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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Contributions of the Nucleus Tractus Solitarius to Ventilatory Acclimatization to Chronic Hypoxia

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Katherine Anne Wilkinson

Committee in charge:

Professor Frank L. Powell, Jr., Chair
Professor Katerina Akassoglou
Professor Tony Yaksh
Professor Jason Yuan
Professor Michael Ziegler

2009

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The Dissertation of Katherine Anne Wilkinson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009

Dedication

For my family and all the friends that made the last five years enjoyable

Epigraph

It is a good morning exercise for a research scientist to discard a pet hypothesis every day before breakfast. It keeps him young.

Konrad Lorenz

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List of Abbreviations

aCSF	Artificial Cerebrospinal Fluid
ACZ	Acetazolamide
ANOVA	Analysis of Variance
AP	Area Postrema
Bw	Body Weight
CH	Chronically Hypoxic
CNS	Central Nervous System
CO_2	Carbon Dioxide
DAPI	4',6-diamidino-2-phenylindole
DMNV	Dorsal Motor Nucleus of the Vagus
ET	Endothelin
ET_{CO_2}	End Tidal Carbon Dioxide
f	Frequency
fR	Neural Burst Frequency
GABA	Gamma-Aminobutyric Acid
Hct	Hematocrit
HCVR	Hypercapnic Ventilatory Response
HVD	Hypoxic Ventilatory Decline
HVR	Hypoxic Ventilatory Response
LC	Locus Coerelius
N	Normoxic

N_2	Nitrogen
NA	Nucleus Ambiguus
NK1R	Neurokinin 1 Receptor
NTS	Nucleus Tractus Solitarius
O_2	Oxygen
Pa_{O_2}	Arterial Oxygen Pressure
Pa_{CO_2}	Arterial Carbon Dioxide Pressure
RTN	Retrotrapezoidal Nucleus
SAP	Saporin
SP	Substance P
SSP	Stable Substance P
VAH	Ventilatory Acclimatization to Hypoxia
V_i	Ventilation
VLM	Ventrolateral Medulla
V_T	Tidal Volume
$\int Phr$	Phrenic Burst Amplitude
$\int Phr \times fR$	Neural Ventilation

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J.R. Mauban, K.A. Wilkinson, C. Schac, J.X. Yuan. Histamine-mediated increase in cytosolic [Ca2+] involve different mechanisms in human pulmonary artery smooth muscle and endothelial cells. *Am J Physiol Cell Physiol.* 290(2): C325-36, 2005 Sep 14.

K. A. Wilkinson, A. P. Maurer, B. F. Sadacca, and B. J. Yates. Responses of feline medial medullary reticular formation neurons with projections to the C5-C6 ventral horn to vestibular stimulation. *Brain Research* 1018: 247-256, 2004.

L. A. Cotter, H. E. Arendt, S. P. Cass, B. J. Jian, D. F. Mays II, C. J. Olsheski, K. A. Wilkinson, and B. J. Yates. Effects of postural changes and vestibular lesions on genioglossal muscle activity in conscious cats. *J Appl Physiol* 96: 923-930, 2004.

I. R. Nourbakhsh, K. Crowley, A. Bhave, E. Hamner, T. Hsiu, A. Perez-Bergquist, S. Richards, and K. Wilkinson. The robotic autonomy mobile robotics course: Robot design, curriculum design and educational assessment. *Autonomous Robots* 18: 103-127, 2005.

ABSTRACT OF THE DISSERTATION

Contributions of the Nucleus Tractus Solitarius to Ventilatory
Acclimatization to Chronic Hypoxia

by

Katherine Anne Wilkinson

Doctor of Philosophy

University of California, San Diego, 2009

Professor Frank L. Powell, Jr., Chair

Following hours to days in hypoxia, a time dependent increase in ventilation occurs, termed ventilatory acclimatization to hypoxia (VAH). Hallmarks of VAH include an increased slope of the isocapnic hypoxic ventilatory response (HVR) and hyperventilation that persists upon return to normoxia (Powell et al., 1998). VAH involves changes in the sensitivity of the carotid body to hypoxia (Nielsen et al., 1988) and the central nervous system to carotid body afferent input (Dwinell &

Powell, 1999). My dissertation tests the hypothesis that plasticity occurs in the Nucleus Tractus Solitarius (NTS), which receives the first synapse from the carotid body afferents (Housley et al., 1987) and also contains CO_2-sensitive cells (Dean et al., 1989). To determine if plasticity in the NTS contributes to VAH, we used two approaches: stimulating CO_2-sensitive cells in the NTS and lesioning a specific type of neuron in the NTS and measuring ventilatory responses to CO_2 and O_2 before and after acclimatization to chronic hypoxia.

Stimulation of the NTS central chemoreceptor cells by focal acidosis increased phrenic nerve activity in anesthetized rats, as reported by other investigators (Coates et al., 1993). The response in chronically hypoxic animals was significantly less than in the normoxic controls in contrast to the increase we predicted to explain the persistent hyperventilation in normoxia with VAH. However, this decreased responsiveness of NTS CO_2-sensitive cells in chronic hypoxia is consistent with cellular data suggesting an increase in the number of cells inhibited by hypercapnia after chronic hypoxia (Nichols et al., 2009).

Our lesion studies targeted neurokinin 1 receptor positive cells (NK1R) in the NTS because similar lesions in other central chemosensitive sites have been shown to reduce the hypercapnic ventilatory response (Nattie & Li, 2002b; Nattie et al., 2004). We measured the ventilatory responses to hypercapnia and hypoxia in normoxic and chronically hypoxic rats following the lesion of NK1R cells in the NTS. No effects of the lesion were seen in normoxic rats. However, ventilation in room air and hypoxia was significantly greater in lesioned animals after chronic

hypoxia. This suggests that NK1R cells in the NTS attenuate the arterial chemoreflex after chronic hypoxia. There was also a non-significant trend for ventilation to increase during hypercapnia in lesioned animals after chronic hypoxia. These studies have established plasticity in the NTS during chronic hypoxia but the exact mechanisms and their contribution to VAH remain to be determined.

Chapter 1: Introduction

1.1 Dissertation Overview

This dissertation is focused on finding a mechanism for ventilatory acclimatization to chronic hypoxia. The two sets of experiments described investigate the hypothesis that a distinct group of CO_2-sensitive cells in the brainstem undergo plasticity during chronic hypoxia and this change explains some of the ventilatory changes that accompany the exposure to chronic hypoxia.

1.2 Control of Breathing

1.2.1 Control of Breathing Overview (Feldman & McCrimmon, 2003)

To maintain homeostasis, appropriate arterial levels of oxygen (Pa_{O_2}) and carbon dioxide (Pa_{CO_2}) must be maintained at all times and 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. Due to the reaction combining CO_2 and water to form carbonic acid, Pa_{CO_2} levels directly affect arterial pH, which must be strictly regulated as it can affect normal protein folding.

The act of breathing is complex and accomplished by the activation of the skeletal respiratory muscles. One of the most important respiratory muscles is the diaphragm, which contracts during inspiration to enlarge the thoracic cavity. This

creates a pressure difference between the lungs and the external environment, thus drawing air into the lungs. In anesthetized animals the nerve innervating the diaphragm, the phrenic, is often recorded as a measure of ventilatory output. Additional respiratory muscles include the inspiratory and expiratory intercostal muscles, abdominal expiratory muscles, and upper airway muscles such as the genioglossus.

Respiratory pre-motor neurons in the brainstem integrate various drives to breathe including those from the respiratory rhythm generators, chemoreceptor systems, and mechanoreceptors. The pre-Bötzinger complex is thought to be the major site of rhythm generation as an injection of muscimol, a $GABA_A$ agonist that inhibits neuronal activity, into the pre-Bötzinger complex leads to a cessation of respiratory activity (Rekling & Feldman, 1998). A second site of rhythm generation has been described in the parafacial respiratory group (Onimaru & Homma, 2003). The peripheral and central chemoreceptor systems sense changes in arterial O_2, CO_2, and pH and alter breathing to maintain constant levels of those important blood gas parameters (for a schematic see Figure 1). The mechanoreceptors monitor lung pressures and volumes and adjust ventilation to limit the work of breathing. The mechanoreceptors also mediate protective reflexes, like coughing to clear foreign objects from the airway. Finally, breathing is also under conscious control and can be modified to accommodate speech or other behaviors. The final respiratory output integrates all of the various drives to breathe to accommodate the conditions the animal is experiencing at any given time.

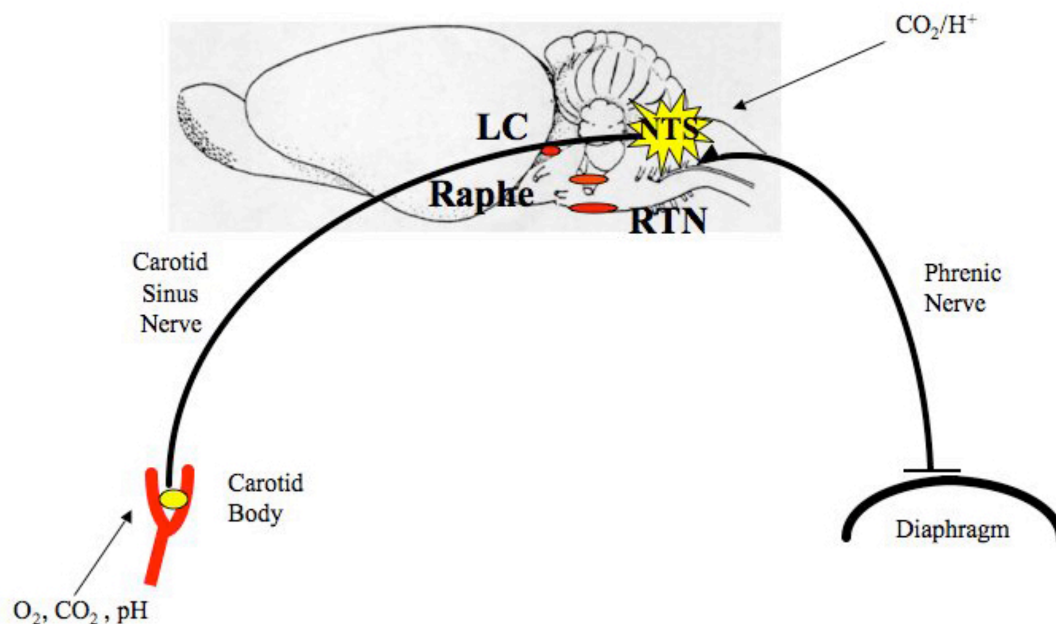

Figure 1: Peripheral and Central Chemoreflexes. Schematic of both the peripheral and central chemoreflexes. ***Peripheral Chemoreflex:*** The carotid body senses changes in arterial O_2, CO_2, and pH and sends afferent information through the carotid sinus nerve, which synapses in the Nucleus Tractus Solitarius (NTS). ***Central Chemoreflex:*** Multiple sites of CO_2-sensitivity are located in the brainstem including the NTS, Locus Coereleus (LC), Raphe, and Retrotrapezoidal Nucleus (RTN). All drives to breathe are integrated in the brainstem and relayed to the respiratory muscles, including the diaphragm via the phrenic nerve.

1.2.2 Peripheral Chemoreceptor System (Gonzalez et al., 1995)

The peripheral chemoreceptors include the aortic and carotid body chemoreceptors. Under most circumstances the carotid body chemoreceptors are the most important and will be the only ones discussed. The carotid bodies mediate both the cardiovascular and ventilatory chemoreflexes. Changes in Pa_{O_2}, Pa_{CO_2}, or arterial pH are sensed by the carotid bodies. The afferent information is relayed through the carotid sinus nerve to the brainstem where it is then integrated with other drives to breathe to modulate ventilation. Decreases in Pa_{O_2} or pH and increases in Pa_{CO_2} all

stimulate ventilation. The carotid bodies are the main Pa_{O_2} sensors, whereas Pa_{CO_2} and pH are also sensed by the central chemoreceptors, which will be discussed below.

The carotid body is located bilaterally at the bifurcation of the common carotid artery. The carotid bodies are highly vascularized and have been estimated to receive the highest blood flow relative to size of any organ in the body, at 1.5 to 2 L/kg*min (Acker & O'Regan, 1981; De Burgh Daly et al., 1954a). This allows the carotid body to sense systemic changes in Pa_{O_2}, Pa_{CO_2}, and pH. There is also evidence that hypotension (Landgren & Neil, 1951; Biscoe et al., 1970), high potassium levels (Jarisch et al., 1952; Linton & Band, 1985), changes in osmolarity (Carpenter & Peers, 1997), and hypoglycemia (Alvarez-Buylla & Roces de Alvarez-Buylla, 1994) are all sensed by the carotid body as well. It has been speculated that the carotid body could act as a polymodal sensor that maintains homeostasis during times of stress (Kumar & Bin-Jaliah, 2007). For the purposes of this thesis, the ability of the carotid body to sense changes in Pa_{O_2}, Pa_{CO_2}, and pH are the most relevant and will be the only functions discussed.

The Type I carotid body glomus cells are electrically excitable and when stimulated depolarize and activate carotid sinus afferent nerve fibers. Surprisingly, the excitatory neurotransmitter between the glomus cells and the carotid sinus afferents has not been identified positively (Iturriaga & Alcayaga, 2004). Hypoxia is sensed through inhibition of membrane potassium channel activity, although the exact mechanism is not known. Direct interaction between O_2 and the ion channels and

indirect modulation through O_2-sensing molecules have both been hypothesized (López-Barneo et al., 2004).

The carotid bodies maintain a tonic output under normoxic conditions (90-100 mm Hg Pa_{O_2}) and only start to be activated under moderate levels of hypoxia (50-60 mm Hg Pa_{O_2}) (López-Barneo et al., 2004). This is consistent with the observation that unlike small changes in Pa_{CO_2}, moderate drops in Pa_{O_2} are necessary to cause a ventilatory response.

1.2.3 Central Chemoreceptor System

In addition to the carotid body chemoreceptors, the brainstem contains cells that sense changes in Pa_{CO_2}. The central ventilatory chemoreflex is the stimulation of breathing by the acidification of the central nervous system (Guyenet et al., 2008).

The first suggestion that the brainstem was CO_2-sensitive came from studies where acidic fluids were perfused into the cerebral ventricles of anesthetized dogs (Leusen, 1954) and conscious goats (Pappenheimer et al., 1965; Fencl et al., 1966). Acidifying the subarachnoid region of the ventrolateral medulla (VLM) caused large increases in ventilation, suggesting the importance of the ventral medullary surface for brainstem chemosensation. Chemosensitive regions in the caudal VLM (Mitchell's area) and the rostral VLM (Loeschke's area) were identified (Loeschcke, 1982; Schlaefke et al., 1970; Mitchell et al., 1963). The intermediate area between the two (Schlaefke's area) was not thought to be chemoreceptive, but cooling the area depressed ventilation and chemosensitivity (Schlaefke et al., 1970; Cherniack et al.,

1979; Millhorn et al., 1982). Subsequently the area of chemosensitivity on the VLM has been identified to be within 800 μm of the surface in the retrotrapezoid nucleus (RTN) (Coates et al., 1993; Mulkey et al., 2004). Additional putative brainstem chemoreceptor sites have also been described and the evidence for them will be discussed below.

Many cells in the body respond to CO_2 or H^+. Whether the signal activating the chemosensitive neurons is CO_2 itself, H^+ ions sensed intracellularly or extracellularly, or bicarbonate ions is not known. It has been speculated that all may play a role in chemosensation and there is some evidence that hypercapnia inhibits pH sensitive K^+ channels, though the specific channel subtype or subtypes relevant to central chemosensitivity have yet to be identified. Putnam and colleagues (2004) have proposed a multiple factors theory of central chemosensitive signaling in which the neuronal response to CO_2/H^+ involves multiple cellular signals and multiple ion channel targets.

To be considered a central chemoreceptor, CO_2/H^+ sensitive cells must also project to a respiratory site and alter ventilation when stimulated. It is hard to definitively prove that a cell is a central chemoreceptor, but Putnam and colleagues (2004) have suggested a few conditions that must be present for a cell to be considered a putative chemoreceptor. First, the cell's firing rate must change in response to changes in CO_2/H^+. Secondly, these changes must occur independent of synaptic inputs. Finally, the cells must be located in an area that can cause changes in ventilation when focally acidified. In brain slices, putative chemoreceptive neurons

have been found in the retrotrapezoidal nucleus (RTN) (Wellner-Kienitz & Shams, 1998; Mulkey et al., 2004), medullary raphe (Richerson, 1995), nucleus tractus solitarius (NTS) (Dean et al., 1989), locus coerelius (LC) (Oyamada et al., 1998), and nucleus ambiguus (NA) (Rigatto et al., 1992). Synaptic blockade was attempted in all of those experiments, but intrinsic chemosensitivity cannot be proven conclusively because of the possibility that electrical coupling by gap junctions and residual chemical neurotransmission might have contributed to the response to CO_2/H^+ in some of the neurons.

Further evidence that the putative chemoreceptor sites identified in vitro are indeed chemoreceptor sites has come from experiments where the sites are focally acidified. Two methods of focal acidification have been used, microinjection of the carbonic anhydrase inhibitor acetazolamide, and microdialysis with a 25% CO_2 equilibrated solution. In anesthetized rats (Coates et al., 1993; Bernard et al., 1996; Xu et al., 2001) and cats (Coates et al., 1993; Coates et al., 1991), acidification by acetazolamide on the ventral medulla, which includes the RTN, the NTS, LC, midline raphe, and fastigial nuclei caused an increase in phrenic nerve activity. In awake animals stimulating the RTN, medullary raphe, and NTS with microdialyzed CO_2 caused an increase in breathing (Li et al., 1999; Nattie & Li, 2001; Nattie & Li, 2002a). There is also evidence that the site of respiratory rhythm generation, the pre-Bötzinger complex, contains CO_2-sensitive cells as well (Solomon et al., 2000).

Focal acidification studies have identified similar putative chemoreceptor sites as the in vitro studies, but have suggested a potentially complicated organization and

state dependence of the response to CO_2. Microdialyzed CO_2 into the RTN caused in increase in ventilation during wakefulness but not sleep (Li et al., 1999). The opposite pattern was shown in the medullary raphe, in that microdialyzed CO_2 only stimulated ventilation during sleep (Nattie & Li, 2001). Microdialysis into the NTS increased ventilation in both sleep and wakefulness (Nattie & Li, 2002a).

Recording from individual RTN neurons in anesthetized rats provided additional evidence for *in vivo* chemosensitivity in the RTN. Firing rate in a subset of RTN neurons was found to increase in response to inspired CO_2. Inhibiting the central pattern generator or removing peripheral chemoreceptor input did not block this hypercapnic response. Hypercapnia did not activate serotonergic cells in the raphe in this preparation (Mulkey et al., 2004).

Comparing the degree of chemoreceptor stimulation between anesthetized and awake rats is difficult because in general anesthesia has a depressant effect on chemoreception. It has been estimated that the chemosensitivity index in the RTN is 7.3 times greater in the awake state than the anesthetized (Nattie & Li, 2006a). Additionally, a much greater change in pH occurs under anesthesia following the focal dialysis of 25% CO_2 (Fencl et al., 1966; Nattie & Li, 2006a). In any case, many putative chemoreceptor sites have been shown to be capable of stimulating ventilation in both the anesthetized and awake state.

In addition to stimulation studies, lesion studies have also been performed to elucidate the roles of the putative central chemoreceptor sites. Many types of lesions in the RTN affect the response to hypercapnia. In anesthetized rats and cats, unilateral

kainic acid or electrolytic RTN lesions decrease the baseline phrenic amplitude and virtually abolish the hypercapnic response, effects that are unchanged following carotid body denervation (Nattie et al., 1994; Nattie & Li, 1994). In awake rats these same unilateral RTN lesions produce no effect on baseline ventilation, but decrease the ventilatory response to 7% CO_2 by 39% (Akilesh et al., 1997). Microdialysis of the $GABA_A$ receptor agonist muscimol into the RTN also decreased the response to inspired CO_2 (Nattie & Li, 2000).

In the NTS, microdialysis of muscimol similarly inhibited the hypercapnic response in both wakefulness and non-REM sleep (Nattie & Li, 2008). In ambient temperatures, the muscimol decreased only the response to hypercapnia and not hypoxia. This suggests that central chemosensitivity and not peripheral CO_2-chemosensitivity was affected by the muscimol. At 30°C, both the hypoxic and hypercapnic responses were decreased. Interestingly, muscimol in the NTS produced a much greater decrease in the hypercapnic response than twice the dose in the RTN did (Nattie & Li, 2008; Nattie & Li, 2000).

In the locus coeruleus of rats, destruction of noradrenergic neurons decreased the ventilatory response to hypercapnia by decreasing tidal volume. No change in baseline ventilation was observed (Biancardi et al., 2008).

These studies provide evidence for the existence of central chemoreceptive sites in the RTN, NTS, LC and medullary raphe. The organization and significance of the multiple sites remains unknown. Two theories for how the central chemoreceptor sites are organized have been advanced: the specialized chemoreceptor and distributed

network theories. The specialized chemoreceptor theory is the oldest and postulates that *in vivo*, the central pattern generator is not pH sensitive and the central chemoreflex is due to one main group of specialized acid-sensitive neurons that drive the network synaptically (Loeschcke, 1982; Guyenet et al., 2008). The distributed theory, in contrast, postulates that there are a large number of chemoreceptor sites and chemosensitivity is due to many types of pH sensitive channels or receptors. Additionally, the distributed network does not assign a hierarchy to the different sites and suggests that different areas may be more important during different states (Nattie & Li, 2006a; Putnam et al., 2004).

Recent proponents of the specialized chemoreceptor theory argue that the RTN is the major site of chemoreception and if anything the other putative chemoreceptor sites only modulate the activity of the RTN (Guyenet et al., 2008). The first chemoreceptor site described was the ventromedullary surface (Loeschcke et al., 1963; Mitchell et al., 1963). Since then, chemosensitivity in the ventrolateral medulla has been located specifically to the RTN and there is much evidence that the RTN plays a large role in CO_2 sensing (Mulkey et al., 2004; Guyenet et al., 2008). The RTN neurons are only mildly modulated by respiration and are robustly activated by pH both *in vivo* and *in vitro* (Mulkey et al., 2004; Putnam et al., 2004). RTN chemosensitivity persists even when the central pattern generator is blocked (Mulkey et al., 2004). Additionally the RTN chemoreceptor cells seem ideally located to sense changes in pH as much of the dendritic length is located in the marginal layer on the

medullary surface near blood vessels (Ballantyne & Scheid, 2001; Bradley et al., 2002; Mulkey et al., 2004).

While the evidence for the importance of the RTN is impressive, there is equally strong evidence that other brainstem sites play an important role in mediating the ventilatory response to CO_2. As described previously, specific stimulation of many sites including the NTS, medullary raphe, and LC can cause an increase in ventilation. Guyenet and colleagues (2008) have argued that the microdialysis probe could have disrupted the neuronal environment and the pH buffering capacity of the surrounding cells, thus leading to a ventilatory response that would not occur under normal circumstances. However, aCSF alone does not cause any changes in ventilation and probe placement in the putative chemoreceptive sites and not surrounding sites is necessary for a ventilatory effect (Nattie & Li, 2006a).

Proponents of the distributed network theory have argued that CO_2-sensing is such an important function that redundancy is to be expected (Nattie & Li, 2006a). Additionally, there is much cellular evidence that many populations of neurons do have CO_2-sensing abilities that are probably mediated by different mechanisms (Putnam et al., 2004). For instance, TASK channels appear to be involved in CO_2-sensation in the medullary raphe cells *in vitro* (Richerson, 2004) but not the RTN (Mulkey et al., 2007). I argue that the evidence for the distributed network theory is stronger as the unimportance of the other sites has not been clearly demonstrated. The experiments in this dissertation support the model that at least one site outside the RTN, the NTS, plays an important role in CO_2-sensing during chronic hypoxia.

1.2.4 Integration of Peripheral and Central Ventilatory Chemoreflexes

The peripheral and central chemoreceptors work together to maintain homeostasis and both systems can sense changes in Pa_{CO_2}. To estimate the relative contributions of the peripheral and central chemoreceptors to the hypercapnic ventilatory response (HCVR), Smith and colleagues (2006) implanted an extracorporeal perfusion circuit into dogs to control the CO_2 stimulus at the peripheral and central chemoreceptors separately. They estimated that about two thirds of the HCVR was due to the central chemoreceptors and one third to the peripheral chemoreceptors. It was also shown that the ventilatory response caused by central chemoreceptor stimulation alone was delayed about 11s from the peripheral response.

The mechanisms by which the peripheral and central chemoreceptors interact to produce the HCVR are not well understood. There is data supporting an additive relationship, where there is no peripheral central interaction, (van Beek et al., 1984; Daristotle & Bisgard, 1989; Clement et al., 1992; St Croix et al., 1996) hyper-additive, where activation of one system increases the gain of the other, (Adams et al., 1978; Robbins, 1988), and hypo-additive relationship, where activation of one system decreases the gain of the other (Smith et al., 1984; Day & Wilson, 2009). A hypo-additive relationship was observed in an *in situ* arterially perfused, vagotomized, decerebrate rat preparation in which brainstem and peripheral chemoreceptors were perfused separately. In that study, lower levels of brain P_{CO_2} increased the frequency of the phrenic response to peripheral chemoreceptor stimulation, either from hypoxia or hypercapnia (Day & Wilson, 2009). A similar hypo-additive result was shown in

an artificially perfused awake goat model (Smith et al., 1984), although Daristotle and Bisgard (1989) showed an additive central-peripheral interaction in awake goats. The data in human subjects is also contradictory and depends on the assumptions made to separate peripheral and central CO_2-sensitivity. Some studies find no interaction between the central and peripheral chemoreceptors (Dahan et al., 1990; Clement et al., 1995) and others an additive interaction (Bellville et al., 1979; Robbins, 1988; Clement et al., 1992).

Regardless of the manner of interaction between the peripheral and central chemoreceptors, there is evidence that tonic input by the carotid bodies is necessary for a complete central response. In patients that underwent carotid body resectioning, the response to CO_2 3 – 6 months post-surgery was decreased by 75%, which is more than would be expected from animal studies that suggest the peripheral chemoreceptors only account for a third of the HCVR (Smith et al., 2006). Plasticity occurred, though, and by 2 years post-surgery the patients' hypercapnic ventilatory responses returned to the pre-surgical values (Dahan et al., 2007). A decrease in the central component of CO_2-sensitivity following carotid body resections was also observed by another group of investigators, but their subjects had 1 to 26 years between surgery and study, which makes their results difficult to compare with Dahan's in terms of post-surgery plasticity (Fatemian et al., 2003). In goats, carotid body denervation caused an initial 60% decrease in sensitivity to CO_2 that returned to normal in only 15 days (Pan et al., 1998), which is much sooner than has been reported in human subjects.

1.3 Ventilatory Acclimatization to Hypoxia

1.3.1 Ventilatory Acclimatization to Hypoxia Overview (Powell et al., 1998)

Ventilation increases as a hyperbolic function of hypoxia, with small changes in Pa_{O_2} having very little effect on ventilation until an inflection point that is dependent on the background Pa_{CO_2} (Weil et al., 1970). Following chronic hypoxia, there is plasticity in the hypoxic ventilatory response (HVR). If measured under poikilocapnic conditions, the slope of the HVR is unchanged but ventilation at all levels of Pa_{O_2} is greater. The slope of the HVR becomes greater in chronic hypoxia if isocapnic conditions are maintained (Aaron & Powell, 1993).

The ventilatory response to hypoxia is dependent upon the time and pattern of hypoxic stimulation (Figure 2). Following 5 to 30 min of sustained hypoxia, ventilation "rolls off" and hypoxic ventilatory decline (HVD) occurs. HVD is characterized by a decrease in ventilation relative to the acute response and this can persist for up to an hour after normoxia is restored (Powell et al., 1998). If hypoxia persists for hours to days, a secondary increase in ventilation termed ventilatory acclimatization to hypoxia (VAH) occurs. Following years of hypoxia, hypoxic desensitization (HD) will occur, such that ventilation decreases compared to subjects who have been exposed to hypoxia for a shorter time. Additionally, the acute hypoxic ventilatory response (HVR) becomes blunted. This dissertation is focused on potential mechanisms for VAH following hours to days of hypoxia.

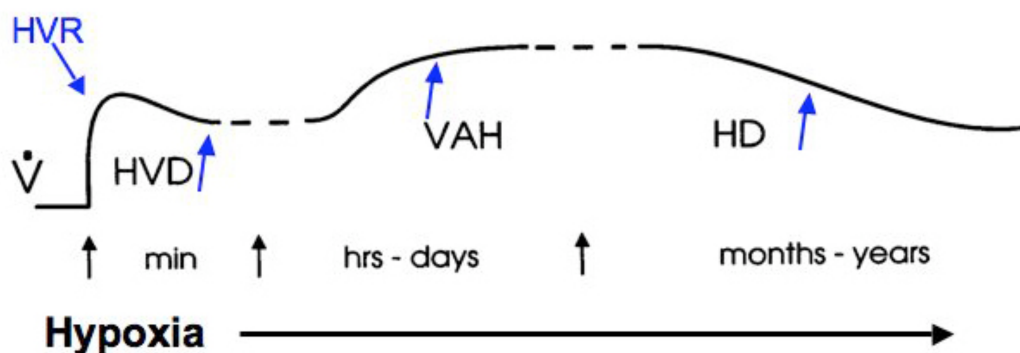

Figure 2: Time Course of Response to Hypoxia. The ventilatory response to hypoxia depends on the length of the hypoxic stimulation. Upon initial exposure to hypoxia, ventilation increases (acute Hypoxic Ventilatory Response or HVR). Following a few minutes in hypoxia, ventilation "rolls off" (Hypoxic Ventilatory Decline or HVD). A secondary increase in ventilation occurs following hours to days in hypoxia (Ventilatory Acclimatization to Hypoxia or VAH). Months to years in hypoxia lead to Hypoxic Decline (HD). HVRs at all time points are indicated by blue arrows. Modified from *Powell et al., 1998.*

The time course for the development of VAH is dependent on the severity of hypoxia and is also species-dependent. Goats develop VAH in as little as 6 hrs (Forster et al., 1981) and in ponies VAH is complete after 24 hrs (Forster et al., 1976). The time course in humans is longer, 4 days in 2900 m (Rahn & Otis, 1949) and 10 days at 4300 m (Forster et al., 1975). At heights of over 8000m upwards of 30 days is required (West, 1988). Rats display a similar time course of VAH during and after chronic hypoxia as humans and are therefore widely used as an animal model to study VAH (Olson & Dempsey, 1978).

VAH is characterized by an increased slope of the isocapnic HVR and also a persistent hyperventilation upon return to normoxia (Figure 3). Ventilation returns to

control with a time course similar to VAH, which is termed ventilatory deacclimatization to hypoxia (VDH) (Dempsey et al., 1979). This hyperventilation leads to a decreased Pa_{CO_2} that is significantly less than the apneic threshold of a normoxic subject.

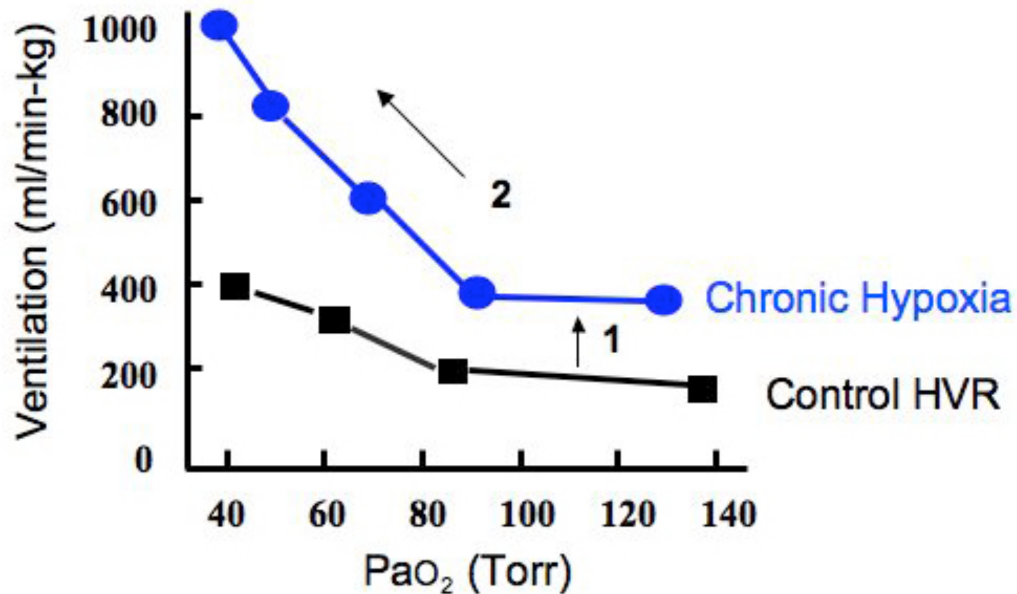

Figure 3: Two Components of Ventilatory Acclimatization to Hypoxia (VAH). VAH is characterized by an increased drive to breathe in normoxia (1) and an increased slope of the isocapnic hypoxic ventilatory response (HVR, 2). Modified from *Aaron & Powell, 1993*.

The plasticity observed during chronic hypoxia is unique. Chronic hypercapnia, which also stimulates ventilation, leads to decreased ventilation during both hypercapnia and normoxia following a few days of hypercapnic exposure (Lai et al., 1981). This is exactly the opposite of what occurs during chronic hypoxia. The known mechanisms mediating VAH will be discussed below as well as the hypotheses being tested in this dissertation.

1.3.2 Theories of VAH

One of the first theories to attempt to explain VAH focused on potential changes in the stimuli for the arterial and central CO_2-chemoreceptors. Severinghaus and colleagues (1963) postulated that active transport of bicarbonate in the cerebrospinal fluid could normalize the cerebrospinal pH and allow for a continued drive to breathe from the central chemoreceptor cells. This would then sum with the hypoxic drive to breathe from the chemoreceptors and lead to the increased ventilation seen in chronic hypoxia as opposed to acute hypoxia, where the lower P_{CO_2} and alkalotic cerebrospinal fluid would inhibit breathing. It was also hypothesized that the renal compensation of arterial pH at later time points would enable cerebrospinal pH compensation to occur more completely (Severinghaus et al., 1963). There was early data that the cerebrospinal pH was normal in subjects following a week at altitude and in a group of high altitude natives (Severinghaus et al., 1963; Severinghaus & Carcelen, 1964). This data was not supported by subsequent work and cerebrospinal pH was shown to be alkaline in chronically hypoxic subjects (Forster et al., 1975).

The other chemical stimuli for breathing also decrease over time in hypoxia and are not able to explain VAH. Pa_{O_2}levels rise and Pa_{CO_2} levels decline as ventilation increases (Rahn & Otis, 1949). Additionally arterial pH in chronic hypoxia is not different from arterial pH during acute hypoxia (Dempsey & Forster, 1982). This suggests that chemoreceptor sensitivity or the processing of the chemoreceptor stimulation, and not the chemical stimulation itself, must explain the time dependent increase in ventilation seen during chronic hypoxia.

1.3.3 Changes in the Carotid Body During Chronic Hypoxia

The carotid body is necessary for the development of VAH and much work has been done to elucidate the plasticity that occurs during chronic hypoxia. Carotid body denervation blocks the development of VAH in cats, dogs, goats, ponies, rats, and sheep (Bouverot et al., 1973; Vizek et al., 1987; Smith et al., 1986; Olson et al., 1988). Following chronic hypoxia, O_2-sensitivity in the carotid body is increased, as is the frequency of carotid sinus nerve firing for a given Pa_{O_2}. Following an hour of sustained hypoxia, the activity of single chemoreceptor fibers in goats steadily increased for at least 4 hours of hypoxia (Nielsen et al., 1988). In cats, the firing rate to acute hypoxia of up to 3 hours did not increase as it had in goats, but the firing rate from cats exposed to 28 days of chronic hypoxia was significantly increased (Barnard et al., 1987). Enhanced peripheral chemoreceptor responsiveness was also observed by recording whole carotid sinus nerve activity in cats exposed to 48 hours of chronic hypoxia and in rats acclimatized for 3-16 days (Barnard et al., 1987; Vizek et al., 1987; Chen et al., 2002b).

The increased HVR in the first hours of chronic hypoxia can be explained entirely by carotid body plasticity. In goats in which the blood supply to the carotid body was isolated, 6 hours of carotid body hypoxia in the absence of systemic hypoxia resulted in a time dependent increase in the HVR (Busch et al., 1985). This effect did not occur following 6 hours of carotid body hypercapnia (Bisgard et al., 1986) or systemic hypoxia alone (Weizhen et al., 1992). At least during the first few hours of chronic hypoxia, the carotid bodies are necessary and sufficient to produce VAH.

The exact mechanism by which carotid body sensitivity changes during chronic hypoxia is not known, but several structural and functional changes have been described. The morphology of the carotid body changes dramatically following chronic hypoxia, with an enlargement of the Type I cells (Mills & Nurse, 1993). This results in a decreased covering of glomus cells by sustentacular cells (Kusakabe et al., 1993), which increases the potential area available for gap junction connections and has been shown to enhance glomus cell sensitivity (Eyzaguirre & Abudara, 1999). However, these morphological changes occur following days in hypoxia and cannot explain the changes in carotid body sensitivity seen after a few hours of hypoxia.

As the exact mechanism of O_2-sensation in the carotid body is unknown, it is hard to determine which changes seen during chronic hypoxia can explain the increase in carotid body sensitivity. Changes in ion channel distribution in the glomus cells that would increase excitability have been observed, including a decreased density of K^+ channels and an increased density of Na^+ and L-type Ca^{2+} channels (Stea et al., 1992; Hempleman, 1995; Hempleman, 1996). Endothelin 1 (ET-1) and its receptor ET_A have been shown to increase hypoxic sensitivity and are both up-regulated in the glomus cells during chronic hypoxia (Chen et al., 2000). Blockade of the ET_A receptor can reverse the increased hypoxic sensitivity of the carotid body during chronic hypoxia (Chen et al., 2002a; Chen et al., 2007). There is also evidence of changes to other neurotransmitter systems including dopamine (Tatsumi et al., 1995; Huey et al., 2000) and acetylcholine (He et al., 2006) during chronic hypoxia, but their importance remains unknown. Regardless of the mechanism, the carotid body

undergoes plasticity that increases its O_2-sensitivity during chronic hypoxia and this is necessary for the development of VAH.

1.3.4 Changes in the Central Nervous System During Chronic Hypoxia

Although the carotid body has been shown to be extremely important in the development of VAH at early time points, other systems also show plasticity. Altered responsiveness in the brainstem respiratory centers during chronic hypoxia was first hypothesized following the observation that the chemoreceptor stimulant doxapram caused an increased ventilatory response in chronically hypoxic subjects (Forster et al., 1974; Dempsey & Forster, 1982). The ventilatory response to doxapram is similarly greater in chronically hypoxic rats (Wilkinson, unpublished observation). Doxapram acts by stimulating the carotid body and also has non-carotid body mediated stimulatory effects on ventilation. The response to doxapram in isolated normoxic and chronically hypoxic carotid bodies is similar (Fidone, unpublished observation) as is the phrenic response to doxapram in carotid body denervated normoxic and chronically hypoxic rats. As both components of doxapram stimulation remain unaltered in chronic hypoxia, this suggests an increased responsiveness of the central nervous system (CNS) respiratory centers in chronic hypoxia. This can be described as an increased CNS gain of the hypoxic ventilatory response (HVR).

More persuasive evidence of an increased CNS gain of the HVR during chronic hypoxia was obtained by comparing the phrenic response to electrical carotid sinus nerve stimulation in normoxic and chronically hypoxic anesthetized rats. Following 7 days of chronic hypoxia, the response to a given carotid sinus nerve

stimulation was greater than in the normoxic animals. The response following 2 days of hypoxia was approaching significance, which is consistent with the idea that CNS plasticity is more important at later time points in chronic hypoxia (Dwinell & Powell, 1999). The mechanism for this CNS plasticity has not been determined, although the activation of O_2-sensitive transcription factors is involved (Powell & Fu, 2008). The relative contributions of the carotid body and the CNS to the later stages of VAH are also unknown and difficult to determine because the CNS changes are at least partially dependent on the afferent input from the carotid bodies.

1.3.5 Other Possible Mechanisms of Plasticity During Chronic Hypoxia

No changes in respiratory motor neuron or muscle function during chronic hypoxia have been observed, but this possibility cannot be ruled out as plasticity in the phrenic motor pool occurs following intermittent hypoxia. Serotonin-mediated plasticity occurs during intermittent hypoxia in which phrenic neural activity is potentiated (phrenic long term facilitation) following multiple bouts of hypoxia. Similar facilitation is not observed after the same time period of hypoxic exposure if it is give in one sustained bout (Baker et al., 2001).

Following the observation that the stimulus for central chemoreceptor activation was unchanged during chronic hypoxia (Forster et al., 1975), the role of central chemoreceptors in VAH has remained largely unstudied. With chronic hypoxia, ventilatory drive in both hypoxia and normoxia is increased so Pa_{CO_2} is lower than normal. Even though the CO_2 set-point has changed, when VAH has fully developed no changes in the slope of the hypercapnic ventilatory (HCVR) response

are observed in either humans or rats. In humans, following 5 days of chronic hypoxia, the slope of the HCVR was the same as in normoxic controls, even though the curve was left shifted in the chronically hypoxic subjects (Somogyi et al., 2005). These results agree with the observation in this thesis that the slope of the HCVR in rats following 7 days of chronic hypoxia is the same as that of normoxic rats (Chapter 2). The slope of the HCVR may change during exposure to chronic hypoxia, though. Following 8 hrs of sustained hypoxia in humans, the slope of the HCVR is increased, with only the peripheral component showing a significantly increased sensitivity to CO_2 (Fatemian & Robbins, 2001). As the slope of the response to CO_2 changes over time in chronic hypoxia, there may be changes in central CO_2-chemosensitivity during chronic hypoxia. This thesis investigates the possibility that plasticity does occur in at least one central chemoreceptor site, the NTS.

1.4 Potential Role of the NTS in Integrating Peripheral and Central Chemoreceptor Information

The NTS is ideally suited to integrate both peripheral and central chemoreceptor input as it receives the first synapse from the carotid body (Housley et al., 1987) and also contains CO_2-sensitive chemoreceptor cells (Dean et al., 1989; Coates et al., 1993). Focal acidification *in vivo* and *in vitro* slice recordings provide evidence for the caudal NTS being a central chemoreceptor site (Coates et al., 1993; Nattie & Li, 2002a; Jansen et al., 1996; Pete et al., 2002; Dean et al., 1989).

1.4.1 Peripheral Chemoreceptor Input to the NTS

There is both anatomical and electrophysiological evidence that the caudal NTS receives the first synapse from the carotid body afferents (Vardhan et al., 1993; Marchenko & Sapru, 2000; Donoghue et al., 1984; Housley & Sinclair, 1988; Housley et al., 1987). In the rat, horseradish peroxidase injected into the carotid sinus nerve produced labeling in the commissural and ventrolateral subnuclei of the NTS, approximately 0.24 to 0.48 caudal to obex (Housley et al., 1987). A similar anatomical location of carotid sinus innervation in the caudal NTS was observed in cats as well (Torrealba & Claps, 1988). Using electrophysiological recordings in cats, chemoreceptor afferents were found that innervated the commissural nucleus at the level of obex and up to 1.5 mm caudal to obex (Donoghue et al., 1984). Functionally, kainic acid lesions of neurons in the caudal NTS decreased the ventilatory response to hypoxia in rats by 67% (Housley & Sinclair, 1988). Using anterograde tracers placed on the carotid body, NTS neurons that receive carotid sinus nerve input can now be identified and recorded in slices, providing more precise anatomical localization information (de Paula et al., 2007).

1.4.2 Central Chemoreception in the NTS

As described previously, microinjection of acetazolamide or microdialysis of CO_2 into the caudal NTS causes an increase in phrenic nerve output (Coates et al., 1993; Nattie & Li, 2002a). Intrinsic responses to CO_2 have also been shown in single cells from the NTS in slice (Dean et al., 1989). Microdialyzed CO_2 increases ventilation in awake rats during both wake and sleep, but the relative importance of

CO_2-sensitive chemoreceptors in the NTS compared to other sites during normal circumstances is unknown (Nattie & Li, 2002a).

Not all cells in the NTS are CO_2-sensitive, but in the caudal NTS 48% of the cells that can be recorded are excited by hypercapnia (Dean et al., 1989). Unfortunately, no marker exists to delineate which cells are chemosensitive and which are not. The Substance P (SP) receptor, Neuronkinin 1 (NK1R), is found in all areas of the brainstem that are putative central chemoreceptor sites, including the NTS (Nakaya et al., 1994), and others have postulated that the NK1R could be a marker of chemosensitive neurons (Nattie & Li, 2002b). Whether the NK1R positive cells are the CO_2-sensitive is not known, but lesions specifically targeting the NK1R cells in the RTN and the raphe have decreased the HCVR in rats and goats (Nattie & Li, 2002b; Nattie et al., 2004; Hodges et al., 2004).

1.5 Specific Aims

The goal of this dissertation is to investigate whether CO_2 sensing in the NTS undergoes plasticity during chronic hypoxia and contributes to VAH. To do this both a NTS specific stimulation and lesion study were completed.

The first set of experiments tests the hypothesis that chronic hypoxia changes the stimulus-response relationship for central CO_2-chemosensitivity in the NTS. Our collaborators have tested this at the cellular level with neural recordings from single chemoreceptor cells in brainstem slices. They found that a greater number of central chemoreceptor cells are inhibited by CO_2 in slices from chronically hypoxic rats as compared to normoxic controls (Nichols et al., 2009). I propose to test whether these

cellular changes can lead to changes in CO_2 processing at the whole animal level in anesthetized rats. The specific experimental design and results are discussed in Chapter 2.

The stimulation experiments will provide information on how the response to CO_2 stimulation changes but during chronic hypoxia, lower not higher CO_2 levels are seen. The second set of experiments investigates how a lesion in the NTS affects the ventilatory response to CO_2. Cells in the NTS containing the NK1R will be specifically killed using a targeted neurotoxin and the effect on room air, hypercapnic, and hypoxic ventilation will be measured in both normoxic and chronically hypoxic animals. This same lesion in the RTN has been shown to decrease the HCVR in normoxic rats, but chronic hypoxia has not been studied (Nattie & Li, 2002b). Finding a change in HCVR in the chronically hypoxic rats would suggest a role for the NTS chemoreceptor cells in VAH. The experimental design and results for this study will be discussed in Chapter 3.

Chapter 2: Effect of Nucleus Tractus Solitarius (NTS) Chemoreceptor Stimulation in Normoxia and Chronic Hypoxia

2.1 Introduction

The organization and significance of the multiple brainstem central chemoreceptor sites is an open question. There is some evidence that different sites can play different roles depending on the state of the animal. For instance, the medullary raphe seems to be more important in sleep (Nattie & Li, 2006a; Nattie & Li, 2001) and the RTN more important during wakefulness or under anesthesia (Li et al., 1999; Guyenet et al., 2008). The goal of this study is to determine whether the NTS chemoreceptor cells could play an important role in resetting the CO_2 set point for ventilation during chronic hypoxia.

One of the hallmarks of ventilatory acclimatization to hypoxia (VAH) is persistent hyperventilation when normoxia is restored. Normoxia removes hypoxic ventilatory drive from the arterial chemoreceptors but hyperventilation continues and decreases arterial CO_2 to levels that would normally inhibit breathing (Powell et al., 1998). The NTS receives the first synapse from the carotid body chemoreceptor afferents (Vardhan et al., 1993; Marchenko & Sapru, 2000; Donoghue et al., 1984; Housley & Sinclair, 1988; Housley et al., 1987) and also contains CO_2-sensitive cells (Coates et al., 1993; Nattie & Li, 2002a; Jansen et al., 1996; Pete et al., 2002). Since the NTS receives information about both O_2 and CO_2, it is ideally situated to mediate ventilatory plasticity during chronic hypoxia. We hypothesize that increased drive

from the NTS chemoreceptor cells during chronic hypoxia contributes to the hyperventilation in normoxia after VAH.

To test the role NTS chemoreceptors play during chronic hypoxia, we stimulated the NTS chemoreceptors in rats acclimatized to normoxia or chronic hypoxia. Acetazolamide was used in an anesthetized model to stimulate the chemoreceptors by creating a focal acidosis and we recorded the phrenic nerve response. This technique was one of the first to provide evidence that multiple putative central chemoreceptor sites identified *in vitro* could have effects *in vivo* on ventilation (Coates et al., 1993). We compared the phrenic response between the normoxic and chronically hypoxic rats and hypothesized that stimulation of the NTS chemoreceptors would produce a larger phrenic response in chronically hypoxic rats.

2.2 Methods

2.2.1 Experimental animals

Male Sprague-Dawley rats (319 – 444 g; Charles River) were housed in standard rat cages in a vivarium and fed ad libitum a standard rat diet. A 12:12-h light-dark cycle was maintained within the vivarium. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

2.2.2 Experimental Groups

Animals were placed in either normoxia or chronic hypoxia. Rats in the chronically hypoxic (CH) group were placed for 7 days in a hypobaric chamber maintained at 0.5 atm (380 mmHg), which approximates exposure to 10% inspired O_2 at sea level and mimics the conditions of chronic hypoxia encountered at ~6,000 m above sea level. Animals, within individual cages, were placed into the hypobaric chamber, and the pressure was lowered from 1.0 to 0.5 atmospheric pressure over a 5-min period. The chamber was opened once daily for about 10 min for regular cage maintenance or when it was necessary to remove animals for experimentation. The hypobaric chamber was maintained in the same vivarium that housed the normoxic control animals (N). No significant differences in body weights between the normoxic and chronically hypoxic groups were observed (N = 371.2 ± 15.4 g; CH = 374.4 ± 10.7 g).

Five of the animals in each group were microinjected with the experimental drug, acetazolamide (ACZ), in the caudal NTS. Four animals in each group served as controls, with half receiving a control injection of artificial cerebrospinal fluid (aCSF) in the caudal NTS and half receiving an ACZ injection in an adjacent area of the brainstem.

2.2.3 Experimental Drugs

Animals received microinjections of either aCSF alone or aCSF with ACZ (Sigma). The aCSF was made of (in mM): NaCl 115; KCl 2.0; KH_2PO_4 2.2; $NaHCO_3$ 25; D-glucose 10; $MgSO_4$ 1.2; $CaCl_2$ 2.5 (1). The concentration of ACZ used was 5 x

10^{-5} M. Both the aCSF and ACZ solutions also included fluorescent microbeads (Polyscience, 0.518 µm diameter) to localize microinjections and were warmed to 38°C and bubbled with 5 % CO_2 in room air.

2.2.4 Surgical Procedures

All animals were initially anesthetized with 5% isoflurane and maintained at 3% isoflurane in a 50% O_2, 50 % N_2 gas mix. A ventral approach was used for bilateral vagotomy and insertion of the tracheal cannula (PE-240 tubing) for artificial ventilation (Model 683 Rodent Ventilatory, Harvard Instruments). The femoral artery and vein were catheterized for intravenous drug infusion and blood pressure and arterial blood-gas measurements using polyethelyene tubing (PE-10 for the artery and PE-50 for the vein). The left phrenic nerve was isolated, cut distally, desheathed, and placed on a custom-made bipolar silver hook electrode. The nerve was immersed in mineral oil to prevent desiccation.

The animal was then placed into a stereotaxic device with earbars (Knopf) and the NTS exposed to allow for microinjection. The NTS was accessed using a dorsal approach. The skin and muscle overlaying the brainstem were incised and retracted using sutures. The dura was then cut at the edge of the skull bone and the brainstem visualized. Upon completion of the surgical procedures, the rat was gradually switched from isoflourane to intravenous urethane over a 20-minute period (1.6 g/kg).

2.2.5 Microinjection of Experimental Drugs

One nL microinjections were made (World Precision Instruments Nanopump) with a glass micropipette (Sutter Instruments P-87; 1B120F-6 capillary glass, WPI); a 10-µm tip diameter was created by gently stabbing the tip through a Kimwipe.

The microinjections were placed using the caudal tip of the area postrema (calamus scriptorius) as a landmark. Injections were made 0.5 mm rostral from calamus scriptorius and 0.5 mm lateral from midline at a depth of 0.4 mm. The injection site corresponds to -13.8 mm caudal to bregma (Paxinos & Watson, 1982).

2.2.6 Phrenic Nerve Signal

The phrenic nerve signal was recorded with a bipolar silver electrode and preamplified (P5 series with HI-Z high impedance probe, Grass), filtered, rectified, and integrated using a moving time averager (model MA-821, CWE; time constant = 50 ms). The raw and integrated phrenic signals were displayed in real time and stored on a computer using an analog to digital data collection program (MP100A, BIOPAC Systems). An example raw and integrated phrenic trace is shown in Figure 4.

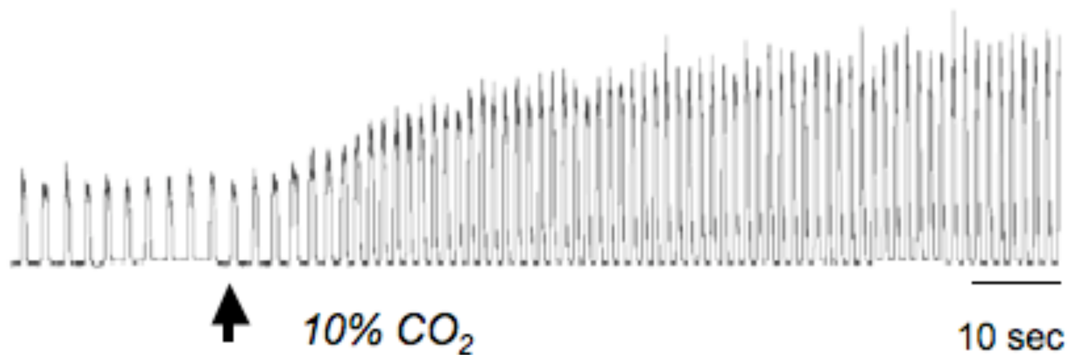

Figure 4: Example Raw and Integrated Phrenic Nerve Trace. Response of phrenic nerve activity to 10% inspired CO_2. Bottom trace is rectified, integrated, and averaged over a 50 ms time constant.

2.2.7 Experimental Protocol

Phrenic nerve activity was allowed to stabilize for 60 min in hyperoxia (FI_{O_2}= 0.50). The apneic threshold, or level of end tidal CO_2 (ET_{CO_2}) that caused phrenic nerve activity to cease, was determined by increasing the ventilator rate and/or tidal volume. Baseline nerve activity was established by increasing PET_{CO_2} to 3 Torr above the apneic threshold with ventilator adjustments. Then a maximum phrenic response to 10% CO_2 was measured and nerve activity was allowed at least 30 min to recover to

baseline. Arterial blood-gas samples were taken during the 10% CO_2 test and during baseline immediately preceding the drug microinjection. Animals were then given either a 1 nL microinjection of ACZ or aCSF into the caudal NTS. Phrenic nerve activity was recorded for 60 min following injection, with arterial blood-gas samples taken at 15 and 60 min. A final 10% CO_2 challenge was administered to determine how drug treatment affected the maximum phrenic response.

2.2.8 Microinjection Localization

Animals were transcardially perfused using 4% paraformaldehyde. The brainstems were removed and postfixed overnight in paraformaldehyde and then cryoprotected in 30% sucrose. The samples were frozen and cut at 50 μm using a cryostat (Reichert-Jung Cryocut 1800). The fluorescent microbeads were located using a fluorescent microscope (Nikon Eclipse E400) to localize the microinjection site. All animals in the ACZ group received injections in the caudal NTS at the level where the area postrema is present. Animals in which an ACZ injection was not centered in this target region were used as control animals.

2.2.9 Data Analysis

Phrenic burst frequency (fR), peak amplitude of integrated phrenic nerve activity (∫Phr), and their product (∫Phr × fR, neural ventilation) were averaged over 10 bursts recorded 5, 15, 30, and 60 min post injection and with 10% CO_2 after injection. The fR was expressed as an absolute value. ∫Phr and ∫Phr × fR were normalized as a percentage of baseline phrenic nerve activity (defined as phrenic nerve activity at

$P_{ET_{CO_2}}$ of 3 Torr above apneic threshold) measured immediately before drug microinjection, as well as to the maximum phrenic activity measured in 10% CO_2 before drug administration.

2.2.10 Statistics

For the phrenic burst frequency, amplitude, and neural ventilatory response to ACZ, a Univariate ANOVA with an LSD Post-Hoc was used. All averages are expressed ± the standard error of the mean. Independent samples t-tests were used to compare body weights, apneic thresholds, and blood-gas values. For all tests $p < 0.05$ was considered significant. All statistics were done using SPSS statistical software.

2.3 Results

2.3.1 Verification of Acclimatization in the Chronically Hypoxic Group

Acclimatization in the CH rats was evidenced by a significantly lower apneic threshold (N = 34.8 ± 0.5; CH = 27.9 ± 1.2) and significantly higher hematocrit (CH = 59.8 ± 2.9; N = 39.8 ± 2.7). Additionally, similar to what is seen in awake, freely behaving rats (Reid & Powell, 2005), the baseline frequency in the CH rats was significantly higher than the normoxic rats (N fR = 36.2 ± 2.0; CH fR = 44.8 ± 2.1 bursts/min). Because phrenic burst amplitude is not an absolute value, it is impossible to compare between the N and CH groups, but it is expected to be higher in the CH rats as well, given the increase in tidal volume observed in acclimatized rats (Aaron & Powell, 1993; Reid & Powell, 2005).

2.3.2 Response to Inspired CO_2 Challenge

When normalized to baseline activity, the phrenic response to a 10% inspired CO_2 challenge was not different between the normoxic and chronically hypoxic animals (N = 388 ± 34%; CH = 333 ± 37%). This agrees with the observation in awake rats that the slope of the hypercapnic ventilatory response is the same in both groups of rats, even though baseline ventilation is approximately 30% higher in the chronically hypoxic rats (Figure 5, see also Aaron & Powell, 1993).

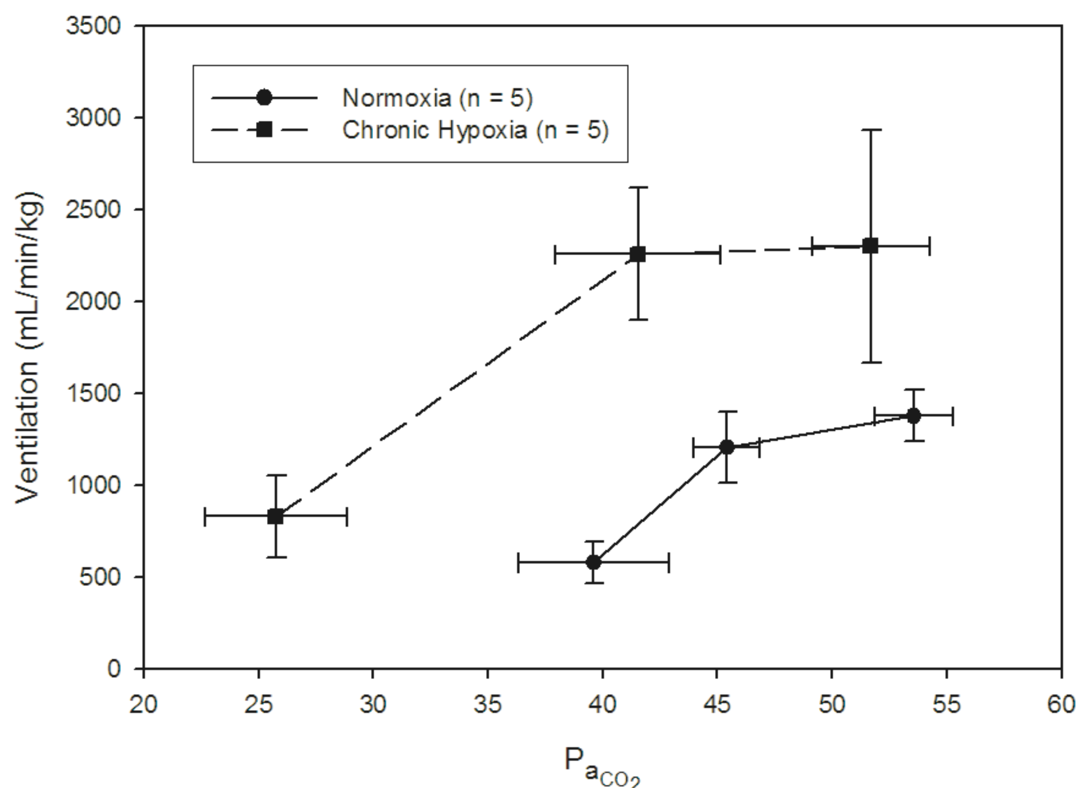

Figure 5: Effect of Chronic Hypoxia on the HCVR in Awake Rats. Ventilation plotted against Pa_{CO_2} at 3 levels of inspired CO_2 in awake rats. Ventilation in the CH rats was higher at all levels of Pa_{CO_2}, but the slope of the HCVR was not significantly different between the two groups.

2.3.3 Location of Drug Microinjections

To be considered a successful ACZ injection in the caudal NTS, the fluorescent microbeads injected with the drug had to be localized between -14.3 to -13.4 mm caudal from bregma (Paxinos & Watson, 1982). This is the area of the NTS in which the Area Postrema is present and corresponds with the area where central chemoreceptor cells are found (Dean et al., 1989). Four ACZ microinjections were located adjacent to the caudal NTS and were included in the control injection group (Figure 6). An additional 4 microinjections of aCSF alone were localized to the caudal NTS (not shown) and used in the control group.

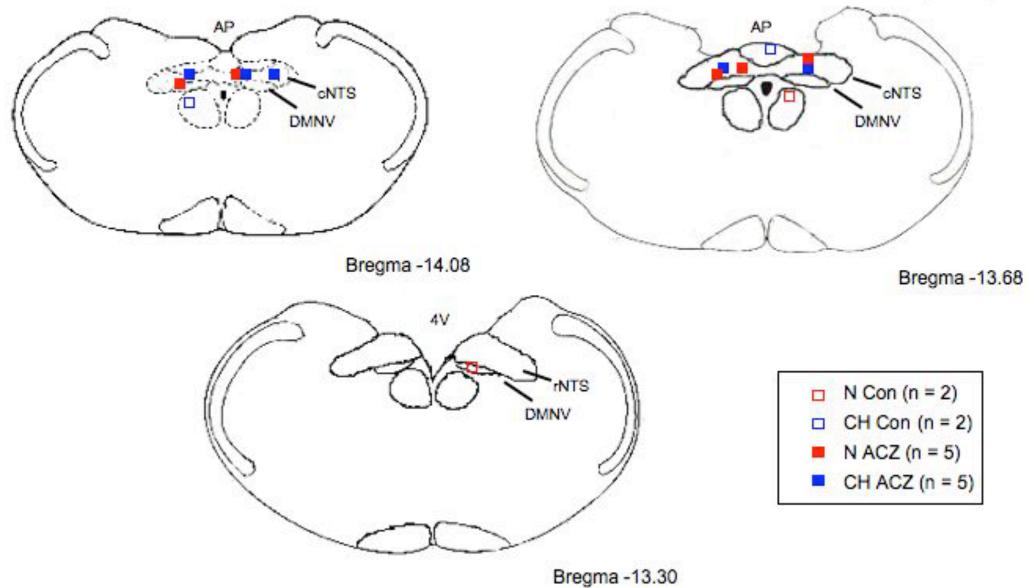

Figure 6: Localization of Acetazolamide (ACZ) Microinjection. Location and effect of ACZ microinjection. Closed squares indicate a phrenic nerve response, open squares indicate no change in phrenic activity. N rats are depicted in red and CH in blue. All effective ACZ microinjections were located in the caudal NTS (cNTS). For simplicity, the 4 artificial cerebrospinal fluid (aCSF) injections that were in the cNTS and caused no change in phrenic activity were not pictured. All aCSF injections were localized to the cNTS. AP = Area Postrema; cNTS = caudal Nucleus Tractus Solitarius; rNTS = rostral Nucleus Tractus Solitarius; DMNV = Dorsal Motor Nucleus of the Vagus; 4V = 4th Ventricle.

2.3.4 Time Course of Response to Acetazolamide

The peak phrenic response to ACZ microinjected in the caudal NTS occurred 30 min following the injection and by 60 min, phrenic activity in approximately half of the animals was returning towards baseline levels. A representative case is shown in Figure 7. The remainder of the analysis was done on the 30 min post-injection time point.

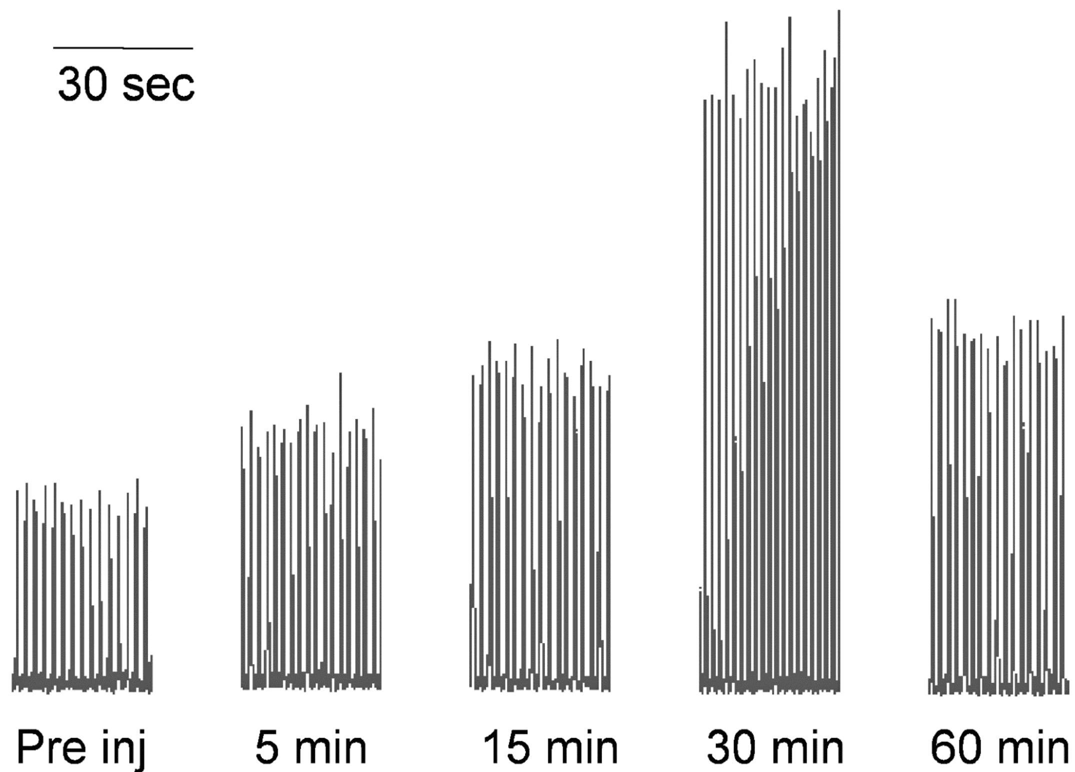

Figure 7: Time Course of Phrenic Response to ACZ. 30 sec integrated phrenic nerve activity traces at 4 time points following ACZ microinjection into the NTS. The peak of the response occurred at 30 min.

2.3.5 Response to Acetazolamide in Normoxic and Chronically Hypoxic Rats

As expected, no change in neural ventilation was observed in the control animals. There were no differences between the control rats that were given the aCSF injection in the caudal NTS or the rats given ACZ in an adjacent location, so both types of animals were pooled for the analysis. Additionally, the N and CH control rats were not significantly different from each other.

In both the N and CH rats, ACZ in the caudal NTS caused an increase in neural ventilation with both ∫Phr and fR elevated in response to the ACZ (Figure 8). The ∫Phr response was the most consistent, with fR tending to vary more over time in the control animals.

Although both groups increase phrenic activity in response to the ACZ, the increase in neural ventilation is significantly greater in the N rats (Figure 8A). This is driven almost entirely by an increased ∫Phr in the N rats (Figure 8B). There is a trend for a greater fR increase in the N animals, but it did not reach significance (Figure 8C).

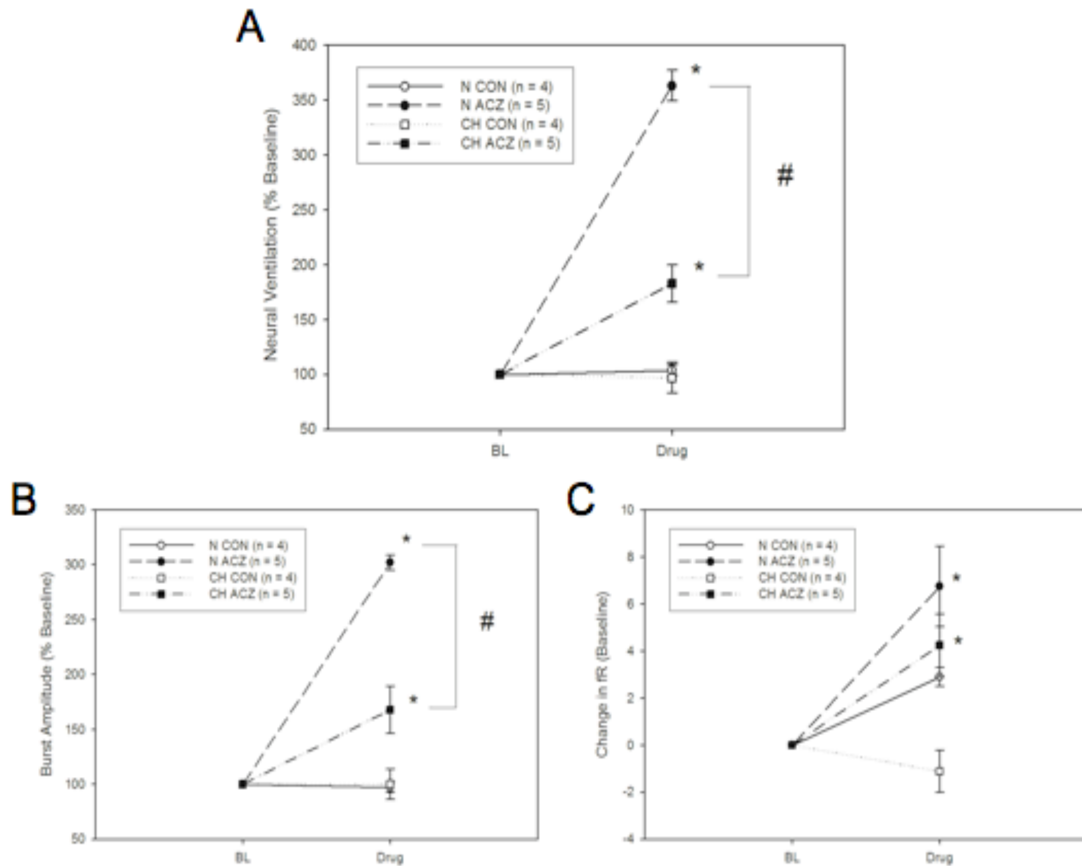

Figure 8: Percent Change in Phrenic Activity in Response to Acetazolamide. Phrenic activity at baseline (BL) and 30 min after ACZ or control (CON) microinjection. All values normalized to BL (=100%). **A**. Phrenic Neural Ventilation **B**. Phrenic Burst Amplitude (∫Phr) **C**. Phrenic Burst Frequency (fR). * $p < 0.05$ from appropriate control; # $p < 0.01$

2.3.6 Neural Activity Still Has Capacity to Increase Following Acetazolamide

Baseline neural ventilation in the CH animals is expected to be higher than the N animals, just as ventilation is in awake animals (Aaron & Powell, 1993). To test the possibility that the phrenic activity in the chronically hypoxic animals reached a maximum value following ACZ microinjection, we measured the effect of increasing inspired CO_2 on neural ventilation in both groups of animals after ACZ microinjection. In all cases, neural ventilation increased compared to the peak response to ACZ at 30 min (Figure 9). Therefore, the dose of ACZ used in this study did not produce a maximal neural ventilatory response.

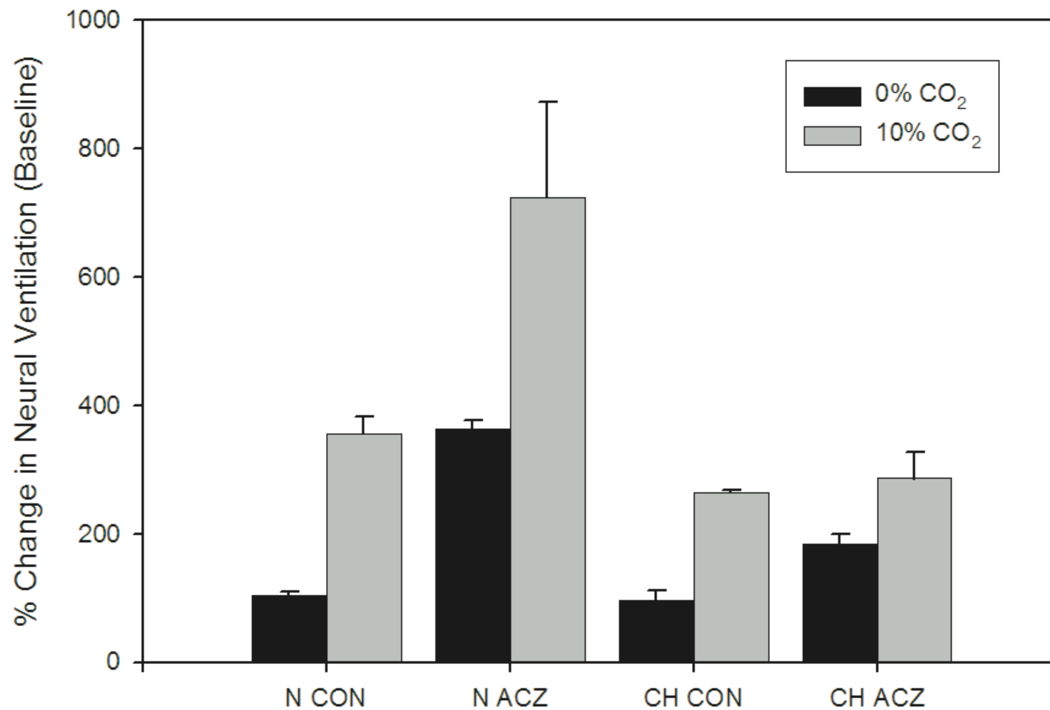

Figure 9: Phrenic Nerve Still Has Capacity to Increase Activity Following Acetazolamide (ACZ). 10% inspired CO_2 increases neural ventilation in normoxic (N) and chronically hypoxic (CH) rats. Significant increases are observed in animals microinjected with aCSF or ACZ outside the cNTS (CON) and in animals with aCZ microinjected in the NTS. Hence, ACZ did not increase ventilation to a level that it could not be further stimulated by CO_2.

2.4 Discussion

2.4.1 Summary of Results

As seen by other groups (Coates et al., 1993), ACZ microinjected into the caudal NTS produced an increase in phrenic activity, with both fR and ∫Phr increasing. This is consistent with results in awake animals where CO_2 microdialyzed in the NTS increased both tidal volume and frequency (Nattie & Li, 2002a). Phrenic activity only

increased if the ACZ microinjection was centered in the caudal NTS. Control injections of aCSF or microinjections of ACZ a few hundred microns outside of the caudal NTS did not affect phrenic activity, demonstrating the specificity of the response (Figure 6). The peak response to microinjected ACZ occurred at 30 min post-injection, which is also similar to what others have observed (Coates et al., 1993) and is consistent with ACZ's mechanism of action to increase PCO_2 and/or $[H^+]$ (Bickler et al., 1988; Teppema et al., 1990).

The new finding of these experiments is that chronic hypoxia decreased phrenic response to NTS chemoreceptor stimulation. This disproves our hypothesis of increased sensitivity in the NTS chemoreceptors with chronic hypoxia. However, the phrenic response to 10% inspired CO_2, which activates both peripheral and all central chemoreceptor sites, was not different in the CH rats when expressed as a percent increase from baseline. This agrees with my observation in awake rats that the slope of the hypercapnic ventilatory response is the same in N and CH rats (Figure 5). It also suggests plasticity in the response to CO_2 stimulation in the NTS during CH and that other chemosensitive sites in the carotid body or CNS compensate for the decreased effect of NTS stimulation in CH rats.

Comparing N and CH animals is often difficult due to the fact that baseline variables are very different between the two animals. Baseline measurements were taken relative to apneic threshold, but even so, baseline ventilation in awake CH rats is much higher than in the N controls. Ventilation during room air breathing is increased approximately 30% by chronic hypoxia in awake rats (Figure 5) so it is important to

consider if the smaller response to ACZ in the NTS is simply an artifact of normalizing to a larger baseline. As mentioned above, it is impossible to measure phrenic nerve activity in absolute units. However, we can scale the baseline neural ventilation at 3 torr above the apneic threshold using the data for ventilation in awake rats. Figure 10 shows that neural ventilation is still significantly less in CH compared to N control rats with NTS chemoreceptor stimulation, even when the baseline neural ventilation is increased for chronic hypoxia. Hence, chronic hypoxia clearly decreases the ventilatory response to NTS chemoreceptor stimulation, in contrast to our original hypothesis.

This data indicates that other ventilatory drives undergo plasticity during chronic hypoxia also. Because the response to inspired CO_2 is similar in the N and CH animals, other chemoreceptor sites may compensate for the lowered NTS response in chronic hypoxia.

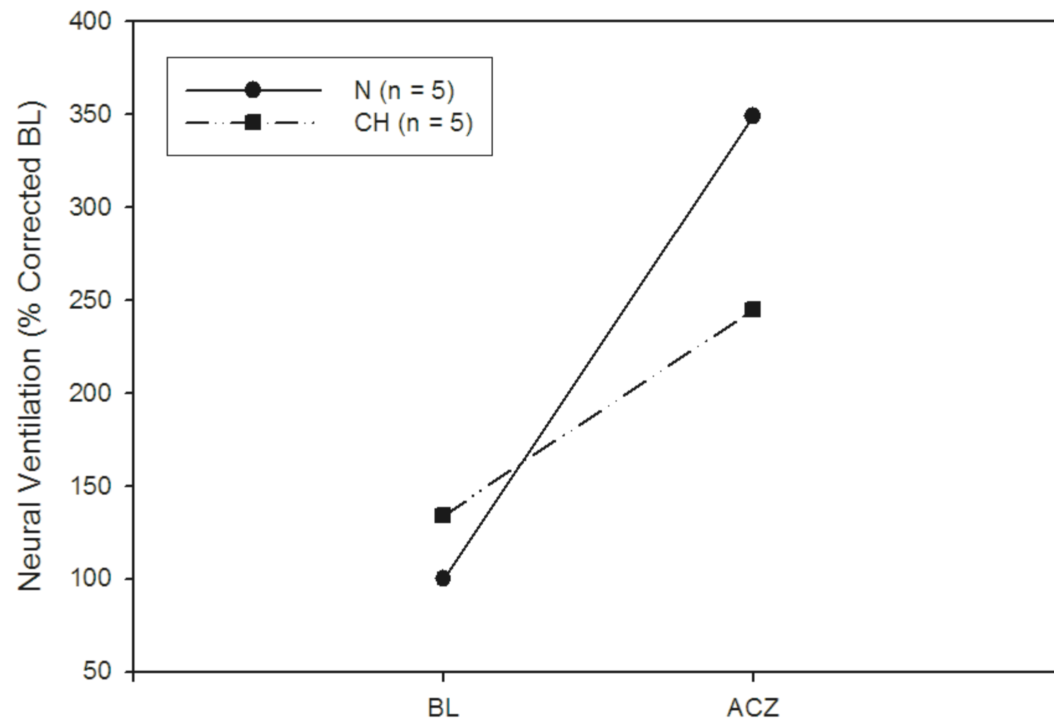

Figure 10: Increased Baseline in Chronic Hypoxia Does Not Explain Decreased Response to Acetazolamide Stimulation. Neural ventilation (∫Phr * fR) increases significantly less with acetazolamide (ACZ) microinjection in chronically hypoxic (CH) versus normoxic control rats (N) even after correcting for the increased baseline predicted in CH rats. The BL value for CH rats was increased relative to N rats by the fractional increase in normoxic ventilation measured in awake CH rats relative to N rats.

2.4.2 Physiological Significance

Our results support findings from isolated chemoreceptor cells in the NTS recorded in brainstem slices that show increased inhibitory effects of hypercapnia during CH (Nichols et al., 2009). In the normoxic rats, 71% of the neurons recorded in the NTS from slices respond to hypercapnia, with 57% of cells being excited and 14% inhibited by CO_2. In the chronically hypoxic slice, the same percentage of neurons

respond to hypercapnia, but significantly more (25%) are inhibited by CO_2 than in normoxia (Nichols et al., 2009). It is unknown whether the cells inhibited by hypercapnia directly inhibit respiratory output or if they are inhibitory interneurons and their activation leads to disinhibition and increased ventilatory output. The simplest model that would reconcile the cellular and whole animal findings would be that the excitatory chemoreceptor cells increase phrenic output and the inhibitory ones decrease phrenic output. If that is the case, there are more inhibitory NTS chemoreceptor cells during chronic hypoxia and would therefore be a decreased phrenic response to stimulation. More studies are necessary to determine the relation of the chemoreceptor cells and the respiratory rhythm generation sites to determine whether this model is correct.

Carotid body chemoreceptors do not increase CO_2-sensitivity during chronic hypoxia (Barnard et al., 1987) although the increased gain of the arterial chemoreflex with chronic hypoxia (Dwinell & Powell, 1999) could increase the ventilatory response to carotid body stimulation by CO_2. The response to RTN chemoreceptor stimulation during chronic hypoxia will be especially interesting to study as neurons from the NTS project to RTN chemoreceptors (Takakura et al., 2006). This means that the NTS is not the only central chemoreceptor site that receives peripheral chemoreceptor input. Sorting out the effects of increased afferent input from other changes with chronic hypoxia on central CO_2-sensitivity in different anatomical sites requires further experiments.

While the response to stimulation of the different chemoreceptor sites may differ with chronic hypoxia, hypocapnia and decreased stimulation occurs during chronic hypoxia. The relevant question therefore is how the central chemoreceptor sites behave during lower Pa_{CO_2} levels. One possibility is that the chemoreceptor cells inhibited by hypercapnia (Nichols et al., 2009) are excited by hypocapnia. This leads to the hypothesis that the increase in this population of cells might provide a drive to breathe during the lower Pa_{CO_2} levels seen during chronic hypoxia and help explain the persistent hyperventilation in normoxia. More studies on the cellular properties and projections of the inhibited NTS chemoreceptors are needed to test this hypothesis.

In summary, this study has provided evidence of a decreased responsiveness to CO_2-stimulation during CH in one central chemoreceptor site, the NTS. The importance of this to acclimatization to chronic hypoxia is unknown but suggests a role for the NTS central chemoreceptors in ventilatory acclimatization to hypoxia.

2.4.2 Critique of Methods

2.4.2.1 Acetazolamide Mechanism of Action

The exact mechanism by which microinjection of AZ produces focal tissue acidosis is not completely understood. An increase in brain P_{CO_2} and H^+ concentration has been demonstrated following intravenous ACZ administration in rabbits and cats (Bickler et al., 1988; Teppema et al., 1990). The acidosis has been suggested to result from the accumulation of metabolically produced H^+ while the increase in brain P_{CO_2}

has been suggested to result from interference with the hydration of CO_2 and impairment of transport in brain capillaries and red blood cells (Severinghaus et al., 1969; Bickler et al., 1988; Teppema et al., 1990). Increases in both H+ and P_{CO_2} have been suggested to be able to activate chemoreceptor cells, but it remains unknown which stimuli is the main mediator for ACZ's activation of the chemoreceptor cells (Putnam et al., 2004).

It was suggested that carbonic anhydrase was necessary for CO_2-sensitivity in central chemoreceptor cells (Dean & Reddy, 1995), but subsequent work has found that was an artifact of the whole cell recording technique and can be prevented by varying the composition of the solution in the patch pipette (Conrad et al., 2009). Additionally, carbonic anhydrase isoforms II and IV are found in non-chemosensitive regions and ACZ does not alter the raphe neuronal firing response to hypercapnia (Wang et al., 2002). Therefore, we do not feel that inhibiting carbonic anhydrase affected the fundamental chemosensing mechanism in the CO_2-sensitive cells.

2.4.2.2 Dose of Acetazolamide Used

The dose of ACZ used in this study is the same as that used by other groups in the fastigial nucleus in rat (Xu et al., 2001) and the pre-Botzinger complex of cats (Solomon et al., 2000; Solomon, 2003). In those studies, slightly larger injection volumes (1-20 nL) were used and also produced an increase in phrenic activity. In the only other study where the NTS in rats was targeted (Coates et al., 1993), the same volume of a slightly lower dose of ACZ (10^{-5} M as compared to 5 x 10^{-5} in this study)

was used. However, this lower dose did not produce a consistent phrenic response in our animals.

Coates and colleagues (1993) measured the tissue acidification in response to 1 nL volumes of 10^{-5} M ACZ injections and found the acidosis to be similar in magnitude to that evoked by increasing end-tidal P_{CO_2} by ~40 Torr. No detectable acidification was present 300 µm from the injection site. As my dose was slightly higher it is likely the acidification was also slightly greater, although the spread should be similar because the injection volumes were the same size. The focal nature of the stimulus is supported by the control ACZ microinjections. Injection sites located a few hundred microns away from the caudal NTS were ineffective and the spread of all included injection sites was centered in the caudal NTS.

2.4.2.3 Differential Effect of Acetazolamide During Chronic Hypoxia

The same dose of ACZ was given to both normoxic and chronically hypoxic animals, but whether that dose results in the same stimulus and pH change in both groups is unknown. ACZ was microinjected under baseline conditions when PET_{CO_2} was 3 Torr above the apneic threshold in both animals. As the apneic threshold is much lower in the CH rats, arterial P_{CO_2} is as well and presumably cerebrospinal pH is more alkaline in the CH rats as was measured in humans (Forster et al., 1975). It is unknown what effect the more alkaline cerebrospinal fluid in the CH rats has on the action of ACZ.

Chapter 3: Effect of Neurokinin 1 Receptor Positive Cell Lesion in the Nucleus of the Solitary Tract During Normoxia and Chronic Hypoxia

3.1 *Introduction*

The previous study addressed plasticity in the response of the central chemoreceptor cells in the NTS to stimulation during chronic hypoxia. The fact that NTS chemoreceptor stimulation had a decreased effect during chronic hypoxia is suggestive of plasticity, but does not address the question of how the central chemoreceptor cells respond to the lower P_{CO_2} values seen during chronic hypoxia. Because of the inability to measure activity below the apneic threshold, the following lesion study was done to further study changes in CO_2-chemosensitivity in the NTS during chronic hypoxia.

Large lesions to the NTS are problematic because of the many sensory systems that synapse in the NTS, including for example, the peripheral chemoreceptors, baroreceptors and vagal afferents (Donoghue et al., 1984; Jordan & Spyer, 1986). Large electrolytic lesions of the NTS cause fatal hypertension if the animals are not immediately treated with pharmacological agents that can block the hypertensive actions of vasopressin and the autonomic ganglionic transmission (Doba & Reis, 1973; Sved et al., 1985). To answer the question of the importance of the NTS central chemoreceptor cells during chronic hypoxia, a lesion of only the CO_2-sensitive cells would ideally be performed. No unequivocal marker of chemoreceptive cells has been

identified yet, but other groups have used a candidate marker to lesion central chemoreceptor sites in the brainstem.

All the putative central chemoreceptor areas contain cells expressing the receptor for Substance P (SP), Neurokinin 1 (NK1R) (Nakaya et al., 1994). The role of NK1R cells in CO_2-sensitivity is not known, but lesions specifically targeting the NK1R cells in chemosensitive areas of the brainstem have decreased the hypercapnic ventilatory response (HCVR) in rats and goats (Hodges et al., 2004; Nattie & Li, 2002b; Nattie et al., 2004).

These studies utilized a novel mechanism of targeting cells containing the NK1R (Wiley & Lappi, 1997). The NK1R is a G-protein linked receptor that is phosphorylated and internalized following ligand binding. Dissociation from the ligand occurs inside the cell and the receptor is then recycled to the cell surface (Mantyh et al., 1995). NK1R internalization provides a mechanism to deliver a neurotoxin into the cell. To target the NK1R cells, SP was conjugated to the ribosomal toxin saporin (SAP), which can only kill cells that it can enter. Hence, SP conjugated SAP (SP-SAP) kills only NK1R cells. A nonsense peptide conjugated to SAP was used as the control drug since it cannot enter any cells.

In the rat, bilateral injections of the RTN with SP-SAP decreased ventilation and tidal volume in both room air and 7% CO_2 (Nattie & Li, 2002b). This effect was present in both sleep and wakefulness. Additionally, the change in ventilation in response to inhaled CO_2 was significantly decreased in the lesioned animals (-17%), leading the authors to posit a role for the NK1R cells in central chemosensitivity in the

RTN. NK1R cell lesion with SP-SAP in the medullary raphe decreased the response to hypercapnia similarly (Nattie et al., 2004). In goats, medullary raphe lesions also decreased the response to CO_2, although it was transient and unlike in rats, room air ventilation was unaffected (Hodges et al., 2004). In rats, larger lesions, which encompass the RTN, medullary raphe, A5 region, and the pre-Bötzinger complex/rostral ventral respiratory group, cause an even more dramatic decrease in CO_2 sensitivity of 61% in wakefulness (Nattie & Li 06). All of these studies suggest that the NK1R cells in multiple chemoreceptor sites are necessary for a normal HCVR.

The goal of these experiments is to determine whether NK1R cell lesion in the NTS affects the HCVR as it does in other brainstem central chemoreceptor sites. Additionally, the effect of the lesion during chronic hypoxia will be studied to determine whether the NK1R cells have a different role during chronic hypoxia.

3.2 Methods

3.2.1 Experimental animals

Male Sprague-Dawley rats (290 – 400 g; Charles River) were housed in standard rat cages in a vivarium and fed ad libitum a standard rat diet. A 12:12-h light-dark cycle was maintained within the vivarium. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental

animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

3.2.2 Experimental Groups

Animals were placed into one of four groups, depending on whether they were given the experimental drug (SSP-SAP) or a control drug (Blank-SAP) and maintained in normoxia (N) or chronic hypoxia (CH). For both N and CH, 11 Blank-SAP and 5 SSP-SAP animals were used. A subset of these animals was measured in both N and CH (Blank n = 4; SSP-SAP n =2). No significant differences in body weights between the groups were observed (Blank-SAP = 346.0 ± 1.65 g; SSP-SAP = 360.4 ± 12.9 g).

3.3.3 Experimental Drugs

The neurotoxin saporin was conjugated with either Stable Substance P or an 11 amino acid nonsense peptide (SSP-SAP and Blank-SAP, Advanced Targeting Systems, San Diego). The Substance P (SP) receptor Neurokinin 1 (NK1R) is internalized following the binding of NK1R ligand, meaning that if saporin is bound to SP it can enter and kill the cell. The Blank-SAP served as the control drug for these experiments, as the nonsense peptide should not bind to any receptors and the saporin will be unable to enter the cells.

3.3.4 Exposure to Chronic Hypoxia

Rats in the CH group were placed for 7 days in a hypobaric chamber maintained at 0.5 atm (380 mmHg), which approximates exposure to 10% inspired O_2 at sea level and mimics the conditions of chronic hypoxia encountered at ~6,000 m

above sea level. Animals, within individual cages, were placed into the hypobaric chamber, and the pressure was lowered from 1.0 to 0.5 atm over a 5-min period. The chamber was opened once daily for about 10 min for regular cage maintenance or when it was necessary to remove animals for experimentation. The hypobaric chamber was maintained in the same vivarium that housed the control animals.

3.3.5 Surgical Preparation

All animals received 200 nL injections of drug (0.013ng/nL), either SSP-SAP or Blank-SAP, into the caudal NTS. Animals were initially anesthetized with 5% isoflurane in O_2 and maintained under anesthesia with 2–2.5% isoflurane in O_2. The skull was shaved and the skin sterilized with betadine and alcohol. The head was placed into a Kopf stereotaxic holder. To visualize the dorsal brainstem, a midline incision was made and the muscle retracted to expose the edge of the skull bone. The head was angled 45°nose-down and the dura cut at the point it connects with the skull. The tip of the area postrema (calamus scriptorius) was then visible and used as a landmark for the injection sites. Microinjections were made (World Precision Instruments Nanopump) with a glass micropipette with a 10-µm tip diameter. A total of 8 injections (25 µL each) were made, with the patency of the micropipette tested after each injection. The first set of injections was made 0.1 mm rostral to the tip of the area postrema bilaterally and the second set 0.4 mm rostral. Injections were made at two depths (0.2 mm and 0.4 mm deep) at every injection site.

Following the injections, rats were maintained in normoxia for 11 days to allow for maximal cell killing, as determined by other groups (Nattie & Li, 2002b)

and confirmed by us in pilot studies. The CH group was then placed into the hypobaric chamber for an additional 7 days of hypoxia, while the N group remained in normoxia for 7 days. Most ventilatory measurements were taken as described below 18 days following the injection, but a subset of animals was measured in normoxia at 11 days prior to being placed in chronic hypoxia (Blank n = 4; SP-SAP n =2).

Two days prior to ventilatory measurements, recovery surgery was performed under isoflurane to implant a temperature telemetry probe into the abdomen (G-2 Emitter, Respironics). Additionally, the femoral artery was cannulated with a custom catheter made of polyethylene tubing (PE-10 joined to PE-50). The catheter was tunneled under the skin to the shoulder area to allow access for blood gas sampling and to prevent the rat from damaging the tubing.

3.3.6 Ventilatory Measurements by Barometric Plethysmography

All ventilatory data was collected on awake, unrestrained animals using the barometric plethysmography modified for continuous flow (Jacky, 1978; Aaron & Powell, 1993). Briefly, barometric plethysmography records the pressure swings caused by the warming and expansion of air when it enters the lungs.

On the experimental day, rats were placed into a 7L, sealed Plexiglass chamber. A thermometer and humidity probe were sealed in the box (Physitemp Thermalert TH-5). An electronic gas-mixer (MFC-4, Sable) was used to regulate the inspired gas concentrations and provide high input impedance. The chamber gas concentrations were measured using a mass spectrometer (MGA 1100, Perkin-Elmer) with the gas exiting the chamber via a vacuum valve (m series, Nupro) to a vacuum

pump. We did not use a reference chamber and compensated for changes in the pressure signal baseline by adjusting the vacuum valve to maintain a chamber pressure near atmospheric level, monitored by water manometer after each change of inspired gases. Respiratory frequency (f) was calculated directly from the ventilation-induced pressure swings (Validyne, MP45). Tidal volume (V_T) was calculated using calibration pulses (1 mL) generated by using a gas-tight syringe and injecting air pulses into the chamber at a rate similar to the rats' frequency following the experimental measurements. Ventilation (V_I) was calculated as the product of f and V_T and normalized for the animal's body weight.

The protocol commenced following an acclimatization period to the box which consisted of 35 minutes in either 21% O_2 for N rats and 10% O_2 for CH rats. Animals were exposed to 10 minutes of each experimental gas mixture before respiratory and arterial blood gas measurements were taken. Experimental protocols for the normoxic and chronically hypoxic rats are shown in Figure 11 and Figure 12.

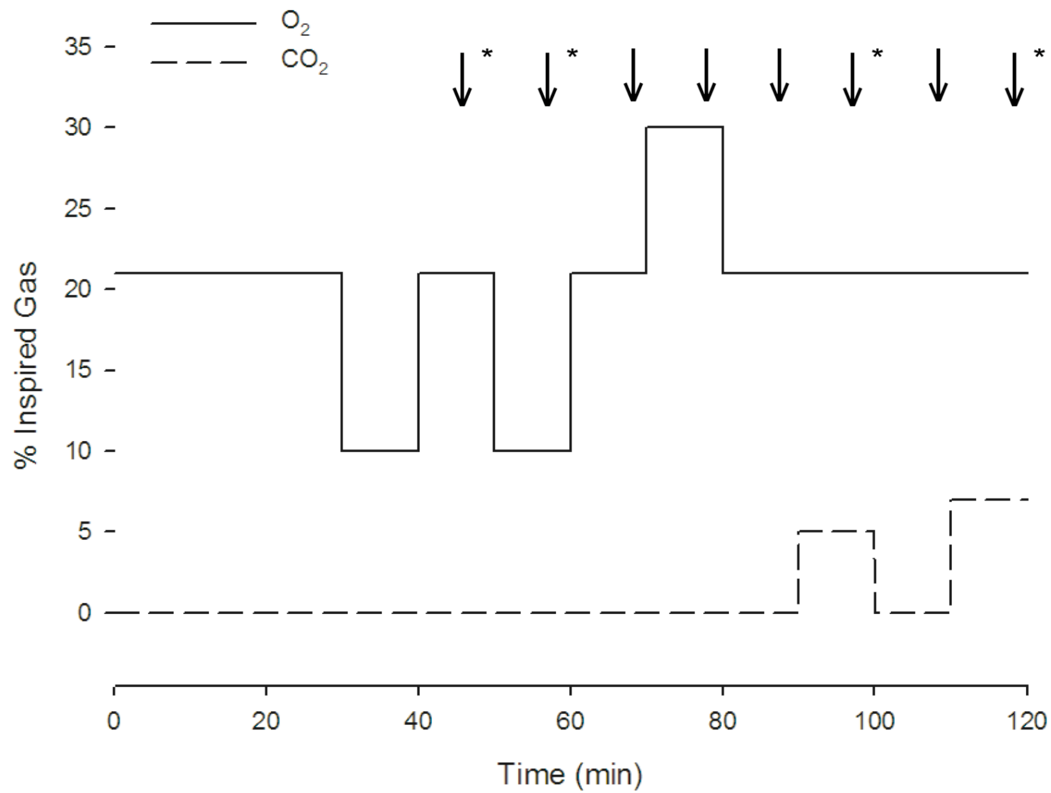

Figure 11: Plethysmograph Data Collection Protocol for Normoxic Rats. O_2 and CO_2 were changed as shown and the balance was N_2. The first 40 min of the protocol acclimates the rat to the plethysmograph. Arrows indicate ventilation measurements made between 10 and 15 minutes at a gas level time point. * indicates arterial blood gas sampling.

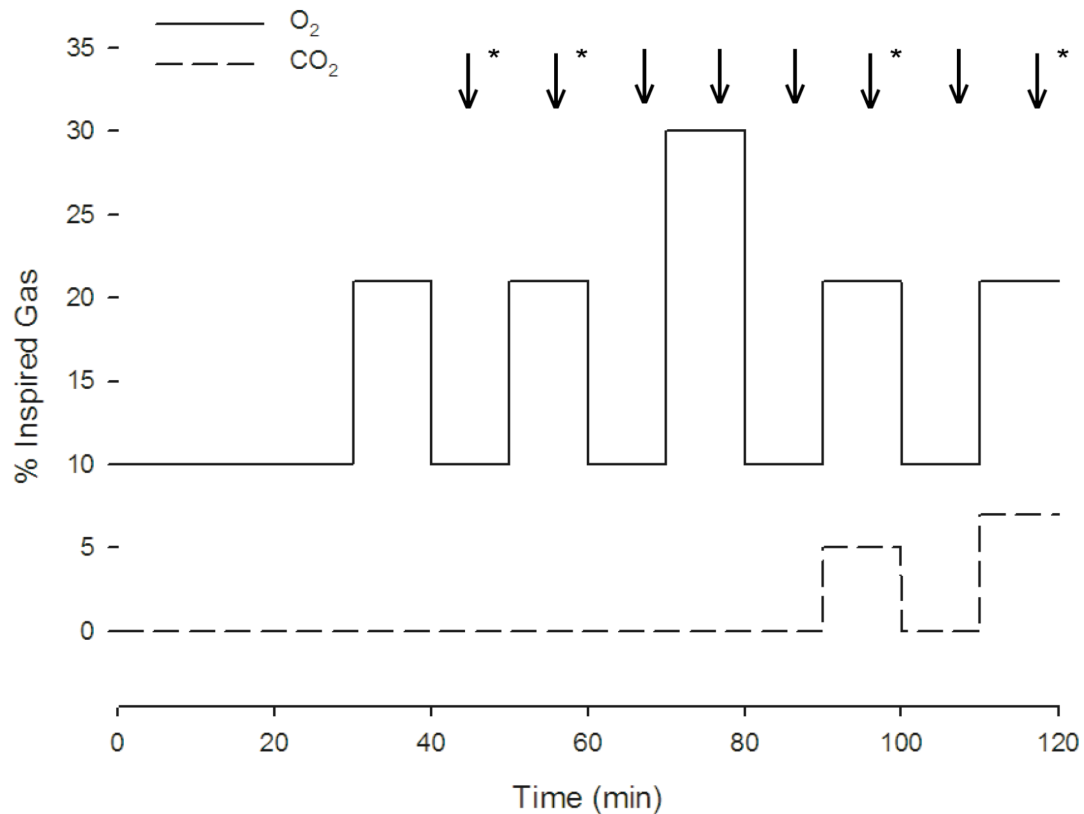

Figure 12: Plethysmograph Data Collection Protocol for Chronically Hypoxic Rats. O_2 and CO_2 were changed as shown and the balance was N_2. The first 40 min of the protocol acclimates the rat to the plethysmograph. Arrows indicate ventilation measurements made between 10 and 15 minutes at a gas level time point. * indicates arterial blood gas sampling.

Arterial blood gases were measured immediately following the 1-minute collection of ventilatory data. Blood samples were taken no more than 4 times during the protocol, with a 0.2 mL sample taken each time. The samples were immediately analyzed on an Instrument Laboratory Synthesis GEM Premier 3000 blood gas machine (San Jose, CA), which returned arterial O_2 (Pa_{O_2}), CO_2 (Pa_{CO_2}), pH, and hematocrit (Hct) values.

3.3.7 Tissue Collection and Preparation

Following the last experimental measure, rats were transcardially perfused with 4% paraformaldehyde and the brainstems removed and post-fixed overnight in paraformaldehyde and then transferred to 30% sucrose and cryoprotected overnight. The brainstems were frozen and sectioned at 30 um using a cryostat (Reichert Jung, Cryocut 1800). The area containing the NTS was then stained using the floating sections method of immunohistochemistry for the NK1 receptor. Primary antibody was applied overnight in a concentration of 1:1000 (Advanced Targeting Systems), followed by 2 hours of secondary antibody application (goat anti-rabbit conjugated to Cy-3; Jackson Immunoresearch). All incubations were done at room temperature on a lab shaker. The sections were then placed on slides and coverslipped using Vectashield mounting media plus DAPI to counterstain the nuclei (Vector Laboratories).

3.3.8 Lesion Quantification

NK1R positive cells were counted in the NTS at four levels using the size of the Area Postrema as a landmark (-14.08, -13.8, -13.5, and -13.3 mm referenced to bregma). The most rostral section was outside of the targeted area. A subset of the sections was counted using a confocal microscope (Olympus FV-1000) where a 3D stack of images was collected and could be used to verify that NK1R immunoreactivity was surrounding a DAPI stained cell nucleus. These sections were also counted using a non-confocal fluorescent microscope (Nikon Eclipse E400). As the cell counts were not significantly different between the two methods, the non-confocal microscope was used for quantification in the majority of the animals. To be considered an NK1R neuron, the cell had to be at least 10 um large, have at least two processes, and the NK1 staining must surround a DAPI stained nuclei (criteria

modified from Potts et al., 2007). Any rat that had less than 30% of the NK1R positive neurons than the average control rat was included as a lesioned animal. No differences were observed between the number of NK1R positive cells in the control N and CH rats, so all control rats were pooled for analysis. All pictures shown of the staining were taken using the Olympus FV-1000 confocal microscope.

3.3.9 Statistics

For each ventilatory (f, V_T, and V_I) and blood gas (Pa_{O_2}, Pa_{CO_2}, pH, Hct) variable a Mixed Factors ANOVA was performed. The between subjects variables were the presence or absence of NK1R cell lesion and chronic hypoxia. When testing the effect on the hypercapnic ventilatory response, percent inspired CO_2 was the within subjects variable and percent inspired O_2 for the hypoxic ventilatory response. All averages are expressed ± the standard error of the mean and $p < 0.05$ was considered significant. All statistical analysis was done on SPSS statistics software and all graphs created using SigmaPlot

3.3 Results

3.3.1 Lesion Verification

In total, very few cell bodies in the NTS were stained for the NK1R. Most of the immunoreactivity was located on cell processes as illustrated in Figure 14C. In the three sections encompassing the target area of the caudal NTS, there was an average of 38.00 ± 1.65 cells. Only animals in which at least 70% of the NK1R positive cells were deleted, leaving less than 11 cells total, were included into the SP-SAP group

(mean of 7.25 ± 1.13 cells). Additionally, NK1R cell number at all levels of the caudal NTS was decreased as shown in Figure 13. Representative staining is shown in Figure 14. Rostral to the obex, no difference in NK1R cell number was observed, suggesting the lesion was localized to the caudal NTS as illustrated in Figure 15.

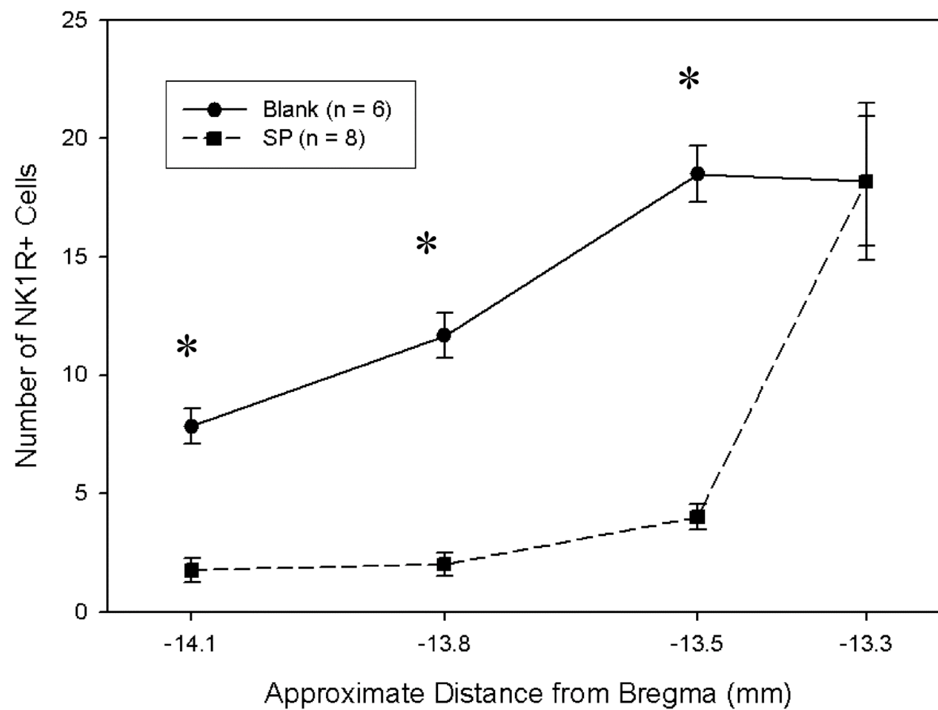

Figure 13: Number of NK1R Positive Cells at 4 Representative Areas of the NTS. The first 3 encompass the caudal NTS under the Area Postrema. The final segment is in the rostral NTS. All animals exhibited at least a 70% decrease in NK1R positive cells. * denotes $p < 0.05$.

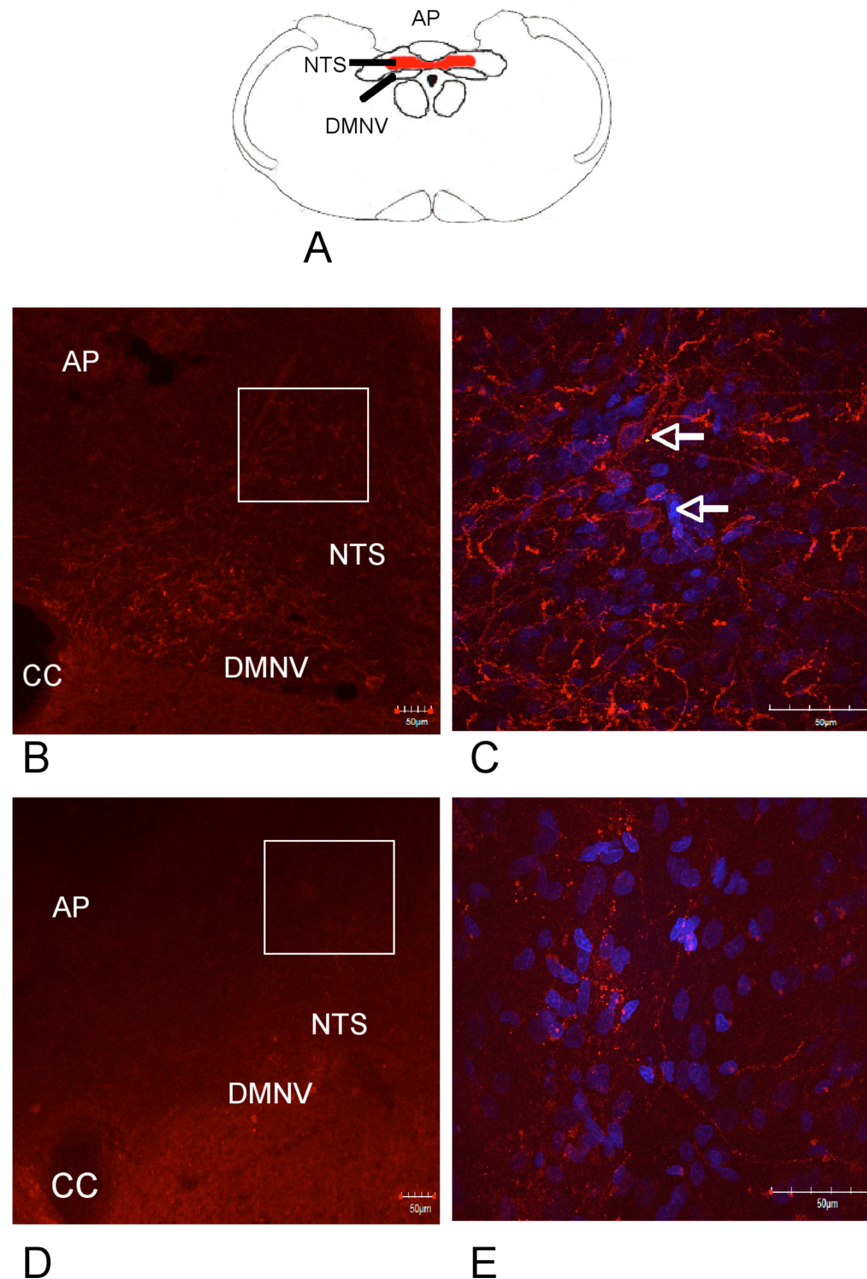

Figure 14: Histochemical Verification of NK1R Cell Lesion.
Immunohistochemistry was used to stain for the NK1R (red). Nuclei are stained with DAPI (blue) in the higher magnification images. A: Location, approximately -13.8 mm from Bregma. Red indicates the caudal NTS. B: Animal injected with control Blank-SAP. C: Higher magnification of caudal NTS of B. NK1R positive cell bodies (arrows) and processes present. D: Animal injected with SP-SAP. E. Higher magnification of caudal NTS of D. No NK1R cell bodies present, some processes present. CC = Central Canal; NTS = Nucleus Tractus Solitarius; DMNV = Dorsal Motor Nuclei of the Vagus; AP = Area Postrema.

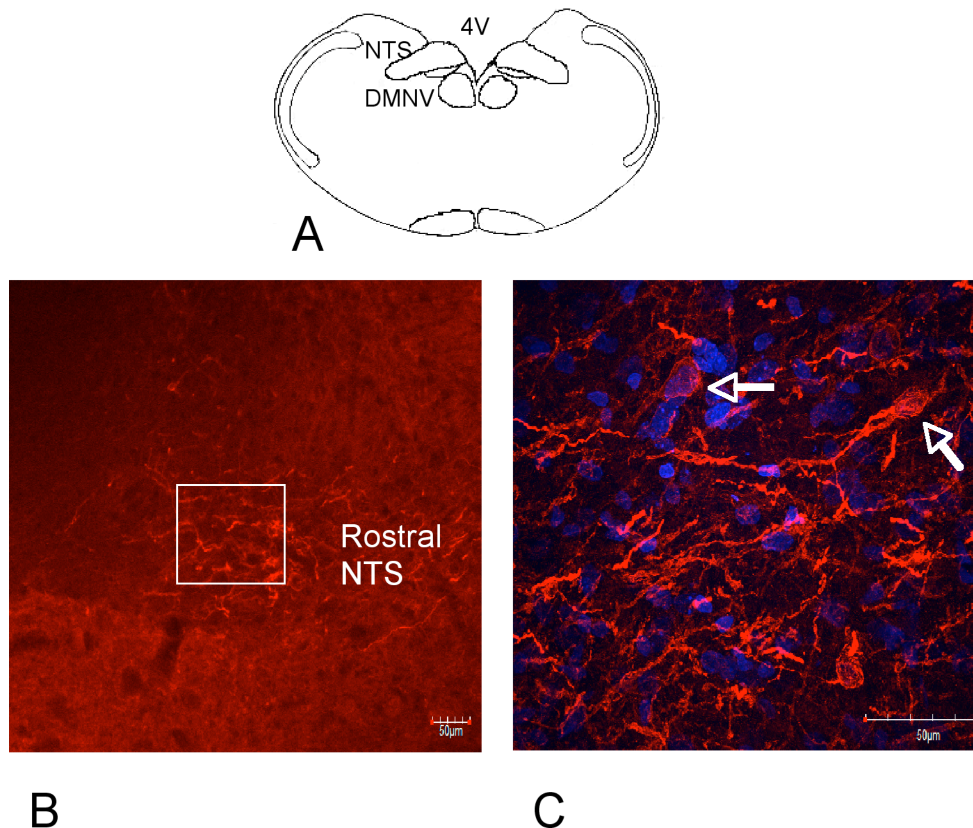

Figure 15: Presence of NK1R Immunoreactivity Rostral to Lesion Site. Immunohistochemistry was used to stain for the NK1R (red). Nuclei are stained with DAPI (blue) in the higher magnification images. A: Location, approximately -13.3 mm from Bregma. B: NTS Rostral to SP-SAP injection site. C: Higher magnification of B. NK1R positive cell bodies (arrows) and processes present. 4V = Fourth Ventricle; NTS = Nucleus Tractus Solitarius; DMNV = Dorsal Motor Nuclei of the Vagus.

3.3.2 Presence of Acclimatization in Chronically Hypoxic Rats

As expected, acclimatization was observed in the CH groups. Frequency, tidal volume, and ventilation were all significantly increased in the CH groups. Additionally Pa_{CO_2} was decreased (Table 1) and hematocrit increased in the CH rats (N = 36.17 ± 4.44; CH = 56.25 ± 3.12).

Table 1: Arterial Blood Gas Measurements During Hypercapnia and Hypoxia.
* denotes $p < 0.05$ from normoxic value. # denotes $p < 0.05$ from Blank value.

10% O_2			
	PO_2	***PCO_2***	***pH***
Blank N (n = 6)	38.17 ±1.70	26.58 ±1.14	7.59 ±0.01
SP N (n = 2)	38.00 ±2.0	23.50# ±0.50	7.63 ±0.00
Blank CH (n = 5)	44.80 ±2.31	20.34* ±1.76	7.51* ±0.01
SP CH (n = 5)	39.40 ±1.94	16.00*# ±0.71	7.52* ±0.01
21% O_2			
	PO_2	***PCO_2***	***pH***
Blank N (n = 6)	96.50 ± 2.88	34.77 ± 1.30	7.47 ± 0.00
SP N (n = 2)	99.50 ±0.50	32.00# ±4.00	7.48 ±0.00
Blank CH (n = 5)	107.25 ± 9.36	25.80* ±2.30	7.44* ±0.01
SP CH (n = 5)	101.40 ±1.94	21.20*# ±1.46	7.44* ±0.01
21% O_2 + 5% CO_2			
	PO_2	***PCO_2***	***pH***
Blank N (n = 5)	123.40 ±8.07	41.24 ±3.48	7.38 ±0.01
SP N (n = 2)	113.50 ±2.50	41.50 ±1.50	7.40 ±0.01
Blank CH (n = 5)	132.40 ±5.16	36.26* ±3.54	7.30* ±0.01
SP CH (n = 3)	120.67 ±3.76	38.00* ±1.15	7.27* ±0.01
21% O_2 + 7% CO_2			
	PO_2	***PCO_2***	***pH***
Blank N (n = 6)	120.00 ±6.10	52.53 ±0.83	7.32 ±0.01
SP N (n = 2)	126.00 ±2.00	51.50 ±0.50	7.33 ±0.02
Blank CH (n = 5)	124.75 ±7.57	45.35* ±2.98	7.24* ±0.02
SP CH (n = 5)	123.50 ±3.23	42.50* ±5.54	7.19* ±0.01

3.3.3 Effect of SP-SAP Lesion on Awake HCVR

Contrary to our hypothesis, the lesion had no significant effect on CO_2 sensitivity on any ventilatory variable in either normoxia or chronic hypoxia (Figure 16). Additionally, no differences were observed in arterial blood gas measurements between the drug groups (Table 1). There was a tendency for V_I to be decreased at the highest level of CO_2 in the N rats, but in chronic hypoxia there was a trend in the opposite direction. Although not significant, V_I tended to be greater after SSP-SAP lesion in CH rats because of a frequency effect.

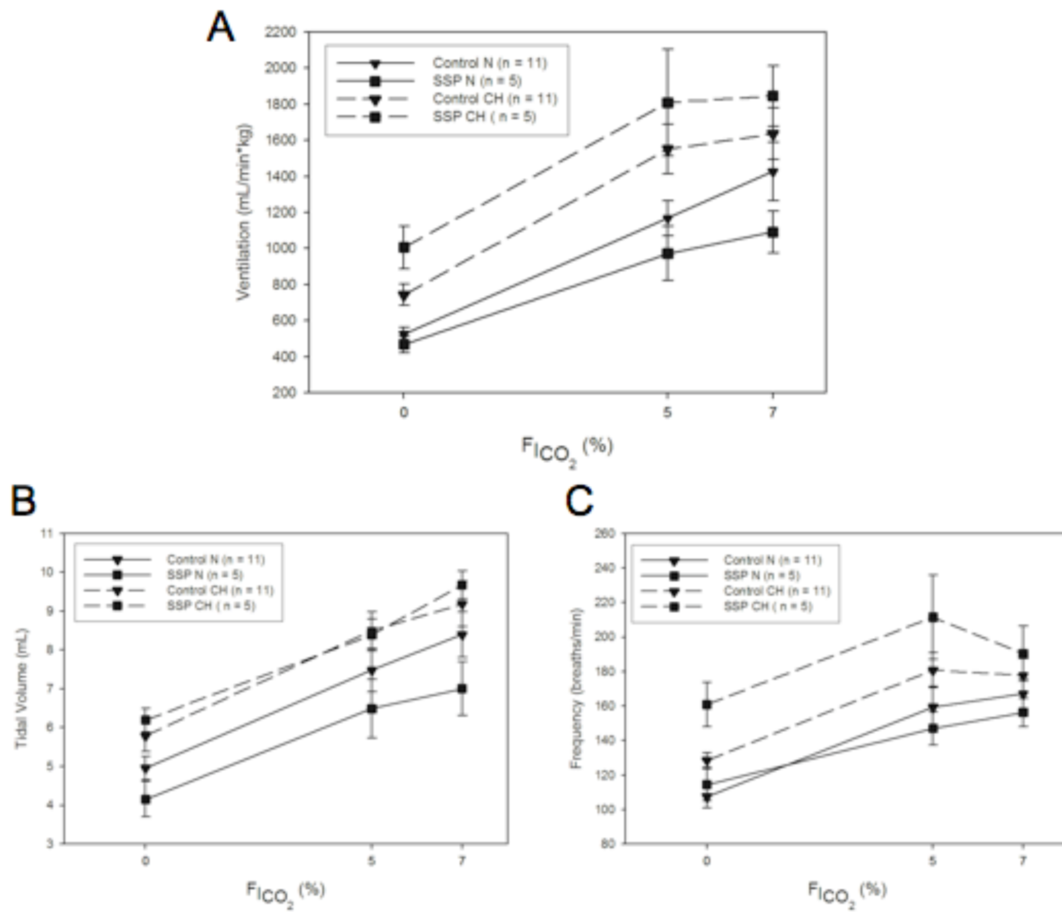

Figure 16: Effect of NK1R Cell Lesion on Awake HCVR. Effect of the NK1R cell lesion on the hypercapnic ventilatory response (HCVR) in normoxic (N) and chronically hypoxic (CH) rats. Stable SP-Saporin lesions (dashed line) in the caudal NTS had no effect on any component of the HCVR in N or CH. A. Ventilation B. Tidal Volume C. Frequency

3.3.4 Effect of SSP-SAP Lesion on Awake HVR

In contrast to the effect of NK1R cell deletion on the hypercapnic response, differences in the ventilatory response to hypoxia were observed, but only in chronic hypoxia. Ventilation in response to hypoxia was significantly increased in the chronically hypoxic SSP-SAP rats but not the normoxic SSP-SAP rats (Figure 17A), which was evidenced by a significant interaction between the drug and chronic hypoxia. This difference was driven by an increased frequency during chronic hypoxia in the lesioned animals (Figure 17C). No effect on tidal volume was observed (Figure 17B). The SP-SAP animals had a lower arterial CO_2 level, which was most pronounced in the chronically hypoxic animals which were also hyperventilating (Table 1).

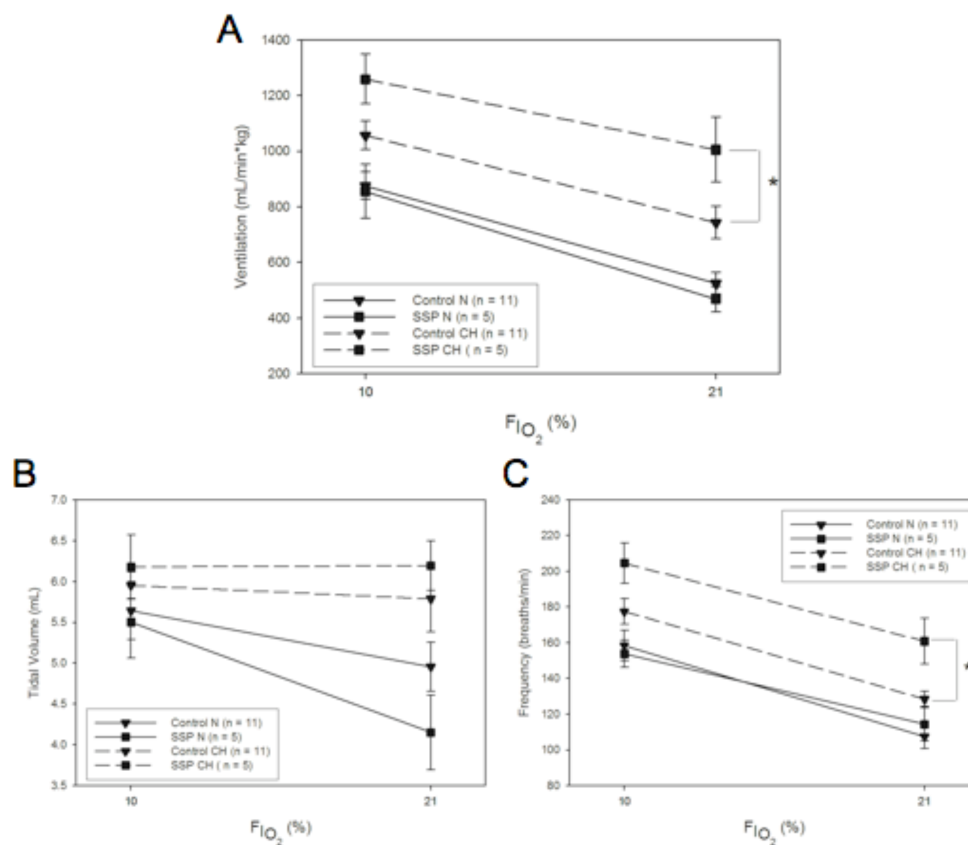

Figure 17: Effect of NK1R Cell Lesion on Awake HVR. Effect of the NK1R cell lesion on the hypercapnic ventilatory response (HVR) in normoxic (N) and chronically hypoxic (CH) rats. Stable SP-Saporin lesions (dashed line) in the caudal NTS had increased ventilation and frequency only in Chronic hypoxia. A. Ventilation B. Tidal Volume C. Frequency. * $p < 0.05$

3.4 Discussion

3.4.1 Summary of Results

Specific NK1R cell lesion in the NTS had no effect on room air, hypercapnic, or hypoxic ventilation in normoxic animals. In contrast, destruction of the NTS NK1R cells during chronic hypoxia resulted in hyperventilation during room air and hypoxic conditions. This hyperventilation was caused by an increased f in the lesioned animals with no effect on V_T. There was a non-significant trend for V_I at 5% CO_2 to be slightly higher in the lesioned CH animals as well.

3.4.2 Effect of the Lesion on the Normoxic Hypercapnic Ventilatory Response

The fact that NK1R cell destruction in the NTS does not diminish the HCVR in normoxia was unexpected. While it has not been determined whether the NK1R cells are chemosensitive, they are present in every site of central chemosensitivity (Nakaya et al., 1994) and lesions of the NK1R cells in both the RTN and medullary raphe have been shown to decrease the normoxic HCVR (Nattie & Li, 2002b; Nattie et al., 2004; Hodges et al., 2004).

There is some evidence that NK1R positive cells in the NTS may be involved in peripheral chemoreceptor circuits in the NTS. The carotid sinus nerve afferents release SP in the caudal NTS in response to hypoxia (Srinivasan et al., 1991), making it likely that the NK1R cells receive peripheral chemoreceptor input. In brain slices

SP increased the firing rate of all cells in the NTS it was applied to, but did not change the chemosensitivity index of any of the NTS chemoreceptor cells (Nichols, 2008). This suggests that SP neurotransmission is not necessary but may modulate CO_2-chemosensitivity in the NTS. The effect of SP on chemosensitivity in other areas has not been determined, but there is no strong evidence for a critical role of NK1R positive cells in the NTS in CO_2-chemosensitivity.

An alternative explanation for the lack of effect of NK1R cell lesion in the NTS on the normoxic hypercapnic response is that the lesion was not large enough. Focal inhibition in the NTS by muscimol does decrease the awake HCVR, but many more cells are affected by muscimol than the lesion used in this study (Nattie & Li, 2008). Very few cell bodies in the NTS are NK1R positive, especially compared with other areas like the RTN (Nattie & Li, 2002b). Animals were only included in the lesioned group if 70% or more of the NK1R positive cells had been killed. This was a larger percentage than necessary in the RTN (36 – 59%) to see a decreased HCVR (Nattie & Li, 2002b), but another group only saw an increase of the apneic threshold in anesthetized animals if 70% of the RTN cells that contain both Phox2B and the NK1R were killed (Takakura et al., 2008). It is therefore possible that even more of the NK1R cells would have to be killed in the NTS to see an effect on the HCVR in normoxia.

3.4.3 Effect of the Lesion in Chronic Hypoxia

Interestingly, the NK1R cell lesion in the NTS did cause hyperventilation in room air and hypoxic ventilation during chronic hypoxia but not in normoxia. This

suggests that the NK1R cells are more active during chronic hypoxia. In the CH lesioned rats, both V_I and f were higher during room air and 10% O_2 breathing. This is somewhat surprising as an increase in respiratory frequency is observed in rats and rabbits following microinjection of SP into the NTS (Chen et al., 1990; Yamamoto et al., 1981; Yamamoto & Lagercrantz, 1985). Since the NK1R is phosphorylated and internalized following ligand binding (Mantyh et al., 1995), there is the potential for desensitization. Five minutes of acute hypoxia (8.5% O2) led to a decrease in NK1R binding in the NTS, which recovered after 60 minutes in normoxia (Mazzone et al., 1997). The authors speculated that hypoxic ventilatory decline (HVD) is partially mediated by changes in SP neurotransmission and the decreased number of receptors on the cell surface. Whether SP neurotransmission undergoes changes during chronic hypoxia as well as in acute hypoxia and specifically if the NK1R are up or down regulated is unknown. Many receptors decrease their number or desensitize following sustained stimulation, but there is evidence in the dorsal horn of the spinal cord that during chronic pain, persistent stimulation causes an up-regulation of the NK1R (Ji et al., 2003). The number of cell bodies positive for the NK1R was unchanged during chronic hypoxia, but that does not preclude a change in receptor number or function during chronic hypoxia.

Another group has shown that NK1R cell lesions in the NTS abolish the depressive effect of somatosensory input on baroreceptor reflex function (Potts et al., 2007). Due to previous work showing that this same effect could also be produced by blocking $GABA_A$ or NK1 receptors (Boscan et al., 2002; Potts et al., 2003), they

postulated that their lesion was killing NK1R positive GABA interneurons. Large NK1R lesions in the NTS block the arterial chemoreflex mediated bradycardia, also suggesting an inhibitory set of NK1R positive neurons (Abdala et al., 2006). As the lesions that produce the inhibitory cardiovascular effects are in the same area as ours, the relevant cells killed in this study may also be GABA interneurons. This would be consistent with the observation that f is increased in the CH lesioned animals.

3.4.4 Potential Model

Our results suggest a role for a group of inhibitory NK1R cells in the NTS during chronic hypoxia. As deletion of the NK1R cells has no effect on normoxic ventilation, the sustained stimulation during chronic hypoxia may up-regulate their activity, something that does occur during chronic pain in the dorsal horn of the spinal cord (Ji et al., 2003). A role for the NK1R in processing peripheral chemoreceptor input is speculated because the carotid body afferents release SP in response to hypoxia (Srinivasan et al., 1991). Additionally, there were no significant changes in ventilation in the lesioned animals during a hypercapnic challenge, when presumably the central chemoreceptors were also activated. To determine if this model is correct, a better understanding of the connectivity of the NK1R cells is necessary, especially whether the peripheral chemoreceptor afferents synapse onto the NK1R cells.

The NK1R and GABA co-localize in the NTS (Potts et al., 2007; Potts, 2006; Boscan et al., 2002; Chen et al., 2009; Potts et al., 1999), but in chronically hypoxic rats there is evidence GABA may be excitatory. Inhibiting GABA neurotransmission during room air breathing in chronic hypoxia actually decreases instead of increases

ventilation (Chung et al., 2006). Additionally, dissociated NTS neurons from CH rats exhibit a reduced sensitivity to $GABA_A$ receptor inhibition (Tolstykh et al., 2004). Not all GABA cells in the NTS contain the NK1R, so it is possible there are two populations of GABA neurons and the NK1R positive GABA cells are still inhibitory during chronic hypoxia.

The fact that the NK1R cells seem to be inhibiting ventilation during chronic hypoxia was unexpected. We were trying to find a mechanism for the increased ventilation during chronic hypoxia but have found a subset of cells that restrain ventilation. Because these cells have been shown to have roles in regulating cardiovascular reflexes, they might also play an important role in cardiovascular control during chronic hypoxia. Activation of the peripheral chemoreflex also activates sympathetic neural activity, which can cause vasoconstriction and increased blood pressure (Kara et al., 2003). Perhaps this population of cells is important in preventing sympathetic over-activation during chronic hypoxia. Blood pressure was not measured in our animals so the effect of the lesion on blood pressure during chronic hypoxia is unknown, although lesion of these cells during normoxia does not change resting blood pressure (Abdala et al., 2006).

In summary, NK1R lesion in the NTS induces hyperventilation during room air and hypoxia in CH rats by increasing respiratory frequency. Unlike similar lesion studies, there is no effect on the HCVR in normoxia or chronic hypoxia. We hypothesize that the NK1R cells in the NTS are inhibitory to breathing and undergo

plasticity to increase their activity during chronic hypoxia, although future studies are necessary to confirm this model.

3.4.5 Critique of Method

The targeted cell killing method in this study has been used by numerous other investigators (Abdala et al., 2006; Nattie & Li, 2006b; Hodges et al., 2004; Nattie & Li, 2002b; Nattie et al., 2004; Takakura et al., 2008; Potts et al., 2007) and has been shown to specifically kill the NK1R positive cells while not altering the total number of cells in the injection site. While the method is specific for cell type, we do not know what role the NK1R cells in the NTS play. In this study we were targeting CO_2-sensitive neurons, but the results argue against NK1R cells being involved in central chemoreception in the NTS. Although we were perhaps not studying what we originally aimed to, the results provide insight into the role of a subset of cells in the NTS during chronic hypoxia.

Additionally, many NK1R positive cells are located in the Dorsal Motor Nucleus of the Vagus (DMNV), which is directly ventral to the caudal NTS. In some animals the NK1R immunoreactivity in the DMNV was also decreased. This suggests some of the effects of the lesion could have been due to loss of DMNV NK1R cells. We believe most of the effects are likely due to the loss of the NTS neurons because that is the area that the peripheral chemoreceptor synapses occur.

Chapter 4: Discussion

4.1 Hypothesis Tested

During chronic hypoxia, a time dependent increase in ventilation (VAH) occurs that allows the animal to function during hypoxia. This hyperventilation increases Pa_{O_2} and lowers Pa_{CO_2}, effects that persist upon return to normoxia. Consequently, the chemical stimuli to breathe decreases over time in hypoxia and their changes cannot explain VAH. Therefore, the processing of these signals or motor systems must be changing. It has been well established that the carotid body becomes more sensitive to changes in O_2 during chronic hypoxia (Gonzalez et al., 1995; Nielsen et al., 1988). Changes in CNS processing also occur at later time points, but the mechanism and site of that plasticity has not yet been determined (Dwinell & Powell, 1999).

One of the hallmarks of VAH is a lowered Pa_{CO_2} and apneic threshold, which could result from a change in CO_2 sensing during chronic hypoxia. One of the earliest theories attempting to explain VAH posited that normalization of arterial and cerebrospinal pH could increase the drive to breathe from the central chemoreceptors (Severinghaus et al., 1963). Subsequent studies found that cerebrospinal pH normalization did not occur during chronic hypoxia (Forster et al., 1975), but few studies have investigated the possibility of central chemoreceptor plasticity during chronic hypoxia. Over the first 8 hrs of VAH, the slope of the hypercapnic ventilatory response increases in humans (Fatemian & Robbins, 2001). Following 5 days of

hypoxia, the slope of the HCVR returns to normal, although ventilation at all levels is increased in the chronically hypoxic subjects (Somogyi et al., 2005). Similarly to humans, we found the slope of the HCVR in rats exposed to 7 days of chronic hypoxia was the same as that of normoxic rats (Chapter 2). This suggests a time dependent change in CO_2-sensation during chronic hypoxia.

This dissertation tested the hypothesis that one of several sites of central CO_2-sensitivity, the Nucleus Tractus Solitarius (NTS) undergoes plasticity during chronic hypoxia. The NTS was studied because it receives the first synapse from the carotid body chemoreceptor afferents (Housley et al., 1987) and also contains CO_2-sensitive cells (Dean et al., 1989), making it an ideal site to integrate both O_2 and CO_2 information. As discussed below, stimulating CO_2-sensitive cells in the NTS and lesioning NK1R positive cells had different effects during chronic hypoxia than normoxia. This suggests a role for the NTS in VAH.

4.2 Summary of Findings

4.2.1 Response of CO_2-Sensitive Neurons in the NTS to Stimulation in Chronic Hypoxia

Focal stimulation of the CO_2-sensitive cells in the NTS by acetazolamide (ACZ) microinjection caused an increase in phrenic nerve activity, as shown by others (Coates et al., 1993). The magnitude of the response in normoxic (N) rats was lower than the phrenic response to 10% inspired CO_2. Stimulation of CO_2-sensitive cells in the NTS by focal acidosis produced a smaller phrenic response in chronically hypoxic (CH) animals than in the N controls (Figure 8). As baseline ventilation in CH rats is

higher than in N controls, we established that all animals still had the capacity to increase phrenic activity to an inspired CO_2 challenge following ACZ microinjection (Figure 9). Additionally, even when we corrected for the increased baseline seen in CH rats, the response to ACZ in the CH rats was still much less than that in the N rats (Figure 10).

This finding was unexpected as we hypothesized that an increased responsiveness to CO_2 from the NTS central chemoreceptor cells would contribute to the hyperventilation seen in VAH. Cellular data from our collaborators suggests a possible explanation for this result, though. A greater percentage of NTS cells from CH slices are inhibited by hypercapnia (27% vs. 14%), though there was not a significant difference in the total number of cells that responded to hypercapnia (N = 54%; CH = 62%; Nichols et al., 2009). The hypercapnia inhibited cells should be excited by lower levels of CO_2 and if they provide a direct excitation to breathe, could mediate the increased ventilation at the lower levels of CO_2 seen in chronic hypoxia (Figure 18B). The increase in the number of CO_2-inhibited cells could explain the decreased phrenic response to ACZ stimulation in the NTS during chronic hypoxia (Figure 18A). More studies are necessary to determine if this model is correct. The circuitry of the NTS central chemoreceptor cells is unknown, and the CO_2-inhibited cells could act not on ventilatory circuits, but could be controlling other functions, for instance cardiovascular responses during chronic hypoxia.

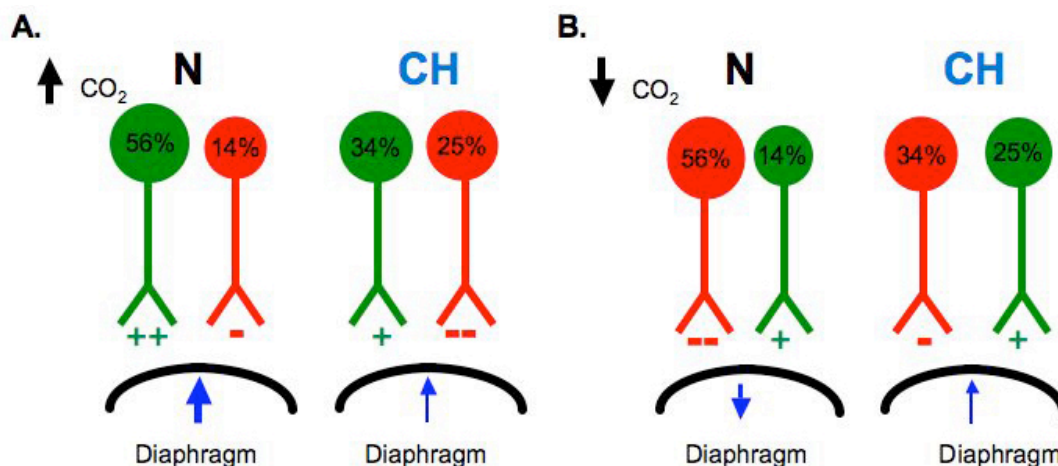

Figure 18: Model for Response of NTS Chemoreceptor Cells to Hypercapnia and Hypocapnia. Hypothesized response to NTS stimulation with **A** hypercapnia and **B** hypocapnia in chronically hypoxic (CH) and normoxic (N) rats. Cells excited by hypercapnia are assumed to be inhibited by hypocapnia and vice versus. This model can explain the decreased responsiveness of the CH rats to acetazolamide stimulation (**A**) as well as the increased drive to breath under hypocapnic conditions in the CH rats (**B**).

4.2.2 Effect of NTS Lesion on Ventilation in Normoxia and Chronic Hypoxia

Lesion of NK1R positive cells in the NTS had no effect on ventilation in N rats during either room air, hypercapnic, or hypoxic breathing. This result was somewhat surprising as NK1R cell lesion in the RTN and medullary raphe decreases the HCVR in awake, normoxic rats (Nattie & Li, 2002b; Nattie et al., 2004). In chronic hypoxia, the lesion caused hyperventilation during room air and hypoxic breathing, but had no significant effect on the HCVR. This suggests that the NK1R cells we killed are not involved in central CO_2-chemoreception as hypothesized, but are instead involved in peripheral chemoreceptor circuits. Alternatively, not enough cells were killed to see

an effect (McKay & Feldman, 2008; Takakura et al., 2008) or the redundancy of the other chemoreceptor sites masked the effect.

The idea that the NK1R sites are involved in peripheral chemoreceptor processing is consistent with the fact that carotid body afferents release the ligand for the NK1R, substance P (SP), during hypoxia (Srinivasan et al., 1991). As no effect of NK1R cell lesion was observed in normoxia, it is possible that constant stimulation during chronic hypoxia may induce plasticity in the NK1R cells. There is precedence for this in the dorsal horn of the spinal cord where constant SP release during chronic pain leads to central sensitization and up-regulation of the NK1R (Ji et al., 2003). No change in the number of NK1R positive cells was found with chronic hypoxia, but that does not preclude a change in the number of receptors found on each cell. More studies would be necessary to further explore a change in receptor number or function during chronic hypoxia.

Other investigators studying the effect of NK1R cell lesion on cardiovascular reflexes have suggested that the NK1R cells are GABA interneurons (Potts et al., 2007). As V_I and f increase in the NK1R lesioned CH rats, inhibition also seems to be removed in our system, though there is some evidence that GABA receptors act in an excitatory manner on ventilation during chronic hypoxia (Chung et al., 2006). Whether one population of NK1R cells in the NTS mediates both the cardiovascular and respiratory effects is unknown. More studies are necessary to determine whether NK1R cells do undergo plasticity during chronic hypoxia and their exact role in VAH.

We propose a model in which the NK1R cells receive direct excitatory input from carotid body afferents, which would provide a mechanism for their increased activity during chronic hypoxia (Figure 19). Additionally, as lesion of these cells inhibits ventilation, we hypothesize that the NK1R cells are inhibitory and influence ventilation through an unknown, but likely indirect pathway. As these cells also appear to be important in cardiovascular regulation (Abdala et al., 2006; Potts et al., 2007), an untested possibility is that these cells also inhibit sympathetic vasoconstriction during chronic hypoxia that would keep blood pressure from rising too dramatically.

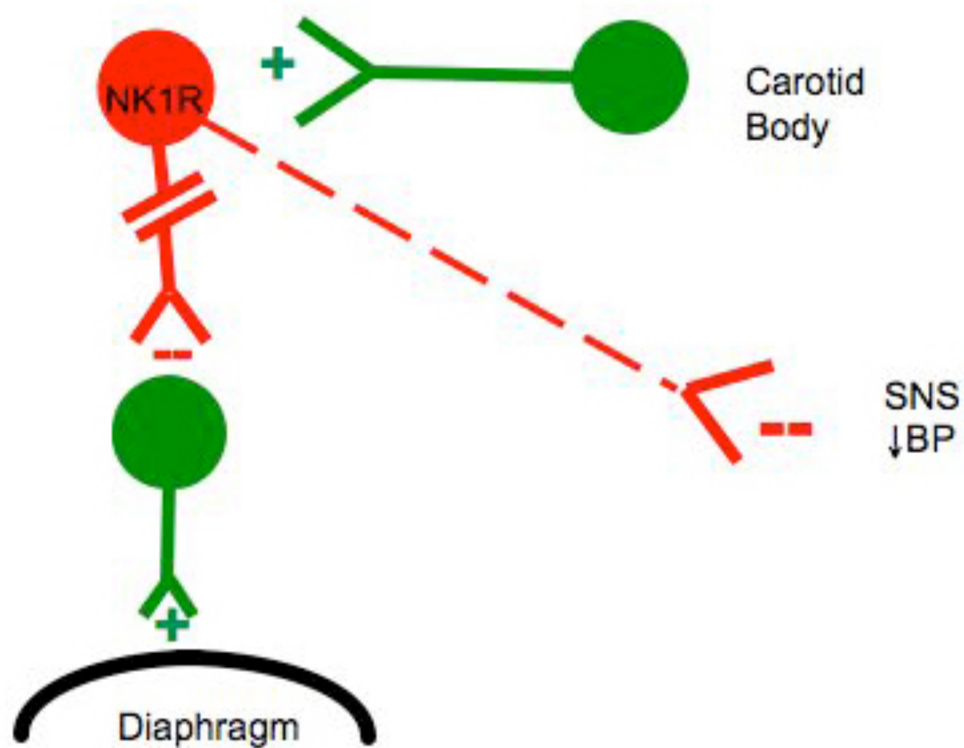

Figure 19: Proposed Model for the Role of Neurokinin 1 Receptor (NK1R) cells in the NTS. Hypothesized circuitry of NK1R cells in the Nucleus Tractus Solitarius (NTS). Carotid body afferents are hypothesized to excite inhibitory NK1R cells, which then inhibit ventilation as seen in our experiments. NK1R cells are also hypothesized to inhibit sympathetic nervous system (SNS) activity and potentially ensure that blood pressure (BP) does not rise too dramatically during chronic hypoxia.

4.3 Significance

The two experiments in this thesis have provided evidence for a role of the NTS in VAH. Specifically, CO_2-sensitivity in the NTS changes during chronic hypoxia, such that focal acidosis leads to a much smaller phrenic response during chronic

hypoxia. Additionally, lesion of the NK1R positive cells only affects ventilation during chronic hypoxia.

The experiments in Chapter 2 are the first to provide evidence in the whole animal that central chemoreceptors in a specific location change their stimulus-response properties during chronic hypoxia. This finding has potential implications for the mediation of the hyperventilation seen following the return to normoxia. The number of hypercapnia inhibited neurons in the NTS increases during chronic hypoxia (25% vs. 14%) and these cells are likely excited by the hypocapnia normally seen in chronic hypoxia (Nichols et al., 2009). The NTS chemoreceptor cells, while responding less to high CO_2 levels, may provide a drive to breathe during low CO_2 levels and contribute to the hyperventilation during VAH.

An unexpected role for the NK1R positive cells in the NTS was found. They were shown not to be important in the awake HCVR in contrast to NK1R cells at other sites of central chemosensitivity, such as the RTN (Hodges et al., 2004; Nattie & Li, 2002b; Nattie et al., 2004). A role for the NK1R cells in peripheral chemoreceptor processing during chronic hypoxia was suggested because CH but not N lesioned animals hyperventilated in room air and hypoxia. There was a non-significant trend for the CH lesioned animals to hyperventilate in hypercapnia, which is consistent with the fact that the central chemoreceptors are responsible for about two-thirds of the hypercapnic response (Smith et al., 2006). These results suggest plasticity in the NK1R cells during chronic hypoxia and that they inhibit ventilation during chronic hypoxia. As these cells have also been implicated in cardiovascular control (Abdala et

al., 2006; Potts et al., 2007), they may be important in controlling blood pressure and other cardiovascular functions during chronic hypoxia. Inhibiting the sympathetic chemoreflex activation during chronic hypoxia so that blood pressure is not increased too much is a potential role for these cells in addition to their inhibition of ventilation (Figure 19).

Although both sets of experiments were targeting the CO_2-sensitive neurons in the NTS, the evidence argues against the NK1R cells being the same population as the CO_2-sensitive neurons studied in Chapter 2. No significant differences in the hypercapnic ventilatory response were seen with NK1R cell lesion, which would be expected following the lesion of CO_2-sensitive cells. Additionally, lesion of the NK1R cells leads to hyperventilation during the return to normoxia in the chronically hypoxic animals, which is exactly the opposite of what would be predicted from our model if the NTS CO_2-sensitive neurons were deleted in the chronically hypoxic rat (Figure 18). It seems more likely that the NK1R positive cells are involved in the peripheral chemoreflex as hyperventilation was observed in room air and hypoxic conditions in the chronically hypoxic rats. More studies are necessary to positively rule out CO_2-sensitivity in the NK1R neurons.

4.4 Future Directions

Both findings for the role of NTS cells during chronic hypoxia were opposite to the hypothesized effects. Therefore much work is needed to fully elucidate the function of the CO_2-sensitive cells in the NTS and cells containing the NK1R in the NTS during VAH. One of the most important things to determine is the connectivity

in the NTS. If the peripheral chemoreceptors synapse onto a subset of the CO_2-sensitive cells, they could be driving the plasticity that switches the cell from being excited to inhibited by hypercapnia. Additionally, whether these cells provide a direct input to ventilation or another system, like the cardiovascular system, remains to be determined. What inputs the NK1R cells receive may help explain the plasticity they undergo during chronic hypoxia and which circuits they project to might explain why inhibition is increased during chronic hypoxia.

Another interesting question is whether the NTS is unique in its response to chronic hypoxia, especially in the plasticity that the CO_2-sensitive cells undergo. The response to inspired CO_2 is similar between N and CH rats (Chapter 2), but whether other central chemoreceptor areas also decrease their responsiveness to CO_2 is unknown. The RTN also receives input from hypoxia-activated cells in the NTS (Takakura et al., 2006) and therefore could also undergo similar plasticity.

In summary, this dissertation has established a role for the NTS in the development of VAH, but the exact mechanisms remain to be determined.

References

Aaron, E. A., & Powell, F. L. (1993). Effect of chronic hypoxia on hypoxic ventilatory response in awake rats. *J Appl Physiol, 74*, 1635-40.

Abdala, A. P., Schoorlemmer, G. H., & Colombari, E. (2006). Ablation of NK1 receptor bearing neurons in the nucleus of the solitary tract blunts cardiovascular reflexes in awake rats. *Brain Res, 1119*, 165-73.

Acker, H., & O'Regan, R. G. (1981). The effects of stimulation of autonomic nerves on carotid body blood flow in the cat. *J Physiol, 315*, 99-110.

Adams, J. M., Attinger, F. M., & Attinger, E. O. (1978). Medullary and carotid chemoreceptor interaction for mild stimuli. *Pflugers Arch, 374*, 39-45.

Akilesh, M. R., Kamper, M., Li, A., & Nattie, E. E. (1997). Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats. *J Appl Physiol, 82*, 469-79.

Alvarez-Buylla, R., & Roces de Alvarez-Buylla, E. (1994). Changes in blood glucose concentration in the carotid body-sinus modify brain glucose retention. *Brain Res, 654*, 167-70.

Baker, T. L., Fuller, D. D., Zabka, A. G., & Mitchell, G. S. (2001). Respiratory plasticity: differential actions of continuous and episodic hypoxia and hypercapnia. *Respir Physiol, 129*, 25-35.

Ballantyne, D., & Scheid, P. (2001). Central chemosensitivity of respiration: a brief overview. *Respir Physiol, 129*, 5-12.

Barnard, P., Andronikou, S., Pokorski, M., Smatresk, N., Mokashi, A., & Lahiri, S. (1987). Time-dependent effect of hypoxia on carotid body chemosensory function. *J Appl Physiol, 63*, 685-91.

Bellville, J. W., Whipp, B. J., Kaufman, R. D., Swanson, G. D., Aqleh, K. A., & Wiberg, D. M. (1979). Central and peripheral chemoreflex loop gain in normal and carotid body-resected subjects. *J Appl Physiol, 46*, 843-53.

Bernard, D. G., Li, A., & Nattie, E. E. (1996). Evidence for central chemoreception in the midline raphé. *J Appl Physiol, 80*, 108-15.

Biancardi, V., Bícego, K. C., Almeida, M. C., & Gargaglioni, L. H. (2008). Locus coeruleus noradrenergic neurons and CO2 drive to breathing. *Pflugers Arch, 455*, 1119-28.

Bickler, P. E., Litt, L., Banville, D. L., & Severinghaus, J. W. (1988). Effects of acetazolamide on cerebral acid-base balance. *J Appl Physiol, 65*, 422-7.

Biscoe, T. J., Bradley, G. W., & Purves, M. J. (1970). The relation between carotid body chemoreceptor discharge, carotid sinus pressure and carotid body venous flow. *J Physiol, 208*, 99-120.

Bisgard, G. E., Busch, M. A., Daristotle, L., Berssenbrugge, A. D., & Forster, H. V. (1986). Carotid body hypercapnia does not elicit ventilatory acclimatization in goats. *Respir Physiol, 65*, 113-25.

Boscan, P., Kasparov, S., & Paton, J. F. (2002). Somatic nociception activates NK1 receptors in the nucleus tractus solitarii to attenuate the baroreceptor cardiac reflex. *Eur J Neurosci, 16*, 907-20.

Bouverot, P., Candas, V., & Libert, J. P. (1973). Role of the arterial chemoreceptors in ventilatory adaptation to hypoxia of awake dogs and rabbits. *Respir Physiol, 17*, 209-19.

Bradley, S. R., Pieribone, V. A., Wang, W., Severson, C. A., Jacobs, R. A., & Richerson, G. B. (2002). Chemosensitive serotonergic neurons are closely associated with large medullary arteries. *Nat Neurosci, 5*, 401-2.

Busch, M. A., Bisgard, G. E., & Forster, H. V. (1985). Ventilatory acclimatization to hypoxia is not dependent on arterial hypoxemia. *J Appl Physiol, 58*, 1874-80.

Carpenter, E., & Peers, C. (1997). Swelling- and cAMP-activated Cl- currents in isolated rat carotid body type I cells. *J Physiol, 503 (Pt 3)*, 497-511.

Chen, C. Y., Bechtold, A. G., Tabor, J., & Bonham, A. C. (2009). Exercise reduces GABA synaptic input onto nucleus tractus solitarii baroreceptor second-order neurons via NK1 receptor internalization in spontaneously hypertensive rats. *J Neurosci, 29*, 2754-61.

Chen, J., He, L., Dinger, B., & Fidone, S. (2000). Pharmacological effects of endothelin in rat carotid body. Activation of second messenger pathways and potentiation of chemoreceptor activity. *Adv Exp Med Biol, 475*, 517-25.

Chen, J., He, L., Dinger, B., Stensaas, L., & Fidone, S. (2002). Role of endothelin and endothelin A-type receptor in adaptation of the carotid body to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol, 282*, L1314-23.

Chen, J., He, L., Liu, X., Dinger, B., Stensaas, L., & Fidone, S. (2007). Effect of the endothelin receptor antagonist bosentan on chronic hypoxia-induced morphological and physiological changes in rat carotid body. *Am J Physiol Lung Cell Mol Physiol, 292*, L1257-62.

Chen, Y., Tipoe, G. L., Liong, E., Leung, S., Lam, S. Y., Iwase, R., et al. (2002). Chronic hypoxia enhances endothelin-1-induced intracellular calcium elevation in rat carotid body chemoreceptors and up-regulates ETA receptor expression. *Pflugers Arch, 443*, 565-73.

Chen, Z., Hedner, J., & Hedner, T. (1990). Substance P in the ventrolateral medulla oblongata regulates ventilatory responses. *J Appl Physiol, 68*, 2631-9.

Cherniack, N. S., von Euler, C., Homma, I., & Kao, F. F. (1979). Graded changes in central chemoceptor input by local temperature changes on the ventral surface of medulla. *J Physiol, 287*, 191-211.

Chung, S., Ivy, G. O., & Reid, S. G. (2006). GABA-mediated neurotransmission in the nucleus of the solitary tract alters resting ventilation following exposure to chronic hypoxia in conscious rats. *Am J Physiol Regul Integr Comp Physiol, 291*, R1449-56.

Clement, I. D., Bascom, D. A., Conway, J., Dorrington, K. L., O'Connor, D. F., Painter, R., et al. (1992). An assessment of central-peripheral ventilatory chemoreflex interaction in humans. *Respir Physiol, 88*, 87-100.

Clement, I. D., Pandit, J. J., Bascom, D. A., Dorrington, K. L., O'Connor, D. F., & Robbins, P. A. (1995). An assessment of central-peripheral ventilatory chemoreflex interaction using acid and bicarbonate infusions in humans. *J Physiol, 485 (Pt 2)*, 561-70.

Coates, E. L., Li, A. H., & Nattie, E. E. (1991). Acetazolamide on the ventral medulla of the cat increases phrenic output and delays the ventilatory response to CO2. *J Physiol, 441*, 433-51.

Coates, E. L., Li, A., & Nattie, E. E. (1993). Widespread sites of brain stem ventilatory chemoreceptors. *J Appl Physiol, 75*, 5-14.

Conrad, S. C., Nichols, N. L., Ritucci, N. A., Dean, J. B., & Putnam, R. W. (2009). Development of chemosensitivity in neurons from the nucleus tractus solitarii (NTS) of neonatal rats. *Respir Physiol Neurobiol, 166*, 4-12.

Dahan, A., DeGoede, J., Berkenbosch, A., & Olievier, I. C. (1990). The influence of oxygen on the ventilatory response to carbon dioxide in man. *J Physiol, 428*, 485-99.

Dahan, A., Nieuwenhuijs, D., & Teppema, L. (2007). Plasticity of central chemoreceptors: effect of bilateral carotid body resection on central CO2 sensitivity. *PLoS Med, 4*, e239.

Daristotle, L., & Bisgard, G. E. (1989). Central-peripheral chemoreceptor ventilatory interaction in awake goats. *Respir Physiol, 76*, 383-91.

Day, T. A., & Wilson, R. J. (2009). A negative interaction between brainstem and peripheral respiratory chemoreceptors modulates peripheral chemoreflex magnitude. *J Physiol, 587*, 883-96.

De Burgh Daly, M., Lambertsen, C. J., & Schweitzer, A. (1954). Observations on the volume of blood flow and oxygen utilization of the carotid body in the cat. *J Physiol, 125*, 67-89.

De Burgh Daly, M., Lambertsen, C. J., & Schweitzer, A. (1954). Observations on the volume of blood flow and oxygen utilization of the carotid body in the cat. *J Physiol, 125*, 67-89.

de Paula, P. M., Tolstykh, G., & Mifflin, S. (2007). Chronic intermittent hypoxia alters NMDA and AMPA-evoked currents in NTS neurons receiving carotid body chemoreceptor inputs. *Am J Physiol Regul Integr Comp Physiol, 292*, R2259-65.

Dean, J. B., & Reddy, R. B. (1995). Effects of intracellular dialysis on CO2/H+ chemosensitivity in brainstem neurons. In C. O. Trouth, R. M. Millis, H. F. Kiwull-Schone, & M. E. Schläfke (Eds.), *Ventral Brainstem Mechanisms and Control of Respiration and Blood Pressure* (pp. 453–461). New York: Dekker.

Dean, J. B., Lawing, W. L., & Millhorn, D. E. (1989). CO2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. *Exp Brain Res, 76*, 656-61.

Dempsey, J. A., & Forster, H. V. (1982). Mediation of Ventilatory Adaptations. *Physiol Rev, 62*, 262-346.

Dempsey, J. A., Forster, H. V., Bisgard, G. E., Chosy, L. W., Hanson, P. G., Kiorpes, A. L., et al. (1979). Role of cerebrospinal fluid [H+] in ventilatory deacclimatization from chronic hypoxia. *J Clin Invest, 64*, 199-205.

Doba, N., & Reis, D. J. (1973). Acute fulminating neurogenic hypertension produced by brainstem lesions in the rat. *Circ Res, 32*, 584-93.

Donoghue, S., Felder, R. B., Jordan, D., & Spyer, K. M. (1984). The central projections of carotid baroreceptors and chemoreceptors in the cat: a neurophysiological study. *J Physiol, 347*, 397-409.

Dwinell, M. R., & Powell, F. L. (1999). Chronic hypoxia enhances the phrenic nerve response to arterial chemoreceptor stimulation in anesthetized rats. *J Appl Physiol, 87*, 817-23.

Eyzaguirre, C., & Abudara, V. (1999). Carotid body glomus cells: chemical secretion and transmission (modulation?) across cell-nerve ending junctions. *Respir Physiol, 115*, 135-49.

Fatemian, M., & Robbins, P. A. (2001). Selected contribution: chemoreflex responses to CO2 before and after an 8-h exposure to hypoxia in humans. *J Appl Physiol, 90*, 1607-14; discussion 1606.

Fatemian, M., Nieuwenhuijs, D. J., Teppema, L. J., Meinesz, S., van der Mey, A. G., Dahan, A., et al. (2003). The respiratory response to carbon dioxide in humans with unilateral and bilateral resections of the carotid bodies. *J Physiol, 549*, 965-73.

Feldman, J. L., & McCrimmon, D. R. (2003). Neural Control of Breathing. In L. R. Squire, F. E. Bloom, S. D. McConnell, J. L. Roberts, N. C. Spitzer, & M. J. Zigmond (Eds.), *Fundamental Neuroscience* Amsterdam: Academic Press.

Fencl, V., Miller, T. B., & Pappenheimer, J. R. (1966). Studies on the respiratory response to disturbances of acid-base balance, with deductions concerning the ionic composition of cerebral interstitial fluid. *Am J Physiol, 210*, 459-72.

Forster, H. V., Bisgard, G. E., & Klein, J. P. (1981). Effect of peripheral chemoreceptor denervation on acclimatization of goats during hypoxia. *J Appl Physiol, 50*, 392-8.

Forster, H. V., Bisgard, G. E., Rasmussen, B., Orr, J. A., Buss, D. D., & Manohar, M. (1976). Ventilatory control in peripheral chemoreceptor-denervated ponies during chronic hypoxemia. *J Appl Physiol, 41*, 878-85.

Forster, H. V., Dempsey, J. A., & Chosy, L. W. (1975). Incomplete compensation of CSF [H+] in man during acclimatization to high altitude (48300 M). *J Appl Physiol, 38*, 1067-72.

Forster, H. V., Dempsey, J. A., Vidruk, E., & Do Pico, G. (1974). Evidence of altered regulation of ventilation during exposure to hypoxia. *Respir Physiol, 20*, 379-92.

Gonzalez, C., Dinger, B. G., & Fidone, S. J. (1995). Mechanisms of Carotid Body Chemoreception. In J. A. Dempsey, & A. I. Pack (Eds.), *Regulation of Breathing 2nd Edition* (pp. 391-472). New York: Marcel Dekker.

Guyenet, P. G., Bayliss, D. A., Mulkey, D. K., Stornetta, R. L., Moreira, T. S., & Takakura, A. T. (2008). The retrotrapezoid nucleus and central chemoreception. *Adv Exp Med Biol, 605*, 327-32.

He, L., Chen, J., Dinger, B., Stensaas, L., & Fidone, S. (2006). Effect of chronic hypoxia on purinergic synaptic transmission in rat carotid body. *J Appl Physiol, 100*, 157-62.

Hempleman, S. C. (1995). Sodium and potassium current in neonatal rat carotid body cells following chronic in vivo hypoxia. *Brain Res, 699*, 42-50.

Hempleman, S. C. (1996). Increased calcium current in carotid body glomus cells following in vivo acclimatization to chronic hypoxia. *J Neurophysiol, 76*, 1880-6.

Hodges, M. R., Opansky, C., Qian, B., Davis, S., Bonis, J., Bastasic, J., et al. (2004). Transient attenuation of CO2 sensitivity after neurotoxic lesions in the medullary raphe area of awake goats. *J Appl Physiol, 97*, 2236-47.

Housley, G. D., & Sinclair, J. D. (1988). Localization by kainic acid lesions of neurones transmitting the carotid chemoreceptor stimulus for respiration in rat. *J Physiol, 406*, 99-114.

Housley, G. D., Martin-Body, R. L., Dawson, N. J., & Sinclair, J. D. (1987). Brain stem projections of the glossopharyngeal nerve and its carotid sinus branch in the rat. *Neuroscience, 22*, 237-50.

Huey, K. A., Brown, I. P., Jordan, M. C., & Powell, F. L. (2000). Changes in dopamine D(2)-receptor modulation of the hypoxic ventilatory response with chronic hypoxia. *Respir Physiol, 123*, 177-87.

Iturriaga, R., & Alcayaga, J. (2004). Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. *Brain Res Brain Res Rev, 47*, 46-53.

Jacky, J. P. (1978). A plethysmograph for long-term measurements of ventilation in unrestrained animals. *J Appl Physiol, 45*, 644-7.

Jansen, A. H., Liu, P., Weisman, H., Chernick, V., & Nance, D. M. (1996). Effect of sinus denervation and vagotomy on c-fos expression in the nucleus tractus solitarius after exposure to CO2. *Pflugers Arch, 431*, 876-81.

Jarisch, A., Landgren, S., Neil, E., & Zotterman, Y. (1952). Impulse activity in the carotid sinus nerve following intra-carotid injection of potassium chloride, veratrine, sodium citrate, adenosine-triphosphate and alpha-dinitrophenol. *Acta Physiol Scand, 25*, 195-211.

Ji, R. R., Kohno, T., Moore, K. A., & Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci, 26*, 696-705.

Jordan, D., & Spyer, K. M. (1986). Brainstem integration of cardiovascular and pulmonary afferent activity. *Prog Brain Res, 67*, 295-314.

Kara, T., Narkiewicz, K., & Somers, V. K. (2003). Chemoreflexes--physiology and clinical implications. *Acta Physiol Scand, 177*, 377-84.

Kumar, P., & Bin-Jaliah, I. (2007). Adequate stimuli of the carotid body: more than an oxygen sensor? *Respir Physiol Neurobiol, 157*, 12-21.

Kusakabe, T., Powell, F. L., & Ellisman, M. H. (1993). Ultrastructure of the glomus cells in the carotid body of chronically hypoxic rats: with special reference to the similarity of amphibian glomus cells. *Anat Rec, 237*, 220-7.

Lai, Y. L., Lamm, J. E., & Hildebrandt, J. (1981). Ventilation during prolonged hypercapnia in the rat. *J Appl Physiol, 51*, 78-83.

Landgren, S., & Neil, E. (1951). Chemoreceptor impulse activity following haemorrhage. *Acta Physiol Scand, 23*, 158-67.

Leusen, I. R. (1954). Chemosensitivity of the respiratory center; influence of CO2 in the cerebral ventricles on respiration. *Am J Physiol, 176*, 39-44.

Li, A., Randall, M., & Nattie, E. E. (1999). CO(2) microdialysis in retrotrapezoid nucleus of the rat increases breathing in wakefulness but not in sleep. *J Appl Physiol, 87*, 910-9.

Linton, R. A., & Band, D. M. (1985). The effect of potassium on carotid chemoreceptor activity and ventilation in the cat. *Respir Physiol, 59*, 65-70.
Loeschcke, H. H. (1982). Central chemosensitivity and the reaction theory. *J Physiol, 332*, 1-24.

Loeschcke, H. H., Mitchell, R. A., Katsaros, B., Perkins, J. F., & Konig, A. (1963). Interaction of intracranial chemosensitivity with peripheral afferents to the respiratory centers. *Ann N Y Acad Sci, 109*, 651-60.

López-Barneo, J., del Toro, R., Levitsky, K. L., Chiara, M. D., & Ortega-Sáenz, P. (2004). Regulation of oxygen sensing by ion channels. *J Appl Physiol, 96*, 1187-95; discussion 1170-2.

Mantyh, P. W., DeMaster, E., Malhotra, A., Ghilardi, J. R., Rogers, S. D., Mantyh, C. R., et al. (1995). Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science, 268*, 1629-32.

Marchenko, V., & Sapru, H. N. (2000). Different patterns of respiratory and cardiovascular responses elicited by chemical stimulation of dorsal medulla in the rat. *Brain Res, 857*, 99-109.

Mazzone, S. B., Hinrichsen, C. F., & Geraghty, D. P. (1997). Substance P receptors in brain stem respiratory centers of the rat: regulation of NK1 receptors by hypoxia. *J Pharmacol Exp Ther, 282*, 1547-56.

McKay, L. C., & Feldman, J. L. (2008). Unilateral ablation of pre-Botzinger complex disrupts breathing during sleep but not wakefulness. *Am J Respir Crit Care Med, 178*, 89-95.

Millhorn, D. E., Eldridge, F. L., & Waldrop, T. G. (1982). Effects of medullary area I(s) cooling on respiratory response to chemoreceptor inputs. *Respir Physiol, 49*, 23-39.

Mills, L., & Nurse, C. (1993). Chronic hypoxia in vitro increases volume of dissociated carotid body chemoreceptors. *Neuroreport, 4*, 619-22.

Mitchell, R. A., Loeschcke, H. H., Massion, W. H., & Severinghaus, J. W. (1963). Respiratory responses mediated through superficial chemosensitive areas on the medulla. *J Appl Physiol, 18*, 519-22.

Mulkey, D. K., Stornetta, R. L., Weston, M. C., Simmons, J. R., Parker, A., Bayliss, D. A., et al. (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci, 7*, 1360-9.

Mulkey, D. K., Talley, E. M., Stornetta, R. L., Siegel, A. R., West, G. H., Chen, X., et al. (2007). TASK channels determine pH sensitivity in select respiratory neurons but do not contribute to central respiratory chemosensitivity. *J Neurosci, 27*, 14049-58.

Nakaya, Y., Kaneko, T., Shigemoto, R., Nakanishi, S., & Mizuno, N. (1994). Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat. *J Comp Neurol, 347*, 249-74.

Nattie, E. E., & Li, A. (1994). Retrotrapezoid nucleus lesions decrease phrenic activity and CO2 sensitivity in rats. *Respir Physiol, 97*, 63-77.

Nattie, E. E., & Li, A. (2001). CO2 dialysis in the medullary raphe of the rat increases ventilation in sleep. *J Appl Physiol, 90*, 1247-57.

Nattie, E. E., & Li, A. (2002). CO2 dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. *J Appl Physiol, 92*, 2119-30.

Nattie, E. E., & Li, A. (2002). Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity. *J Physiol, 544*, 603-16.

Nattie, E. E., Li, A., Mills, J., & Huang, Q. (1994). Retrotrapezoid nucleus muscarinic receptor subtypes localized by autoradiography. *Respir Physiol, 96*, 189-97.

Nattie, E. E., Li, A., Richerson, G., & Lappi, D. A. (2004). Medullary serotonergic neurones and adjacent neurones that express neurokinin-1 receptors are both involved in chemoreception in vivo. *J Physiol, 556*, 235-53.

Nattie, E., & Li, A. (2000). Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. *J Appl Physiol, 89*, 153-62.

Nattie, E., & Li, A. (2006). Central chemoreception 2005: a brief review. *Auton Neurosci, 126-127*, 332-8.

Nattie, E., & Li, A. (2006). Neurokinin-1 receptor-expressing neurons in the ventral medulla are essential for normal central and peripheral chemoreception in the conscious rat. *J Appl Physiol, 101*, 1596-606.

Nattie, E., & Li, A. (2008). Muscimol dialysis into the caudal aspect of the Nucleus tractus solitarii of conscious rats inhibits chemoreception. *Respir Physiol Neurobiol, 164*, 394-400.

Nichols, N. L., Wilkinson, K. A., Powell, F. L., Dean, J. B., & Putnam, R. W. (2009). Chronic Hypoxia Suppresses the CO2-response of Solitary Complex (SC) Neurons from Rats. *Respir Physiol Neurobiol,*

Nielsen, A. M., Bisgard, G. E., & Vidruk, E. H. (1988). Carotid chemoreceptor activity during acute and sustained hypoxia in goats. *J Appl Physiol, 65*, 1796-802.

Nishino, T., Mokashi, A., & Lahiri, S. (1982). Stimulation of carotid chemoreceptors and ventilation by doxapram in the cat. *J Appl Physiol, 52*, 1261-5.

Olson, E. B., & Dempsey, J. A. (1978). Rat as a model for humanlike ventilatory adaptation to chronic hypoxia. *J Appl Physiol, 44*, 763-9.

Olson, E. B., Vidruk, E. H., & Dempsey, J. A. (1988). Carotid body excision significantly changes ventilatory control in awake rats. *J Appl Physiol, 64*, 666-71.

Onimaru, H., & Homma, I. (2003). A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci, 23*, 1478-86.

Oyamada, Y., Ballantyne, D., Mückenhoff, K., & Scheid, P. (1998). Respiration-modulated membrane potential and chemosensitivity of locus coeruleus neurones in the in vitro brainstem-spinal cord of the neonatal rat. *J Physiol, 513 (Pt 2)*, 381-98.

Pan, L. G., Forster, H. V., Martino, P., Strecker, P. J., Beales, J., Serra, A., et al. (1998). Important role of carotid afferents in control of breathing. *J Appl Physiol, 85*, 1299-306.

Pappenheimer, J. R., Fencl, V., Heisy, S. R., & Held, D. (1965). Role of cerebral fluids in control of respiration as studied in unanesthetized goats. *Am J Physiol, 208*, 436-50.

Paxinos, & Watson (1982). *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press.

Pete, G., Mack, S. O., Haxhiu, M. A., Walbaum, S., & Gauda, E. B. (2002). CO(2)-induced c-Fos expression in brainstem preprotachykinin mRNA containing neurons. *Respir Physiol Neurobiol, 130*, 265-74.

Potts, J. T. (2006). Inhibitory neurotransmission in the nucleus tractus solitarii: implications for baroreflex resetting during exercise. *Exp Physiol, 91*, 59-72.

Potts, J. T., Fong, A. Y., Anguelov, P. I., Lee, S., McGovern, D., & Grias, I. (2007). Targeted deletion of neurokinin-1 receptor expressing nucleus tractus solitarii neurons precludes somatosensory depression of arterial baroreceptor-heart rate reflex. *Neuroscience, 145*, 1168-81.

Potts, J. T., Fuchs, I. E., Li, J., Leshnower, B., & Mitchell, J. H. (1999). Skeletal muscle afferent fibres release substance P in the nucleus tractus solitarii of anaesthetized cats. *J Physiol, 514 (Pt 3)*, 829-41.

Potts, J. T., Paton, J. F., Mitchell, J. H., Garry, M. G., Kline, G., Anguelov, P. T., et al. (2003). Contraction-sensitive skeletal muscle afferents inhibit arterial baroreceptor signalling in the nucleus of the solitary tract: role of intrinsic GABA interneurons. *Neuroscience, 119*, 201-14.

Powell, F. L., & Fu, Z. (2008). HIF-1 and ventilatory acclimatization to chronic hypoxia. *Respir Physiol Neurobiol, 164*, 282-7.

Powell, F. L., Milsom, W. K., & Mitchell, G. S. (1998). Time domains of the hypoxic ventilatory response. *Respir Physiol, 112*, 123-34.

Putnam, R. W., Filosa, J. A., & Ritucci, N. A. (2004). Cellular mechanisms involved in CO(2) and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol, 287*, C1493-526.

Rahn, H., & Otis, A. B. (1949). Man's respiratory response during and after acclimatization to high altitude. *Am J Physiol, 157*, 445-62.

Reid, S. G., & Powell, F. L. (2005). Effects of chronic hypoxia on MK-801-induced changes in the acute hypoxic ventilatory response. *J Appl Physiol, 99*, 2108-14.

Rekling, J. C., & Feldman, J. L. (1998). PreBötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu Rev Physiol, 60*, 385-405.

Richerson, G. B. (1995). Response to CO2 of neurons in the rostral ventral medulla in vitro. *J Neurophysiol, 73*, 933-44.

Richerson, G. B. (2004). Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci, 5*, 449-61.

Rigatto, H., Fitzgerald, S. C., Willis, M. A., & Yu, C. (1992). In search of the central respiratory neurons: II. Electrophysiologic studies of medullary fetal cells inherently sensitive to CO2 and low pH. *J Neurosci Res, 33*, 590-7.

Robbins, P. A. (1988). Evidence for interaction between the contributions to ventilation from the central and peripheral chemoreceptors in man. *J Physiol, 401*, 503-18.

Schlaefke, M. E., See, W. R., & Loeschcke, H. H. (1970). Ventilatory response to alterations of H+ ion concentration in small areas of the ventral medullary surface. *Respir Physiol, 10*, 198-212.

Severinghaus, J. W., & Carcelen, A. (1964). Cerebrospinal fluid in man native to high altitude. *J Appl Physiol, 19*, 319-21.

Severinghaus, J. W., Hamilton, F. N., & Cotev, S. (1969). Carbonic acid production and the role of carbonic anhydrase in decarboxylation in brain. *Biochem J, 114*, 703-5.

Severinghaus, J. W., Mitchell, R. A., Richardson, B. W., & Singer, M. M. (1963). Respiratory control at high altitude suggesting active transport regulation of CSF pH. *J Appl Physiol, 18*, 1155-66.

Smith, C. A., Bisgard, G. E., Nielsen, A. M., Daristotle, L., Kressin, N. A., Forster, H. V., et al. (1986). Carotid bodies are required for ventilatory acclimatization to chronic hypoxia. *J Appl Physiol, 60*, 1003-10.

Smith, C. A., Jameson, L. C., Mitchell, G. S., Musch, T. I., & Dempsey, J. A. (1984). Central-peripheral chemoreceptor interaction in awake cerebrospinal fluid-perfused goats. *J Appl Physiol, 56*, 1541-9.

Smith, C. A., Rodman, J. R., Chenuel, B. J., Henderson, K. S., & Dempsey, J. A. (2006). Response time and sensitivity of the ventilatory response to CO2 in unanesthetized intact dogs: central vs. peripheral chemoreceptors. *J Appl Physiol, 100*, 13-9.

Solomon, I. C. (2003). Focal CO2/H+ alters phrenic motor output response to chemical stimulation of cat pre-Botzinger complex in vivo. *J Appl Physiol, 94*, 2151-7.

Solomon, I. C., Edelman, N. H., & O'Neal, M. H. (2000). CO(2)/H(+) chemoreception in the cat pre-Bötzinger complex in vivo. *J Appl Physiol, 88*, 1996-2007.

Somogyi, R. B., Preiss, D., Vesely, A., Fisher, J. A., & Duffin, J. (2005). Changes in respiratory control after 5 days at altitude. *Respir Physiol Neurobiol, 145*, 41-52.

Srinivasan, M., Goiny, M., Pantaleo, T., Lagercrantz, H., Brodin, E., Runold, M., et al. (1991). Enhanced in vivo release of substance P in the nucleus tractus solitarii during hypoxia in the rabbit: role of peripheral input. *Brain Res, 546*, 211-6.

St Croix, C. M., Cunningham, D. A., & Paterson, D. H. (1996). Nature of the interaction between central and peripheral chemoreceptor drives in human subjects. *Can J Physiol Pharmacol, 74*, 640-6.

Stea, A., Jackson, A., & Nurse, C. A. (1992). Hypoxia and N6,O2'-dibutyryladenosine 3',5'-cyclic monophosphate, but not nerve growth factor, induce Na+ channels and

hypertrophy in chromaffin-like arterial chemoreceptors. *Proc Natl Acad Sci U S A, 89*, 9469-73.

Sved, A. F., Imaizumi, T., Talman, W. T., & Reis, D. J. (1985). Vasopressin contributes to hypertension caused by nucleus tractus solitarius lesions. *Hypertension, 7*, 262-7.

Takakura, A. C., Moreira, T. S., Colombari, E., West, G. H., Stornetta, R. L., & Guyenet, P. G. (2006). Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO2-sensitive neurons in rats. *J Physiol, 572*, 503-23.

Takakura, A. C., Moreira, T. S., Stornetta, R. L., West, G. H., Gwilt, J. M., & Guyenet, P. G. (2008). Selective lesion of retrotrapezoid Phox2b-expressing neurons raises the apnoeic threshold in rats. *J Physiol, 586*, 2975-91.

Tatsumi, K., Pickett, C. K., & Weil, J. V. (1995). Possible role of dopamine in ventilatory acclimatization to high altitude. *Respir Physiol, 99*, 63-73.

Teppema, L. J., Rochette, F., & Demedts, M. (1990). Effects of acetazolamide on medullary extracellular pH and PCO2 and on ventilation in peripherally chemodenervated cats. *Pflugers Arch, 415*, 519-25.

Tolstykh, G., Belugin, S., & Mifflin, S. (2004). Responses to GABA(A) receptor activation are altered in NTS neurons isolated from chronic hypoxic rats. *Brain Res, 1006*, 107-13.

Torrealba, F., & Claps, A. (1988). The carotid sinus connections: a WGA-HRP study in the cat. *Brain Res, 455*, 134-43.

van Beek, J. H., Berkenbosch, A., de Goede, J., & Olievier, C. N. (1984). Effects of brain stem hypoxaemia on the regulation of breathing. *Respir Physiol, 57*, 171-88.

Vardhan, A., Kachroo, A., & Sapru, H. N. (1993). Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. *Am J Physiol, 264*, R41-50.

Vizek, M., Pickett, C. K., & Weil, J. V. (1987). Increased carotid body hypoxic sensitivity during acclimatization to hypobaric hypoxia. *J Appl Physiol, 63*, 2403-10.

Wang, W., Bradley, S. R., & Richerson, G. B. (2002). Quantification of the response of rat medullary raphe neurones to independent changes in pH(o) and P(CO2). *J Physiol, 540*, 951-70.

Weil, J. V., Byrne-Quinn, E., Sodal, I. E., Friesen, W. O., Underhill, B., Filley, G. F., et al. (1970). Hypoxic ventilatory drive in normal man. *J Clin Invest, 49*, 1061-72.

Weizhen, N., Engwall, M. J., Daristotle, L., Pizarro, J., & Bisgard, G. E. (1992). Ventilatory effects of prolonged systemic (CNS) hypoxia in awake goats. *Respir Physiol, 87*, 37-48.

Wellner-Kienitz, M. C., & Shams, H. (1998). Hyperpolarization-activated inward currents contribute to spontaneous electrical activity and CO2/H+ sensitivity of cultivated neurons of fetal rat medulla. *Neuroscience, 87*, 109-21.

West, J. B. (1988). Rate of ventilatory acclimatization to extreme altitude. *Respir Physiol, 74*, 323-33.

Wiley, R. G., & Lappi, D. A. (1997). Destruction of neurokinin-1 receptor expressing cells in vitro and in vivo using substance P-saporin in rats. *Neurosci Lett, 230*, 97-100.

Xu, F., Zhang, Z., & Frazier, D. T. (2001). Microinjection of acetazolamide into the fastigial nucleus augments respiratory output in the rat. *J Appl Physiol, 91*, 2342-50.

Yamamoto, Y., & Lagercrantz, H. (1985). Some effects of substance P on central respiratory control in rabbit pups. *Acta Physiol Scand, 124*, 449-55.

Yamamoto, Y., Lagercrantz, H., & von Euler, C. (1981). Effects of substance P and TRH on ventilation and pattern of breathing in newborn rabbits. *Acta Physiol Scand, 113*, 541-3.