NEURONS PROJECTING TO THE PARAVENTRICULAR NUCLEUS OF THE
HYPOTHALAMUS ARE ACTIVATED AND CONTRIBUTE TO CARDIORESPIRATORY
RESPONSES TO SYSTEMIC ACUTE HYPOXIA

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NEURONS PROJECTING TO THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ARE
ACTIVATED AND CONTRIBUTE TO CARDIORESPIRATORY RESPONSES TO SYSTEMIC ACUTE
HYPOXIA

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Dedication

For my family, I love you.

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LIST OF NOMENCLATURE

ANOVA: Analysis of variance
AP: area postrema
CS: calamus scriptorius
CSN: carotid sinus nerve
CVLM: caudal ventrolateral medulla
DMX: dorsal motor nucleus of the vagus
DRG: dorsal respiratory group
FG: Fluoro-Gold
GABA: gamma-aminobutyric acid
GAD67: glutamic acid decarboxylase isoform 67
HR: heart rate
IR: immunoreactive
IX/X: Ninth and Tenth Cranial nerves
MAP: mean arterial pressure
NA: nucleus ambiguous
NDS: normal donkey serum
nTS: nucleus tractus solitarii
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
TH: tyrosine hydroxylase
VLM: ventrolateral medulla
VRG: ventral respiratory groups
XII: Hypoglossal Nerve/Nucleus
7N: Facial Nerve/Nucleus

CHAPTER 1: INTRODUCTION

1.1 Dissertation overview

Neural control of the cardiorespiratory system maintains constant homeostasis under baseline conditions and in response to diverse environmental challenges by integrating a wide variety of sensory afferent inputs and controlling a multitude of efferent projections. This dissertation will seek to identify some of the key neural pathways and cellular phenotypes that are involved in adaptations to acute hypoxia. Three studies (Chapter 2-4) investigate the overall hypothesis that activation of neurons projecting to the paraventricular nucleus of the hypothalamus occurs during hypoxia and recruitment of this pathway has a functional role in maintenance of normal cardiorespiratory responses to hypoxia.

1.2 Overview of cardiorespiratory and autonomic function

Few central nervous system functions are more critical to life than maintenance of breathing, blood pressure, and heart rate. Neural control of the cardiorespiratory system ensures that the heart maintains cardiac output and the lungs preserve ventilation to meet the metabolic demands of all the organs in the body. The sympathetic innervations of the heart and blood vessels, when activated, increases heart rate and cardiac contractility, and causes vasoconstriction. On the other hand, parasympathetic vagal innervation inhibits cardiovascular function, resulting in decreased heart rate and cardiac contractility. During the fight-or-flight response, an increase in sympathetic activity is prominent, whereas parasympathetic activity is

increased for stimulation of "rest-and-digest" activities in the body (91, 114).

Ventilation requires the coordination of two main muscle groups: inspiratory and expiratory muscles, to produce rhythmic inspiration and expiration. These muscles include the diaphragm, intercostal and abdominal muscles (27). Cell bodies of the motor neurons that innervate the respiratory muscles are located in the spinal cord. The diaphragm is innervated by the phrenic nerve, originating from the phrenic motor nucleus at spinal level C3-C5; intercostal motor nerves originate between T1-T12 and abdominal motor nerves originate between T4-L3 (108).

A general overview of neural control of the cardiorespiratory system is illustrated in **Figure 1.1**. There are two main sets of sensory receptors shown. The baroreceptors are mechanosensors that sense arterial blood pressure. The chemoreceptors sense oxygen content and pH/CO₂ of arterial blood. The location of these receptors are in very restricted, specialized areas (carotid sinuses, aortic arch, and carotid bodies) where they can easily sense blood pressure changes as well as alterations in arterial oxygen and carbon dioxide levels. Action potentials generated in neurons associated with these receptors travel through the nodose and petrosal ganglia, which contain the cell bodies of neurons traversing to the nucleus tractus solitarii (nTS), the first synapse of afferent information in the central nervous system. The baroreceptor reflexes, activated during changes in mean arterial pressure, and the chemoreceptor reflexes, activated during low arterial oxygen (hypoxia) and an increased acidity (acidosis) help the body make vital cardiovascular and ventilatory adjustments, including control of blood pressure, heart rate, and increases in ventilation (57, 204).

Neural Control of the Cardiorespiratory System

Figure 1.1 –

Schematic illustration of the neural control of the cardiorespiratory system. Primary sensory afferents involved in cardiorespiratory regulation (e.g. baro- and chemoreceptors) which terminate in the brain (specifically the nTS). The efferent motor (gray) and the parasympathetic (blue), and sympathetic (red) divisions of the autonomic nervous system work together to determine blood pressure, heart rate and ventilation. Adapted from: Abboud, F. Am J Physiol Regul Integr Comp Physiol., 2010 (1)

1.3 Overview of the respiratory system and chemoreflex function

Ventilation requires the coordination of two main muscle groups: inspiratory and expiratory. During inspiration, which is initiated by the contraction of the diaphragm and supported by external intercostal muscles, the lungs expand and air flows in. Expiration, a passive process at rest, occurs as the lungs and chest wall return to their original positions, forcing air out (29, 219, 242).

To maintain homeostasis, appropriate arterial levels of oxygen (PaO_2) and carbon dioxide (PaCO_2) must be maintained under a variety of conditions. The respiratory system controls these variables by altering ventilation to control the amount of air (i.e. O_2) inspired and metabolic waste (i.e. CO_2) expired. There are two groups of respiratory-related chemoreceptors: peripheral (or arterial) chemoreceptors which monitor the O_2 and CO_2/pH levels in the arterial blood and central chemoreceptors which monitor pH or CO_2 levels in the cerebral spinal fluid (CSF) surrounding the brainstem. The peripheral chemoreceptors are located in the carotid (at the bifurcation of the common carotid artery) and aortic bodies, while the central chemoreceptors are located, predominately, on the surface of the ventrolateral medulla but have also been found in other areas of the brainstem (76). These two sets of chemoreceptors constitute part of the important peripheral and central chemoreflexes that are vital to regulating pulmonary ventilation (68).

1.3.1 The peripheral chemoreflex

In rodents, the carotid body chemoreceptors are the main receptors and will be the focus for this introduction. The carotid bodies mediate primarily ventilatory

chemoreflexes but can also modulate the cardiovascular baroreflex. The carotid bodies are highly vascularized and receive a very high blood flow relative to its size. The carotid body is composed of several cell types. Glomus cells are the chemosensitive cells that primarily detect changes in arterial PO_2 , but also sense changes in blood pH and PCO_2 (2, 32). They stimulate afferent nerves to increase their firing rate in response to low arterial PO_2 or high PCO_2 . The afferent information is relayed through the carotid sinus nerve via the glossopharyngeal nerve to the brainstem (24) where it is then integrated with a multitude of other afferent inputs.

Decreases in PaO_2 or pH and increases in $PaCO_2$ all stimulate primarily increases in ventilation (204), but also influence arousal, release of hormones (e.g. vasopressin, adrenocorticotrophic hormone), and hemodynamic and autonomic adjustments that can compensate for the peripheral vasodilatory effect of hypoxia (87, 98). These autonomic adjustments help redistribute blood flow to essential organs such as the brain, heart and kidneys (155). For example, the systemic hemodynamic responses to hypoxia in rats consist of an increase in cardiac output and a decrease in peripheral vascular resistance due to systemic vasodilation (99). Experiments evaluating selective redistribution of blood flow have found that there are significant increases in blood flow to the brain, respiratory muscles, and the liver. For example, there is an increase in cerebral blood flow and in the number of perfused brain arterioles and capillaries of conscious rats during acute exposure to 10% oxygen (124). The increase in blood flow to vital organs is made at the expense of organs in the gastrointestinal tract, spleen and pancreas, and to some extent, skin, fat and bone (137, 154). Blood flow to other organs,

such as non-respiratory muscles remains relatively unchanged (137). Interestingly, renal blood flow appears relatively unchanged during hypoxia, possibly because of autoregulation of the renal circulation (102, 137). In summary, the redistribution of blood flow ensures that the rate of oxygen supply to the brain, respiratory muscles and liver does not decrease, but actually increases above the levels observed in normoxia to ensure proper oxygenation to these organs (137).

Less research has evaluated differential regional control of efferent sympathetic nerve activity responses during hypoxia. Collectively, evidence from human and animal literature indicates an increase in sympathetic nerve activity during chemoreflex stimulation, but variable experimental conditions (anesthetized vs unanesthetized, type of chemoreflex stimulation, etc) makes it difficult to compare studies. Increases in renal (190) and splanchnic (152) nerve activity are evident in rats exposed to acute systemic hypoxia. In one study, simultaneous recordings were made from three sympathetic beds (cutaneous, splanchnic, and cardiac sympathetic nerve activity) in anesthetized artificially ventilated rabbits with neuromuscular blockade. This study revealed differential regional changes. Under hypoxic conditions, cutaneous, and cardiac sympathetic nerve activities were increased while splanchnic nerve activity was unchanged (107). Interestingly, in humans, the net effect of acute systemic hypoxia is also vasodilation even though there are reflex increases in muscle sympathetic nerve vasoconstrictor activity (233). In summary, redistribution of blood flow and modulation of sympathetic nerve activity during chemoreflex stimulation provides an important

mechanism by which organs in the body can adjust to meet cardiorespiratory demands during hypoxia.

Finally, adaptations to hypoxia include important neuroendocrine responses. Hormones such as vasopressin, oxytocin and corticotrophin releasing hormone not only act systemically to affect chemoreflex responses but also as neurotransmitters within the nervous system. For example, hypoxia results in a rise in circulating levels of vasopressin in several species, including the rat (188, 239). Vasopressin release has also been reported in human subjects exposed to high altitude (44, 174). Release of vasopressin is thought to mediate some of the cardiovascular responses to hypoxia, the most significant effect appearing to be the direct stimulation of vascular vasopressinergic V_{1A} receptors. This results in peripheral vasoconstriction, thus limiting the evoked fall in arterial pressure during hypoxia (148, 239). Within the central nervous system, vasopressin-containing PVN neurons have projections to the pre-Bötzinger complex, phrenic motoneurons and RVLM (116, 119). Vasopressin type 1A receptors are expressed on sympathetic preganglionic neurons and microinjection of vasopressin in these regions increases respiration, blood pressure and heart rate, supporting the idea that vasopressin can affect autonomic, respiratory and cardiovascular functions through a central nervous system mechanism (116, 117). Another hormone released during exposure to hypoxia is oxytocin (120). Similar to vasopressin, oxytocin can have systemic effects and also influence neural control as a neurotransmitter. Although the exact role of oxytocin during hypoxia is unknown, it does have natriuretic properties and therefore could affect systemic fluid balance (43). Also,

oxytocin can act as a neurotransmitter at many of the same sites that vasopressin does (117), including at the level of sympathetic preganglionic neurons (187). Collectively, neuroendocrine release in response to hypoxia plays an important role in maintaining overall homeostasis.

1.3.2 The central chemoreflex

In contrast to peripheral chemoreceptors, which primarily sense low PaO_2 to regulate breathing, central chemoreceptors adjust ventilation by responding to changes in brain pH and/or PCO_2 . Central chemoreceptive neurons are activated by increases in interstitial pH and/or CO_2 (170). pCO_2 detection by central neurons is presumably mediated by the effect of pH on ion channels and perhaps, other forms of proton receptors in the cell (88, 185). When excess arterial CO_2 diffuses across the blood brain barrier, the result is a decrease in pH, thereby stimulating the central chemoreceptors that project to respiratory and sympathetic control regions to modulate breathing and increase sympathetic nerve activity, respectively (76, 170, 171). Although the exact location of central chemoreceptors has not been fully identified, several potential sites in the central nervous system have been described. Neurons within brain regions such as the ventral medullary surface, the nTS, locus coeruleus, the medullary raphe, the rostral aspect of the ventral respiratory group (the pre-Bötzinger complex), the retrotrapezoid nucleus and the paraventricular nucleus of the hypothalamus are all able to sense changes in PCO_2 (167, 171). The exact network by which these neurons connect to premotor neurons and modulate breathing and sympathetic nerve activity is unclear (167). Overall, accumulation of CO_2 in the brain during hypercapnia activates

central chemoreceptors that, in addition to peripheral chemoreceptors, have direct effects on ventilation and sympathetic nerve activity.

1.4 Central nervous system involvement in cardiorespiratory control

Thus far, the focus has been on normal chemoreflex function. Equally important however, are the precise neural mechanisms that generate and maintain normal breathing/autonomic control and the plasticity of these pathways, allowing organisms to adapt to cardiorespiratory-related stresses. Therefore, this part of the review briefly explains the current understanding of the neural pathways that mediate the main response elicited by peripheral chemoreceptor stimulation and how they are modulated. It will focus on the regions that are specifically investigated in this dissertation: the nucleus tractus solitarius (nTS), caudal ventrolateral medulla (CVLM), rostral ventrolateral medulla (RVLM) and paraventricular nucleus of the hypothalamus (PVN). **Figure 1.2** shows the neuroanatomy of the complex networks involved in cardiorespiratory control, including several brain regions that, although not addressed in this dissertation, should not be overlooked when considering respiratory rhythm generation (i.e. retrotrapezoid nucleus/parafacial respiratory group, Bötzinger complex, pre-Bötzinger complex, caudal and rostral ventral respiratory groups).

Figure 1.2 –

Neuroanatomy of the brainstem cardiorespiratory control network. Sagittal view of the rat brainstem, highlighting the main groups of CNS neurons important for respiratory, sympathetic and parasympathetic control in mammals. Anatomically, neuronal circuitries controlling respiratory functions located in the medulla oblongata are within or in close proximity to cardiovascular networks. 7N, facial nucleus; Amb, nucleus ambiguus; Bo, Bötzinger complex; CVRG, caudal ventral respiratory group; CVLM, caudal ventrolateral medulla; DMX, dorsal motor nucleus of the vagus; LC, locus coeruleus; LRT, lateral reticular nucleus; nTS, nucleus tractus solitarius; PrBo, pre-Bötzinger complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; RVRG, rostral ventral respiratory group; RVLM, rostral ventrolateral medulla. Adapted from: Moreira, T. et al. Braz J Med Biol Res. 2011. (168)

1.4.1 The first synapse of peripheral cardiorespiratory reflexes: the nucleus tractus solitarius (nTS)

Signals from carotid and aortic bodies are sent to the nTS via the glossopharyngeal and vagus nerves, respectively. The nTS is considered the gateway to the autonomic nervous system and respiratory control as it integrates sensory/visceral afferent information across many organ systems including the cardiovascular and respiratory systems (15). For example, the nTS is the site of the first central synapse for the vitally important homeostatic cardiorespiratory reflexes such as the arterial baroreflex and chemoreflex. Furthermore, the nTS is also the site of terminations of other respiratory-related sensory afferents, including slow and rapidly adapting pulmonary stretch receptors and bronchopulmonary C fibers (58, 135).

Anatomically, the nTS is organized such that different visceral afferents generally synapse in distinct anatomic locations. For example, afferents from the arterial baroreceptors and cardiac baroreceptors synapse primarily dorsomedially, while arterial chemoreceptors terminate ventrally and laterally in the nTS. Overlap of the sensory inputs does occur however, especially in the caudal nTS (6). Additionally, some nTS neuronal phenotypes are not confined to cytoarchitecturally-distinct subnuclei. Among these are a group of noradrenergic (A2; catecholamine-producing neurons), which form a long linear column through the nTS and project extensively to higher-order central autonomic cell groups such as PVN (205). In addition to visceral afferent information, the nTS also receives input from other brain regions including the cortex, amygdala, perifornical regions, the PVN, parabrachial nucleus, the pons, caudal medullary raphe

nuclei, area postrema, and the ventrolateral medulla, many of which have been shown to modulate signal transmission within the nTS (57, 199). Afferent fibers to the nTS release a wide variety of neurotransmitters, including amino acids, monoamines, and neuropeptides, all of which can modulate signals leaving the nTS (147).

Within the nTS there is great complexity. The nTS contains about 42,000 neurons and more than a million synapses. There is an intricate network of excitatory and inhibitory neurons within the nTS. nTS neurons themselves also express a diverse array of neurotransmitters, neuromodulators and receptors for these transmitters and hormones (7). In fact, the nTS contains nearly all of the neurotransmitters and neuromodulators present within the CNS (e.g. dopamine, serotonin, glycine, adenosine, substance P). Glutamate and gamma-aminobutyric acid (GABA), are the major excitatory and inhibitory neurotransmitters, respectively, in the nTS and are believed to be critical in cardiorespiratory regulation. Overall, the characteristics of transmission within the nTS and also of nTS neurons that project to other brain regions have the potential to modulate efferent output.

The nTS projects to various regions known to play an important role in cardiorespiratory control. These regions include the IML cell column, the RVLM and CVLM, the medullary raphe nuclei, the parabrachial complex in the pons, the midbrain, the PVN, the lateral hypothalamic area, and other forebrain nuclei (57). Some of these efferents terminate directly on sympathetic and parasympathetic preganglionic neurons. Also, extensive monosynaptic excitatory projections to the phrenic nucleus in

the spinal cord have been identified from the neurons in the nTS (75). The nTS also provides important excitation to the ventral respiratory groups.

With regard to integration of the peripheral chemoreflex and respiratory function, there is both anatomic and electrophysiological evidence that neurons in the nTS receive direct input from carotid body afferents and they respond to hypoxia (42, 77, 235). Studies using Fos immunohistochemistry have shown that system hypoxia (21, 71, 104, 234), as well as stimulation of afferent fibers of the carotid sinus nerve (72), increase activation of nTS neurons. Projections from the nTS provide several pathways by which peripheral chemoreceptor activation increases ventilation and sympathetic activity. The nTS sends excitatory projections directly to the RVLM to produce an overall increase in presympathetic neuronal activity during hypoxia (167). Hypoxia also activates nTS glutamatergic neurons that innervate the retrotrapezoid nucleus (RTN), a nucleus containing pH sensitive neurons that may integrate peripheral and central chemoreception (171). In addition, the nTS is thought to innervate and activate a ventilatory central pattern generator (CPG) in the pre-Bötzinger complex (167). Collectively, these observations suggest that the nTS is a complex integrative center that can influence respiratory and autonomic outflow both through local brainstem circuits or through projecting to other central autonomic and motor sites.

1.4.1a Phenotypes evaluated in the nTS

In the present studies, we examined two neuronal phenotypes in the nTS known to modulate synaptic activity and alter cardiorespiratory function: GABAergic and catecholaminergic neurons.

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. GABAergic neurons are distributed throughout the nTS, and most GABAergic neurons are second-order neurons that receive direct, excitatory input from the solitary tract plus additional indirect polysynaptic input that can be excitatory or inhibitory (7, 14, 159) . Their activity is vital to the maintenance of cardiorespiratory homeostasis. For example, microinjection of GABA_A or GABA_B receptor agonists, into the nTS results in an increase in arterial pressure and sympathetic nerve activity (28) and can reduce the gain of baroreflex mediated bradycardia (33). Conversely, microinjection of the GABA_A receptor antagonists into the nTS decreases baseline arterial pressure and sympathetic nerve activity and GABA_B receptor antagonists augment the excitatory response of nTS neurons to increasing arterial pressure (83, 225). These studies suggest a tonic inhibitory action of GABA in the nTS with regard to arterial baroreflex function.

GABAergic cells play an important role in chemoreflex regulation. For example, GABAergic cells not only project from nTS to the ventrolateral medulla, but there are also GABAergic interneurons within the nTS that inhibit second-order nTS neurons receiving afferent cardiorespiratory information (58, 74, 223). Following sustained visceral afferent stimulation, Fos studies indicate that inhibitory neurons actually account for a large proportion of all the nTS neurons that are activated (38, 243). With regard to the peripheral chemoreflex, microinjection of a selective GABA uptake inhibitor into the nTS attenuates increases in respiratory movement (measured by the expansion of the dorsal thorax), respiratory rate, and changes in arterial blood pressure

that result from carotid chemoreceptor stimulation in rats. These increases in respiration can be antagonized by prior microinjection of a GABA_A antagonist, but not of a GABA_B antagonist, suggesting that endogenous GABA can inhibit the chemoreflex by preferentially acting on GABA_A receptors (226). Interestingly, intravenous administration of GABA_A agonists reduce resting phrenic nerve activity and blood pressure and also inhibit carotid chemoreceptor-induced increases in phrenic nerve activity. This suggests that GABA has the ability to blunt important parts of chemoreflex responses. Furthermore, the effects of intravenous administration of GABA_A agonists on the chemoreceptor reflex can be reversed by microinjection of GABA antagonists in the nTS. This supports the concept that GABA receptors in the nTS play a role in the carotid chemoreceptor reflex pathway that traverses through the nTS (227). In summary, GABAergic neurons are an important neuronal phenotype that can modulate nTS activity and may directly affect propagation of cardiovascular and respiratory signals through the nTS. Despite this background, less is known about the activation of GABAergic neurons in the nTS during acute systemic hypoxia and if GABAergic neurons in the nTS innervate forebrain regions such as the PVN. Therefore, in part of this study, we determined if GABAergic nTS neurons project to the PVN and whether they are activated by hypoxia.

One specific nTS neuronal phenotype that has been studied in terms of mediating autonomic and neuroendocrine responses to cardiorespiratory challenges (9, 37, 69, 115, 193), including hypoxia (71, 104, 126, 234) are catecholaminergic neurons. The catecholamine biosynthetic pathway, summarized in *Figure 1.3 (left)*, highlights the

initial and rate limiting enzyme in their production, tyrosine hydroxylase (TH). The presence of TH is often used as a marker for catecholaminergic neurons. *Figure 1.3 (right)* illustrates the extensive projections of catecholaminergic neurons. TH-positive neurons are located throughout the entire nTS, with noradrenergic (A2) neurons overlapping with adrenergic cells (C2 cells) at the rostral portion of the nTS (10, 110). Catecholaminergic neurons in the nTS receive direct input from visceral afferents and project to various medullary and supramedullary nuclear regions, including those that influence respiration and autonomic control (8). Silencing of the catecholaminergic neurons in the nTS results in elevation in arterial pressure, indicating that they play a chronically suppressing arterial pressure. Interestingly, decreasing catecholaminergic neurons in the nTS also affected respiration, water intake, and urine output, as well as feeding, emphasizing that catecholaminergic neurons in the nTS have multi-functional roles (66). Of particular interest are the neurons in the A2 cell group that project to the paraventricular nucleus of the hypothalamus (PVN) where they densely innervate parvocellular neurons. Several studies have shown that catecholaminergic nTS neurons are involved in the control of breathing (31, 72) and cardiovascular (55, 193, 211) function. Therefore, our interest lies in the role of catecholaminergic neurons during activation of the chemoreflex, both in peripheral and central chemoreception. Current studies examined catecholaminergic neurons in the nTS to determine if they are activated by hypoxia and also if they project to the PVN.

Figure 1.3 – Catecholamines in the central nervous system.

Left) Pathway for catecholamine biosynthesis and its enzymatic steps. Right) Catecholaminergic neurons and their major projections in the rat brain (drawings on the midsagittal plane of the brain). Norepinephrine (solid triangles and lines in red) and epinephrine (open triangles and lines in green) pathways are depicted as well as the major groups of catecholamine synthesizing neurons and the brain nuclei in which they reside. A1, A2, A4, A5, and A7, noradrenergic neurons; C1, C2, and C3, adrenergic neurons; CVLM, caudal ventrolateral medulla; nTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla; LC, locus coeruleus. adapted from Kvetnansky et al. *Physiol Rev.* 2009. (139)

1.4.2 The presympathetic neurons and sympathetic nerve activity generation: the rostral ventrolateral medulla (RVLM)

The sympathetic nerve activity response to stimulation of peripheral chemoreceptors is excitatory (87, 92), and therefore, a brief description of the nucleus involved in tonic and baseline sympathetic tone generation is warranted. The RVLM is the primary source of excitation to the sympathetic preganglionic neurons in the spinal cord and are therefore often referred to as “pre-sympathetic” neurons. The RVLM is also a key nucleus for the final integration of many cardiorespiratory reflexes (57, 89). The predominant response of most RVLM presympathetic neurons to carotid body stimulation (using potassium cyanide or brief hypoxia) in rodents is activation (132, 192). Consistent with this observation, a large proportion (up to 70%) of C1 neurons in the RVLM express Fos in conscious mammals after exposure to hypoxia (72, 104). Furthermore, increases in sympathetic nerve activation elicited by carotid body stimulation is severely attenuated after selective lesions of the C1 neurons (211). The classical pathway between the carotid bodies and the RVLM involves a single projection from the nTS and this has been considered the main pathway involved in peripheral chemoreflex activation (4, 41, 204).

1.4.3 A critical component of the CNS pathways involved in cardiorespiratory reflexes: the caudal ventrolateral medulla (CVLM)

The CVLM is an essential component of the brainstem circuitry best known for its role in reflex control of arterial blood pressure. However, as with other areas initially thought to have only one function, the CVLM is more than a simple relay of

baroreceptor reflex function. In the baroreflex, inhibitory GABAergic CVLM neurons receive excitatory input from the nTS. These CVLM neurons, in turn, project to the RVLM and inhibit neurons that innervate sympathetic preganglionic neurons. This pathway modulates sympathetic nerve activity and ultimately maintains arterial pressure in a normal range. The CVLM also contains a group of noradrenergic neurons (A1 neurons) which are interspersed with GABAergic CVLM neurons. These cells are unique in that they synthesize catecholamines and they have different anatomic targets than GABAergic neurons. A1 neurons influence cardiorespiratory function through projections to both the hypothalamus and limbic centers, which in turn affect neuroendocrine and autonomic regulation and coordinate appropriate behavioral responses, respectively (36, 59, 121).

The role of the CVLM in peripheral chemoreflex function has been equivocal. For example, inhibition of the CVLM does not alter the tonic sympathoexcitation induced by hypoxia, suggesting the CVLM is not required for increased sympathetic nerve discharge during chemoreflex stimulation (130). Other studies, however, indicate CVLM neurons exhibit hypoxia-induced activation (71, 144, 234) and contribute to the respiratory coupling of RVLM neuronal activity and sympathetic nerve activity during hypoxia (151, 164). Taken together, data suggest that participation of CVLM neurons in the chemoreflex is critical in homeostatic responses to hypoxia.

1.4.4 Integration and modulation of cardiorespiratory control: the paraventricular nucleus of the hypothalamus (PVN)

Anatomic location of the PVN. The PVN is an important integrative nucleus located within the hypothalamus. It is located bilaterally between the fornix and the third ventricle. The PVN is bordered by the thalamus dorsally and anterior hypothalamic nucleus ventrally. In the rat, the PVN extends approximately 1 mm in the rostral - caudal direction and is approximately 1 mm at its most lateral extension.

Structural organization of the PVN. The PVN contains a heterogeneous group of neurons consisting of magnocellular and parvocellular neurons which are either intermingled with each other or segregated into groups within the nucleus (**Figure 1.4**). The magnocellular neurons are composed mostly of large neurons and can be subdivided into the anterior, medial and posterior groups (229). The majority of the magnocellular PVN neurons synthesize and store vasopressin (AVP) and oxytocin and release these hormones via the neurohypophyseal tract via projections to the posterior pituitary gland (230).

The parvocellular PVN neurons are subdivided into several subnuclei; namely, the periventricular, anterior, medial, ventral, lateral and dorsal subdivisions which consist predominantly of small to medium sized neurons (6 to 13 μm in diameter). The majority of the anterior, medial and periventricular parvocellular neurons are involved in the hypothalamic-pituitary adrenal-cortical axis via projections to the anterior pituitary (30, 100), but parvocellular nuclei are also known to be extremely diverse.

PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

Figure 1.4 –

Overview of the structural organization of the PVN.

Coronal view of the PVN illustrating the main projections from the PVN and the phenotype of the corresponding subnuclei. Figure based on literature in the Central Regulation of Autonomic Function, 2nd edition, Chapter 3 and 4 (147).

They express more than thirty recognized neurotransmitters/neurohormones including oxytocin, AVP, somatostatin, corticotrophin releasing hormone, thyrotrophin releasing hormone, and dopamine. Thus, these neurons regulate the release of hormones, which affect the functions of various peripheral organs including the adrenal cortex, the thyroid gland, etc (13). On the other hand, parvocellular neurons located in the dorsal, ventral and lateral sub-divisions of the PVN are known to send projections to various autonomic nuclei that are critical for autonomic and cardiorespiratory regulation. This will be discussed in further detail in the forthcoming sections of this introduction.

Afferent projections to the PVN (Figure 1.5). Detailed anatomical and biochemical specificity of afferent inputs to the PVN are important in understanding the coordinated output of this nucleus (160, 206). Autonomic regions within the brainstem, involved in the regulation of the cardiorespiratory system, project to the PVN. These regions include the nTS, RVLM, CVLM, area postrema (AP), parabrachial nucleus, locus coeruleus and the raphe nuclei (3, 94, 113). The PVN also receives input from a number of forebrain regions including the olfactory bulb, and amygdala, the subfornical organ (SFO) and the tissues lining the third ventricle, which include the median preoptic nucleus and organum vasculosum of the lamina terminalis (5, 229).

Interestingly, the PVN is densely and differentially innervated by catecholaminergic terminals. Adrenergic (C1 and C2) and noradrenergic (A1, A2, A6) cell groups in the VLM and nTS project to the PVN. Brainstem noradrenergic (A1, A2, A6) cell groups provide the majority of this catecholaminergic input (205). Electron microscopy and immunocytochemical techniques have been used to show that these adrenergic/noradrenergic cell groups project to specific subdivisions of the PVN. Direct projections from the A1 region are found primarily in the magnocellular division of the PVN known to contain vasopressinergic and oxytocin neurons. Projections from the A2 region are distributed throughout both magnocellular and parvocellular divisions of the PVN, with no preferential distribution into regions where AVP- or oxytocin- containing cells are concentrated. However, prominent projections from the A2 catecholaminergic neurons are observed in the parvocellular division of the PVN, an area known to contain corticotrophin releasing hormone. Afferent fibers originating from the locus coeruleus

(A6) are distributed almost entirely around the third ventricle, which is the region of the PVN that contains dopamine-, somatostatin-, and thyrotropin-releasing-hormone-containing neurons (52, 206). These preferential innervations imply that each of the three brainstem noradrenergic cell groups have the ability to modulate the activity of anatomically and chemically distinct groups of neurosecretory and pre-autonomic neurons.

**Figure 1.5 –
Main neuronal afferents to the PVN.**

A1/A2/A6: noradrenergic cell groups; ARC: arcuate nucleus; BNST: bed nucleus of the stria terminalis; C1/C2: adrenergic cell groups; DM: dorsomedial nucleus; LC: locus coeruleus; nTS: nucleus tractus solitarius; PM: premammillary nucleus; PB: parabrachial nucleus; RN: raphe nuclei; SCN: suprachiasmatic nucleus; SFO: subfornical organ; IX: glossopharyngeal nerve; X: vagus. Figure outlines adapted from *The Brain in Stereotaxic Coordinates*, 2007 (177) and based on Liposits, Z, *Crit Rev Neurobiol* 1993

Efferent projections from the PVN (Figure 1.6). The extensive network of efferent projections from the PVN emphasizes the role of the PVN in the regulation of the cardiorespiratory system. The PVN projects to many brain regions including the brainstem and spinal cord (16, 46, 145, 186). Interestingly, many of the brain regions that send afferent input to the PVN receive reciprocal efferent projections from the PVN. Also, many of these brain nuclei are known to be involved and are important in the regulation of the cardiorespiratory system (55, 186, 189, 194). Some of these important autonomic nuclei are the nTS, RVLM, ventral respiratory groups and the intermediolateral cell column, where phrenic motor nuclei and the sympathetic pre-ganglionic motor neurons are located (203). The latter is of particular interest as PVN neurons that directly project to the phrenic motor nuclei and sympathetic pre-ganglionic motor neurons located in the IML provide the anatomical framework that enables the PVN to regulate ventilation and sympathetic nerve activity directly. Furthermore, spinally-projecting neurons in the PVN are also known to send collaterals to the RVLM (186), which contains neurons that project to the IML of the spinal cord (112) and is important for the tonic generation of sympathetic nerve activity. Hence, the projection of PVN neurons to the RVLM provides the PVN with the ability to influence sympathetic nerve activity indirectly. Such organizational complexity of PVN anatomical connections suggests it has the capacity to both directly and indirectly influence breathing and sympathetic nerve activity.

Figure 1.6 –

Main neuronal efferents from the PVN.

AP: area postrema; APit and PPit: anterior and posterior pituitary; DX: dorsal nucleus of the vagus nerve; IML: intermedio-lateral column; ME: median eminence; OVLT: organum vasculosum of the lamina terminalis; LC: locus coeruleus; nTS: nucleus tractus solitarius; PAG: Periaqueductal gray; PB: parabrachial nucleus; PVN: paraventricular nucleus; SFO: subfornical organ; VLM: ventrolateral medulla; VRG: ventral respiratory groups; PMN: phrenic motor nucleus; X: vagus. Figure outlines adapted from *The Brain in Stereotaxic Coordinates*, 2007 (177) and based on Liposits, Z, *Crit Rev Neurobiol* 1993

1.5 Role of catecholamine inputs to the PVN in integration of cardiorespiratory reflexes

The preceding sections have highlighted that the PVN contains the anatomical framework to mediate changes in ventilation, cardiovascular control, and sympathetic nerve activity. This section highlights the physiological roles of the PVN in the reflex responses elicited by disturbances in oxygen saturation. Various studies imply the important role of the PVN in the control of breathing, sympathetic nerve activity and neuroendocrine function, and in modulation of these parameters in response to chemoreflex activation. Stimulation of the peripheral chemoreflex using acute hypoxia or potassium cyanide induces Fos-immunoreactivity within PVN neurons (149), including magnocellular (217) and pre-autonomic neurons (47). Activation (67, 248) or disinhibition (208) of the PVN leads to an increase in respiratory rate, mean arterial pressure, and heart rate. In contrast, electrolytic lesions of the PVN depress ventilation (191) and PVN lesion or blockade attenuates the arterial pressure, sympathoexcitatory, and phrenic nerve responses evoked by stimulation of arterial chemoreceptors (175, 190). PVN projections to the pituitary gland are also implicated in vasopressin and ACTH release due to hypoxia (79, 144).

Despite the well-known importance of the PVN in hypoxia-elicited responses, the direct pathways by which the PVN receives chemoafferent information remain to be fully elucidated. The PVN receives a very dense catecholaminergic innervation (80) as mentioned above, but, only a few studies have evaluated the contribution of catecholaminergic inputs to the PVN. For example, in vitro experiments indicate that

stimulation of alpha-adrenergic receptors increases the excitability of spinally-projecting PVN neurons by augmenting glutamatergic and attenuating GABAergic neurotransmission (16, 48). Even fewer studies provide evidence regarding the functional role of catecholamine release in the PVN during hypoxia. Kubo et al. (136) indicated that blockade of adrenergic receptors in the PVN blunts cardiovascular responses and vasopressin release due to carotid chemoreceptor stimulation, suggesting that catecholaminergic inputs to the PVN are involved in mediation of chemoreceptor induced pressor responses. Also, unilateral ibotenic acid lesions restricted to the caudal two thirds of the VLM cell group significantly reduced the activation of vasopressin, corticotropin releasing hormone, and oxytocin PVN cell types following hypoxia (82, 217). However, whether this function requires direct afferent input from the CVLM, including a catecholaminergic input, was not determined.

Thus, although adrenergic inputs may participate in cardiovascular and neuroendocrine responses during hypoxia, the source of catecholaminergic inputs to the PVN was not evaluated in these studies. Furthermore, previous studies did not address the ventilatory responses to hypoxia and if they were affected by blockade of adrenergic receptors in the PVN. Hypoxia increases respiratory rate and sympathetic nerve activity but it is not known if this increase is mediated through adrenergic inputs to the PVN. Collectively, dense catecholaminergic inputs to the PVN and data from the preceding work provides a clear rationale for investigation of the functional role of these inputs during activation of the chemoreflex. The present study provides evidence

that catecholaminergic input to the PVN from hypoxia-activated neurons may be a pathway that contributes to cardiorespiratory responses.

1.6 Overview of Techniques used to explore the central nervous system pathways involved in chemoreflex function

1.6.1 Fos: A Marker of Neuronal Activation

Immunohistochemical identification of Fos protein has become a widely accepted marker for acute neuronal activation in the central nervous system (55). This technique is a useful tool to localize cells and cell groups that are activated following acute exposure to hypoxia or hypercapnia. The *c-fos* proto-oncogene is an immediate early gene and encodes a DNA-binding protein (Fos) that functions as a component of the mammalian transcription factor, activator protein-1. Immediate early genes are genes that are rapidly transcribed and activated within minutes following the presence of a stimulus. In neurons, the stimulus that results in immediate early gene expression may be synaptic excitation, a hormone, or any other stimulus that leads to neuronal depolarization (56, 169). In the present study, we utilized the activation of the *c-fos* gene (occurring immediately with the onset of hypoxia), by detecting the protein product of this gene (the Fos protein, which takes 30-90 minutes to be expressed). Thus, we used the presence of Fos protein due to activation of *c-fos* to detect increased neuronal activity in the immediate physiological responses to hypoxia.

There are several advantages as well as limitations of using *c-fos* expression as a marker for neuronal activation (55, 134). The *c-fos* functional mapping technique is

widely utilized because it is relatively easy to carry out, using conventional immunohistochemical procedures to reveal Fos-positive cells. It can be combined with retrograde or anterograde tracing techniques or immunohistochemistry for other markers, making it is possible to determine afferent or efferent projections of activated (Fos immunoreactive) neurons, receptors expressed by those neurons, and if the neuron is of a certain phenotype. If studies are designed appropriately, the technique can be useful to study the effect of acute specific stimuli in conscious animals in many brain regions. Under baseline conditions *c-fos* expression is low, providing a qualitative comparison to animals exposed to a stimulus (55).

It is important to remember certain limitations, however. Fos is not expressed in all neurons, and neurons that are inhibited will not express Fos (55). Quantifiable Fos expression in most neurons typically requires a strong and sustained stimulus of about 30-90 minutes. In the present studies, although activation of neurons and physiological responses occurred within minutes of the exposure to hypoxia, in order to assess neuronal activation in our immunohistochemical studies (Chapter 2 and 3), we utilized an acute hypoxia exposure of three hours to be able to detect Fos protein expression. Finally, because a sustained stimulus is required, it may be difficult to ascertain if Fos expression in neurons following a stimulus is a direct or indirect result of that stimulus. For example, especially in conscious animals, a sustained period of hypoxia may be a direct consequence of the stimulus itself, or of indirect changes (such as changes in blood pressure) that results from the primary stimulus (37, 55, 134).

Despite these limitations, the *c-fos* functional mapping technique is sensitive enough to label individual neurons and is by far the most effective marker of neuronal activation that can be used with a wide variety of experimental conditions. The combination of this technique in conjunction with retrograde labels was used extensively throughout this work.

1.6.2 Retrograde Tracers:

To identify projection neurons in this study, we utilized two retrograde tracers: Fluoro-Gold (FG) and cholera toxin B (CtB). Retrograde tracers are a powerful tool in identifying projections from a particular region of the brain to another (236). Combined with immunolabeling techniques that characterize the phenotype of a neuron, it provides strong evidence for the role of a specific phenotype in a specific neuronal pathways.

FG is an extremely stable compound and a weak base that enters into lysosomes of axons, neural terminals or cell bodies by passive diffusion. Once in the neuronal lysosome, FG is protonated and the positively charged molecule remains trapped within the cell. Retrograde migration of lysosomes along the cellular cytoskeleton results in labeling of the neuron soma (35, 240). In this study, we visualized FG by its intrinsic fluorescent nature. A consideration in the current experiments is there are reports suggesting FG can be taken up by fibers of passage, leading to labeling in areas that do not have terminals within the FG microinjected region (54, 243).

In our first study, we used two retrograde tracers, FG and CtB. FG was used to label PVN projecting neurons and we compared this to RVLM projecting cells, labeled by

CtB. CtB, a relatively newer tracer, is a plant lectin and has a high affinity to specific sugars on cellular membranes such as monosialogangliosides in neurons. Upon binding of these sugars on the neuronal membrane, it is internalized and retrogradely transported to the soma. Similar to FG, it is taken up by axon terminals, damaged nerve fibers, and axons of passage (62, 129, 146). We utilized an antibody against CtB to visualize this tracer within the cell. In conclusion, we used FG and CtB in conjunction with immunohistochemistry to identify specific neuronal populations, specifically the phenotype of output neurons from the nTS and CVLM. Furthermore, we determined whether these projection neurons are activated in response to hypoxia.

1.6.3 Retrograde Lesion: Selective depletion of noradrenergic and adrenergic neurons by anti-dopamine beta hydroxylase saporin (DSAP)

The DSAP conjugate (an anti-dopamine beta hydroxylase antibody conjugated to a ribosomal toxin saporin) is an immunotoxin that was developed as a means to selectively target neurons that express dopamine beta hydroxylase on their plasma membrane. This includes noradrenergic and adrenergic, but not dopaminergic, neurons (245). Dopamine beta hydroxylase is exteriorized during exocytosis by fusion of the vesicle containing the enzyme to the outer membrane. It therefore acts as an extracellular membrane receptor for the internalization of DSAP. Once inside the cell, saporin irreversibly inactivates the 60S subunit of ribosomes, preventing protein synthesis and eventually killing the cell (221). Previous studies have shown that DSAP can effectively reduce noradrenergic and adrenergic cells when injected into the region

of the terminals of the neurons (for example, noradrenergic neurons projecting to the PVN) (23, 25, 65, 196).

1.6.4 Whole Body Plethysmography

The most common way to non-invasively measure ventilation in awake, unrestrained animals is by using whole-body plethysmography. The theory for this method is based on measuring pressure changes when a gas is expanded or compressed within a rigid chamber, i.e. the plethysmograph, as the animal breathes. This measurement is determined by Boyle's Law, which describes the correlation between the pressure and volume of a gas. This law is written as follows: $P_1 \times V_1 = P_2 \times V_2$, where the subscripts "1" and "2" indicate the first and second condition of the gas and the left side of the equation is equal to the right. Based on this law, if temperature is held constant, there is an inverse relationship between the volume and pressure of a gas. Therefore, when a gas is in a closed container and is compressed, the volume of the gas decreases. As the air is compressed, however, the pressure of the gas inside the container increases simultaneously. Therefore, the product of volume and pressure before and after the gas is compressed remains constant (45). This law is applied to plethysmography as follows: air expands when it is warmed and humidified moving through the animal's lungs during inspiration. This expansion of air causes an increase in pressure within the plethysmograph. Changes in pressure within the plethysmograph are measured via a pressure transducer, which is connected to the plethysmograph. Breathing is therefore measured as the change in pressure associated with the change in volume that occurs when inspired air is warmed and humidified during inspiration.

Finally, the signal from the pressure transducer was converted from an analogue to digital signal, filtered, and amplified (231).

The acute hypoxic exposures in these experiments were performed under poikilocapnic conditions. Therefore, arterial PCO_2 was not maintained at any given level and presumably changes as the animal adjusts minute ventilation. The increase in breathing during hypoxia is caused by the decrease in inspired (and arterial) PO_2 via peripheral chemoreflex activation. However, as the animal increases ventilation it also increases the rate of CO_2 release. This causes arterial CO_2 levels to fall. Thus, the level of breathing during acute poikilocapnic hypoxia results from lowered O_2 levels (which increase breathing) and lower arterial CO_2 levels (which decrease breathing). Therefore, we would expect the increase in breathing observed during poikilocapnic hypoxia would not be as great compared to an exposure where only the O_2 level was being changed. Overall, the hypoxic ventilatory responses measured in this study are indicative of changes in both O_2 and CO_2 rather than O_2 alone (242). However, poikilocapnic hypoxia is a form of physiological hypoxia encountered in nature (i.e., when animals ascend to high altitudes; (241). Isocapnic hypoxia (in which blood CO_2 levels are experimentally maintained at a constant level) allows for an examination of the “isolated” hypoxic ventilatory response (with no confounding effects of CO_2) but it is considered an experimental condition (182).

1.7 Specific Aims and Hypotheses

In recent years, there has been a growing body of evidence demonstrating that the PVN is important in the modulation of the sympathetic nervous system and cardiovascular function. It is unclear, however, how the PVN is involved in the cardiorespiratory responses that are initiated in response to disturbances in body homeostasis during systemic exposures to hypoxia. Also, neural pathways by which the PVN receives chemoafferent information have not been investigated. Hypoxia elicits chemoreflexes affecting ventilation, blood flow redistribution and neuroendocrine responses, all of which could be modulated by the PVN. This dissertation investigated chemoafferent input to the PVN and the contribution of these inputs to cardiorespiratory control. *The overall hypotheses for the current studies are that PVN-projecting cells are activated in response to acute systemic hypoxia and these cells contribute to cardiorespiratory responses elicited by peripheral and central chemoreflex stimulation.* The three main aims of this study are:

Aim 1:

Identify cells in the nTS that are activated following progressive increases in hypoxia and determine the phenotype of these cells. In addition, identify subpopulations of nTS neurons that project to the PVN using retrograde tracer techniques.

Hypothesis: nTS neurons that project to the PVN are activated in response to hypoxia and PVN-projecting nTS neurons are activated primarily at higher intensities of hypoxia (lower levels of inspired oxygen).

Rationale: As described above, the PVN receives projections from a variety of brain regions important in cardiorespiratory control including direct anatomical connections from the nTS. Most PVN-projecting nTS neurons receive polysynaptic connections from visceral afferents and therefore may require a stronger stimulus compared to monosynaptically connected pathways (15). Because catecholaminergic neurons are important in cardiorespiratory reflexes and a substantial number project from the nTS to the PVN, we determined if PVN-projecting nTS neurons activated by hypoxia were catecholaminergic. Furthermore, GABAergic neurons are found throughout the nTS, and inhibition at the level of the nTS is important in modulating cardiorespiratory responses. Therefore, we determined if GABAergic nTS neurons project to the PVN and whether nTS GABAergic neurons are activated by hypoxia.

Aim 2:

Identify cells in the CVLM that are activated following progressive increases in hypoxia and determine if these cells are catecholaminergic. In addition, identify the connectivity of CVLM neurons to the PVN using retrograde tracer techniques.

Hypothesis: CVLM neurons that project to the PVN are activated in response to more severe hypoxia.

Rationale: The PVN receives catecholaminergic input from the caudal ventrolateral medulla. CVLM neurons are activated by chemoreflex stimulation and it has been suggested that the CVLM contributes to neuroendocrine responses to hypoxia, especially at higher intensities of hypoxia. However, whether CVLM cells, in particular catecholaminergic CVLM neurons with direct projections to the PVN, become activated

in response to systemic hypoxia and if they are important in overall ventilatory and sympathetic responses during hypoxia is unknown.

Aim 3:

Determine if catecholaminergic neurons projecting to the PVN play a role in cardiorespiratory responses to peripheral and central chemoreflex activation in conscious animals

Hypothesis: Compared to control rats, animals with lesioned catecholamine projections to the PVN have attenuated ventilatory and cardiovascular responses to acute hypoxia and hypercapnia.

Rationale: We have limited understanding of the pathways by which chemoreflex afferent information is relayed to the PVN. Interestingly, a large proportion of activated PVN-projecting neurons are catecholaminergic, suggesting they might have a very specific function associated with responses to chemoreflex activation. However, whether catecholaminergic input to the PVN plays a functional role in respiratory responses to hypoxia is currently unknown and warrants further investigation.

**CHAPTER 2: Hypoxia activates nucleus tractus solitarii neurons projecting
to the paraventricular nucleus of the hypothalamus**

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2.1 ABSTRACT

Peripheral chemoreceptor afferent information is sent to the nucleus tractus solitarius (nTS), integrated, and relayed to other brain regions to alter cardiorespiratory function. The nTS projects to the hypothalamic paraventricular nucleus (PVN), but activation and phenotype of these projections during chemoreflex stimulation is unknown. We hypothesized that activation of PVN-projecting nTS neurons occurs primarily at high intensities of hypoxia. We assessed ventilation and cardiovascular parameters in response to increasing severities of hypoxia. Retrograde tracers were used to label nTS PVN-projecting neurons and, in some rats, RVLM-projecting neurons. Immunohistochemistry was performed to identify nTS cells that were activated (Fos-immunoreactive, Fos-IR), catecholaminergic, and GABAergic following hypoxia. Conscious rats underwent 3hrs normoxia (n=4, 21% O₂) or acute hypoxia (12, 10, or 8% O₂; n=5 each). Hypoxia increased ventilation and the number of Fos-IR nTS cells (21%, 13±2; 12%, 58±4; 10%, 166±22; 8%, 186±6). Fos expression after 10% O₂ was similar whether arterial pressure was allowed to decrease (-13±1 mmHg) or was held constant. The percentage of PVN-projecting cells activated was intensity-dependent, but contrary to our hypothesis, PVN-projecting nTS cells exhibiting Fos-IR were found at all hypoxic intensities. Notably, at all intensities of hypoxia, approximately 75% of the activated PVN-projecting nTS neurons were catecholaminergic. Compared to RVLM-projecting cells, a greater percentage of PVN-projecting nTS cells was activated by 10% O₂. Data suggest that increasing hypoxic intensity activates nTS PVN-projecting cells, especially catecholaminergic, PVN-projecting neurons. The nTS to PVN catecholaminergic

pathway may be critical even at lower levels of chemoreflex activation and more important to cardiorespiratory responses than previously considered.

2.2 INTRODUCTION

The arterial chemoreceptor reflex responds to decreases in arterial oxygen levels and regulates ventilation, sympathetic nerve activity, and blood pressure. This reflex is critical in physiological adaptations to acute and chronic hypoxia, and also in pathophysiological conditions such as obstructive sleep apnea (OSA), hypertension, and heart failure (114, 198, 215). The first synapse of chemoreceptor afferent nerves in the central nervous system occurs in the nucleus tractus solitarii (nTS). The nTS is a highly integrative nucleus, and chemoreceptor input is modulated within the nucleus before output neurons relay this information to brain regions involved in autonomic and respiratory regulation (6, 204). The phenotype and activity of these output neurons thus are critical in shaping cardiorespiratory reflex responses.

The nTS sends monosynaptic projections directly to the rostral ventrolateral medulla (RVLM) (126, 132) and this is thought to be the primary pathway involved in chemoreflex regulation of sympathetic nerve activity and breathing (91, 126, 131, 132). However, the contributions of other areas in the brain to chemoreflex responses have not been fully characterized. A brain region that plays an important role in autonomic and neuroendocrine integration is the paraventricular nucleus of the hypothalamus (PVN) (117, 229). PVN neurons are activated by hypoxia and hypercapnia (21, 63, 149). In addition, the PVN modulates cardiorespiratory function, including blood pressure

(136, 175), sympathetic nerve activity (46), and respiratory timing and activity (118, 150, 209), via direct projections to the RVLM (12, 13, 46, 94), spinal cord (13, 189, 222), phrenic motor neurons and neurons located in the pre-Botzinger complex (150). There is also evidence that the PVN is required for full expression of chemoreflex responses. Lesion (175) or interruption of neuronal activity in the PVN (190) blunts the magnitude and duration of the pressor and sympathoexcitatory responses evoked by chemoreflex activation. Taken together, these data support the concept that the PVN plays an important role in central processing of the arterial chemoreflex. However, the source of input carrying chemoreceptor afferent information to the PVN remains to be established.

The PVN receives projections from a variety of brain regions important in cardiorespiratory control (206), including direct anatomical connections from the nTS (195). Because the nTS receives afferent input from the peripheral chemoreceptors, we hypothesized that nTS neurons that project to the PVN are activated in response to hypoxia. Previous studies have shown that most PVN-projecting nTS neurons receive polysynaptic connections from visceral afferents. These neurons exhibit variable synaptic responses to solitary tract activation that include small excitatory post synaptic currents and high rates of failed synaptic transmission (15). Thus, successful transmission of peripheral sensory afferent signals from the nTS to the PVN is less likely and may require a stronger stimulus compared to monosynaptically connected pathways. We therefore hypothesized that PVN-projecting nTS neurons would be activated primarily at higher intensities of hypoxia (lower levels of inspired oxygen).

This study used Fos immunohistochemistry combined with retrograde tracers to examine activation of PVN-projecting nTS neurons by increasing hypoxic intensity. Because catecholaminergic neurons are important in cardiorespiratory reflexes (217) and a substantial number project from the nTS to the PVN (206, 209), we determined if PVN-projecting nTS neurons activated by hypoxia were catecholaminergic. Furthermore, GABAergic neurons are found throughout the nTS (11, 38, 78) and inhibition at the level of the nTS is important in modulating cardiorespiratory responses (83). Therefore, we determined if GABAergic nTS neurons project to the PVN and whether they are activated by hypoxia.

Our findings indicate that increasing the intensity of acute (three hours) hypoxia activates progressively more nTS cells, including PVN-projecting neurons. In contrast to our hypothesis, nTS neurons projecting to the PVN were activated even at the lowest intensity of hypoxia studied. With more severe hypoxic stimuli, there was further recruitment of these cells, especially catecholaminergic, PVN-projecting neurons. These results suggest that PVN-projecting nTS neurons contribute to cardiorespiratory responses and play an increasingly important role as chemoreflex stimulation intensifies.

2.3 METHODS

2.3.1 Animals

Experiments were performed on adult male Sprague–Dawley rats (280-350 g, n=43, Harlan, Indianapolis, IN, USA) that were maintained on a twelve hour light–dark

cycle with food and water provided *ad libitum*. All experiments were done in accordance with the “Guiding Principles for the Care and Use of Vertebrate Animals” established by the American Physiological Society and were approved by the University of Missouri Institutional Animal Care and Use Committee. Rats were allowed at least seven days to acclimate to their surroundings prior to experimental procedures.

2.3.2 Surgical procedures

For protocols requiring prior instrumentation or treatment, rats were anesthetized with Isoflurane (AERane, Baxter, Deerfield, IL, USA; 5% for induction and 2–2.5% for maintenance). All procedures were performed using aseptic technique. Rats were treated post operatively with fluids (3 ml, 0.9% saline s.c.), Baytril (2.5 mg/kg i.m., Bayer, Shawnee Mission, KS, USA), and Buprenex (0.03 mg/kg s.c., Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) to maintain hydration status, prevent infection, and for pain management, respectively. Upon recovery from anesthesia animals were returned to their cages.

Microinjection of retrograde tracers. Rats (n=22) were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA), and a midline incision (~ 2 cm) was made along the dorsal surface of the skull. Muscle and fascia were bluntly dissected to visualize bregma and lambda, which were then positioned in the horizontal plane. A small drill hole was made in the skull and the dura cut for introduction of a single barrel glass pipette (OD 10-20 μ m) filled with the retrograde tracer Fluoro-Gold (FG, 2% in deionized water, Fluorochrome Inc., Denver, CO, USA). The target stereotaxic coordinates for PVN microinjections were: 1.8 - 2.0 mm caudal to bregma, $\pm$ 0.5 mm lateral from the midline

and 7.8 mm ventral to the dura. Fluoro-Gold (60-90 nL) was microinjected bilaterally into the PVN using a custom built pressure injection system. The volume of retrograde tracer injected was quantified by monitoring the movement of the meniscus within the pipette using a 150× microscope (Rolyn Optics, Corvina, CA, USA) with a calibrated eyepiece micrometer.

In three animals, PVN injections were performed as described above, and microinjections were also made bilaterally in the RVLM. In these animals, we used two retrograde tracers, FG and cholera toxin B (CtB, 1% in deionized water, List Biological Laboratories, Inc., Campbell, CA). The site of injection (PVN or RVLM) for the individual tracers was varied. In these animals, a catheter was inserted into the aorta via the femoral artery for monitoring arterial pressure and was removed after the surgery. For RVLM injections, the dorsal surface of the medulla was exposed via a limited occipital craniotomy and calamus scriptorius (CS) visualized. The head was then deflected downward until CS was 2.4 mm posterior to interaural 0, positioning the medulla in a horizontal position (126, 166). Target stereotaxic coordinates for the RVLM were: 0.7 - 0.8 mm rostral and 1.6 - 1.8 mm lateral to CS, and 3.6 - 4.2 mm ventral to the dorsal surface of the brain. The RVLM was identified functionally by pressor responses (≥ 15 mmHg) to microinjection of L-glutamate (10 mM, 30 nL) through one barrel of a double barreled pipette. Without moving the pipette, the retrograde tracer (30 nL) was microinjected through the second barrel.

Retrograde tracers were injected over one minute and the pipette remained in the tissue for at least five minutes to minimize movement of tracer up the injection

tract. The pipette was then removed and the incision site closed. The animals were allowed 7 - 10 days for surgical recovery and for transport of the retrograde tracer. During this period, daily clinical examination and body weight measurements were performed.

Blood pressure measurements. To assess mean arterial blood pressure (MAP) and heart rate (HR) changes in response to acute hypoxia, a separate group of rats (n=11) without injection of retrograde tracer was instrumented with a telemetry device (TA11PA-C40, Data Sciences International, USA). Via a midline incision (3 - 4 cm), the abdominal aorta was visualized and the catheter probe of the telemetry device was inserted. The site was sealed with a cellulose patch and tissue adhesive. The transmitter was secured to the abdominal muscle using nonabsorbable suture, and the skin incision closed. Animals were allowed to recover for at least 10 days. Arterial pressure, HR, and the strength and quality of the telemetry signal were evaluated daily after surgery. Five days before immunohistochemistry experiments in which MAP was maintained constant during acute hypoxia (see below), four of these animals also were instrumented with a femoral venous catheter.

In a separate group of animals (n=5) catheters (PE10 fused to PE50) were placed in the femoral artery and vein for measurement of arterial pressure and drug administration, respectively. Catheters were secured, tunneled subcutaneously and exteriorized at the back of the neck then filled with heparinized saline (10 units/mL). Animals were allowed 5 days for recovery before plethysmography experiments in which MAP was held constant.

2.3.3 Oxygen saturation and respiratory reflex responses to increasing severity of hypoxia

Ventilation was assessed in conscious, freely moving rats (n=6) by whole body plethysmography. Animals were placed in a Plexiglas plethysmography chamber (Model: 600-2400-001, Data Sciences International, USA) that contained inlet and outlet ports to permit airflow through the chamber. The animal and reference chambers were connected to a differential pressure transducer (Validyne Engineering Corp., Northridge, CA, USA; model: DP 45-34) to measure respiratory parameters (PowerLab Data Acquisition System, ADInstruments, Colorado Springs, CO, USA). We used a leaky, flow-through plethysmography chamber and the pressure signal recorded was proportional to volume changes (50); body temperature and metabolic rate were not quantified. In order to obtain a measure of tidal volume (tidal volume index), we integrated the area under the inspiratory pressure curve and normalized to body weight. Ventilatory parameters measured included: respiratory rate (RR, breaths per min), tidal volume index and minute ventilation index (RR x tidal volume index). Oxygen saturation was measured using a collar pulse oximeter (MouseOx, Starr Life Sciences Corp., USA). All animals were acclimatized to the chamber environment for two to three hours at least five times prior to the experiment. On the day of the experiment, rats were weighed and allowed to acclimate in the chamber for 60 minutes. Ventilation and oxygen saturation were monitored continuously at baseline and during exposure (ten minutes) to 21, 12, 10 and 8% O₂-balanced N₂. Ventilatory parameters were measured from an average of about 20 consecutive breaths independent of sniffs, sighs or

movement artifacts within the last minute of exposure to each level of oxygen (125, 127). This protocol was repeated after a 30 minute recovery period breathing room air. The order of gas exposure was varied to produce a balanced design.

Maintenance of arterial pressure. Preliminary experiments indicated that exposure to acute hypoxia decreased MAP. To evaluate if changes in MAP affected respiratory responses, we measured the hypoxic ventilatory response independent of changes in MAP in five animals. Respiratory variables were measured using plethysmography as described above during normoxia, acute hypoxia (10% inspired O₂) and acute hypoxia during infusion of the vasoconstrictor phenylephrine (PE; 0.6 mg/mL, 0.1-0.5 mL/hr, i.v.) through the venous catheter in order to maintain MAP constant.

2.3.4 nTS neuronal activation to increasing severity of hypoxia

Five to seven days after injection of retrograde tracer, conscious rats in their home cages were acclimated to the hypoxia chamber (Biospherix Inc., Redfield, NY, USA) for 1-3 hours per day for 2 days to allow them to become accustomed to the environmental stimuli associated with the chamber. On the day of the experiment, conscious rats (n=22) with previous PVN or PVN and RVLN microinjections were allowed to acclimate for 30 minutes in the chamber (21% O₂). The gas mixture then was adjusted to bring the air in the chamber to: 21% O₂ (normoxic control, n=4), 12% O₂ (n=5), 10% O₂ (n=5) or 8% O₂ (n=5) and maintained at that percentage for three hours via a negative feedback control system, similar to previous studies (21, 55, 126, 234). Double labeled animals (n=3) were only exposed to 10% O₂. Gas levels and temperature were monitored throughout the protocol.

Maintenance of arterial pressure. To evaluate neuronal activation due to acute hypoxia, independent of changes in arterial pressure, in a separate group of rats, MAP and HR were monitored continuously using telemetry during two hours of normoxia (21% O₂; n=7), 10% O₂ (n=8), or 10% O₂ with infusion of PE (0.6 mg/mL, 0.1-0.5 mL/hr, i.v.; n=4) to maintain MAP constant. In seven of these rats, MAP and HR were measured under more than one condition, with at least five days between experiments. Following experiments in which MAP was held constant, animals were perfused and brains processed for Fos immunohistochemistry. Fos expression in these animals was compared to animals in which MAP was not held constant.

2.3.5 nTS immunohistochemistry

Transcardial perfusion and tissue preparation. Immediately following exposure to hypoxia, animals were deeply anesthetized with Isoflurane and transcardially perfused with heparinized, Dulbecco's Modified Eagle Medium (Sigma, 125 mL, pH 7.4) bubbled with oxygen, followed by 4% paraformaldehyde (PFA, Sigma, 500 mL, pH 7.4). The brains were removed and post-fixed in 4% PFA overnight. Thirty micron hindbrain sections were cut on a vibrating microtome (VT 1000S; Leica, Germany).

Immunohistochemistry was performed on every sixth section (separated by 180 µm).

Immunohistochemistry protocols. Two sets of immunohistochemistry protocols were performed on tissue from animals with PVN microinjections of retrograde tracer. Fos-immunoreactivity (IR) was used as a marker for cells within the nTS that were activated after hypoxia (55). Neurons that projected to the PVN were identified by the presence of retrograde tracer. Tyrosine hydroxylase (TH), the rate limiting enzyme in

catecholamine synthesis, was used as a marker for catecholaminergic neurons.

GABAergic neurons were identified using immunoreactivity for glutamic acid decarboxylase isoform 67 (GAD67), a GABA synthesizing enzyme.

For Fos and GAD67, free floating coronal sections at room temperature were rinsed (3×10 min) in 0.01 M PBS (phosphate buffered saline, pH 7.4) and incubated in 0.3% hydrogen peroxide (30 minutes) to quench endogenous peroxidases before preblocking (30 minutes) with 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc., West Grove, PA, USA) in 0.01 M PBS. Sections were rinsed and incubated (72 hours) in antibodies against GAD67 (mouse anti-GAD67, 1:5000; MAB5406, Millipore Inc., Billerica, MA) and Fos (rabbit anti-Fos, 1:3000; PC38, Calbiochem; Darmstadt, Germany) with 3% NDS in 0.01 M PBS. Sections were then rinsed and incubated for two hours in donkey anti-mouse biotinylated IgG (1:300; Jackson ImmunoResearch) and Cy3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) with 3% NDS in 0.01 M PBS. The sections were again washed and incubated in a series of steps for Tyramide Signal Amplification (one hour Streptavidin-HRP, 15 min TSA-biotin using TSA kit from Perkin Elmer SAT700001). Finally, sections were rinsed, then incubated with neutravidin/avidin Oregon Green (1:300, Invitrogen Oregon Green® 488, Carlsbad, CA) with 3% NDS in 0.01 M PBS for 2 hours. After a final rinse, the sections were mounted on gel-coated slides, air-dried, coverslipped with ProlongGold (Invitrogen, P36930), and sealed with nail polish. Immunohistochemistry involving GAD67 was always performed within 1 week of perfusion of the tissue.

For Fos/TH immunohistochemistry, free floating sections underwent a series of rinse/blocking steps as described above. Sections were incubated for 24 hours in 1% NDS and 0.3% Triton-0.01M PBS containing primary antibodies against Fos (rabbit anti-Fos, 1:3000, Calbiochem) and TH (mouse anti-TH, 1:1000, Millipore Inc.). The following day, sections were rinsed then incubated for two hours in Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG (1:200 Jackson ImmunoResearch) with 1% NDS in 0.3% Triton-0.01M PBS. Sections then were rinsed and mounted on gel-coated slides as described above. In addition to animals with PVN microinjections of retrograde tracers, tissue from rats in which MAP was maintained constant during hypoxia also underwent this protocol.

In PVN and RVLM microinjected animals the retrograde tracers FG and CtB were visualized using guinea pig anti-FG (Protos Biotech, New York, NY, USA, 1:500) and goat anti-cholera toxin B subunit (List Biological Laboratories, Inc., 1:2000), respectively. Sections from these animals also underwent a Fos/TH immunohistochemistry protocol similar to that described above. Briefly, sections were incubated for 24 hours in a solution containing antibodies against FG, CtB, Fos and TH. They then underwent a two hour incubation in AMCA-conjugated donkey anti-guinea pig IgG, Cy2-conjugated donkey anti-goat IgG, Cy5-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG (all from Jackson ImmunoResearch).

Antibody specificity. Individual immunohistochemistry protocols were conducted simultaneously on tissue from hypoxic and normoxic animals and in each protocol at least one section served as a control (was not incubated in a primary or secondary

antibody). Previous experiments verified optimal primary and secondary antibody concentrations. Antibodies were verified for their specificity by the vendor indicated using western blots (anti-Fos) or preincubation with immunogen (anti-FG), or by our laboratory (5) and others (28) (anti-TH or anti-GAD67, respectively) using western blot. In addition, as in previous studies (1), we confirmed the specificity of the FG and CtB antibodies in pilot studies in which we compared the intrinsic fluorescence of FG or fluorescent-conjugated CtB with that of the appropriate antibody. An exact match of the antibody with the intrinsic fluorescence was observed.

Verification of microinjection sites. In all animals with retrograde tracer injections, the forebrain or brainstem was sectioned and examined to verify the location of PVN or RVLM injection sites, respectively. A standard brain atlas (58) was used to anatomically verify the location of each injection site. Only the animals in which the microinjections were localized to the PVN (and RVLM in double labeled animals) were used for immunohistochemistry protocols.

2.3.6 Microscopy and image analysis

Image acquisition. Brainstem sections were examined using an Olympus epifluorescent microscope (BX51). Filter sets for AMCA, Oregon Green or Cy2, Cy3, Cy5, and Fluoro-Gold were used as appropriate to visualize positive labeling. The image was brought into focus using the filter set for Cy3 (Fos-IR). Images in this same focal plane were then captured under each filter set using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ, USA). Images subsequently were combined and analyzed with Image J (ver. 1.41, NIH) using a custom made plugin (GAIA Group,

Novato, CA, USA, <http://gaiag.net/index.html>). Image brightness and contrast only were adjusted for image clarity.

nTS analysis. For immunohistochemical analysis, we evaluated the caudal nTS, the primary site of cardiorespiratory afferent projections. The section containing the caudal pole of the area postrema was identified and designated as calamus scriptorius (defined as “0”). Seven sections of the nTS were examined extending from caudal to rostral (-540 to +540 μm relative to calamus scriptorius). Because in some animals the injections were not centered on the PVN bilaterally and since the projection from nTS to PVN is primarily ipsilateral (207), we evaluated the nTS unilaterally, ipsilateral to the appropriate injection site. On the digital image of each section, the region of the nTS was outlined unilaterally and cells were counted manually using Image J. Two individuals blinded to the experimental protocol performed the counting and counts for each section were averaged. The following criteria were used to identify positively labeled cells: Fos-IR was identified as round or ovoid-shaped nuclear staining with a visible nucleolus. GAD67-IR somas exhibited cytoplasmic staining surrounding a blank nucleus. TH-IR cells exhibited cytosolic labeling with visible processes and a blank nuclear region. FG or CtB-positive cells displayed bright or punctate cytosolic labeling.

When the above criteria were met under more than one filter set, the cells were considered double-labeled. Cells that contained positive signal under all three filter sets were considered triple-labeled. Immunohistochemical data were analyzed as the total (sum) of counts from all sections examined within the nTS, and also at each caudal-rostral level of the nTS evaluated. In experiments studying the role of changes in MAP

on expression of Fos due to hypoxia, Fos-IR in specific subregions (commissural, medial, ventrolateral, ventral, dorsolateral, and central subnuclei) of the nTS also was evaluated by comparing sections to a standard brain atlas (177).

2.3.7 Statistical analysis.

Statistical analyses were performed using SigmaPlot (11.0, Systat Software, San Jose, CA, USA) . All data are presented as mean $\pm$ SE. Significance was accepted at $p \leq 0.05$. A one-way repeated measures ANOVA was used to compare the number of Fos, TH, GAD67 and FG labeled cells, co-labeling, and percentages of co-labeling among the three intensities of hypoxia and normoxia. The percent of co-labeling was calculated by dividing the number of co-labeled cells by the total number of cells of each individual phenotype. For example, (Fos and FG co-labeled cells)/(total number of FG labeled cells) X 100 = the percent of projecting cells activated. A one-way repeated measures ANOVA was also used to evaluate respiratory changes in response to increasing intensity of hypoxia. Caudal-rostral and subregional distributions of labeling were analyzed by two-way repeated measures ANOVA. Blood pressure and heart rate responses to hypoxia were analyzed with a two-way repeated measures ANOVA. When appropriate, ANOVAs were followed by post-hoc analysis using Fisher's LSD test.

2.4 RESULTS

2.4.1 Chemoreflex respiratory responses during increasing intensities of hypoxia

In conscious rats, exposure to increasing hypoxia progressively decreased arterial oxygen saturation, accompanied by an intensity-dependent increase in respiratory rate, tidal volume

index, and minute ventilation index. The response of an individual rat to activation of the chemoreflex with three intensities of acute hypoxia is shown in Figure 1A. Respiratory variables increased within the first minute of hypoxic challenge and were graded with the intensity of hypoxia. Group data are shown in Figure 1B-E. There was a progressive decrease in arterial blood oxygen saturation and graded increases in respiratory rate, tidal volume index and minute ventilation index to increasing hypoxia. All parameters returned to baseline within 5 minutes when rats were allowed to recover at 21% O₂. The increase in minute ventilation index was due to both increased respiratory rate and tidal volume index.

2.4.2 Immunohistochemistry of the nTS after graded hypoxia

Hypoxia increases Fos immunoreactivity in the nTS. Fos-immunoreactivity (IR) was used to examine activation of nTS cells by graded hypoxia. Figure 2A includes examples of Fos-IR in brainstem sections containing the nTS from animals that were exposed to three hours of normoxia (21% O₂) or increasing intensities of hypoxia (12, 10, 8% inspired O₂). On the right, a diagrammatic representation and bright field image depict the regions of the nTS shown in the photomicrographs. In control animals exposed only to normoxia (21% O₂) the nTS contained very few Fos-IR cells, indicating a low level of basal Fos expression. The number of Fos-IR cells increased in relation to the intensity of hypoxia. Mean data showing the increase in total number (sum) of all Fos-IR cells counted in seven sections of the nTS are depicted in Figure 2B. Significant increases in Fos-IR in the nTS were apparent at 12% O₂. Further intensifying the hypoxic stimulus produced a progressive increase in the number of cells activated.

A distinct caudal-rostral pattern of activation in response to hypoxia was seen in the nTS. Two-way repeated measures ANOVA revealed main effects of hypoxic intensity

and caudal-rostral level but the interaction ($p=0.07$) did not reach statistical significance. A greater proportion of Fos-IR was localized in the caudal nTS and decreased in more rostral sections. The greatest expression of Fos was observed at $-540\text{ }\mu\text{m}$ caudal to CS while the least amount of Fos-IR was seen at $540\text{ }\mu\text{m}$ rostral to CS (Figure 2C).

2.4.3 Maintaining arterial blood pressure during acute hypoxia does not affect the hypoxic ventilatory response or nTS Fos-IR

Because preliminary studies indicated that acute hypoxia produced moderate depressor responses which may alter respiration and Fos-IR via interactions with the baroreflex, the hypoxic ventilatory response and nTS neuronal activation were compared in rats after acute hypoxia ($10\%\text{ O}_2$) or hypoxia with MAP held constant by PE infusion ($10\%\text{ O}_2 + \text{PE}$). Normoxia did not alter MAP or HR (Figure 3A and B). Acute hypoxia in conscious rats induced an early increase in HR, and a sustained depressor response which was prevented by simultaneous infusion of PE. Maintaining MAP constant did not alter oxygen saturation ($10\%\text{ O}_2$: $70.1\pm0.9\%$; $10\%\text{ O}_2 + \text{PE}$: $70.5\pm1.0\%$) or ventilatory responses to hypoxia, including respiratory rate ($10\%\text{ O}_2$: 148 ± 9 ; $10\%\text{ O}_2 + \text{PE}$: 144 ± 8 breaths per minute), tidal volume index ($10\%\text{ O}_2$: 0.447 ± 0.02 ; $10\%\text{ O}_2 + \text{PE}$: 0.454 ± 0.01 arbitrary units), and minute ventilation index ($10\%\text{ O}_2$: 67 ± 7 ; $10\%\text{ O}_2 + \text{PE}$: 66 ± 4 arbitrary units).

The total number of Fos-IR nTS neurons following hypoxia was similar whether MAP was allowed to fall ($10\%\text{ O}_2$: 184 ± 9) or was held constant ($10\%\text{ O}_2 + \text{PE}$: 176 ± 6). In addition, the caudal-rostral distribution of Fos-IR in the nTS was similar between groups (Figure 3C). To further evaluate potential differences in Fos expression, we also

compared the distribution of Fos-IR cells within nTS subnuclei of the two hypoxic groups (Figure 3D) and the number of activated catecholaminergic nTS cells. In both groups, Fos-IR was significantly greater in the medial, dorsolateral and ventrolateral nTS compared to other subnuclei, with the greatest number of Fos-IR cells in the medial subnucleus. Importantly, Fos-IR in all subregions was similar whether arterial pressure was allowed to fall during hypoxia or was maintained constant. The number of activated catecholaminergic cells also was similar between the hypoxic groups (10% O₂: 57±3; 10% O₂ + PE: 54±7). Thus, the modest decrease in MAP did not alter the hypoxic ventilatory response nor nTS neuronal activation due to 10% hypoxia.

2.4.4 Neuronal phenotypes activated by acute hypoxia

Because the PVN plays an important role in modulation of cardiorespiratory responses to chemoreflex activation, and the nTS projects to the PVN, we examined whether hypoxia resulted in Fos-IR in nTS cells projecting to the PVN (FG labeled). We also evaluated whether activated and/or PVN-projecting neurons were catecholaminergic (TH-IR) or GABAergic (GAD67-IR). Labeling for all of these phenotypes was observed in the nTS. As expected, the number of TH-IR and GAD67-IR cells was independent of severity of hypoxia (Table 1).

Verification of injection sites. Before retrograde labeling in the nTS was evaluated, histological verification of microinjection sites was performed in all animals. Figure 4A contains representative photomicrographs from two individual animals that received FG injections in the PVN (left) or RVLM (right). Schematics (177) of the PVN (B) or RVLM (D) illustrate the center of injection sites in all the animals. For RVLM

microinjections, injection sites were also identified functionally by pressor responses (17+ 2 mmHg) to microinjection of glutamate (10 mM, 30 nL) before injection of the retrograde tracer. Only the animals in which the microinjections were located within the PVN (and RVLM in double labeled animals) were used in immunohistochemistry studies. Location of injections (Figure 4B and C) and the number of retrogradely labeled (FG) cells (Table 1) were similar in animals from the normoxic and all hypoxic groups.

PVN-projecting nTS neurons are activated by hypoxia. Microinjection of the retrograde tracer FG into the PVN resulted in labeling at all levels of the nTS evaluated. The caudal-rostral distribution of PVN-projecting cells within the nTS was similar in all groups, with more PVN-projecting neurons at caudal levels of the nTS and fewer more rostrally (data not shown). Further characterization of PVN-projecting nTS neurons revealed that almost none of the PVN-projecting neurons was GABAergic (FG + GAD67-IR, Table 1). However, combined data indicate that almost half ($47\pm 2\%$) of PVN-projecting neurons (FG) were co-labeled with TH-IR (FG+TH).

Increased expression of Fos-IR in PVN-projecting nTS neurons was observed as hypoxic intensity was increased. Figure 5A includes examples of Fos-IR (pseudo-colored red) and FG labeling (pseudo-colored blue) in the nTS of animals that were exposed to normoxia (21% O₂) and each intensity of hypoxia. There were very few PVN-projecting cells that were activated after breathing 21% O₂. Note the increase in co-labeling of Fos-IR and FG at increased intensities of hypoxia.

The number of PVN-projecting nTS cells that co-expressed Fos was significantly increased at all levels of hypoxia, with greater Fos-IR at higher intensities. Exposure to

hypoxia significantly increased the number of nTS cells co-labeled with FG and Fos-IR to 11 ± 1 , 30 ± 2 and 34 ± 3 cells, for 12, 10, and 8% O₂, respectively, compared to normoxic animals (3 ± 1). The number of FG and Fos-IR co-labeled cells was significantly greater at 10% and 8% compared to 12% O₂; however there was no difference between 10 and 8% O₂. We also examined the percentage of PVN-projecting nTS cells that co-expressed Fos (Figure 6A). For all intensities of hypoxia, the percent of PVN-projecting cells that was activated was significantly increased compared to normoxia. Furthermore, breathing either 10% or 8% O₂ increased Fos-IR compared to 12% O₂ but there was no difference between the two highest intensities of hypoxia.

Catecholaminergic nTS neurons are activated by hypoxia. Catecholaminergic (TH-IR) cells were found throughout the caudal-rostral extent of the nTS. Combined data indicate that nearly a third ($31\pm2\%$) of catecholaminergic nTS cells were PVN-projecting (TH-IR and FG co-labeled; Table 1). There were no differences among groups in the number of cells exhibiting co-labeling for TH-IR and FG labeling.

Co-localization of Fos-IR in catecholaminergic neurons was observed in the nTS after exposure to every intensity of hypoxia. Figure 5B contains photomicrographs of the nTS showing labeling for catecholaminergic (TH-IR, pseudo-colored green) and activated cells (Fos-IR, pseudo-colored red) from normoxic and hypoxic animals at three intensities of hypoxia. There were very few TH-IR cells that were activated following normoxia. Note that the number of co-labeled cells increased with exposure to higher intensities of hypoxia.

The number of catecholaminergic nTS cells that was activated (TH- and Fos-IR) increased significantly at all intensities of hypoxia. Compared to normoxia (4 ± 2 cells) the number of co-labeled cells increased significantly to 21 ± 3 , 57 ± 3 and 69 ± 2 cells for 12, 10, and 8% O₂, respectively. There was a graded effect of hypoxia, with each level of hypoxia activating significantly more catecholaminergic neurons compared to lower intensities. We also examined TH- and Fos-IR co-labeling as a percentage of the number of catecholaminergic nTS cells (Figure 6B). At all intensities of hypoxia, the percent of catecholaminergic cells exhibiting Fos-IR was significantly greater than after normoxia. Furthermore, at each increasing intensity of hypoxia there was a significantly greater percent of TH-IR cells that was activated.

PVN-projecting catecholaminergic neurons are activated by hypoxia. Fos-IR due to hypoxia in catecholaminergic vs. non-catecholaminergic PVN-projecting nTS neurons also was examined. Figure 5C includes photomicrographs showing examples of PVN-projecting catecholaminergic nTS cells that also exhibited Fos-IR in animals exposed to normoxia or increasing hypoxia. There were few triple labeled cells (Fos-IR, TH-IR, FG) in the normoxic animals, while the number of cells that were triple-labeled increased with hypoxia. The number of PVN-projecting catecholaminergic nTS cells that was activated increased significantly at each level of hypoxia (21%: 3 ± 1 , 12%: 11 ± 1 , 10%: 25 ± 4 , 8%: 34 ± 4). Figure 6C includes mean data indicating the percent of PVN-projecting catecholaminergic or non-catecholaminergic nTS cells that was activated by hypoxia. At all intensities of hypoxia, the percent of FG and TH-IR co-labeled cells that also exhibited Fos-IR was significantly greater than normoxia, and activation was graded with intensity

of hypoxia. After breathing 8% O₂, approximately two-thirds of PVN-projecting catecholaminergic cells were activated. Compared to catecholaminergic cells, there were significantly fewer non-catecholaminergic PVN-projecting cells activated at each intensity of hypoxia. Of all activated PVN-projecting nTS neurons, at each intensity of hypoxia, the majority (approximately 75%) were catecholaminergic.

Activation of GABAergic neurons by hypoxia. GABAergic neurons were observed throughout the nTS and there was no difference in the caudal-rostral distribution of GAD67-IR cells between normoxic and hypoxic animals (data not shown). Almost no GABAergic cells also projected to the PVN (Table 1). Photomicrographs showing examples of cells in the nTS with GAD67-IR (pseudo-colored green) and Fos-IR (pseudo-colored red) from normoxic and hypoxic animals at three intensities of hypoxia are included in Figure 5D. Activated GABAergic neurons were observed in the nTS after exposure to all intensities of hypoxia. There were very few GAD67-IR cells that were also Fos-IR after breathing 21% O₂ (1 ± 0) and the number of co-labeled cells increased with hypoxia (12%: 8 ± 1 , 10%: 16 ± 2 , 8%: 14 ± 3). The percent of GABAergic nTS cells that was also Fos-IR after hypoxia is shown in Figure 6D. At all intensities of hypoxia, the percent of GAD67-IR cells that was co-labeled with Fos-IR was significantly greater than normoxia. Unlike the other activated phenotypes, however, maximum percent activation of GABAergic cells occurred after breathing 10% O₂ and, although changes were small, the percent of activated GABAergic cells decreased significantly at 8% ($14 \pm 2\%$) compared to 10% O₂ ($19 \pm 3\%$).

2.4.5 PVN-projecting vs. RVLN-projecting nTS neurons

Previous studies from our laboratories (126) determined that hypoxia (10% O₂) results in Fos expression in RVLM-projecting nTS neurons. In the current study, animals received microinjections of retrograde tracers in both the PVN and RVLM and we compared directly the activation by hypoxia (10% inspired O₂) of nTS neurons that projected to these two regions. Immunohistochemical analysis (Figure 7A and B) revealed separate populations of PVN- and RVLM-projecting nTS neurons that were activated (Fos-IR) by hypoxia. Fewer nTS cells projected to the PVN (63±3) than to the RVLM (158±7) and no cells were identified that projected to both the PVN and RVLM. Following hypoxia, the number of RVLM-projecting cells that was Fos-IR (40±3) was significantly greater than the number of co-labeled PVN-projecting cells (29±2). However, relative to the specific population of projecting cells, the percent of PVN-projecting nTS cells activated by hypoxia (10% O₂) was significantly greater than the percent of RVLM-projecting cells activated (Figure 7C).

2.5 DISCUSSION

This study determined whether a direct pathway between the nTS and PVN is activated by increasing severity of hypoxia. We hypothesized that PVN-projecting nTS neurons are activated by hypoxia, but because the majority of these neurons receive weak, polysynaptic inputs from visceral afferents, (15), a strong hypoxic stimulus would be required to activate them. Data indicate that even the lowest intensity of hypoxia studied (12% inspired O₂) activated nTS neurons that project to the PVN. Furthermore, increasing the hypoxic intensity progressively augmented activation of nTS neurons,

including PVN-projecting neurons. Catecholaminergic PVN-projecting neurons were particularly sensitive to hypoxia. These neurons may therefore play a more important role in cardiorespiratory responses to chemoreflex activation than previously understood.

2.5.1 Increasing intensity of hypoxia augments ventilatory responses and activation of nTS neurons

Because increasing the severity of hypoxia produced progressive increases in ventilation and neuronal activation in the nTS, it is likely that the activated cells are involved in chemoreflex function. The pattern of activation, with more Fos-IR in the caudal nTS, is consistent with previous studies showing that this area of the nTS is innervated by carotid body afferent fibers (77). These results also support previous work evaluating Fos expression in the nTS after stimulation of the chemoreflex in rats exposed to two to three hours of moderate to severe hypoxia (21, 104, 126, 234).

The progressive increase in Fos-IR in the nTS due to increasing hypoxia is most likely related primarily to chemoreflex activation rather than the influence of other afferents such as the arterial baroreceptors. In this study, hypoxia (10% O₂) resulted in a brief increase in heart rate and a small, sustained decrease in arterial pressure that occurred after five minutes. While some studies report an increase in MAP due to chemoreflex activation (18, 48, 104), our results are consistent with work in which systemic hypoxia resulted in relatively small decreases (10-20 mmHg) in arterial pressure (71, 153, 154, 184, 217). The decrease in MAP may be due to hypoxia-mediated vasodilation, either directly or indirectly via the production of vasodilator

metabolites such as adenosine, carbon dioxide, or nitric oxide (153, 154). Importantly, both the hypoxic ventilatory response and the number and distribution of Fos-IR nTS cells following hypoxia was similar whether MAP was allowed to decrease or was held constant. Thus it is unlikely that baroreflex responses to the modest decrease in MAP contributed substantially to hypoxia-induced Fos expression in the nTS. Of course, it is possible that the same nTS neurons are activated by both chemoreflex and blood pressure changes; however separate populations of neurons appear to be activated by these distinctly different stimuli (48, 162, 163). Although we cannot eliminate the possibility that other factors could contribute, taken together, our data suggest that neuronal activation in the nTS after acute hypoxia was predominantly due to chemoreflex activation.

2.5.2 Involvement of PVN-projecting nTS neurons in the peripheral chemoreflex pathway

The PVN plays an important role in the modulation of breathing and sympathetic nerve activity induced by chemoreflex activation. Microinjection of excitatory amino acid or adrenergic antagonists into the PVN of anesthetized rats (136) or prior bilateral electrolytic lesion of the PVN in awake rats (175) significantly blunts the immediate pressor response following peripheral chemoreceptor activation with injection of potassium cyanide or inorganic phosphate. Furthermore, blockade of the PVN using lidocaine attenuates the arterial pressure, renal sympathetic nerve activity, and phrenic nerve response evoked by potassium cyanide-induced stimulation of arterial

chemoreceptors (190). An important question not addressed by previous studies is the source of input to the PVN during chemoreceptor stimulation.

Retrogradely labeled PVN-projecting cells were observed in all caudal-rostral levels of the nTS evaluated. The greatest number of PVN-projecting neurons was found in the caudal nTS and gradually decreased more rostrally. A consideration in the current experiments is that, although some studies indicate that FG is not taken up by fibers of passage (35, 210), there are reports suggesting otherwise (54, 243). Therefore, it is possible that some FG-labeled nTS neurons may project to brain regions other than the PVN via fibers that course through the PVN. Nevertheless, the current findings are in consensus with previous studies demonstrating anatomical connections between the nTS and PVN (51, 52, 101, 206). Also consistent with previous studies indicating that a large proportion of PVN-projecting cells in the nTS are catecholaminergic (51, 101, 206), we found that almost half of PVN-projecting nTS neurons were TH-IR. In contrast, almost none of the PVN-projecting neurons were GABAergic. As expected, the number and phenotype of PVN-projecting nTS neurons in the present study were similar among groups, independent of exposure to hypoxia.

The observation that acute hypoxia produced an intensity-dependent activation of PVN-projecting nTS neurons suggests that a direct pathway from the nTS to the PVN is important in mediating cardiorespiratory responses to hypoxia. This does not eliminate a potential role for other pathways however. The PVN not only receives direct projections from the nTS, but also from the ventrolateral medulla (52), circumventricular organs (5, 17), parabrachial complex and the periaqueductal gray

(161, 176). All of these projections could be important in conveying information contributing to autonomic and neuroendocrine responses elicited by hypoxia. Nevertheless, the graded and substantial activation of PVN-projecting nTS neurons in the current experiments suggests an important role for the nTS-PVN pathway in chemoreflex responses.

Because in vitro studies indicate that the fidelity of transmission of visceral afferent information to PVN projecting nTS neurons is relatively low (15), we expected that severe hypoxia might be required to activate these neurons. However, PVN-projecting nTS neurons were activated by even the lowest intensity of hypoxia examined. It is possible that the mildest hypoxic stimulus (12% inspired O₂) used in this study was of sufficient intensity, particularly when applied for a period of three hours, to activate these polysynaptically connected nTS neurons. Alternatively, there is a subpopulation of PVN-projecting nTS neurons that are monosynaptically connected to visceral afferents (15), and these neurons may be activated at the lower intensities of chemoreflex activation. With increasing severity of hypoxia, nTS neurons receiving polysynaptic inputs may be recruited. Interestingly, the overall activation of PVN-projecting nTS neurons appeared to be maximal when breathing 10% O₂, with no further increase due to more intense hypoxia. It is possible that these polysynaptic connections will serve as a low-pass filter, limiting activation of PVN-projecting neurons. Further studies evaluating activation of nTS neurons following exposure to a greater range of hypoxic intensities are required to address these questions.

2.5.3 Phenotypes of nTS neurons in the peripheral chemoreflex pathway

Catecholaminergic neurons. Brainstem catecholaminergic neurons have been implicated in mediating adaptive autonomic and neuroendocrine responses to cardiorespiratory challenges (9, 37, 69, 193), including hypoxia (71, 104, 126, 234). We confirmed and extended this previous work, showing that increasing severity of hypoxia produced a progressive increase in activation of nTS catecholaminergic neurons and that a substantial proportion of these neurons project to the PVN. Remarkably, of PVN-projecting catecholaminergic cells, about thirty percent were activated by moderate hypoxia (12% O₂) and activation of these cells more than doubled with more severe hypoxic challenges. In contrast to PVN-projecting neurons as a whole, the subpopulation of catecholaminergic PVN-projecting neurons exhibited progressive activation at all intensities of hypoxia. Significantly fewer non-catecholaminergic PVN-projecting neurons were activated by hypoxia. Thus, hypoxia preferentially activates catecholaminergic PVN-projecting nTS cells and these neurons may specifically contribute to chemoreflex responses throughout the range of hypoxic challenges.

The catecholaminergic inputs to the PVN presumably relay sensory information and influence respiratory, neurosecretory and/or autonomic responses during chemoreflex activation, likely by activation of PVN adrenergic receptors (136). For example, stimulation of alpha adrenergic receptors increases the excitability of spinally-projecting PVN neurons by augmenting glutamatergic and attenuating GABAergic neurotransmission (39, 140). Furthermore, blockade of PVN adrenergic receptors blunts

the cardiovascular response to carotid chemoreceptor stimulation with inorganic phosphate (136). Thus, adrenergic inputs may augment sympathetic responses during hypoxia, although the source of adrenergic inputs to the PVN was unknown. Together, data from the current study indicate that catecholaminergic projections from the nTS to the PVN are activated by hypoxia, and thus these nTS projection neurons may influence cardiorespiratory responses to chemoreflex activation.

Interestingly, the transcription factor *Phox2b* is expressed in a population of catecholaminergic neurons of the nTS, but rarely in neurons that are GAD67-IR (111). Furthermore, *Phox2b* is expressed in a group of brainstem neurons involved in chemoreception, including hypoxia-sensitive nTS neurons that project to the retrotrapezoid nucleus, and it has been suggested that *Phox2b* defines a series of neurons critical to both central and peripheral chemoreception (30, 75). Whether *Phox2b* is expressed in activated catecholaminergic neurons that project to the PVN remains to be clarified.

GABAergic neurons. GABAergic neurons are interspersed throughout the nTS and contribute to cardiorespiratory regulation (78, 83, 109). They serve as local interneurons to modulate visceral afferent input (14, 135), project to other brainstem regions and the spinal cord (11, 223, 243), and are activated following visceral afferent stimulation (38, 243). Consistent with previous studies, (11, 14, 78, 223), we found GAD67-IR neurons distributed throughout the nTS. However, none of these GABAergic neurons projected to the PVN, suggesting that the nTS is not a major source of direct inhibitory input to the PVN. This is in contrast to findings that a proportion of nTS

projections to other brain regions important in cardiorespiratory regulation, including the RVLM and CVLM, are GABAergic (11, 38, 228, 243).

Although GABAergic nTS neurons were activated by all intensities of hypoxia examined, activation was appreciably less than the other phenotypes studied. In contrast to other phenotypes, recruitment of GABAergic neurons peaked at lower levels of stimulation and decreased with more severe hypoxia. It is possible that this apparent decrease in activation of inhibitory neurons at higher intensities of hypoxia may result in disinhibition, contributing to further activation of other cellular phenotypes and augmentation of ventilation. Overall, evidence from the current study supports a role for GABAergic neurons in the modulation of chemoreflex function within the nTS.

2.5.4 Activation of PVN- vs. RVLM-projecting nTS neurons during hypoxia

Previous work from our laboratory (126) and others (104) demonstrates that RVLM-projecting cells in the nTS are activated by hypoxia (10% O₂). However, the relative importance of the PVN- vs. RVLM-projecting pathways from the nTS is not known. We found that virtually no nTS cells projected to both the RVLM and PVN, consistent with the concept that few nTS neurons send collateral projections to different brain regions (101). However, both types of projection neurons were activated by a near maximal hypoxic stimulus (10% O₂). At this intensity of hypoxia, the proportionate activation of the PVN-projecting pathway was greater than the classical nTS to RVLM chemoreflex pathway. Thus, the nTS to PVN pathway also may be vital in mediating the full expression of chemoreflex responses. Future experiments will compare these pathways at less severe hypoxic intensities to determine if differential

activation of RVLM-projecting versus PVN-projecting nTS neurons occurs at various levels of hypoxia.

2.6 PERSPECTIVES AND SIGNIFICANCE

These studies evaluated the neural pathways involved in cardiorespiratory responses to arterial chemoreceptor activation, particularly neurons projecting from the nTS to PVN. PVN-projecting nTS neurons were activated by all levels of hypoxia evaluated. Strikingly, catecholaminergic PVN-projecting neurons were preferentially activated by hypoxia compared to non-catecholaminergic neurons. Despite the substantial number of GABAergic neurons in the nTS, essentially none of them projected to the PVN. Finally, we considered the possibility of differential activation of brain region specific nTS projection neurons and found that the proportion of PVN-projecting nTS neurons activated during hypoxia was greater than the proportion projecting to the RVLM. The projection from the nTS to the PVN may be critical in mediating cardiorespiratory responses to hypoxia even during mild to moderate hypoxia. Thus, the PVN, activated by an nTS to PVN catecholaminergic pathway, may be more important to integrated chemoreflex responses than previously thought.

2.7 ACKNOWLEDGEMENTS

We would like to thank Sarah A. Friskey for her outstanding technical expertise.

Figure 2.1 –

Ventilatory responses to increasing intensities of hypoxia. (A) 10 second raw tracings from a representative rat, showing the changes in oxygen saturation (top) and breathing (bottom) in response to chemoreflex activation using three intensities of acute hypoxia (12, 10, and 8% inspired O₂). (B-E) Group data (n=6); Effects of graded intensities of hypoxia upon respiratory variables: (B) Arterial oxygen saturation, (C) Respiratory rate, (D) Tidal Volume Index and (E) Minute Ventilation Index. Measurements reflect the average from 20 breaths during the last minute of exposure to each hypoxia intensity. p<0.05; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. Ventilatory parameters during periods of recovery are denoted by open circles.

Figure 2.2 –

Increasing intensity of hypoxia augments Fos-IR in the nTS. (A) Photomicrographs taken from coronal sections at the postremal level of the nTS. Images depict Fos-IR nuclei (pseudo-colored red) following exposure to normoxia (upper left) and increasing intensities of acute hypoxia (12% O₂, 10% O₂, 8% O₂). White outlines identify area of the nTS where cells IR for Fos were counted. On the right, diagrammatic representation and bright field image illustrating the region of the nTS shown in the photomicrographs. (B) Total number of Fos-IR cells counted in all seven sections of the nTS following exposure to normoxia (n=4) and each intensity of acute hypoxia (n=5 each). Note the graded increase in number of Fos-IR cells in response to increasing hypoxia. (C) Caudal-rostral distribution of Fos-IR nTS cells for each intensity of acute hypoxia. Two-way repeated measures ANOVA indicated significant main effects of hypoxic intensity and caudal-rostral level. "0" represents calamus scriptorius (the caudal-most portion of the area postrema). p<0.05; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. In (C), significance represents post hoc analysis of main effect of hypoxic intensity. nTS: nucleus tractus solitarii, AP: Area Postrema, TS: Solitary tract, DMX: Dorsal motor nucleus of the vagus. Scale bars=100 μm (photomicrographs) and 1mm (bright field).

Figure 2.3 -

Maintenance of arterial blood pressure during acute hypoxia does not affect nTS Fos-

IR. Mean arterial pressure (MAP, A) and heart rate (HR, B) during a period of 120 minutes of normoxia (21% O₂, n=7), systemic hypoxia without (10% O₂, n=8) or with phenylephrine infusion (10% O₂ + PE, n=4). The period of hypoxia began at time "0". PE infusion prevented the hypoxia-induced depressor response. (C) Caudal-rostral distribution and (D) subregional distribution of Fos-IR cells in the nTS in response to hypoxia without (10% O₂) or with (10% O₂ + PE) MAP maintained at normoxic levels. There were no significant differences in caudal-rostral or subregional distribution of Fos-IR cells between groups. In A, *, p<0.05; 10% O₂ vs. 21% and 10% O₂ + PE. In B, *, p<0.05; 10% O₂ and 10% O₂ + PE vs. 21% O₂. In D, for both 10% O₂ and 10% O₂ + PE, p<0.05; SolM (a) > SolDL (b) > SolVL (c) > SolC, SolV, SolCe. SolC: commissural nTS, SolM: medial nTS, SolVL: ventrolateral nTS, SolV: ventral nTS, SolDL: dorsolateral nTS, SolCe: central nTS.

Figure 2.4 –

Microinjection sites were identified and verified anatomically. (A) Epifluorescent photomicrographs of representative injection site of 60 nL of 2% FG injected bilaterally in the PVN (left) or 30 nL of FG in the RVLM (right). Arrows indicate the center of the injection sites. (B) Location of PVN injection sites unilaterally, ipsilateral to the side on which labeled cells in the nTS were counted. Filled circles represent hypoxic animals and open circles normoxic animals. Grey circles represent animals that had a retrograde tracer injected in both the PVN and RVLM. (C) Location of all RVLM injection sites, plotted unilaterally, ipsilateral to the side counted. Numbers indicate the location in millimeters caudal to Bregma. Amb: nucleus ambiguus, f: fornix, 3V: third ventricle, opt: optic tract, 4V: fourth ventricle. Scale bars=100 μ m

Figure 2.5 –

Phenotypes of nTS neurons activated following increasing intensities of acute hypoxia.

Representative merged photomicrographs showing labeling for Fos (pseudo-colored red) and (A) FG (pseudo-colored blue), (B) TH (pseudo-colored green), (C) FG and TH,

and (D) GAD67 (pseudo-colored green) in the nTS from animals exposed to normoxia (21% O₂), 12% O₂, 10% O₂, or 8% O₂. In a given section, images for each fluorophore were taken in the same focal plane. Landmarks in the region of the nTS are labeled in photomicrographs from animals breathing 21% O₂. Abbreviations are the same as in Figure 2. Examples of Fos-IR cells co-labeled with FG, TH, FG and TH (triple labeled cell), or GAD67 are denoted by arrows. Higher magnification of boxed areas in some panels also shows co-labeling of Fos with FG and/or TH. Scale bars=100 μ m (A,B,C), 50 μ m (D).

Figure 2.6 –

Relative activation of nTS neuronal phenotypes in response to increasing intensities of acute hypoxia. The percent of nTS cellular phenotypes that also were activated (Fos-IR) following exposure to normoxia (n=4) or increasing intensities of acute hypoxia (12% O₂, 10% O₂, 8% O₂; n=5 each): (A) PVN-projecting [(Fos+FG)/(FG) X100], (B) catecholaminergic [(Fos+TH)/(TH) X100], (C) PVN-projecting catecholaminergic [(Fos+TH+FG)/(TH+FG) X100] or PVN-projecting noncatecholaminergic [(Fos+(FG and TH-negative))/(FG and TH-negative) X100] and (D) GABAergic [(Fos+GAD67)/(GAD67) X100] nTS neurons. (C) Solid bars, PVN-projecting catecholaminergic cells; striped bars, PVN-projecting non-catecholaminergic cells. Within the catecholaminergic group of PVN projecting neurons there was a graded effect of hypoxia. Within the noncatecholaminergic group (C) there was no difference between 10% and 8% O₂. p<0.05; for a specific phenotype, * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. †, p<0.05 vs. catecholaminergic cells within each hypoxic group.

Figure 2.7 –

Acute hypoxia activates RVLM- and PVN-projecting nTS neurons. (A) Representative merged photomicrograph showing immunoreactivity and labeling for Fos (pseudo-colored red), TH (pseudo-colored green), PVN-projecting (pseudo-colored blue), and RVLM-projecting (pseudo-colored gray) cells in the nTS from an animal exposed to acute hypoxia (10% inspired O₂). (B) Higher magnification of boxed region in photomicrograph (A) above showing co-labeled cells. Arrow: activated catecholaminergic cell, Arrow head: activated RVLM-projecting cell, Double arrow head: activated PVN-projecting catecholaminergic cell. (C) The percent of RVLM- or PVN-projecting cells co-labeled with Fos-IR (n=3). *, $p \leq 0.05$, PVN-projecting versus RVLM-projecting. PVN: paraventricular nucleus of hypothalamus, RVLM: rostral ventrolateral medulla. Scale bar = 100 μ m

Table 2.1 –

Table 2.1. Total number of positively labeled cells counted in seven nTS sections.

	21% O ₂	12% O ₂	10% O ₂	8% O ₂
# FG cells	84 $\pm$ 7	76 $\pm$ 7	85 $\pm$ 8	93 $\pm$ 10
# TH cells	145 $\pm$ 15	148 $\pm$ 4	147 $\pm$ 6	149 $\pm$ 4
# GAD67 cells	106 $\pm$ 16	68 $\pm$ 11	83 $\pm$ 10	98 $\pm$ 16
# FG + TH cells	51 $\pm$ 4	39 $\pm$ 3	44 $\pm$ 6	51 $\pm$ 6
# FG + GAD67 cells	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 1	1 $\pm$ 1

PVN-projecting (FG; FG labeled), catecholaminergic (TH; TH-IR), GABAergic (GAD67; GAD67-IR) and colabeled cells in normoxic animals (n=4) and in animals exposed to one of three levels of hypoxia (n=5 each). Values are mean $\pm$ S.E.

**CHAPTER 3: Acute systemic hypoxia activates hypothalamic
paraventricular nucleus-projecting catecholaminergic neurons in the
caudal ventrolateral medulla**

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3.1 ABSTRACT

Hypoxia activates catecholamine neurons in the caudal ventrolateral medulla (CVLM). The hypothalamic paraventricular nucleus (PVN) modulates arterial chemoreflex responses and receives catecholaminergic projections from the CVLM, but it is not known whether the CVLM-PVN projection is activated by chemoreflex stimulation. We hypothesized that acute hypoxia (AH) activates PVN-projecting catecholaminergic neurons in the CVLM. Fluorogold (FG, 2%, 60-90 nL) was microinjected into the PVN to retrogradely label CVLM neurons. After recovery, conscious rats underwent three hours of normoxia (21% O₂, n=4) or AH (12, 10, or 8% O₂; n=5 each group). We used Fos-immunoreactivity (IR) as an index of CVLM neuronal activation and tyrosine hydroxylase (TH)-IR to identify catecholaminergic neurons. Positively labeled neurons were counted in six caudal-rostral sections containing CVLM. Hypoxia progressively increased the number of Fos-IR CVLM cells (21%: 19 ± 6; 12%: 49 ± 2; 10%: 117 ± 8; 8%: 179 ± 7; p<0.001). Catecholaminergic cells co-labeled with Fos-IR in the CVLM were observed following 12% O₂ and further increases in severity of hypoxia caused markedly more activation. PVN-projecting CVLM cells were activated following more severe hypoxia (10% and 8% O₂). A large proportion (89 ± 3%) of all activated PVN-projecting CVLM neurons were catecholaminergic, regardless of hypoxia intensity. Data suggest that catecholaminergic, PVN-projecting CVLM neurons are particularly hypoxia-sensitive and these neurons may be important in the cardiorespiratory and/or neuroendocrine responses elicited by the chemoreflex.

3.2 INTRODUCTION

Peripheral chemoreflex activation by systemic hypoxia increases ventilation, sympathetic nerve activity, and secretion of vasopressin and adrenocorticotrophic hormone (ACTH). These respiratory, autonomic, and endocrine responses are critical for the body's homeostatic mechanisms and maintain adequate tissue oxygen levels (99, 154). Decreases in the partial pressure of oxygen in arterial blood are sensed by chemoreceptors located in the carotid bodies and afferent information is relayed through the carotid sinus nerve to the brainstem (162). The nucleus tractus solitarius (nTS) is the first site of chemoreceptor afferent integration and modulation (7, 204). A monosynaptic projection from the nTS to the rostral ventrolateral medulla (RVLM) is thought to be the primary pathway involved in arterial chemoreflex function (87). However, other brainstem and forebrain nuclei also contribute importantly to chemoreflex function (117), but the central nervous system pathways and their relative importance in responses to systemic, acute hypoxia remain unclear.

The paraventricular nucleus of the hypothalamus (PVN) is an important integrative center containing neurons that regulate both autonomic and neuroendocrine function (229). Magnocellular neurons in the PVN regulate release of vasopressin and oxytocin from the posterior pituitary while parvocellular PVN neurons are involved in autonomic nervous system and respiratory function, and secretion of corticotropin-releasing hormone (13, 94, 189). Neurons in the PVN receive multiple inputs and send direct projections to brain regions important in regulation of sympathetic outflow (3, 206, 209) and respiratory drive (248). Oxytocin- and

vasopressin-containing PVN parvocellular neurons project to the RVLM, pre-Botzinger complex, phrenic motor nucleus and the intermediolateral cell column (IML), and both neuropeptides modulate ventilation (117). PVN neurons are activated by hypoxia and hypercapnia (21, 63), and may play a role in chemoreflex function. Lesion or inhibition of the PVN decreases ventilation and attenuates cardiovascular and sympathetic responses to chemoreflex activation (175, 190), indicating the PVN is required for full expression of the peripheral chemoreceptor reflex. While these data indicate the PVN is important in central processing of chemoreceptor information, the neural pathways and neurotransmitters that convey peripheral chemoreceptor input to the PVN have not been fully delineated.

Catecholaminergic inputs to the PVN may influence autonomic responses during chemoreflex activation, as blockade of adrenergic receptors in the PVN blunts the cardiovascular response to carotid chemoreceptor stimulation (136). Previous work from our laboratory (122) indicates that hypoxic stimuli activate nTS cells with projections to the PVN, especially catecholaminergic PVN-projecting neurons. The PVN also receives projections, including catecholaminergic input, from the caudal ventrolateral medulla (CVLM; (52, 207). These projections have been implicated in mediating neuroendocrine responses to hemorrhage (97) and stress (61), and could also play a role in responses to hypoxia. CVLM neurons are activated by chemoreflex stimulation (151, 152, 217) and it has been suggested that the CVLM contributes to neuroendocrine responses to hypoxia (217). In addition, a population of hypoxia-sensitive CVLM neurons responds to changes in arterial pressure (152), and the

decrease in pressure observed during hypoxia may influence activation of these neurons. However, it is not known whether PVN-projecting CVLM cells, in particular catecholaminergic CVLM neurons with direct projections to the PVN, become activated in response to systemic hypoxia. Furthermore, whether the recruitment of CVLM neurons during hypoxia is influenced by hypoxia-induced changes in arterial pressure has not been determined.

In this study, we used retrograde labeling to directly evaluate the caudal to rostral activation of PVN-projecting CVLM neurons due to increasing severity of acute hypoxia. Second, we determined if these CVLM projection neurons are catecholaminergic. Finally, because acute hypoxia decreases arterial pressure and changes in pressure affect CVLM neuronal activation, we also assessed if hypoxia-induced decreases in pressure influenced activation of these neurons during hypoxia. We hypothesized that the direct projection from CVLM catecholamine cells to the PVN is activated during hypoxia. Furthermore, since the peripheral chemoreceptors do not project directly to the CVLM, and thus afferent information must traverse several synapses before activating CVLM neurons, we hypothesized that these neurons would require higher intensities of hypoxia for activation. Our findings indicate that increasing the intensity of acute systemic hypoxia activates progressively more CVLM cells including PVN-projecting catecholaminergic neurons. These results support the role of PVN-projecting CVLM neurons in physiological responses to hypoxia.

3.3 METHODS

Studies were performed in 23 male Sprague Dawley rats (280-340g). The rats were housed in a 12 hour light/dark cycle at a constant temperature and humidity (22°C and 40%, respectively). Animals were given food and water *ad libitum*. All experimental procedures were conducted in accordance with APS guidelines and the NIH "Guide for the Care and Use of Laboratory Animals," and were approved by the University of Missouri Animal Care and Use Committee. Tissue from animals used in the current experiments was also used in previously published studies to evaluate effects of hypoxia on nTS neurons (122).

3.3.1 Surgical and microinjection procedures

The distribution and activation of neural inputs to the PVN from the CVLM was investigated by injecting the retrograde tracer Fluoro-Gold (FG, 2% in deionized water, Fluorochrome Inc., Denver, CO, USA) into the PVN using techniques similar to procedures previously published for retrograde tracer and microinjection experiments (122, 126, 156). All surgeries were performed under aseptic conditions. Anesthetized animals (Isoflurane; Baxter Healthcare Corporation, Deerfield, IL, USA; 5% for induction and 2–2.5% for maintenance) were placed in a stereotaxic frame, the dorsal surface of the skull exposed and a small hole made to access the brain. Coordinates for PVN microinjections were: 1.8 - 2.0 mm caudal to bregma, $\pm$ 0.5 mm lateral from the midline and 7.8 mm ventral to the dura. FG (60-90 nL) was microinjected bilaterally into the PVN. Incisions were closed, rats were treated with post-operative antibiotics, and returned to their home cage for at least two weeks of recovery. To measure blood

pressure in response to acute hypoxia, in a separate group of rats (n=4), telemetry devices (TA11PA-C40, Data Sciences International, USA) were implanted via the abdominal aorta and animals were allowed to recover for at least 10 days. Arterial pressure, heart rate (HR), and the strength of the telemetry signal were evaluated daily after surgery. Five days before immunohistochemistry experiments in which MAP was maintained constant during acute hypoxia, animals were also instrumented with a femoral venous catheter for drug administration.

3.3.2 Hypoxia Experimental Procedures

Prior to the experiment, conscious rats in their home cages were acclimated to the hypoxia chamber (Biospherix Inc., Redfield, NY, USA) for 1-3 hours per day for 2 days to allow them to become accustomed to the environmental stimuli associated with the chamber. On the day of the experiment, conscious rats with previous PVN tracer microinjections were allowed to acclimate for 30 minutes in the chamber (21% O₂). The gas mixture then was adjusted to bring the air in the chamber to: 21% O₂ (normoxic control; n=4), 12% O₂, 10% O₂, or 8% O₂ (n=5 each group) and maintained at that percentage for three hours via a negative feedback control system, similar to previous studies (21, 122, 126, 234).

3.3.3 Maintenance of arterial blood pressure during hypoxia

In the animals implanted with telemetry devices (n=4), two sets of blood pressure measurements were obtained, each during three hours of hypoxia. These animals were also acclimatized to the hypoxic chamber as above but only underwent hypoxia exposure at 10% O₂ for three hours. In the initial hypoxic exposure, blood

pressure was continuously measured and recorded using telemetry. After two weeks, animals were instrumented with a femoral venous catheter and then allowed one week for recovery. Then, animals were again exposed to three hours of hypoxia at 10% O₂, but this time, blood pressure was maintained constant throughout the hypoxic period by infusing phenylephrine (0.6 mg/mL, 0.1-0.5 mL/hour, i.v.). Animals were perfused immediately following the experiment. Fos expression in these animals was compared to the group of FG-microinjected animals that were exposed to 10% hypoxia only.

3.3.4 Immunohistochemistry (IHC)

Immediately following hypoxia experiments, animals were transcardially perfused with Dulbecco's Modified Eagle Medium (Sigma, 125 mL, pH 7.4) bubbled with oxygen, followed by 0.01M phosphate buffered 4% paraformaldehyde (PFA, Sigma, 500 mL, pH 7.4). After postfixation, coronal sections were cut at 30µm (brainstem) or 40µm (forebrain) with a vibratome (VT 1000S; Leica, Germany). In all animals with retrograde tracer injections, forebrain sections were examined to verify the location of PVN injection sites using a rat brain stereotaxic atlas (177). Only animals in which the microinjections were localized to the PVN were used for immunohistochemistry protocols. A double labeling technique similar to that previously described (122, 126) was used to identify immunoreactivity (IR) for Fos (a marker for neuronal activation) and tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis, a marker for catecholaminergic neurons. Briefly, sections were incubated overnight in 1% NDS and 0.3% Triton-0.01M PBS containing primary antibodies against Fos (rabbit anti-Fos, 1:3000, Calbiochem) and TH (mouse anti-TH, 1:1000, Millipore Inc.). The following day,

sections were rinsed then incubated for two hours in Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG (1:200 Jackson ImmunoResearch) with 1% NDS in 0.3% Triton-0.01M PBS and coverslipped. In additional tissue from a subset of three of the rats subjected to 10% O₂, a primary antibody against phenylethanolamine N-methyl transferase (sheep anti-PNMT, 1:2000, Millipore) was included in the primary antibody solution and Cy5-conjugated donkey anti-sheep IgG secondary antibody (1:200, Jackson ImmunoResearch) was used. IHC controls included omission of primary or secondary antibodies. Because so little PNMT was observed in the CVLM sections included in the current study, we verified the validity of our immunohistochemical technique by examining the next two more rostral sections, which would be expected to include both A1 and C1 neurons. In these sections, $23 \pm 9\%$ of all TH neurons were also PNMT-IR. In addition, evaluation of the rostral RVLM indicated that a large proportion ($94 \pm 2\%$) of the TH-IR neurons also exhibited PNMT-IR. Antibody specificity for Fos (216), TH (11) and PNMT (81) have been verified previously. Neurons in the CVLM that projected to the PVN were identified by the intrinsic fluorescence of retrograde tracer.

Microscopy and image analysis. We evaluated the CVLM based on location relative to clearly recognizable anatomic structures including: lateral to the pyramidal tracts and inferior olives, medial to the spinal trigeminal tracts, dorsal to the lateral reticular nuclei, and ventral to the nucleus ambiguus (177). Brainstem sections were examined using an Olympus epifluorescent microscope (BX51). Filter sets for Cy2, Cy3, Cy5 or Fluoro-Gold were used to visualize positive labeling. Image brightness and

contrast only were adjusted for image clarity. Images in the same focal plane were captured under each filter set using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ, USA). Images subsequently were combined and analyzed with Image J (ver. 1.41, NIH) using a custom made plugin (GAIA Group, Novato, CA, USA, <http://gaiag.net/index.html>). Two individuals blinded to the experimental protocol performed the counting and counts for each section were averaged. The following criteria were used to identify positively labeled cells: Fos-IR nuclei were identified as highly condensed stained regions, with an ovoid shape and frequently with a clearly discernible unstained nucleolus. TH- and PNMT- positive cells were recognizable by staining of the cytoplasm, visible processes and a blank nuclear region. FG-positive cells displayed bright or punctate cytosolic labeling. When the above criteria were met under more than one filter set, the cells were considered double- or triple-labeled. Counts of positively labeled cells were made in six caudal to rostral levels of the CVLM: -15.00 to -14.10 mm relative to bregma (corresponding to -540 to +360 μ m relative to calamus scriptorius).

3.3.5 Data analysis

Immunohistochemical data were analyzed as the total (sum) of counts from all sections and also at each caudal to rostral level of the CVLM. The percent of co-labeled CVLM neurons was calculated by dividing the number of co-labeled cells by the total number of cells of each individual phenotype. For example, the percent of projecting cells activated was calculated as: (Fos and FG co-labeled cells)/(total number of FG labeled cells) X 100. Statistical analyses were performed using SigmaPlot (12.3, Systat

Software, San Jose, CA, USA). All data are presented as mean $\pm$ SE. Statistical significance was set at a p value of ≤ 0.05 . One-way repeated measures ANOVA was used to compare the total number of neurons with a given phenotype, co-labeling and percentages of co-labeling (e.g., TH-IR neurons expressing Fos) among experimental groups. Caudal-rostral distributions of labeling among hypoxic groups were analyzed by two-way repeated measures ANOVA; average distributions were analyzed by one-way repeated measures ANOVA. When appropriate, ANOVAs were followed by post-hoc analysis using Fisher's LSD test.

3.4 RESULTS

3.4.1 PVN-projecting CVLM cells have a distinct distribution and many are catecholaminergic

Microinjection sites for FG within the PVN were verified histologically in all animals used in this study. Figure 1A depicts a representative PVN injection site; a summary of all sites was presented previously (122). An example of retrograde labeling in the CVLM is shown in Figure 1B. Examination of the distribution of PVN-projecting cells in the CVLM indicated a distinct pattern, with a gradual increase in the number of labeled cells from caudal to rostral (Figure 1C). Two-way repeated measures ANOVA revealed that the three most rostral sections examined in the CVLM contained a significantly greater number of PVN-projecting cells compared to more caudal sections. In contrast, catecholaminergic (TH-IR) cells were evenly distributed throughout the CVLM, similar to previous reports (52, 60, 206). Overall, about two-thirds ($67 \pm 3\%$) of all

PVN-projecting CVLM neurons were catecholaminergic. The distribution of catecholaminergic PVN-projecting neurons was similar to that of FG labeled cells; the greatest proportion of TH- and FG co-labeled cells also occurred at intermediate and rostral levels of the CVLM (Figure 1D). As expected, hypoxia had no effect on total number (Table) or distribution (Figure 1) of FG, TH or their co-labeling in the CVLM.

3.4.2 Graded systemic hypoxia progressively activates CVLM cells

We used Fos-immunoreactivity (IR) to determine activation of CVLM cells by increasing intensities of hypoxia. Figure 2A-D includes examples of activated cells (Fos-IR, pseudo-colored red) in brainstem sections containing the CVLM from animals that were exposed to three hours of normoxia (21% O₂) or different intensities of hypoxia (12, 10, 8% inspired O₂). In control animals, there were very few Fos-IR cells in the CVLM, suggesting a low level of acute activation under normoxic conditions. Increasing the intensity of hypoxia augmented the number of Fos-IR cells. Mean data showing the increase in total number of activated cells in the CVLM are depicted in Figure 2E (left). Significant activation of CVLM cells was first apparent after breathing 12% O₂. Greater intensities of hypoxia increased the number of Fos-IR neurons in the CVLM, with the greatest activation following exposures to 8% O₂. Evaluation of the caudal to rostral extent of the CVLM indicated that activation of cells was evenly distributed in all hypoxia groups, with an intensity-dependent effect of hypoxia evident throughout (Figure 2E, right).

3.4.3 Hypoxia activates catecholaminergic and PVN-projecting cells in the CVLM

Although exposure to hypoxic gas mixtures induces Fos expression in CVLM catecholamine cells (217), the projections of these hypoxia-sensitive CVLM cells are not known. To investigate the phenotype of activated CVLM cells, we evaluated whether they were catecholaminergic (TH-IR) and/or PVN-projecting (FG labeled).

Catecholaminergic CVLM cells are activated following graded hypoxia

Figure 3 includes examples of activated (Fos-IR) and catecholaminergic cells (TH-IR, pseudo-colored green) in brainstem sections containing the CVLM from animals exposed to three hours of normoxia (21% O₂) or different intensities of hypoxia (12, 10, 8% inspired O₂). Very few TH-IR cells were activated following normoxia (Figure 3A). Activation of catecholaminergic cells occurred in response to 12% O₂ (Figure 3B) and further increases in hypoxia caused markedly more activation (Figure 3C, D). The mean number of catecholaminergic CVLM cells activated (Fos- and TH- co-labeled) by exposure to hypoxia is shown in Figure 3E (left). Compared to normoxia, the number of co-labeled cells increased significantly after breathing 12% O₂ and increased further with greater intensity of hypoxia. The greatest number of activated catecholaminergic cells was reached at 10% and 8% inspired O₂. Analysis of activation of catecholamine cells through the caudal-rostral extent of the CVLM indicated a main effect of hypoxia intensity but no effect of caudal to rostral level. Thus, catecholaminergic cells throughout the CVLM were activated by hypoxia with no distinct recruitment pattern (Figure 3E, right).

The percent of CVLM catecholaminergic cells that were activated by hypoxia is shown in Figure 3F and indicates that increasing hypoxia produced greater activation of TH-IR neurons. Furthermore, a considerable proportion of TH-IR cells ($71 \pm 4\%$) was activated at the highest intensity of hypoxia studied. Similar to absolute numbers, (Figure 3E) examination of the caudal to rostral distribution as a percent of catecholamine cells activated (data not shown), indicated an intensity dependent effect but no distinct caudal to rostral pattern of activation.

Based upon their caudal location within the medulla, we would expect the majority of catecholaminergic cells evaluated to be noradrenergic (A1) rather than adrenergic (C1) neurons. Nevertheless, examination of TH-IR does not allow us to unequivocally distinguish between these two subgroups. Therefore, in a subgroup ($n=3$) of animals exposed to 10% O₂, we also evaluated PNMT-IR, which would be observed only in the C1 neurons. At the caudal-rostral levels of the medulla examined in the current study, only $1.7 \pm 1.7\%$ of the TH-IR neurons also exhibited PNMT-IR. Thus, approximately 98% of the catecholaminergic neurons examined in the current studies belong to the A1 cell group. Furthermore, none of the activated TH-IR cells at these levels expressed PNMT.

Hypoxia activates PVN-projecting CVLM cells

Representative pseudo-colored photomicrographs of FG- and Fos-IR co-labeled cells within the CVLM of animals that were exposed to normoxia (21% O₂) and increasing intensity of hypoxia are shown in Figure 4A-D. Very few PVN-projecting cells exhibited Fos-IR after exposure to normoxia but activation of FG-labeled cells became

apparent at more severe hypoxic intensities. Mean data showing activation of PVN-projecting CVLM cells in response to acute hypoxia are shown in Figure 4E and F. Whether evaluated as the absolute number or as a percent of FG-labeled cells, breathing 12% oxygen did not activate PVN-projecting cells compared to normoxia. However, the number of PVN-projecting CVLM cells that co-expressed Fos was significantly increased at 10 and 8% hypoxia, with 8% oxygen producing the greatest activation of these cells. Interestingly, 8% O₂ activated over half of all PVN-projecting cells (Figure 4F).

With respect to the caudal to rostral extent of Fos-IR in the CVLM, two-way ANOVA revealed a significant main effect of hypoxia (Figure 4E, right). As with the total number (Figure 4E, left), there was no significant effect of exposure to 12% O₂, but a progressive increase was observed at 10% and 8% O₂. There was a trend ($p=0.08$) for more co-labeled cells to be localized caudally (Figure 4E, right). In addition, because the overall distribution of PVN-projecting CVLM cells was such that there were fewer in the most caudal CVLM sections analyzed (Figure 1C), the percent of PVN-projecting cells activated caudally was significantly more prominent at caudal levels (-15.00 through -14.64) compared to more rostral regions (14.10, -14.28 and -14.46) relative to bregma.

Hypoxia preferentially activates catecholaminergic PVN-projecting cells in the CVLM.

Of the activated PVN-projecting CVLM neurons (presented in Figure 4E, left; Fos + FG), almost all were also catecholaminergic (Fos + FG + TH). This was independent of hypoxia severity, and on average $89 \pm 3\%$ of activated projecting cells were also catecholaminergic. Therefore, we examined specifically the activation of PVN-projecting

catecholaminergic cells. Photomicrographs illustrating activation of PVN-projecting catecholaminergic neurons are presented in Figure 5 for normoxia (A) and all hypoxia intensities (B-D). Although there was little activation of these cells following normoxia or exposure to 12% O₂, increasing severity of hypoxia produced substantially more Fos-IR in PVN-projecting catecholaminergic neurons (Figure 5 C, D). The mean number of triple labeled (Fos-, TH- and FG co-labeled) cells (Figure 5E, left) was not significantly altered in response to 12% inspired O₂. However, labeling was significantly greater at 10% and 8% hypoxia compared to both 21% and 12% O₂ levels. Independent of caudal to rostral distribution, within the CVLM (Figure 5E, right) activation was greater in animals exposed to 10 and 8% O₂. Similar to above, because there were fewer PVN-projecting catecholaminergic CVLM cells in the most caudal CVLM sections analyzed (Figure 1D), the percent of PVN-projecting catecholaminergic cells activated was significantly more prominent at the caudal levels -15.00 through -14.64 compared to rostral levels -14.10 and -14.46 relative to bregma. Up to 88% of PVN-projecting catecholaminergic neurons was activated in the most caudal sections of the CVLM after inspiration of 8% oxygen.

We also compared directly the activation of PVN-projecting CVLM neurons with respect to their phenotype (catecholaminergic or non-catecholaminergic; Figure 5F). The percentage of catecholaminergic PVN-projecting neurons (TH- and FG-co-labeled, solid bars) activated by exposure to 12% inspired O₂ was similar to normoxia. However, there was substantial activation of these neurons in response to breathing 10% or 8% O₂. A large proportion ($71 \pm 7\%$) of PVN-projecting catecholaminergic cells was

activated at the most intense hypoxic stimulus. In contrast, compared to the substantial number of catecholamine neurons activated, hypoxia-induced Fos expression was less in non-catecholaminergic PVN-projecting CVLM neurons following exposure to 12, 10, and 8% O₂ (Figure 5F, striped bars). Fos-IR in the non-catecholaminergic PVN-projecting neurons was not significantly different from normoxia after exposure to any intensity of hypoxia.

3.4.4 Hypoxia activates non-phentyped CVLM neurons

Hypoxia also activated cells in the CVLM of unknown phenotype (Fos-IR only cells). There was a significant increase in the number of these cells at 10 and 8% hypoxia (10% O₂: 60 ± 8 ; 8% O₂: 91 ± 6) compared to normoxia (11 ± 3) and 12% hypoxia (21 ± 2). Thus, a substantial proportion of Fos-IR CVLM neurons was neither catecholaminergic nor projected to the PVN.

3.4.5 Proportional activation of CVLM cells remains constant with increasing intensities of hypoxia.

We also considered the entire activated cell population in the CVLM and evaluated the proportion of the total number of activated cells made up by each phenotype. Interestingly, although the total number of activated CVLM cells increased with increasing severity of hypoxia (Fig. 2E), the proportion of this total number comprised by each phenotype remained the same. For example, about 50% of all activated CVLM cells was catecholaminergic, independent of hypoxia severity (12% O₂: $55 \pm 5\%$; 10% O₂: $49 \pm 6\%$; 8% O₂: $43 \pm 2\%$). Thus, although the number of activated catecholaminergic cells was augmented with each increase in hypoxia, the proportion of

the overall number of Fos-IR cells that was TH-positive was similar, independent of hypoxia intensity. In addition, of all activated CVLM cells, approximately 30% projected to the PVN, and this percentage also was not influenced by intensity of hypoxia (12% O₂: 30 ± 4%; 10% O₂: 25 ± 6%; 8% O₂: 30 ± 5%). Remarkably, of these activated PVN-projecting cells, almost all were catecholaminergic (12% O₂: 94 ± 4%; 10% O₂: 95 ± 4%; 8% O₂: 81 ± 8%) at all intensities of hypoxia. Finally, a similar percent of all Fos-IR cells was un-phenotyped (neither catecholaminergic nor PVN-projecting), again independent of hypoxia severity (12% O₂: 44 ± 5%; 10% O₂: 54 ± 10%; 8% O₂: 51 ± 4%). Thus, although hypoxia activates progressively more cells with each increase in hypoxia intensity, at each intensity, a similar proportion of each phenotype was activated.

3.4.6 Activation of CVLM neurons by hypoxia is not dependent on changes in arterial pressure

Results indicate an increase in activation of PVN-projecting CVLM neurons after a hypoxic stimulus. It is well known that CVLM neurons respond to changes in blood pressure (37, 38, 212) and some CVLM neurons are activated by decreases in blood pressure (37, 53, 55, 143). Previous studies indicate that acute hypoxia in conscious rats produced moderate depressor responses (122, 154) which could potentially alter Fos-IR in the CVLM. To evaluate the effect of arterial pressure on CVLM neuronal activation during hypoxia, in a separate group of animals PE was infused (10% O₂ + PE; 0.6 mg/mL, 0.1-0.5 mL/hour, i.v.) to hold MAP constant during hypoxia. Fos-IR in these animals was compared to Fos-IR in the group of rats that were exposed to acute hypoxia only (10% O₂ group). In animals implanted with telemetric devices, exposure to 10% O₂ decreased

MAP from 106 ± 3 to 87 ± 4 mmHg at 30 minutes, and the decrease in pressure was maintained throughout the hypoxic period (93 ± 2 mmHg at the end of hypoxia). Infusion of phenylephrine during exposure to hypoxia prevented the depressor response to hypoxia (108 ± 6 to 115 ± 7 mmHg at 30 minutes) and MAP was maintained throughout (106 ± 1 at the end of hypoxia). Figure 6 includes photomicrographs showing Fos- and TH-IR from animals in which blood pressure was not controlled (Figure 6A) or was held constant (Figure 6B). Group data revealed that there was no significant difference in total number of cells activated (Figure 6C) or caudal to rostral distribution (data not shown) of Fos-IR between groups. In addition, the number (Figure 6D) and distribution (data not shown) of activated CVLM catecholaminergic cells also was similar whether MAP was allowed to decrease during hypoxia or was held constant. These results suggest that the modest decreases in MAP during 10% acute hypoxia did not alter the number of CVLM neurons activated.

3.5 DISCUSSION

Acute hypoxia activates peripheral chemoreceptors and elicits physiological responses to promote enhanced gas exchange, maintain cardiovascular function and preserve overall homeostasis. The complex central nervous system pathways involved in the chemoreflex have not been delineated fully. The current studies show that hypoxia progressively activates CVLM neurons, and, contrary to our initial hypothesis, activation occurred even at relatively low intensities of hypoxia (12% O₂). Hypoxia activated a large proportion (up to 71%) of catecholaminergic CVLM neurons, suggesting

an important role of these neurons in cardiorespiratory reflexes. Importantly, CVLM neurons that directly project to the PVN are also activated by hypoxia but require a more intense hypoxic stimulus to be recruited. The majority ($89 \pm 3\%$) of hypoxia-sensitive PVN-projecting CVLM neurons was catecholaminergic, providing an anatomic and phenotypic substrate for a role of the CVLM - PVN pathway in chemoreflex responses. Finally, we found that hypoxia-induced decreases in blood pressure did not alter the number of activated CVLM neurons.

Classically, the CVLM has been shown to contribute to the arterial baroreflex pathway via its inhibition of pre-sympathetic RVLM neurons (212). However, the role of the CVLM in peripheral chemoreflex function has been equivocal. While CVLM inhibition does not alter the tonic sympathoexcitation induced by hypoxia (130), others have shown that CVLM neurons exhibit hypoxia-induced activation (71, 234) and contribute to the respiratory coupling of RVLM neuronal activity and sympathetic nerve activity during hypoxia (151, 164). Our data support and extend the concept that the CVLM may contribute to chemoreflex function, even at low intensities of hypoxia. The mildest stimulus examined (12% O₂) produced Fos-IR in the CVLM and increasing intensities of hypoxia progressively activated CVLM cells, which parallels previously observed decreases in oxygen saturation and increases in ventilation (122). Interestingly, a substantial proportion of hypoxia-activated CVLM neurons was catecholaminergic, with severe hypoxia activating ~70% of these TH-IR neurons. Collectively, the observations that CVLM neurons, and specifically catecholaminergic CVLM cells, are activated early in

hypoxia and are further activated by more intense stimuli suggest these neurons may fulfill a significant functional role in mediating hypoxic-related responses.

Interestingly, CVLM catecholaminergic neurons may also be important for long-term adaptations to hypoxia and in respiratory diseases. Partial acclimatization to long-term hypoxia is associated with TH upregulation in the ventrolateral medulla (200) and chronic intermittent hypoxia (an experimental model of obstructive sleep apnea), increases noradrenergic terminal densities in brain regions that receive input from A1 neurons in the CVLM (165, 201). Thus, increased noradrenergic input from A1 neurons to other regions of the brain may contribute to hypertension and augmented cardiorespiratory reflexes in chronic intermittent hypoxia. Furthermore, impairment of the transcription factor Phox2b, which is expressed largely in noradrenergic neurons (106, 247), has been linked to several respiratory pathologies characterized by hypoventilation and impaired responses to hypoxia and hypercapnia, suggesting that catecholaminergic cells are critical to both central and peripheral chemoreception (111, 224, 238). Thus, CVLM catecholamine neurons may be essential for not only acute, but also chronic respiratory system challenges.

Chemoreflex activation increases sympathetic and respiratory activity through multisynaptic central pathways which originate in the nTS. While projections from the nTS to RVLM contribute (87), the central pathways that elicit the multifactorial response to hypoxia remain poorly understood. The PVN plays an important role in control of breathing, sympathetic nerve activity and neuroendocrine function, and contributes to their modulation in response to chemoreflex activation (46, 150, 190). Activation (67,

248) or disinhibition (208) of the PVN increases respiratory rate, mean arterial pressure, and heart rate. Stimulation of peripheral chemoreceptors induces Fos-immunoreactivity within PVN neurons (21), including magnocellular (217) and pre-autonomic neurons (47). In contrast, lesion or blockade of the PVN depresses ventilation and attenuates arterial chemoreceptor mediated responses including increased arterial pressure, sympathetic and phrenic nerve activity (175, 190). PVN projections to the pituitary gland also participate in vasopressin and ACTH release due to hypoxia (79, 144). Thus, activation of the PVN contributes to the robust and varied cardiorespiratory response to hypoxia.

The PVN receives perhaps the most dense catecholaminergic innervation of any region in the CNS (80). Functionally, blockade of adrenergic receptors in the PVN blunts cardiovascular responses and vasopressin release due to carotid chemoreceptor stimulation (136), suggesting that catecholaminergic inputs to the PVN are important in eliciting chemoreceptor induced pressor responses. Given that a large proportion of catecholaminergic innervation to the PVN arises from the noradrenergic cell group of the CVLM (206, 229), we evaluated whether these cells exhibit hypoxia-induced Fos-IR. An important and novel finding in the present study is that a relatively large proportion of PVN-projecting CVLM neurons was activated by hypoxia, suggesting that the CVLM is an important source of chemoreflex related input to the PVN. In contrast to PVN-projecting neurons from the nTS, which were activated after breathing 12% O₂, the hypoxic threshold for activation of PVN-projecting CVLM neurons was greater (10% O₂). Thus, the CVLM to PVN pathway may be less sensitive to hypoxic stimulation and a more

intense stimulus is needed to recruit this pathway, perhaps via activation of the nTS to CVLM pathway. However, once activated, PVN-projecting CVLM neurons are recruited further as severity of hypoxia increases. Functionally, the combination of these two hypoxia sensitive pathways to the PVN could increase the responsiveness of the chemoreflex over a wide range of hypoxic stimuli. For example, more severe hypoxia intensities (7 – 9% O₂) are required to increase plasma vasopressin (188), suggesting that a relatively strong chemoreflex stimulus is needed to initiate vasopressin secretion. The caudal CVLM has strong projections to magnocellular cells in the PVN (52) and lesions encompassing this region reduce activation of vasopressin and oxytocin PVN cell types following hypoxia (82, 217). Thus, recruitment of PVN-projecting CVLM neurons in response to a strong hypoxic stimulus, as shown in the current experiments, could be an important mechanism for promoting vasopressin secretion during intense hypoxia.

Of all PVN-projecting neurons, there was a specific sensitivity of catecholaminergic PVN-projecting neurons to increasing severity of hypoxia. In fact, almost all hypoxia-activated PVN-projecting cells were catecholaminergic, independent of hypoxic intensity, indicating that hypoxia preferentially activates catecholaminergic rather than non-catecholaminergic PVN-projecting CVLM cells. As such, we might expect that a larger proportion of all activated cells would be catecholaminergic. However, when all activated cells in the CVLM were considered, progressively severe hypoxia recruited a greater number of each cell type (e.g. catecholaminergic or PVN-projecting) in proportion to the overall number of CVLM cells activated. Thus, rather than sequentially recruiting different phenotypes of cells, increasing intensity of hypoxia

recruited more of each phenotype proportionally. This is an interesting finding, suggesting that the approach to counter more severe external hypoxia stimuli is to recruit more cells overall, rather than different neuronal phenotypes.

Independent of hypoxia severity, approximately half of all CVLM cells activated in response to hypoxia were neither catecholaminergic nor PVN-projecting. Some of these neurons may be involved in chemoreflex function but could relay chemoreceptor-related information to brain regions other than the PVN. For example, GABAergic barosensitive CVLM neurons respond to hypoxia and likely project to the RVLM (151) to influence presympathetic RVLM neurons (164) and respiratory related changes in sympathetic activity (152). Pontine groups that play a role in respiratory rhythmogenesis also receive projections from cells in the CVLM (86, 90). The CVLM has projections to the thoracic spinal cord which terminate on intercostal, sympathetic preganglionic, and phrenic motor neurons (70, 93, 246), supporting their involvement in respiratory control. Taken together, these data suggest that CVLM neurons are a heterogeneous population with the ability to influence a variety of other brain regions and physiological responses to hypoxia.

Acute hypoxia in conscious rats produces moderate depressor responses. In addition, CVLM cells receive convergent baroreceptor and chemoreceptor inputs (144, 152) and CVLM neurons respond to decreases in blood pressure (37, 53, 55). We therefore evaluated the effect of decreases in arterial pressure during hypoxia on CVLM neuronal activation. Because overall CVLM activation and activation of catecholaminergic neurons following hypoxia was similar whether MAP was allowed to

decrease or was held constant, we propose that recruitment of CVLM neurons in response to acute hypoxia was predominantly due to chemoreflex activation and not the modest decrease in MAP. We cannot eliminate the alternative that in the same CVLM neurons, hypoxia and changes in blood pressure resulted in a different magnitude of cell activation compared to hypoxia alone, as Fos-IR indicates activation, but not the magnitude of activation. Nevertheless, the similarity in results for the effects of hypoxia alone and hypoxia with blood pressure held constant on activation of CVLM neurons indicates that the decrease in blood pressure during hypoxia in the current experiments did not activate additional neuronal populations.

3.6 PERSPECTIVES AND SIGNIFICANCE

One of the most fundamental functions of the brain is the control of breathing. The central nervous system respiratory network helps regulate oxygen supply to the body and responds to acute, chronic and intermittent decreases in body oxygen levels. Given the evidence that chemoreflex function contributes to acclimatization to high altitude (198), exercise (220), and also to autonomic imbalances in various diseases (114, 183, 214), it is becoming increasingly important to fully understand the central nervous system pathways and mechanisms involved during hypoxia. Because the CVLM to PVN pathway projects both to parvocellular and magnocellular regions of the PVN (52), the present study suggests this pathway may be important to all facets of the chemoreflex: cardiorespiratory, sympathetic nerve activity, and neuroendocrine responses to acute hypoxia. Moreover, the CVLM-PVN pathway may provide a robust

influence given the large proportion of this pathway activated by hypoxia when compared to other pathways known to be activated by cardiorespiratory stimuli. For example, only 10–15% of nTS cells that display Fos-IR in response to hypoxia also project to the RVLM (126), 12–13% of CVLM- projecting nTS neurons express Fos in response to arterial baroreceptor activation (243) and approximately 15% of RVLM-projecting PVN neurons are activated by osmotic challenges (112). Overall, the relatively large proportion of catecholaminergic PVN-projecting cells in the CVLM that are activated by hypoxia suggests these neurons might be critical in relaying chemoreflex afferent information to the PVN. They are also ideally situated for facilitating the autonomic and humoral responses during hypoxia, for they not only receive input from regions with direct input from chemoreflex afferents (such as the nTS), but also contain projection neurons that densely innervate the PVN. As a major integrative center, the PVN may have a significant role in receiving and sending hypoxic-related signals from and to other central pathways especially in response to more severe hypoxia. Although the CVLM has been studied extensively for its role in the arterial baroreflex via projections to the RVLM, given the present data, the CVLM should be considered for its important role in the chemoreflex as well, through projections which include the PVN.

Figure 3.1 -

Caudal-rostral distribution of PVN-projecting neurons in the CVLM. A) Epifluorescent photomicrograph merged with a bright field image showing a representative injection site of 60 nL of 2% FG injected bilaterally in the PVN. B) Schematic representation of the medulla with the area containing the CVLM outlined in the red dashed box. Below, a bright field image at the same level of the brainstem as above (left) and representative FG labeling found in CVLM neurons (right). C, D) Caudal to rostral distribution of the number of C) PVN-projecting cells (FG-labeled) and D) PVN-projecting catecholaminergic (FG and TH co-labeled) cells in the CVLM following graded hypoxia. Statistical results for both Two-Way RM ANOVA (factors: hypoxia intensity and caudal-rostral level) and One-Way RM ANOVA (evaluation of mean data, independent of hypoxic intensity; red line) are as follows: $p \leq 0.05$; $\dagger$ vs. -15.00, $\dagger\dagger$ vs. -15.00, -14.82, and -14.64, relative to Bregma. Calamus scriptorius (the caudal-most portion of the area postrema) is at approximately -14.4mm relative to bregma. Abbreviations: 3V: third ventricle; 12: hypoglossal nucleus; Amb: Nucleus ambiguus; CVLM: Caudal ventrolateral medulla; f: fornix; IO: inferior olive; LRT: lateral reticular nuclei; nTS: nucleus tractus solitarius; PVN: paraventricular nucleus of the hypothalamus; py: pyramidal tract; Sp, SpC: spinal trigeminal tract and nucleus. Scale bars = 250 μ m.

Figure 3.2 -

Increasing intensities of hypoxia progressively activated CVLM cells. Representative photomicrographs (coronal sections) show activation (Fos-IR nuclei, pseudo-colored red) in the CVLM after normoxia (A) and exposure to increasing levels of acute hypoxia (B-D). Inset: High magnification image of the area outlined. E) Left: Total number of Fos-IR cells in six sections of the CVLM in response to increasing intensities of acute hypoxia (21% O₂, n= 4; 12, 10, 8% O₂, n= 5). Right: number of activated (Fos-IR) cells throughout the caudal to rostral extent of the CVLM. Two-way repeated measures ANOVA indicated no effect of caudal-rostral level but significant main effects of hypoxic group. Statistical results are as follows: p<0.05; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. Scale bars = 250µm.

Figure 3.3 -

Acute hypoxia progressively increased activation of catecholaminergic CVLM cells.

Representative merged photomicrographs showing labeling for Fos-IR (red) and TH-IR (pseudo-colored green) cells in the CVLM after normoxia (A) and increasing intensity of acute hypoxia (B-D). Insets: higher magnification of boxed area including examples of cells with double labeling. E) The number of activated CVLM catecholaminergic (Fos and TH co-labeled) neurons in response to increasing intensities of hypoxia (left) and the caudal to rostral distribution of activated catecholaminergic cells in the CVLM (right). Two-way repeated measures ANOVA indicated significant main effect of hypoxic intensity but not CVLM level. F) The percent of CVLM catecholaminergic cells that were activated following exposure to normoxia or increasing intensities of acute hypoxia determined by: $[(\text{Fos} + \text{TH}) / (\text{TH})] \times 100$. Statistical results are as follows: $p \leq 0.05$; * vs. 21% O₂; ** vs. 21 and 12% O₂. Scale bars = 250 μm .

Figure 3.4 -

Increasing intensities of hypoxia progressively activated PVN-projecting CVLM neurons.

Representative merged photomicrographs showing Fos-IR (red) and FG labeling (pseudo-colored blue) in the CVLM from individual animals exposed to normoxia (A) and different intensities of hypoxia (B-D). Higher magnification of boxed areas shows co-labeling of Fos and FG. E) Total number of PVN projecting CVLM cells that were activated (Fos-IR and FG-labeled) by hypoxia (left) and caudal-rostral distribution of activated PVN projecting CVLM cells for each intensity of acute hypoxia (right). Two-way repeated measures ANOVA indicated a significant main effect of hypoxic intensity but not caudal-rostral level. F) The percent of PVN-projecting CVLM cells that were activated following exposure to normoxia or increasing intensities of acute hypoxia: $[(\text{Fos} + \text{FG}) / (\text{FG})] \times 100$ followed a similar pattern. In E) and F), statistical results are as follows: $p < 0.05$; * vs 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. Scale bars = 250 μm .

Figure 3.5 -

Increasing intensities of hypoxia preferentially activated catecholaminergic PVN-projecting CVLM neurons.

Merged photomicrographs illustrate that the majority of activated PVN-projecting CVLM neurons were catecholaminergic. Activated (Fos-IR, red), catecholaminergic (TH-IR, green) and PVN-projecting (FG labeled, blue) cells are shown together from individual animals exposed to normoxia (A) and three different intensities of hypoxia (B-D). Boxed areas show higher magnification of triple labeled cells. E) Total number of triple labeled CVLM cells in response to increasing intensities of hypoxia (left) and caudal-rostral distribution of activated catecholaminergic-PVN projecting CVLM cells for each intensity

of acute hypoxia (right). Two-way repeated measures ANOVA indicated significant main effects of hypoxic intensity but not caudal-rostral level. F) Relative activation of CVLM PVN-projecting cells in response to increasing intensities of acute hypoxia. The percent of activated PVN-projecting catecholaminergic $\left[\frac{((\text{Fos}+\text{FG}+\text{TH}))}{(\text{FG}+\text{TH})}\right] \times 100$; solid bars] or PVN-projecting noncatecholaminergic $\left[\frac{((\text{Fos}+(\text{FG and TH-negative})))}{(\text{FG and TH-negative})}\right] \times 100$; striped bars]. Within the catecholaminergic group of PVN projecting neurons there was a graded effect of hypoxia. Within the noncatecholaminergic group there was no difference among groups. Statistical results are as follows: $p \leq 0.05$; for a specific phenotype, ** vs. 21 and 12% O_2 . †, $p \leq 0.05$ vs. catecholaminergic cells within each hypoxic group. Scale bars = 250 μm .

Figure 3.6 -

Changes in MAP during chemoreflex stimulation do not alter the number of activated CVLM neurons overall or of activated catecholaminergic CVLM neurons.

Representative photomicrographs of coronal CVLM sections which have been immunolabeled for the detection of Fos- and TH-IR. Sections are from animals subjected to (A) 10% O_2 or (B) 10% O_2 + phenylephrine (PE) to maintain blood pressure during hypoxia. Number of Fos-IR cells (C) or cells double labeled with Fos- and TH-IR (D) counted in CVLM after hypoxia alone (10% O_2) or with MAP maintained constant (10% O_2 + PE). Scale bars = 250 μm .

Table 3.1 -

Table 3.1. Total number of positively labeled cells counted in six CVLM sections.

	21% O ₂	12% O ₂	10% O ₂	8%O ₂
# of PVN-projecting cells	99±15	99±6	98±10	100±11
# of catecholaminergic cells	96±9	102±4	106±16	108±2
# of colabeled cells	66±3	73±5	60±9	58±5

Catecholaminergic (TH-IR), PVN-projecting (FG labeled), and colabeled cells in normoxic animals (21% O₂; n=4) and in animals exposed to three levels of hypoxia (n=5 each). Values are mean ± S.E. The number of PVN-projecting and catecholaminergic neurons was similar among all groups.

CHAPTER 4: Catecholaminergic neurons projecting to the paraventricular nucleus (PVN) of the hypothalamus are essential for cardiorespiratory adjustments to hypoxia

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4.1 ABSTRACT

Brainstem catecholamine neurons modulate sensory information and participate in control of cardiorespiratory function. These neurons have multiple projections, including to the PVN, which contributes to cardiorespiratory and neuroendocrine responses to hypoxia. We have shown that PVN-projecting catecholaminergic neurons are activated by hypoxia, but the function of these neurons is not known. To test the hypothesis that PVN-projecting catecholamine neurons participate in responses to respiratory challenges, we injected IgG saporin (control, n=6) or anti-dopamine beta-hydroxylase saporin (DSAP, n=6) into the PVN to retrogradely lesion catecholamine neurons projecting to the PVN. After 2 weeks, respiratory (plethysmography) measurements were made in awake rats during normoxia, increasing intensities of hypoxia (12%, 10%, 8% O₂), and hypercapnia (5% CO₂/95% O₂). DSAP decreased the number of tyrosine hydroxylase immunoreactive cells counted in VLM (-67%) and nTS (-56%). DSAP significantly decreased breathing rate during normoxia and all intensities of hypoxia. Tidal volume and minute ventilation (V_E) index also were impaired at higher hypoxic intensities (10, 8% O₂; e.g. V_E at 10% O₂: IgG = 181±22, DSAP = 91±4 arbitrary units). Depressed ventilation in DSAP rats was associated with significantly lower arterial O₂ saturation at all hypoxic intensities. PVN DSAP also reduced ventilatory responses to 5% CO₂ (V_E: IgG = 176±21, DSAP = 84±5 arbitrary units). Data indicate that catecholamine neurons projecting to the PVN are critical for peripheral and central chemoreflex respiratory responses and for maintenance of arterial oxygen levels during hypoxic stimuli.

4.2 INTRODUCTION

Peripheral chemoreflex activation by systemic hypoxia produces highly integrated respiratory, autonomic, behavioral, and endocrine responses (47, 79, 90, 239). Together these responses are critical for homeostasis, serving to restore, protect and maintain tissue oxygenation throughout the body. The central nervous system pathways involved in responses to acute hypoxia are complex. Peripheral chemoreceptor afferent nerves from the carotid bodies enter the central nervous system and project onto neurons located in the nucleus tractus solitarii (nTS) (42, 48, 162). Chemoreceptor afferent input is modulated and integrated in the nTS and then sent to the rostral ventrolateral medulla (126, 131, 204). This projection is considered the primary pathway mediating cardiorespiratory chemoreflex responses.

Another brain region that may play an important role in the integrated response to hypoxia is the paraventricular nucleus of the hypothalamus (PVN). PVN neurons are activated by chemoreflex stimulation, including hypoxia and hypercapnia (21, 63, 104). The PVN modulates cardiorespiratory function, including blood pressure (136, 175), sympathetic nerve activity (46), and respiratory timing and activity (150, 191, 208, 209, 248), via direct projections to the rostral ventrolateral medulla (12, 112), spinal cord (189, 222), phrenic motor nucleus and pre-Botzinger complex (118). Furthermore the PVN is required for full expression of chemoreflex responses, since lesion (175) or interruption of neuronal activity in the PVN (190) blunts the magnitude and duration of the pressor and sympathoexcitatory responses evoked by chemoreflex activation.

The pathways by which chemoreflex afferent information is relayed to the PVN are less well known. The PVN receives catecholaminergic input from the adrenergic/noradrenergic regions of the nTS (A2/C2 cell group), the ventrolateral medulla (VLM; A1/C1 cell group), and the locus coeruleus (A6 cell group) (51, 52, 205). All of these catecholaminergic populations are responsive to a variety of cardiorespiratory stimuli (9, 37, 69, 193). For example, noradrenergic neurons of the A1 region in the caudal ventrolateral medulla (CVLM) are activated by hypoxia (123, 217) and lesion of this medullary region attenuates vasopressin and adrenocorticotropin hormone responses to hypoxia (26). Furthermore, blockade of adrenergic receptors in the PVN blunts the cardiovascular response to acute chemoreflex stimulation in spinally transected rats (136).

Recent studies from our laboratory revealed that acute hypoxia activates PVN-projecting neurons from both the nTS and the CVLM. Importantly, a large proportion of these projection neurons is catecholaminergic (122, 123), suggesting they might have a very specific function associated with responses to chemoreflex activation. However, the extent to which catecholaminergic input to the PVN plays a functional role in respiratory responses to acute hypoxia is currently unknown. This study was designed to test the hypothesis that PVN-projecting catecholaminergic neurons participate in baseline cardiorespiratory function and contribute to cardiorespiratory responses elicited by hypoxia or hypercapnia. To test this hypothesis, we retrogradely lesioned PVN-projecting catecholaminergic neurons by injecting anti-dopamine beta-hydroxylase saporin (DSAP) into the PVN of rats and measured ventilation, arterial blood pressure

and heart rate during normoxic, hypoxic and hypercapnic conditions. The primary finding of the present study is that lesion of PVN-projecting catecholaminergic neurons reduced ventilatory responses to increasing intensities of systemic hypoxia and also to hypercapnia, suggesting that these neurons play a significant role in baseline respiration and peripheral and central chemoreflex function. Overall, the present study demonstrates the importance of catecholaminergic inputs to the PVN in mediating chemoreflex-induced responses.

4.3 METHODS

4.3.1 Animals

Adult male Sprague-Dawley rats were obtained from Harlan Laboratories. Animals were individually housed in a temperature and humidity controlled vivarium. The animals were maintained on a 12 hour light, 12 hour dark cycle with food and water available ad libitum. All experimental animal protocols were approved by the University of Missouri Institutional Animal Care and Use Committee, and conform to National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of twelve male Sprague-Dawley rats (250–350 g) were assigned to two groups: lesion with PVN microinjection of anti-dopamine beta-hydroxylase–saporin (DSAP; n=6) or control with IgG-saporin (n=6).

4.3.2 Surgical Procedures

Two weeks before hypoxia experiments, rats were anesthetized with Isoflurane (AErane, Baxter, Deerfield, IL, USA; 5% for induction and 2–2.5% for maintenance). The following procedures were performed using aseptic technique:

Telemetry device placement for cardiovascular measurements. To assess continuous mean arterial blood pressure (MAP) and heart rate (HR) changes in response to acute hypoxia, all rats were instrumented with a telemetry device (TA11PA-C40, Data Sciences International, USA) placed in the abdominal aorta as previously described (122). Briefly, following induction of anesthesia, a midline incision was made, the abdominal aorta visualized and the catheter probe of the telemetry device was inserted. The site was sealed with a cellulose patch and tissue adhesive. The transmitter was secured to the abdominal muscle using non-absorbable suture, and the skin incision closed.

Immunotoxin lesions. During the same surgery, microinjections of anti-dopamine beta-hydroxylase conjugated to saporin (DSAP, Advanced Targeting Systems, 42 ng in 200 nl in phosphate buffer, pH 7.4, n = 6) or a control IgG antibody conjugated to saporin (IgG, Advanced Targeting Systems, 42 ng in 200 nl in phosphate buffer, pH 7.4, n = 6), was microinjected bilaterally into the PVN. An important feature of DSAP is that, unlike many other saporin conjugates, it is retrogradely transported (25, 181, 196, 213, 244). We therefore used DSAP to selectively eliminate catecholaminergic neurons that project to the PVN. Injections were made using a custom pressure injection system through a stereotaxically positioned single barrel glass micropipette. The coordinates for injections localized to the PVN were: 1.8 - 2.0 mm caudal to bregma, $\pm$ 0.5 mm lateral

from the midline and 7.6-7.8 mm ventral to the dura, similar to previous work from our laboratory and others (122, 123, 142, 156). The concentration and volume of DSAP and IgG injected at each site were determined from previous experiments using similar protocols (23, 65). Rats were treated post operatively with fluids (3 ml, 0.9% saline s.c.), Baytril (2.5 mg/kg i.m., Bayer, Shawnee Mission, KS, USA), and Buprenex (0.03 mg/kg s.c., Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) to maintain hydration status, prevent infection, and for pain management, respectively. Upon recovery from anesthesia animals were returned to their cages. At least two weeks were allowed for degeneration of the targeted neurons and for recovery from abdominal and intracranial surgery (236, 245). During this period, daily clinical examination, body weight measurements and the strength of the telemetry signal were evaluated.

4.3.3 Plethysmography for ventilatory assessment.

Ventilation was assessed in conscious, unrestrained rats by whole body plethysmography, as previously described (50, 122, 125, 127). Typically, one DSAP and one IgG-treated rat were placed in adjoining whole body plethysmography chambers and experiments run simultaneously using the same gas sources. Chambers (Data Sciences International, USA) include both an inlet port for administration of test gases and an outlet port to allow airflow through the chamber; each chamber was connected to a differential pressure transducer (Validyne MP45, Validyne Engineering Corp., USA) and to a reference chamber. The recorded pressure signal is proportional to volume changes and allowed us to obtain a measure of tidal volume (tidal volume index) by integrating the area under the inspiratory pressure curve. Ventilatory measurements

were recorded using a data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO, USA) and included: respiratory rate (RR, breaths per min), tidal volume index and minute ventilation index (RR x tidal volume index). We also examined the number and amplitude of augmented breaths. Tidal volume index, minute ventilation index, and augmented breath amplitude were normalized to body weight. Oxygen saturation was measured using a collar pulse oximeter (MouseOx, Starr Life Sciences Corp., USA).

Baseline ventilation was assessed before surgical procedures described above and on the day of the experiment examining chemoreflex function; cardiovascular parameters were measured on the day of the experiment only because telemetry devices were implanted during the surgery. Animals were acclimatized to the chamber environment before the actual experiments. On the day of the experiment, animals were allowed to acclimate to the plethysmograph while breathing room air. Ventilation, oxygen saturation, arterial blood pressure and heart rate were monitored continuously during room air breathing, and during exposure to 20 minutes normoxia (21% O₂), and hypoxia (12, 10 and 8% O₂) to assess peripheral chemoreflex function. To evaluate central chemoreflex responses, rats were exposed to hyperoxia (100% O₂) to minimize peripheral chemoreceptor input, followed by hypercapnia (95% O₂/5% CO₂). The order of gas exposure was varied to produce a balanced design except 100% O₂ was always followed by hypercapnia to isolate central chemoreflex responses. For experiments examining responses to hypoxia, mixtures of gases were regulated by a gas blender that provided precise control of oxygen concentrations (Hypoxydial, Starr Life Sciences Corp.,

USA), which were confirmed by an O₂ analyzer; hypercapnia experiments utilized compressed gas tanks. The ventilatory response during these exposures was quantified by analyzing an average of approximately twenty consecutive breaths excluding sniffs, augmented breaths or movement artifacts. Augmented breaths were determined from their comparatively large inspiratory and expiratory flows, which could be easily discerned from the background of normal breathing, appearing as episodic spikes in the trace (Figure 4A). Because of their relatively infrequent occurrence, frequency of augmented breaths was not analyzed during normoxia, and was expressed as the number of augmented breaths per 10 seconds during hypoxic exposures.

4.3.4 Assessment of DSAP lesion and Immunohistochemistry

After chemoreflex testing was completed, animals were transcardially perfused with heparinized, Dulbecco's Modified Eagle Medium (Sigma, 125 mL, pH 7.4) followed by 4% paraformaldehyde (PFA, Sigma, 500 mL, pH 7.4). Standard immunohistochemical approaches were used to evaluate the efficacy of DSAP lesions. Brains were removed and post-fixed overnight in 4% paraformaldehyde followed by coronal sectioning on a vibratome for immunohistochemical staining of tyrosine hydroxylase (mouse anti-TH, 1:1000, Millipore Inc), the rate limiting enzyme in catecholamine synthesis, which is commonly used as a marker for catecholaminergic neurons (126, 180). One of every six sections was incubated overnight in 1% normal donkey serum (NDS) and 0.3% Triton-0.01M PBS containing the primary antibody against TH. The following day, sections were rinsed and incubated for two hours in Cy2-conjugated donkey anti-mouse IgG (1:200 Jackson ImmunoResearch) with 1% NDS in 0.3% Triton-0.01M PBS. Sections were

then mounted on slides and coverslipped for microscopic evaluation. Histological sections used in figures were captured using an Olympus epifluorescent microscope (BX51). Images were analyzed with Image J (ver. 1.41, NIH) using a custom made plugin (GAIA Group, Novato, CA, USA, <http://gaiag.net/index.html>). In some cases, brightness and contrast only were altered to achieve uniformity among photomicrographs. To evaluate the effects of DSAP injections on catecholaminergic neurons, two individuals blinded to experimental groups counted the number of TH immunoreactive (TH-IR) cells and results were averaged. Cells were counted over the range of -14.70 to -9.48 mm relative to bregma in the following specific nuclei based on an anatomic brain atlas (177): the nTS (A2 cell group), the ventrolateral medulla (VLM; A1/C1 cell group), and the locus coeruleus (LC; A6 cell group). For each region of interest the number of sections counted and the caudal to rostral length evaluated were not different between the groups. All cells with distinct cytosolic labeling, visible processes, and a blank nuclear area in each region of interest were counted.

4.3.5 Statistics

Statistical analyses were performed using SigmaPlot (12.3, Systat Software, San Jose, CA, USA). All data are presented as mean $\pm$ standard error and statistical significance was set at $p \leq 0.05$. Total counts of TH-IR neurons were compared between IgG and DSAP treatment by t-test. Caudal-rostral distributions of TH labeling were analyzed by two-way repeated measures analysis of variance (ANOVA). A Pearson correlation was used to calculate R^2 values, slope, and significance of correlations between peak ventilatory responses and number of cells remaining in the nTS and VLM.

A two-way repeated measures ANOVA was performed to examine differences in cardiorespiratory responses of the two groups (IgG and DSAP injection) to various levels of inspired O₂ or CO₂. T-tests were used to compare the slopes of respiratory variables plotted against oxygen saturation. When interactions between group and oxygen level occurred, ANOVAs were followed by post-hoc analysis using Fisher's LSD test.

4.4 RESULTS

4.4.1 Lesion of PVN-projecting catecholaminergic neurons decreases the number of TH-IR cells in the brainstem

In the current studies, we used DSAP (181, 196) to specifically lesion PVN-projecting catecholaminergic neurons. To validate the efficacy of the toxin we analyzed TH-IR in the brainstem, specifically the regions of the nTS (A2), VLM (A1/C1), and LC (A6). Figure 1 depicts the decreased number of nTS and VLM catecholamine neurons after DSAP injections. Schematic representations of the nTS and VLM regions shown in the photomicrographs are presented in Figure 1A; Figure 1B includes corresponding coronal sections showing TH-IR in IgG control rats (top row) and rats injected with DSAP into the PVN (bottom row). DSAP injections eliminated a large proportion of TH immunolabeling within these sites. Quantitative analysis indicated that in the nTS, DSAP decreased the number of TH-IR cells by 57% and in the VLM by 69% (Figure 1C). There was no significant change in the number of TH-IR cells in the LC (IgG: 26 ± 5 , DSAP: 23 ± 6 cells).

4.4.2 PVN-projecting catecholamine neurons contribute to baseline ventilation and blood pressure

Initial experiments examined the effects of lesioning PVN-projecting catecholaminergic neurons on basal cardiorespiratory parameters. Prior to surgical procedures, body weight, oxygen saturation and ventilation during normoxic breathing were not different between rats assigned to IgG control or DSAP lesioned groups (Table 1). Two weeks after IgG injections, respiratory rate, tidal volume, and minute ventilation were not significantly altered. In contrast, DSAP-injected animals exhibited a significantly decreased respiratory rate while breathing room air compared to pre-surgery and to the control group. There was no significant difference in body weight, tidal volume, and minute ventilation between groups, although body weight increased in both groups after two weeks. In addition, DSAP injections into the PVN resulted in increased baseline mean arterial pressure compared to controls. Because telemetric devices were implanted during the same surgery as PVN injections, pre-surgical measurements of MAP and heart rate were not obtained.

4.4.3 PVN-projecting catecholamine neurons contribute to cardiorespiratory responses to hypoxia

We evaluated the role of PVN-projecting catecholaminergic neurons in responses to hypoxia using plethysmography and telemetry, by comparing hypoxic responses of IgG control animals with those of lesioned animals. Representative recordings of plethysmography-acquired respiratory responses in individual rats injected with IgG (top) or DSAP (bottom) to normoxia (21% O₂) or activation of the peripheral

chemoreflex with three intensities of acute hypoxia are shown in Figure 2A. In IgG control animals, exposure to increasing hypoxia resulted in progressive increases in respiratory rate and tidal volume. Respiratory variables increased within the first minute of hypoxic challenge and remained elevated throughout the hypoxia exposures. Although DSAP-treated animals increased respiratory rate, tidal volume and minute ventilation above their baseline ventilation, these responses to hypoxia were blunted compared to control animals. Group data for respiratory variables are shown in Figure 2B-E. In response to increasing hypoxia both groups exhibited progressive decreases in arterial blood oxygen saturation and increases in respiratory rate, tidal volume index and minute ventilation index. In the DSAP group however, oxygen saturation was less compared to IgG control animals at each intensity of hypoxia. Despite lower oxygen saturation and presumably partial pressures of oxygen, DSAP-treated animals exhibited similar ventilatory responses to 12% O₂ and blunted increases in respiratory rate, tidal volume and minute ventilation to higher intensities of hypoxia.

The mean arterial pressure and heart rate responses to graded intensities of hypoxia are shown in Figure 2F and G. In both groups, systemic hypoxia evoked a decrease in arterial blood pressure, although blood pressure in DSAP treated animals was higher during all intensities of hypoxia. In IgG control animals, heart rate increased during hypoxia, reaching statistical significance at 8% O₂. In contrast, the increase in heart rate was blunted in rats treated with DSAP.

Because oxygen saturation during hypoxia was different between groups, we also evaluated the respiratory response relative to a given oxygen saturation (Figure

3A-C). In both groups a decline in oxygen saturation was associated with a progressive increase in respiratory rate, tidal volume and minute ventilation. However, the slope of these relationships was significantly reduced in DSAP treated rats, compared to controls (Table 2) indicating an attenuated response to a given arterial oxygen saturation. Overall, these results indicate that bilateral lesioning of PVN-projecting catecholaminergic neurons markedly attenuated cardiorespiratory responses to hypoxia.

A common feature of chemoreflex responses is the occurrence of augmented breaths taken by the animal during hypoxia. Therefore, we also evaluated the number and amplitude of augmented breaths during hypoxia. Figure 4A contains raw flow traces obtained from individual rats injected with IgG (top) or DSAP (bottom), shown in compressed time scale. An example of a spontaneous augmented breath with an expanded time scale is also shown. Augmented breaths were more prevalent in the IgG rat compared to DSAP. The mean frequency of augmented breaths recorded for all animals is displayed in Figure 4B. Augmented breaths were rarely observed during normoxia and data were not analyzed. Both groups displayed augmented breaths in response to 12% O₂. In IgG-injected animals, the frequency increased further at 10% and 8% O₂. However, in DSAP-injected animals frequency was not significantly increased further until exposure to 8% O₂. Furthermore, the frequency was significantly less in DSAP compared to control IgG treated animals while breathing 10% and 8% O₂. Figure 4C depicts the average amplitude of these augmented breaths. There was no significant effect of hypoxia intensity on the amplitude of augmented breaths. However, amplitude

was significantly less in DSAP treated animals. These results indicate that the occurrence of augmented breaths during hypoxia is dependent, in part, on catecholaminergic input to the PVN.

4.4.4 Peak ventilatory responses are correlated with the number of PVN-projecting catecholaminergic cells in animals with DSAP injections

In DSAP-treated rats the peak minute ventilation (8% O₂) was significantly correlated with the number of TH-IR neurons counted in the both the nTS and the VLM (Figure 5). Thus, the fewer PVN-projecting catecholaminergic neurons remaining in a given DSAP rat, the more attenuated was their ventilatory response to hypoxia. In contrast, within the IgG-injected animals, there was no correlation between these two variables (data not shown). This supports the view that hypoxia-mediated activation of catecholaminergic neurons in both medullary regions relays chemoreflex information to the PVN, and noradrenergic input to the PVN is critical in mediating ventilatory responses to hypoxia.

4.4.5 PVN-projecting catecholamine neurons contribute to cardiorespiratory responses to hypercapnia

To analyze the importance of PVN-projecting neurons in central chemoreceptor function we used 100% O₂ to minimize peripheral chemoreceptor input prior to exposure to 5%CO₂/95% O₂ to activate predominantly central chemoreceptors (84, 128). Figure 6A shows respiratory flow traces during hyperoxia (100% O₂; left) followed by hypercapnia (5% CO₂/95% O₂; right) in individual rats with control (IgG) or DSAP injections in the PVN. Figure 6B-D includes mean data and indicates that, relative to

breathing 100% O₂, both groups exhibited increased respiratory rate, tidal volume, and minute ventilation under hypercapnic conditions. DSAP animals were significantly less responsive to hypercapnia compared to control animals with respect to all ventilatory parameters. Ventilation during inhalation of 100% O₂, was similar in both groups.

4.5 DISCUSSION

The present study provides new findings regarding cardiorespiratory control by PVN-projecting catecholaminergic neurons in the brainstem. Substantial depletion of neurons in the primary catecholaminergic regions in the caudal medulla (A1/A2, C1/C2), depressed respiratory rate and increased mean arterial blood pressure during normoxic conditions, suggesting that these pathways contribute to baseline ventilation and cardiovascular control. In addition, lesioning catecholaminergic projections to PVN reduced ventilatory responses to increasing intensities of systemic hypoxia, and reduced the ability to maintain O₂ saturation. Responses to central chemoreflex activation with hypercapnia also were impaired. These findings indicate that PVN-projecting catecholaminergic neurons play a key role in cardiorespiratory adjustments to both peripheral and central chemoreflex challenges and support the importance of the PVN in baseline and reflex control of respiration.

The current studies used the anti-dopamine beta hydroxylase saporin conjugate (245) to selectively lesion catecholamine cells that project to the PVN and specifically examine the role of these neurons in cardiorespiratory function. Similar to previous studies (23, 105, 196), in the current experiments, catecholamine cell numbers in the

nTS and VLM were decreased significantly by PVN injections of DSAP, while little depletion of catecholaminergic neurons in the LC was observed (73, 179). In the more rostral VLM, which contains primarily cells that project to the spinal cord (95, 205), there was correspondingly less reduction in catecholaminergic neurons due to DSAP injections. Importantly, results are consistent with our previous studies (122, 123), in which we found that approximately 50% of nTS and 70% of VLM catecholaminergic neurons project to the PVN. This corresponds closely with the percent of catecholaminergic neurons that were lesioned in the present study (57% for the nTS and 69% for the VLM). Therefore, the data indicate that we were able to selectively lesion PVN-projecting catecholaminergic neurons.

Previous studies in mice with *Phox2a* deletions (237) and in rats with widespread DSAP lesions due to administration into the fourth ventricle (139) indicate that catecholaminergic neurons in general provide a tonic stimulus to intrinsic breathing frequency. The current experiments confirm and extend these findings to indicate that in conscious rats catecholaminergic projections specifically to the PVN are critical to baseline respiratory rate, likely via their PVN projections. Although recent studies (19) in which overall catecholamine synthesis in nTS was reduced with shRNA indicate no effect on basal respiratory frequency, catecholamine synthesis would be reduced in both PVN-projecting and non-projecting nTS neurons, so that there may be off-setting or nonspecific effects. In addition, the role of co-transmitters in catecholaminergic neurons cannot be eliminated as these neurons presumably remained functional and could release neurotransmitter upon activation. Nevertheless, it is possible that PVN-

projecting catecholamine neurons independent of nTS (likely from the ventrolateral medulla) contribute to basal effects on breathing frequency. Future experiments are required to confirm the role of nTS vs. VLM catecholaminergic neurons. In contrast to baseline effects on frequency, lesion of PVN-projecting catecholaminergic neurons had no effect on resting tidal volume. These data suggest that the neurocircuitry controlling basal tidal volume (such as pontine regions and lung afferents) has distinct inputs and neural pathways that do not require catecholaminergic projections to the PVN.

Different from effects on baseline breathing, lesion of PVN-projecting catecholaminergic neurons increased resting MAP. The mechanisms contributing to this elevation in MAP were not specifically addressed in our experiments but there are several possible explanations. One possibility is that these catecholaminergic neurons could contribute to tonic inhibition of PVN neurons that send excitatory projections to the RVLM and spinal cord. Alternatively, a loss of noradrenergic neurons in the nTS (A2 neurons) may be responsible for the elevated resting arterial pressure. Although reducing nTS catecholamine synthesis has no effect on resting pressure (19), selective depletion of catecholamine cells in the nTS leads to hypertension for several days followed by marked chronic lability of arterial pressure (218). Furthermore, A2 lesions also attenuate reflex bradycardic responses to a pressor stimulus, implying the importance of A2 neurons in arterial baroreflex function (232). Taken together, catecholaminergic inputs to the PVN are critical in tonic control of breathing and blood pressure. Additional studies are required to determine the specific mechanisms contributing to blood pressure effects, and whether different populations of PVN-

projecting catecholaminergic neurons affect basal ventilatory and cardiovascular control.

Previous work from our laboratory indicates that hypoxia produces significant activation of PVN-projecting catecholaminergic neurons in both the nTS and CVLM, particularly at greater hypoxia intensities (122, 123). However, the functional relevance of these projections was unknown. A major finding of the present study is that lesion of PVN-projecting catecholaminergic neurons significantly impaired respiratory responses to peripheral chemoreflex activation. Increasing intensities of hypoxia in IgG-injected control animals decreased oxygen saturation accompanied by robust increases in ventilation; however, peak ventilatory responses in DSAP injected rats were significantly attenuated. Importantly, blunted ventilation produced physiological consequences, including lower oxygen saturation. Furthermore, at a given oxygen saturation, ventilatory responses were markedly attenuated in DSAP compared to IgG control rats. Taken together, these data demonstrate the crucial role of the PVN and catecholaminergic input to the PVN in eliciting an appropriate response to increasing hypoxia. While these catecholaminergic neurons could project to multiple brain regions, prior work has shown that at least in the nTS, most neurons, including catecholaminergic neurons, project primarily to only one region, including the PVN (101). These projections may provide a specific pathway by which chemoreflex information is integrated.

Although other studies have implicated PVN-projecting neurons in hypoxia induced responses, none has definitively shown that catecholaminergic input is required

for functional ventilatory responses to acute chemoreflex stimulation. For example, unilateral ibotenic acid lesions of the brainstem including the caudal two thirds of the VLM catecholamine cell group significantly reduced activation of PVN vasopressin, CRH, and oxytocin cell types following hypoxia (217). However, these lesions were not specific to catecholamine neurons and ventilatory responses were not examined. Knockdown of overall catecholamine synthesis in nTS had no effect on basal respiratory frequency but reduced ventilatory and cardiovascular responses to chronic intermittent hypoxia (19); neither direct PVN projections nor acute hypoxic responses were examined. In addition, while blockade of adrenergic receptors in the PVN blunts cardiovascular responses and vasopressin release to carotid chemoreceptor stimulation in spinally transected rats (136), neither the source of this input nor ventilatory responses were addressed. The present work shows for the first time that catecholaminergic inputs to PVN are involved in respiratory responses to chemoreflex activation.

Augmented breaths increase in frequency during hypoxia (157, 158) and are thought to be important in preventing atelectasis, which otherwise may result in collapsed and hypoventilated regions of the lung. In the absence of occasional augmented breaths, lung compliance gradually decreases and efficacy of gas exchange worsens (22, 172). Therefore, an occasional augmented breath is important to maintenance of effective lung function. Interestingly, PVN activity changes dynamically during phasic respiratory events, and thus may contribute to the progression of augmented breaths (133). In the current study, lesion of PVN-projecting

catecholaminergic neurons from the nTS and/or VLM significantly blunted the frequency and amplitude of augmented breaths during progressive hypoxia, suggesting that such inputs and the PVN are necessary for these breaths. Thus, catecholaminergic projections to the PVN may be important to prevent transient respiratory disturbances at the level of the lungs during hypoxia.

As previously described (122, 153), acute hypoxia decreases mean arterial pressure, an effect that progresses with hypoxia intensity. Interestingly, catecholamine neurons projecting to the PVN are activated in response to cardiovascular challenges such as hypovolemia and osmotic stimuli (65). However, the involvement of the PVN in cardiovascular adaptations specifically to hypoxia has not been evaluated thoroughly. In the present study, while baseline MAP was higher in rats with lesions of PVN-projecting catecholaminergic neurons, both groups exhibited similar decreases in blood pressure to hypoxia, suggesting this depressor response does not require PVN-projecting catecholaminergic neurons. Interestingly, however, an increase in heart rate to hypoxia was observed only in control animals, implying that adrenergic input to the PVN is involved in certain cardiovascular responses to hypoxia. Overall, novel results from this study indicate that PVN-projecting catecholaminergic neurons may influence not only respiratory, but also cardiovascular responses to hypoxia.

In regard to central chemoreflexes, prior observations indicate that a widely distributed loss of catecholaminergic neurons decreases breathing frequency and blunts central chemoreceptor responses (132, 139, 237). Although global inhibition of the PVN with lidocaine, which would be expected to eliminate both excitatory and inhibitory

influences, does not alter cardiorespiratory responses to central chemoreflex activation with 10% CO₂ (190), PVN neurons (including vasopressin and oxytocin neurons projecting to the RVLM and spinal cord) are activated by hypercapnia (63, 96, 118, 144, 150, 173, 178, 234). Novel results from the current study indicate that lesion of PVN-projecting catecholaminergic neurons significantly attenuated breathing frequency, tidal volume and minute ventilation during exposure to systemic hypercapnia (5% CO₂). These data demonstrate that catecholaminergic neurons participate in central chemoreceptor ventilatory responses and extend previous work to indicate that catecholaminergic projections to the PVN may be particularly important. Collectively, the present and previous findings suggest that the CO₂ chemosensory system is composed of many interconnected neurons that have the ability to affect regulation of autonomic, endocrine and behavioral responses. Our findings demonstrate for the first time that catecholaminergic input to the PVN is part of the hypercapnia-sensing network.

4.6 PERSPECTIVES AND SIGNIFICANCE

In summary, we conclude that PVN-projecting brainstem catecholaminergic neurons provide a tonic excitatory drive that maintains baseline respiratory rate, but tonically decreases arterial pressure. Whether different populations of these neurons influence cardiovascular and respiratory function currently is not known. PVN-projecting catecholaminergic neurons also contribute to peripheral and central chemoreflex

control of ventilation. The present findings confirm that the cardiorespiratory responses to hypoxia and hypercapnia are dependent, in part, on hindbrain catecholamine neurons that project to the PVN, as lesions of these neurons attenuated peripheral and central chemoreflex responses. Furthermore, these effects are physiologically relevant, in that blunted hypoxic ventilatory responses were associated with greater decreases in O₂ saturation. Previous work has focused on catecholaminergic neurons and the PVN separately. We now provide evidence that catecholaminergic projections to the PVN likely provide a link integrating respiratory, cardiovascular, and neuroendocrine responses to chemoreflex stimulation.

Figure 4.1 -

DSAP injection into the PVN reduces catecholaminergic neurons in both nTS and VLM regions. A) Schematic representation of the areas containing the nTS (left) and VLM (right) corresponding to photomicrographs below. B) Photomicrographs of TH-positive cells within the nTS (left panels) and VLM (right panels) in representative control (top) and DSAP-treated animals (bottom). DSAP lesions reduced TH-IR in both regions. Scale bar = 250 μ m. C) Total number of TH-positive neurons counted within the nTS (left) and VLM (right) in control (open bars) and DSAP-treated rats (filled bars). * $p \leq 0.05$, DSAP vs. IgG controls. VLM: Ventrolateral Medulla; nTS: nucleus tractus solitarii; 10: dorsal motor nucleus of the vagus; AP: area postrema; CC: central canal; TS: solitary tract.

Figure 4.2 -

Lesions of PVN-projecting catecholaminergic neurons resulted in blunted ventilatory responses to hypoxia.

A) Raw plethysmography records showing changes in pressure (which are proportional to tidal volume) from individual awake IgG control- (top) and DSAP-injected rats (bottom) during normoxia and three intensities of hypoxia (10 seconds each). Group data (n=6) showing effect of graded hypoxia on B) Oxygen saturation, C) Respiratory Rate, D) Tidal Volume Index, E) Minute Ventilation Index, F) Mean Arterial Pressure, and G) Heart Rate in IgG (open circles) vs DSAP-treated animals (filled circles) animals. Two-way repeated measures ANOVA indicated significant interactions of hypoxia intensity and group in all respiratory variables and a main effect of hypoxia for mean arterial pressure. DSAP injections significantly decreased respiratory rate during normoxia and blunted ventilatory responses to increasing intensities of hypoxia, despite greater decreases in oxygen saturation. $p < 0.05$; * vs. IgG; † vs. 21% O₂; †† vs. 21 and 12% O₂; ††† vs. 21, 12, and 10% O₂ within a group.

Figure 4.3 -

Correlation of ventilatory responses and oxygen saturation in IgG vs. DSAP-injected animals. A) Respiratory rate, B) tidal volume index, and C) minute ventilation index responses relative to oxygen saturation in IgG- (open circles) vs. DSAP-injected animals (filled circles). The slope of each correlation was significantly less in DSAP-injected animals compared to control. $p \leq 0.05$; * vs. IgG.

Figure 4.4 -

Lesion of PVN-projecting catecholaminergic neurons reduced the number of augmented breaths during hypoxia. A) Representative raw flow traces from a control (top) and DSAP-lesioned (bottom) animal while breathing 8% O₂. The augmented breaths appear as “spikes” in the traces with compressed time scales (five minutes). In the inset, the time scale is expanded to show the details of an augmented breath and how they can be identified in the normal underlying breathing rhythm. There was a decreased prevalence of augmented breaths in the DSAP compared with IgG treated animal. The mean B) number of augmented breaths over ten minutes and the C) amplitude of augmented breaths in response to hypoxia. With decreasing O₂, control animals show an increase in the number of augmented breaths. In DSAP animals there were significantly fewer augmented breaths at 10 and 8% O₂. Two-way repeated measures ANOVA indicated significant interactions of hypoxia intensity and group in the frequency of augmented breaths. There was also a main effect of group on the amplitude of augmented breaths. $p < 0.05$; * vs. IgG; † vs. 12% O₂; †† vs. 8% O₂.

Figure 4.5 -

Peak respiratory responses during hypoxia in DSAP rats are significantly correlated with the average number of remaining TH positive neurons. Peak minute ventilation index responses (y-axis) achieved during hypoxia (8% O₂) in DSAP animals are significantly correlated with the average number of remaining TH neurons counted within the nTS (A) and VLM (B). R² and *p* values (Pearson Correlation) are included on each graph. There were no correlations between the number of catecholaminergic cells and peak minute ventilation responses in control animals.

Figure 4.6 -

The central chemoreflex response is blunted after lesioning of catecholaminergic cells projecting to the PVN. Plethysmographic traces showing breathing during 100% O₂ (left) and 5% CO₂/95% O₂ (right) in individual control vs. DSAP animals. Group data (n=6) showing effect of 100% O₂ compared to 5% CO₂/95% O₂ on B) Oxygen Saturation, C) Respiratory Rate, D) Tidal Volume Index, and E) Minute Ventilation index in control (open bars) vs. DSAP animals (filled bars). There was a significant increase in all ventilatory parameters except oxygen saturation between 100% O₂ and 5% CO₂/95% O₂ in both groups (+). However the response was blunted in DSAP vs. IgG injected animals. p<0.05; * IgG vs. DSAP; † vs. 100% within a group.

Table 4.1

Table 4.1. Effects of IgG or anti-D β H saporin (DSAP) microinjections into the PVN on baseline cardiorespiratory variables

	pre-injection		post-injection	
	IgG	DSAP	IgG	DSAP
Body Weight (grams)	241 $\pm$ 23	230 $\pm$ 16	279 $\pm$ 4 [#]	302 $\pm$ 14 [#]
Oxygen Saturation (%)	95.7 $\pm$ 0.2	95.9 $\pm$ 0.4	95.9 $\pm$ 0.6	95.7 $\pm$ 0.4
Respiratory Rate	95 $\pm$ 5	94 $\pm$ 3	88 $\pm$ 2	76 $\pm$ 1 ^{*#}
Tidal Volume Index	0.39 $\pm$ 0.05	0.43 $\pm$ 0.01	0.47 $\pm$ 0.03	0.41 $\pm$ 0.05
Minute Ventilation Index	37 $\pm$ 4	40 $\pm$ 0.9	41 $\pm$ 6	31 $\pm$ 5
Mean Arterial Pressure			112 $\pm$ 1	122 $\pm$ 1.5 [*]
Heart Rate			353 $\pm$ 7	367 $\pm$ 15

All values are means $\pm$ SE. Pre-injection values were not significant between groups (n=6 each). After DSAP injection, baseline respiratory rate was decreased and mean arterial pressure was increased. $p \leq 0.05$; * vs. IgG. [#] vs. pre-injection. Mean arterial pressure and heart rate were measured using implanted telemetric devices. Therefore, no pre-surgical measurement were made.

Table 4.2

Table 4.2. Slope and R² values for the correlation between ventilatory responses and oxygen saturation in IgG vs DSAP-injected animals.

	IgG	DSAP
Slope		
Respiratory Rate	-1.92 $\pm$ 0.37	-0.88 $\pm$ 0.09 [*]
Tidal Volume	-0.03 $\pm$ 0.01	-0.009 $\pm$ 0.0006 [*]
Minute Ventilation	-4.96 $\pm$ 1.03	-1.38 $\pm$ 0.06 [*]
R ²		
Respiratory Rate	0.89 $\pm$ 0.03	0.90 $\pm$ 0.03
Tidal Volume	0.83 $\pm$ 0.02	0.79 $\pm$ 0.08
Minute Ventilation	0.89 $\pm$ 0.02	0.85 $\pm$ 0.04

Slope and R² values were calculated for the relationship of ventilatory responses and oxygen saturation shown in Figure 3. The slope of each correlation was significantly less in DSAP-injected animals compared to control. $p < 0.05$; * vs. IgG.

CHAPTER 5: DISCUSSION

Historically, the focus in cardiorespiratory control, especially the generation and maintenance of respiration, has been on the brainstem. Recently, however, regions rostral to the medulla and pons are being recognized as important modulatory regions, adjusting sympathetic and respiratory outflow during various physiological and pathophysiological states (30). The PVN is a key integrative nucleus in the hypothalamus that is critical to homeostasis. It is unique in that it affects both autonomic and neuroendocrine function. The PVN contains neurons that have direct outputs to autonomic regions and sympathetic preganglionic neurons that contribute to maintaining overall sympathetic nerve activity (13). Neurons in the PVN also secrete hormones such as corticotropin-releasing hormone, thyrotropin-releasing hormone, vasopressin and oxytocin (145). The PVN is involved in a wide variety of physiological functions including food intake, responses to stress, adjusting metabolic rate, thermoregulation, and regulation of the autonomic nervous system (65, 67, 103, 141, 202). However, fewer studies have focused on the role of the PVN in the regulation of ventilation during baseline conditions and cardiorespiratory stressors such as hypoxia. Furthermore, little is known about how respiratory afferent information is conveyed to the PVN. This dissertation therefore examined the responses elicited during hypoxia and also investigated the role of several key autonomic sites in the brainstem with connections to the PVN. Collectively, it demonstrated a critical role for PVN-projecting neurons in baseline control of respiration and also in the responses elicited by peripheral and central chemoreflex stimulation. Additionally, it provided evidence that

activation of catecholaminergic inputs is one mechanism by which the PVN receives afferent information. A general schematic of this work is presented in *Figure 5.1*, illustrating the novel concept that adrenergic/noradrenergic input from brainstem regions to the PVN could be important contributors to modulation of chemoreflex responses.

In **AIM 1 and 2** we address the activation of nTS and CVLM neurons in response to hypoxia. While previous studies have demonstrated that these brainstem regions respond to a chemoreflex stimulus (21, 234), we provide the first evidence that many PVN-projecting neurons are activated by hypoxia. Furthermore, analysis of PVN-projecting neurons in particular reveals that most of these hypoxia-activated neurons are also catecholaminergic. Collectively these studies suggest that adrenergic input to the PVN may play a critical role in responses, including cardiorespiratory responses, to chemoreflex stimulation.

Since hypoxia activated such a large percentage of catecholaminergic PVN-projecting neurons (**AIM 1 and 2**), we wanted to determine if adrenergic projection to the PVN plays a role in modulating central and peripheral chemoreflexes. In **AIM 3** we examined, *in vivo*, the role of catecholaminergic input by eliminating PVN-projecting catecholaminergic cells with dopamine beta hydroxylase conjugated to saporin. The results from this part of the dissertation demonstrate that catecholaminergic input contributes to baseline respiratory rate and mean arterial blood pressure. In addition, lesion of adrenergic input to the PVN resulted in significant attenuation in the ventilatory response to higher intensities of hypoxia and hypercapnia, suggesting that

these pathways contribute to normal chemoreflex function. Collectively, our data demonstrate a previously unrecognized role for catecholaminergic inputs to the PVN in the regulation of cardiorespiratory control.

5.1 PVN-projecting neurons are activated during acute hypoxia

The present work utilized a combination of plethysmography, telemetry, and immunohistochemistry to evaluate the response of conscious animals to hypoxia. We used three intensities of hypoxia (12, 10, and 8% O₂) to stimulate the chemoreflex. Under these hypoxic conditions, oxygen saturation progressively decreased, leading to an increase in respiration, tidal volume, and minute ventilation. Furthermore, we found that acute hypoxia in conscious rats induced an early increase in heart rate, and a sustained depressor response.

Hypoxia activates nTS and CVLM cells

We found that in conscious animals, neuronal activation was progressively increased within the nTS and CVLM following graded intensities of hypoxia. This finding is based on the increased production of Fos (a marker of neuronal activity) following hypoxia. This is also in agreement with earlier studies from other laboratories, evaluating Fos expression in the nTS and CVLM after stimulation of the chemoreflex in rats exposed to moderate to severe hypoxia (21, 71, 104, 126, 234). Therefore, both the present and previous studies demonstrate that the nTS and CVLM may contribute to responses to hypoxia. We also show that the decrease in blood pressure during hypoxia does not contribute to an increased number or distribution of activated cells in the nTS

or CVLM. This suggests it is unlikely that the decrease in blood pressure could account for the increased activation of nTS and CVLM neurons during hypoxia.

Hypoxia activates PVN-projecting nTS and CVLM cells

One of the most important findings of the present study is that nTS and CVLM neurons projecting to the PVN were activated following exposure to hypoxia. We originally hypothesized that pathways projecting to the PVN might be recruited at higher hypoxia intensities, compared to the more classical nTS-RVLM pathway which would be activated first. However, we found that even during inspiration of 12% O₂, PVN-projecting nTS neurons are activated, suggesting that this intensity of hypoxia is sufficient to recruit PVN-projecting neurons and the threshold for activation of these neurons might be at a similar intensity to other pathways. To test this hypothesis, additional studies should be performed with a wider range of hypoxic intensities.

Remarkably, a significant number of PVN-projecting neurons (up to 39% in the nTS and 52% in the CVLM) exhibited Fos following 8% O₂. This proportion of activated PVN-projecting neurons is higher than that of several other pathways and stimuli studied to date, suggesting that hypoxia is a very effective stimulus to activate this pathway. For example, as mentioned in Chapter 3, only 10–15% of nTS cells that display Fos-IR in response to 10% O₂ hypoxia also project to the RVLM (126), 12–13% of CVLM-projecting nTS neurons express Fos in response to baroreceptor activation (243) and approximately 15% of RVLM-projecting PVN neurons are activated by osmotic challenges (112). This suggests that the PVN-projecting neurons in the nTS and CVLM

make a potentially large contribution to the cardiorespiratory and neuroendocrine responses initiated by hypoxia.

Interestingly, we found several differences between the activation of nTS compared to CVLM neurons that projected to the PVN following the three intensities of hypoxia. In the nTS, PVN-projecting neurons were significantly activated following a relatively mild hypoxic stimulus (12% O₂). In contrast, the threshold for activation of PVN-projecting CVLM neurons was greater, as Fos-IR was observed only following exposure to more severe hypoxia intensities (10% O₂). Thus, the CVLM to PVN pathway may be less sensitive to hypoxic stimulation and a more intense stimulus is needed to recruit this pathway. However, in the CVLM, PVN-projecting neurons are additionally recruited as severity of hypoxia increases. Functionally, the combination of these two hypoxia sensitive pathways to the PVN could increase the responsiveness of the chemoreflex over a wide range of hypoxic stimuli.

Hypoxia preferentially activates PVN-projecting nTS and CVLM cells that are catecholaminergic

In the present investigation, there was a preferential activation of catecholaminergic neurons that projected to the PVN following exposure to hypoxia. Indeed, the percentage of activated PVN-projecting neurons that was also catecholaminergic was 75% in the nTS and 89% in the CVLM. This finding suggests that catecholamine neurons projecting to the PVN that are activated following hypoxia are critical to chemoreflex integration. These findings are also in agreement with previous observations that catecholaminergic neurons projecting to the PVN are likely involved in

chemoreflex function. For example, blockade of adrenergic receptors in the PVN blunts the increases in blood pressure during carotid chemoreceptor stimulation (136). It has also been suggested that the CVLM contributes to neuroendocrine responses to hypoxia (217). However, the extent to which catecholaminergic cells, in particular catecholaminergic neurons with direct projections to the PVN, become activated in response to systemic hypoxia and how they affect ventilatory responses was unknown until now.

Projections from the nTS: Catecholaminergic vs. Non-catecholaminergic

An observation specifically with regard to the nTS was made when we compared the diverse projections of this nucleus. In this dissertation we provide evidence that during hypoxia, the majority of PVN-projecting activated neurons are catecholaminergic. However, the nTS also sends direct projections to the CVLM and RVLM. Interestingly, previous work from our laboratory and others have shown that almost no RVLM-projecting nTS neurons are catecholaminergic (126) and very few cells projecting to the CVLM also contain TH (11, 38). Together, our findings indicate that the catecholaminergic phenotype within the nTS may represent a preferential projection target to the PVN. This phenotypic organization within the nTS may strongly impact afferent signals directed to specific autonomic or neuroendocrine regions, which can ultimately affect the outcome of chemoreflex responses very early in the reflex pathway.

PVN-projecting catecholaminergic neurons could potentially contribute to many responses elicited by hypoxia

Given that catecholamine projections to the PVN are extremely dense, the effect of these projections may be quite extensive. Activation of the PVN can elicit sympathoexcitatory and ventilatory effects. Furthermore, it has been demonstrated that various stressors, including immobilization, cold, hemorrhage, and pain, can cause norepinephrine release in the PVN which triggers hypothalamic-pituitary adrenal axis activity (increases in plasma adrenocorticotrophic hormone, vasopressin, oxytocin and corticosterone), suggesting there is a positive correlation between adrenergic content in the PVN and neuroendocrine release (20, 34, 65, 138, 197). Although investigated with several stimuli, the role of catecholaminergic input to the PVN during hypoxia has not been fully elucidated, particularly effects on cardiorespiratory outputs. It is possible that release of epinephrine or norepinephrine onto the neurons in the PVN that project to the spinal cord, phrenic motonuclei, the pre-Botzinger complex, and to the RVLM, regions critical for the regulation of sympathetic nerve activity and ventilation, could contribute to the cardiorespiratory responses to hypoxia. This effect would contribute to the increases in respiratory rate and re-distribution of blood flow to enable oxygen delivery to all tissues as oxygen saturation declines. Activation of the hypothalamic-pituitary adrenal axis during chemoreflex stimulation could serve to maintain further homeostasis during hypoxic conditions. Taken together, the PVN is a very plausible region within the central nervous system through which catecholamines may influence autonomic, respiratory and neuroendocrine function during exposure to hypoxia.

5.2 PVN-projecting catecholaminergic neurons contribute to baseline cardiorespiratory control and responses to acute hypoxia

Basal cardiorespiratory control

As mentioned previously, the PVN has significant connections with brainstem nuclei that control cardiorespiratory output. Ascending, descending, and reciprocal projections from the PVN and the medulla, all of which have the ability to modulate respiratory outflow, are known to exist. The role that the PVN plays in basal cardiorespiratory control has been investigated by very few studies that activated or disinhibited the activity of PVN neurons. These studies showed that activation (67, 248) or disinhibition (208) of the PVN leads to an increase in respiratory rate, mean arterial pressure, and heart rate and suggest that the PVN provides an excitatory influence over sympathetic and ventilatory drive.

As such a large proportion of PVN-projecting neurons in the nTS and CVLM are adrenergic/noradrenergic and these were activated during hypoxia (AIM 1 and 2), we sought to determine if catecholaminergic input may play a role in relaying this information to the PVN and if the input is required for chemoreflex responses. It was found that lesion of catecholaminergic input to the PVN significantly reduced respiratory rate and increased arterial blood pressure. This implies that there is a catecholaminergic input to the PVN, mediating baseline control of breathing and blood pressure. This finding also provides a strong rationale for investigating the role of adrenergic inputs to the PVN during cardiorespiratory stressors such as hypoxia.

Responses to hypoxia

Even fewer studies to date have considered whether the PVN is involved in hypoxia. For example, lesion (175) or interruption of neuronal activity in the PVN (190) blunts the magnitude and duration of the pressor, sympathoexcitatory and phrenic nerve activity responses evoked by chemoreflex activation with potassium cyanide. These results suggest that neurons in the PVN facilitate the cardiorespiratory response to hypoxia. However, no studies have investigated the role of the PVN in response to acute systemic hypoxia or have examined the input conveying chemoreflex information. Therefore, the present study contributes several novel findings to what is known about the PVN, its role in respiratory control and also the central pathways providing input. We found that lesion of PVN-projecting catecholaminergic neurons resulted in significant deficits in peripheral chemoreflex function, particularly in response to more severe hypoxic stimuli. Interestingly, this finding is consistent with our immunohistochemical studies in which there is significant activation of PVN-projecting neurons in both the nTS and CVLM, especially at more severe hypoxia intensities (122). We suggest that the PVN may be a site in the brain in which norepinephrine/epinephrine, acting at (nor)adrenergic receptors, can elicit increases in ventilation and sympathetic nerve activity during hypoxia.

Responses to hypercapnia

Another significant discovery of the present study is that after lesion of PVN-projecting catecholaminergic neurons, there was a blunted response to central chemoreflex stimulation, suggesting that these neurons are involved in the ventilatory response elicited during hypercapnia. Supporting the findings in the present studies,

there is evidence from other laboratories showing involvement of the PVN in the central chemoreflex. These include observations that the PVN contains CO₂ sensitive cells (64) and vasopressin and oxytocin containing neurons that project to the pre-Botzinger complex and phrenic motoneurons are activated during hypercapnia (119, 150). However, our findings are novel as they demonstrate that adrenergic input to the PVN is part of this hypercapnia-sensing network.

5.3 Potential Future Studies

Taken together, the combination of these three studies clearly supports an important role of PVN-projecting neurons in maintaining cardiorespiratory regulation at baseline and in response to peripheral and central chemoreflex stimulation. As with all studies, however, interesting questions arise as a result of the findings. These include further elucidation of all the features of chemoreflex responses and careful examination of adrenergic receptors/mechanisms within the PVN that affect these responses. For example, while our studies have shown both baseline respiration and ventilatory chemoreflex responses are dependent in part on catecholaminergic input to the PVN, we did not examine other aspects of the response such as the release of various hormones. It has been shown that during more severe hypoxia (10%, 8% O₂), there are increases in vasopressin, ACTH, and corticosteroids (188). The extent to which PVN noradrenergic input is required for the release of these hormones is not currently known. Furthermore, how hormonal release during hypoxia could potentially affect the same brain regions we investigated in this work warrants further investigation.

This study showed that while both the nTS and CVLM send projections to the PVN, it appears that the nTS pathway reaches threshold before the CVLM pathway. Therefore, one major question that arises is the extent to which these two pathways contribute to differential responses during hypoxia. The anatomic segregation of noradrenergic input to the PVN provides a substrate to answer this question. We would anticipate that the nTS, projecting prominently to the parvocellular division of the PVN known to contain pre-autonomic and CRH-containing neurons, might be involved in initial increases in ventilation and sympathetic nerve activity (Figure 5.1). This part of the pathway may contribute to hypoxic responses even at relatively mild intensities. In contrast, the CVLM, which sends noradrenergic projections primarily to magnocellular regions of the PVN, might be more critical to coordinating the neuroendocrine responses to hypoxia, which are more noticeable at higher intensities of hypoxia.

Further studies are also required to determine the receptors and mechanisms within the PVN that coordinate these functional responses. α_1 , α_2 and β adrenergic receptors are present in the PVN (121). If these adrenergic receptors are located on pre-autonomic or neuroendocrine cells, release of catecholamines in the PVN could potentially mediate the responses to hypoxia and hypercapnia. With regard to the peripheral chemoreflex, studies suggest the α receptor, not the β receptor, may mediate the release of hormones (40) and pressor responses (136) during acute hypoxic stress. Interestingly, recent *in vitro* studies have begun to examine the effect of catecholamines on cells in the PVN and median preoptic nucleus, which also receives a dense norepinephrine innervation from brainstem autonomic centers. In spinally-

projecting PVN neurons, for example, activation of $\alpha 1$ adrenergic receptors increases excitability by augmenting glutamatergic and attenuating GABAergic neurotransmission (39, 140). The presynaptic $\alpha 2$ adrenergic receptors also attenuate GABAergic inputs to PVN neurons projecting to the spinal cord, likely augmenting cardiorespiratory responses, (140). As illustrated in Figure 5.1, activation of adrenergic receptors (through the release of norepinephrine within the PVN) could contribute to altered glutamate and GABA release and therefore regulation of sympathetic outflow and neuroendocrine release during hypoxia. Taken together, further studies should be done to address the interaction between catecholamine input to the PVN, how this affects the delicate balance of glutamate and GABA in the PVN, and also how this ultimately impacts autonomic and neuroendocrine responses during hypoxia.

One important technical consideration in the current studies is that the use of TH-IR as a marker for catecholaminergic neurons and the use of DSAP for lesioning these cells limits the differentiation between adrenergic and noradrenergic inputs to the PVN. The nTS and the VLM contain a mixture of adrenergic and noradrenergic cells (51, 205), and in the first study we did not differentiate between these catecholaminergic populations. However, the comparison of these populations could elucidate if the PVN receives projections from both types of neurons or if there is a preference for one type of projection. In Chapter 3, we were able to utilize the combination of TH-IR and phenylethanolamine N-methyltransferase-IR (specific for adrenergic neurons) to compare adrenergic to noradrenergic projections from the VLM to the PVN. In this part of the study, we found that 98% of the catecholaminergic CVLM cells were

noradrenergic rather than adrenergic. Therefore, based on our current findings, we hypothesize that norepinephrine release, rather than epinephrine, might be the main catecholamine playing a role in modulating output from the PVN.

Finally, little is known about catecholaminergic inputs to the PVN and their role in chronic pathological respiratory conditions such as sleep apnea. Interestingly, alterations of catecholaminergic function have been implicated in several genetic respiratory diseases including sudden infant death syndrome, congenital central hypoventilation syndrome and Rett syndrome (49, 115). We show in this dissertation that adrenergic/noradrenergic neurons projecting from brainstem nuclei to the PVN and the lack of these inputs is sufficient to induce respiratory disturbances, but how this neural mechanism contributes to some of the above mentioned pathologies is not well understood. Hopefully, the connection between the physiological function of the PVN-projecting pathways with the catecholaminergic phenotype will benefit the understanding of the respiratory pathologies.

In conclusion, the present study has demonstrated a key role of the PVN in the acute response to hypoxia and demonstrates that catecholaminergic neurons projecting to the PVN contribute to chemoreflex responses. Future studies will be needed to provide further insight into the mechanism by which catecholamines influence output from the PVN during hypoxia and whether this pathway also contributes to common respiratory pathologies.

Figure 5.1 – Schematic diagram showing afferent and efferent neural pathways to and from the PVN that could modulate responses to peripheral chemoreflex stimulation. The nTS and CVLM contain a population of GABAergic (red circle) and catecholaminergic (green circle) neurons, of which only catecholaminergic neurons project to the PVN. Noradrenergic (NA) as well as adrenergic (A) afferent innervation from the nTS and VLM modulates the activity of the PVN via α_1 and α_2 receptors which augment glutamate release and attenuate GABA release. PVN neurons project to the ventral respiratory groups and the phrenic motor nucleus regulating respiration. In addition, PVN neurons can directly influence sympathetic nerve activity via a PVN-IML pathway, and indirectly via a PVN-RVLM pathway. Finally, neurons in the PVN are responsible for release of important hormones that help maintain fluid balance during hypoxia as well as maintain overall homeostasis in the incidence of a stress. Overall, nTS and CVLM neurons are activated during relatively low intensities of hypoxia (+). Via activation of nTS PVN-projecting adrenergic pathways, cardiorespiratory adaptations to hypoxia can be made very quickly and in response to low intensities of hypoxia (+). In response to higher intensities of hypoxia (++), additional PVN-projecting neurons are recruited from the CVLM, allowing for further strengthening of cardiorespiratory responses and also contributing to the initiation of additional neuroendocrine responses.

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Appendix (Supplemental Figures)

Figure 6.1-

Caudal to rostral distribution of catecholaminergic (TH labeled) cells in the CVLM following graded hypoxia. Two-way repeated measure ANOVA showed that there was no significant effect of hypoxia group or caudal-rostral distribution on the number of cells. Caudal to rostral CVLM levels are indicated relative to bregma. Calamus scriptorius (the caudal-most portion of the area postrema) is at approximately -14.4mm relative to bregma.

Figure 6.2 -

Caudal to rostral distribution of the percent of A) catecholaminergic, B) PVN-projecting and C) PVN-projecting catecholaminergic (FG and TH labeled) cells activated in the CVLM following graded hypoxia. Two-way repeated measures ANOVA indicated significant main effect of hypoxic intensity and in some cases CVLM level. $p \leq 0.05$; † vs. 14.10 and -14.28; †† vs. 14.10, -14.28 and -14.46. $p \leq 0.05$; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂.

Figure 6.3 -

The percent activated PVN-projecting that were catecholaminergic calculated as $[(Fos+TH+FG)/(Fos+FG)]$ in the nTS (top) and CVLM (bottom). Independent of hypoxia severity, about 75% of nTS and 90% of CVLM activated PVN-projecting cells were also catecholaminergic, indicating that hypoxia preferentially activates catecholaminergic rather than non-catecholaminergic PVN-projecting nTS and CVLM cells and the proportion does not change with severity of hypoxia.

Figure 6.4 -

A) The number of all CVLM activated cells compared to the number of CVLM cells that were only Fos-IR (non-phentyped cells; dashed bars). B) Independent of hypoxia severity, about 50% of all activated cells in the CVLM were nether TH-IR or FG labeled. $p \leq 0.05$; * vs. 21% O_2 ; ** vs. 21 and 12% O_2 ; *** vs. 21, 12, and 10% O_2 .

Figure 6.5 -

A) The number of all CVLM activated catecholaminergic cells (from Figure 3.3) compared to the number of CVLM cells that were only Fos and TH-IR (non-FG cells). B) The percent of all activated cells that were catecholaminergic did not change with hypoxia severity; On average, $45 \pm 3\%$ of all activated cells in the CVLM were TH-IR. $p \leq 0.05$; * vs. 21% O_2 ; ** vs. 21 and 12% O_2 .

Figure 6.6 -

A) The number of all CVLM activated PVN-projecting cells (from Figure 3.4) compared to the number of CVLM cells that were only Fos and FG labeled (non-TH-IR). B) The percent of all activated cells that were PVN-projecting did not change with hypoxia severity; On average, $27 \pm 2\%$ of all activated cells in the CVLM were PVN-projecting. $p \leq 0.05$; * vs. 21% O_2 ; ** vs. 21 and 12% O_2 ; *** vs. 21, 12, and 10% O_2 .

Figure 6.7 -

Pie graph illustrating the activation profile in the CVLM. Overall, the strategy to adapt to hypoxia is to activated more of the same populations of cells, not different percentages of these populations.

Figure 6.8 -

Caudal-rostral distribution of A) Fos-IR and B) Fos-IR and TH-IR cells in the CVLM in response to hypoxia without (10% O₂; open circles) or with MAP (10% O₂ + PE; filled circles) maintained at normoxic levels. There were no significant differences in caudal-rostral distribution of Fos-IR or co-labeled cells between groups.

% of TH cells lost with DSAP injections vs. % of PVN projecting cells that are TH

Figure 6.9 -

% of TH cells lost after DSAP injections (Study 3) vs. the % of PVN projecting cells that were also TH-IR (Study 1 and 2) in the nTS (left) and VLM (right). The similarity of these percentages suggest an inner consistency between our studies.

Figure 6.10 -

Correlation in IgG animals of peak minute ventilation responses (to 8% O₂) to the number of TH-IR cells remaining in the nTS (top) and VLM (bottom). Unlike in the DSAP injected animals (Figure 4.4) where there was a significant correlation, in the IgG group, there was no correlation between peak ventilatory responses and the number of cells.

Figure 6.11 -

Average time to the maximal responses following initiation of a hypoxia challenge (calculated as the percent maximal change from baseline). The time to max respiratory rate was significantly less in DSAP injected animals (top). There were no differences in the time to max tidal volume and overall minute ventilation between IgG and DSAP groups. Overall, respiratory adaptations to hypoxia occurred within about 5 minutes.

VITA

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