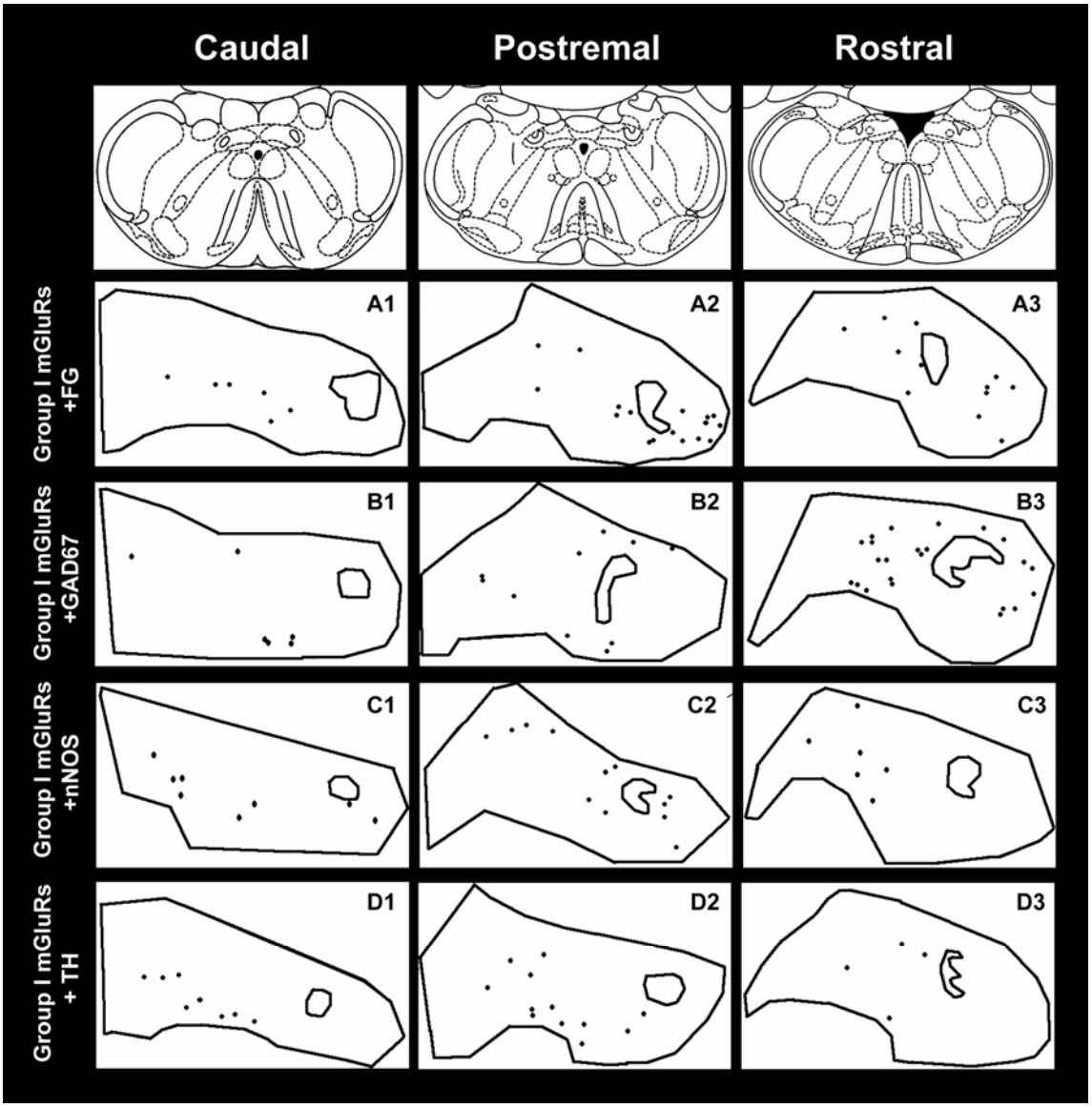

Figure 8: **Anatomic location of Group I mGluR colabeled cells in the NTS.** Representative diagrammatical images of Group I mGluR colabeled neurons. Images depict Group I mGluRs and FG (A), Group I mGluRs and GAD67 (B), Group I mGluRs and nNOS (C), and Group I mGluRs and TH (D) immunolabeling and CVLM retrogradely labeled neurons. A1-E1: Caudal NTS represents sections that include calamus scriptorius (CS), and those sections through 360 μm caudal to CS. A2-E2 represents sections in the postremal NTS, at the level of and containing the AP. A3-E3 represents the rostral NTS, sections rostral to the AP, with the NTS still located on the floor of the fourth ventricle. This range includes sections through 540 μm rostral to the rostral margin of AP. Colabeling occurred at all rostral-caudal levels of the NTS but was not restricted to any particular subregions.

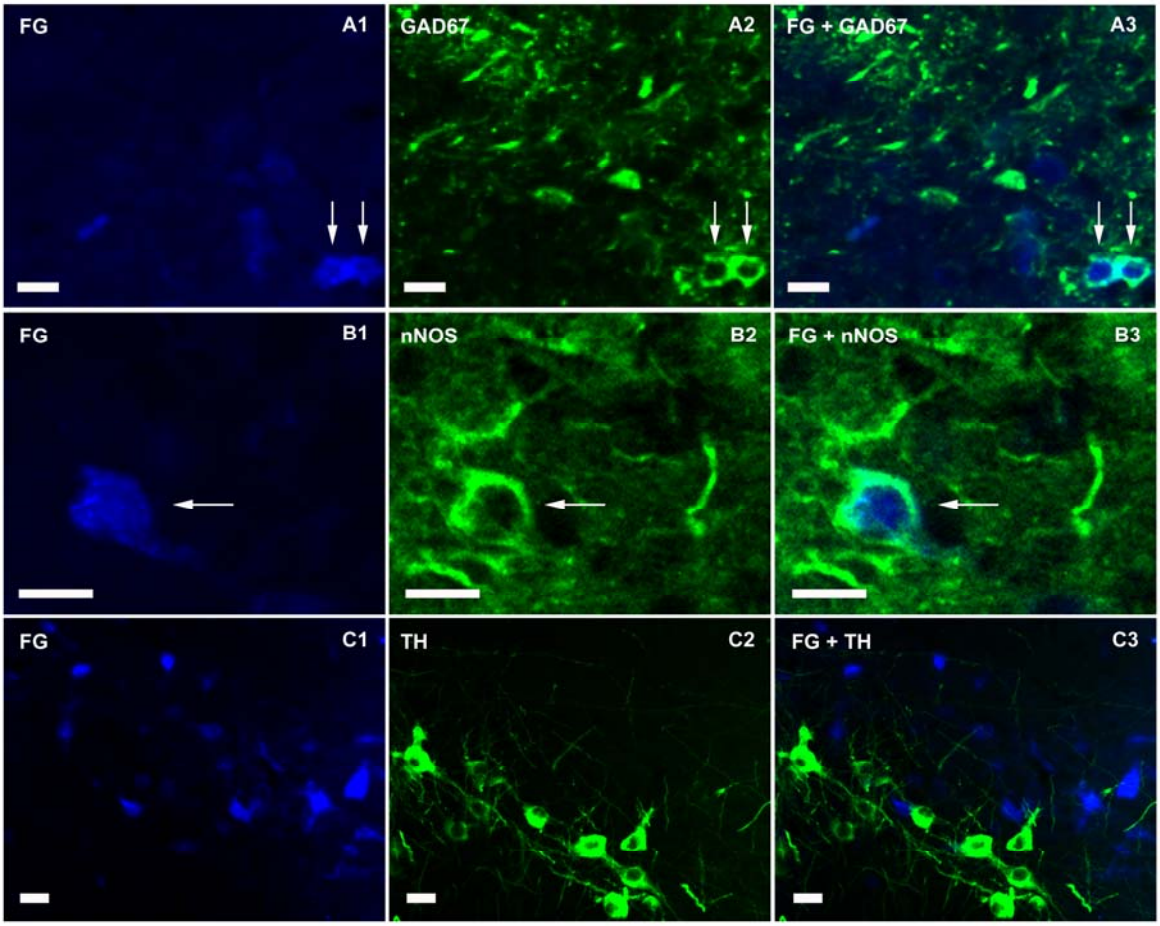

Figure 9: **Colabeling of FG with other neuronal phenotypes.** Pseudocolored photomicrographs of FG alone (A1-C3), GAD67 (A2), nNOS (B2), TH (C2) and merged images of FG with each label (A3-C3). Cells that are colabeled are indicated by the white arrows. Images are taken from the NTS, from areas surrounding the solitary tract. No colabeling was seen in the majority of FG and TH containing neurons. Scale bar is 25 μm.

Subpopulations of CVLM projecting neurons are GABAergic or nitroxidergic. Virtually no CVLM projecting neurons appear to be catecholaminergic.

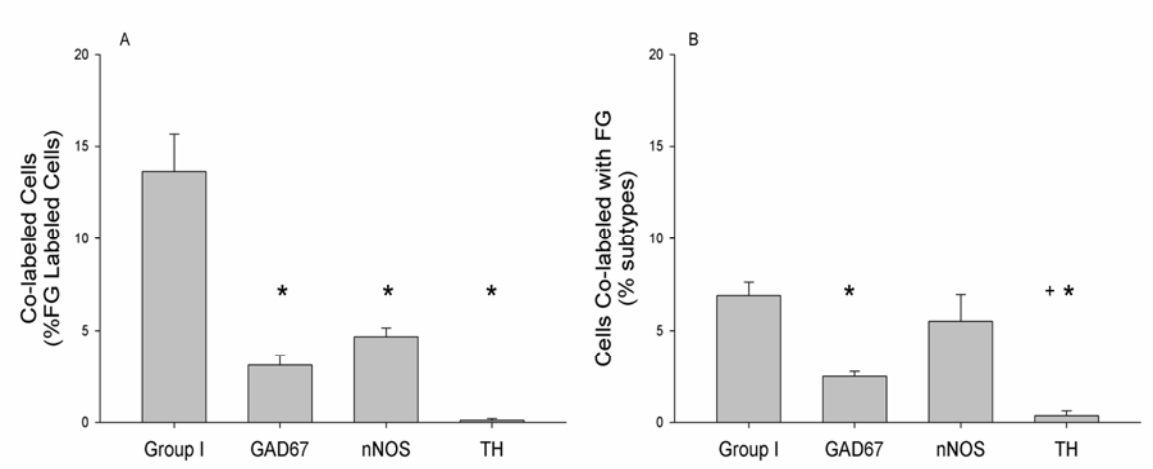

Figure 10: **Percent co-labeling of CVLM-projecting cells in the NTS.** FG cells colabeled with Group I mGluRs, GAD67, nNOS, or TH, expressed as (A) a percentage of the number of FG cells; (B) a percentage of the specific subtype of labeled cells. Bars are mean ± SE. * $p<0.05$ vs. Group I mGluRs; + $P<0.05$ vs. nNOS.

Though some of the CVLM projecting neurons are GABAergic or nitroxidergic, a majority of CVLM projecting neurons are not GABAergic, nitroxidergic, or catecholaminergic. In this study, CVLM projecting neurons receive the greatest influence from Group I mGluRs.

different from the percent of Group I mGluR cells colabeled with FG. Though colabeling of FG and nNOS was relatively low, these two types were distributed in the same subnuclei, and in some cases, in close proximity. There was very little colabeling of FG and TH within the NTS (3 colabeled cells of ~270 total TH labeled cells) and the percent of colabeling was significantly less than that of Group I mGluRs or nNOS with FG.

Triple-Labeling of Group I mGluR and Different Neurochemical Phenotypes of CVLM Projecting Cells:

Although few, we did observe some triple labeled cells. No TH-IR cells were labeled for both Group I mGluRs and FG. Typically, there were 4-5 GAD67-IR cells per animal that expressed Group I mGluRs and also projected to the CVLM. This constituted ~20% of the GAD67-IR neurons that expressed Group I mGluRs, ~30% of GAD67-IR cells that projected to the CVLM, and ~10% of CVLM projecting neurons that expressed Group I mGluRs. The total number of triple labeled GAD67 neurons was less than 1% of each of the respective single labeled cells.
There were 10-15 nNOS containing cells per animal that expressed Group I mGluRs and also projected to the CVLM. This constituted ~25% of the nNOS neurons that expressed Group I mGluRs and ~17% of CVLM projecting neurons that expressed Group I mGluRs. Interestingly, ~47% of the nNOS-IR neurons that projected to the CVLM also were Group I mGluR-IR. The total number of triple-labeled nNOS neurons was below 2% of each of the respective single labeled cells.

Functional and Histological Identification of CVLM Injection Sites:

In all animals that received FG microinjections the CVLM was functionally identified by cardiovascular responses to Glu (10mM, 30 nL) injection. Microinjection of glutamate into the CVLM resulted in a depressor response (-21 ± 3 mmHg) and a decrease in HR (-34 ± 8 bpm). Prior to immunohistochemical protocols, a series of medullary slices from each animal was examined to anatomically define the site of the FG microinjection. Injection sites were found to be 0.54 to 1.08 mm rostral to CS (Fig. 11A), with the injections centered within the region of the CVLM (115). Figure 11B is a representative dark field photomicrograph of the FG injection site indicating the center of the injection within the CVLM. Figure 11C is a pseudocolored photomicrograph depicting the spread of FG around the midpoint of the injection site.

To further assess our CVLM injection sites, additional experiments were performed to ensure that CVLM injections were in a region required for depressor responses to activation of the NTS. This group of animals (n=4) received microinjections of L-Glu (10 mM, 30 nL) into the NTS before, during, and after blockade of ionotropic glutamate receptors in the CVLM with microinjections of Kynurenic acid, (KYN, 40 mM, 15 nL). Locations for CVLM microinjections were determined by responses to L-Glu as described above. Unilateral microinjection of L-Glu into the NTS significantly reduced MAP and HR (Con, Figs. 12A, 12B). Ipsilateral microinjection of KYN (40 mM, 15 nL) into the CVLM had no significant effect on MAP or HR (data not shown). KYN microinjection into the CVLM did, however, abolish the cardiovascular responses to Glu injection into the NTS (Kyn-

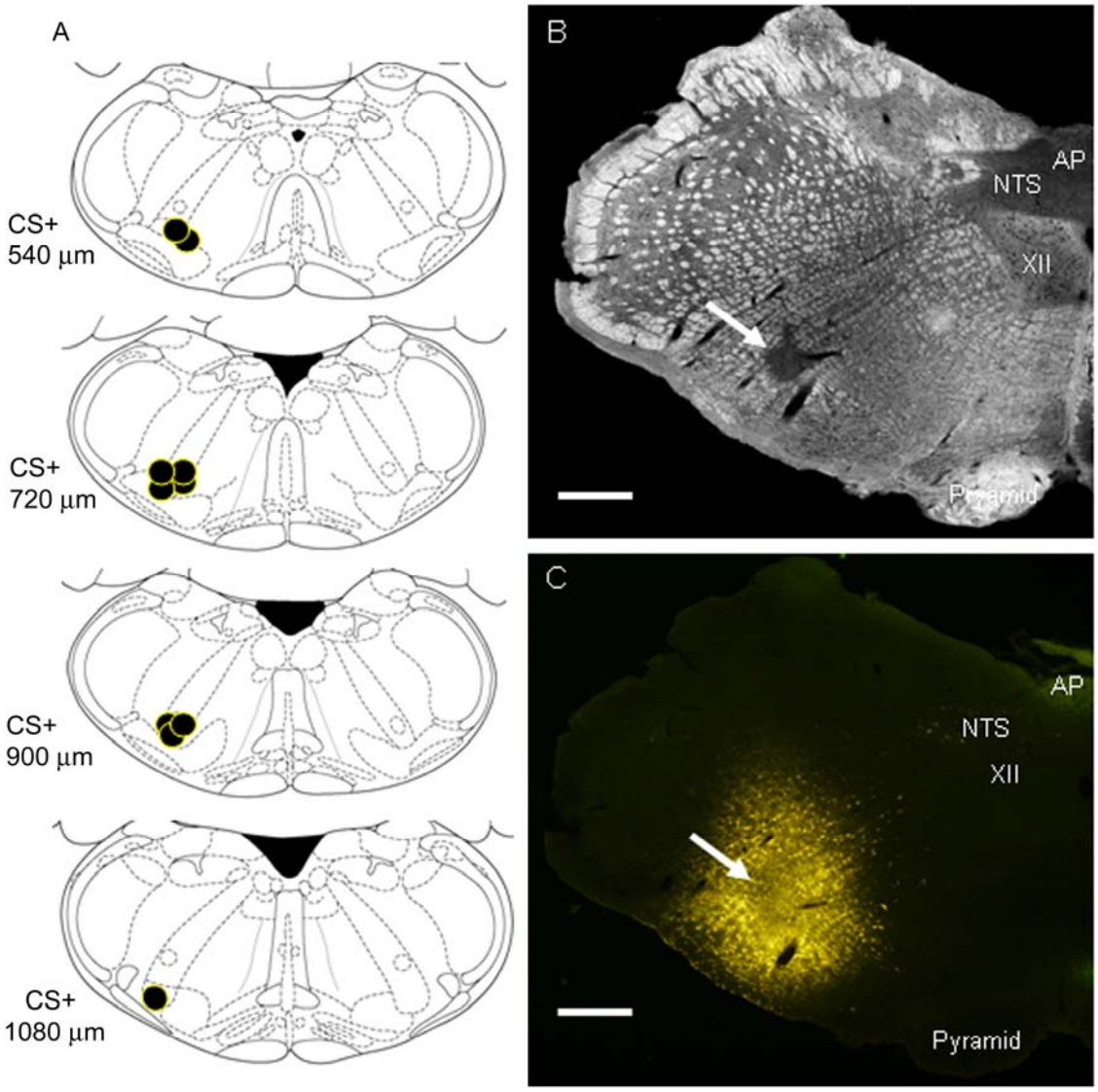

Figure 11: **Histological verification of CVLM injection sites**. (A) Locations of the center of FG microinjections. Black dots represent the midpoint of the FG microinjection site for animals used in this study. (B) Dark field photomicrograph of a representative FG injection site. (C) Pseudocolored photomicrograph of same section in (B) showing FG injection site and labeling in the NTS. Section is located 540 μm rostral to calamus scriptorius. Arrow points to center of injection site. AP: area postrema; NTS: nucleus of the solitary tract; XII: Hypoglossal nucleus. Scale bar is 500 μm.

These results verify that FG was microinjected into the CVLM and FG labeled neurons in the NTS can be presumed to project to the baroreflex region of the CVLM.

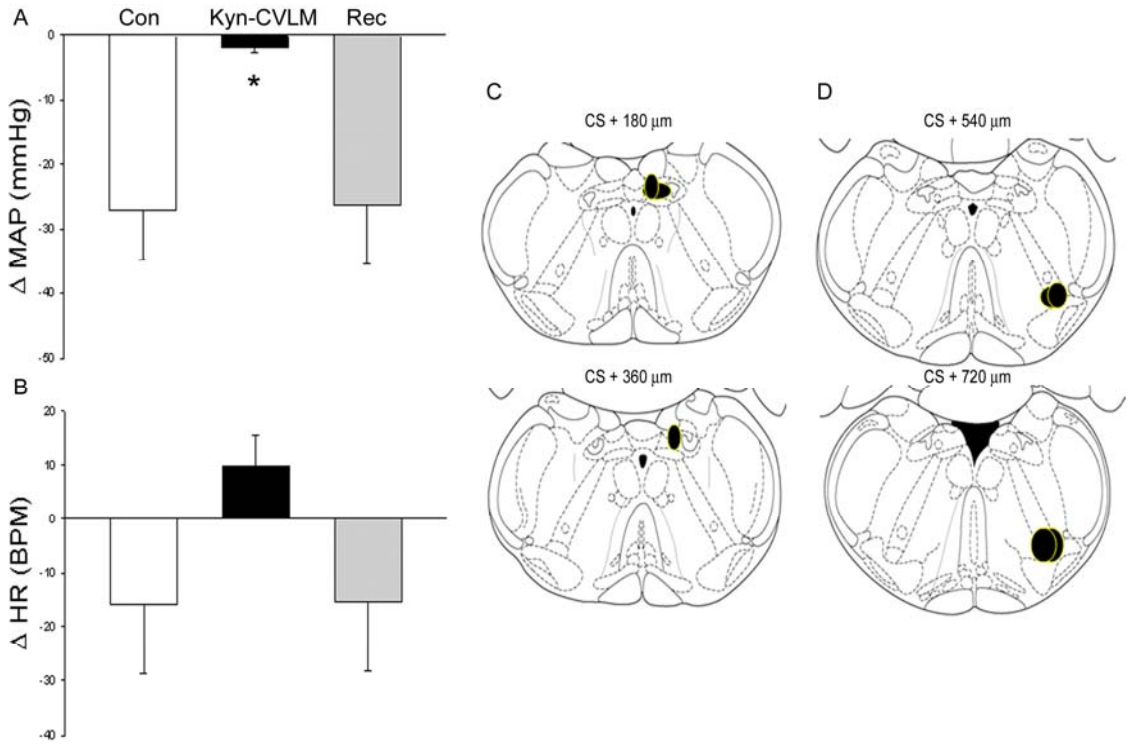

Figure 12: **Pharmacological and histological verification of CVLM injection sites**. The response to glutamate microinjection into the NTS is blocked by ionotropic glutamate receptor blockade in the region of the CVLM. Change in mean arterial pressure (MAP, A) or heart rate (HR, B) due to glutamate (10 mM; 30 nL) microinjection into the NTS under control conditions (Con), in the presence of kynurenate (Kyn-CVLM) and following recovery (Rec). Bars are mean ± SE; * p=0.05. The location of Glu microinjections in the NTS (C) and Glu or KYN microinjections in the CVLM (D) of the four animals in this set of experiments.

These results verify that the microinjection protocol used in this and further studies can be used locate coordinates of the CVLM that correspond to the baroreflex region.

CVLM, Figs. 12A, B). Within 20-40 minutes, the response to microinjection of Glu recovered to pre-KYN microinjection responses (Rec, Figs. 12A, B). Histological analysis of the brainstems indicated that NTS injection sites (Fig. 10C) corresponded with NTS regions known to receive baroreceptor afferent projections (9; 31; 98; 138). In addition, injection sites for KYN within the CVLM
(Fig. 10D) were similar to injection sites for FG in the immunohistochemical experiments.

Discussion:

This study examined the distribution of Group I metabotropic glutamate receptors (mGluRs) in the nucleus of the solitary tract (NTS) on four separate neuronal subtypes: CVLM projecting neurons, retrogradely labeled with Fluoro-Gold (FG); GABAergic neurons, indicated by immunoreactivity for glutamic acid decarboxylase (GAD67); nitroxidergic neurons, indicated by neuronal nitric oxide synthase (nNOS); and catecholaminergic neurons, indicated by tyrosine hydroxylase (TH) immunoreactivity. The primary findings were that Group I mGluRs are expressed throughout the NTS and are present on a variety of cell types known to influence autonomic nervous system function within the NTS. The percent of CVLM projecting Group I mGluR-IR cells was similar to the percent containing nNOS. A greater proportion of Group I mGluR-IR cells projected to the CVLM compared to the percent that also contained GAD67 or TH. Furthermore, a greater percentage of CVLM projecting NTS cells expressed Group I mGluRs compared to those that contained GAD67, nNOS or TH. Thus, Group I mGluRs in the NTS could influence

autonomic function by affecting cells that then project to the CVLM or by modulating the activity of other NTS cells known to influence cardiovascular regulation.

Metabotropic Glutamate Receptors:

G-protein coupled mGluRs are located throughout the central nervous system (32; 117; 118; 128). mGluRs are divided into three different subgroups, based on amino acid sequence homology and signal transduction mechanisms. Group I mGluRs include receptor types 1α and 5, and generally activate phospholipase C. Group II includes receptor types 2 and 3, and are negatively coupled to adenylate cyclase. Group III includes receptor types 4, 6, 7, and 8 and are also negatively coupled to adenylate cyclase. Group I mGluRs are generally found postsynaptically, while group II and group III mGluRs are localized primarily presynaptically (32; 117; 118; 128).

mGluRs modulate synaptic transmission within the NTS (9; 30; 44; 52; 74; 118; 128; 134) and activation of Group I mGluRs within the NTS influences autonomic function (48; 49; 104). Microinjections of non-specific mGluR agonists and Group I mGluR selective agonists result in reductions in mean arterial pressure (MAP), heart rate (HR) and sympathetic nerve activity (SNA). Thus, activation of Group I mGluRs mimics the response to glutamate microinjection or excitatory amino acid release within the NTS (48; 49).

Similar to previous studies (63), our data indicate that Group I mGluRs are expressed throughout the NTS. The greatest expression was at the level of and rostral to the area postrema. The presence of a greater expression of Group I

mGluRs in the postremal region of the NTS may suggest a more pronounced role for modulating synaptic transmission in these regions. Interestingly, postremal NTS is a region from which microinjection of Group I mGluR agonists elicits depressor responses and sympathoinhibition (48; 49; 104).

Group I mGluRs were also expressed, although at relatively low levels, on each phenotype of neuron that we examined. Among the cell types evaluated, the percentage of colabeling of Group I mGluRs with CVLM projecting (FG) neurons was higher than most other phenotypes. Furthermore, a greater percentage of the CVLM-projecting neurons expressed Group I mGluRs than any of the other markers examined. Most of the evidence indicates that Group I mGluRs increase cell excitability (32; 117; 118; 128). Therefore, colabeling of Group I mGluRs with CVLM projecting (FG) neurons is consistent with previous work showing that Group I mGluR agonists microinjected into the NTS reduce MAP and SNA, presumably by activation of the CNS baroreflex pathway (48; 49; 104). Thus, one mechanism by which stimulation of Group I mGluRs in the NTS could inhibit sympathetic nervous system function is by increased excitation of these CVLM-projecting neurons.

There was a trend for nNOS and TH containing cells in the NTS to exhibit greater Group I mGluR-IR compared to GAD67 containing cells. This is consistent with previous work in which microinjection of NO donors into the NTS decreases MAP and SNA (88; 111; 146). Activation of nNOS containing neurons by Group I mGluRs would likely increase production and release of NO within the NTS, resulting in decreased MAP and SNA. TH neurons in the NTS project to a variety of other brain regions involved in regulation of the autonomic nervous system, including

the paraventricular nucleus of the hypothalamus (69; 75; 76), parabrachial nucleus (69), central nucleus of the amygdala (123), and the nucleus paragigantocellularis (123). Group I mGluRs could influence autonomic function by modulating the activity of these neurons. Taken together, the data are consistent with the idea that Group I mGluRs are expressed on cells in the NTS that contribute to pathways involved with autonomic regulation.

Group I mGluRs are generally thought to be located postsynaptically (32; 117; 118; 128). Within the NTS, studies *in vitro* suggest that activation of mGluRs produces postsynaptic effects, including depolarization, activation of an inward current, potentiation of ionotropic glutamate receptor evoked currents, inhibition of $GABA_A$ receptor mediated currents, and activation of a Na^+/Ca^{2+} exchanger (44; 52-54; 134). These studies demonstrate that mGluRs can influence NTS cellular activity postsynaptically, likely through Group I receptors. Also, studies utilizing RT-PCR have shown that, compared to the NTS, expression of Group I mGluR mRNA is lower (30) or not present (72) in nodose ganglia, which contain cell bodies of visceral afferents. In addition, Chen et al (30) reported that Group II and Group III mGluRs, but not Group I mGluRs, inhibit glutamate release from the presynaptic terminal of labeled afferent neurons. Taken together, these data are consistent with the concept that Group I mGluRs are located predominantly postsynaptically rather than presynaptically on visceral afferent terminals. Nevertheless, a recent study (74) suggested that presynaptic Group I mGluRs in the NTS can regulate the release of GABA or glutamate. In the current study, much of the Group I mGluR labeling appeared to be membrane associated. Although we could not determine with

certainty whether this labeling was pre- or postsynaptic, it met our criteria for cellular labeling. There also was heavy punctuate labeling throughout the NTS, which we did not quantify but which was consistent with terminal labeling. Thus, it is possible that both presynaptic and postsynaptic Group I mGluR immunoreactivity was present and the current data are consistent with the presence and effects of Group I mGluRs, both presynaptic and postsynaptic, within the NTS.

CVLM Projecting Neurons:

The NTS serves as the termination point for multiple visceral afferents (9; 31; 105; 155), including arterial baroreceptors. Baroreflex-mediated sympathoinhibition and depressor responses then require a projection from the NTS to the CVLM. Baroreceptor afferent input undergoes substantial integration within the NTS. Thus, the phenotype and neuronal influences on NTS neurons projecting to the CVLM are critical to the autonomic effects of NTS activation.

In the current study, microinjections of FG into the CVLM produced a pattern of labeling within the NTS generally similar to what has been reported previously for CVLM projecting NTS neurons (55; 69; 157). FG labeling was strongest in the medial subregion of the caudal NTS and in subregions surrounding the tractus solitarius in the postremal and rostral NTS. The CVLM is a heterogeneous nuclear region that lacks defined boundaries and is in close proximity with other regions of the ventral medulla that influence cardiorespiratory regulation. Presumably any NTS cells that project to the CVLM, independent of function, would be labeled with FG. Several lines of evidence suggest our FG injections were centered in regions of the

CVLM involved with arterial baroreflex function. The range of our injection sites was similar to regions of the CVLM shown to be required for baroreflex control of blood pressure (55; 69; 73; 102; 125; 131) and Glu (30 nL) microinjected into the CVLM produced a depressor response, with only a modest decrease in HR. Importantly, microinjection of small volumes of KYN (15 nL) into the same coordinates of the CVLM as above blocked the depressor response due to Glu microinjection into the NTS. Thus, although we did not directly evaluate their function, our results are consistent with the concept that at least some of our NTS neurons that project to the CVLM likely contribute to arterial baroreflex function.

A small percentage of CVLM projecting NTS neurons appeared to be GABAergic, as indicated by colabeling of FG and GAD67. This is consistent with previous work (157) demonstrating a GABAergic projection from NTS to CVLM. However, the level of GAD67 and FG colabeling in our study was lower than previously reported (157). The reasons for this discrepancy are not clear, but may be due to differences in experimental approaches. Weston et al (157) used *in situ* hybridization to label GAD67 mRNA, iontophoresis of FG, and an amplification technique to detect FG. In contrast, we examined GAD67 protein using immunohistochemistry, used microinjection to apply the retrograde tracer, and used FG autofluorescence to identify CVLM projecting neurons. Regardless of these differences, in both the previous work (157) and the present study, relatively few neurons that project to the CVLM were GABAergic.

As previously shown, nNOS immunoreactivity (77; 86; 90; 91; 93; 147) was detectable in the NTS. The pattern of distribution of FG and nNOS labeling within

the NTS was similar and the two labels were often in close proximity. Given the well established role of NO as a diffusible neuromodulator (41; 113; 145), these results suggest that NO released within the NTS could modulate activity of CVLM projecting neurons. In addition, a small degree of colabeling of FG and nNOS was observed. Thus, NO may also influence activity of the CVLM directly via neurons from the NTS that release NO within the CVLM.

The percentage of colabeling of FG and TH was negligible. In the five sets of NTS tissue that were immunohistochemically processed, we counted only three CVLM projecting neurons that also contained TH. NTS catecholaminergic neurons, contained in the A2 neural group, have been shown to project to a variety of brain regions (69; 75; 76; 123), although only a small number appear to project to the CVLM (69). The percentage of FG and TH colabeling in our study was less than that observed by Hermes et al (2006). This variation may be due to several factors, including the amounts of tracer microinjected into the CVLM. Hermes et al (69) utilized 50-80 nL of FG, whereas we injected 15 nL. It is likely that our lower volume FG injections in the CVLM accounts in part for less colabeling of FG and TH than previously reported (69).

Triple-Labeled Neurons:

We also examined Group I mGluR immunoreactivity on phenotypically different CVLM projecting NTS neurons. There were few triple labeled neurons detected in any of the immunohistochemical groups. There were no triple-labeled Group I mGluR, FG, and TH neurons. This is not surprising, as the number of

catecholaminergic NTS neurons that projected to the CVLM was very small. Similarly, there were only a few triple-labeled GAD67 neurons, consistent with the finding that relatively few CVLM-projecting neurons in the NTS were positively labeled for GAD67. Also, because microinjection of Group I mGluR agonists into the NTS results in a depressor response (consistent with activation of CVLM neurons) it is not surprising that a low percentage of CVLM-projecting neurons expressed both Group I mGluRs and GAD67.

Of interest are triple-labeled neurons for Group I mGluRs, nNOS, and FG. Nearly half of nNOS neurons that projected to the CVLM also expressed Group I mGluRs. Furthermore, a fourth of the NTS neurons that expressed Group I mGluRs and were nNOS-IR also projected to the CVLM. Microinjection of Group I mGluR agonists (48; 49; 104) and NO donors (146) into the NTS both result in depressor responses and the current data provide an anatomical substrate by which these modulators may interact. For example, Group I mGluRs could decrease arterial pressure in part either by activating nNOS cells that then release NO within the NTS or by activating nNOS neurons that project to CVLM and release NO within the CVLM. Future studies are required to address these possibilities.

Technical Considerations:

In the present study, we used immunohistochemistry to identify different neurochemical phenotypes. The use of strict criteria for determining positively labeled cells versus nonlabeled or partially labeled cells minimizes the possibility of false positives. This may also lead to underestimation of positively labeled cells. In

some cases, the use of multiple sets of tissue from the same animals served as their own controls. Group I mGluRs and FG were counted in each immunohistochemical pairing. The number and distribution of cells positively labeled for Group I mGluRs or FG was similar among the different immunohistochemical protocols, providing evidence of the reproducibility of our results.

The antibody that was used to detect Group I mGluRs recognizes both Group I subtypes, mGluR 1α and mGluR 5. It has been suggested that mGluR 5 can be located both presynaptically and postsynaptically, whereas mGluR 1α is found primarily postsynaptically. According to the criteria used, NTS cells were considered to be positive for Group I mGluRs, and labeling appeared to be membrane-associated. However, future studies using electron microscopy are required to definitively determine whether this labeling is pre-or postsynaptic.

Summary:

Group I mGluRs are expressed throughout the NTS and are present on a variety of cell types known to influence autonomic nervous system function within the NTS. Cells expressing Group I mGluRs were more likely CVLM projecting or nitroxidergic (nNOS) compared to GABAergic (GAD67) or catecholaminergic (TH). Regarding NTS cells that project to the CVLM, a greater percentage expressed Group I mGluRs compared to those that contained nNOS, GAD67, or TH. Interestingly, a high proportion of nNOS-IR CVLM projecting cells expressed Group I mGluRs. This study provides anatomical evidence supporting a role of Group I mGluRs in the modulation or activation of autonomic neurons in the NTS.

Chapter 3: Increases and Decreases in Arterial Pressure Result in the Activation of Phenotypically Different Populations of Neurons in the Nucleus of the Solitary Tract (NTS)

Background:

The simplest model of the central pathways of the arterial baroreflex consistent with current data is shown in figure 1. Based on this model, increases in arterial pressure would increase baroreceptor afferent activity and activate neurons within the NTS. This has been shown to occur in numerous studies. In contrast, decreases in arterial pressure would reduce baroreceptor and might be expected to result in reduced NTS activation. However, this does not appear to be the case. Periods of hypotension also appear to activate neurons within the NTS. The specific NTS neurons activated by hypotensive stimuli are not known, but it is possible that decreases in pressure activate a separate population of neurons in the NTS in comparison to increases in pressure. This study will determine the level of activation of specific populations of NTS neurons by examination of the protein Fos, a marker of neuronal activation. After acute periods of hypertension and hypotension, the activation of GABAergic, nitroxidergic, and catecholaminergic neurons will be examined. Further, the activation of CVLM projecting neurons in response to increases and decreases in pressure will also be examined.

Abstract:

The NTS is the termination site of multiple visceral afferents, including the arterial baroreceptors. Increases in arterial pressure activate second order baroreflex neurons in the NTS. However, hypotension also activates NTS neurons. We hypothesized that different populations of neurons are activated by increases vs. decreases in arterial pressure. Male Sprague-Dawley rats (n=17) received microinjections of the retrograde tracer Fluoro-Gold (2%, 15 nL) into the caudal ventrolateral medulla and were instrumented with arterial and venous catheters. After 5 days, animals were administered the vasoconstrictor phenylephrine (PE: 15 μg/kg/min, n=6), the vasodilator diazoxide (Diaz: 50 mg/kg, supplement 12.5 mg/kg, n=6), or saline (control, n=5), i.v. and monitored for 90 min. Rats were perfused at the conclusion of drug administration, brains cut into 30 μm coronal sections, and sections immunohistochemically treated for activated (Fos) neurons and GABAergic (GAD67), nitroxidergic (nNOS) or catecholaminergic (TH) neurons. Fos expression was significantly enhanced by increases (PE, 207 ± 30) and decreases (Diaz, 174 ± 32) in arterial pressure compared to saline (31 ± 5). Hypertension resulted in significantly greater activation of CVLM projecting (Fluoro-Gold containing) neurons (consistent with baroreflex activation) in comparison to hypotension and control, and a significantly greater number of nitroxidergic neurons compared to control. In contrast, both hypertension and hypotension resulted in significantly greater activation of TH containing neurons compared to control. Thus, phenotypically separate populations of NTS neurons are activated by hypertension and hypotension.

Introduction:

The nucleus of the solitary tract (NTS) is a highly heterogeneous nuclear region that receives and processes input from multiple visceral afferents (7-9; 31; 36; 98; 138), including the arterial baroreflex. Increases in arterial blood pressure activate mechanosensitive baroreceptors on the aortic arch and carotid sinuses and increase afferent information conveyed to the NTS (7-9; 31; 36; 98; 138). These increases in baroreceptor afferent input result in activation of NTS neurons which then process the information from the baroreceptors and convey it through output neurons to the caudal ventrolateral medulla (CVLM). Inhibitory neurons from the CVLM then project to the rostral ventrolateral medulla (RVLM), reducing RVLM neuronal activity, decreasing sympathetic nerve activity and buffering the increase in arterial pressure (AP) (7-9; 31; 36; 98; 138).

NTS activation in response to increases in arterial pressure has been well studied (7-9; 31; 36; 98; 138). Electrophysiological studies *in vivo* and *in vitro* confirm that baroreceptor afferent stimulation activates NTS neurons (126; 142; 161; 162). In addition, increased arterial pressure results in augmented expression of Fos, the protein product of the immediate early gene *c-fos* and a marker for neuronal activation 7-9; 31; 36; 93; 130). Fos expression in response to hypertension is located in regions of the NTS where baroreceptor afferents terminate 7-9; 31; 36; 93; 130). Fos immunoreactivity is eliminated by denervation of the arterial baroreceptors (7-9; 31; 36; 98; 138). Further studies indicate that activated neurons include a variety of phenotypes, including glutamatergic, GABAergic, catecholaminergic and nitroxidergic neurons (27; 108; 157). Interestingly, studies

have shown that decreasing arterial pressure either pharmacologically by administration of a vasodilator or through hemorrhage also results in activation of NTS neurons (25; 26; 35; 58; 122). This is a particularly interesting observation, as decreasing arterial pressure reduces baroreceptor afferent activity and therefore would be expected to decrease NTS neuronal activity. It is possible that increases and decreases in arterial pressure result in the activation of different neuronal populations in the NTS. Although some studies have attempted to classify the phenotype of these cells (25; 26; 35), additional experimentation is required to effectively clarify which neurons are activated by increases and/or decreases in arterial pressure.

We compared the response of three major phenotypes of neurons (GABAergic, nitroxidergic, and catecholaminergic) in the NTS known to be involved in autonomic function. Further, through the use of a retrograde tracer, we also examined neurons activated by hypertensive or hypotensive stimuli to determine if those neurons project to the CVLM. We hypothesized that increases and decreases in arterial pressure would result in activation of phenotypically different populations of neurons within the NTS. Specifically, we hypothesized that hypertension results in activation of a greater number of CVLM projecting and nNOS containing NTS neurons. We further hypothesized that hypotension results in a greater number of GABAergic and catecholaminergic NTS neurons activated.

Methods:

Animals:

Experiments were performed according to the guidelines in the NIH "Guide for the Care and Use of Laboratory Animals." The University of Missouri Animal Care and Use Committee approved all procedures and protocols. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, n=16) weighing 275-325 grams were used. Rats were housed within an in-house animal facility on a 12 hour day/night cycle. Temperature and humidity were held constant at 72°F and 40%, respectively, and food and water were available ad libitum. All rats were given at least seven days to acclimate to the surroundings prior to any experimental procedure.

Surgical Procedures:

Surgeries were performed using aseptic technique. Rats were anesthetized with Isoflurane (AErane, Baxter [5% in 100% O_2, 2L per minute for induction and maintenance at 2-2.5%]). Catheters (PE10 fused to PE50) were inserted into the descending aorta via the femoral artery and into the inferior vena cava via the femoral vein. Catheters were run under the skin and exited through a small incision in the back of the neck. The arterial catheter was connected to a pressure transducer and the arterial pressure signal was measured using a DC amplifier (ADInstruments, Colorado). Mean arterial pressure (MAP) and heart rate (HR) were determined electronically using a PowerLab Data Acquisition System (ADInstruments, Colorado) connected to a Pentium computer. The rat was placed in a Kopf stereotaxic device, the dorsal surface of the medulla was exposed, and

calamus scriptorius (CS) was identified. The head was then deflected downward so that inter-aural zero was 2.4 mm rostral to CS (55; 110) placing the brainstem on the horizontal plane.

Microinjection Procedures:

After determining stereotaxic coordinates for CS, a double-barreled glass pipette (OD 20-30 μm), with one barrel containing L-glutamate (Glu, 10 mM) and the second filled with Fluoro-Gold (FG) (2% in dH_2O), was advanced to the initial target stereotaxic coordinates for the CVLM (0.2 mm caudal and 2.0-2.2 mm relative to CS, and 2.0-2.6 mm ventral to the dorsal surface of the brain; brain stem positioned horizontally). Similar to our previous studies (chapter 2), we confirmed the location of the CVLM functionally through depressor responses (> –20 mmHg) to microinjection of glutamate (10 mM, 30 nL), in combination with a bradycardia of < –40 bpm. Following functional identification of the CVLM, FG (15 nL) was microinjected into the same site through the other barrel of the pipette. Microinjections were made using a custom made pressure injection system and fluid levels in the pipette were visualized through a 150x microscope with a calibrated eyepiece micrometer. Injection volumes were quantified by measuring the movement of the meniscus within the pipette.

Following the FG microinjection the pipette remained in the medullary tissue for five minutes to minimize movement of FG up the injection tract. After removal of the pipette, the leg, neck and back areas were sutured closed. Animals were given post-operative injections of Baytril (0.03 mL) and Buprenex (0.6 mg/mL) i.m. to

prevent infection and for pain management, respectively. After recovery from anesthesia they were returned to their cages.

Drug Infusion:

Three to five days prior to and every day after FG microinjection, rats were trained for three hours per day to sit in the experimental room in a cage containing their own bedding. Five days after the FG microinjection, conscious rats were placed in the experimental cage and the arterial catheter was connected to a pressure transducer for recording arterial pressure, MAP and HR (see above). The femoral vein catheter was connected to a syringe for drug administration. The animal was given 60-90 minutes in the experimental cage to establish stable baseline hemodynamic parameters. Animals then received either: 1) infusion of phenylephrine (PE: 15 μg/kg/min i.v., n=6) for 90 minutes or 2) a bolus injection of Diazoxide (Diaz: 50 μg/kg i.v., n=6) with supplements (12.5 μg/kg) at 30 and 60 minutes. These concentrations were used to increase (PE) MAP 40-50 mmHg, and decrease (Diaz), MAP 35-45 mmHg. In control experiments (n=5), animals were infused with saline (0.9%, 10 μl/min),

Transcardial Perfusion:

At the conclusion of the experiment, animals were deeply anesthetized with Beuthanasia (Schering-Plough, Union, NJ, 0.5 ml/kg) and transcardially perfused with Dulbecco's Modified Eagles Medium (Sigma, St Louis, 100 mL) and 4% paraformaldehyde (Sigma, 500 mL), as previously described (Chapter 2). Brainstem

sections (30 μm) were cut on a vibrating microtome in a 1 in 6 series. If immunohistochemistry was performed within one week, sections were stored at 4°C in 0.1M PBS. All other sections were stored in cryoprotectant at -20°C. One series of sections was mounted on gel coated slides and coverslipped with Vectashield (Vector, Burlingame, VT) to verify the midpoint of the FG microinjection within the CVLM.

Immunohistochemistry:

Protocols:

Sections were treated with a modified immunohistochemical protocol (50) (Chapter 2) to visualize Fos-, GAD67-, TH-, and nNOS-immunoreactive (IR) cells. Preliminary experiments were conducted to determine optimal primary and secondary antibody concentrations. The specific steps of the protocol were dependent on the protein visualized. Briefly, each immunohistochemical protocol was a pairing of primary antibodies for Fos (rabbit, 1:3000, Santa Cruz, Santa Cruz, CA) with antibodies for GAD67 (mouse 1:5000, Chemicon, Temecula, CA), nNOS (mouse, 1:2000, Santa Cruz) or TH (mouse, 1:500, Chemicon). Fos antibodies were visualized with Cy3-conjugated donkey anti-rabbit IgG, 1:200, Jackson, West Grove, PA). nNOS and TH were visualized with Cy2-conjugated donkey anti-mouse IgG, 1:300, Jackson). GAD67 was amplified with goat anti-mouse biotinylated IgG (2 hours, 1:200, Vector, Burlingame, VT), Streptavidin-Horseradish Peroxidase (1 hour, 1:200, PerkinElmer, Boston, MA) and TSA-biotin (15 minutes, 1:100, PerkinElmer) and visualized with Neutravidin/avidin Oregon Green (1:300, Molecular Probes,

Carlsbad, CA). In addition, FG was present in each series and was visualized using its autofluorescence. All incubations in the immunohistochemical protocols were performed at room temperature on a shaker in sterile 24 well plates (Vector). Fos and GAD67 sections were incubated for 70-72 hours, while Fos and nNOS or Fos and TH sections were incubated for 46-48 hours. Sections were incubated in secondary antibodies (conjugated to Cy2, Cy3, or Neutravidin/avidin Oregon Green) for 2 hours. In most cases, individual immunohistochemistry protocols were conducted simultaneously on tissue from separate animals receiving phenylephrine, diazoxide and saline treatment.

Sections were mounted on gel-coated slides and air dried. Fos and GAD67 labeled sections were coverslipped with Vectashield (Vector). Fos and nNOS or TH labeled sections were coverslipped with ProLong Gold (Molecular Probes). All slides were then sealed with clear nail polish.

Antibody Specificity:

In each protocol, primary antibodies were withheld from single sections to serve as controls. Examples of controls and positive labeling are shown in Figure 13. Antibody specificity for Fos (Santa Cruz), and nNOS (Santa Cruz), was verified by the vendor by western blot. Specificity for GAD67 was verified previously (50). The mouse anti-TH antibody was not verified by the vendor, but it has been used in numerous published reports (16; 65; 137; 143). Characteristics of positive-immunoreactivity (-IR) have been shown previously for Fos (23; 27; 157), nNOS (47; 159), and GAD67 (50).

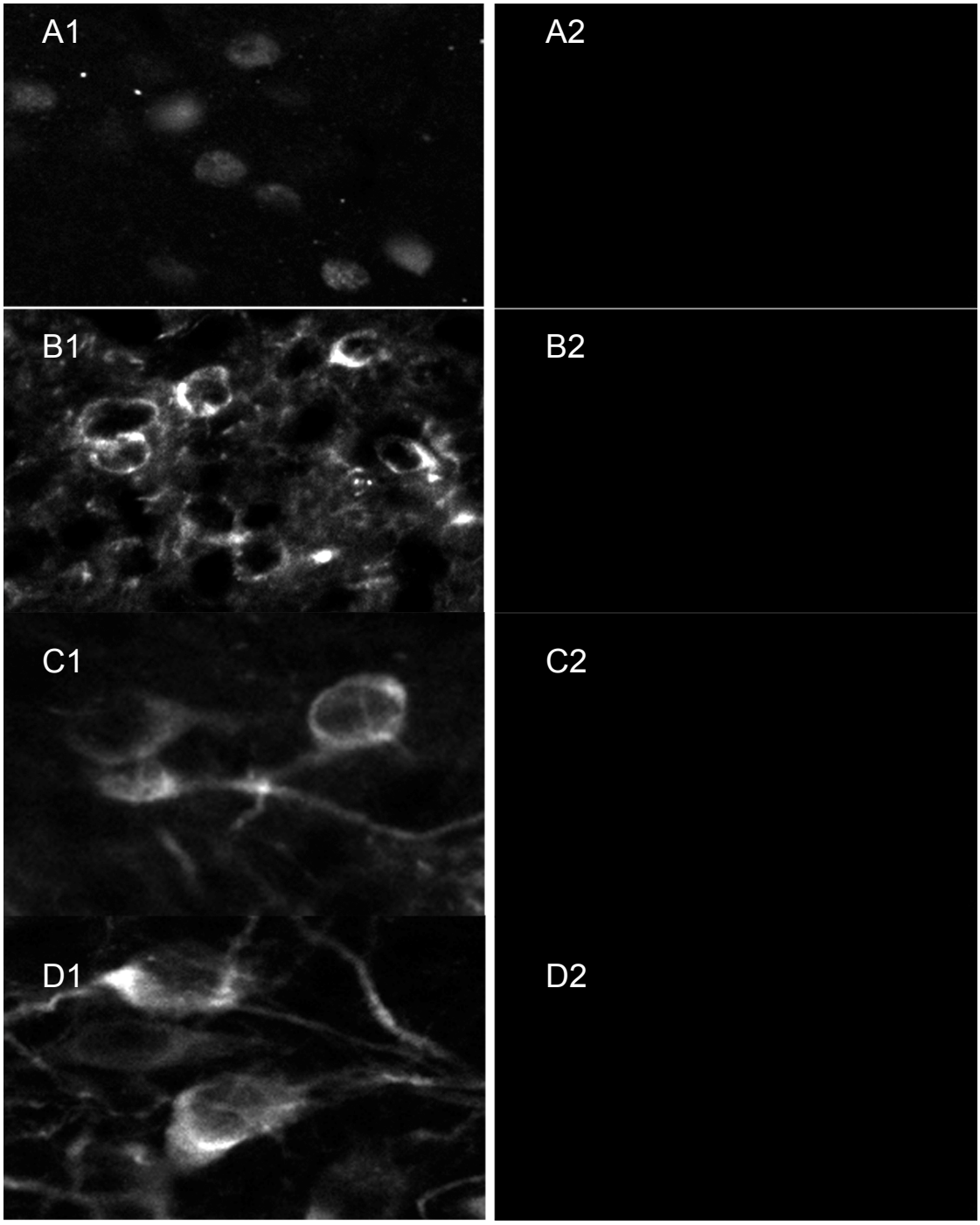

Figure 13: **Validation of immunohistochemical protocols.** Epifluorescent photomicrographs of immunopositive labeling. Images depict positive labeling for Fos-IR (A1), GAD67-IR (B1), nNOS-IR (C1), and TH-IR (D1), or the appropriate non-primary control (A2-D2).

Images were taken of positive IR of each type, and then the microscope was moved to a control section without altering the camera exposure time. The lack of staining in non-primary controls provides evidence validating the immunohistochemical protocols used.

Microscopy and Image Analysis:

Sections were examined with an Olympus microscope (BX51), using a 2-axis motorized stage (Ludl Electonic Products Ltd, Hawthorne, NY). Separate filter sets for Oregon Green 488 or Cy2 [ex. λ480 nm; em. λ510 nm], Cy3 [ex. λ550 nm; em. λ570 nm], and Fluoro-Gold [ex. λ330 nm; em. λ515 nm] were used to visualize positive immunoreactivity. Images of cells were captured with a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ) and analyzed with the Neurolucida (ver. 7.5, MicroBrightField, Willison, VT) software package.

In all immunohistochemical series, the section containing CS, the caudal pole of area postrema, was determined. The NTS, ipsilateral to the FG injection site, was then examined in sections that were -180 μm caudal and 180, 540, and 900 μm rostral to CS. Images captured of these areas were evaluated as described previously (Chapter 2). Briefly, sections and relevant nuclear regions were outlined. The top and bottom of the section were determined, and single images were taken at one micron increments for each filter through the entirety of the section. All cells were counted by an individual blinded to the experimental protocol. Criteria used to identify positively labeled cells were as follows: Fos-positive cells appeared as solid round objects, representing nuclear staining. GAD67, nNOS, and TH staining consisted of complete cytosolic staining with a visibly empty nuclear region. FG staining was visualized as either a bright completely filled cytosol with a blank nuclear region, similar to GAD67, nNOS, and TH, or punctate labeling surrounding the empty nuclear region. If cells exhibited these characteristics in two or three of

the filter sets within the same focal plane, then those cells were considered double- or triple-labeled, respectively. For representative images of single, double and triple labeling, single images were taken with the appropriate filters, imported into Photoshop (ver. 7.0 Adobe Systems, San Jose, CA) and combined. Image brightness and contrast only were adjusted for clarity.

Data Analysis:

Counts of single, double, and triple labeled cells of each section were analyzed using the software package NeuroExplorer (ver. 4.5, MicroBrightField) and exported into a spreadsheet (Excel 11.656, Microsoft, Redmond, WA). The data were analyzed for each individual section and the total of four sections throughout the NTS for each immunohistochemical group. Also, the percentage of double-labeled cells of a specific pairing was determined relative to the total number of each of the individual single labeled cells of that pair. Percentages were calculated by dividing the number of colabeled cells by the total number of the single labeled cells. The total number of triple labeled cells of each immunohistochemical pairing was used to calculate the percent of double-labeled cells that were triple labeled. The distribution of single, double and triple labeled cells was examined using NeuroExplorer, with subnuclear regions determined by comparing our sections to a standard atlas (115) when necessary. All data were graphed using SigmaPlot (8.0, SPSS, Chicago, IL).

Statistics:

All data are presented as mean ± SE. Changes in MAP and HR in response to saline, PE, or Diaz treatments were analyzed by two-way repeated measures (RM) ANOVA. Total Fos and FG and the Fos and FG colabeling were analyzed with two-way RM ANOVA, while the distribution of GAD67, nNOS and TH single labels and their colabeling with Fos was analyzed with one-way ANOVA. When appropriate, ANOVA's were followed by a Student-Neuman-Keuls method post-hoc analysis. Statistical analyses were performed using Sigma Stat (3.5,SPSS, Chicago, IL) software and significance was accepted at $P < 0.05$.

Results:

Identification of CVLM:

Glutamate microinjection into the caudal ventrolateral medulla (CVLM) resulted in a reduction in MAP (-22.9 ± 1.3 mmHg) and in HR (-28.9 ± 4.6 BPM). Histological examination of the FG injection sites (Fig 14) indicates the midpoints of the injections were between 500μm and 1000μm rostral to calamus scriptorius (CS), in the region of the ventrolateral medulla. Injection sites were similar for all of the treatment groups.

Hemodynamic Responses:

There were no significant differences among the three drug treatment groups in baseline MAP (saline: 115.1 ± 7; PE: 113.2 ± 3; Diaz: 108.5 ± 6 in mmHg) or HR (saline: 376.5 ± 29; PE: 349.5 ± 15; Diaz: 344.7 ± 2 in BPM). Infusion of PE (n=5)

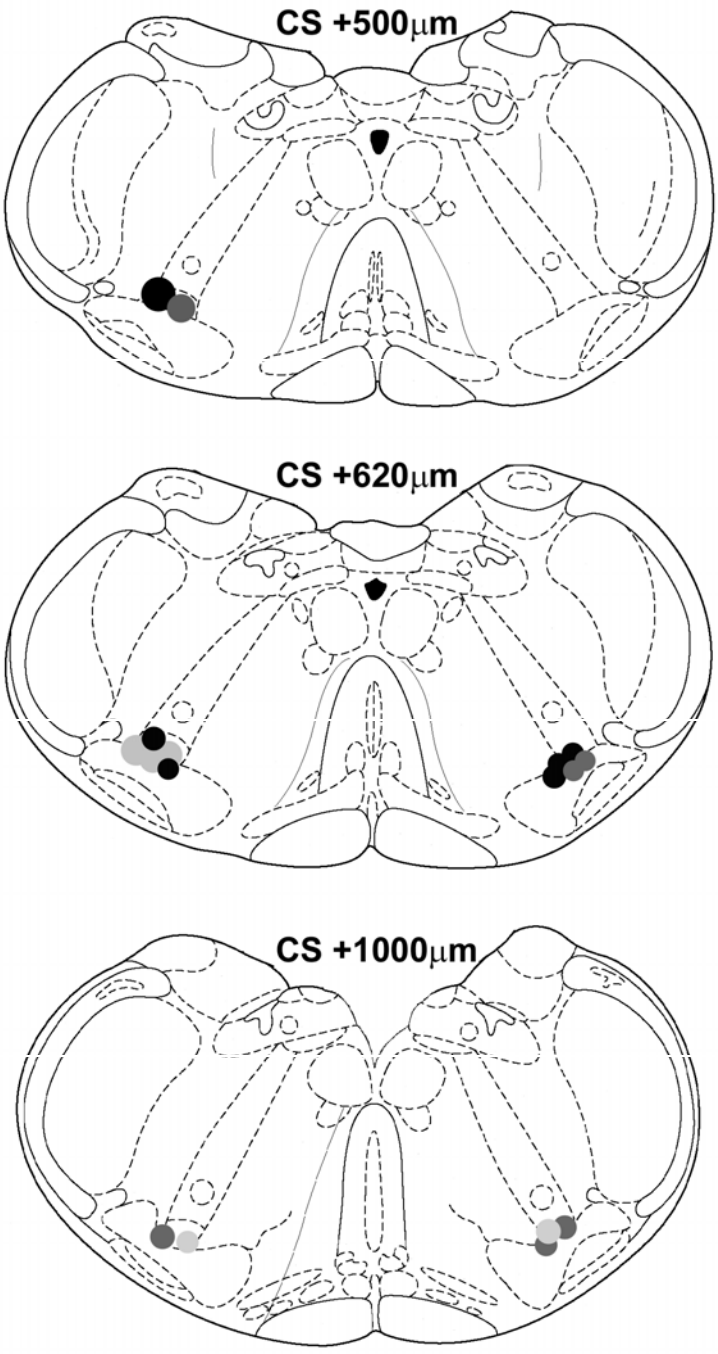

Figure 14: **Histological verification of FG injection sites.** Dots indicate the midpoint of the FG injection in Saline (light grey, n=5), PE (black, n=6) and Diaz (dark grey, n=6) treated rats in sections located 500 μm (A), 620 μm (B), and 1000 μm (C) rostral to calamus scriptorius (CS). Representative sections are taken from Paxinos and Watson (106).

Similar to Figure 12, this confirms that the FG was microinjected into the CVLM. Further, there were no differences between the treatment groups in relation to the midpoints of FG microinjection.

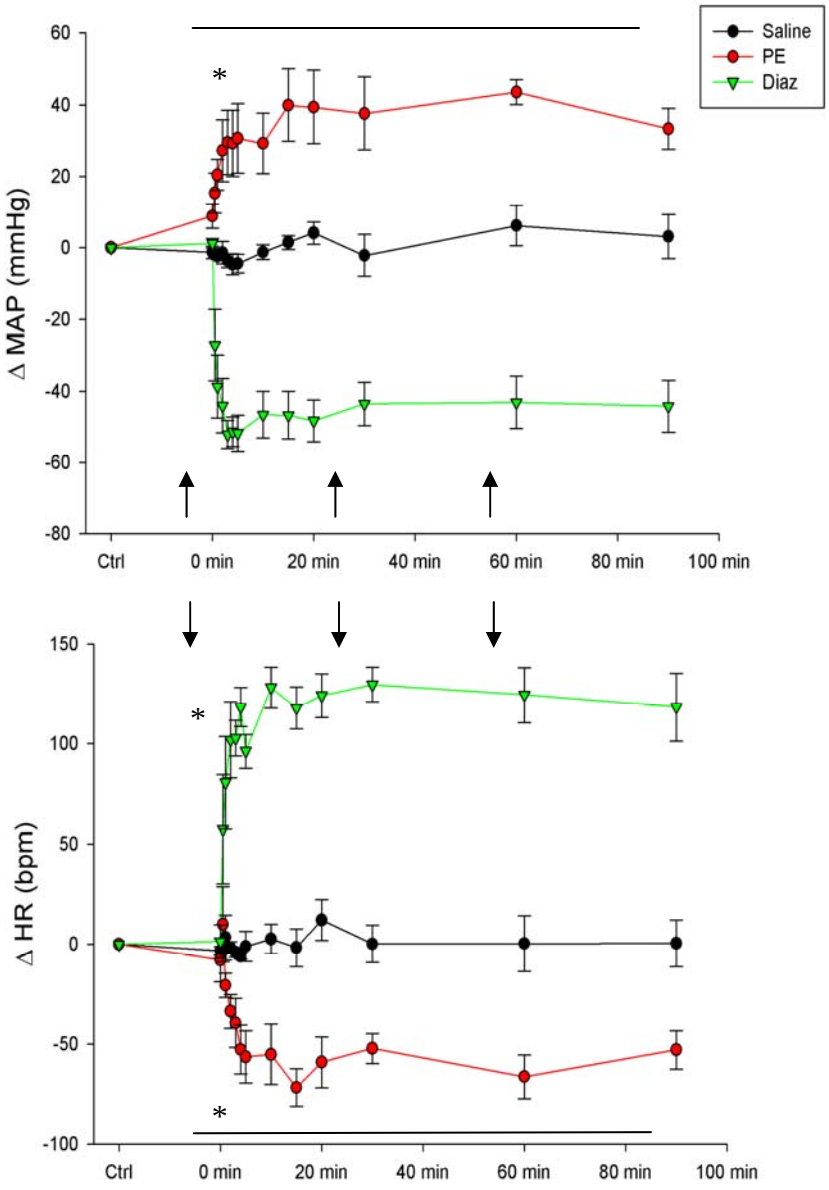

Figure 15: **Hemodynamic responses to drug treatment.** Change in MAP (A) and HR (B) in conscious rats in response to Saline [n=4; black], Phenylephrine (PE) [n=5; red] or Diazoxide (Diaz) [n=5; green] administration. Infusion of PE resulted in a sustained significant increase in MAP and a reflex decrease in HR. Conversely, administration of Diaz produced sustained significant decreases in MAP and a reflex increase in HR. Infusion of saline produced no significant change in MAP or HR. Bar represents period of PE infusion. Arrows represent time points for bolus Diaz injections. * is $P<0.05$ vs. saline time point through remainder of treatment time.

These data show that increases or decreases in pressure were sustained for the entirety of the drug administration. Saline produced no significant hemodynamic response.

a reflex decrease in HR (Fig 15B, P<0.05 vs. saline) beginning at 2 minutes after the start of PE infusion. Conversely, bolus injections of Diaz (n=5) resulted in a sustained significant decrease in MAP (Fig 15A, P<0.05) and reflex increase in HR (Fig 15B, P<0.05) starting at 1 minute post injection. Saline infusion (Fig 15A, B, n=4), did not result in a significant change vs. the control period.

Immunohistochemistry:

Fos-Immunoreactivity:

The total number of Fos-IR neurons in four sections of the NTS in each treatment group is shown in Figure 16A and the rostral-caudal distribution is shown in Figure 16B. Increases or decreases in MAP due to infusion of PE or injections of Diaz, respectively, over the 90 minute time frame resulted in significant increases of Fos-IR in the NTS vs. saline treatment. Further, the overall levels of Fos-IR in response to hypertension and hypotension were similar (PE: 207 ± 30, Diaz: 174 ± 32). There was, however, a trend (p = 0.08) for a greater number of Fos-IR in PE treated animals than Diaz treated animals in the sections 180 μm caudal to CS and 180 μm rostral to CS (Fig 16B). Figure 17 is a diagrammatic representation of the distribution of Fos-IR in single saline (1), PE (2), or Diaz (3) treated animals at four levels of the NTS. Fos-IR after saline treatment was low and randomly distributed within the NTS, with the labeling not localized to a specific subnuclear region at any of the four rostral-caudal levels. Fos-IR in response to PE

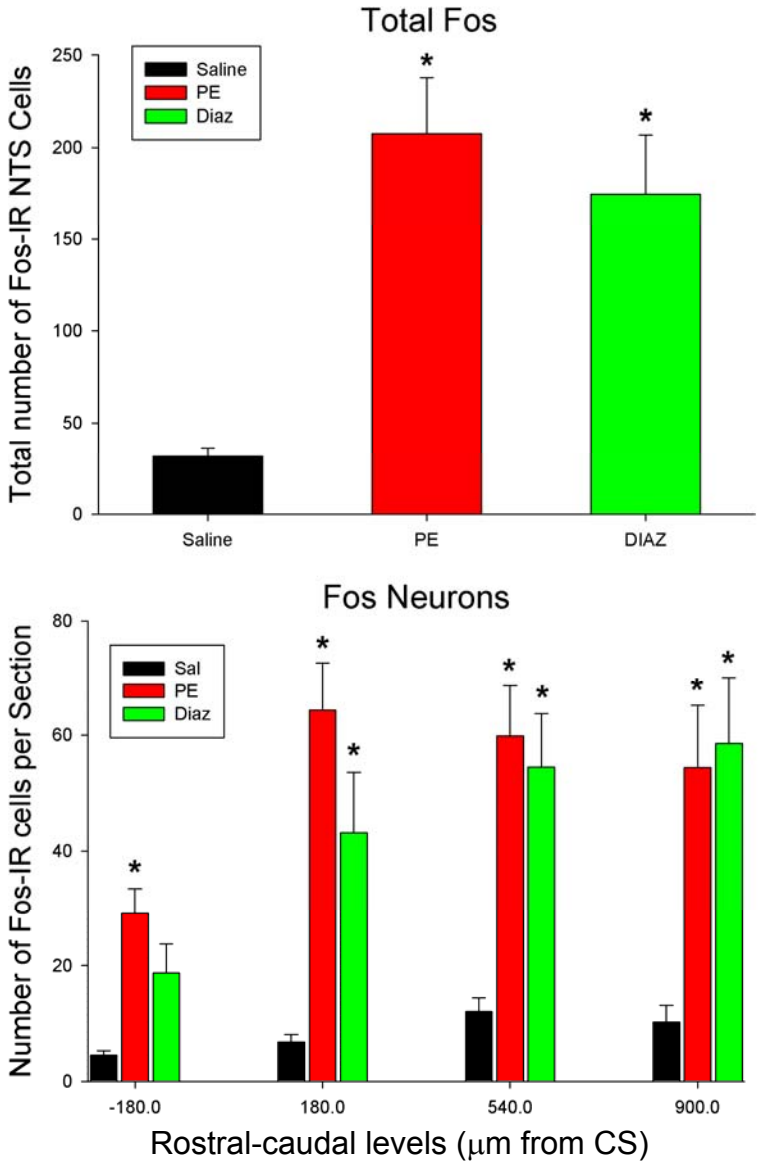

Figure 16: **Activation of NTS neurons.** Comparison of Fos-IR neurons in the NTS between drug treatment groups. A. Total Fos-IR in Saline (black), PE (red), and Diaz (green) treated animals at four levels of the NTS. B. Rostral-caudal distribution (relative to calamus scriptorius, CS) of Fos-IR in the NTS after treatment with saline (black), PE (red) or Diaz (green). There was a trend (P=0.08) for the number of Fos-IR neurons to be greater in the NTS from animals subjected to hypertension vs. hypotension in sections 180 μm caudal to CS and 180 μm rostral to CS. * P<0.05 vs. Saline treatment.

These data show (A) that there is a generally similar level of activation in the NTS in response to either hypertension or hypotension. Also, hypertension and hypotension activated a significantly greater number of neurons in comparison to saline treatment. (B) A rostral-caudal variation may exist between activated neurons in the NTS in response to hypertension and hypotension.

and Diaz treatments resulted in some labeling in similar areas, but was localized to specific subnuclear regions as well. For example, at 180 and 540 μm rostral to CS, the subnuclear distribution of Fos-IR neurons appeared to be different. In brains from PE treated animals, Fos-IR was apparent in the dorsolateral subnuclear region and medial to the solitary tract. In contrast, Fos-IR in Diaz treated animals was localized primarily in the medial and subpostremal subnuclear regions (Fig 17). In addition, in sections 900 μm rostral to CS, Fos-IR in Diaz treated animals was present predominantly in the medial and subventricular subnuclear regions whereas PE treatment resulted in Fos-IR in the medial, ventrolateral, and lateral subnuclear regions.

GAD67-IR, nNOS-IR, TH-IR and FG Containing Cells:

Single neurons positively labeled for FG, GAD67-, nNOS-, and TH-IR were counted in tissue from animals receiving each of the treatments.

There were no significant differences among saline, PE, or Diaz treated groups in the number of cells stained for any of these markers (Table 2). These data indicate the reliability of our immunohistochemical protocols. The subnuclear locations of FG labeling (157), GAD67-IR (50; 157), nNOS (22; 46; 47; 90-94), and TH (69; 75; 76; 123) were all similar to locations described previously.

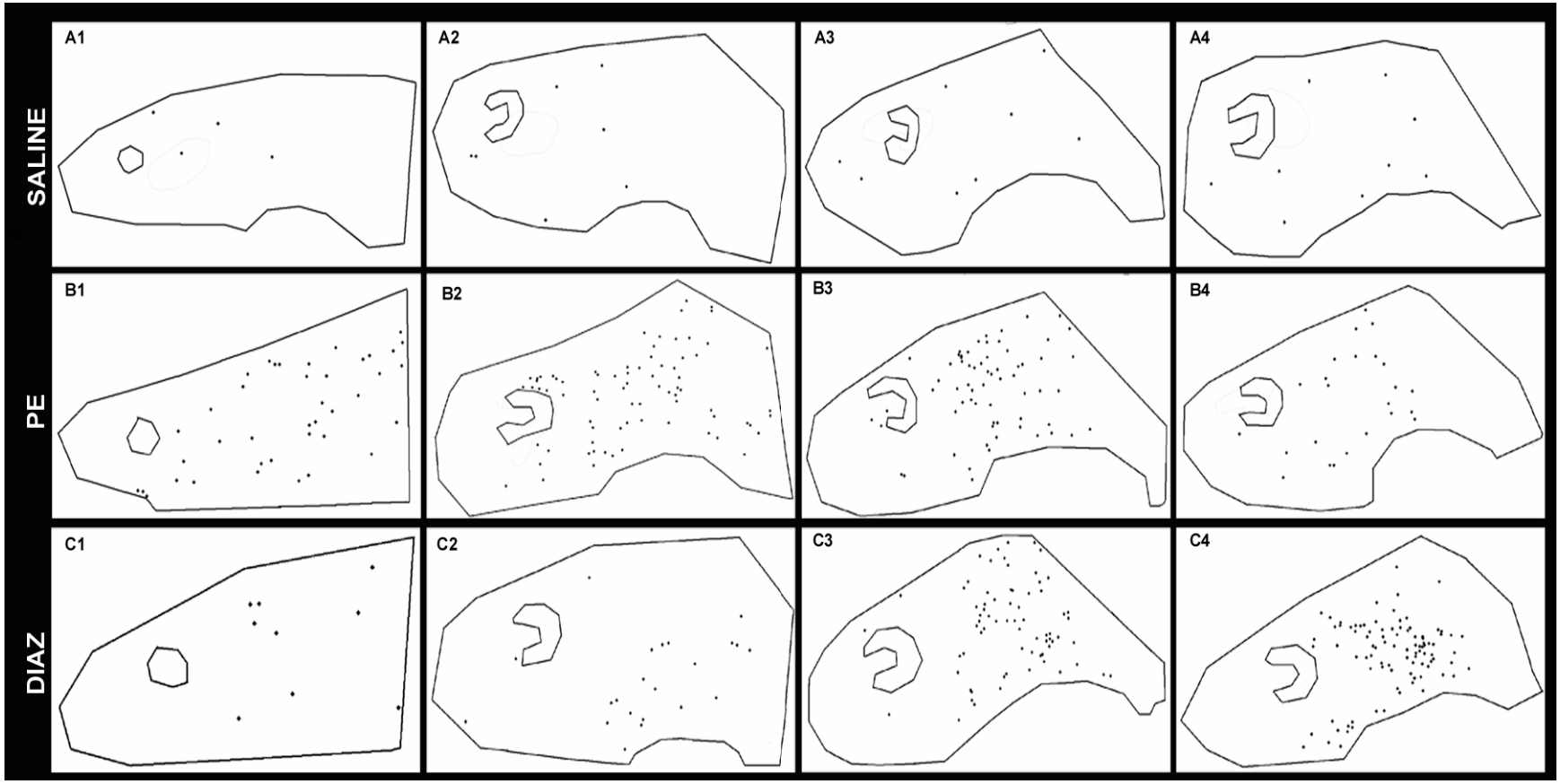

Figure 17: **Distribution of activated neurons in the NTS in response to hypertension or hypotension.** Diagrammatic representation of Fos distribution at four rostral-caudal levels of the NTS. A) Saline treated animals. B) PE treated animals. C) Diaz treated animals, at levels 1) 180 μm caudal to calamus scriptorius (CS). 2) 180 μm rostral to CS. 3) 540 μm rostral to CS. 4) 900 μm rostral to CS.

These data indicate a variation in distribution of activated neurons in the NTS in response to these stimuli. In hypertensive animals, neurons located in the dorsomedial subnuclear region and surrounding the solitary tract were activated, whereas this area was virtually devoid of activation in response to hypotension. Further, there were more activated neurons in the medial subregion in response to decreases in pressure than with increases in pressure. Activation by saline was low and not localized to any specific subnuclear region.

Drug Treatment	FG	GAD67	nNOS	TH
Saline	154.1 ± 24.8	154.4 ± 25.3	125.6 ± 27.1	88.6 ± 8.6
PE	168.8 ± 30	252 ± 48.37	123.2 ± 16.1	89 ± 6.3
Diaz	170.6 ± 19.2	224.3 ± 53.6	119.3 ± 9.5	81.7 ± 7.7

Table 2**: Single labeled neurons in the NTS**. The total number of single labeled FG or GAD67-, nNOS-, and TH-IR neurons in each drug treatment group. There were no significant differences in total counts among drug treatment groups.

Fos-IR and FG Colabeling:

In saline treated animals, the number of cells colabeled with Fos-IR and FG was minimal (Fig 18A). PE treatment resulted in a significantly greater number of NTS cells colabeled with FG and Fos-IR cells compared to saline or Diaz treatment (Fig 18A). Diaz did not alter the number of colabeled NTS cells compared to saline. The percent of Fos-IR cells that projected to the CVLM (labeled with FG) also was significantly greater in tissue from animals treated with PE compared to that from Diaz or saline treated rats (Fig 18B). Similarly, of CVLM-projecting (FG positive) cells, the percent exhibiting Fos-IR in PE treated animals was greater than that in Diaz or saline treated animals (Fig 18C). The majority of the neurons colabeled with Fos-IR and FG in response to PE was located in the dorsolateral subnuclear region, with some colabeling in the area medial and dorsal to the ST. Colabeling of Fos-IR and FG positive neurons in the Diaz and saline treatment groups was dispersed throughout the four sections examined. Pseudocolored photomicrographs of Fos-IR from PE (Fig 19A) and Diaz (Fig 19D) treated animals show examples of colabeling with FG positive neurons (Fig 19B, E) in the dorsolateral subnuclear region of the NTS, and 540 μm rostral to CS. Examples of colabeling are shown in merged images in Figure 19C and 19F.

Fos-IR and GAD67-IR Colabeling:

There were few NTS cells colabeled for Fos-IR and GAD67-IR in tissue from saline treated animals (Fig 20A). Animals treated with PE or Diaz exhibited a slight but not significant increase in the number of cells colabeled with GAD67-IR and Fos-

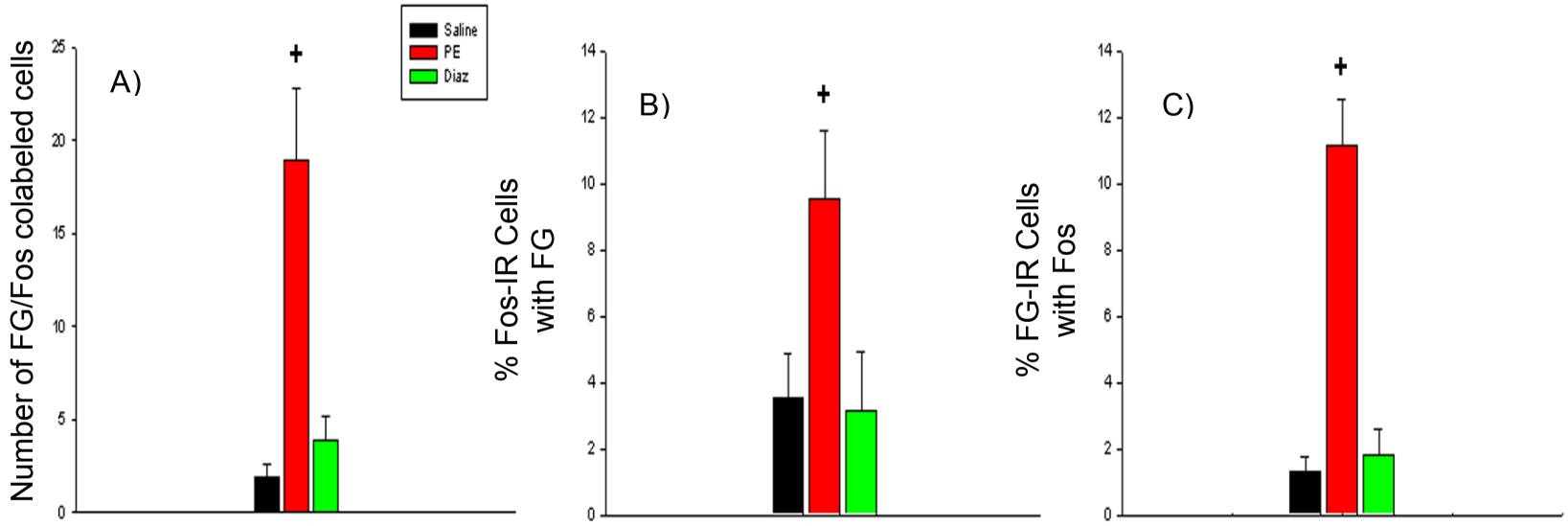

Figure 18: **Colabeling of Fos-IR and CVLM projecting cells in the NTS.** A) Total # of cells colabeled with Fos-IR and FG following administration of Saline (black), PE (red), or Diaz (green). B) Percent of Fos-IR neurons that colabeled with FG. C) Percent of FG neurons that colabeled with Fos-IR. + $P < 0.05$ vs. saline and Diaz.

These data suggest that CVLM projection neurons are activated in response to hypertension but not hypotension.

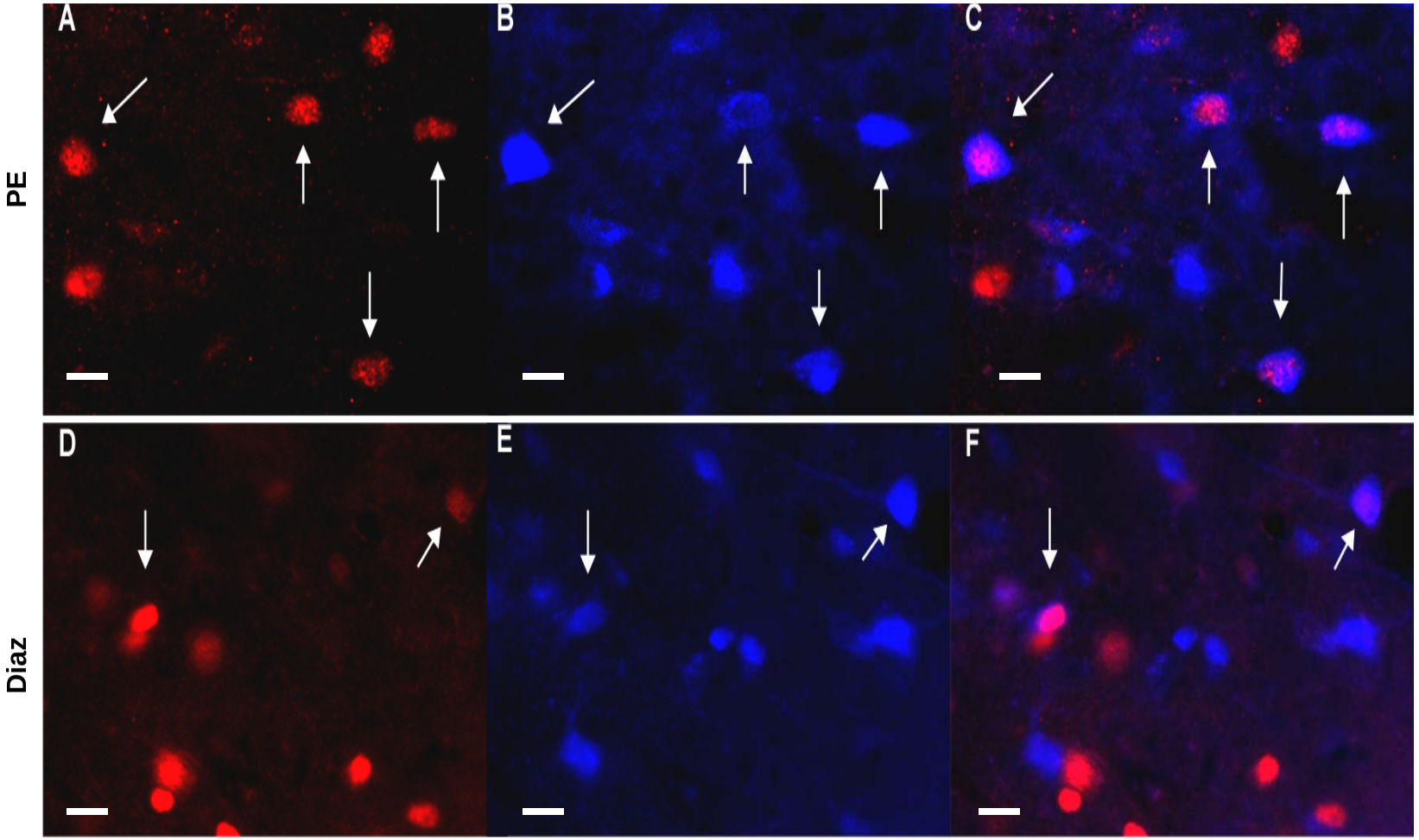

Figure 19: **Images of Activated CVLM projecting cells.** Pseudocolored photomicrographs of the NTS after PE (top) and Diaz treatment (bottom). A and D: Fos-IR; B and E: CVLM projecting cells; C and F: Merged images. Images are Dorsal and medial to the solitary tract, 180 μm rostral to CS. White arrows denote colabeled cells. Bar is 25 μm.

IR in comparison to the saline treated animals (Fig 20A). When evaluating Fos-IR cells, there was no difference among groups in the percent of Fos-IR cells that colabeled with GAD67-IR (Fig 20B). Similarly, the percent of GAD67-IR cells that colabeled with Fos-IR (Fig 20C) was not different among groups. Pseudocolored photomicrographs of Fos-IR and GAD67-IR from PE (Fig 21A,B) and Diaz (Fig 21D,E) treated animals show labeling in the medial region of the NTS, and 180 μm rostral to CS. Examples of colabeling are shown in merged images in Figure 21C and 21F.

Fos-IR and nNOS-IR Colabeling:

Saline treatment resulted in little Fos-IR and nNOS-IR colabeling in the NTS (Fig 22A). PE treatment produced a significant increase in the number of Fos-IR and nNOS-IR colabeled cells compared to the saline treated group (Fig 22A). The percent of Fos-IR cells in the NTS that colabeled with nNOS-IR (Fig 22B) and the percent of nNOS-IR cells that colabeled with Fos-IR neurons (Fig 22C) was significantly greater after PE treatment compared to saline. In Diaz treated animals, there was no statistical difference in the percent of Fos-IR cells that colabeled with nNOS-IR in comparison to saline treated animals (Fig 2B). However, the percent of nNOS-IR NTS cells that colabeled with Fos-IR neurons (Fig 22C) in Diaz treated animals was significantly greater compared to saline treated animals. Also, there were no significant differences between PE and Diaz treated animals in total number of Fos- and nNOS-IR colabeled cells (Fig 22A) or the percent of either Fos-IR

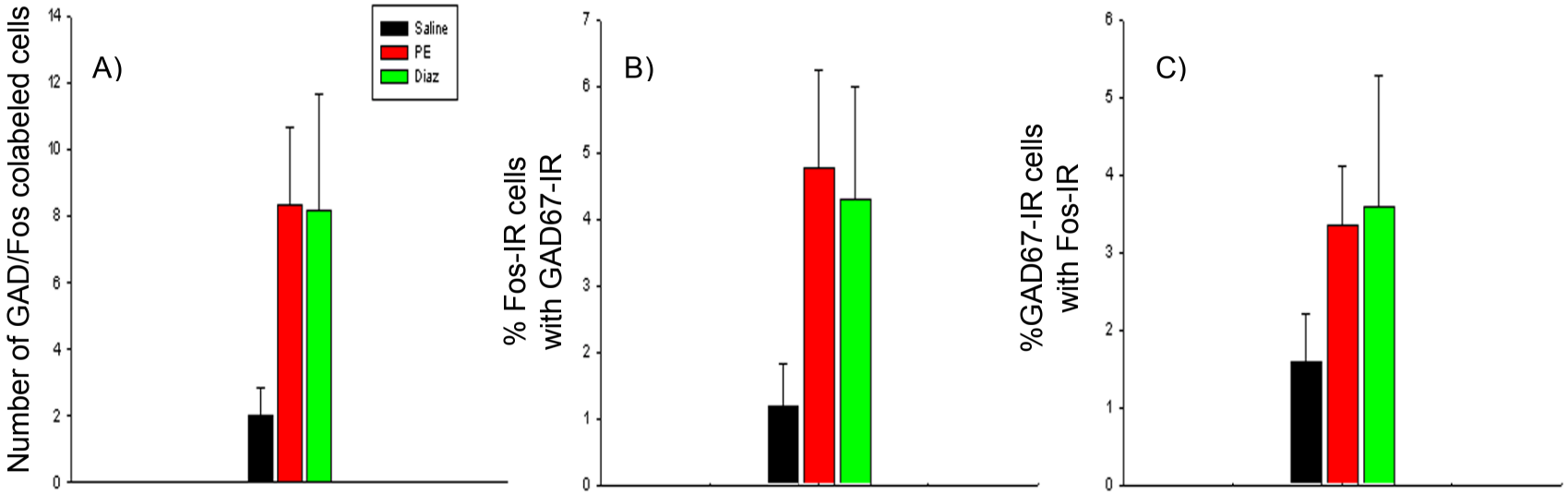

Figure 20**: Colabeling of Fos-IR and GABAergic cells in the NTS.** A) Total # of cells colabeled with Fos-IR and GAD67-IR following administration of Saline (black), PE (red), or Diaz (green). B) Percent of Fos-IR neurons that colabeled with GAD67-IR. C) Percent of GAD67-IR neurons that colabeled with Fos-IR.

These data suggest there is no difference in GABAergic activation in response to hypertension or hypotension.

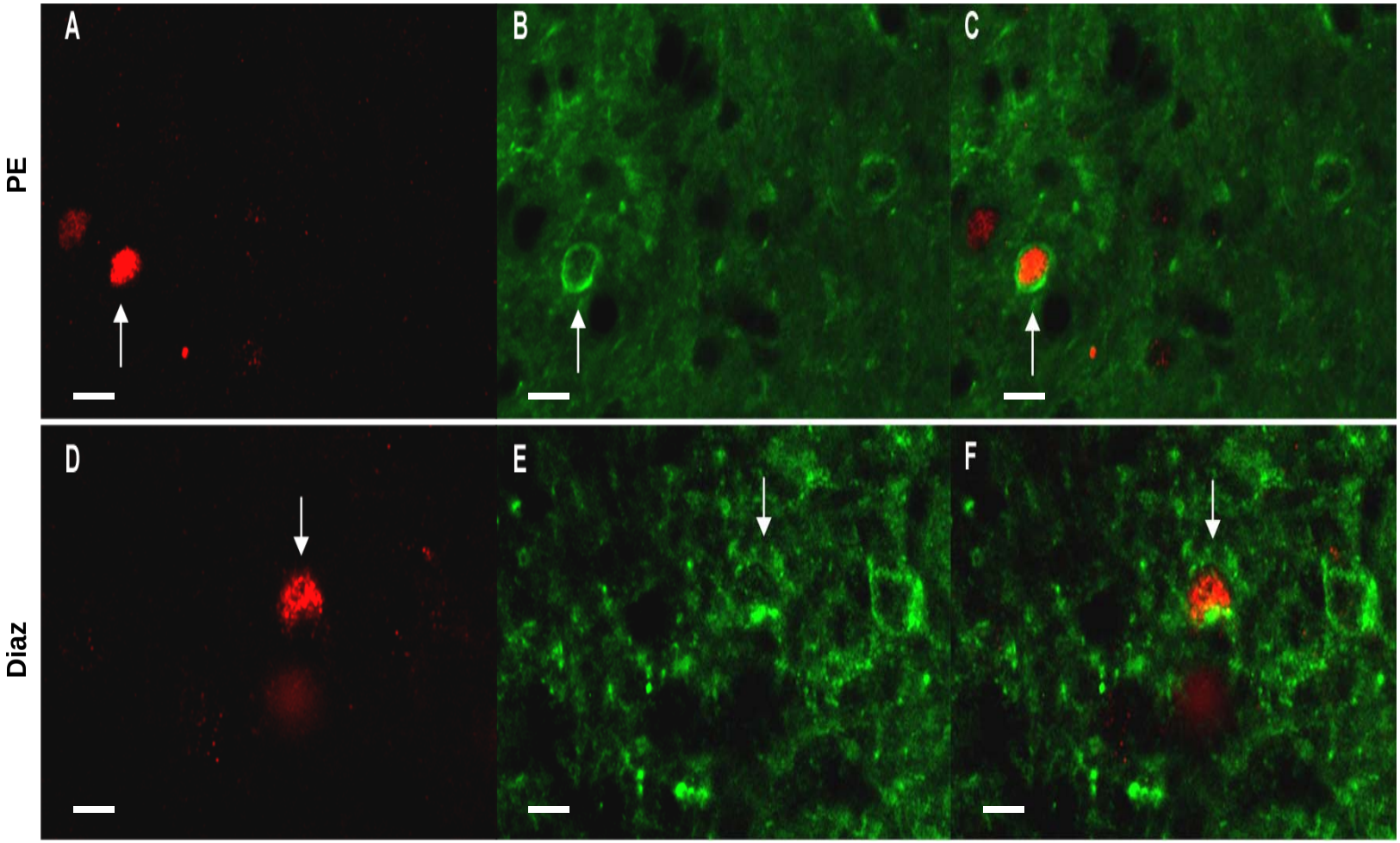

Figure 21: **Images of Activated GABAergic cells.** Pseudocolored photomicrographs of the NTS after PE (top) and Diaz treatment (bottom). A and D: Fos-IR; B and E: GAD67-IR; C and F: Merged images. Images are ventral and medial to the solitary tract, 180 μm rostral to CS. White arrows denote colabeled cells. Bar is 25 μm.

or nNOS-IR cells colabeled with the other (Fig 22B, 22C). However, examination of the distribution of Fos- and nNOS-colabeled cells within the NTS suggested that in PE treated animals, the population of nNOS neurons located in the dorsolateral subnuclear region of the NTS was activated, while in Diaz treated animals the nNOS neurons in the medial and subpostremal subnuclear regions of the NTS were activated (Fig 17B2 & B3). In both PE and Diaz treatments the postremal NTS (180 and 540 μm rostral to CS) contained the highest number of colabeled neurons. Pseudocolored photomicrographs of Fos-IR and nNOS positive neurons from PE (Fig 23A,B) and Diaz (Fig 23D,E) treated animals show examples of labeling in the dorsolateral subnuclear region of the NTS, and 540 μm rostral to CS. Examples of colabeling are shown in merged images in Figure 23C and 23F.

Fos-IR and TH-IR Colabeling:

Animals that received saline treatment had little Fos-IR and TH-IR colabeling (Fig 24A). Animals treated with PE or Diaz had a significantly greater number of colabeled Fos-IR and TH-IR cells (Fig 24A) compared to rats given saline (Fig 24A). There were no differences among treatments in the percent of total Fos-IR cells that colabeled with TH-IR neurons (Fig 24B). However, of the TH-IR cells in the NTS, there was a significantly greater percent that colabeled with Fos-IR in PE and Diaz treated animals than in saline treated animals (Fig 24C). Similar levels of Fos-IR and TH-IR colabeling were seen in the catecholaminergic neuronal populations in both the PE and Diaz treated animals. Pseudocolored photomicrographs of Fos-IR from PE (Fig 25A) and Diaz (Fig 25D) treated animals

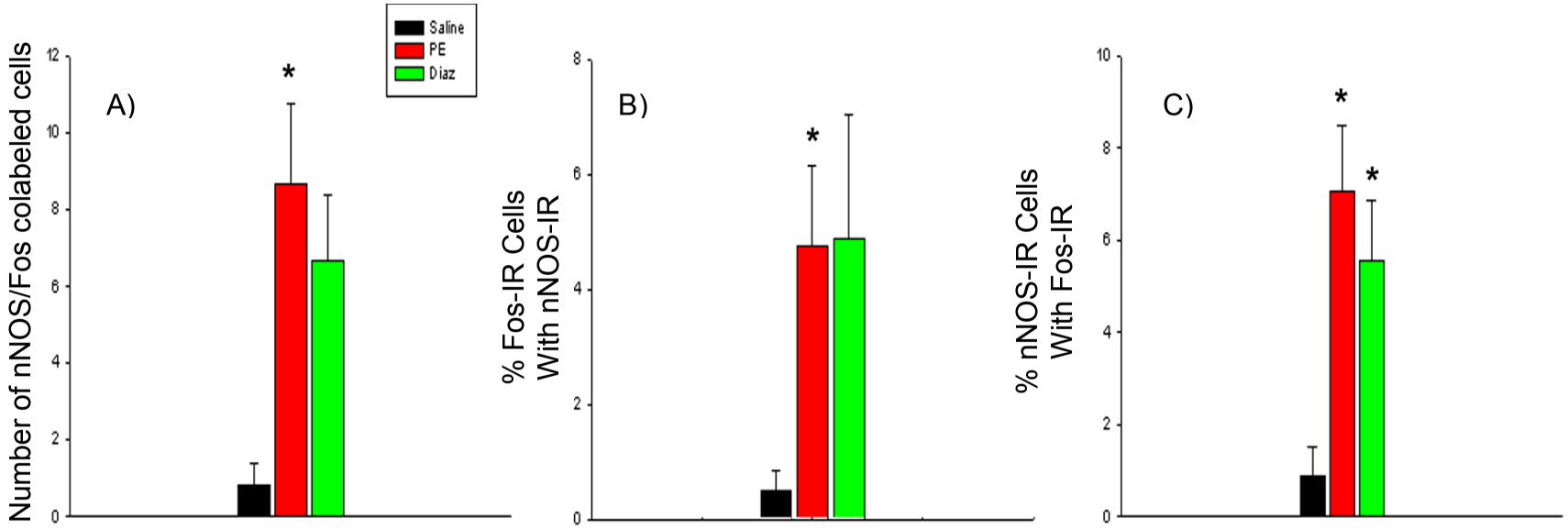

Figure 22: **Colabeling of Fos-IR and nitroxidergic cells in the NTS.** A) Total # of cells colabeled with Fos-IR and nNOS-IR following administration of Saline (black), PE (red), or Diaz (green). B) Percent of Fos-IR neurons that colabeled with nNOS-IR. C) Percent of nNOS-IR neurons that colabeled with Fos-IR. $P < 0.05$ vs. Saline

These data suggest that a significantly greater number of nitroxidergic neurons in the NTS are activated in response to hypertension in comparison to control. Further, hypertension and hypotension activated a greater percentage of nitroxidergic neurons in comparison to control.

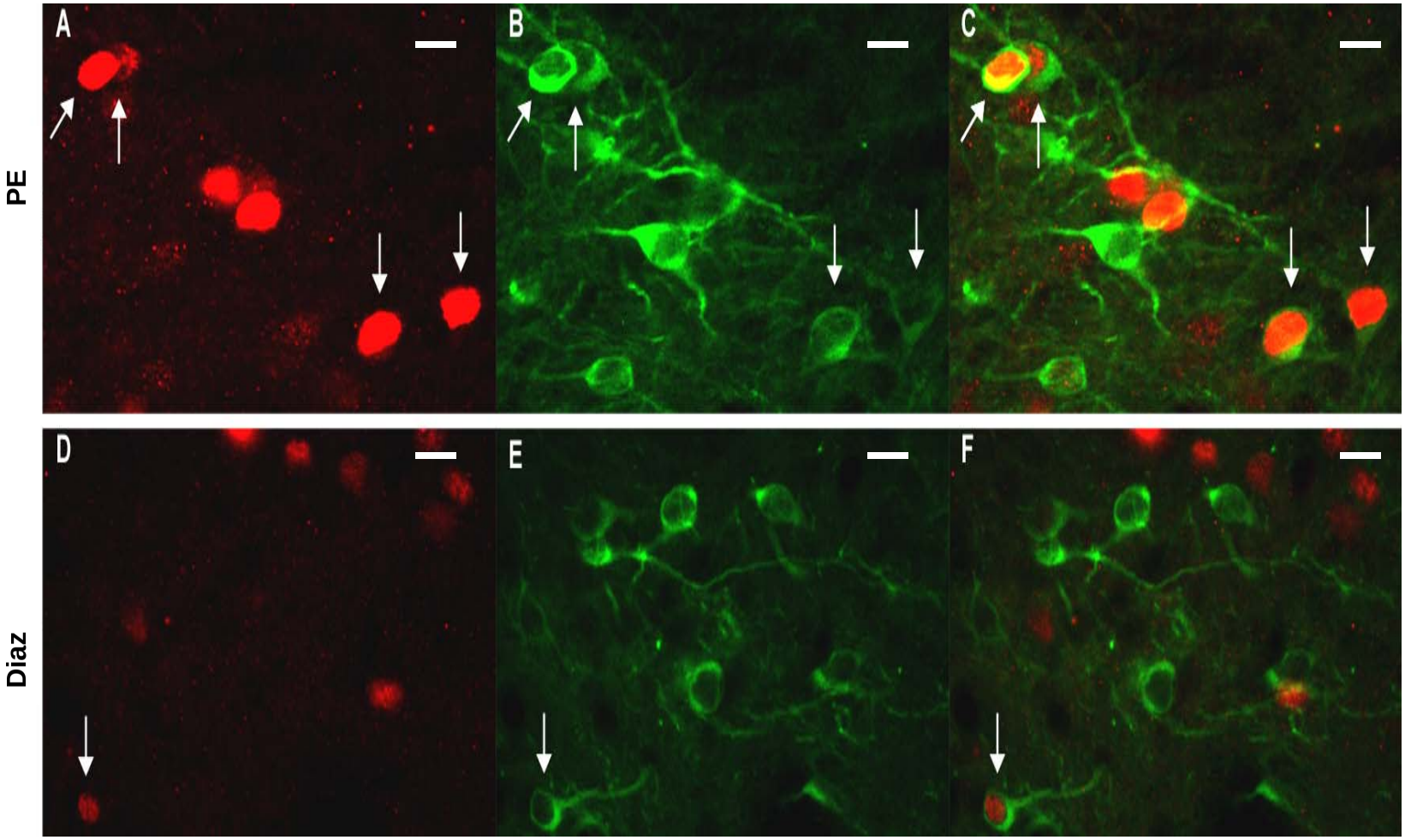

Figure 23: **Images of activated nitroxidergic cells.** Pseudocolored photomicrographs of the NTS after PE (top) and Diaz treatment (bottom). A and D: Fos-IR; B and E: nNOS-IR; C and F: Merged images. Images are Dorsal and medial to the solitary tract, 180 μm rostral to CS. White arrows denote colabeled cells. Bar is 25 μm.

show examples of colabeling with FG positive neurons (Fig 25B,E) in the medial subnuclear region of the NTS, dorsal and lateral to the dorsal motor nucleus of the vagus, and 540 μm rostral to CS. Examples of colabeling are shown in merged images in Figure 25C and 25F.

Discussion:

This study compared the activation of specific phenotypes of neurons in the nucleus of the solitary tract (NTS) after acute periods of hypertension or hypotension. Through the use of retrograde labeling, neurons that project from the NTS to the caudal ventrolateral medulla (CVLM) were examined. Data from this study suggest that acute hypertension results in activation of a significant number of CVLM projecting NTS neurons. In contrast, in response to hypotension the number of activated CVLM projecting neurons was not different from control. Data also suggest that the number of activated GABAergic, nitroxidergic, and catecholaminergic neurons by acute hypertension or hypotension was similar, although there was a difference in the subnuclear distribution of the activated nitroxidergic cells. Therefore, activation of NTS neurons by hypertension or hypotension results in differential activation of CVLM projecting neurons and nitroxidergic neurons, with similar GABAergic and catecholaminergic activation.

Identification of Activated Neurons within the NTS:

Based on the current model of baroreflex function (Chapter 1, Fig. 1), increases in arterial pressure activate baroreceptors in the wall of the aortic arch and

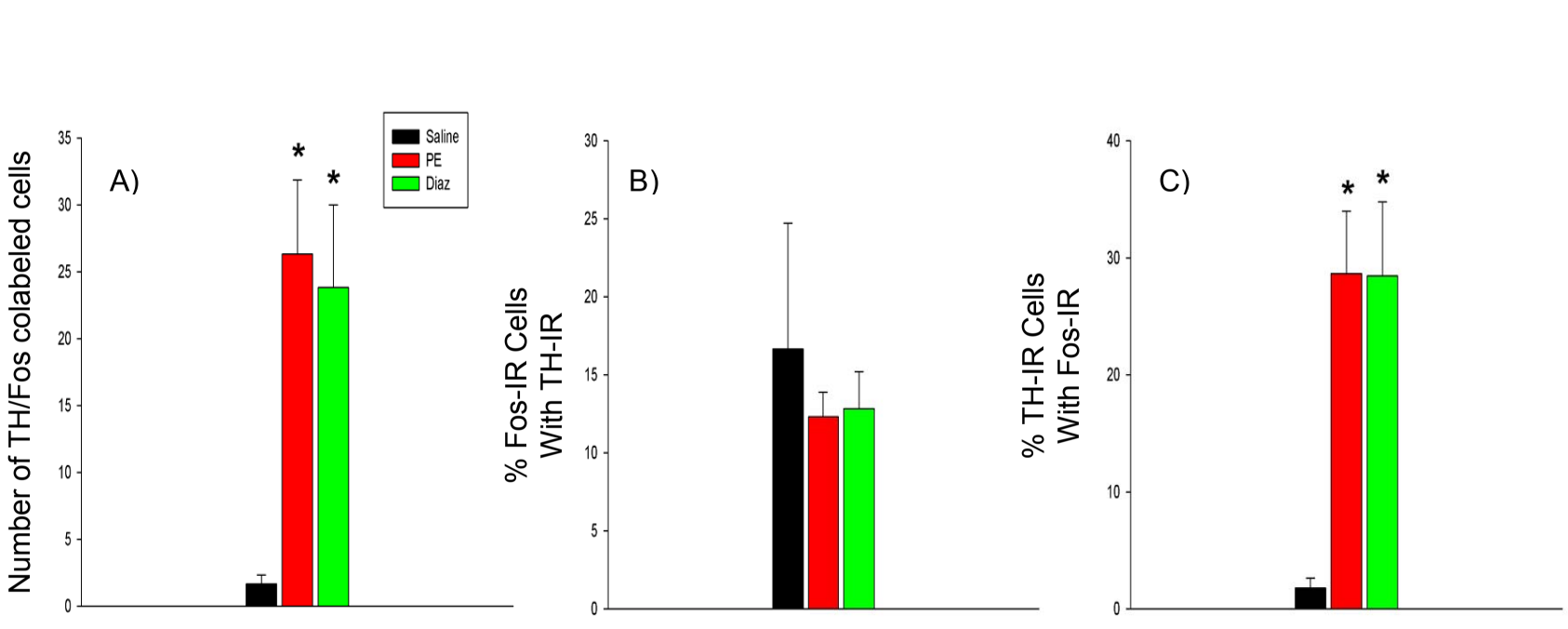

Figure 24: **Colabeling of Fos-IR and catecholaminergic cells in the NTS:** A) Total # of cells colabeled with Fos-IR and TH-IR following administration of Saline (black), PE (red), or Diaz (green). B) Percent of Fos-IR neurons that colabeled with TH-IR. C) Percent of TH neurons that colabeled with Fos-IR. * $P < 0.05$ vs. Saline

These data suggest that catecholaminergic cells are activated in response to hypertension and hypotension in similar numbers.

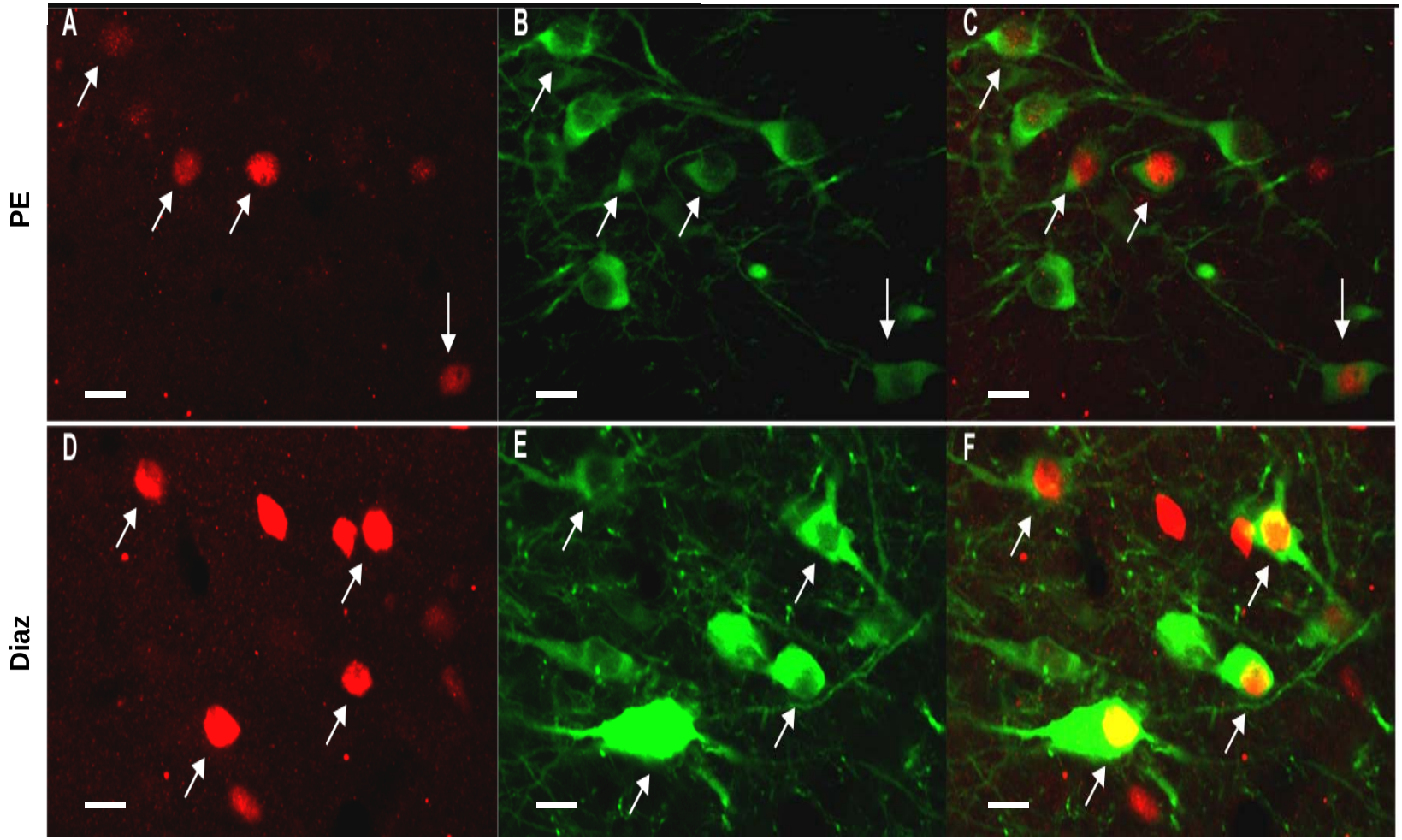

Figure 25: **Images of activated catecholaminergic cells:** Pseudocolored photomicrographs of the NTS after PE (top) and Diaz treatment (bottom). A and D: Fos-IR; B and E: TH-IR; C and F: Merged images. Images are Dorsal and medial to the solitary tract, 180 μm rostral to CS. White arrows denote colabeled cells. Bar is 25 μm.

carotid sinus which then relay excitatory signals to the NTS. NTS neurons in turn excite cardiovascular neurons in the CVLM. These CVLM neurons send an inhibitory projection to the rostral ventrolateral medulla (RVLM). The RVLM is a tonically active region that projects to the spinal cord and controls the sympathetic nervous system. Therefore, increases in arterial pressure inhibit activity of the RVLM, reducing sympathetic activity and arterial pressure (7-9; 31; 36; 98; 138). Based on this model, decreases in arterial pressure would decrease baroreceptor afferent activity, leading to reduced NTS and CVLM activity. This would disinhibit the RVLM, increasing sympathetic nerve activity and arterial pressure. Thus, acute hypertension would result in activation of cells in the NTS, whereas acute hypotension would be expected to result in reduced NTS cell activation (7-9; 31; 36; 98; 138).

Interestingly, this is not the case. Acute hypertension and hypotension both result in activation of NTS cells. In studies in which animals underwent sinoaortic denervation, the number of neurons in the NTS activated by hypertension and hypotension was reduced to near control levels (7-9; 31; 36; 98; 129; 130; 138). This suggests that activation of NTS neurons by hypertension and hypotension may be baroreceptor mediated. However, other reflexes cannot be completely ruled out as the source of activated neurons in the NTS after hypotension because sinoaortic denervation also removes the arterial chemoreflex afferents (3). Stimulation of the arterial chemoreflex, through hypoxia or hypercapnia, results in activated neurons in the commissural and postremal NTS (15). It is possible that activated neurons with hypotension may be the result of multiple afferent inputs. Also, peripheral

administration of an angiotensin II antagonist, sarile, reduces the total number of activated neurons in the NTS in response to hypotension (26). This suggests that a hormonal response may also contribute to the increased activation of the NTS in response to hypotension.

Activation of Neurons in the NTS by Hypertension or Hypotension:

Saline infusion did not alter arterial pressure or heart rate during the 90 minute experimental period. Although saline infusion was associated with a small number of Fos-IR cells in the NTS, activated neurons were randomly distributed within the NTS. No definitive pattern could be detected in any of the immunohistochemical groupings from the five control animals and the number of activated cells was significantly less than that observed in tissue from animals in which arterial pressure was altered. Therefore, activation of NTS neurons in response to hypertension or hypotension most likely was not due to the environment of the experimental procedure or the change in blood volume during drug infusion.

Throughout the four rostral-caudal levels of the NTS, similar numbers of Fos-IR neurons were observed in both PE and Diaz treated animals, and this was significantly greater than following saline treatment. Though there were no significant differences in Fos-IR between PE or Diaz treated animals in individual sections, a trend ($P=0.08$) did exist for Fos-IR in PE treated animals to be higher than the Fos-IR from Diaz treated animals in sections 180 μm caudal to calamus scriptorius (CS) and 180 μm rostral to CS. The distribution of the Fos-IR was similar to previously reported data for PE treated animals (26; 27; 157). Comparison of the

Fos-IR in Diaz treated animals to previously reported distributions in other forms of hypotension, was also similar (25; 35; 58; 122).

There was also a distinct subnuclear separation of the Fos-IR due to the different stimuli. PE treatment resulted in staining in the dorsomedial subnuclear regions and surrounding the solitary tract of the postremal NTS, whereas staining in those regions was absent in the animals treated with Diaz. Further, hypotension produced more Fos-IR in the medial and subpostremal subnuclear regions in the postremal NTS and the medial region of the rostral NTS compared to hypertension. The pattern of Fos-IR in the NTS was similar to that seen previously in response to hypertension (26; 27; 122; 157) or to hypotension (25; 35; 58; 122).

Colabeled Neurons:

Fos-IR and FG Containing Cells:

Labeling of the CVLM projecting neurons, those containing FG, was similar to that previously reported (157). There was no difference in the number of CVLM projecting cells in the NTS in any of the drug treatment groups. There were few colabeled Fos-IR and FG containing cells in the saline treated animals. Increases in arterial pressure resulted in Fos expression in CVLM-projecting NTS neurons, and the percent of colabeling of Fos-IR and FG containing neurons in PE treated animals was similar to levels reported by Weston et al (157). The significant difference between activation of CVLM projecting neurons in response to hypertension and hypotension is striking. The regions of the NTS in which the FG containing cells are located contain relatively few Fos-IR in the Diaz treated animals. With respect to the

generally accepted pathway of the arterial baroreflex, it would be surprising to see any staining in this area in response to hypotension, where the arterial baroreceptors terminate (7-9; 31; 36; 98; 138).

Of interest is the relatively low percent of colabeling of FG and Fos-IR cells that was seen in response to increases in pressure in this study and previously (157). Recent data have suggested that all CVLM projecting cells in the NTS are second order neurons (13), showing constant waveforms, little change in latencies, and few failed synaptic responses to activation of the solitary tract. Bailey et al (8) also described these CVLM projecting neurons as receiving axosomatic contacts from peripheral afferents (by stimulating the solitary tract). There are still questions, however, regarding the number of CVLM projecting cells that may have afferent synapses from the baroreceptors. Further, Zhang and Mifflin (162) have demonstrated that relatively few neurons in NTS demonstrated an excitatory-inhibitory current profile that is common to the second order neurons in the NTS. It is possible that although CVLM projecting neurons are second order neurons, *in vivo* activation of those neurons during increases in arterial pressure is less than *in vitro* activation of neurons due to solitary tract stimuli. Further studies are needed to determine the extent of activation of these neurons.

Fos-IR and GAD67-IR:

GABAergic neurons in the NTS play a pivotal role in modulation of synaptic activity. Microinjection of muscimol, a $GABA_A$ receptor agonist, or baclofen, a $GABA_B$ receptor agonist, bilaterally into the NTS results in an increase in arterial

pressure and sympathetic nerve activity (17; 18; 106; 144) and can reduce the baroreceptor mediated gain of bradycardia (21). Also, blockade of $GABA_A$ or $GABA_B$ receptors augments the excitatory response of NTS neurons during increases of arterial pressure, which would increase the sympathoinhibitory response to arterial baroreflex stimulation or glutamate microinjection (142). Microinjection of the $GABA_A$ receptor antagonist bicuculline into the NTS results in decreases in arterial pressure and sympathetic nerve activity (162). These studies suggest a tonic inhibitory action of GABA on the NTS neurons. It would be expected, then, for GAD67-IR NTS neurons to be activated in response to hypertensive stimuli, not only because of the constant release of GABA (106), but also because GABAergic neurons have been shown to receive baroreceptor afferent information (12). The number of activated GABAergic cells found in the NTS in hypertensive animals was relatively low, in comparison to previously reported levels (27; 108; 157). One possible explanation is that previous studies used *in situ* hybridization to detect mRNA for GAD67 (27; 157), in comparison to our detection of protein. Another possibility could be that during the relatively prolonged change in arterial pressure baroreceptors or central regions of the baroreflex undergo resetting, which could alter the level of neural activation.

Fos-IR and nNOS-IR:

Nitric oxide (NO) is a potent neuromodulator throughout the CNS (41; 81; 82; 147). Within the NTS, NO causes a depressor response (46; 82; 154). nNOS as been shown to be present only in neurons in the NTS (92) and activation of those

neurons and release of NO can lead to increased synaptic activity and Fos production in surrounding neurons (28). It is unknown, however, if hypotension activates nNOS containing cells in the NTS. Treatment with PE or Diaz resulted in similar numbers of nitroxidergic cells activated. However, there was an anatomical separation of that colabeling. In response to increases in pressure, colabeling of Fos-IR and nNOS-IR was present primarily in the dorsomedial subnuclear region, the region that contains arterial baroreceptor afferent terminals. In response to decreases in pressure, Fos-IR and nNOS-IR colabeling was primarily in the medial and subpostremal subnuclear region, in the region of the catecholaminergic cells. This suggests that separate populations of nNOS may be preferentially activated, depending on the stimulus.

Chan and Sawchenko (27) reported that over half of the nNOS-IR cells in the dorsomedial subnuclear region of the postremal NTS (region II in their report) were activated by increases in arterial pressure. Visual examination of that region in the postremal NTS sections in this work suggests similar levels of activation, though the system for determining the distribution was different. Chan and Sawchenko also did not examine labeling in the rostral NTS, which contains considerable nNOS-IR, especially in the central subnuclear region. It is therefore possible that inclusion of this NTS region resulted in an overall reduction in the percent of colabeling in our studies in comparison to others. Though there were nNOS-IR cells that colabeled with FG, there was no triple labeling of Fos-IR and nNOS-IR with FG. This suggests that nNOS in this region may stimulate other neurons in the region, in response to hypertension, but also suggests that hypertension does not result in the release of

NO into the CVLM from the axon terminals of nitroxidergic NTS cells that project to the CVLM.

To our knowledge, the colabeling of nNOS-IR with Fos-IR in response to hypotension has not been reported previously. Colabeled Fos-IR and nNOS-IR cells in Diaz treated animals were located mainly in the medial subpostremal subnuclear regions of the postremal NTS, regions that contain extensive catecholaminergic cell immunoreactivity. There is little to no colabeling of nNOS and TH neurons in the NTS (82; 137). However, nNOS activation and release of NO in this area may increase the activity of the catecholaminergic neurons, similar to the potential role of nNOS neurons on CVLM projecting cells seen in the PE treated animals. As this study did not examine triple labeled Fos-IR, nNOS-IR, and TH-IR, additional work is need to determine if those nNOS-IR cells activated with Diaz treatment are catecholaminergic cells.

Fos-IR and TH-IR:

The catecholamine neurons in the NTS are important output neurons, much like the CVLM projecting neurons. Catecholamine neurons project to multiple medullary and supramedullary nuclear regions, including the paraventricular nucleus of the hypothalamus, and the rostral ventrolateral medulla, nuclear regions that can increase sympathetic nerve activity and increase arterial pressure (7-9; 36; 59; 69; 98; 123; 143). Previous data have suggested that these neurons are activated during periods of hypotension (25; 26; 35; 138). Though previous studies have examined Fos-IR and catecholaminergic cells in PE treated animals, these studies

did not compare the activation of catecholaminergic neurons in response to hypertension in comparison to hypotension .

The distribution of TH-IR cells was similar to that in previous studies (75; 76; 137; 143). The Fos-IR and TH-IR colabeling in Diaz treated animals was considerable, with nearly a third of all catecholaminergic neurons counted colabeled with Fos-IR. These colabeled neurons were located primarily in the medial subnuclear region of the postremal NTS. Of interest is the similar colabeling of Fos-IR and TH-IR in PE treated animals. The level of colabeling in the PE treated animals is in contrast to levels previously reported. Though Chan and Sawchenko (27) did report some colabeling of TH-IR and Fos-IR neurons in the medial subnuclear region of the postremal NTS in response to hypertension, their percent of colabeling was lower than reported here. Examination of that region in the current study suggests that a large portion of the catecholaminergic neurons of the postremal NTS are activated by hypertension. The possibility exists that the prolonged infusion of PE and subsequent arterial pressure elevation may have resulted in the activation of other visceral reflexes, as previous studies maintained the hypertensive response for a shorter period. Of interest is the observation that both increases and decreases in arterial pressure resulted in extensive colabeling in the medial subnuclear region of the postremal NTS. A possibility is that the same catecholaminergic cells are being activated in response to both hypertension and hypotension. It is apparent that further studies are needed to attempt to elucidate this.

Experimental Paradigm Used to Alter Pressure:

Use of PE and Diaz to alter arterial pressure is a well established experimental paradigm, as is the labeling of Fos protein to evaluate neuronal activation. However, there are some differences in our protocol in comparison to previous reports. Some studies used a 25 minutes infusion period for PE (23; 24; 26; 27; 157) and then pressure was allowed to return to control for 75 minutes prior to sacrificing the animals. The current study used a protocol of 90 minutes for the infusion period of PE, sacrificing animals at the completion of that protocol. It is well established that Fos-immunoreactivity (IR) peaks at ninety minutes after the initiation of the stimulus (26; 37-39; 68; 116; 124). One question is related to the use of the 25 minutes time frame for infusions (157), as the Fos-IR found in animals could be due to the increase in arterial pressure, from PE infusion, or the subsequent decrease in pressure with the removal of PE. In our study, PE was infused for ninety minutes and resulted in a prolonged significant increase in arterial pressure and a significant reflex decrease in heart rate throughout the infusion period. Perfusing animals at the completion of the infusion period helped to ensure that any Fos-IR detected was due to the increase in blood pressure from PE infusion. Similar reasoning applies to Fos-IR observed in Diaz treated animals, which received bolus supplemental injections to maintain the significant decrease in arterial pressure and significant reflex increase in heart rate throughout the experimental period.

Summary:

This study presents data suggesting that acute (90 minute) increases or decreases in arterial pressure result in the activation of similar numbers of NTS neurons, though the anatomical distribution of these neurons varies. Hypertension resulted in a greater number of CVLM projection neurons activated in comparison to hypotension. However, activation of GABAergic, nitroxidergic, and catecholaminergic was similar. Regionally, separate populations of nitroxidergic cells were activated in response to increases or decreases in arterial pressure, suggesting a role for NO in enhancing synaptic transmission in the neurons surrounding those cells. Further studies are needed to discern the similarities of catecholaminergic cell activation.

Chapter 4: Expression of Group I mGluRs on Neurons Activated by Hypertension or Hypotension in the NTS:

Background:

The initial studies presented here (Chapter 2) show that Group I mGluRs are expressed on several different neuronal phenotypes within the NTS. In addition, their expression on NTS neurons that project to the CVLM is greater than that on the other phenotypes examined. Furthermore, the data in Chapter 3 indicate that CVLM projecting NTS neurons are activated by increases in arterial pressure, but not by decreases in pressure. Taken together, these data are consistent with the concept that Group I mGluRs contribute to activation of neurons that are involved in baroreflex sympathoinhibitory responses to hypertensive stimuli. A logical extension of these studies is to evaluate the expression of Group I mGluRs on NTS neurons activated in response to increases and decreases in arterial pressure. A proposal to examine this question is presented in the following chapter.

Introduction:

The nucleus of the solitary tract (NTS) is a highly integrative region of the dorsal medulla oblongata that serves as the termination site for multiple visceral afferents and receives projections from multiple regions of the brain (7-9; 31; 36; 98; 138). Afferent signals that are received in this nuclear area are integrated and sent to several parts of the brain important in regulation of the autonomic nervous system

(7-9; 31; 36; 98; 138). There are numerous cell types that can modulate signals within the NTS, including excitatory and inhibitory interneurons, peptidergic neurons and neurons that produce and release nitric oxide (7-9; 87; 150). There are also a large number of transmitters and receptors that are found both pre- and postsynaptically and can modulate synaptic transmission (7-9; 87; 106; 126; 150; 161). Metabotropic glutamate receptors (mGluR) are a class of receptors that play an important role in synaptic transmission. mGluRs are separated by their sequence homology and second messenger systems into three separate groups (10; 32; 117; 118; 128). Group I mGluRs are located primarily postsynaptically and tend to increase cell excitability (52; 54). Also, electrophysiological recordings have shown that mGluRs increase NTS cell excitability *in vitro* (52; 54)) and *in vivo* (136), likely due to activation of Group I mGluRs. Therefore, Group I mGluRs may activate neurons within the NTS that are part of the arterial baroreflex (10; 32; 117; 118; 128). Consistent with this concept, microinjection of the broad spectrum mGluR agonist 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid (ACPD) or the Group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) results in a decrease in arterial pressure (AP) and heart rate (HR), and sympathetic nerve activity (SNA) (48; 49; 104).

Group I mGluRs are expressed in the NTS (63; 72) and are expressed on neurons that project from the NTS to the CVLM (Chapter 2 and 3). It is not known, however, whether those neurons within the NTS that are activated by increases in pressure (23; 24; 26; 27; 58; 107-109; 122; 135; 157), express Group I mGluRs. This study will examine the expression of Group I mGluRs on neurons that have been activated by increases in arterial pressure due to phenylephrine infusion. Also,

the retrograde tracer Fluoro-Gold (FG) will be used to identify those neurons in the NTS that project to the CVLM. Previous studies have also shown that NTS neurons are activated after decreases in arterial pressure (25; 26; 35; 58; 122). A separate set of animals will receive injections of the vasodilator diazoxide to lower arterial pressure along with FG labeling of NTS to CVLM projecting neurons. We hypothesize that an equal number of NTS cells activated by hypertension or hypotension express Group I mGluRs. However, there will be a greater number of cells that are activated, express Group I mGluRs, and project to the CVLM in response to hypertension compared to hypotension.

Methods:

Animals:

Experiments will be performed according to the guidelines in the NIH "Guide for the Care and Use of Laboratory Animals." The University of Missouri Animal Care and Use Committee has approved all procedures and protocols. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, n=17) weighing 275-325 grams will be used. Rats will be housed within an in-house animal facility on a 12 hour day/night cycle. Temperature and humidity are held constant at 72°F and 40%, respectively, and food and water will be available ad libitum. All rats will be given at least seven days to acclimate to the surroundings prior to any experimental procedure.

Instrumentation:

All surgical procedures will be done under aseptic conditions. Animals will be anesthetized with Isoflurane (AErane, Baxter [5% in 100% O_2, 2L per minute, and maintained at 2-2.5%]), and catheters (PE50 fused to PE10) will be inserted through the femoral artery and vein, to the descending aorta and inferior vena cava, respectively. These catheters will be run under the skin and exit through a small incision in the neck. The arterial catheter will be connected to a pressure transducer, which in turn is connected to a DC amplifier (ADInstruments, Colorado). Arterial pressure signals will be evaluated electronically to determine mean arterial pressure (MAP) and HR with the PowerLab Data Acquisition System (ADInstruments), which is connected to a Pentium computer. Rats will then be placed into a Kopf stereotaxic device. The dorsal medullary surface will be exposed and calamus scriptorius (CS) visualized. After determining inter-aural zero, the head will be defected downward until CS is 2.4 mm caudal to inter-aural zero. This will place the brainstem in the horizontal plane.

Microinjection of Retrograde Label:

To determine the location of CVLM, cardiovascular responses to microinjection of the excitatory amino acid L-glutamate (Glu) will be used. A double-barreled glass pipette (OD 20-30 μm) will be filled with Glu in one barrel and FG (2% in dH_20) in the other. The pipette will be placed into the hindbrain at the initial target coordinates of the CVLM (1.8-2.0 mm ventral and 0.2 mm caudal to CS and 2.4 mm ventral to the dorsal surface, with the brainstem in a horizontal position).

Microinjections of Glu (10mM, 30 nL) will be used to functionally locate the CVLM. Criteria will include a depressor response of 20-25 mmHg and less than 40 bpm decrease in HR in response to microinjection of Glu. After the CVLM is localized, FG (15 nL) will be injected into the same site through the other barrel of the pipette. Microinjections of Glu and FG will be made with a custom made pressure injection system and the fluid levels in the pipette will be visualized through a 150x microscope with a calibrated eyepiece micrometer. The microinjected volume will be quantified by observing movement of the meniscus within the pipette. The pipette will remain in the medullary tissue for five minutes to prevent the FG from traveling up the injection tract. After the removal of the pipette, the head, neck and leg areas will be sutured closed and cleaned and the catheters will be sealed closed. All animals will receive post-operative injections of Baytril (0.03 mL) and Buprenex (0.6 mg/mL) i.m. to prevent infection and for pain management, respectively. Animals will be returned to their cages and observed for two hours, then returned to the in-house animal colony.

Drug Treatments:

Seven to ten days prior to the experiment, except for the day of surgery, animals will be trained to sit within an experimental cage in an isolated room in the laboratory for three hours. The experimental cage will contain bedding from that animal's home cage. Five days post-FG injection, the arterial catheter will be connected to the pressure transducer for MAP and HR measurements (see above) and the venous catheter will be connected to a syringe for drug administration. After

a period of 90 minutes for establishment of baseline hemodynamic parameters, animals will receive infusions of saline (0.9%, n=5) or phenylephrine (PE: 15 μg/kg/min i.v., n=6) for 90 minutes, or a bolus injection of Diazoxide (Diaz: 50 μg/kg i.v., n=6) with supplements (12.5 μg/kg) at 30 and 60 minutes. These concentrations will be used to increase (PE) MAP 40-50 mmHg, and decrease (Diaz), MAP 35-45 mmHg.

Transcardial Perfusion:

At the conclusion of the experiment, animals will be euthanized with Beuthanasia (Schering-Plough, Union, NJ, 0.5 ml/kg) and transcardially perfused with Dulbecco's Modified Eagles Medium (Sigma, St Louis, 100 mL) and 4% paraformaldehyde (PFA, Sigma, 500 mL), as previously described (Chapter 2 and 3). Brains will be postfixed in PFA for 18-24 hours. Brainstem sections (30 μm) will be cut on a vibrating microtome in a 1 in 6 series. One series of sections will be mounted onto gel-coated slides and coverslipped with Vectashield (Vector, Burlingame, VT). These sections will be used to verify the midpoint of the FG microinjection in that animal. All other sections will be stored in cryoprotectant at -20°C.

Immunohistochemistry:

Sections will be treated with a modified immunohistochemical protocol (Chapter 2) to visualize Group I mGluRs and Fos containing cells. Preliminary experiments will be done prior to these immunohistochemical experiments to

determine the primary and secondary antibody concentrations that provide the greatest signal to noise ratio. A mouse anti-mGluR 5/1α (Neuromab, Davis, CA) will be visualized with donkey anti-mouse Cy2 (1:200, Jackson, West Grove, PA). This will be paired with a rabbit anti-c-fos (1:3000, Santa Cruz, Santa Cruz, CA), which will be visualized with a donkey anti-rabbit Cy3 (1:200, Jackson). FG fluoresces and will not require antibody treatment.

All rinses will take place within 24 well rinse plates with 0.1M PBS, while antibody treatments will take place with 48 well sterile plates. Sections will be initially rinsed in a 10% pre-blocking solution (10% normal donkey serum [NDS, Jackson] and PBS with 0.3% Triton X-100 [TR-PBS, Sigma, St Louis]) for 30 minutes. After a 30 min rinse, sections will be incubated for 48 hours in a cocktail of the primary antibodies with 3% NDS and TR-PBS. After the primary treatment period, sections will be rinsed for 30 minutes, and then treated with a cocktail of secondary antibody with 3% NDS and TR-PBS for 2 hours. Sections will be rinsed a final time for 30 minutes and then mounted onto gel coated slides. Slides will be coverslipped with Vectashield (Vector) and sealed with clear nail polish.

Antibody Specificity:

With each protocol, primary antibodies will be withheld from single sections for controls. Antibody specificity for Fos (Santa Cruz), has been verified by the vendor. Characteristics of positive-immunoreactivity (-IR) have been shown previously for Fos (23; 27; 157) (Chapter 3), and Group I mGluR (Neuromab, Davis, CA).

Data Acquisition and Analysis:

Sections will be examined with an Olympus microscope (BX51), using a 2-axis motorized stage (Ludl Electronic Products Ltd, Hawthorne, NY). Filter sets for Oregon Green 488 or Cy2 [ex. λ480 nm; em. λ510 nm], Cy3 [ex. λ550 nm; em. λ570 nm], and Fluoro-Gold [ex. λ330 nm; em. λ515 nm] will be used to visualize positive immunoreactivity (Chapters 2 and 3). Images of cells will be captured with a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Images will be analyzed with the Neurolucida (ver. 7.5, MicroBrightField, Willison, VT) software package.

In each set of tissue to be examined, the section that contains CS will be determined, as the section that contains the caudal most area of the area postrema. The NTS, ipsilateral to FG injection site, will be examined in sections 180 μm caudal and 180, 540, and 900 μm rostral to CS. The entire section, NTS, solitary tract, hypoglossal nucleus, and area postrema, if present, will be outlined. After the top and bottom of the section are identified, image stacks 1 μm apart will be taken with each filter set, without movement of the section. Image stacks will be analyzed for positive labeling of Group I mGluRs, Fos, and FG. Positive labeling will require: Fos positive immunoreactivity (IR) filling the entire nucleus of cell; Group I mGluR-IR encompassing the entire soma membrane; FG positive labeling of the soma and possible labeling of projections. In each case, labeling will occur in one focal plane for a cell to be counted. In the case in which multiple positive signals are seen in cells under different filter cubes in the same focal plane, that cell will be counted as double or triple labeled.

Counts of single, double and tripled labeled neurons will be analyzed with the software package NeuroExplorer (ver. 4.5, MicroBrightField) and exported into a spreadsheet (Excel 11.656, Microsoft, Redmond, WA). The data will be analyzed for each individual section and the total of the four sections for each drug treatment (saline, PE, and Diaz) group. Also, the percentage of double-labeled cells relative to the number of each of the single labeled cells will be determined. Percentages will be calculated by dividing the number of colabeled cells by the total number of the single labeled cells. The number of triple labeled cells of each immunohistochemical pairing will be used to calculate the percent of any double-labeled cells that are also triple labeled. The distribution of single, double and triple labeled cells will be examined using NeuroExplorer, with subnuclear regions determined by comparing our sections to a standard atlas (115). All data was graphed using SigmaPlot (8.0, SPSS, Chicago, IL).

Single images of single and any double or triple labeled neurons will be taken with the microscope (see above) with the addition of the DSU spinning disk (Olympus). Images will be imported into Adobe Photoshop (ver. 7.0 Adobe Systems, San Jose, CA) and combined, using only changes to image brightness and contrast for the purpose of improving image clarity.

Statistical Analysis:

All data will be expressed as mean ± SE. Comparisons among drug treatment groups will be done with one way ANOVA, with any post hoc analyses done using a Student-Newman-Keuls post-hoc analysis. Statistical analysis will be

done with Sigma Stat (3.5,SPSS, Chicago, IL) software and significance will be accepted as a $P<0.05$.

Expected Results:

Similar to previous studies, sustained increases or decreases in arterial pressure due to treatment with PE or Diaz, respectively, will result in similar levels of neuronal activation, as indicated by the number of Fos-IR cells, throughout the rostral-caudal extent of the NTS. Saline infusion will result in minimal Fos-immunoreactivity that will occur randomly throughout the NTS. Group I mGluRs are expressed throughout the rostral-caudal extent of the NTS, and within most of the subnuclear regions. With the high number of neurons that are activated in the NTS due to increases or decreases in pressure, we hypothesize that a high number of Group I mGluRs would colabel with neurons activated by both increases or decreases in pressure. There may, however, be a separation on the rostral-caudal and subnuclear distribution of that colabeling. We expect the colabeling of Group I mGluR-IR and Fos-IR in hypertensive animals to peak at the postremal NTS, while Group I mGluR-IR and Fos-IR in hypotensive animals will be greatest in the rostral NTS. In addition, as there would likely be a lower number of colabeled Fos and FG neurons after hypotensive compared to hypertensive stimuli, the number of triple labeled neurons in hypotensive animals would also be low, in comparison to hypertensive animals.

Rationale:

Microinjection of specific Group I mGluR agonists into the NTS results in decreases in MAP, HR (104) and SNA (48; 49), mimicking the effects of Glu microinjection and baroreflex activation. Further, anatomical evidence indicates the expression of Group I mGluRs on neurons that project from the NTS to the CVLM, the next relay in the baroreflex arc, to a greater extent than other cell types examined (Chapter 2). Taken together, the data suggest that neurons activated by increases in blood pressure may also express Group I mGluRs. However, because hypotension also results in similar levels of NTS neuronal activation (25; 26; 35; 122) (Chapter 3) and Group I mGluRs are heterogeneously expressed throughout the NTS (63) (Chapter 2), there could also be colabeling of Fos and Group I mGluRs in Diaz treated animals. The levels of colabeling of Fos and Group I mGluRs may be similar in PE and Diaz treated animals. However, cells that are activated by hypertension or hypotension are located in different subnuclear regions within the NTS (58; 122) (Chapter 3). For example, PE treated animals show extensive staining in the dorsomedial region of the postremal NTS (23; 24; 26; 27; 58; 107-109; 122; 135; 157) (Chapter 3) that is not found in Diaz treated animals (26; 122) (Chapter 3). Therefore, it is likely that the subnuclear regions that contain colabeling of Fos and Group I mGluR may be different.

It has also been shown that increases in arterial pressure activate a greater number of CVLM projecting neurons than decreases in pressure (Chapter 3). Because previous work indicates that few Fos and FG colabeled neurons will likely be present in Diaz treated animals, the number of triple labeled cells presumably

would also be low. The total number of Fos and FG colabeled cells in PE-treated animals and Group I mGluR and FG colabeling is relatively low in comparison to the number of single labeled Fos, FG, or Group I mGluR NTS neurons (157) (Chapter 2 and 3). Nevertheless, the two colabeled pairings can be found in similar subnuclear regions. Group I mGluR expression on CVLM projecting neurons and activation of CVLM projecting neurons in response to hypertension were localized to the dorsomedial subnuclear region of the NTS. It could be presumed that those two colabeled pairs would again be found in the same subnuclear region in the same animal. Further, experiments suggest that activation of Group I mGluRs in the NTS mimics the response of arterial baroreflex activation (104). This collection of data would suggest that there may be a relatively high incidence of triple labeled Group I mGluR, Fos, and FG neurons in response to hypertension. Also, the locations of those triple labeled neurons would be in discrete subnuclear regions, in the postremal NTS surrounding the solitary tract, as this it the area where both the arterial baroreceptor afferents terminate (7-9; 31; 36; 98; 138) and the highest levels of FG labeling are located (157) (Chapter 2 and 3).

Chapter 5: Discussion

The overall goal of this research is to understand the arterial baroreflex and the role of different neurons and different neurotransmitter/receptor systems in the processing of baroreceptor information into and out of the NTS. Specifically, these studies focused on Group I mGluRs, output neurons from the NTS to the caudal ventrolateral medulla (CVLM), and some of the neuronal phenotypes that are activated in response to acute periods of hypertension or hypotension.

The impact of these studies is three fold. Group I mGluRs, which, when activated in the NTS result in sympathoinhibition and a depressor response, are preferentially expressed on NTS neurons that project to the CVLM. Also, a relatively high percentage of CVLM projecting NTS neurons express Group I mGluRs. This suggests that a major effect of Group I mGluRs in the NTS is to directly modulate the excitability of the baroreflex output neurons. Another important finding was that CVLM projecting neurons are activated by increases but not decreases in arterial pressure. Thus, acute hypertension resulted in activation of a separate population of NTS neurons in comparison to neurons activated in response to acute hypotension. There has been substantial confusion in the literature regarding the fact that both increases and decreases in arterial pressure activate NTS neurons but produce directionally opposite reflex responses. The current data provide information that may begin to explain this apparent anomaly. Finally, these studies provide the first evidence that a subpopulation of CVLM projecting NTS neurons is nitroxidergic. Previous studies indicate that CVLM projecting neurons are glutamatergic or GABAergic (157) but little was known concerning the role of nitroxidergic neurons.

Based on our results, it is possible that activation of CVLM projecting NTS neurons not only can release glutamate or γ-aminobutyric acid (GABA) in the CVLM, but may also release nitric oxide, possibly in conjunction with glutamate.

The nucleus of the solitary tract (NTS) is the termination point for multiple visceral and central afferents (7; 9; 36; 59; 84; 98; 138). Information from these afferents is integrated and modulated by a myriad of interneurons, neurotransmitters and neuromodulators, and both pre- and postsynaptic receptors. The net result of this integration is disseminated by output projections to areas of the brain that influence cardiovascular, respiratory, hormonal, and stress related neural activity (7; 9; 36; 59; 84; 98; 138). Understanding the role of neuromodulation within the NTS during normal physiological or pathophysiological conditions is vital to understanding alterations to cardiovascular reflex function during those conditions.

One modulatory class of receptors located in the NTS is the Group I mGluRs. Group I mGluRs are G-protein coupled receptors that generally are postsynaptic and modulate neuronal activity by increasing the potential excitability of a cell (10; 32; 117; 118; 128). Group I mGluRs have been shown to evoke a depressor response and sympathoinhibition when activated in the NTS (104). The phenotype of the neurons that express and may be activated by Group I mGluRs in the NTS is unknown.

GABAergic, nitroxidergic, and catecholaminergic neurons were evaluated in this study because each of these phenotypes has been demonstrated to play a role in modulating synaptic activity or affecting cardiovascular function within the NTS. GABA, the main inhibitory neurotransmitter in the CNS, tonically inhibits

neurotransmitter release through presynaptic activation of $GABA_B$ receptors and reduces neuronal activity postsynaptically through activation of inhibitory $GABA_A$ receptors (18; 106). Nitroxidergic neurons release NO, a gaseous neuromodulator that is produced from L-arginine in response to increases in intracellular calcium. NO activates intracellular second messengers that in turn influence cellular excitability within the NTS (82). Finally, catecholaminergic neurons in the NTS receive input from and project to other medullary and supramedullary nuclear regions, including those that influence the autonomic nervous system (69; 75; 76; 123; 143).

To date, most studies of the NTS have examined inputs to the NTS and neuronal responses to those inputs. An important aspect of the current studies was the focus (using the retrograde label Fluoro-gold) on NTS output neurons that project to the CVLM. Information that is carried on this projection is the integration of afferent information from the arterial baroreceptors and any modulation that occurs within the NTS. Therefore, activity of these neurons represents the ultimate effect of the NTS on baroreflex control of the sympathetic nervous system. Little is known regarding the properties of these neurons, including their phenotypes and the complement of receptors they express. The current studies examined three different cell types known to be involved in cardiovascular control to provide information relative to the phenotype of the CVLM-projecting NTS neurons. We also evaluated whether these neurons are activated in response to increases or decreases in arterial pressure.

These studies were conducted in normal animals and provide important information relative to NTS function. Information obtained from normal animals can then be used to understand variation in animals that have undergone physiological or pathophysiological alterations to cardiovascular function.

Expression of Group I Metabotropic Glutamate Receptors on Phenotypically Different Cells within the Nucleus of the Solitary Tract in the Rat:

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors that are expressed within the CNS and traditionally have been separated into three groups by amino acid sequence homology and second messenger activation (10; 32; 117; 118; 128). The current study focused on Group I mGluRs in the NTS. These receptors are primarily postsynaptic and serve to increase cellular excitability, through second messenger activation of calcium channels (32; 117), the sodium/calcium exchanger (134), and inhibition of potassium channels (10; 32; 117; 118; 128).

Previous data indicate that Group I mGluRs are expressed in the NTS (63). Anatomic and molecular studies have shown the expression of Group I mGluRs in the NTS, but not in the nodose ganglion (72), and therefore presumably not in terminals of the aortic depressor nerve. Further, electrophysiological studies suggest that Group I mGluRs activate neurons that receive baroreceptor input (52-54). Finally, microinjection of Group I mGluR agonists into the NTS mimics the responses to arterial baroreflex activation (48; 49; 104; 153). Together, the evidence

suggests that Group I mGluRs may influence arterial baroreflex regulation of the sympathetic nervous system. There is, however, little information regarding what type of neurons express Group I mGluRs in the NTS. This information is necessary to fully elucidate the functional role of Group I mGluRs in the NTS.

In the current study, immunohistochemistry for Group I mGluRs was paired with markers for GABAergic (GAD67), nitroxidergic (nNOS), and catecholaminergic (TH) neurons individually. This allowed us to determine if these different cell types express Group I mGluRs. The localization of CVLM projecting (157), GABAergic (50; 140; 157), nitroxidergic (92-94; 137), and catecholaminergic (69; 75; 76) neurons was similar to previous reports. In addition, Group I mGluRs were widely expressed throughout the NTS in this study, although the receptors were more concentrated in some subnuclear regions of the NTS in comparison to other subnuclear regions.

Expression of Group I mGluRs on Different Neuronal Phenotypes:

Of the cells examined, CVLM projecting neurons had the highest number of cells that also exhibited Group I mGluR-IR. Because Group I mGluRs tend to be excitatory, this is consistent with previous data showing that microinjection of Group I mGluR specific agonists into the NTS results in a depressor response and sympathoinhibition (48; 49; 104; 153). The percent of the CVLM projecting neurons expressing Group I mGluRs was under twenty percent, suggesting that Group I mGluRs likely do not affect all of the CVLM projecting neurons. It is not surprising that only a portion of the CVLM projecting NTS neurons would express Group I

mGluRs, since current data (Chapter 3) and a recent study (157) showed that only a relatively small number of CVLM projecting neurons were activated by increases in arterial pressure. The current study did not ascertain whether the CVLM projecting NTS neurons that express Group I mGluRs are activated by baroreceptor stimulation. Additional studies utilizing electrophysiological techniques or immunohistochemical labeling of Fos (Chapter 4) will be required to make this determination. Nevertheless, to our knowledge, these are the first data suggesting that Group I mGluRs in the NTS may produce decreases in arterial pressure and sympathetic nerve activity by directly activating output neurons that project to the sympathoinhibitory region of the CVLM.

Cells expressing GAD67, the enzyme that catalyses the conversion of glutamate to GABA and is a marker for GABAergic neurons, exhibited the least amount of colabeling with Group I mGluR-IR. This is consistent with data showing that microinjection of Group I mGluR agonists into the NTS (48; 49; 104) does not produce a pressor response or sympathoexcitation, as would be expected if inhibitory GABAergic neurons were activated. However, it is possible that an effect of Group I mGluRs on GABAergic neurons was masked by an overriding depressor response. Also, as Group I mGluRs have also been shown to inhibit the activity of calcium channels and activate potassium channels in some cases (32; 117), it is possible that Group I mGluRs expressed on GABAergic cells can inhibit their activity in this manner, thereby producing decreases in arterial pressure and sympathetic nervous system activity.

Activation of nitroxidergic neurons likely results in the release of NO, which in turn could influence the activity of that neuron or of surrounding neurons (82; 154). Group I mGluR activation can lead to increases in intracellular calcium, which would stimulate the synthesis and release of NO from a nitroxidergic neuron. Colabeling of Group I mGluR-IR and nNOS-IR occurred in the dorsomedial subnuclear region of the NTS, the primary area of baroreceptor afferent termination. Activation of baroreceptors would result in release of glutamate from afferent terminals and activation of ionotropic glutamate receptors on second order neurons. Activation of Group I mGluRs on these neurons and the subsequent increase in intracellular calcium could further excite the cell. This effect would be slower than a cell activated by ionotropic glutamate receptors, due to the G-protein coupled response of the mGluR. Therefore, we propose that NO production from Group I mGluR activation could result in a greater magnitude or prolongation of the response of baroreflex second order neurons, compared to the response of ionotropic glutamate receptor activation alone.

Tyrosine hydroxylase is the initial and rate limiting enzyme in the catecholamine cascade, converting the amino acid tyrosine into L-DOPA (75; 76) and cells containing TH are considered to by catecholaminergic. Catecholaminergic neurons project from the NTS to multiple nuclear regions that influence cardiovascular activity, including the rostral ventrolateral medulla and the paraventricular nucleus of the hypothalamus (69; 75; 76; 123; 143). The level of colabeling of TH-IR and Group I mGluR-IR was low. This was not surprising,

because Group I mGluRs on these neurons might be anticipated to produce a pressor response and sympathoexcitation (69; 75; 76; 123; 143).

CVLM projecting NTS Cells:

This study also examined the phenotype (GABAergic, nitroxidergic, and catecholaminergic neurons) of CVLM projecting cells. A previous study suggested that a majority of CVLM projecting neurons were glutamatergic, with the remainder of the CVLM projecting neurons being either GABAergic or unidentified (157). The current data suggest that some of the unidentified CVLM projecting neurons may be nitroxidergic. It is possible that these neurons produce nNOS in the NTS, and then the enzyme migrates to the axon terminals within the CVLM where NO is synthesized and released. The NO released can influence neurons located within the CVLM, including the GABAergic neurons involved in the baroreflex. In support of this concept, NO has been shown to excite cells within the CVLM (158), consistent with a sympathoinhibitory response.

In this study colabeling of CVLM projecting and GABAergic cells occurred at a lower level than previously described (157). While the reasons for this discrepancy are not completely clear, it may be due to the variation of methods used to visualize GABAergic neurons. Weston et al (157) used *in situ hybridization* to label GAD67 mRNA, and labeled their Fluoro-Gold with a primary and secondary antibody complex. In contrast, we labeled GAD67 protein, and visualized Fluoro-Gold utilizing its fluorescent characteristics. These differences may account for the smaller percent of CVLM projecting and GABAergic cells that colabeled in this study.

For example, it is possible that some cells express GAD67 mRNA (and therefore would be visualized by Weston et al (157)), but do not synthesize the protein (and therefore would not be visualized in our studies). There also were very few CVLM projecting and catecholaminergic colabeled cells. This is consistent with previous studies, as these two cell types have been suggested to be separate populations that project to anatomically different areas of the brain (75; 76; 123).

The majority of the Group I mGluR expressing neurons were not double labeled with GABAergic, nitroxidergic, or catecholaminergic neurons. It is likely that most of these neurons are glutamatergic, as a majority of the neurons within the NTS are glutamatergic (9; 157). Previous studies indicate that there is little colabeling in the NTS between nitroxidergic and catecholaminergic neurons (82; 137). The colabeling of GABAergic and nitroxidergic neurons and GABAergic and catecholaminergic in the NTS has not been extensively examined. Future studies would be needed to determine if Group I mGluRs are expressed on NTS neurons that are both GABAergic and nitroxidergic or both GABAergic and catecholaminergic, as our experiments did not examine immunohistochemical grouping of Group I mGluRs, GABAergic, and nitroxidergic neurons or Group I mGluRs, GABAergic, and catecholaminergic neurons.

There were few triple labeled neurons found in this study. Nonetheless, an interesting finding was that nearly half of the Group I mGluR expressing cells that projected to the CVLM (therefore, contained FG) were also nitroxidergic.
The proposed action of NO in the CVLM may be related to the triple labeling of Group I mGluRs, nitroxidergic and CVLM projecting cells. For example, activation of

these cells by Group I mGluRs in the NTS could release NO within the CVLM to assist in the activation of cells in that region (158). The relatively high percent of these neurons suggests that this may be an important mechanism by which NO can modulate arterial baroreceptor activity. The very small number of CVLM projecting cells that expressed Group I mGluR and were TH-IR was consistent with previous data that suggest CVLM projecting and catecholaminergic neurons are separate populations of neurons.

Finally, approximately 20% of Group I mGluR expressing GABAergic neurons projected to the CVLM. This number is unexpectedly high in light of the physiological effects of activation of Group I mGluRs. One possible explanation is that Group I mGluRs may have a postsynaptic role in excitation of GABAergic neurons that was not detected in whole animal or electrophysiological studies. However, GABAergic CVLM projecting neurons were not activated in response to hypertension (Chapter 3 and Weston et al (157) or hypotension (Chapter 3)). It is possible that Group I mGluRs inhibit GABAergic neurons that project to the CVLM, by increasing intracellular potassium (by activation of K^+ channels). This could be examined with a preparation similar to that used by Bailey et al (12), in which transgenic mice are engineered to produce green fluorescent protein on GABAergic neurons. This allows for their ready identification in the brainstem slice preparation. By labeling the CVLM projecting neurons in these mice with a retrograde label such as FG, it would be possible to use a single cell recording technique, in horizontal sections, to record from identified GABAergic CVLM projecting neurons. Application of small amounts of Group I mGluR agonists to those GABAergic CVLM projecting

neurons, while recording electrical activity of the cell, would determine if the Group I mGluRs produce an inhibitory or excitatory response in GABAergic CVLM projecting neurons.

In summary, this study indicates that Group I mGluRs are expressed preferentially on CVLM projecting neurons compared to GABAergic, nitroxidergic, or catecholaminergic NTS neurons. Localization of Group I mGluRs on CVLM projecting NTS neurons is consistent with their role in reducing arterial pressure and sympathetic nerve activity. Thus, these results provide an anatomic substrate by which Group I mGluRs may produce their physiological effects within the NTS. Alternatively, Group I mGluRs may modulate a variety of NTS neurons, i.e. interneurons, that could then modulate baroreflex second- or higher order neurons. Also of interest is the number of Group I mGluR expressing cells that colabel with the other cell types examined. Thus, the effect of these receptors may be relatively widespread and they may play a role in other systems within the NTS in addition to the arterial baroreflex.

Increases and Decreases in Arterial Pressure Result in Activation of Phenotypically Different Populations of Neurons in the Nucleus of the Solitary Tract (NTS):

The NTS serves as the termination point for the arterial baroreceptors, and is integral in the regulation of blood pressure on a beat by beat basis (7-9; 31; 36; 98; 138). Increases in arterial pressure activate mechanoreceptors in the walls of the aortic arch and carotid sinus (7; 9; 98; 138). Information from those receptors is

then carried by the aortic depressor nerve and carotid sinus nerve via the vagus and glossopharyngeal nerves, respectively, to the NTS (Figure 1). Within the NTS, afferent information is integrated and ultimately sent to the CVLM (7-9; 31; 36; 98; 138). Neurons that are activated in the NTS due to stimulation of the arterial baroreceptors are identifiable by examining the expression of Fos, a marker of neuronal activation (37-39; 68; 124). Numerous studies have observed Fos expression in the NTS after acute bouts of hypertension due to infusion of the α-adrenergic receptor agonist phenylephrine (PE) (23; 24; 26; 27; 58; 107-109; 122; 135; 157). Paradoxically, acute bouts of hypotension also result in the activation of neurons in the NTS (25; 26; 35; 58), and the levels of Fos-IR in response to hypotension are similar to those due to hypertension (26; 122). The current study examined the activation of GABAergic, nitroxidergic, or catecholaminergic NTS neurons and CVLM projecting neurons in response to both hypertension and hypotension. The goal of this study was to test the hypothesis that hypertension and hypotension activate separate populations of neurons within the NTS.

Animals received Fluoro-Gold microinjections in the CVLM and were administered saline, PE, or the vasodilator diazoxide (Diaz). PE resulted in a sustained increase in arterial pressure accompanied by a reflex decrease in heart rate. Diaz produced a sustained decrease in arterial pressure associated with a reflex increase in heart rate. Saline infusion did not alter arterial pressure or heart rate in comparison to the baseline period. Saline infusion also resulted in few Fos-IR neurons overall, and few CVLM projecting, GABAergic, nitroxidergic, or catecholaminergic neurons were activated in response to saline.

Hypertension and hypotension resulted in a similar overall level of NTS neuronal activation, although variations in the phenotype and distribution of those activated neurons were observed. In response to hypotension, little Fos-IR was observed in the dorsomedial subnuclear region and surrounding the solitary tract in the postremal NTS or within the entirety of the commissural NTS. This is important, as these are primary regions in which the arterial baroreceptors terminate. In addition, elimination of baroreceptor afferent input by sinoaortic denervation (SAD) reduces the levels of Fos-IR to control levels in hypotensive animals (122). These data suggest that NTS neuronal activation during decreases in arterial pressure is due to baroreceptor afferents. However, as the arterial chemoreflex is also eliminated by sinoaortic denervation, it is possible that arterial chemoreceptors may be stimulated during hypotension, resulting in activation of neurons in the NTS (3). It has been suggested that hypotension could result in a reduction of blood flow through the carotid body, resulting in reduced oxygen delivery. This could be interpreted as a hypoxic state, activating chemoreceptors (85; 103; 141). Activation of the arterial chemoreceptors results in sympathoexcitation and pressor responses (100), which are eliminated after SAD (70). Previous studies have shown that hypoxia and hypercapnia both result in activation of NTS neurons. The localization of these neurons is the commissural and caudal postremal NTS, which is an area in which there were low levels of activation in response to hypotension in our study (57; 70; 78; 148). However, other data indicate that hypoxia results in widespread neuronal activation throughout the NTS (20; 71). Therefore, the possibility that stimulation of the arterial chemoreflex during periods of acute hypotension as

described in this study contributed to the observed NTS neuronal activation can not be ruled out. Future studies would be required to eliminate this possibility.

An interesting aspect of this study in comparison to others was the time frame of hemodynamic alteration. Previous studies that imposed both acute bouts of hypertension (23; 24; 26; 27; 157) and hypotension (26; 122) used a time period of 25-60 minutes for the hemodynamic stimulus and the animals were sacrificed 90 minutes after the initiation of stimulus. It has been determined that *c-fos* activation and subsequent Fos expression peaks at 90-120 minutes (37-39; 68; 124). Therefore, it is possible that Fos-IR in the previous studies may be related to the change in arterial pressure either due to drug treatment or to the return of pressure to baseline levels following drug treatment. In the current study changes in pressure were maintained for ninety minutes. Animals were euthanized at the end of the drug treatment, to prevent any neural activation, and therefore Fos expression, due to arterial pressure being returned to baseline levels. Therefore, it is likely that any Fos detected in these animals was due to the induced change in arterial pressure.

An important finding in this study is that CVLM projecting neurons were activated in response to hypertension but not hypotension. As FG in the NTS represents those neurons that project to the arterial baroreflex region of the CVLM, these data are consistent with the current model of baroreflex function (Fig.1). CVLM projecting NTS neurons, activated in response to hypertension, presumably excite neurons in the CVLM (as suggested by the absence of GAD67 in those activated projecting neurons) (Chapter 3) (157). Schreihofer and Guyenet (131) demonstrated that barosensitive CVLM neurons are excited by increases in arterial

pressure. Therefore, non-GABAergic neurons that project to the CVLM should be activated in response to hypertension. Our results are in line with this conclusion, and further, provide new evidence that these neurons are not activated in response to hypotension.

There were a similar number of activated nitroxidergic NTS neurons in response to both hypertensive and hypotensive stimuli. However, the subnuclear regions that exhibited colabeling to these stimuli were different. In response to hypertension, nNOS-IR and Fos-IR colabeling was located in the dorsomedial subnuclear region of the postremal NTS, in the same region as double labeled Fos-IR and FG cells. We suggest that activation of the nitroxidergic cells in this area releases NO, which may subsequently influence the surrounding CVLM projecting neurons. This is supported by microinjection studies that show administration of NO donors into the NTS causes a depressor response and sympathoinhibition, and blockade of NOS activity through microinjection of NOS inhibitors increases arterial pressure and sympathetic nerve activity (60). In response to hypotension, however, the Fos-IR and nNOS-IR colabeling was located in the medial and subpostremal subnuclear regions of the postremal NTS. This area corresponds to a population of catecholaminergic neurons that have been labeled in these studies (Chapters 2 and 3) and described in previous reports (75; 76; 137). As these neurons project to the paraventricular nucleus of the hypothalamus and rostral ventrolateral medulla (69; 75; 76; 123; 143), regions that lead to sympathoexcitation and increased arterial pressure, it is possible that the NO that is released from these nitroxidergic (nNOS-containing) cells during decreased arterial pressure can increase the activity of the

surrounding catecholaminergic (TH containing) cells, contributing to a sympathoexcitatory response.

The number of GABAergic cells that were activated by hypertension and hypotension was similar, and those colabeled cells were found in similar subnuclear regions of the NTS. We initially had hypothesized that GABAergic NTS neurons would be preferentially activated in response to hypotension, thus contributing to the reflex sympathoexcitatory response to hypotensive stimuli. The significance of activation of inhibitory cells within the NTS in response to both increases and decreases in arterial pressure is not clear. However, it may be related to the general importance of inhibition within the central nervous system in terms of shaping neuronal responses (1). Although directionally opposite, both of the responses to these different stimuli would need to be precise in nature so as to appropriately respond to changes in arterial pressure.

The total number of GAD67-IR and Fos-IR colabeled cells observed in response to hypertension was less than previously described (27; 157). The methodology employed for detection of GAD67-containing cells was different in this study and previous experiments, as indicated earlier. Nevertheless, the difference in the percent of GAD67-IR cells that colabeled with Fos-IR was greater than what might be expected due solely to differences in detection techniques. It is possible that the time frame of PE infusion, 90 minutes in this study vs. 25 min in previous studies, may be a critical factor. The increase in arterial pressure coupled with the subsequent return to baseline may result in the activation of neurons not solely due to the increase in pressure. Data from this experiment (chapter 3) have

demonstrated that GABAergic neurons were activated by both increases and decreases in pressure. It is therefore possible that the return to baseline (after 25 minutes of hypertension) in the study by Weston et al (157) could result in activation of GABAergic neurons activated due to both the increase in arterial pressure and the return to baseline. This could explain the discrepancy between the percent colabeling of activated GABAergic neurons described in this study (Chapter 3) and Weston et al (157).

Finally, the number of activated catecholaminergic neurons in both hypertension and hypotension was similar. The catecholaminergic cells of the NTS project to several regions of the brain, including cardiovascular areas that influence arterial pressure and sympathetic nerve activity (69; 75; 76; 123; 143). In response to hypotension, activation of these cells could contribute to the reflex increase in sympathetic nervous system activity. Previous studies have demonstrated that hypotension results in the activation of dopamine-β-hydroxylase (another enzyme in the catecholamine synthetic cascade) containing cells, but the level of activation of these catecholaminergic cells in response to hypotension was less than what was seen in the current study (Chapter 3) (35). It is possible that the variation in activated catecholaminergic neurons is due to the differences in enzymes that were used as to identify those catecholaminergic neurons in this study in comparison to Curtis et al (35). The number of catecholaminergic cells that were activated by hypertension was similar to hypotension, but higher than previously reported (27). Though there were differences in the time frame of PE infusion, this does not explain the activation of neurons that would further increase arterial pressure or sympathetic

activity. As the catecholaminergic neurons also project to areas that control stress, specifically the amygdala, it is possible that the experimental environment may be stressful to the animal. This is unlikely, however, as saline animals expressed significantly lower levels of activated catecholaminergic cells, and animals were trained to sit in the recording cages prior to the drug treatment period. It is possible that the prolonged variation in arterial pressure induced a stress reaction. Examination of Fos in the forebrain would determine if prolonged alteration in pressure activated regions of the brain known to be involved in stress responses. Experiments utilizing baroreceptor denervation would also provide insight into whether the observed effects were stress-related or pressure related.

Interestingly, there were no triple labeled neurons in this study. However, some double labeled neurons were found in close proximity to each other. For example, nitroxidergic neurons activated in response to increases in pressure were located in the same subnuclear region as CVLM projecting neurons activated in response to increases in pressure. In some sections, these two sets of colabeled neurons were adjacent to one another (an activated nitroxidergic neuron “on top of” an activated CVLM projecting neuron). In response to decreases in pressure, activated nitroxidergic neurons were located in the same subnuclear region as catecholaminergic neurons also activated in response to decreases in pressure. In both hypertensive and hypotensive animals, therefore, the proximity of these colabeled neurons to one another suggests that NO (released in response to neuronal activation) may influence the activity of the surrounding CVLM projecting or catecholaminergic neurons, respectively. Thus, NO may contribute to the arterial

baroreflex sympathoinhibitory response (CVLM projecting neurons) during hypertension or to activation of neurons that increase arterial pressure (catecholaminergic) during hypotensive stimuli. There were also Fos-IR cells in both hypertensive and hypotensive animals that did not colabel with any other marker. These may represent glutamatergic interneurons within the NTS or neurons that project to regions of the brain other than the CVLM that were also not GABAergic, nitroxidergic, or catecholaminergic.

This study examined the NTS after acute hypertension and hypotension to determine if separate populations of NTS neurons were activated in response to these stimuli. Results suggest that hypertension activated CVLM projecting neurons and nitroxidergic neurons in the area of the CVLM projecting cells. In contrast, hypotension did not activate CVLM projecting NTS neurons and activated nitroxidergic neurons in the area of the catecholaminergic neurons. Similar levels of GABAergic and catecholaminergic cells were activated by both hypertensive and hypotensive stimuli. The data suggest that hypotension-induced neural activation is focused to catecholaminergic neurons, with possible further activation of those cells through the release of NO. Further studies are needed to determine the phenotype of the remaining Fos-IR cells.

Expression of Group I mGluRs on Neurons Activated by Hypertension or Hypotension in the NTS:

An important question raised by the results of our completed studies is whether Group I mGluRs are expressed on NTS neurons activated in response to

baroreflex stimuli. We thus proposed an experiment (Chapter 4) to address this question and integrate the results of the first study with the results of the second. Our initial experiments told us that Group I mGluRs are preferentially expressed on CVLM projecting NTS neurons, while the second study showed that CVLM projecting cells are activated after periods of hypertension but not in response to hypotension. In addition, previous data have shown that Group I mGluR agonists, when microinjected into the NTS, reduce arterial pressure and sympathetic nerve activity (48; 49; 104). There is, however, no evidence suggesting that Group I mGluRs are expressed on neurons that are activated in response to hypertension. We hypothesize that activated neurons that project to the CVLM also express Group I mGluRs. If so, this would be consistent with experiments reporting depressor and sympathoinhibitory responses to NTS microinjection of Group I mGluR agonists. Also, hypotension results in activation of catecholaminergic neurons (26; 35), which also express Group I mGluRs (Chapter 2). It is vital to determine if Group I mGluRs are expressed on catecholaminergic neurons that are activated by hypotension. This would further determine if Group I mGluRs play a role in the reflex activation of the catecholaminergic NTS neurons. Because of limitations of the current immunohistochemical protocols (only a limited number of markers can be visualized in the same tissue), catecholaminergic cells can not be labeled in the same protocol. However, because the catecholaminergic cells are located in a specific region of the NTS and that region has been well documented (Chapter 2 & 3) (75; 76), any colabeling that occurs with Group I mGluR-IR and Fos-IR in Diaz treated animals could be compared with catecholaminergic neurons from previous studies (75; 76;

137). Experiments could then be designed specifically to evaluate the role of these neurons (for example, by eliminating FG injections from those studies and replacing it with an additional antibody complex such as TH. This would allow for labeling of Group I mGluRs, Fos, and TH). In this case, little would be lost by eliminating FG because there is virtually no colabeling of FG and TH-IR. Further experiments could be designed using an additional filter cube to visualize an additional fluorescent secondary antibody (for example, conjugated to Cy5) to reduce current technical limitations of immunohistochemical protocols. It would be possible then to examine the expression of Group I mGluRs, activated neurons, and catecholaminergic or nitroxidergic neurons, to determine if Group I mGluRs are expressed on catecholaminergic or nitroxidergic neurons activated by increases or decreases in arterial pressure.

Because of the heterogeneous expression of Group I mGluRs throughout the NTS, and the similar number of NTS activated neurons in response to hypertension and hypotension, we expect that there will be similar levels of colabeling of Group I mGluR-IR and Fos-IR in both treatment groups. There will, however, be rostrocaudal and subnuclear variations among colabeled groups, similar to the differences seen with single labeled Fos-IR cells. Also, we anticipate some triple labeling of Group I mGluR-IR, Fos-IR, and CVLM projecting neurons in PE treated animals, while there likely will be little to no triple labeling in Diaz treated animals. This would further confirm that Group I mGluRs have a role in the activation and modulation of synaptic activity of arterial baroreflex neurons in the NTS. This would

demonstrate that Group I mGluRs may be involved in directly influencing arterial baroreflex activity through the NTS projection to the CVLM.

In summary, the main impact of these studies is three fold. The first is that Group I mGluRs are expressed on multiple cell types within the NTS, but exhibit preferential expression on neurons that project to the CVLM, therefore raising the possibility that these receptors are important to arterial baroreflex function. Group I mGluR expression on GABAergic, nitroxidergic, and catecholaminergic neurons suggests that these receptors play a widespread role in the NTS. They may influence baroreflex modulatory neurons within the NTS, or may have a role in affecting neurons other than those that regulate arterial pressure. The second is that though hypertension and hypotension result in generally similar levels of activation in the NTS, there are differences in neuronal activation within the NTS both anatomically and phenotypically. The fact that CVLM projecting neurons are activated in response to hypertension but not hypotension, and the regional difference of nitroxidergic cell activation in response to hypertension or hypotension suggest that acute increases or decreases in arterial pressure activate distinct populations of neurons. This variation in neuronal activation most likely accounts for the different reflex responses. The significance of activation of similar numbers of GABAergic and catecholaminergic neurons is not clear. The similar number of GABAergic cells activated by both stimuli may be due to the importance of inhibition or the extended period of arterial pressure shift, and the similar number of catecholaminergic neurons activated may be due to the same set of neurons being activated. Finally, the third impact will depend on the results of the third study. If the

hypotheses are confirmed, this would suggest that Group I mGluRs may not only play a role in baseline autonomic regulation, but may be integral to arterial baroreflex function. In addition, they may contribute to the activation of neurons in response to hypertension and also to hypotension.

Future Studies:

Though these studies provide new information to better understand the role of Group I mGluRs in the NTS, and what populations of neurons are activated in response to hypertension and hypotension, many questions remain. In the following paragraphs I will propose experiments that will clarify remaining questions from the studies above, and pose new questions in relation to what I have learned.

One area of interest from the second study is the similar number of catecholaminergic cells that are activated in response to hypertension and hypotension. As Appleyard et al (11) have described, catecholaminergic neurons in the NTS receive monosynaptic connections from visceral afferents, which can be stimulated through the solitary tract. It is possible that during hypertension and hypotension individual cells may be activated by both stimuli. To determine this, animals will be instrumented similarly to those described in the methods of Chapter 3. Rats will be treated with PE or Diaz to increase or decrease arterial pressure, respectively, or saline as a control as described in Chapter 3. In these experiments, animals will be returned to their home cages after the treatment period. On the next day, animals will receive a second treatment with saline, PE, or Diaz, but not the same drug as the first treatment period. After this second treatment period, animals

will be sacrificed. Fos-IR will be identified, as will the immunoreactivity of Fos-B. Fos-B is similar to Fos, but while Fos expression peaks between 90 minutes to 2 hours, and dissipates after 4 hours, Fos-B does not reach its peak expression until 6-8 hours, and remains high until 48 hours after application of a stimulus (116). Therefore, Fos-B would label any neurons in the NTS that were activated in response to changes of arterial pressure on the first day, and Fos would label any neurons that are activated with the drug treatment on the second day. Immunohistochemical examination of the immunoreactivity of these two proteins, along with catecholaminergic neurons, could provide answers to the question if the same catecholaminergic cell is activated in response to increases and decreases in pressure. A caveat to the proposed study is that with the change of pressure on the first day, the return of pressure to control levels may also result in activation of neurons. Preliminary studies would be done to determine if the return to baseline pressure levels activated neurons in the NTS. Two separate groups of animals for each treatment would be instrumented for drug infusion and recording of arterial pressure (similar to experiments in Chapter 3). Individual animals would be infused with PE or Diaz for 90 minutes. At the conclusion of the infusion period, animals would be either sacrificed immediately or after a period of 24 hours. Fos numbers in the NTS would be compared after 90 minutes or 24 hours, to determine if a greater number of Fos-B neurons were present in comparison to Fos. A greater number of Fos-B neurons could suggest that the return to baseline resulted in additional activation of NTS neurons. An additional preliminary experiment would be to expose the same animal on consecutive days to the same treatment (either hypertension or

hypotension), and then immunohistochemically treat sections for Fos and Fos-B. Neurons that were not colabeled for Fos and Fos-B and were labeled with Fos-B only may again suggest that neurons are activated as a result of arterial pressure returning to baseline levels. A high percentage of colabeled neurons would suggest that the return to baseline pressure on the first day does not affect the expression of Fos B evaluated the second day.

Additionally, questions remain in regard to Group I mGluRs and neurons that project to the CVLM. Studies presented here have demonstrated that Group I mGluRs are expressed on CVLM projecting neurons, and the final study will determine if Group I mGluRs are expressed on CVLM projecting neurons that are activated by hypertension. It remains to be seen if CVLM projecting neurons in the NTS can be activated by Group I mGluRs. Though it has been well established that activation of Group I mGluRs in the NTS produces reductions in arterial pressure and sympathetic activity (48; 49; 104), no studies have examined the effect of Group I mGluR specifically on efferent neurons. One important aspect of this project was examining the output neurons from the NTS to the CVLM, because that projection is the culmination of afferent input as well as integration and modulation within the NTS. This proposed experiment would use the whole cell patch clamp technique in a horizontal medullary slice. CVLM projecting neurons in the NTS would be identified with latex fluorescent beads (preliminary studies have shown difficulty recording from neurons labeled with Fluoro-Gold). CVLM projecting neurons that responded to stimulating the solitary tract, which contains the baroreceptor afferents (73), would be tested. The specific Group I mGluR agonist 3,5-

dihydroxyphenylglycine (DHPG) would be picospritzed onto the cell, and any variation in cellular activity would be noted. Picospritzing of DHPG would insure a discrete application to the cell that is being recorded. This method would allow us to determine if Group I mGluRs are capable of activating CVLM projecting neurons within the NTS.

Finally, the arterial baroreflex is a highly plastic reflex, in which the effectiveness of the reflex can be altered due to acute or chronic physiological or pathophysiological conditions (Fig. 2). One possible alteration of the baroreflex is a loss in gain, e.g. a reduction in the reflex change in sympathetic output due to alterations in arterial pressure. This reduction in gain is seen in many situations, including cardiovascular deconditioning (62). Cardiovascular deconditioning is a result of prolonged bed rest or periods of microgravity (62), and alterations of the central component of the arterial baroreflex have been proposed as a cause for this reduction in gain. As mGluRs have a role in modulation of synaptic function (10; 32; 48; 49; 104; 117; 118; 128) and alterations of mGluR expression and activity have been found in several central nervous system diseases (101; 128), alterations in mGluR expression may be involved in loss of arterial baroreflex gain. An initial experiment to examine this phenomenon would be to determine if there is any alteration in the mRNA and/or protein levels in the nodose ganglia, NTS, CVLM, and RVLM after cardiovascular deconditioning. To achieve a state of cardiovascular deconditioning, the rat 14 day hind limb unloaded model will be used. Punches of brainstem regions from hind limb unloaded and control rats will be used to measure mRNA and total protein levels. The nodose ganglia will be taken as well as an index

of the presynaptic receptors on baroreceptor afferent terminals (72). This experiment would effectively describe any alteration in mGluR mRNA or protein levels in cardiovascularly relevant regions of the brainstem due to cardiovascular deconditioning, and serve as a stepping stone for further studies examining the role of mGluRs in the reduction of arterial baroreflex gain.

Significance:

The metabotropic glutamate receptors have been shown to play a role in regulation of synaptic transmission, either through increasing or decreasing synaptic activity. It is not surprising that a variation in expression, or the lack of expression, of an mGluR subtype is considered a contributing factor to certain neurological disorders. Group I mGluRs have been shown to be vital in preventing excitotoxic cell death, and reduced Group I mGluR activity has been shown in amyotrophic lateral sclerosis (ALS; Lou Gehrig's Disease), Huntington's disease, and Parkinson's disease (101; 128). Alterations in Group II mGluR function may be an underlying cause of epilepsy and increases in psychotic episodes, while variations in Group III mGluR expression patterns are found in inflammatory disorders, such as multiple sclerosis (101; 128). Finally, Group I and II mGluRs have a role in the development of Alzheimer's disease (101; 128). It is possible that other neural regions that express mGluRs may experience similar debilitating effects if the expression or function of mGluRs is altered.

Changes in synaptic transmission due to mGluRs in the NTS could contribute to alterations of the arterial baroreflex that occur in response to acute (e.g. exercise,

stress) or chronic (e.g. pregnancy, cardiovascular deconditioning, and water deprivation) physiological or pathophysiological conditions. Previous experiments in our laboratory and others have demonstrated that activation of Group I mGluRs in the NTS results in a decrease in arterial pressure and sympathetic nerve activity, similar to activation of the baroreflex (48; 49; 104; 153). The NTS cells involved and mechanisms mediating these responses have not been fully delineated. Figure 26 is a schematic diagram depicting expression of Group I mGluRs on NTS neurons examined in the current study. Our results indicate that Group I mGluRs are expressed on all NTS neuronal phenotypes examined including neurons that project to the CVLM, nitroxidergic neurons, which synthesize nitric oxide (NO), GABAergic neurons, which produce the inhibitory neurotransmitter GABA, and catecholaminergic neurons, which influence the activity of other nuclear regions in the medulla and forebrain. The major region of the NTS receiving baroreceptor afferents overlaps the region of CVLM projecting neurons (dotted line). Within this region Group I mGluRs were expressed mainly on CVLM projecting and nitroxidergic neurons. Expression of these receptors on CVLM projecting neurons, as detailed above, could contribute to the depressor and sympathoinhibitory responses to microinjection of Group I mGluR agonists into the NTS (48; 49; 104; 153). Furthermore, these data suggest that Group I mGluRs may have a role in arterial baroreflex function, possibly contributing to baroreflex mediated sympathoinhibition. Group I mGluR expression was also observed to a relatively high extent on NTS neurons containing nNOS. We propose that activation of Group I mGluRs on nitroxidergic neurons in the NTS induces release of NO which then influences

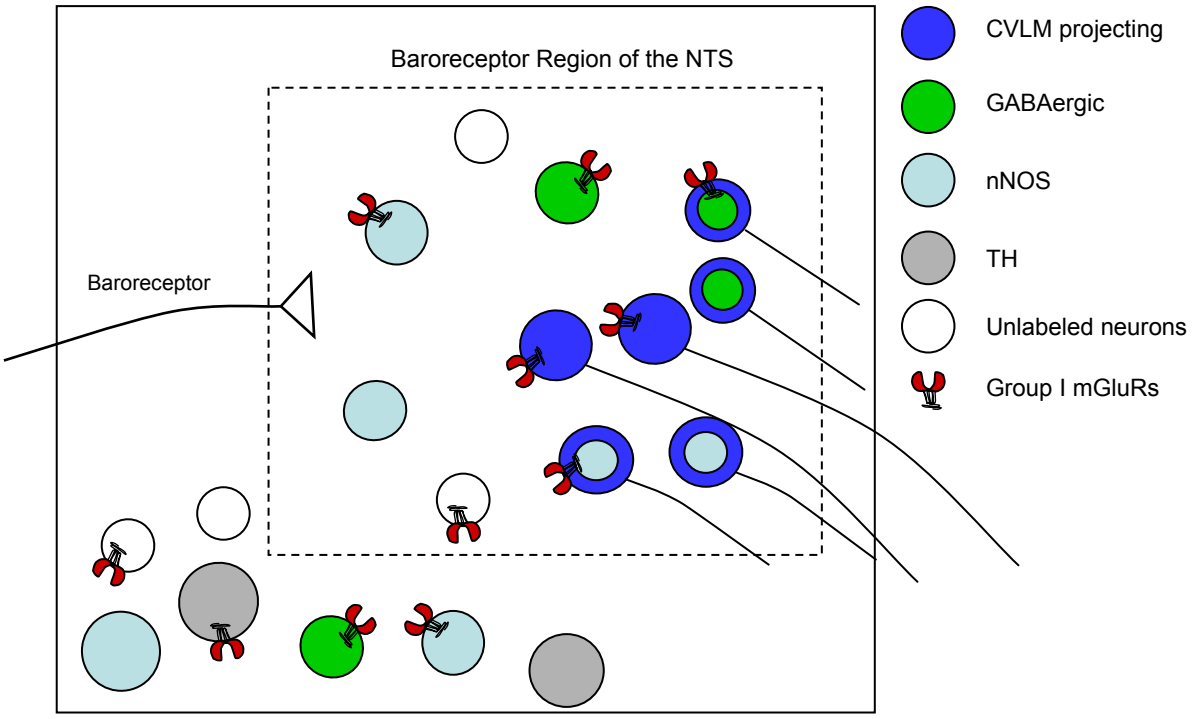

Figure 26: **Expression of Group I mGluRs on neurons in the NTS**. A model depicting the proposed expression of Group I mGluRs within the NTS (solid box). Group I mGluRs were expressed on all phenotypes of neurons examined, with additional expression on unlabeled cells. Group I mGluRs were expressed on neurons that projected to the CVLM, including GABAergic and nitroxidergic neurons that and were located in the region of arterial baroreceptor termination (dashed line). Note that the number or percentage of different neuronal phenotypes is not reflected in the model.

surrounding neurons. For example, activation of nitroxidergic neurons in the vicinity of CVLM projecting neurons could enhance the activation of those CVLM projecting neurons (Fig. 27), resulting in a reduction in arterial pressure. Such a mechanism could also contribute to the depressor and sympathoinhibitory effects of microinjection of Group I mGluR agonists into the NTS. In addition, these data provide the anatomic substrate by which Group I mGluRs may influence arterial baroreflex activity through release of NO, either within the NTS or possibly by activating nitroxidergic neurons that project to the CVLM.

Expression of Group I mGluRs on GABAergic and catecholaminergic neurons also was observed, although to a relatively lesser extent. The significance of expression on these cells is less apparent. As discussed earlier, activation of $GABA_A$ or $GABA_B$ receptors in the NTS results in increases in arterial pressure and sympathoexcitation, while inhibition of those receptors can result in decreases in arterial pressure and sympathoinhibition. Also, catecholaminergic neurons have been shown to project to regions that cause pressor responses and sympathoexcitation (69; 75; 76; 123; 143). One possible role of Group I mGluRs expressed on non-CVLM projecting GABAergic neurons may be involved in a negative feedback loop, to prevent over excitation of neurons in the NTS. Alternatively, depending on downstream cellular mechanisms, Group I mGluRs on GABAergic and catecholaminergic neurons may actually inhibit those neurons, through activation of potassium channels or inhibition of calcium channels (10; 32; 117; 118; 128). Of course, it is possible that Group I mGluRs that are expressed on

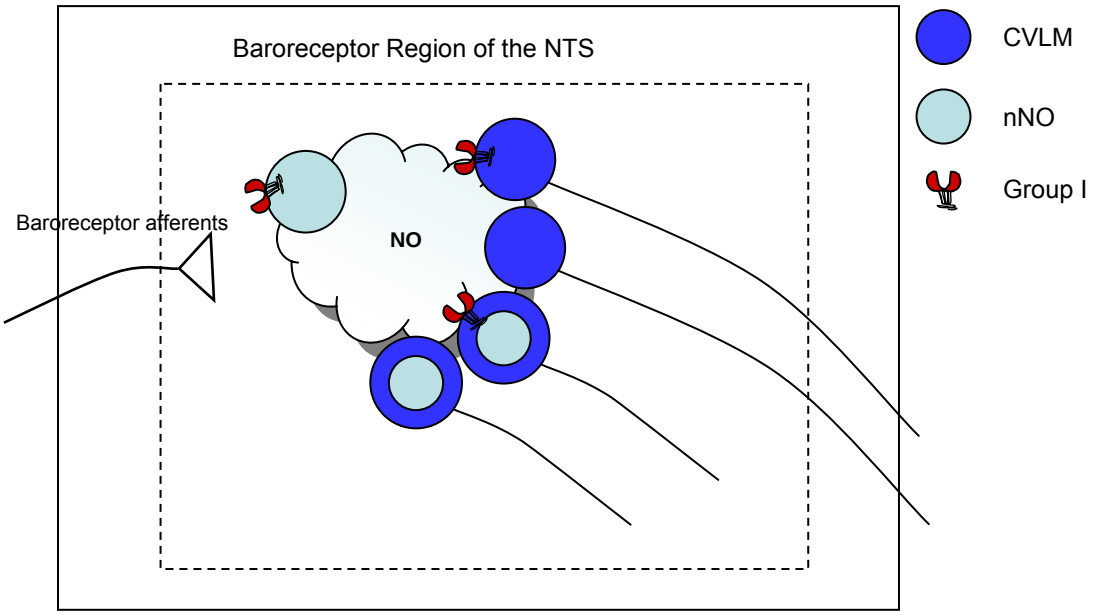

Figure 27: **Possible role of Group I mGluRs in arterial baroreflex function**. A model depicting the proposed action of Group I mGluRs on arterial baroreflex function in the NTS in the area of the arterial baroreceptor termination and CVLM projecting neurons (dashed line). Glutamate from baroreceptor afferents (or possibly other sources) activates Group I mGluRs on CVLM projecting neurons, which results in reduced arterial pressure and sympathetic nerve activity. Additionally, Group I mGluRs on nitroxidergic neurons could be activated. This induces the release of NO (cloud), which could activate the CVLM projecting neurons that do or do not express Group I mGluRs.

GABAergic and catecholaminergic neurons in the NTS are not related to autonomic regulation and serve another function in the NTS.

Previous reports have shown that it is possible to record from NTS neurons that are GABAergic (12) or catecholaminergic (11), identified by green fluorescent protein, and record from neurons that are retrogradely labeled from the CVLM (13). Further experiments could evaluate the function of Group I mGluR expression on identified GABAergic, catecholaminergic, and CVLM projecting neurons. Application of small amounts of Group I mGluR agonists to these neurons, in concert with electrophysiological recordings, would provide important information regarding the role of Group I mGluRs on these specific phenotypes of neurons.

The current data provide the anatomic substrate by which Group I mGluRs could influence baseline autonomic regulation and contribute to normal baroreflex function. Whether changes in Group I mGluR function play a role in altered arterial baroreflex states is not known. For example, it is possible that under certain circumstances, expression of Group I mGluRs could be reduced. This could decrease the excitation of CVLM projecting neurons from the NTS during increases in arterial pressure. Because these neurons are involved in baroreflex function, inhibitory tone from the CVLM to the RVLM would be reduced, maintaining sympathetic nerve activity at an increased level. Additionally, as Group I mGluRs could act to prolong the excitatory signal of the baroreflex, they also may be involved in resetting of the arterial baroreflex.

An interesting question regarding the role of Group I mGluRs is the exact time frame in which these receptors are activated. Specifically, when glutamate is

released into the NTS in response to an increase in arterial pressure, are Group I mGluR activated immediately, or do the Group I mGluRs require accumulation of released neurotransmitter in order to be activated (in comparison to ionotropic glutamate receptors), resulting in a delayed response? The location of receptor could be the cause for a delayed response, as the receptor can be expressed outside the synaptic cleft (10; 32; 117; 118; 128). This also could be due to Group I mGluRs being expressed on interneurons, which could then activate CVLM projecting neurons. Another question is related to the source of glutamate that activates the Group I mGluRs. For example, is glutamate released from the baroreceptor afferent terminals, from interneurons that themselves serve as the second order neurons, or from afferents from other brain regions? The answers to these questions await further experiments.

A novel aspect of these studies was the examination of CVLM projecting neurons and the phenotypes of those cells. As alluded to above, the projection from the NTS to the CVLM is critical because the information that is carried on this projection is the culmination of afferent input into the NTS and any modulation that occurs within the NTS. The overwhelming majority of CVLM projecting neurons have been found to be glutamatergic, with the remaining CVLM projecting neurons being GABAergic or unknown (157). The current study (Chapter 2) examined an additional phenotype, nitroxidergic neurons. We discovered that a percentage of the CVLM projecting neurons were nitroxidergic. This observation suggests that NTS neurons that produce NO could possibly release it from their terminals in the CVLM. It is questionable if these nitroxidergic neurons participate in arterial baroreflex

responses, since data from Chapter 3 showed a lack of nitroxidergic CVLM projecting neurons that were activated in response to increases in pressure. However, there were several activated nitroxidergic neurons in the same area as activated CVLM projecting neurons (Fig. 27). It is possible then that the main role of nitroxidergic neurons in the NTS within the baroreflex is to produce and release NO within the NTS itself, activating surrounding neurons. Expression of Group I mGluRs on nitroxidergic neurons may contribute to a role for those neurons in long term arterial baroreflex function. As NO release in the NTS results in a depressor response and sympathoinhibition (60), baroreflex resetting may be associated with a reduction in nNOS efficiency or activity.

Arterial baroreflex function is a pivotal foundation of homeostasis. Numerous studies have examined the pathways involved in the baroreflex, and the activation of specific brain regions in response to increases in arterial pressure or simulated arterial baroreflex activation (by direct stimulation of the aortic depressor nerve; (162). These studies have shown that activation of the NTS in response to hypertension is subregion specific within the NTS (23; 24; 26; 27; 58; 107-109; 122; 135; 157). Studies have also examined the role of the NTS in response to hypotension (25; 26; 35; 58). Contrary to what would be expected from the commonly accepted model of arterial baroreflex function (Fig. 1), reduced arterial pressure also results in activation of the NTS. Previous studies have examined primarily the phenotypes of neurons activated in response to hypertension (27; 157). The current study (Chapter 3) is the first direct examination of NTS neuronal phenotypes activated in response to both increases and decreases in arterial

pressure in the same experimental paradigm. Data presented in chapter 3 are illustrated in figures 28 and 29. NTS (solid box) neurons are activated in response to hypertension (Fig. 28). CVLM projecting and nitroxidergic neurons activated in response to hypertension were localized to the primary region of baroreceptor afferent termination (dotted line), while activated catecholaminergic neurons were localized to other regions of the NTS. GABAergic and unlabeled neurons activated in response to hypertension were present in both areas. Figure 29 details the response to hypotension in the NTS (solid box). Two major differences between the effect of hypertension and hypotension were observed (Figs 28 and 29). These include the difference in phenotypes of activated neurons and the location of activated neurons. Hypotension does not activate neurons that project to the CVLM (Fig 29) while these neurons were activated by increases in pressure (Fig 28). This suggests that neurons activated in the NTS in response to hypotension are not part of the traditional central arterial baroreflex pathway, though they may receive information from the fibers of the solitary tract. It is possible that neurons activated in response to hypotension are part of an unknown pathway involved with the arterial baroreflex. For example, it has been shown that there is a tonic GABAergic tone in the NTS (106; 162). This GABAergic inhibition may be the result of baroreceptor afferent input. If so, the tonic inhibitory activity of NTS GABAergic neurons could cease during periods of hypotension, resulting in the disinhibition of higher order neurons. These higher order neurons may project to regions of the brain that contribute to reflex sympathoexcitation in response to decreases in arterial pressure.

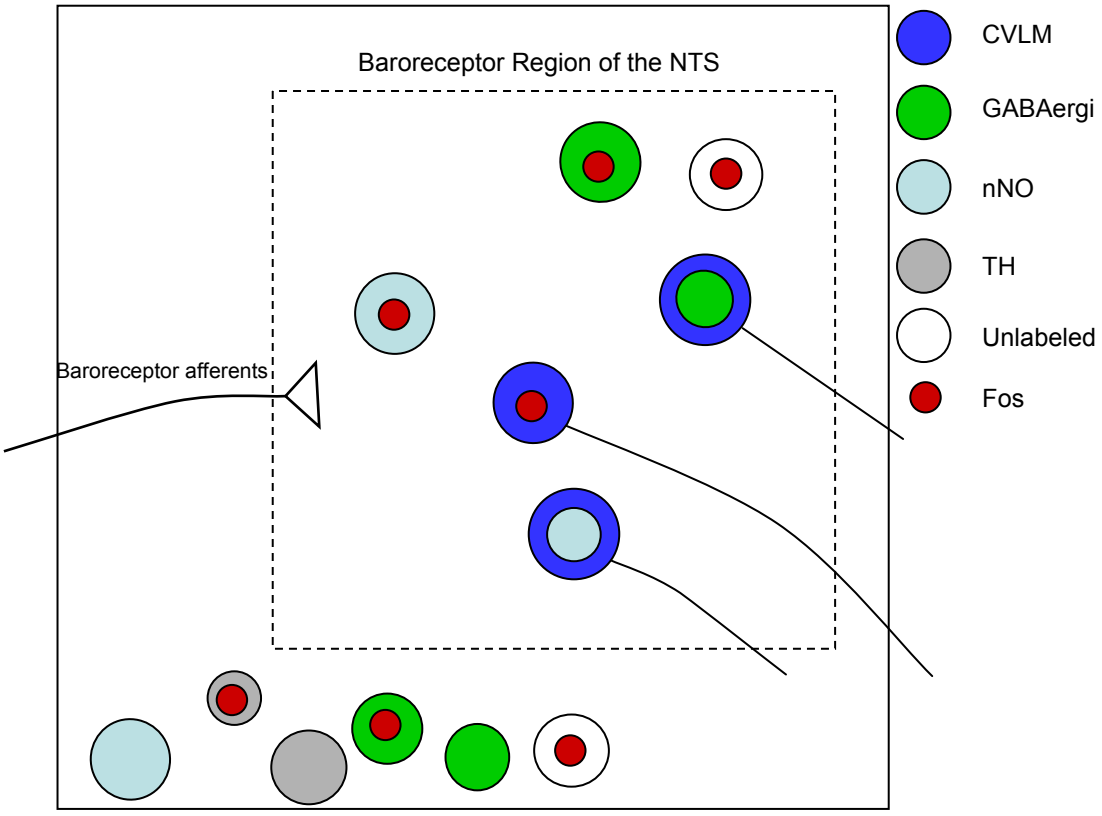

Figure 28: **Activation of NTS neurons in response to hypertension**. A model depicting the proposed activation of NTS (solid line) neurons in response to increases in arterial pressure. Hypertension resulted in activation of all phenotypes examined and unlabeled cells. However, activation of specific populations of labeled neurons was differentially localized. In the region of arterial baroreceptor termination (dashed line), CVLM projecting cells were activated as were nitroxidergic neurons. Activated catecholaminergic neurons were located outside the primary region of arterial baroreceptor termination, and GABAergic neurons were activated in both the region of baroreceptor termination and outside it. Nitroxidergic and GABAergic neurons that projected to the CVLM were not activated in response to increases in pressure.

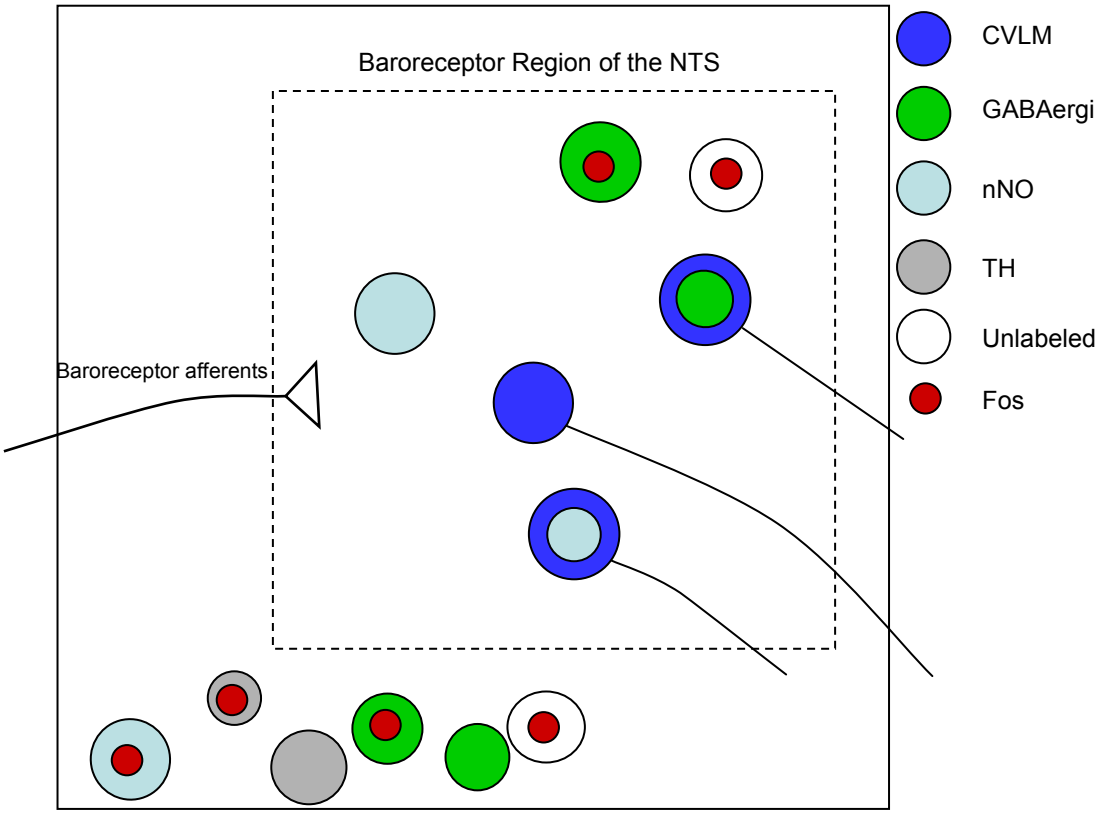

Figure 29: **Activation of NTS neurons in response to hypotension**. A model depicting the proposed activation of NTS (solid line) neurons in response to decreases in arterial pressure. Hypotension activated all phenotypes of neurons examined, as well as unlabeled cells. Activated neurons were localized primarily outside of the region of arterial baroreceptor termination, except for some activated GABAergic and unlabeled neurons. Activated catecholaminergic, nitroxidergic, GABAergic, and unlabeled neurons were located in other regions. There were no triple labeled neurons activated in response to hypotension.

This raises the possibility that additional central pathways of the sympathetic arm of the arterial baroreflex exist, beyond what was described in figure 1.

Nitroxidergic neurons activated in response to decreases in pressure were located in regions outside of the area of baroreceptor afferent termination (Fig. 29, dashed line). In contrast, in hypertension activated nitroxidergic neurons were in the primary region of baroreceptor termination (Fig. 28). This suggests different roles for nitroxidergic neurons with each experimental procedure.

It is possible that other reflexes are activated in response to hypotension, stimulating neurons in the NTS. One in particular is the arterial chemoreflex. Arterial chemoreflex fibers are intermixed with arterial baroreflex fibers (3), and removal of these fibers (sinoaortic denervation) prevents neuronal activation in response to both hypertension and hypotension (70). Thus, it is possible that at least some of the Fos observed in the NTS in response to hypotension is due to chemoreceptor activation. Future studies will be needed to be performed to address this possibility. Blocking chemoreceptor input to the NTS, by denervating the carotid body prior to hypotension, would remove any neurons that are activated by chemoreceptors while maintaining the arterial baroreflex input.

Overall, these studies provide novel information on the possible role of Group I mGluRs in the NTS, the phenotype of neurons that project from the NTS to the CVLM, and the phenotypes of neurons that are activated in response to hypertension or hypotension. As with most studies, the questions that are answered are outnumbered by the new questions that arise. The role of Group I mGluRs most likely extends beyond that of purely involvement in arterial baroreflex function. How

Group I mGluRs modulate GABAergic and catecholaminergic neurons in the NTS may provide clues to a role in modulation of other reflexes. Also, examination of neurons activated in response to hypotension did not provide a complete picture of why there is activation of NTS neurons under these circumstances. Further studies are needed to determine the role of activated GABAergic and catecholaminergic neurons in response to hypotension and why there are similar levels of activation in response to hypertension in comparison to hypotension. These questions require techniques beyond immunohistochemistry, necessitating electrophysiological and molecular biological approaches to tease out those answers.

Technical Considerations:

As in all studies, there are certain aspects of these experiments that must be considered when interpreting the data. In this section, those aspects will be addressed. Initially I will discuss the microinjection technique used for locating the CVLM and subsequent microinjection of Fluoro-Gold. Application of drugs into neuronal tissue commonly is achieved through iontophoresis or pressure injection. Iontophoresis uses application of electrical current to inject charged molecules into tissue. This has the advantage of avoiding pressure or volume artifacts, but the actual amount or dose of the substance administered is not known. In contrast, pressure injection allows for exact measurements of injected volume and therefore the actual dose of a substance injected. For this reason we used pressure microinjection in our experiments. By injecting very small volumes (15-30 nL in the current studies), with measured pressure pulses, pressure artifacts are avoided.

To functionally identify the CVLM, by microinjection of glutamate, specific criteria were adopted. The goal was to verify that the pipette tip was located in the depressor region of CVLM by observation of an adequate depressor response. We also wanted to verify that the pipette was not in the nucleus ambiguus (which controls parasympathetic activity to the heart and is located in close proximity to the CVLM) by limiting the bradycardic response that was considered acceptable. Therefore, in these studies, we used a cutoff in the reduction of arterial pressure (greater than -20 mmHg) and heart rate (less than -40 bpm). We recognize that eliminating depressor responses of slightly less than -20 mmHg may result in the possible loss of data by excluding animals that may actually have had an injection in the CVLM, but simply exhibited smaller responses. Nevertheless, we felt that identification of the correct region of the CVLM was critical to this study and chose to risk losing data in order to enhance the likelihood of correct placement of the Fluoro-Gold injection.

Immunohistochemical approaches have been widely used and accepted as a valid means for identification of specific protein markers. One limitation of immunohistochemistry is that it is difficult to evaluate possible variations of expression of those markers when comparing different biological states. Nevertheless, immunohistochemistry has many advantages that made it idea for these studies. In contrast to molecular biology procedures (such as RT-PCR or Western blots from tissue punches), immunohistochemistry can detail the presence of markers in defined subnuclear regions. It also is possible to evaluate the relative location of one type of marker or cell with respect to others (e.g. nitroxidergic

neurons in close proximity to CVLM projecting neurons). Furthermore, it is possible to determine if the same cell contains two or more different proteins. This information can provide important clues as to the function of these cells.

When using immunohistochemical approaches, it is critical to employ great care when counting cells. In these studies, positively labeled neurons were counted under strict guidelines, to prevent over counting (or false positives). Because of the different nature of the protein markers examined, different staining patterns are expected. Fos protein is localized to the nucleus, so positive staining would require the entirety of the nucleus to be labeled, with a solid, round-like pattern. Conversely, GAD67, nNOS, and TH are all enzymes localized to the cytosol. Therefore, staining would encompass the soma with a blank area, indicating the unstained nucleus. Finally, membrane bound receptors such as Group I mGluRs would present as rings of positive labeling, with a mostly clear middle region (some staining of internalized receptors is to be expected). Colabeling of neurons can be used to determine if two separate protein markers are found in the same individual neuron. Using specific criteria for determining colabeling is also essential, again to prevent false positives. Additionally, in the case of animals from Chapter 3, counts were made blindly, with the counter unaware as to the drug (PE, Diaz, or saline) given to a particular animal until counting was completed.

In these studies, colabeling of Group I mGluRs with other markers (Chapter 2) provided insight into the possible role of Group I mGluRs in the NTS by determining which neurons expressed Group I mGluRs. Colabeling of activated neurons (Chapter 3) allowed for examination of the population of neurons activated

in response to hypertension or hypotension. Finally, colabeling of CVLM projection neurons (Chapter 2) offers suggestions on which cells project, and how information in the NTS is conveyed to the CVLM. These studies used immunohistochemistry to evaluate specific populations of neurons in concert with the examination of the anatomic expression of those populations. It would not be possible to ascertain this level of resolution from tissue punches containing heterogeneous population of cells, as would be used for RT-PCR or Western blot analysis.

In chapter 3, the populations of neurons activated in response to increases or decreases in pressure were examined. The advantages and disadvantages of the use of Fos immunohistochemistry to visualize those activated neurons were outlined in Chapter 1 ("Fos as a Marker of Neuronal Activation"). In these studies (Chapter 3), animals were subjected to the alteration of pressure for ninety minutes, and then euthanized. This paradigm is different from most previous studies that increased pressure for a short amount of time (25 minutes, the minimum time needed for Fos development), and then allowed arterial pressure to return to baseline for a period of time before the animal was euthanized. We believe that our approach is an improvement over the shorter protocols used to increase pressure, as the subsequent return to baseline (decreases in pressure) would allow possible neuronal activation in response to both increases in pressure (through PE infusion) and decreases in pressure (the return to baseline levels). By sustaining the change in pressure through the entire experimental period, in concert with a controlled environment, any neurons that are activated (and subsequently visualized through immunohistochemical labeling for Fos) in the brain are likely activated due to the

increase or decrease in pressure. We believe that this approach of avoiding "off responses" should be considered in any experiments utilizing immunohistochemical labeling for Fos.

An important point to be considered in this and other studies is the classification of receptor subtypes and certain synthetic enzymes. For example, metabotropic glutamate receptors are typically divided into three groups, by amino acid sequence homology and intracellular second messenger activity. Of interest is the fact that Group II and Group III mGluRs share several similar characteristics. Both groups inhibit neurons through the same intracellular mechanisms (negatively coupled to adenylate cyclase, inhibiting the production of cAMP), and both are normally found presynaptically. Nevertheless, the amino acid sequence homology and pharmacological characteristics of these two groups are quite different. In future work describing the classification of mGluRs, utilization of structural and pharmacological characteristics may be a more valid approach, as these characteristics are more specific than those used traditionally.

Another issue for consideration is that of nomenclature for the different isozymes of nitric oxide synthase (NOS). NOS is the enzyme necessary to produce nitric oxide. The three isoforms of NOS are termed endothelial, neuronal, and inducible NOS. Endothelial NOS (eNOS) was first identified in endothelial cells. However, despite its name, eNOS is found in neurons and glia in the CNS as well as other structures (92; 99). Similarly, nNOS can be found in both neurons and glia cells in the brain, as well as in peripheral tissues such as skeletal muscle (99; 133; 139). Finally, inducible NOS has been found to be constitutively expressed in a

variety of tissues (151). In future studies and publications, identifying NOS isoforms by number (neuronal-1, inducible-2, and endothelial-3) would avoid the nomenclature inconsistencies and confusion.

Summary:

As previously stated in this document, the nucleus of the solitary tract (NTS) is a heterogeneous nuclear region that has a role in regulating nearly all of the autonomic functions involved in homeostasis. The exact details concerning the modulation of input signals and the resulting output information from the NTS are not completely understood, but likely are critical to alterations in autonomic function under a variety of conditions. This project examined a known class of modulatory receptors, Group I metabotropic glutamate receptors (mGluRs), and the expression of Group I mGluRs on different phenotypes of neurons within the NTS. We presented data suggesting Group I mGluRs are present on neurons likely involved in arterial baroreflex function and suggest that therefore these receptors are likely involved in modulation of that specific reflex. Further, we examined the neurons in the NTS that were activated in response to increases or decreases in arterial pressure. Data presented suggest that increasing and decreasing arterial pressure result in activation of separate populations of neurons. This dissertation provides valuable information regarding the expression of receptors and distribution of specific neurons within the NTS in normal animals. This information can be compared in future experiments examining the NTS under different physiological or

pathophysiological conditions to determine what, if any, alterations in Group I mGluR expression or activation of NTS neurons occur.

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Vita

James Robert Austgen, second son of John and Mary Austgen, was born on April 13th, 1978 in East Chicago, IN. Spending three years in Hammond, IN, James moved to Muscatine, Iowa in 1981, where he graduated from Muscatine High School in May, 1996. James spent two years at Muscatine Community College, for which he received an AS in Biology. James then transferred to Augustana College, Rock Island, IL. There he received an AB in Biology and Pre-Medical Studies in 2000. James joined the graduate program of the Dept. of Physiology at the University of Missouri in 2001. He received his Master's of Science in Physiology under J. Thomas Cunningham, PhD in 2003 and his Doctor of Philosophy in Physiology under Eileen M. Hasser, PhD in 2008, from the Dept of Medical Pharmacology and Physiology. James is currently a Post-Doctoral Fellow with David D. Kline, PhD in the Dep. of Biomedical Science at the University of Missouri. James is engaged to be married in the fall of 2008, to Katherine R. Gray