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The Effects of Chronic Hypoxia and Substance P on the Chemosensitive Response of Individual Nucleus Tractus Solitarius (NTS) Neurons from Adult Rats

Nicole L. Nichols
Wright State University

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THE EFFECTS OF CHRONIC HYPOXIA AND SUBSTANCE P ON THE
CHEMOSENSITIVE RESPONSE OF INDIVIDUAL NUCLEUS TRACTUS
SOLITARIUS (NTS) NEURONS FROM ADULT RATS

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy (Biomedical Sciences)

By

NICOLE LOUISE NICHOLS
B.S., Otterbein College, 2003

2008
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY NICOLE LOUISE NICHOLS ENTITLED THE EFFECTS OF CHRONIC HYPOXIA AND SUBSTANCE P ON THE CHEMOSENSITIVE RESPONSE OF INDIVIDUAL NUCLEUS TRACTUS SOLITARIUS (NTS) NEURONS FROM ADULT RATS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

__________________________
Robert W. Putnam, Ph.D.
Dissertation Director

__________________________
Gerald M. Alter, Ph.D.
Director, Biomedical Sciences Ph.D. Program

__________________________
Joseph F. Thomas, Jr. Ph.D.
Dean, School of Graduate Studies

Signatures of Committee on Final Examination

__________________________
Robert W. Putnam, Ph.D.

__________________________
Francisco J. Alvarez, Ph.D.

__________________________
Jay B. Dean, Ph.D.

__________________________
David L. Goldstein, Ph.D.

__________________________
James E. Olson, Ph.D.
Nichols, Nicole Louise. Ph.D., Biomedical Sciences Program, Wright State University, 2008. The effects of chronic hypoxia and substance P on the chemosensitive response of individual nucleus tractus solitarius (NTS) neurons from adult rats.

The chemosensitive responses of individual nucleus tractus solitarius (NTS) neurons from neonatal rats have been extensively studied, but few studies have examined the chemosensitive responses of NTS neurons from adult rats. In addition, environmental conditions have been used to mimic respiratory diseases/disorders in rats to study how the cellular responses of individual neurons change to regulate breathing during pathological conditions. Lastly, it has been shown that substance P release increases in response to hypoxia from peripheral afferents that primarily terminate in the caudal NTS. We studied the effect of chronic hypoxia (CHx) and substance P on the response to hypercapnia of individual NTS neurons in control and CHx-adapted adult rats by simultaneously measuring the intracellular pH (pHi) and firing rate responses to hypercapnia using fluorescence imaging microscopy and the blind whole cell patch clamp technique. We found that NTS neurons from control adult rats have a lower steady state pH, similar intrinsic responses to hypercapnic acidosis, and a similar response to isohydric hypercapnia (decreased ΔpH and induced pH recovery) compared to neonates. In CHx rats, we found that the percentage of NTS neurons activated by hypercapnia decreased and the percentage that were inhibited by hypercapnia increased with no change in their magnitude of response as compared to NTS neurons from control rats. Additionally, these changes appear to be intrinsic. Substance P significantly increased
the basal firing rate and caused a significant decrease in steady state pHᵢ in NTS neurons in control and CHx-adapted animals, although the increase in firing rate caused by substance P was significantly smaller in CHx-adapted animals. In contrast, substance P had no effect on the percentage of neurons that respond or the magnitude of the response of NTS neurons to hypercapnic acidosis. In conclusion, intrinsic chemosensitivity established early in life for NTS neurons is maintained throughout adulthood, and is not dependent on substance P. Also, CHx causes a suppression of the adult chemosensitive response of NTS neurons that is not dependent on substance P. Finally, we have identified a model system to study the mechanism of how chemosensitive neurons are inhibited by hypercapnia.
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DEDICATION

I would like to dedicate this thesis to my parents Carolyn L. Nichols, Sidney A. Thorpe, and William F. Nichols because without them, I would not be where I am today. I would also like to dedicate this thesis to my brother William F. Nichols Jr., who will always be in my heart and whom I only hope is proud of what I have accomplished.
CHAPTER I

INTRODUCTION
Breathing is a complex physiological process that is regulated by chemoreceptors found centrally and peripherally. Peripheral chemoreceptors are found in the carotid bodies and aortic bodies and mostly sense O₂ (Nattie, 2006a; 2006b). Central chemoreceptors are located in several brainstem regions and are the predominant CO₂ sensors (Nattie, 2006a; 2006b). Central chemoreceptors contain CO₂-sensitive neurons, whose firing rate either increases, decreases, or does not change in response to acute hypercapnia (Putnam et al., 2004). Central chemoreceptors have mostly been studied in neonatal rats (Nattie, 2006b), and very few have been studied in adult rats (Dean et al., 1989; 1990). Specifically, we have found that nucleus tractus solitarius (NTS) neurons respond to hypercapnia with a maintained acidification as well as an increase in integrated firing rate. Hypercapnic-activated NTS neurons (40-50%) from neonates have a chemosensitivity index (for definition see page 8) of about 150% (Conrad et al., 2008). It is not known whether these chemosensitive properties of NTS neurons are maintained into adulthood, or show developmental changes. Thus, whether studies in neonates reflect studies in adults still remains a debate.

In addition, it is unknown how the activity of central chemoreceptors changes in diseases and disorders such as chronic obstructive pulmonary disease (COPD), congestive heart failure (CHF), and sleep apnea to regulate ventilation. For instance, studies have been conducted looking at the effect of chronic hypercapnia both at the whole animal level and cellular level (Nichols et al., 2008c; Putnam et al., 2008). It was found that there was a suppression of the whole animal ventilatory response to hypercapnia, but the chemosensitive response of NTS neurons was either increased or not changed (Nichols et al., 2008c; Putnam et al., 2008). A number of pathological
conditions, including the ones mentioned above, CHF and COPD, involve chronic hypoxic (CHx) states. In addition, studies have been conducted where the effect of CHx on the whole animal ventilatory response was studied (Aaron and Powell, 1993; Powell et al., 1998; Reid and Powell, 2005; Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998), but no cellular chemosensitive studies have been done. Additionally, it is known that peripheral chemoreceptor afferents terminate in the caudal NTS (Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003), and activity of these afferents increase in response to CHx (Powell, 2007). This begs the question of whether the response of NTS neurons are similarly activated by chronic exposure to hypoxia.

Lastly, the role of substance P on ventilation has been studied, and has been found to have an excitatory effect on ventilation. When the effect of substance P was inhibited, breathing was decreased. This implies that substance P must play an important role in modulating ventilation. Also, substance P release from the peripheral afferents is known to increase in response to hypoxia, implying that substance P will have an increased effect on NTS neurons. However, the effect of substance P on cellular chemosensitivity of NTS neurons has not been studied. In this thesis, I will be studying the effect of CHx and substance P on the chemosensitive response of individual NTS neurons from adult rats.
CHAPTER II

LITERATURE REVIEW
Control of Ventilation

Many physiological systems are regulated by higher centers in order to maintain homeostasis. Ventilation is a complex process that is controlled by structures located both peripherally and centrally, and is controlled by a negative feedback loop (Fig. 1). The negative feedback loop for the control of breathing is activated or inhibited when there are changes in the stimuli including $O_2$, $CO_2$ or extracellular pH ($pH_o$) which are detected by sensors called chemoreceptors (Fig. 1). The chemoreceptors will then send information to the central pattern generator which is believed to reside (at least in part) in the pre-Bötzinger region found in the brain, which patterns motor output to the effectors or the breathing muscles (diaphragm and intercostal muscles) to either increase or decrease breathing (Fig. 1). The activation of breathing muscles will then result in altered ventilation (either through changes in rate, tidal volume or both) and thereby increase or decrease the blood gases ($O_2$ and $CO_2$) to return blood gases and $pH_o$ to normal values (Fig. 1).

Chemoreceptors are located peripherally and centrally. The peripheral chemoreceptors are found in the aortic and carotid bodies and mainly sense changes in $O_2$, but have also been found to detect changes in $CO_2$ (Gonzalez et al., 1994; Nattie, 2006b). A central chemosensitive neuron will be defined as a neuron whose firing rate changes reversibly in response to a change of $CO_2$ and is found in a brainstem region that increases minute ventilation when focally acidified. Central chemoreceptors are found in multiple regions in the brainstem and mainly sense changes in $CO_2$ and $pHi$. In addition, neurons from the pre-Bötzinger complex (Ramirez et al., 1998; Solomon et al., 2000b), rostral ventrolateral medulla (VLM) (Nolan and Waldrop,
Figure 1:

**A.** Diagram of the structures proposed to be involved in the control of breathing. This feedback loop includes the central pattern generator (integrator). The integrator then sends information through efferents or motor neurons to the effectors or the breathing to either increase or decrease breathing. The breathing muscles will then increase or decrease the blood gases (O₂ and CO₂ which directly changes pHᵢ) via changing alveolar ventilation. Changes in O₂, CO₂ or pHᵢ (stimuli) are detected by sensors which are called chemoreceptors (both peripheral and central). The chemoreceptors will then send information back to the integrator via neural networks to reset breathing and to return the stimuli to their initial conditions. **B.** The integrator may be a medullary region known as the pre-Bötzinger complex. The effectors are the phrenic motor nerve (serving the diaphragm) and the intercostal nerves (serving the intercostal muscles). Peripheral chemoreceptors largely consist of the glomus cells of the carotid body (as well as the aortic bodies) and central chemoreceptors are believed to involve a distributed network of CO₂-sensitive neurons throughout the brainstem.
Figure 1:

A. 

B.
1993; Sun and Reis, 1994; Mazza et al., 2000) and from the nucleus tractus solitarius (NTS) (Pascual et al., 2002) have also been found to detect changes in O2. Chemoreceptors located centrally have been found in the NTS, ventrolateral medulla, retrotapezoid nucleus (RTN), medullary raphé, locus coeruleus, fastigial nucleus of the cerebellum, and the pre-Bötzingr region (Fig. 2) (Miles, 1983; Dean et al., 1989; 1990; Wellner-Kienitz and Shams, 1998; Mulkey et al., 2004b; Ritucci et al., 2005b; Richerson, 1995; Oyamada et al., 1998; Filosa et al., 2002; Solomon et al., 2000a; Martino et al., 2007). Chemosensitivity is defined in the same way in neurons from all regions. This involves measuring what percentage of neurons in a given region respond to hypercapnia and the magnitude to which they respond, calculated as chemosensitivity index (CI) (Wang and Richerson, 1999). Chemosensitive neurons have a fairly standard response to hypercapnia, exhibiting a maintained acidification during the hypercapnic exposure (Putnam et al., 2004; Ritucci et al., 1997) and either increased (Filosa et al., 2002; Ritucci et al., 2005b) or decreased (Richerson, 1995; Huang et al., 1997; Conrad et al., 2008) firing rates. Another standard definition of a chemosensitive neuron is that it must have an altered magnitude of firing rate in response to acute hypercapnia of at least 20% (either above or below control firing rate), as determined from calculations of the CI (100 X 10^(log[FR15−log FR5]0.2 / (pH5−pH15))) (Wang and Richerson, 1999), where pH5 and FR5 are the pHo and firing rate at 5% CO2, respectively, and pH15 and FR15 are the pHo and firing rate at 15% CO2, respectively. The CI is measured by the firing rate change in response to hypercapnia for a given change in pHo. As mentioned before, all neurons do not respond the same. Specifically, neurons from chemosensitive regions can be activated by hypercapnia, inhibited by hypercapnia, or not changed at all in response to hypercapnia.
Figure 2:

Sagittal view of the brainstem that represents proposed sites of central chemosensitivity that are found in the rat. Central chemoreceptors are found in multiple regions in the brainstem. Central chemoreceptors located centrally are depicted in gray and include the NTS, rostral ventral respiratory group (rVRG), retrotrapezoid nucleus (RTN), locus coeruleus (LC), and the fastigial nucleus of the cerebellum (FN). Notice the red circle in the caudal NTS, which is the area of the NTS known to play a role in ventilation. Areas not depicted in gray, but are known sites of central chemosensitivity include the medullary raphé and the pre-Bötzinger region. Other regions shown for landmarks include the nucleus ambiguus (AMB), pontine nucleus (Pn), superior olive (SO), facial nucleus (VII), and root of the facial nucleus (7N). Dashed vertical line demarcates the boundary between the pons (to the left) and the medulla (to the right).
Figure 2:
Most studies have looked at central chemosensitivity in neonates, and very few have studied neurons from adults (see Dean et al., 1989; 1990). Studies done in neonates have found that regions sensing CO₂ do not respond identically. For example, CO₂ activates most LC neurons (>80%), only about 50% of NTS neurons, and only 18% of medullary raphé neurons (Oyamada et al., 1998; Filosa et al., 2002, Conrad et al., 2008; Richerson, 1995). In addition, regions that respond to CO₂ do not respond with the same magnitude of change in firing rate (chemosensitivity index-CI; Wang and Richerson, 1999). For example, LC neurons have a low CI (125%; Hartzler et al., 2007; 2008) where RTN neurons have a very high CI (~250%; Ritucci et al., 2005b) in response to hypercapnia. As of now, it is not clear why there are so many areas that contain central chemoreceptors. It has been hypothesized this might be because: 1) redundancy, or 2) each area has a special function. Another possibility is that there may only be one area as recently suggested by a review that challenged the idea that there are multiple sites of central chemosensitivity (Guyenet et al., 2008). These authors proposed that the site of central chemosensitivity exists only in the RTN or the ventral medulla discounting other areas. In contrast, others (Nattie and Li, 2008) suggest that neurons from at least 3 areas play a critical role in central chemosensitivity including neurons from the LC, raphé, and RTN, which when focally acidified or ablated alter ventilation. Also, these areas contain chemosensitive neurons as determined by in vitro studies (Nattie and Li, 2006). For instance, ablation of the LC led to a 64% decrease in the ventilatory response to CO₂ in conscious animals. The NTS was initially suggested to be a site involved in central chemosensitivity by the work of Miles (1983), and it has further been suggested to be an important chemoreceptive site by the work of Dean et al. (1990) and Nattie and Li.
Clearly, there are multiple sites of chemoreception and the reason for multiple sites still remains a question.

One way chemosensitivity has been studied is to look at how ventilation changes over development. The developmental ventilatory response to inspired CO₂ has been studied at the whole animal level. It has been found that the whole animal ventilatory response is either triphasic or biphasic. The triphasic response consists of a high response early that wanes at P8-P10, that then increases to adult levels at P14 (Stunden et al., 2001). On the other hand, the biphasic response consists of a minimal response until around P14 that then increases to adult levels from P14-P18 or 19 (Davis et al., 2006). Regardless, both studies indicate a postnatal developmental change in the response to hypercapnia that switches to an adult form at or after P14. Cellular studies looking at the development of central chemosensitivity have been conducted in the medullary raphé, RTN, LC and the NTS. In the NTS, it has been found that neuronal responses to hypercapnia are fully intrinsic (not dependent on synaptic input) after P10 (Conrad et al., 2008), where as in LC neurons the chemosensitive response is intrinsic early followed by a decrease in the chemosensitive response (Hartzler et al., 2007). Central chemosensitivity is completely developed at birth and does not change over development in the RTN, although the intrinsic chemosensitivity was not studied (Ritucci et al., 2005b). Medullary raphé neurons have a low chemosensitive response early that then increases after P12, however intrinsic chemosensitivity was not studied (Wang and Richerson, 1999). However, none of these cellular studies of the development of the chemosensitive response correlate with the whole animal studies done looking at the developmental ventilatory response to CO₂. This indicates that either these
chemosensitive regions are not involved in the organismic ventilatory response, which is unlikely, or that network properties exist between these regions that require about 2-3 weeks to develop fully (Putnam et al., 2005).

Usually cells in the body exhibit recovery mechanisms from acidification. The normal pHᵢ response of neurons from chemosensitive regions to acute hypercapnia is a maintained acidification with pHᵢ returning to its initial value after a return to normocapnia (Ritucci et al., 1997; Wiemann et al., 1998; Nottingham et al., 2001). There is no recovery during the hypercapnic challenge because the Na⁺/H⁺ exchanger (NHE), the pHᵢ recovery mechanism following acidification in medullary neurons (Ritucci et al., 1997; Putnam, 2001), is inhibited by the extracellular acidification (Ritucci et al., 1998). The normal pHᵢ response to hypercapnia in neurons from non-chemosensitive regions is an acidification followed by alkalinizing pHᵢ recovery during maintained hypercapnia (Ritucci et al., 1997; Nottingham et al., 2001). When acute hypercapnia is removed, these neurons exhibit an alkalinizing overshoot, which is consistent with pHᵢ recovery during the acidifying hypercapnia (Boron and DeWeer, 1976).

It is thought that the mechanism for the response of neurons activated by hypercapnia involves a decrease in intracellular pH (pHᵢ) and/or a decrease in extracellular pH (pHₑₒ) (Putnam et al., 2004). Specifically, it is thought that the elevated CO₂ will cross the cell membrane, hydrate and rapidly dissociate into HCO₃⁻ and H⁺ (Putnam et al., 2004) (Fig. 3). The increase in H⁺ (decrease in pHᵢ and/or pHₑₒ) is thought to inhibit one or more K⁺ channels and depolarize the neuron, ultimately resulting in an increase in neuronal firing rate (Putnam et al., 2004). Likely candidates for K⁺ channels that are inhibited in response to hypercapnia include those that are sensitive to changes
Figure 3:
The classical model for a neuron that is activated by hypercapnic acidosis. It is thought that CO₂ crosses the cell membrane, hydrates, and rapidly dissociates into HCO₃⁻ and H⁺. The increase in H⁺ (decrease in pHᵢ and/or pHₒ) is thought to inhibit a K⁺ channel and depolarize the neuron to ultimately lead to an increase in neuronal firing rate. At the same time, there is a decrease in pHₒ that inhibits the NHE, which leads to a maintained acidification of the chemosensitive neuron.
Figure 3:

\[ \text{carbonic anhydrase} \]

\[ \text{CO}_2 \ + \ H_2O \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{HCO}_3^- \ + \ H^+ \]

\[ \text{K}^+ \text{ channel} \]

\[ \text{firing rate} \]
in pH (intracellular and/or extracellular) within the physiological range. These candidates for K\(^+\) channels include the inwardly rectifying K\(^+\) channel family (K\(_{IR}\)) (Schulte and Fakler, 2000; Xu et al., 2000), voltage-sensitive K\(^+\) channel family (K\(_{v}\)) (Berger et al., 1998), TWIK-related K\(^+\) channels (TREK) (Miller et al., 2004; Honoré, 2007), and TWIK-related acid-sensitive K\(^+\) channels (TASK) (Bayliss et al., 2001; Duprat et al., 1997).

The mechanism that decreases the firing rates of some chemosensitive neurons during hypercapnia is unknown. It is clear that inhibited neurons must elaborate different ion channels than activated neurons. In addition, we do not know the role of inhibited channels in chemoreception and control of ventilation. It may be that the mechanism for inhibited neurons also includes a decrease in pH\(_i\) and decrease in pH\(_o\), but not activates different K\(^+\) channels to lead to a hyperpolarization and ultimately decreased firing rate. One possible candidate for a K\(^+\) channel that may get activated in response to hypercapnia is the calcium-activated K\(^+\) channel (specifically BK) (Wellner-Kienitz et al., 1998). This channel is directly activated by elevated intracellular Ca\(^{2+}\), and it has been found that Ca\(^{2+}\) can be increased in response to hypercapnia (Filosa and Putnam, 2003; Putnam et al., 2004). Thus, activation of BK channels may lead to hyperpolarization of the membrane and inhibit firing rate. Another K\(^+\) channel that may be involved is TREK1. It has been found that TREK1 channels are activated by decreased pH\(_i\) (Honoré, 2007), thus it can be hypothesized that activation of TREK1 channels leads to hyperpolarization and decreased firing rate.

An important question is what is the necessary signal required for neurons to be activated by hypercapnia? The candidates for signals that have been studied in depth so
far include a decrease in pH, decrease in pHo, or CO2 itself. To examine the role of these signals, one study used solutions that manipulated one or more of these signals (Fig. 4) and then measured the response of firing rate of LC neurons from neonates (Filosa et al., 2002). These solutions included isohydric hypercapnia (increased CO2 and decreased pH with no change in pHo), isocapnic acidosis (decreased pH and decreased pHo with no change in CO2), acidified HEPES (decreased pH and pHo, with no CO2 added), propionate (decreased pH with no change in CO2 or pHo), and hypercapnic acidosis (decreased pH, decreased pHo and increased CO2). Isohydric hypercapnia specifically examined the role of pHo and it was found that although there was a smaller acidification with pH recovery (since NHE exchange is no longer inhibited by decreased pHo), there was still an increase in firing rate (Filosa et al., 2002) (Fig. 5). Isocapnic acidosis specifically examined the role of CO2 and it was found that although there was no change in CO2, there was still an increase in firing rate (Filosa et al., 2002) (Fig. 5). Acidified HEPES also specifically examined the role of CO2 and it was found that the firing rate increased even more compared to solutions that had CO2 present (Filosa et al., 2002) (Fig. 5). Propionate specifically looked at the role of CO2 and pHo together and found that firing rate increased very little (Filosa et al., 2002) (Fig. 5). Hypercapnic acidosis specifically looked at the role of all three signals and found that there was a maintained acidification and an increase in firing rate (Filosa et al., 2002) (Fig. 5). These results showed a positive correlation between the degree of acidification and the magnitude of increased firing rate (Fig. 5), indicating that a change of pH might be a necessary chemosensitive signal. However, the correlation was not perfect. As depicted by the red arrows, very different changes in pH resulted in a similar change in firing rate caused by
Figure 4:

A summary of the solutions used to manipulate one or more of the signals proposed to change firing rate of chemosensitive neurons that were used by Filosa et al. (2002). These solutions included hypercapnic acidosis (decreased pH$_i$, decreased pH$_o$ and increased CO$_2$), isohydric hypercapnia (increased CO$_2$ and decreased pH$_i$ with no change in pH$_o$), acidified HEPES (decreased pH$_i$ and pH$_o$ with no CO$_2$ added), propionate (decreased pH$_i$ with no change in CO$_2$ or pH$_o$), and isocapnic acidosis (decreased pH$_i$ and decreased pH$_o$ with no change in CO$_2$).
Figure 4:

<table>
<thead>
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<th></th>
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<th>$\text{CO}_2$</th>
<th>$\text{pH}_i$</th>
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<tbody>
<tr>
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<td>↑</td>
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<tr>
<td>ISOHYDRIC HYPERCAPNIA</td>
<td>—</td>
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<tr>
<td>ACIDIFIED HEPES</td>
<td>↓</td>
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<tr>
<td>PROPIONATE</td>
<td>—</td>
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<tr>
<td>ISOCAPNIC ACIDOSIS</td>
<td>↓</td>
<td>—</td>
<td>↓ SLOW</td>
</tr>
</tbody>
</table>
Figure 5:

Summary of the findings of Filosa et al. (2002) where solutions were used to manipulate one or more of the signals proposed to change firing rate of LC neurons.
Figure 5:

\[ Y = 7.47X - 0.67 \]
\[ r = 0.87 \]

both isohydrmic hypercapnia and isocapnic acidosis (Fig. 5). Further, as depicted by the blue arrows, two solutions have a similar change in pH\(_{i}\) caused by isohydrmic hypercapnia and propionate, but these solutions caused very different changes in firing rate (Fig. 5). This suggests that a change in pH\(_{i}\) maybe an adequate signal to change firing rate, but is not a necessary signal to change firing rate. However, notice that none of these solutions looked at the effect of not changing pH\(_{i}\) while changing CO\(_{2}\) and/or pH\(_{o}\). Recently, a technique was developed where pH\(_{i}\) can be clamped while exposing a neuron to hypercapnia in order to measure the role of pH\(_{i}\) specifically in chemosensitive signaling (Hartzler et al., 2008). It was found that when pH\(_{i}\) was held constant, there was still a firing rate change induced by hypercapnia (Hartzler et al, 2008). In conclusion, none of the studied signals thus far appears to be a necessary signal for a response to hypercapnia, since firing rate still responds when one or more signals are manipulated. Thus, a multiple factors theory has been developed that includes multiple signals (\(\Delta\)pH\(_{i}\), \(\Delta\)pH\(_{o}\), \(\Delta\)Ca\(^{2+}\), etc.) affecting multiple ion channels in order to change firing rate (Filosa and Putnam, 2003; Putnam et al., 2004) (Fig. 6). In addition, these factors could be different among the different chemosensitive regions such that the percentage of sensitive neurons and CI varies among these regions.

For example, 30-50% of caudal NTS neurons are CO\(_{2}\)-excited where as about 80-90% of locus coeruleus neurons are CO\(_{2}\)-excited (Huang et al., 1997; Conrad et al., 2008; Filosa et al., 2002; Oyamada et al., 1998). In addition, neurons from different regions have different values for their CI. For example, NTS neurons have a value of CI of about 150% whereas LC neurons have a value of about 125% (Conrad et al., 2008; Filosa et al., 2002). These differences may be due to different regions contributing to ventilatory
Figure 6:

The multiple factors model of chemosensitive signaling that has been proposed for a change in firing rate in response to hypercapnic acidosis. In this model, an acid stimulus such as hypercapnic acidosis (HA), results in multiple signals, including decreased pH$_i$, decreased pH$_o$, and increased CO$_2$. These signals are proposed to affect multiple ion channel targets, including various K$^+$ channels (TASK and tetraethylammonium (TEA)-sensitive K$^+$ channels) as well as Ca$^{2+}$ channels. It is proposed that the overall effect of these multiple signals affecting multiple channels may determine the magnitude of the increase in firing rate of the chemosensitive neuron.
Multiple Factors Model of Chemosensitive Signaling

Stimulus:

Signals:

Targets:

Response:

control in different states (e.g. sleep vs. awake vs. anesthetized). Specifically, it has been found that focal acidification causes a chemoreceptor response that was specific to wakefulness in the RTN (Li et al., 1999), specific to NREM sleep in the medullary raphé (Nattie and Li, 2001), and was present in both wakefulness and sleep in the caudal NTS (Nattie and Li, 2002a). It is not even clear what aspect of a chemosensitive region is most important for impacting the respiratory network, the CI of individual neurons or the percentage of neurons in the region that are chemosensitive. For instance, only half of the neurons from the NTS are CO₂ excited but individual neurons have a CI of about 150%, whereas nearly all LC neurons are CO₂ excited but they have a CI of only 125%. The output of CS neurons is believed to affect the neurons that set the respiratory rhythm (pre-Bötzinger neurons, Mulkey et al., 2004b; Shao and Feldman, 2005) which in turn control the major muscle of breathing (phrenic motor nerve serving the diaphragm, Yokota et al., 2001). Thus, chemosensitive neuronal activation by hypercapnia can lead to a homeostatic increase in ventilation, resulting in a return to normocapnia.

Since it is known that regions involved in central chemosensitivity receive input from other areas, studies have been done to measure whether or not the chemosensitive response of neurons within these regions is intrinsic or not. One way to study the intrinsic response is to look at the impact of chemical transmission. The solution that has been popularly used is synaptic blockade solution (increased Mg²⁺ and low Ca²⁺) (Dean et al., 1990; Dean et al., 1997, Huang et al., 1997; Hartzler et al., 2007; Conrad et al., 2008). This solution is hypothesized to block the presynaptic Ca²⁺ influx and thus block the release of neurotransmitters from vesicles. Another way to block chemical
transmission is to use a cocktail of receptor antagonists for neurotransmitters that are known to affect the region of interest (Mulkey et al., 2006; 2007). In addition, these synaptic blockade solutions also reveal what the tonic input is to the region of interest *in vitro*. In the LC, it has been shown that synaptic blockade solution causes basal firing rate to increase, which suggests that individual LC neurons receive tonic inhibitory input. NTS neurons from neonates have a decrease in firing rate when exposed to synaptic blockade solution, which suggest they receive tonic excitatory input. Synaptic blockade medium reduced tonic firing of NTS neurons in adult rats (Dean et al., 1990), suggesting a balance favoring excitatory input to NTS neurons from rats of all ages.

Despite this effect of chemical synaptic blockade on tonic firing properties, chemical transmission does not appear to be required for the chemosensitive response. It has been found that when chemical transmission was blocked, central chemosensitivity of individual neurons from the LC, medullary raphé, NTS and RTN did not change (Dean et al., 1990; Richerson, 1995; Kawai et al., 1996; Dean et al., 1997, Huang et al., 1997; Mulkey et al., 2004b; Hartzler et al., 2007; Conrad et al., 2008).

In addition to chemical synapses, hypercapnic responses of individual neurons in the network could be transmitted via gap junction mediated electrical transmission. Gap junctions are made from proteins called connexins, and connexin expression has a complex developmental pattern that varies with the specific connexin (Dermietzel et al., 1989; Belliveau and Naus, 1995; Solomon et al., 2001). Connexins have been found in neurons (Cx26, Cx32, and Cx36) (Solomon et al., 2001; Solomon, 2003). Chemosensitive neurons from the NTS have been shown to be extensively gap junction coupled (Dean et al., 1997; Huang et al., 1997) and to contain, both in neonates and
adults, connexins Cx26, Cx32, Cx36 (Solomon et al., 2001; Solomon, 2003). The role of gap junctions in the chemosensitive response has been studied in two areas, the NTS and the LC. These studies used the drug carbenoxolone which is known to inhibit gap junctions (Dean et al., 2001). Other effects of carbenoxolone have been found including inhibition of a key enzyme in glucocorticoid metabolism (Zhang et al., 2006), uncoupling of oxidative phosphorylation (Pivato et al., 2006), inhibition of voltage-gated Ca\(^{2+}\) channels (Vessey et al., 2004) as well as other non-specific effects (Rouach et al., 2003). It was found that carbenoxolone affected the chemosensitive response in both LC and NTS neurons. In LC neurons from neonates, it was found that both the percentage of responsive neurons and CI were decreased throughout postnatal development by carbenoxolone (Nichols et al., 2008a). In neonatal NTS neurons, carbenoxolone only decreased the percentage of activated NTS neurons from neonatal rats younger than P10 (Conrad et al., 2008; Nichols et al., 2008a). These data suggest that gap junctions play a role in the early development of chemosensitive neurons within the LC and NTS. Further it has been hypothesized that gap junctions may exist in these areas early in development to couple intrinsically chemosensitive neurons to non-chemosensitive neurons which could then result in a greater response of the LC and NTS to chemosensitivity in general (Conrad et al., 2008). This type of hypothesis has been proposed in the pre-Bötzinger region where NK1 receptor expressing neurons have been proposed to be gap junction-coupled to neurons not expressing NK1 receptors in order for these neurons to respond to substance P with a depolarization even though they do not express NK1 receptors (Hayes et al., 2007). On the other hand, it is not clear if gap junction coupling completely disappears in older neonates (>P10) or if the role of gap junction coupling in
chemosensitive and non-chemosensitive NTS neurons from neonates <P10 promotes the transformation of some non-chemosensitive neurons into chemosensitive neurons in neonates >P10 (Conrad et al., 2008). In summary, it is clear that once an NTS neuron becomes intrinsically chemosensitive in neonates >P10, gap junctions do not play a role in the chemosensitive response of those neurons.

Modulation of ventilation by CHx

The ventilatory responses to respiratory stimuli are plastic and can be modified by altered environmental conditions. Exposure to chronic altered gas tensions can alter the ventilatory response to respiratory stimuli. For example, exposure to chronic hypercapnia tends to reduce the ventilatory response to acute hypercapnia (Rezzonico and Mortola, 1989). Further, exposure to CHx results in an increase in the hypoxic ventilatory response (HVR), i.e. the ventilatory response to acute hypoxia (Aaron and Powell, 1993; Powell et al., 1998; Reid and Powell, 2005). Interestingly, it appears that CHx also increases the hypercapnic ventilatory response (HCVR), i.e. the ventilatory response to acute hypercapnia (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998). Also, plasticity has been found in other studies looking at effects of either chronic or intermittent hypoxia (Reeves and Gozal, 2005; Reeves et al., 2006; Berner et al., 2007; Szdzy and Mortola, 2007). However, the cellular basis for these forms of plasticity is not well understood.

The general effects of CHx include increased hematocrit, hypertension, right ventricular hypertrophy, and hyperventilation (Hunter et al., 1974; Rabinovitch et al., 1979) as well as an increase in the HVR (Aaron and Powell, 1993; Powell et al., 1998; 2000; Reid and Powell, 2005). Specifically, it has been shown that CHx increases the
normoxic ventilation and that it increases ventilatory sensitivity to hypoxia (Powell et al., 1998). It was shown that the increased HVR could not be fully explained by changes in the carotid body (Bisgard and Neubauer, 1995), suggesting involvement of central adaptations to CHx. Further, it has been suggested that the chronic activation of peripheral chemoreceptors may lead to an increased CNS gain after CHx (Powell et al., 1998; Dwinell and Powell, 1999). Hypoxia is largely sensed by peripheral chemoreceptors in the carotid body. It is known that the carotid body has an increased response to O₂ after CHx (Smith et al., 1986; Bisgard, 2000), so it is likely that sensory afferent output will tonically increase (Powell, 2007). Increased afferent input into the caudal NTS could lead to altered ventilation (Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003). Alternatively, plasticity within the NTS might be involved in ventilatory plasticity associated with CHx. This is supported by preliminary evidence showing that when NTS neurons were focally acidified in animals adapted to CHx, the HCVR increased compared to control animals (Powell et al., personal communication).

In summary, it has been shown that plasticity of ventilation occurs after CHx (Szdzuy and Mortola, 2007) and the HCVR is also increased after CHx (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998). The NTS contains neurons sensitive to CO₂ (Dean et al 1989; 1990; 1997; Huang et al., 1997; Conrad et al., 2008), so it is possible that plasticity occurs in the NTS after CHx through modification of the properties of chemosensitive neurons from the NTS. However, no one has studied changes in the chemosensitive response of caudal NTS neurons after CHx.
Nucleus tractus solitarius (NTS)

The NTS region is found in the dorsal medulla oblongata and the caudal pons (Fig. 2). NTS neurons are involved in modulating physiological processes including gustatory, cardiovascular, and ventilatory responses. It is thought that the caudal NTS is primarily involved in modulating ventilatory function whereas the rostral NTS is primarily involved in modulating cardiovascular and gustatory function (Coates et al., 1993; Nattie and Li, 2002a; Lawrence and Jarrot, 1996; Ambalavanar et al., 1998). Specifically, in the NTS, it was found that when the rostral region was focally acidified there was a small increase in ventilation, and when the caudal region of the NTS was focally acidified there was a large increase in ventilation (Nattie and Li, 2002a). NTS neurons are thought to be involved in central chemosensitivity and are known to respond to acute hypercapnia (Dean et al., 1989; 1990). Further, NTS neurons that are activated by hypercapnia do not depend on chemical transmission since their chemosensitive response is unchanged in the presence of synaptic block medium, but activated NTS neurons from <P10 animals depend on electrical transmission to have a chemosensitive response since the gap junction blocker carbenoxolone significantly decreased the percentage activated (Conrad et al., 2008). However, the majority of these experiments have been done in neonatal rats. There are few studies that have been done in adult rats, and these only looked at the firing rate response of NTS neurons to hypercapnia, and did not measure the pH response or the magnitude to which the firing rate increased in response to hypercapnic acidosis (Dean et al., 1989; 1990). Thus, it is not clear how representative the previous studies of the chemosensitive response of NTS neurons from neonatal rats are compared to NTS neurons from adult rats.
NTS neurons have specific properties that change before reaching their mature form which include changes in morphology, electrophysiology, action potentials, ion channels and neurotransmitters (Putnam et al., 2005). Morphological changes that NTS neurons undergo include soma size and dendritic length increasing, while spine density decreases over development into adulthood (Vincent and Tell, 1999).

Electrophysiological changes that NTS neurons undergo include the time constant and input resistance both reaching their maximum at P21, but then they both decrease as the neurons mature (Vincent and Tell, 1997). Once NTS neurons mature into their adult form, NTS neurons were found to have a mean rheobase current (the current needed to induce an action potential) that was twice the mean rheobase current seen in NTS neurons from neonates (Vincent and Tell, 1997). Thus, since input resistance decreases and rheobase increases in NTS neurons from adults, it can be suggested that it is easier for current to pass through the membrane of adult NTS neurons. This may further suggest that the number or the activity of ion channels also increases in adult NTS neurons. Other electrophysiological changes that have been studied include action potential waveforms (Vincent and Tell, 1997). It has been found that the amplitude of action potentials of NTS neurons at birth increase until they reach their maximal peak at P14, but then the amplitude decreases to a steady amplitude in NTS neurons from adults (Vincent and Tell, 1997).

Additionally, NTS neurons undergo changes in neurotransmitter currents, including both glutamatergic and GABAergic currents, before reaching their mature adult form (Kawai and Senba, 2000). It has been found that NTS neurons exhibit both spontaneous glutamatergic and GABAergic currents early in development, which is taken
as an indicator of activity in the local circuit. As neurons differentiate and become mature, they exhibit either spontaneous excitatory or inhibitory current (Kawai and Senba, 2000). Further, the density of glutamate receptors increases from birth to P9, but then decreases thereafter (Rao et al., 1997). This latter finding would suggest that a strong excitatory input exists until P9, which then decreases into adulthood in NTS neurons.

Glutamate has been implicated in CHx-induced plasticity. It has been found that when NMDA receptors are blocked by systemic application of MK-801 (an NMDA receptor antagonist), the HVR was eliminated during CHx (Reid et al., 2005). However, one problem with systemic blockade of NMDA receptors was that glutamate effects on the NTS could not be distinguished from glutamate effects on other sites (Reid et al., 2005). When MK-801 was microinjected into the NTS of CHx adult rats, there was a decrease in the ventilatory response to acute hypoxia, but no effect was seen on the HCVR (Powell et al., personal communication). Therefore, in the NTS, glutamate (NMDA receptor) seems to be important for the ventilatory response to hypoxia but not the response to hypercapnia. Thus, we will not be testing the role of glutamate in this study since we will be focusing on the response of NTS neurons to hypercapnia.

Other neurotransmitters and a neuropeptide that may play a role in the NTS include those released from the carotid body. As mentioned earlier, the glomus cells of the carotid body are known to send output to NTS neurons (Finley and Katz, 1992). It is known that glomus cells sense hypoxia and activate the carotid sinus nerve to cause action potentials to be sent along the afferent nerve, which then terminates in the caudal NTS (Finley et al., 1992). Carotid body stimulation can result in the release of ATP,
acetylcholine, noradrenaline, substance P, and dopamine (Gonzalez, 1995). Studies have been conducted on the role of dopamine and substance P after CHx. It was found that dopaminergic modulation of the HVR was changed by CHx, which included fine-tuning the CNS gain of the HVR in order to respond to the change in O2 level caused by CHx (Huey et al., 2000). However, this modulation did not explain the increase in the HVR (Huey et al., 2003). On the other hand, preliminary evidence has suggested that substance P has a role in the ventilatory response (Wilkinson et al., 2006). Further, the carotid sinus nerve is thought to project to two types of neurons in the NTS, which then can take one of three pathways to the phrenic motor nerve to directly or indirectly modulate ventilation (Finley and Katz, 1992) (Fig. 7). The first pathway begins with the carotid sinus nerve projecting to a post-synaptic NTS neuron which is a bulbospinal neuron projecting to the phrenic motor nerve (Dobbins and Feldman, 1994) (Fig. 7, red). The second pathway begins with the carotid sinus nerve projecting contralaterally to a post-synaptic NTS neuron which projects to another NTS neuron (Fig. 7, green). The second NTS neuron is the bulbospinal neuron that then projects to the phrenic motor nerve (Fig. 7, green). The third pathway includes the carotid sinus nerve projecting contralaterally to a post-synaptic NTS neuron that is a propriobulbar neuron that projects to the ventral respiratory group (VRG) (Fig. 7, blue). The post-synaptic VRG neuron is the bulbospinal neuron that then projects to the phrenic motor nerve (Dobbins and Feldman, 1994) (Fig. 7, blue). All of these pathways ultimately project to the phrenic motor nerve located in the cervical region of the spinal cord. The phrenic motor nerve then projects to the major respiratory muscle, the diaphragm, to modulate ventilation.
Figure 7:

Suggested pathways occurring after the glomus cells of the carotid bodies are stimulated by hypoxia. The glomus cells of the carotid bodies then activate the carotid sinus nerve (CSN) to cause action potentials to be sent along this afferent nerve. The CSN then projects to 2 types of neurons in the NTS which can then take one of three pathways:

**Red:** The CSN projects to a post-synaptic NTS neuron which is a bulbospinal neuron projecting directly to the phrenic motor nerve (PMN) located in the cervical level of the spinal cord. **Green:** The CSN projects contralaterally to a post-synaptic NTS interneuron which projects to another NTS neuron. The second NTS neuron is the bulbospinal neuron that then projects to the PMN. **Blue:** The CSN projects contralaterally to a post-synaptic NTS neuron which is a propriobulbar neuron that projects to the VRG. The post-synaptic VRG neuron is the bulbospinal neuron that then projects to the PMN. Notice that substance P is released from the carotid bodies in response to hypoxia and that we have hypothesized that NK1 receptors are present on NTS neurons. It is unknown which NTS neuron expresses the NK1 receptor, and it is unknown if these NTS neurons are chemosensitive.
Type 1 cells or glomus cells in carotid bodies

Low O₂

Lungs

Diaphragm

Substance P

CSN

NK1

VRG

NTS

PMN

NK1

Figure 7:
(Dobbins and Feldman, 1994) (Fig. 7, black). Thus, alteration of NTS neurons might mediate the changes in the ventilatory response to both hypoxia and hypercapnia in animals adapted to CHx, which may include binding of substance P. Although we will not be testing these pathways, we will be testing the response of NTS neurons to acute hypercapnia and the role that substance P might play in this response.

**Substance P**

The majority of substance P is stored and released from carotid body afferent neurons whose cell bodies are located in the proximal petrosal ganglion and jugular ganglion (Finley et al., 1992). The neuropeptide is packaged in large, dense-core vesicles in the cell bodies of the carotid body afferent neuron and then transported down the pre-synaptic axon via microtubules to the synaptic terminal (Purves et al., 2004). Substance P is then released via a Ca$^{2+}$-dependent mechanism (Squire et al., 2003) and binds with high affinity to the metabotropic NK1 receptor (Regoli et al., 1994; Medhurst and Hay, 2002; Wang and Marvizon, 2002; Roosterman et al., 2004). It is known that the NK1 receptor is a G-protein-linked receptor. Specifically, the binding of the ligand substance P to the NK1 receptor will cause phosphorylation of the G-protein. G-protein activation will then trigger internalization of the receptor-ligand complex and dissociation of the ligand from the receptor. This mechanism is completed with the aid of β-arrestins which will then mediate the recycling of the receptor to the cell surface (Wang and Marvizon, 2002; Roosterman et al., 2004). In addition, it is known that the NK1 receptor is susceptible to desensitization. Therefore, repeated bouts of a stimulus may downregulate the response to substance P (Mazzone and Geraghty, 2000). Once substance P binds to its respective receptor, it is inactivated enzymatically by substance P endopeptidases.
It is not known if the post-synaptic NTS neurons that bind
substance P are CS or not and thus it is unclear what the effects of substance P release
might be on ventilatory responses to hypercapnia.

NK1 receptors have been found in areas involved in breathing including the pre-
Bötzingener region, RTN, medullary raphé, locus coeruleus, caudal NTS, caudal ventral
medulla and rostral nucleus ambiguus (Nakaya et al., 1994). It has been postulated that
NK1 receptors can be used as markers of chemosensitive neurons or neurons involved in
the system response to CO₂ (Nakaya et al., 1994). Substance P binding neurons in the
pre-Bötzingener region have been shown to be preferentially the rhythm-generating neurons
(Monteau et al., 1996; Gray et al., 1999; 2001; Pilowsky and Feldman, 2001; Guyenet et
al., 2002; Manzke et al., 2003; Stornetta et al., 2003; Pagliardini et al., 2005; Hayes et al.,
2007). In addition, when saporin (ribosomal toxin that kills cells) conjugated to
substance P was used to lesion pre-Bötzingener neurons, it was found that breathing rhythm
was disrupted \textit{in vivo} (Gray et al., 2001; McKay et al., 2005). Also, it has been found
that substance P accentuates respiratory rhythm \textit{in vitro} and depolarizes every pre-
Bötzingener neuron that was recorded intracellularly (Gray et al., 1999; Murakoshi et al.,
1985, Pena and Ramirez, 2004). Thus, in the pre-Bötzingener region, NK1 receptors can be
used to identify neurons involved in the control of breathing. Another region involved in
ventilation that contains neurons expressing NK1 receptors is the RTN. It has been found
that when saporin (complexed to substance P) was microinjected into the RTN, of an
adult rat, the HCVR (both frequency and tidal volume) was decreased (Nattie and Li,
2002b). In a different area, the caudal NTS, it has been found that when substance P was
either injected intraventricularly (Hedner et al., 1984; Yamamoto and Lagercrantz, 1985)
or microinjected into the NTS (Chen et al., 1990a) respiratory frequency increased. Also, when substance P was applied by microiontophoresis into the NTS, excitatory effects were found (Morin-Surun et al., 1984). Thus, it seems reasonable to postulate that substance P may play a role in both rhythm-generation of ventilation and central chemosensitivity.

One study has shown that endogenous release of substance P occurs after chronic hypoxia in the rostral ventrolateral medulla. Specifically, it was found that NK1 receptors are involved in the response to hypoxia, there is endogenous release of substance P, and when a NK1 antagonist was injected into rat pups, the pups could not hold a sustained response to hypoxia (Wickström et al., 2004). Furthermore, it has been found that when substance P is released in response to hypoxia, it binds with high affinity to the metabotropic NK1 receptor on NTS neurons (Mazzone and Geraghty, 2000). As stated previously, it has been shown that CHx increases the HCVR (Bisgard et al. 1986; Engwall and Bisgard 1990; Fatemian and Robbins 1998), and it has been shown that carotid body stimulation increases substance P release (Mifflin, 1997). Thus, a possible role for substance P in NTS responses to hypercapnia appears reasonable to postulate. It is known that when substance P binds to NK1 receptors on NTS neurons from neonatal and adult rats, ventilation increases (Monteau et al., 1996; Mazzone and Geraghty, 2000). Preliminary evidence has been found that when saporin complexed to substance P was microinjected in the NTS of an adult rat, the HCVR was decreased (Wilkinson et al., 2006). Preliminary evidence has also suggested that NK1 receptor expression in the NTS (measured by Western blot analysis) increases after CHx (Wilkinson et al., 2006). These
data imply that substance P in the NTS may be playing a role in the plasticity of the
HCVR in animals adapted to CHx.

Taken together, these data suggest that while the exact mechanism of
ventilatory plasticity in response to acute hypoxia and hypercapnia after adapting
rats to CHx is not known, involvement of neurons from the NTS and possible
changes in substance P neurotransmission in these neurons are likely candidates.

Significance

CHx is commonly experienced during a variety of pathological conditions,
including congestive heart failure, chronic obstructive pulmonary disease, stroke, and
sleep apnea. The ability of CHx to alter ventilatory patterns is well known, but the neural
bases of these effects are poorly understood. The present work addresses changes in the
properties of neurons from a critical brainstem region, the NTS, which is involved in
control of breathing and numerous cardiovascular parameters. Our work should lead to a
greater understanding of the changes in intrinsic neuronal properties as well as shifts in
major neurotransmitter systems within the NTS and should yield insight into causes of
ventilatory plasticity induced by CHx.
CHAPTER III

HYPOTHESIS & SPECIFIC AIMS
Hypothesis: Chronic hypoxia (CHx) results in an increased responsiveness of chemosensitive NTS neurons to acute hypercapnia, which we hypothesize is mediated by an increased responsiveness of these neurons to substance P.

Specific Aims

For all these aims, an individual NTS neuron within a brainstem slice from adult rat will be identified as either being chemosensitive (CS) or non-chemosensitive (non-CS).

Aim 1. Identification and characterization of the effect of acute hypercapnia on the firing rate and intracellular pH (pHi) of individual CS and non-CS NTS neurons from adult rats.

It is hypothesized that NTS neurons from adult rats will have a similar response to acute hypercapnia as that seen in NTS neurons from neonatal rats, i.e. about 50% of neurons activated by hypercapnia, a small percentage inhibited by hypercapnia, with the remainder of neurons having no firing response to hypercapnia. In addition, acute hypercapnia will result in a maintained acidification of NTS neurons from adult rats. To test this we will simultaneously measure both the pHi and firing rate responses to acute hypercapnia (both hypercapnic acidosis and isohydric hypercapnia) of individual CS and non-CS NTS neurons from control adult rats in the absence and presence of chemical and electrical synaptic blockade medium (SNB).

Aim 2. The effect of CHx on the cellular responses of individual CS and non-CS NTS neurons to acute hypercapnia.
It is hypothesized that NTS neurons from CHx-adapted adult rats will have an increased chemosensitive response to acute hypercapnia (relative to controls), i.e. a greater percentage of NTS neurons will be activated by acute hypercapnia and the magnitude of their response, as determined by CI, will be increased. To test this we will simultaneously measure both the pH_i and firing rate responses to acute hypercapnia of individual CS and non-CS NTS neurons from control and CHx-adapted rats in the absence and presence of chemical SNB.

**Aim 3. The effect of CHx on the responses of individual CS and non-CS NTS neurons to substance P.**

It is hypothesized that the increased responsiveness of chemosensitive NTS neurons from CHx-adapted adult rats is mediated by an increased responsiveness of these neurons to substance P. We will use SNB to isolate individual CS and non-CS NTS neurons. The effect of added substance P on the pH_i and firing rate responses of these neurons will be tested. Further, in the presence of substance P, the response of pH_i and firing rate to acute hypercapnia will be determined. With the use of immunohistochemistry, we will determine the presence of NK1 receptors in various types of NTS neurons. Finally, we will study the effect of an NK1 antagonist on the response of NTS neurons to acute hypercapnia. These studies will serve to indicate whether there is an endogenous release of substance P in response to acute hypercapnia and to determine if there are any non-specific effects of the NK1 antagonist.
CHAPTER IV

GENERAL METHODS
Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01). All experiments were conducted on Sprague-Dawley adult male rats (P50-P90).

Chronic Adaptation to Hypoxia

Adult male Sprague-Dawley rats (~P50) were exposed to hypobaric hypoxia (0.5 ATA of 21% O₂ which is approximately 0.1ATA), which we term chronic hypoxia (CHx), for ≥ 7 days. Sixteen groups of 5-6 adult male rats each were adapted to CHx and the hematocrit and body weight were both recorded for each animal prior to euthanasia.

Briefly, 5-6 Sprague-Dawley male adult rats were placed in a plexiglas chamber (Fig. 8) (cylinder 91.4 cm long and 27.9 cm in diameter) in which they were exposed to approximately 0.1 ATA (0.5 ATA of 21% O₂) starting at P50. O₂ was monitored with an electrode connected to an O₂ gauge (Teledyne Analytical Instruments, Industry, CA) and pressure was monitored with a differential electronic manometer (HHP91, Omega, Stamford, CT). All animals were maintained on a 12:12 light:dark cycle and had free access to food and water. Chamber pressure was interrupted for 20-30 minutes 3 times a week for cleaning and replacement of food and water. Each group was left in the chamber for 7 days and then exposure continued until each animal was sacrificed and tested. For controls, 5-6 male adult rats were placed in the chamber with a continuous flow of room air (21% O₂). Temperature and relative humidity were recorded.
Figure 8:

Picture of the plexiglas chamber that was used to adapt adult rats to hypobaric hypoxia for $\geq 7$ days.
continuously for all groups with HOBO HO8 RH recorders (Onset Computer Corporation, Bourne, MA) placed in the plexiglas chamber. In order to measure hematocrit, blood samples were collected after decapitation by filling heparinized capillary tubes (6 samples per animal) with blood from the carotid artery. These samples were then spun for 3-5 minutes at maximum speed (13,460Xg using a hematocrit centrifuge (IEC)).

**Solutions**

Artificial cerebral spinal fluid (aCSF) contained the following (in mM): 124 NaCl, 5.0 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 26 NaHCO₃, and 10 glucose, and was equilibrated with 95% O₂/5% CO₂ (extracellular pH, pH₀ ~ 7.45 at 37°C). This aCSF solution has a somewhat elevated K⁺ concentration in order to enhance basal spontaneous activity (Dean et al., 1990). Hypercapnic acidotic (HA) solution was identical to aCSF, but was equilibrated with 85% O₂/15% CO₂ (pH₀ ~ 6.8-6.9 at 37°C).

Synaptic blockade (SNB) solution was modified from aCSF with 0.2 mM CaCl₂ and 11.4 mM MgSO₄ to block chemical synapses (Dean et al., 1990; Conrad et al., 2008). Carbenoxolone (CARB-100 μM) was added to SNB solution to block electrical transmission (Dean et al., 2001; Conrad et al., 2008). Isohydric hypercapnic (IH) solution was modified from aCSF with NaHCO₃ increased to 77 mM and NaCl decreased to 73mM in order to keep pH₀ and osmotic pressure the same. The whole cell patch intracellular solution contained (in mM): 130 K⁺-gluconate, 10 KCl, 10 HEPES, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, and 2 ATP, (pH = 7.45 at room temperature). This solution was used to reduce potential washout of the chemosensitive response (Filosa and Putnam, 2003; Conrad et al., 2008). The high K⁺/nigericin solution that was used for calibration
(Thomas et al., 1979) in the imaging studies contained (in mM): 104 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 25 N-methyl-D-glucamine (NMDG)-HEPES, 25 K-HEPES, 10 glucose, and 0.004 nigericin titrated with KOH or HCl to a pH value ranging from 6.2-8.6. The NH₄Cl solution was modified from aCSF with 104 mM NaCl in order to adjust for the added 20 mM NH₄Cl. 0 Na⁺ solution contained the following in mM: 124 NMDG-Cl, 3 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, and 10 glucose. 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS, pyranine) (Invitrogen, Eugene, OR) was added to the whole cell patch intracellular solution. 5 mg of substance P methyl ester (purchased from American Peptide, Sunnyvale, CA and Sigma, St. Louis, MO) was prepared in 3.7 mL of 100 mM acetic acid and then aliquots of 50 μL were stored at -20°C until needed. An entire 50 μL aliquot of substance P was added to 50 mL of SNB solution to give a final concentration of 1 μM substance P for experiments. 5 mg of L-703,606 oxalate salt (NK1 antagonist) (purchased from Alexis Biochemicals, San Diego, CA and Sigma, St. Louis, MO) was prepared in 8.35 mL of ddH₂O and then aliquots of 50 μL were stored at 4°C until needed. An entire 50 μL aliquot of L-703,606 oxalate salt was directly added to 50 mL of SNB solution to give a final concentration of 1 μM L-703,606 oxalate salt for experiments. All chemicals were purchased from Sigma (St. Louis, MO) except where indicated.

**Brain Slice Procedure**

Adult male Sprague-Dawley rats (P50-P90) were anesthetized with a brief exposure to CO₂ (100 %), which was followed by rapid decapitation. The brainstem was then removed and submerged in aCSF equilibrated with 5% CO₂ / 95% O₂ gas mixture. Transverse slices (300 μm) were prepared on a vibratome (Pelco 101, series 1000)
beginning at the obex and extending rostrally for ~ 1 mm, as previously described (Mulkey et al., 2003) (Fig. 9). Slices were allowed to recover for at least 1 hour at room temperature in aCSF equilibrated with a 5% CO₂ / 95% O₂ gas mixture. Individual slices for study were placed in a superfusion chamber on the stage of an upright Nikon Optiphot-2 microscope. Slices were immobilized with a nylon grid and superfused at ~2.4 ml/min with aCSF equilibrated with a 5% CO₂ / 95% O₂ gas mixture (pH ~ 7.45 at 37°C).

Individual neurons from the nucleus tractus solitarius, NTS, were then studied. The experimental protocol for studies consisted of a 5 minute exposure to aCSF equilibrated with 5% CO₂ / 95% O₂, a 10-15 minute exposure to aCSF equilibrated with 15% CO₂ / 85% O₂, (pH ~ 6.8-6.9) and a 5-10 minute exposure to aCSF equilibrated with 5% CO₂ / 95% O₂. This protocol was then repeated in the same neuron with either SNB solution or SNB + CARB solution. We hyperpolarized the membrane potential before exposing the neuron to hypercapnia in order to accurately measure the chemosensitive response in the presence of SNB or SNB + CARB. For the IH experiment, the response to HA was first tested and then returned to normocapnia before testing the response to IH. The protocol used for aCSF was also used to study neuronal responses to 1μM substance P in the presence of SNB (prolonged exposure in the presence of substance P equilibrated with 5% CO₂ / 95% O₂ was ≥ 8 minutes). For studies looking at the effect of the NK1 antagonist, we first looked at the effectiveness of the NK1 antagonist blocking substance P actions. This was tested after a 5 minute exposure to SNB equilibrated with 5% CO₂ / 95% O₂, followed by a 5 minute exposure to the NK1 antagonist in SNB equilibrated with 5% CO₂ / 95% O₂, and lastly a 5 minute exposure to the NK1 antagonist plus
Figure 9:

The left picture shows brain slices, containing the NTS, from adult (P57) and neonatal rats (P1) (cerebellum still attached to slices from neonatal rats). Neonatal slices are on the left, and adult slices are on the right from caudal (top) to rostral (bottom). Notice that the NTS can be found on more slices in adult rats compared to neonates. The slices shown in the red rectangle represent the slices that are kept, and the slices that are shown in the yellow rectangle represent the slices used for study. In the right picture, the NTS and the dorsal motor nucleus is depicted by the red circles.
Figure 9:

Neonates | Adults
---|---
Caudal

Rostral

NTS
substance P in SNB equilibrated with 5% CO₂ / 95% O₂. The chemosensitive response
was then studied in the presence of the NK1 antagonist and in the presence of the NK1
antagonist plus substance P. The protocol for these experiments included a 5 min
exposure to SNB equilibrated with 5% CO₂ / 95% O₂, followed by a 5 min exposure to
the NK1 antagonist in SNB equilibrated with 5% CO₂ / 95% O₂. The chemosensitive
response then was tested by giving a 10-15 min exposure to the NK1 antagonist in SNB
equilibrated with 15% CO₂ / 85% O₂, and a 5-10 min exposure to the NK1 antagonist in
SNB equilibrated with 5% CO₂ / 95% O₂. This was followed by a 5 min exposure to the
NK1 antagonist plus substance P in SNB equilibrated with 5% CO₂ / 95% O₂. The
chemosensitive response was then tested again in the presence of the NK1 antagonist plus
substance P in SNB using the same protocol as above.

**Fluorescence Microscopy**

pHᵢ was measured as previously described (Ritucci et al., 2005b). Individual NTS
neurons were loaded with 1 mM of the pH-sensitive dye, pyranine, through a whole cell
patch pipette (Fig. 10). Loaded neurons were excited and alternately exposed for 1 s to
light of wavelength 450 ± 10 and 410 ± 10 nm using a Sutter Lambda 10-2 filter wheel.
Emitted fluorescence at 515 ± 10 nm was collected and processed using MetaFluor
7.1.4.0 software (Meta Imaging), and the 450/410 fluorescence ratios (Rᵢ) were
determined. A calibration curve was generated using the high K⁺/nigericin technique
(Thomas et al., 1979). Rᵢ values measured during the experiment were divided by the Rᵢ
value at pH 7.4 measured at the beginning of each experiment to yield values for
normalized Rᵢ (Nᵢ). A calibration of Nᵢ versus pHᵢ was then constructed, which
produced a calibration curve with the following equation: pH = 7.4969 + log (Nᵢ -
Figure 10:

An example of a pyranine-loaded NTS neuron from an adult rat. Notice the recording pipette coming in on the left, which forms a seal on the soma membrane. Throughout the experiment, pyranine loads the soma and dendrites, allowing intracellular pH to be recorded.
\[0.2003)/(2.0194 - N_0); r^2 = 0.99 \text{ (n = 27).} \] A pH_i versus time plot was made in Microsoft Excel, and the pH_i recovery rate from CO_2-induced acidification of an individual neuron was estimated by the slope of a linear fit to the pH_i versus time trace during acute hypercapnia (at least 5 points starting at minimum pH_i).

**NH_4Cl prepulse technique**

We performed an NH_4Cl prepulse, which is a technique to acidify cells, to study the pH_i responses of NTS neurons from adult rats. The control response of pH_i to an NH_4Cl prepulse is a rapid alkalinization followed by an acidification (Boron and De Weer, 1976; Ritucci et al., 1997). The alkalinizing effect is due to NH_3 diffusing into the cell along its gradient and combining with an H^+ ion to form NH_4^+. Once equilibrium of NH_3 is reached, pH_i will plateau. However, NH_4^+ can also enter the cell and dissociate into NH_3 and H^+, which causes a plateau acidification (Boron and De Weer, 1976; Putnam, 2001). When NH_4Cl is removed and replaced with aCSF, intracellular NH_4^+ will dissociate into H^+, which acidifies the cell, and NH_3, which will diffuse out of the cell. The net result is an intracellular acidification to a pH_i value lower than the initial pH_i. The cell then exhibits pH_i recovery back towards it initial value of pH_i. We determined buffering power by performing the same prepulse experiment and then replaced this solution with 0 Na^+ solution. Once pH_i reached a minimum value and plateaued, the 0 Na^+ solution was removed and was replaced with aCSF. Neuronal buffering power was estimated form the change of pH_i upon removal of NH_4Cl in 0 Na^+ solution using the technique of Boron (1977).

**pH_i regulation studies**
We also wanted to study pH$_i$ recovery mechanisms of NTS neurons from adult rats. We had success using 0 Na$^+$ solution and found that the pH$_i$ recovery mechanism was primarily Na$^+$-dependent. It is possible that other pH$_i$ recovery mechanisms exist since pH$_i$ recovery was not completely inhibited in all neurons by the 0 Na$^+$ solution, so we attempted to study other mechanisms using amiloride (sodium hydrogen exchanger inhibitor), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA: sodium hydrogen exchanger inhibitor), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS: HCO$_3^-$-Cl exchanger inhibitor). However, since these inhibitors are light sensitive and seem to absorb light at a wavelength that is close to the excitation wavelength of pyranine (pH$_i$-sensitive dye), the inhibitors interfered with our pH$_i$ measurements (pyranine). In the past, the pH-sensitive dye 2′-7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used successfully to study pH$_i$ recovery mechanisms in NTS neurons from neonates (Ritucci et al., 1996; 1997; 1998). Thus, we will study the other pH$_i$ recovery mechanisms using BCECF and will be publishing those results as a separate study.

**Electrophysiological Studies**

The blind whole-cell patch clamp technique was used to measure neuronal membrane potential ($V_m$) and integrated firing rate as described previously (Blanton et al. 1989). The experimental setup that was used has been previously described (Dean et al. 1997; Huang et al. 1997; Filosa et al. 2002; Conrad et al., 2008). Briefly, a whole cell patch pipette (5 MΩ) was fabricated from borosilicate glass using a Narishige PP-830 dual stage pipette puller. The pipette was filled with whole cell patch solution (see above). Positive pressure was maintained on the pipette while a -0.1nA pulse (30 msec, 5 Hz) was applied. Tip impedance increased as the pipette approached a neuron, which
was indicated by a 1-2 mV downward deflection. Negative pressure was applied to the pipette to obtain a giga-ohm seal, brief suction was applied to the pipette to rupture the membrane, and then $V_m$ and integrated firing rate were measured throughout the experiment. Integrated firing rate (Hz) was determined from the $V_m$ trace in 10 s bins using a window discriminator (FHC model 700B), and analyzed using pClamp 8.2 software. Viable neurons had a stable $V_m$ of between -40 and -60 mV and fired action potentials that crossed through zero.

Sharp-tip recordings were used to assure that our whole cell recordings did not cause washout of the electrical response to hypercapnic acidosis as previously described (Dean and Reddy, 1995; Richerson, 1995; Mulkey et al., 2003). Sharp-tip electrodes were also fabricated from borosilicate glass, filled with 3 M potassium acetate solution, and were pulled to a tip resistance of ~ 100-150 M$\Omega$ using a one-stage Flaming/Brown micropipette puller (P87, Sutter Instrument). Sharp-tip recordings began once a stable membrane potential and firing rate were reached.

**Electrophysiological data analysis**

Membrane potential and firing rate were recorded on a personal computer using Axoscope pClamp 9.0 interface and software (Axon Instruments, Inc.). The firing rates during normocapnia and hypercapnia were collected by a multichannel slope/height window discriminator (FHC Model 700B) using 10 second bins to define the integrated firing rate in Hz (action potentials/second). The firing rates and $V_m$ were then averaged using MATLAB 7.0.

In order to quantify the magnitude of the firing rate response to hypercapnia, we calculated the chemosensitivity index (CI) using the equation of Wang and Richerson
(1999) in Excel (Microsoft Office, 2003). In words, CI is the percentage of the initial firing rate upon a 0.2 pH unit change in pHo. A CI value of 100% indicates a nonchemosensitive neuron, a CI of 200% indicates a neuron that is activated by hypercapnia with a doubling of firing rate for a 0.2 pH unit extracellular acidification, and a CI of 50% indicates a hypercapnia-inhibited neuron whose firing rate is halved for a 0.2 pH unit extracellular acidification.

Individual neurons were categorized as activated, nonchemosensitive, or inhibited. Under hypercapnic conditions, neurons having a CI of 120% or more were considered activated, neurons whose CI was between 80-120% were considered nonchemosensitive, and neurons having a CI of less than 80% were categorized as inhibited.

Immunohistochemistry

Adult rats were first anesthetized with pentobarbitol (100 μg/g) and then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH-7.4). The brainstem was isolated and fixed overnight in 4% paraformaldehyde, and then kept in 30% sucrose until sectioning (50 μm) on a cryostat (Microm, Walldorf, Germany). Sections were collected on slides (VWR Vistavision™ Histobon®). Tissue sections were washed in PBST (0.01 M; Phosphate Buffered Saline and 0.1% Triton), incubated for 1 hour in normal donkey serum (1:10 in PBST) to reduce nonspecific staining, and incubated overnight (4°C) with rabbit polyclonal anti-NK1 antibody (1:750, Chemicon International) and mouse monoclonal anti-NeuN (1:1000, Chemicon International) diluted in PBST. After washing in PBST, the sections were incubated for 2 hours with cyanine 3 (Cy3)-conjugated donkey anti-rabbit (1:50; Jackson ImmunoResearch
Laboratories, West Grove, PA) diluted in PBST to identify anti-NK1 and fluorescein isothiocyanate (FITC)-donkey anti-mouse FITC (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBST to identify neurons. Tissue sections were then washed in PBS, dried, and coverslipped with Vectashield (H1000, Vector Laboratories, Inc., Burlingame, CA). Mounted sections were examined with a Fluoview 1000 microscope (Olympus Optical, Tokyo, Japan) (20X or 60X objective) using Argon (FITC) and Krypton (Cy3) lasers and Fluoview software. For Cy3 fluorescence, excitation was set to 560 nm, and emitted fluorescence was collected between 580-680 nm. For FITC fluorescence, excitation was set to 488 nm, and emitted fluorescence was collected between 500-555 nm. A series of confocal optical sections (z step 0.5 μm) were obtained to assess extent of NK1 receptor labeling and the degree to which it colocalized with NeuN immunolabeling.

Identified neurons

After a recording in which the neuronal response to hypercapnic acidosis was identified, a slice was fixed by first storing it in 4% paraformaldehyde overnight, followed by submerging the slice in 30% sucrose overnight. The 300 μm slice was then thin sliced (20 μm) on a cryostat. Pictures were taken initially to identify the pyranine-labeled neuron using a microscope equipped with a filter for FITC. These sections were then kept at -20 °C until further processing. Tissue sections were washed in PBST, incubated for 1 hour in normal donkey serum to reduce nonspecific staining, and incubated overnight (4°C) with rabbit polyclonal anti-NK1 antibody (1:750, Chemicon International) diluted in PBST. After washing in PBST, the sections were incubated for 2 hours with Cy3-conjugated donkey anti-rabbit (1:50; Jackson ImmunoResearch
Laboratories, West Grove, PA) diluted in PBST to identify anti-NK1. Tissue sections were then washed in PBS, dried, and coverslipped after the application of Vectashield. Mounted sections were the imaged as stated above using a Fluoview 1000 microscope (20X or 60X objective and then zoomed for higher magnification) using Argon (FITC) and Krypton (Cy3) lasers and Fluoview software. A series of confocal optical sections (z step 0.5 μm) were obtained to assess extent of NK1 receptor labeling and the degree to which it colocalized with the pyranine-labeled neuron.

We tried this technique on 26 identified neurons. Although we could detect positive NK1 receptor labeling in other neurons, we could not detect NK1 receptor labeling on identified neurons (Fig. 11). We only detected NK1 receptors on one NTS neuron from a CHx-adapted animal (Fig. 12). In addition, one neuron had extensive dendritic processes, which also displayed a lack of NK1 receptors (Fig. 13). We are unsure of exactly why we could not detect NK1 receptors on labeled neurons, but we presume that the pyranine signal was too bright to detect NK1 receptors on the soma membrane. It is possible that the use of a different fluorophore to identify NTS neurons would cause less interference with visualization of NK1 receptors.

Statistical Analysis

Values are reported as means ± S.E.M. $\chi^2$ tests were used to compare differences between the percentages of neurons that respond to hypercapnia. Student’s t-tests were used to compare differences between two means with a level of significance at $P<0.05$. Differences between three or more means were determined by one-way ANOVA. If significant differences existed, then multiple comparisons were done using Tukey's method with a level of significance of $P<0.05$. 
Figure 11:

NK1 receptor staining and pyranine loaded neuron (red arrow) taken at 60X2 on a confocal microscope. The figure shows a neuron (from a control animal) that was non-chemosensitive.  

A. Shows superimposed image.  

B. Shows only channel for Cy3 turned on.  It is observed that there are no NK1 receptors on the pyranine-loaded neuron, which is adjacent to an NTS neuron with positive NK1 receptor immunolabeling (thick white arrow).
Figure 11: Non-chemosensitive - control
Figure 12:

NK1 receptor staining and pyranine loaded neuron (red arrow) taken at 60X1 on a confocal microscope. The figure shows a neuron (from a CHx animal) that was non-chemosensitive. A. Shows superimposed image. B. Shows only channel for Cy3 turned on. It is observed that there are NK1 receptors on the pyranine-loaded neuron. Also, another NTS neuron with positive NK1 receptor immunolabeling is shown (thick white arrow).
Figure 12:

Non-chemosensitive - CHx

A.

[Image: Microscope image with green and red markers, scale bar 20 μm]

B.

[Image: Microscope image with green and red markers, scale bar 20 μm]
Figure 13:

This figure shows a pyranine loaded neuron taken at 60X1.5 on a confocal microscope. The figure shows a neuron (from a control animal) that was inhibited. It is observed that there are no NK1 receptors on the pyranine-loaded neuron. Notice the extensive loading of the soma (depicted by the red arrow) and the dendritic processes (depicted by the white arrows).
Figure 13: Inhibited - Control

[Image of a neuronal cell with labeled parts: Soma and Dendritic processes]
CHAPTER V

Characterization of the chemosensitive response of individual nucleus tractus solitarius (NTS) neurons from adult rats
**Introduction**

Putative central chemosensitive neurons have mostly been studied in neonatal rats in various localized regions within the brainstem, including the nucleus tractus solitarius (NTS) (Dean et al., 1989; 1990; Mulkey et al., 2003; Conrad et al., 2008), ventrolateral medulla (VLM) (Wellner-Kienitz and Shams, 1998), retrotrapezoid nucleus (RTN) (Mulkey et al., 2004b; Ritucci et al., 2005b), medullary raphe (Richerson, 1995), pre-Bötzinger region (Solomon et al., 2000a), and the locus coeruleus (LC) (Oyamada et al., 1998; Filosa et al., 2002). All of these regions increase ventilation when focally acidified (Coates et al., 1993; Nattie, 2001). Specifically, in the NTS, it was found that when the rostral region was focally acidified there was a small increase in ventilation, and when the caudal region of the NTS was focally acidified there was a large increase in ventilation (Nattie and Li, 2002a). All of these regions also contain neurons whose firing rate increases when exposed to hypercapnia (Putnam et al., 2004). The mechanism believed to be responsible for this increase in firing rate is that increases in CO₂ result in a maintained fall of intracellular (pHi) (Ritucci et al., 1997; Filosa et al., 2002; Putnam et al., 2004), leading to depolarization and thereby an increase in firing rate (Filosa et al., 2002; Wang et al., 2002; Filosa and Putnam, 2003; Putnam et al., 2004). Thus, many studies of chemosensitive neurons focused on their pHᵢ responses to hypercapnia.

The neonatal NTS neuronal response to hypercapnia has been well characterized. While the neonatal preparation offers several technical advantages, concerns remain that due to developmental effects the findings in neonates are not representative of adult chemosensitive neurons. The response of adult NTS neurons to hypercapnic acidosis has received less attention (Dean et al., 1989, 1990; Mulkey et al., 2004a) and has not been
well quantified with respect to the percentage and magnitude of response or with respect to the response of pH_i to hypercapnia.

In the present study, we examined the cellular responses to hypercapnia of NTS neurons from adult rats including pH_i and firing rate responses. We found that NTS neurons from adult rats had a maintained acidification with a lack of pH_i recovery in response to hypercapnia, which is similar to what has been found in NTS neurons from neonates (Mulkey et al., 2004a, Ritucci et al., 1997), and that the percentage of NTS neurons activated or inhibited by acute hypercapnia, and the magnitude of these responses were unchanged in adult rats, compared to neonates (Conrad et al., 2008).

Preliminary reports of these findings have previously been published (Nichols et al., 2007).
Methods

Solutions

Artificial cerebral spinal fluid (aCSF) contained the following (in mM): 124 NaCl, 5.0 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 26 NaHCO₃, and 10 glucose, and was equilibrated with 95% O₂/5% CO₂ (extracellular pH, pH₀ ~ 7.45 at 37°C). Hypercapnic acidotic (HA) solution was identical to aCSF, but was equilibrated with 85% O₂/15% CO₂ (pH₀ ~ 6.8-6.9 at 37°C). Synaptic blockade (SNB) solution was modified from aCSF with 0.2 mM CaCl₂ and 11.4 mM MgSO₄ in order to block chemical synapses (Dean et al., 1990; Conrad et al., 2008). Carbenoxolone (CARB-100 μM) was added to SNB solution in order to block electrical transmission (Dean et al., 2001; Conrad et al., 2008). Isohydric hypercapnic (IH) solution was modified from aCSF with NaHCO₃ increased to 77 mM and NaCl decreased to 73 mM in order to keep pH₀ and osmotic pressure the same. The whole cell patch intracellular solution contained (in mM): 130 K⁺-gluconate, 10 KCl, 10 HEPES, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, and 2 ATP, (pH = 7.45 at room temperature). This solution was used to reduce potential washout of the chemosensitive response (Filosa and Putnam, 2003; Conrad et al., 2008). The high K⁺/nigericin solution that was used for calibration (Thomas et al., 1979) in the imaging studies contained (in mM): 104 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 25 N-methyl-D-glucamine (NMDG)-HEPES, 25 K-HEPES, 10 glucose, and 0.004 nigericin titrated with KOH or HCl to a pH value ranging from 6.2-8.6. The NH₄Cl solution was modified from aCSF with 104 mM NaCl in order to adjust for the added 20 mM NH₄Cl. 0 Na⁺ solution contained the following in mM: 124 NMDG-Cl, 3 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, and 10 glucose. 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt
(HPTS, pyrane) (Invitrogen, Eugene, OR) was added to the whole cell patch intracellular solution. All chemicals were purchased from Sigma (St. Louis, MO) except where indicated.

Slice preparation

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01). Adult male Sprague-Dawley rats (P50-P90) were anesthetized with a brief exposure to CO2 (100 %), which was followed by rapid decapitation. The brainstem was then removed and submerged in aCSF equilibrated with 5% CO2 / 95% O2 gas mixture. Transverse slices (300 μm) were prepared on a vibratome (Pelco 101, series 1000) beginning at the obex and extending rostrally for ~ 1mm, as previously described (Mulkey et al., 2003). Slice were allowed to recover for at least 1 hour at room temperature in aCSF equilibrated with a 5% CO2 / 95% O2 gas mixture. Individual slices for study were placed in a superfusion chamber on the stage of an upright Nikon Optiphot-2 microscope. Slices were immobilized with a nylon grid and superfused at ~2.4 ml/min with aCSF equilibrated with a 5% CO2 / 95% O2 gas mixture (pH ~ 7.45 at 37°C).

Individual neurons from the nucleus tractus solitarius, NTS, were then studied. The experimental protocol for studies consisted of a 5 minute exposure to aCSF equilibrated with 5% CO2 / 95% O2, a 10-15 minute exposure to aCSF equilibrated with 15% CO2 / 85% O2, (pH ~ 6.8-6.9) and a 5-10 minute exposure to aCSF equilibrated with
5% CO₂ / 95% O₂. This protocol was then repeated in the same neuron with either SNB solution or SNB + CARB solution. For the IH experiment, the response to HA was first tested and then returned to normocapnia before testing the response to IH.

**Measurement of intracellular pH**

pHᵢ was measured as previously described (Ritucci et al. 2005b). Individual NTS neurons were loaded with 1mM of the pH-sensitive dye, pyranine, through a whole cell patch pipette. Loaded neurons were excited and alternately exposed for 1 s to light of wavelength 450 ± 10 and 410 ± 10 nm using a Sutter Lambda 10-2 filter wheel. Emitted fluorescence at 515 ± 10 nm was collected and processed using MetaFluor 7.1.4.0 software (Meta Imaging), and the 450/410 fluorescence ratios (R₄₅₀) were determined. A calibration curve was generated (Fig. 14) using the high K⁺/ nigericin technique (Thomas et al., 1979). 

R₄₅₀ values measured during the experiment were divided by the R₄₅₀ value at pH 7.4 measured at the beginning of each experiment to yield values for normalized R₄₅₀ (N₄₅₀). A calibration of N₄₅₀ versus pHᵢ was then constructed (Fig. 14), which produced a calibration curve with the following equation: pH = 7.4969 + log(N₄₅₀ - 0.2003)/(2.0194 - N₄₅₀); r² = 0.99 (n = 27). A pHᵢ versus time plot was made in Microsoft Excel, and the pHᵢ recovery rate from CO₂-induced acidification of an individual neuron was estimated by the slope of a linear fit to the pHᵢ versus time trace during acute hypercapnia (at least five points starting at minimum pHᵢ) (a sample pHᵢ recording of an adult NTS neuron can be seen in the second panel of Fig. 16).

**Electrophysiological studies**

The blind whole-cell patch clamp technique was used to measure neuronal membrane potential (Vₘ) and integrated firing rate as described previously (Blanton et al. 1989).
Figure 14: The calibration curve for pyranine in individual NTS neurons from adult rats.

Each point represents an Nf value at a given pH for a single NTS neuron. Rf values measured during the experiment were divided by the Rf value at pH 7.4 measured at the beginning of each experiment, which then gave Nf. A sigmoidal curve was fit to the data over a range of pH from 6.2 to 8.6. The equation for this curve (Methods) was used to convert Nf values into pHi.
Figure 14
The experimental setup that was used has been previously described (Dean et al. 1997; Huang et al. 1997; Filosa et al. 2002; Conrad et al., 2008). Briefly, a whole cell patch pipette (5 MΩ) was fabricated from borosilicate glass using a Narishige PP-830 dual stage pipette puller. The pipette was filled with whole cell patch solution (see above). Positive pressure was maintained on the pipette as a -0.1nA pulse (30 msec, 5 Hz) was applied. Tip impedance increased as the pipette approached a neuron, which was indicated by a 1-2 mV downward deflection. Negative pressure was applied to the pipette to obtain a giga-ohm seal, brief suction was applied to the pipette to rupture the membrane, and then $V_m$ and integrated firing rate were measured throughout the experiment. Integrated firing rate (Hz) was determined from the $V_m$ trace in 10s bins using a window discriminator (FHC model 700B), which was then analyzed using pClamp 8.2 software. Viable neurons had a stable $V_m$ of between -40 and -60 mV and fired action potentials that crossed through zero. The electrophysiological response to hypercapnia was quantified using two measures: percentage of neurons whose firing rate was reversibly altered by acute hypercapnia, and the magnitude of the firing rate response to acute hypercapnia, calculated as the chemosensitivity index or CI according to the equation of Wang and Richerson (1999). A neuron was designated as activated if its CI was greater than 120% or inhibited if its CI was less than 80%.

Sharp-tip recordings were used to assure that our whole cell recordings did not cause washout of the electrical response to hypercapnic acidosis as previously described (Dean and Reddy, 1995; Richerson, 1995; Mulkey et al., 2003). Sharp-tip electrodes were also fabricated from borosilicate glass, filled with 3M potassium acetate solution, and were pulled to a tip resistance of ~ 100-150 MΩ using a one-stage Flaming/Brown
micropipette puller (P87, Sutter Instrument). Sharp-tip recordings began once a stable membrane potential and firing rate were reached.

Statistical Analysis

Values are reported as means ± S.E.M. $\chi^2$ tests were used to compare differences between the percentage of neurons that respond to hypercapnia. Student’s t-tests were used to compare differences between two means with a level of significance at $P<0.05$. Differences between three or more means were determined by one-way ANOVA. If significant differences existed, then multiple comparisons were done using Tukey's method with a level of significance of $P<0.05$. 
Results

*pH*<sub>i</sub> in NTS neurons from adult rats

The steady state *pH*<sub>i</sub> in NTS neurons from adult rats is 7.30 ± 0.03 (n=27), significantly less alkaline than the value of 7.49 ± 0.02 previously reported for NTS neurons from neonatal rats (Ritucci et al., 1997). The NH<sub>4</sub>Cl prepulse technique was used to study the *pH*<sub>i</sub> responses of NTS neurons from adult rats. The normal response of *pH*<sub>i</sub> to an NH<sub>4</sub>Cl prepulse is a rapid alkalinization followed by an acidification (Boron and DeWeer, 1976; Ritucci et al., 1997). We found that the response to NH<sub>4</sub>Cl of NTS neurons from adult rats (Fig. 15, red trace) was similar to that of NTS neurons from neonatal rats (Ritucci et al., 1997; 1998). Adult NTS neurons exhibited a rapid *pH*<sub>i</sub> recovery from an NH<sub>4</sub>Cl prepulse induced acidification that returned towards the initial *pH*<sub>i</sub> (Fig. 15, red trace). This recovery is entirely due to Na<sup>+</sup>-dependent pH regulating transporters (Putnam, 2001) since it was reversibly inhibited by 0 Na<sup>+</sup> solutions (Fig. 15, blue trace). These findings are consistent with Na<sup>+</sup>/H<sup>+</sup> exchange playing a predominant role in *pH*<sub>i</sub> recovery in NTS neurons from adults as it does in NTS neurons from neonates (Ritucci et al., 1997; 1998). However, we were not able to more fully study *pH*<sub>i</sub> recovery in adult NTS neurons since the pH transporter inhibitors amiloride and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) interfered with pyranine fluorescence. We were able to estimate neuronal buffering power from the change of *pH*<sub>i</sub> upon removal of NH<sub>4</sub>Cl in 0 Na<sup>+</sup> (Fig. 15, blue trace) using the technique of Boron (1977). This leads to an estimate of total buffering of 35 mM/pH unit, which includes HCO<sub>3</sub><sup>-</sup> buffering and intrinsic buffering. We estimated buffering due to HCO<sub>3</sub><sup>-</sup>
Figure 15: The pH$_i$ response of NTS neurons from adult rats to an acidification induced by an NH$_4$Cl prepulse followed by either aCSF or 0 Na$^+$ solution. The control experiment (NH$_4$Cl prepulse followed by aCSF) is shown by the red circles. The neuron exhibited recovery from NH$_4$Cl-induced acidification back to the initial pH$_i$. The blue squares show the 0 Na$^+$ experiment. In 0 Na$^+$ solution the neuron had a maintained acidification with pH$_i$ recovery inhibited. Upon replacing 0 Na$^+$ solution with aCSF, pH$_i$ recovered rapidly back towards initial pH$_i$. 
as 25 mM/pH unit, meaning that intrinsic buffering is about 10 mM/pH unit in NTS neurons from adults, considerably less than the value of 46 mM/pH unit for intrinsic buffering in NTS neurons from neonates (Ritucci et al., 1998).

**pH$_i$ and electrophysiological response to acute hypercapnia of NTS neurons from adult rats in aCSF**

A sample recording of the pH$_i$ and electrophysiological response to acute hypercapnia (15% CO$_2$) of an NTS neuron from an adult rat is shown in Fig. 16. Each neuron was given a 5 minute exposure to 5% CO$_2$, a 10 minute exposure to 15% CO$_2$, and a final 5 minute exposure to 5% CO$_2$. NTS neurons from adult rats showed a similar pH$_i$ response to 15% CO$_2$ compared to NTS neurons from neonatal rats, acidifying by $0.25 \pm 0.011$ pH unit compared to $0.26 \pm 0.006$ pH unit (Mulkey et al., 2004a). NTS neurons from adult rats exhibited a similar pattern of pH$_i$ response to maintained hypercapnia that was previously reported for NTS neurons from neonatal rats (Ritucci et al., 1997). NTS neurons from adult rats exhibited a lack of pH$_i$ recovery (-0.007 ± 0.002 pH unit/min; $n = 69$). Upon return to normocapnia, the pH$_i$ of NTS neurons returned to initial values with no apparent overshoot (Fig. 16), consistent with a lack of pH$_i$ recovery during hypercapnia (Boron and De Weer, 1976; Ritucci et al., 1997).

We also measured the integrated firing rate response to acute hypercapnia in NTS neurons from adult rats. NTS neurons were classified (based on their integrated firing rate response to hypercapnia) as activated (firing rate increased by > 20%), inhibited (firing rate decreased by > 20%), or non-chemosensitive (firing rate changed by < 20%). A sample trace of an activated NTS neuron from an adult rat is shown in Fig. 16. We assessed neuronal responses to acute hypercapnia in two ways, determining the
**Figure 16:** The pHᵢ and firing rate response of an individual NTS neuron from an adult rat that was activated by hypercapnic acidosis. The top panel shows the experimental protocol used. The second panel shows sample fluorescence images of the NTS neuronal cell body in response to each solution. The third panel shows the pHᵢ response of the NTS neuron to hypercapnic acidosis over time, which was a maintained acidification with a lack of pHᵢ recovery. Notice that once the hypercapnic solution was removed, pHᵢ returned back towards initial pHᵢ. The fourth panel shows the firing rate response to hypercapnic acidosis of the NTS neuron over time, which increased in response to hypercapnic acidosis and returned towards initial firing rate once hypercapnic acidosis was removed. The bottom panel shows 10 second samples of action potentials taken at the time points indicated in the fourth panel. Notice that action potential frequency increased with hypercapnic acidosis, and then decreased back towards initial frequency once hypercapnia was removed.
Figure 16

Control Hypercapnia Control

![Graph showing pH and firing rate over time](image)

- **pH**
  - 7.4
  - 7.3
  - 7.2
  - 7.1
  - 7.0
  - 6.9
  - 6.8

- **Firing rate (Hz)**
  - 3
  - 2
  - 1
  - 0

- **Time (minutes)**
  - 0 10 20 30 40

- **5 sec 20 mV**

Legend:
- 1
- 2
- 3
percentage of neurons that respond to and the magnitude of their response to acute
hypercapnia. We found that NTS neurons from adult rats were 57% activated (n = 32) 
(Fig. 20), 9% inhibited (n = 5) (Fig. 20), and 34% non-chemosensitive (n = 19) (data not 
shown). The magnitude of the response of NTS neurons to hypercapnia was quantified 
by calculating the chemosensitivity index (CI) (Wang and Richerson, 1999). Average CI 
for activated NTS neurons from adult rats was 177 ± 8% and for inhibited NTS neurons 
from adult rats was 63 ± 10%. Activated neurons in 5% CO₂ had an average basal 
integrated firing rate of 0.6 ± 0.13 Hz that increased to 2.0 ± 0.33 Hz in response to 15% 
CO₂, and returned to initial values of 0.6 ± 0.12 Hz upon return to 5% CO₂. Inhibited 
neurons had an integrated firing rate of 0.9 ± 0.30 Hz in 5% CO₂, which fell to 0.3 ± 0.08 
Hz in response to 15% CO₂, and returned to 0.5 ± 0.24 Hz upon return to 5% CO₂. Non-
chemosensitive neurons had an integrated firing rate of 1.1 ± 0.42 Hz in 5% CO₂, which 
did not change in 15% CO₂ (1.0 ± 0.30 Hz), and remain unchanged upon return to 5% 
CO₂ (0.5 ± 0.11 Hz).

We used sharp-tip recordings to see if our whole cell recordings caused washout 
of the electrical response of these neurons to hypercapnia. It was found that 49% of NTS 
neurons from adult rats were activated (21/43) with a CI of 133 ± 5% while none were 
inhibited (data not shown). The percentage activated with sharp-tip recordings (49%) is 
similar to the findings from the whole cell recordings (57%). However, the CI of NTS 
neurons activated by hypercapnic acidosis recorded using sharp-tip electrodes (133 ± 5%) 
was lower than the CI found for activated NTS neurons using the whole cell pipettes (177 
± 8%). We have no explanation for why the CI is higher for activated NTS neurons using 
the whole cell technique compared to the sharp-tip technique, but it may be due to a
higher background leak current with sharp tip electrodes. Nevertheless, it is clear that using the whole cell solution does not cause complete washout of the response of adult NTS neurons to hypercapnia. Thus, as previously reported for LC neurons from neonates (Filosa and Putnam, 2003), whole cell pipettes can be used to study the chemosensitive response of NTS neurons.

*pHi and electrophysiological response to acute hypercapnia of NTS neurons from adult rats in SNB and SNB + CARB*

We next wanted to measure the intrinsic response to hypercapnia of NTS neurons from adult rats. Thus, we first blocked chemical transmission using synaptic blockade medium (SNB). A sample recording of the basal firing rate response to SNB is seen in Fig.17A, which shows that basal firing rate increases in response to SNB. Also, notice that excitatory post-synaptic potentials are eliminated in the presence of SNB (Fig. 17A inset and Fig. 17B). The average basal firing rate in aCSF of NTS neurons from adult rats (n = 14) increases significantly when medium is changed from aCSF to SNB (0.7 ± 0.22 Hz vs. 1.6 ± 0.5 Hz, respectively) (Fig. 17C). These data suggest that NTS neurons from adult rat brainstem slices receive tonic inhibitory input. Alternatively, the decreased external Ca\(^{2+}\) in SNB could suppress the Ca-dependent K\(^{+}\) channel, resulting in increased firing rate. Regardless, SNB had no effect on basal pHi (7.16 ± 0.03 pH units in aCSF versus 7.17 ± 0.03 pH units in the presence of SNB).

We determined the effect of SNB on NTS neuronal responses to hypercapnia. In order to compare hypercapnia-induced effects on firing rate, we wanted to start from nearly the same initial firing rate as in aCSF. Thus, in the presence of SNB, before the neuron was exposed to hypercapnia, the firing rate was decreased back towards initial
**Figure 17: A:** A sample basal firing rate response of an NTS neuron to SNB. Notice that basal firing rate increases in response to SNB. The inset shows that the presence of SNB (bottom) suppresses the post-synaptic potentials (greater than 5 mV deflection) that can be seen in the absence of SNB (top). **B:** A sample truncated action potential in the absence (left) and presence of SNB (right). Notice that the excitatory post-synaptic potentials seen on the left (signified by arrows) are eliminated by SNB shown on the right. **C:** The average basal firing rate of adult NTS neurons in the presence of aCSF and SNB. The “*” indicates that the firing rate in SNB is significantly greater than the firing rate in aCSF (P = 0.0469). The height of a bar represents the mean firing rate with error bar of 1 SEM.
Figure 17A

Figure 17B

Figure 17C
firing rate by injecting negative DC current (Fig. 18). It was found that SNB did not significantly affect the magnitude of acidification caused by hypercapnia (0.21 ± 0.02 pH units in the presence of aCSF and 0.15 ± 0.03 pH units in the presence of SNB) (n = 18). Additionally, there was still a lack of pHi recovery from hypercapnia-induced acidification in the presence of SNB (-0.015 ± 0.003 pH unit/min in aCSF and -0.006 ± 0.003 pH unit/min in the presence of SNB) (Fig. 18). The integrated firing rate response to HA in NTS neurons from adult rats also did not change in the presence of SNB. Details of the firing rate response to hypercapnia in SNB of NTS neurons from adult rats will be given below. Fig. 18 shows a sample trace of a hypercapnia-activated NTS neuron from an adult rat in the presence of SNB.

We also studied the response to hypercapnia of NTS neurons from adult rats in solutions in which both chemical synaptic transmission (SNB) was blocked and electrical transmission was blocked (using carbenoxolone, CARB). Fig. 19 shows a sample pHi and electrophysiological response to acute hypercapnia in the presence of SNB + CARB from an NTS neuron. Before the hypercapnic response was measured in the presence of SNB + CARB, the firing rate was decreased back towards initial firing rate by injecting negative DC current. We found that the acidification induced by hypercapnia was significantly larger in the presence of SNB + CARB (0.52 ± 0.06 pH unit in the presence SNB + CARB; n = 17) (P=0.0002). There was still a lack of pHi recovery in response to hypercapnia in SNB + CARB in NTS neurons from adult rats (-0.011 ± 0.005 pH unit/min) (Fig. 19). Upon return to normocapnia, the pHi of NTS neurons returned to initial values with no apparent overshoot (Fig. 19), consistent with a lack of pHi recovery during hypercapnia (Boron and De Weer, 1976; Ritucci et al., 1997). The integrated
**Figure 18:** The pH$_i$ and firing rate response of an individual NTS neuron from an adult rat that was activated by hypercapnic acidosis in the presence of SNB. The top panel shows the experimental protocol used. The second panel shows the pH$_i$ response of the NTS neuron to hypercapnic acidosis over time, which was not changed by SNB (i.e. a maintained acidification with a lack of pH$_i$ recovery). Notice that once the hypercapnic solution was removed, pH$_i$ returned back towards initial pH$_i$. The third panel shows the firing rate response to hypercapnic acidosis over time of the NTS neuron, which increased in response to hypercapnic acidosis and returned back towards initial firing rate once the hypercapnic acidosis was removed. Notice that we hyperpolarized the V$_m$ (indicated by hyp) by injecting negative DC current to bring the firing rate back towards initial firing rate before exposing the neuron to hypercapnia. The bottom panel shows 10 second samples of action potentials taken at the time points indicated in the fourth panel. Notice that action potential frequency increased with hypercapnic acidosis, and then decreased back to initial frequency once hypercapnia was removed.
Figure 18

Hypercapnia

SNB

SNB

pH

Firing rate (Hz)

10 min

1

2

3

hyp 1

hyp 2

hyp 3

10 min

20 mV

5 sec
**Figure 19:** The pHᵢ and firing rate response of an individual NTS neuron from an adult rat that was activated by hypercapnic acidosis in the presence of SNB + CARB. The top panel shows the experimental protocol used. The second panel shows the pHᵢ response to hypercapnic acidosis over time of the NTS neuron. Notice the similar pattern of pHᵢ response to hypercapnic acidosis as in SNB (Fig. 5) but the much larger hypercapnia-induced acidification in SNB + CARB. The third panel shows the firing rate response of the NTS neuron to hypercapnic acidosis over time, which increased reversibly in response to hypercapnic acidosis. The bottom panel shows 10 second samples of action potentials taken at the time points indicated in the fourth panel. Notice that action potential frequency increased with hypercapnic acidosis, and then decreased back to initial frequency once hypercapnia was removed.
**Figure 19**

The figure depicts the effects of hypercapnia on pH and firing rate. The graph shows a timeline with SNB + CARB and Hypercapnia conditions. The pH values are plotted on the y-axis, ranging from 6.5 to 7.5, and the firing rate is shown on the x-axis. Arrows indicate significant changes in pH and firing rate. The graph also includes a scale for time (5 min) and voltage (5 sec, 20 mV).
firing rate response to HA in NTS neurons from adult rats did not change in the presence of SNB + CARB (n = 16). Fig. 19 shows a sample trace of an activated NTS neuron in the presence of SNB + CARB from an adult rat, which shows a reversible increase in firing rate in response to hypercapnia. The details of the firing rate responses are presented below.

Fig. 20 summarizes the percentage of NTS neurons from adult rats that activated or inhibited by hypercapnia in aCSF (white bar), SNB (gray bar), or SNB + CARB (black bar). We found that SNB by itself (only chemical transmission blocked; 57%) and SNB + CARB (which blocks both chemical and electrical transmission; 56%) had no effect on the percentage of NTS neurons from adult rats that were activated by hypercapnia (aCSF = 57%). However, the percentage of NTS neurons from adult rats that were inhibited by hypercapnia in aCSF vs. in SNB (p<0.001) or SNB + CARB (p<0.001) were significantly different based on a χ² test. However, very few NTS neurons overall are inhibited by hypercapnia (aCSF; 5/56 neurons inhibited; SNB; 1/14 neurons inhibited; SNB + CARB; 3/16 neurons inhibited) so we are not sure how meaningful these differences are. At any rate, under all conditions, relatively few (10-20%) NTS neurons from adult rats are inhibited by hypercapnia. Thus, in terms of percentages of NTS neurons from adults that respond to hypercapnia, the responses measured in aCSF appear to be intrinsic responses, and not dependent on chemical or electrical synaptic transmission.

Fig. 21 summarizes the magnitude of the response to hypercapnia (determined as CI) in activated NTS neurons from adult rats in aCSF (white bar), SNB (gray bar), and SNB + CARB (black bar). We found that the CI of activated NTS neurons in aCSF (177 ± 8%) was the same as the CI in SNB (162 ± 14%) and in SNB + CARB (189 ± 18%).
Figure 20: Summary of the percentage of NTS neurons from adult rats that respond to hypercapnic acidosis. We found that the % activated in the presence of only aCSF (white bar) was not changed by SNB (gray bar) or SNB + CARB (black bar). The percentage of inhibited neurons (9% in aCSF) was decreased by SNB (7%) and increased by SNB + CARB (19%). However, these results are based on a very low n value of inhibited NTS neurons. An “*” indicates values that are statistically significant for values of aCSF (P < 0.001). N values are denoted on each bar and height of bars represent the mean values.
Figure 20
**Figure 21:** Summary of the CI of NTS neurons from adult rats that are activated in response to hypercapnic acidosis. We found that the CI of activated NTS neurons from adult rats in the presence of aCSF only (white bar) was not affected by SNB (gray bar) or SNB + CARB (black bar). Height of bar represents mean CI and error bar represents 1 SEM.
Figure 21

Chemosensitivity Index (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemosensitivity Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>150</td>
</tr>
<tr>
<td>SNB</td>
<td>175</td>
</tr>
<tr>
<td>SNB + CARB</td>
<td>220</td>
</tr>
</tbody>
</table>
summary, in adult rats, both with respect to the magnitude and the percentage of NTS neurons that respond to hypercapnia, the responses seen in aCSF appear to be intrinsic and not dependent on chemical or electrical synaptic input.

\[ \text{pHi and electrophysiological responses to isohydric hypercapnia of NTS neurons from adult rats} \]

Lastly, we measured the pHi and electrophysiological response to isohydric hypercapnia (IH) of NTS neurons from adult rats. A sample record of the pHi and electrophysiological response to isohydric hypercapnia from an NTS neuron from an adult rat is shown in Fig. 22. All experiments with IH were done in the presence of SNB throughout. NTS neurons from adult rats exhibited a similar pattern of pHi response to IH as previously reported for NTS neurons and locus coeruleus neurons from neonatal rats (Ritucci et al., 1997; Filosa et al., 2002). This includes a small hypercapnia-induced acidification followed by pHi recovery, with pHi exhibiting an alkaline overshoot upon return to normocapnia (Fig. 22). This was accompanied by a reversible increase in firing rate induced by hypercapnia (Fig. 22). On average, activated NTS neurons from adult rats showed a smaller pHi response to isohydric hypercapnia (0.11 ± 0.03; n = 10) than NTS neurons from adult rats in response to hypercapnic acidosis (0.21 ± 0.04; n = 7) (Fig. 23A). Isohydric hypercapnia induced pH recovery in 13/19 NTS neurons from adult rats (0.011 ± 0.002 pH unit/min) with 6 neurons having no pH recovery from hypercapnia-induced acidosis (Fig. 23B). This response is very different from the pH recovery of NTS neurons from adult rats to hypercapnic acidosis, where only 4/19 neurons showed at most a slight recovery (0.003 ± 0.004 pH unit/min) (Fig. 23B).

We could not assess the magnitude of neuronal firing rate responses to IH using
Figure 22: The pHᵢ and firing rate response of an individual NTS neuron from an adult rat that was activated by isohydric hypercapnia in the presence of SNB. The top panel shows the experimental protocol used. The second panel shows the pHᵢ response to isohydric hypercapnia over time of the NTS neuron. Notice that there is pHᵢ recovery during isohydric hypercapnia and that once the hypercapnic solution was removed, there was an alkaline overshoot in pHᵢ that returned back to a value above initial pHᵢ. The third panel shows the firing rate response to isohydric hypercapnia over time of the NTS neuron, which increased reversibly in response to isohydric hypercapnia. The bottom panel shows 10 second samples of action potentials taken at the time points indicated in the fourth panel. Notice that action potential frequency increased with isohydric hypercapnia, and then decreased back to initial frequency once the hypercapnic solution was removed.
Figure 22

SNB

Isohydric Hypercapnia

pH 7.25
7.20
7.15
7.35
7.30
7.25
7.20
7.15
7.10

Firing rate (Hz)

1 2 3

1 2 3

5 sec
20 mV

2 min
**Figure 23: A:** The average magnitude of acidification that is induced by either hypercapnic acidosis or isohydric hypercapnia in CO₂-activated NTS neurons from adult rats. Note that IH induced a statistically smaller (*) acidification than HA (P < 0.0001).

**B:** The average rate of pHi recovery from hypercapnia-induced acidification for NTS neurons from adult rats in response to hypercapnic acidosis and isohydric hypercapnia. Very few (4/19) NTS neurons exhibited at most a small pHᵢ recovery from HA-induced acidification whereas a majority (13/19) of NTS neurons showed clear pHᵢ recovery for IH-induced acidification. Of the 13/19 NTS neurons that exhibited pHᵢ recovery in response to the IH-induced acidification, 10/13 NTS neurons exhibited an alkaline overshoot in response to IH removal. In all cases the height of a bar represents either the mean ΔpHᵢ or the mean pHᵢ recovery rate with the error bar being 1 SEM.
Figure 23A

![Graph showing the change in pH (ΔpH) for HA and IH conditions.](graph.png)

Figure 23B

![Graph showing the rate of recovery in pH units per minute for HA and IH conditions.](graph.png)
CI since CI values are normalized to the change in pH\textsubscript{o} and pH\textsubscript{o} does not change with IH. Thus, we quantified the neuronal response to IH by measuring the change in firing rate that is induced by both HA and IH. IH induced an increase in firing rate from 1.3 ± 0.21 Hz to 4.4 ± 0.57 Hz (n=11) (P<0.0001) and hypercapnic acidosis induced an increase in firing rate from 0.5 ± 0.12 Hz to 3.0 ± 0.96 Hz (n=8) (P=0.0167). The increase in firing rate induced by IH (3.1 ± 0.51 Hz) and HA (2.5 ± 0.86 Hz) was not different for activated NTS neurons from adult rats (Fig. 24). We also could look at the percentage of NTS neurons that responded to the two conditions. It was found that 54% of NTS neurons from adult rats were activated in HA (n = 8) and 73% were activated in IH (n = 11), while 13% were inhibited in both cases (n = 2). There was no statistical difference either for the percentage activated or the percentage inhibited by HA or IH.
Figure 24: The average firing rate change induced by either hypercapnic acidosis or isohydric hypercapnia. Both hypercapnic solutions caused a significant increase in firing rate ($P = 0.0167$ for HA and $P < 0.0001$ for IH), but there was no significant difference in the change of firing rate induced by IH compared to HA. The height of a bar represents the mean firing rate and the error bar is 1 SEM.
Figure 24

The figure shows a bar graph comparing the change in firing rate (Hz) between HA and IH conditions. The y-axis represents the firing rate change, and the x-axis represents the conditions HA and IH. The graph indicates a higher firing rate in the IH condition compared to the HA condition.
Discussion

We studied the cellular properties of NTS neurons from adult rats and their intrinsic responses to hypercapnia. The main findings of this study are that compared to NTS neurons from neonatal rats, NTS neurons from adult rats: 1) have a lower initial pHᵢ; 2) have similar responses to IH (reduced acidification, pHᵢ recovery, and increased firing rate); 3) have a similar intrinsic firing rate response to HA. Thus, the chemosensitive response of NTS neurons from adult rats is quite similar to the chemosensitive response of NTS neurons from neonatal rats, indicating that the chemosensitive response of NTS neurons in adults is established early in neonatal development.

*PHi responses of NTS neurons from adult rats*

We found that NTS neurons from adult rats have a steady state pHᵢ (~7.30) that is 0.2 pH unit lower than NTS neurons from neonatal rats (~7.50) (Ritucci et al., 1997). In neonates, steady state pHᵢ of NTS neurons is largely determined by alkalinizing Na⁺/H⁺ exchange (Ritucci et al., 1998). A major role for Na⁺/H⁺ exchange in pHᵢ regulation in NTS neurons from adults is consistent with our finding that all recovery from acidification is Na⁺ dependent (Fig. 15), although we cannot rule out a contribution from Na⁺ driven HCO₃⁻ dependent transports (Putnam, 2001). NTS neurons from neonates are unique among the medullary neurons studied in largely lacking acidifying Cl⁻/HCO₃⁻ exchange (Ritucci et al., 1998), which seemed to account for their very alkaline values of pHᵢ. The lower steady state value of pHᵢ in NTS neurons from adults could result if adult neurons had increased expression of Cl⁻/HCO₃⁻ exchange. Using our experimental approach, we were not able to assess the possible role of HCO₃⁻ dependent transporters
since the commonly used inhibitors (amiloride and DIDS) interfered with fluorescence at the excitation wavelength for pyranine. The role of HCO$_3^-$ transporters in pH$_i$ regulation in NTS neurons from adults is being determined by an ongoing study using BCECF.

*Responses to hypercapnic acidosis and isohydric hypercapnia of NTS neurons from adult rats*

Studies of the pH$_i$ response to hypercapnic acidosis (HA) in NTS neurons from neonatal rats have found a ΔpH$_i$ of 0.26 ± 0.006 pH unit (Mulkey et al., 2004a) in response to HA and a maintained acidification with a lack of pH$_i$ recovery from that acidification (Ritucci et al., 1997; Wiemann et al., 1998; Nottingham et al., 2001). There is no recovery during the HA challenge because the Na$^+$/$H^+$ exchanger (NHE), the predominante pH$_i$ recovery mechanism following acidification in medullary neurons (Ritucci et al., 1997; Putnam, 2001), is inhibited by the extracellular acidification (Ritucci et al., 1998). To our knowledge, there are no studies looking at the pH$_i$ response to HA in individual NTS neurons from adult rats. In the current study, we found that the acidification induced by HA was 0.25 ± 0.011 pH unit, which is almost identical to what was seen in neonates (Mulkey et al., 2004a). We also found that NTS neurons from adult rats lacked pH$_i$ recovery during the HA exposure (Fig. 16). This lack of pH$_i$ recovery was also seen in neurons from neonates (Ritucci et al., 1997; Wiemann et al., 1998; Nottingham et al., 2001). We conclude that the pH$_i$ response to HA in adult rats is similar to neonates and thus does not change during development.

The electrical response to HA has been studied in caudal NTS neurons from neonatal rats. In neonates, about 50% of caudal NTS neurons are activated with a chemosensitivity index (CI) of about 150% and about 10-15% are inhibited with a CI of
57 ± 6% (Conrad et al., 2008). Studies done on NTS neurons from adult rats have only looked at the percentage of neurons that were activated by HA (Dean et al., 1989; Dean et al., 1990; Miles, 1983; Mulkey et al., 2003). In a mix of caudal and more rostral slices, 35% of NTS neurons from adult rats were found to be activated in response to HA (Dean et al., 1990), while in caudal slices only, 53% of NTS neurons from adult rats were activated in response to HA (Mulkey et al., 2003). The current study examined NTS neurons exclusively taken from the caudal region of the NTS from adult rats. We found very similar results to the results from the neonatal studies (Conrad et al., 2008) and the results from Mulkey et al. (2003) in that 57% were activated with a CI of 177 ± 8% and 9% were inhibited with a CI of 63 ± 10%. Thus, we conclude that the electrical response to HA of NTS neurons from adult rats is quite similar to that of NTS neurons from neonates, implying that the electrical response of NTS neurons to hypercapnia also does not appear to change during development. However, while the change of pH\textsubscript{i} and the increase in firing rate in response to hypercapnia are the same in neonates and adults, adults have a lower initial pH\textsubscript{i} than neonates. To the degree of the pH\textsubscript{i} determines firing rate, these findings imply that there has been a shift in pH\textsubscript{i} sensitivity in adult NTS neurons to a lower value of pH\textsubscript{i} compared to neonates.

We also studied the response of NTS neurons from adult rats to a different hypercapnic stimulus, IH, in which pH\textsubscript{o} is maintained constant. The response of IH has been studied in NTS neurons from neonatal rats (Ritucci et al., 1997). IH causes a smaller acidification than HA and unlike the lack of pH\textsubscript{i} recovery seen during HA, there was pH\textsubscript{i} recovery during the IH exposure. When IH was removed, NTS neurons exhibited an alkalinizing overshoot (Ritucci et al., 1997). We found that the pH\textsubscript{i} response
to IH of NTS neurons from adults was similar to the response of neonates, namely a smaller acidification, during IH, and an alkaline overshoot upon return to aCSF (Fig. 22). The presence of pHᵢ recovery with IH-induced acidification but not with HA-induced acidification suggests that as with neonates, pHᵢ recovery mechanism(s) are present in NTS neurons from adult rats but that these mechanism(s) are inhibited by extracellular acidification (Ritucci et al., 1997; 1998).

NTS neurons from adult rats increase their firing rate in response to IH (Fig. 22). We do not have a firing rate response to IH for NTS neurons from neonatal rats, but it is instructive to compare the responses to IH vs. HA for NTS neurons from adults. Despite a smaller change in pHᵢ induced by IH than HA (Fig. 23A), the increase in firing rate is similar (Fig. 24). This pattern of a smaller ΔpHᵢ with IH than with HA but a similar firing rate response to the two acid challenges has also been observed in neurons from the LC (Filosa et al., 2002) and the RTN (Ritucci et al., 2005). Interestingly, it has been shown that there is not a unique relationship between the fall of pHₒ and increased ventilation (Eldridge et al., 1985). These data clearly indicate that the magnitude of the fall of pHᵢ (and pHₒ) are not the sole determinants of the firing rate response of chemosensitive neurons to acid challenges, suggesting that chemosensitive signaling involves multiple factors (Putnam et al., 2004).

Intrinsic chemosensitivity of NTS neurons from adult rats

A major concern with our previous measurements of the hypercapnic response of NTS neurons in slices bathed in aCSF is whether the neuron that we are studying is intrinsically chemosensitive or not. It is possible that the neuron increases its firing rate in response to hypercapnia due to increased excitatory chemical synaptic input or through
direct electrical coupling with another neuron that is intrinsically responding to increased CO$_2$. The effects of chemical synaptic transmission has been assessed in NTS neurons from both neonates (Huang et al., 1997; Conrad et al., 2008) and adults (Dean et al., 1990) using synaptic block medium (SNB). SNB alone has been shown to decrease the initial spontaneous firing rate of NTS neurons from both neonates (Conrad et al., 2008) and adults (Dean et al., 1990). In the current study with adults, however, we found that SNB results in a marked increase in spontaneous firing rate (Fig. 17). The differences in these findings with the previous studies in adults may be due to the inclusion of more rostral slices in the earlier studies. It may be that the local circuit differs in the caudal NTS vs. the more rostral NTS. Differences with the findings done with adults may exist because of the use of dorsal medullary slices by Dean et al. (1990). In that study, the ventral half of the slice was removed. It is possible in our study that the inhibitory input arises from the ventral portion of the slice and would thus not be evident with the preparation used by Dean et al. (1990). Our findings suggest that in intact medullary slices, NTS neurons from neonates receive, on balance, excitatory input while NTS neurons from adults receive inhibitory input.

SNB has little effect on the response to hypercapnia of NTS neurons from rats of all ages. SNB was shown not to alter the pH$_i$ response to hypercapnia of NTS neurons from neonates (Martino et al., 2007) and we found here no effect in NTS neurons from adult rats. Both the magnitude and percentage of the firing rate responses to hypercapnia were similarly largely unaffected by SNB in NTS neurons from neonates (Conrad et al., 2008) although Huang et al. (1997), using more rostral slices, found that SNB reduced the percentage of activated NTS neurons from 34% to 25% (Huang et al., 1997). In
adults, we find no effect of SNB on the pH\textsubscript{i} and firing rate response of NTS neurons to hypercapnia (Figs. 20 and 21), which agree with previous findings that SNB reduced the percentage of adult NTS neurons that are activated by hypercapnia by at most 10% (Dean et al., 1990). Thus, it seems that the response of chemosensitive neurons to hypercapnia does not require synaptic input in NTS neurons from rats of all ages.

In the current study, we also measured the chemosensitive response of caudal NTS neurons from adult rats in the presence of SNB + CARB in order to remove electrical as well as chemical synaptic transmission. Chemosensitive NTS neurons from neonates are preferentially gap junction coupled (Dean et al., 1997; Huang et al., 1997) and connexins (the proteins that form gap junctions) are expressed in NTS neurons from both neonates and adults (Solomon et al., 2001; Solomon, 2003). Carbenoxolone has been shown to effectively uncouple chemosensitive neurons (Dean et al., 2001) and is used widely to block gap junctions (Rekling and Feldman, 1997; Dean et al., 2001; Parisian et al., 2004), although it is known to have other effects (Rouach et al., 2003; Vessey et al., 2004; Pivato et al., 2006). Surprisingly we found that the hypercapnia-induced intracellular acidification was significantly larger in SNB + CARB (Fig. 19) in NTS neurons from adults, although we previously reported no differences in NTS neurons from neonates (Martino et al., 2007). We do not know the basis for this carbenoxolone effect on acidification in adult NTS neurons. It is noteworthy that this increased acidification induced by hypercapnia is not accompanied by a larger firing rate response, further indicating the lack of a simple relationship between pH\textsubscript{i} and firing rate in NTS neurons (Putnam et al., 2004). Regardless, in NTS neurons from adult rats, neither the magnitude nor the percentage of neurons responding to hypercapnia was
affected by SNB + CARB. In NTS neurons from neonates the percentage of NTS neurons that were activated by hypercapnia was decreased by carbenoxolone in rats aged P1-P10, but carbenoxolone had no effect in older neonates (Conrad et al., 2008; Nichols et al., 2008a). These findings suggest that except in very young neonates, the response of NTS neurons to hypercapnia is intrinsic and does not rely on chemical or electrical synaptic input.

**Significance**

This is the first extensive study of intrinsic chemosensitivity in NTS neurons from adult rats. The key finding of this study combined with an earlier study on NTS neurons from neonatal rats (Conrad et al., 2008), is that after about neonatal day P10, chemosensitive NTS neurons are fully developed and express an intrinsic response to hypercapnia. This is of importance for studies of the basis of cellular chemosensitive signaling, which require intrinsically chemosensitive neurons. This pattern of early maturity of chemosensitive NTS neurons is in contrast to the early developmental pattern of the whole animal ventilatory response to inspired CO₂. The exact pattern of this development is uncertain, being either triphasic with a minimum point at about P5-P10 (Stunden et al., 2001) or involving a dramatic increase in the hypercapnic ventilatory response from P15-P20 (Davis et al., 2006). This lack of a match of early development of intrinsic chemosensitivity in NTS neurons vs. the early response to CO₂ could be explained in a couple of different ways. Since central chemosensitivity is a distributed property involved in numerous chemoreceptive sites (Nattie and Li, 2008), it is possible that some other site plays an important role in early development. Sites that have been suggested to be involved in the early response to CO₂ are two catecholaminergic regions...
including the locus coeruleus (Hartzler et al., 2007) and adrenal chromafin cells (Muñoz-Cabello et al., 2005). The other possibility is that while intrinsic chemosensitivity of chemosensitive NTS neurons is fully developed at birth, the interaction of these neurons with the respiratory network requires a week or two to develop fully. Thus, future work delineating the development of the respiratory network will be crucial for evaluating the last possibility.
CHAPTER VI

Chronic hypoxia suppresses the chemosensitive response of individual nucleus tractus solitarius (NTS) neurons from adult rats
**Introduction**

Ventilation is regulated in part by cells called chemoreceptors. The two main stimuli that control breathing and activate these cells are hypoxia (low O$_2$) and hypercapnia (high CO$_2$). Hypoxia is largely sensed by peripheral chemoreceptors in the carotid body.

Output of peripheral chemoreceptors goes to the caudal portion of the nucleus tractus solitarius (NTS) and ultimately can lead to altered ventilation (Lopez-Barneo, 2003). Hypercapnia is largely sensed in the brainstem by central chemoreceptors residing in a variety of areas including the NTS, ventrolateral medulla, retrotrapezoid nucleus, medullary raphé, locus coeruleus, and the pre-Bötzinger region (Dean et al., 1989; 1990; Wellner-Kienitz and Shams, 1998; Mulkey et al., 2004b; Ritucci et al., 2005b; Richerson, 1995; Oyamada et al., 1998; Filosa et al., 2002; Solomon et al., 2000a). These neurons have a fairly standard response to hypercapnia, exhibiting a maintained acidification during the hypercapnic exposure (Putnam et al., 2004; Ritucci et al., 1997) and either increasing (Filosa et al., 2002; Ritucci et al., 2005b) or decreasing (Richerson, 1995; Huang et al., 1997; Conrad et al., 2008) their firing rates. Not all neurons within a region are chemosensitive (CS). In the NTS for instance, between 30-50% of the neurons are CO$_2$ excited, about 10-15% are CO$_2$ inhibited, and the remainder show no firing rate response to hypercapnia (Huang et al., 1997; Conrad et al., 2008; Nichols et al., 2008a; 2008b). The output of chemosensitive neurons is believed to affect the neurons that set the respiratory rhythm (Mulkey et al., 2004b; Shao and Feldman, 2005) and that control the major muscles of breathing (e.g. phrenic motor nerve; Yokota et al., 2001). Thus, activation of chemosensitive neurons by systemic hypercapnia can lead to a homeostatic increase in ventilation, resulting in a return to normocapnia.
One way to study central chemosensitivity is to adapt animals to an environmental condition and then investigate changes at the cellular level. For example, exposure to chronic hypercapnia reduces the ventilatory response to acute hypercapnia (Rezzonico and Mortola, 1989; Putnam et al., 2008), but does not induce any changes in the NTS neuronal response to hypercapnia (Nichols et al., 2008c). In addition, exposure to chronic hypoxia (CHx) results in an increase in the hypoxic ventilatory response (HVR) to acute hypoxia (Reid and Powell, 2005). Interestingly, it appears that CHx also increases the hypercapnic ventilatory response (HCVR) (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998). The cellular bases for these forms of plasticity are not well understood and no prior study has examined the effect of CHx on cellular chemosensitivity.

General effects of CHx on rats include increased hematocrit, hypertension, right ventricular hypertrophy, and hyperventilation (Hunter et al., 1974; Rabinovitch et al., 1979) including an increase in the HVR (Aaron and Powell, 1993; Powell et al., 1998; 2000). It was shown, however, that the increased HVR could not be fully explained by changes in the carotid body (Bisgard and Neubauer, 1995), suggesting involvement of central adaptations to CHx. Further, it has been suggested that the chronic activation of peripheral chemoreceptors may lead to an increased CNS gain after CHx (Powell et al., 1998; Dwinell and Powell, 1999). Since the carotid body output goes to the caudal NTS (Donoghue et al., 1984; Finley and Katz, 1992; Finley et al., 1992; Mifflin, 1992) and the caudal NTS (as opposed to the rostral NTS) is known to play a role in ventilation (Nattie and Li, 2002a), it seems likely that the caudal NTS is involved in ventilatory plasticity.
associated with CHx. Thus, alteration of NTS neurons might mediate the changes in ventilatory response to both hypoxia and hypercapnia in animals adapted to CHx.

In this study, we tested the hypothesis that the effects of CHx on the ventilatory response to hypercapnia are mediated by an increased CO₂ responsiveness (percentage and magnitude as determined by the chemosensitivity index (CI)) of neurons within the NTS, a known site for central chemosensitivity. Our findings did not support this hypothesis. The main findings of this study are that CHx decreases the percentage of NTS neurons that are activated and increases the percentage that are inhibited by hypercapnia. We saw the same changes in percentages of NTS neurons from CHx-adapted animals in the presence of chemical synaptic block medium, suggesting these changes are due to changes in intrinsic neuronal properties. We found that CHx does not affect the CI.

A preliminary report of these findings has previously been published (Nichols et al., 2008d).
Methods

Chronic Adaptation to Hypoxia

Adult male Sprague-Dawley rats (~P50) were exposed to hypobaric hypoxia (0.5 ATA of 21% O₂, which is approximately 0.1 ATA), which we term chronic hypoxia (CHx), for ≥ 7 days. Sixteen groups of 5-6 adult male rats each were adapted to CHx and the hematocrit and body weight were both recorded for each animal prior to euthanasia. Briefly, 5-6 Sprague-Dawley male adult rats were placed in a plexiglas chamber (cylinder 91.4 cm long and 27.9 cm in diameter) in which they were exposed to approximately 0.1 ATA (0.5 ATA of 21% O₂) starting at P50. O₂ was monitored with an electrode connected to an O₂ gauge (Teledyne Analytical Instruments, Industry, CA) and pressure was monitored with a differential electronic manometer (HHP91, Omega, Stamford, CT). All animals were maintained on a 12:12 light:dark cycle and had free access to food and water. Chamber pressure was interrupted for 20-30 minutes 3 times a week for cleaning and replacement of food and water. Each group was left in the chamber for 7 days and then exposure continued until each animal was sacrificed and tested. For controls, 5-6 male adult rats were placed in the chamber with a continuous flow of room air (21% O₂). Temperature and relative humidity were recorded continuously for all groups with HOBO HO8 RH recorders (Onset Computer Corporation, Bourne, MA) placed in the plexiglas chamber. Temperature ranged from 17-23° C and relative humidity ranged from 23-60%. In order to measure hematocrit, blood samples were collected after decapitation by filling heparinized capillary tubes (6 samples per animal) with blood from the carotid artery. These samples were then spun for 3-5 minutes at maximal speed (13,460Xg using a hematocrit centrifuge (IEC)). It was found that the change in
hematocrit above control induced by CHx was significant for each group of rats (Fig. 25) (47.2 ± 0.2% for controls (n=44) which increased 18.7 ± 0.8% for CHx animals) (n=79) (P<0.0001), and body weight was significantly lower for rats adapted to CHx (269 ± 8g for control animals and 230 ± 3g for CHx animals) (P<0.0001). The change in hematocrit that was induced by CHx is similar to the findings of Villafuerte et al. (2007). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01).

Solutions
Artificial cerebral spinal fluid (aCSF) contained the following (in mM): 124 NaCl, 5.0 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 26 NaHCO₃, and 10 glucose, and was equilibrated with 95 % O₂/ 5% CO₂ (pH ~ 7.45 at 37°C). Synaptic blockade (SNB) solution was modified from aCSF with 0.2 mM CaCl₂ and 11.4 mM MgSO₄ in order to block chemical synapses. The whole cell patch intracellular solution contained (in mM): 130 K⁺-gluconate, 10 KCl, 10 HEPES, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, and 2 ATP, (pH = 7.45 at room temperature) (Filosa and Putnam, 2003). The high K⁺/nigericin solution that was used for calibration in the imaging studies contained (in mM): 104 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 25 N-methyl-D-glucamine (NMDG)-HEPES, 25 K-HEPES, 10 glucose, and 0.004 nigericin titrated with KOH or HCl to a pH value of 7.4. The pH-sensitive fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (1 mM) (HPTS, pyranine) was added to the whole cell patch intracellular solution and
Figure 25: Hematocrit was recorded in both chamber control adult rats and adult rats adapted to CHx to verify that hypoxia had been achieved in the adult rats adapted to CHx. The hematocrit was 47 ± 0.21% for chamber control animals. The average change in hematocrit (18.7 ± 0.81% for all CHx animals (n=79) (P<0.0001)) was significantly increased in all sixteen groups of adult rats adapted to CHx. The height of each bar represents the mean change in hematocrit for each group adapted to CHx and the error bars represent 1 SEM.
Figure 25

![Graph showing Δ Hematocrit (%) vs. Group (＃)](image-url)

- Δ Hematocrit (%) on the y-axis.
- Group (＃) on the x-axis.
- Bars represent data for each group with error bars indicating variability.
was purchased from Invitrogen (Eugene, OR). All chemicals were purchased from Sigma (St. Louis, MO) except where indicated.

**Slice preparation**

Slices were prepared and used as previously described (Nichols et al., 2008b). Briefly, control and CHx adult male rats were anesthetized with a brief exposure to CO₂ (100 %), which was followed by rapid decapitation. The brainstem was then removed and submerged in aCSF equilibrated with 5% CO₂ / 95% O₂ gas mixture. Transverse slices (300 μm) were prepared on a vibratome (Pelco 101, series 1000) beginning at the obex and extending rostrally for ~ 1mm and were allowed to recover for at least 1 hour at room temperature in aCSF equilibrated with 5% CO₂ / 95% O₂. Individual slices for study from control and CHx adult male rats were placed in a superfusion chamber on the stage of an upright Nikon Optiphot-2 microscope. Slices were immobilized with a nylon grid and superfused at ~2.4 ml/min with aCSF equilibrated with 5% CO₂ / 95% O₂ (pH 7.45 at 37° C).

Individual neurons from NTS were then studied. To study the response of these neurons to hypercapnia, we used a protocol that consisted of a 5 minute exposure to aCSF equilibrated with 5% CO₂ / 95% O₂, a 10-15 minute exposure to aCSF equilibrated with 15% CO₂ / 85% O₂, and a 5-10 minute exposure to aCSF equilibrated with 5% CO₂ / 95% O₂. For SNB studies, this protocol was then repeated in the same neuron with SNB solution.

**Imaging of Fluorescent Neurons**

pHᵢ was measured as previously described (Ritucci et al., 2005b). Individual NTS neurons were loaded with 1mM of the pH-sensitive dye, pyranine, through a whole cell
patch pipette. Loaded neurons were excited and alternately exposed for 1s to light of wavelength 450 ± 10 and 410 ± 10 nm using a Sutter Lambda 10-2 filter wheel. Emitted fluorescence at 515 ± 10 nm was collected and processed using MetaFluor 7.1.4.0 software (Meta Imaging), and the 450/410 fluorescence ratios (R_{fl}) was determined. We used a one point calibration (pH of 7.4) using the high K+/nigericin technique (Thomas et al., 1979), to determine initial pH of NTS neurons from adult rats that had been adapted to CHx. R_{fl} values measured at the beginning of the experiment were divided by the R_{fl} value at pH 7.4 measured at the end of each experiment, which gave normalized R_{fl} (N_{fl}). We used the equation (pH = 7.4969 + log (N_{fl} -0.2003)/(2.0194 - N_{fl}); r^2 = 0.99) (Nichols et al., 2008b) to convert N_{fl} into pH. A pH versus time plot was made in Microsoft Excel, from which the pH recovery rate from CO2-induced acidification of an individual neuron could be estimated from the slope of a linear fit to the pH versus time trace during acute hypercapnia (at least 5 points starting at minimum pH).

**Electrophysiological Studies**

The blind whole-cell patch clamp technique was used to measure neuronal membrane potential (V_m) and integrated firing rate as described previously (Blanton et al. 1989; Nichols et al., 2008b). The experimental setup that was used has been previously described (Dean et al., 1997; Huang et al., 1997; Filosa et al., 2002; Conrad et al., 2008; Nichols et al., 2008b). Briefly, a whole cell patch pipette (5 MΩ) was fabricated from borosilicate glass using a Narishige PP-830 dual stage pipette puller. The pipette was filled with whole cell patch solution (see above). Positive pressure was maintained on the pipette while a -0.1nA pulse (30 msec, 5 Hz) was applied. Tip impedance increased as the pipette approached a neuron, which was indicated by a 1-2 mV downward
deflection. Negative pressure was applied to the pipette to obtain a giga-ohm seal, brief suction was applied to the pipette to rupture the membrane, and then $V_m$ and integrated firing rate were measured throughout the experiment. Integrated firing rate (Hz) was determined from the $V_m$ trace in 10s bins using a window discriminator (FHC model 700B), which was then analyzed using pClamp 8.2 software. Viable neurons had a stable $V_m$ of between -40 and -60 mV and fired action potentials that crossed through zero. The electrophysiological response to hypercapnia was quantified using two measures: percentage of neurons activated by acute hypercapnia, and the magnitude of the firing rate response to acute hypercapnia, calculated as the chemosensitivity index or CI according to the equation of Wang and Richerson (1999). A neuron was designated as activated if its CI was greater than 120% or inhibited if its CI was less than 80%.

**Statistical Analysis**

Values are reported as means ± S.E.M. $\chi^2$ tests were used to compare differences between the percentages of neurons that respond to hypercapnia. Student’s t-tests were used to compare differences between two means with a level of significance of $P<0.05$. Differences between three or more means were determined by one-way ANOVA. If significant differences existed, then multiple comparisons were done using Tukey's method with a level of significance of $P<0.05$. 

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Results

*pH*\textsubscript{i} and electrophysiological responses to acute hypercapnia of NTS neurons from control and CHx-adapted adult rats in aCSF

The initial *pH*\textsubscript{i} of NTS neurons from control (7.30 ± 0.03; n = 27) and CHx-adapted (7.35 ± 0.06 pH units; n = 12) adult rats did not differ. Both values for adults were somewhat less alkaline than previously reported values from neonates (7.49 ± 0.02; Ritucci et al., 1997), as previously noted (Nichols et al., 2008b).

A sample recording of the *pH*\textsubscript{i} and firing rate response to acute hypercapnia of a CO\textsubscript{2}-activated NTS neuron from a control adult rat is shown in Fig. 26. This shows a typical response to hypercapnia (15% CO\textsubscript{2}) with a maintained acidification without *pH*\textsubscript{i} recovery and a reversible increase in firing rate. A similar response of *pH*\textsubscript{i} and firing rate to hypercapnia in an activated NTS neuron from adult rats adapted to CHx is shown in Fig. 27. On average, NTS neurons from adult rats adapted to CHx showed a similar *pH*\textsubscript{i} response to 15% CO\textsubscript{2} compared to NTS neurons from control adult rats, acidifying by 0.26 ± 0.013 pH units (CHx, n = 89) compared to 0.28 ± 0.018 pH units (control, n = 47) (data not shown). NTS neurons from control adult rats exhibited a lack of *pH*\textsubscript{i} recovery (-0.005 ± 0.001 pH unit/min; n = 47) (data not shown). Similarly, NTS neurons from adult rats adapted to CHx exhibited a lack of *pH*\textsubscript{i} recovery (-0.009 ± 0.002 pH unit/min; n = 89) (data not shown). Upon return to normocapnia, the *pH*\textsubscript{i} of NTS neurons from control and CHx adapted adult rats returned to initial values with no apparent overshoot (Figs. 26 and 27), consistent with a lack of *pH*\textsubscript{i} recovery during hypercapnia (Boron and De Weer, 1976; Ritucci et al., 1997).
Figure 26: The pH\textsubscript{i} and firing rate responses of an individual NTS neuron that was activated by hypercapnic acidosis from a chamber control adult rat. The top panel shows the experimental protocol used. The second panel shows the pH\textsubscript{i} response to hypercapnic acidosis over time of the NTS neuron, which was a maintained acidification with a lack of pH\textsubscript{i} recovery. Notice that once the hypercapnic solution was removed, pH\textsubscript{i} returned back towards initial pH\textsubscript{i}. The bottom panel shows the firing rate response to hypercapnic acidosis over time of the NTS neuron, which was a reversible increase in firing rate in response to hypercapnic acidosis.
Figure 26

The figure shows the changes in pH and firing rate over time under different conditions.

- **Control** condition shows a relatively stable pH level.
- **Hypercapnia** condition causes a significant decrease in pH.
- **Control** condition returns after the hypercapnia period.

The firing rate also shows variations, with a peak during the hypercapnia phase and a return to baseline levels afterward.
**Figure 27:** The pHᵢ and firing rate responses of an individual NTS neuron that was activated by hypercapnic acidosis from an adult rat adapted to CHx. The top panel shows the experimental protocol used. The second panel shows the pHᵢ response to hypercapnic acidosis over time of the NTS neuron, which was a maintained acidification with a lack of pHᵢ recovery. Notice that once the hypercapnic solution was removed, pHᵢ returned back towards initial pHᵢ. The bottom panel shows the firing rate response to hypercapnic acidosis over time of the NTS neuron, which was a reversible increase in firing rate in response to hypercapnic acidosis.
Figure 27

Graph showing the effect of Hypercapnia on pH and Firing rate over time. The pH decreases during Hypercapnia, followed by a recovery period back to control levels. Similarly, the firing rate increases during Hypercapnia and decreases back to control levels.
We measured the integrated firing rate response to acute hypercapnia in NTS neurons from adult rats. NTS neurons were classified (based on their integrated firing rate response to hypercapnia) as activated (firing rate increased by > 20%) (Figs. 26 and 27), inhibited (firing rate decreased by > 20%) (Figs. 28 and 29), or non-chemosensitive (firing rate changed by < 20%) (sample not shown). Fig. 28 shows the typical response of a control NTS neuron that is inhibited by hypercapnia with a typical maintained intracellular acidification and a reversible reduction of firing rate. A similar response is seen in an inhibited NTS neuron from a CHx-adapted rat (Fig. 29). NTS neurons, both activated and inhibited from CHx animals respond similarly to NTS neurons from control animals, indicating that CHx does not induce a major change in the way that NTS neurons respond to hypercapnia.

We assessed neuronal responses to acute hypercapnia in two ways, determining the percentage of neurons that respond to and the magnitude of their response to acute hypercapnia. Previously, we found that NTS neurons from adult rats were: 57% activated (n = 32) and 9% inhibited (n = 5) (Nichols et al., 2008b) by hypercapnia. In this study we found that our controls showed similar percentages with 46% activated (n = 18; Fig. 30A) and 8% inhibited (n = 3; Fig. 30B). Interestingly, we found that these percentages were significantly (P<0.001) altered in NTS neurons from CHx-adapted neurons, with a decrease in the percentage activated to 34% (n = 30; Fig. 30A) and an increase in the percentage inhibited to 28% (n = 24; Fig. 30B). The magnitude of the response of NTS neurons to hypercapnia was quantified by calculating the chemosensitivity index (CI) (Wang and Richerson., 1999). Previously, we showed that the average CI for activated and inhibited NTS neurons from adult rats was 177 ± 8% and
**Figure 28:** The pH\textsubscript{i} and firing rate responses of an individual NTS neuron that was inhibited by hypercapnic acidosis from a chamber control adult rat. The top panel shows the experimental protocol used. The second panel shows the pH\textsubscript{i} response to hypercapnic acidosis over time of the NTS neuron, which was a maintained acidification with a lack of pH\textsubscript{i} recovery. Notice that once the hypercapnic solution was removed, pH\textsubscript{i} returned back towards initial pH\textsubscript{i}. The bottom panel shows the firing rate response to hypercapnic acidosis over time of the NTS neuron, which reversibly decreased in response to hypercapnic acidosis.
Figure 28

[Graph showing pH and firing rate over time with control and hypercapnia periods.]
**Figure 29:** The pH$_i$ and firing rate responses of an individual NTS neuron that was inhibited by hypercapnic acidosis an adult rat adapted to CHx. The top panel shows the experimental protocol used. The second panel shows the pH$_i$ response to hypercapnic acidosis over time of the NTS neuron, which was a maintained acidification with a lack of pH$_i$ recovery. Notice that once the hypercapnic solution was removed, pH$_i$ returned back towards initial pH$_i$. The bottom panel shows the firing rate response to hypercapnic acidosis over time of the NTS neuron, which reversibly decreased in response to hypercapnic acidosis.
Figure 29

- **pH<sub>i</sub>**
  - Control
  - Hypercapnia
  - Control

- **Firing rate (Hz)**
  - Time (min)

Graph showing the changes in pH<sub>i</sub> and firing rate over time during control and hypercapnia phases.
Figure 30: **A**: The percentage of NTS neurons from control adult rats (white bar) and adult rats adapted to CHx (black bar) that were activated by hypercapnic acidosis. The N values are denoted on each bar. Notice that the percentage activated by hypercapnic acidosis is significantly decreased by CHx (* = P < 0.001). **B**: The percentage of NTS neurons from control adult rats (white bar) and adult rats adapted to CHx (black bar) that were inhibited by hypercapnic acidosis. The N values are denoted on each bar. Notice that the percentage inhibited by hypercapnic acidosis is significantly decreased by CHx (* = P < 0.001). The total number of activated or inhibited (N) neurons in each group is given in the bar. **C**: The chemosensitivity index of NTS neurons from control adult rats and adult rats adapted to CHx that were activated by hypercapnic acidosis. **D**: The chemosensitivity index of NTS neurons from control adult rats and adult rats adapted to CHx that were inhibited by hypercapnic acidosis. Notice that the CHx does not affect the chemosensitivity index of NTS neurons activated (**C**) or inhibited (**D**) by hypercapnic acidosis. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM.
Figure 30

A. Percentage (%)

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<th>Control</th>
<th>Chronic hypoxia</th>
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<td>18/39</td>
<td>30/87</td>
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B. Percentage (%)

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<th>Control</th>
<th>Chronic hypoxia</th>
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<td>3/39</td>
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C. Chemosensitivity Index (%)

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<th>Control</th>
<th>Chronic hypoxia</th>
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D. Chemosensitivity Index (%)
63 ± 10% respectively (Nichols et al., 2008b). In this study, we found similar control values for CI for activated (166 ± 11%; Fig. 30C) and for inhibited (45 ± 15%; Fig. 30D) NTS neurons from adult rats. Values of CI were unchanged in CHx-adapted rats, with values of 160 ± 6% (Fig. 30C) and 55 ± 3% (Fig. 30D) for activated and inhibited NTS neurons from adult rats, respectively. Activated NTS neurons in 5% CO$_2$ from control and CHx-adapted rats had a similar basal integrated firing rate (0.76 ± 0.10 Hz and 0.56 ± 0.07 Hz respectively) that increased significantly in response to 15% CO$_2$ (2.42 ± 0.56 Hz and 1.58 ± 0.19 Hz respectively), and returned towards initial values of 1.37 ± 0.39 Hz (control) and 0.88 ± 0.12 Hz (CHx) upon return to 5% CO$_2$ (data not shown). There were no significant differences in firing rates between control and CHx values in activated NTS neurons at any level of CO$_2$. Inhibited NTS neurons from control and CHx rats had a similar basal integrated firing rate in 5% CO$_2$ (0.55 ± 0.13 Hz and 0.94 ± 0.22 Hz respectively) that fell significantly in response to 15% CO$_2$ (0.03 ± 0.03 Hz and 0.32 ± 0.10 Hz respectively), and returned towards initial values of 0.79 ± 0.54 Hz (control) and 0.90 ± 0.19 Hz (CHx) upon return to 5% CO$_2$ (data not shown). The firing rate in response to 15% CO$_2$ was significantly lower for control rats compared to CHx rats (P=0.0055) and was the only significant difference in firing rates between control and CHx values. Non-chemosensitive neurons from control and CHx animals had a similar basal integrated firing rate in 5% CO$_2$ (1.75 ± 0.28 Hz and 1.09 ± 0.24 Hz respectively), which did not change significantly in 15% CO$_2$ (1.69 ± 0.29 Hz and 1.12 ± 0.23 Hz respectively), and remain unchanged upon return to 5% CO$_2$ (1.60 ± 0.26 Hz and 1.26 ± 0.24 Hz respectively) (data not shown).
pH, and firing rate responses to acute hypercapnia of NTS neurons from control and CHx-adapted adult rats in synaptic blockade medium

We measured the impact of chemical synaptic transmission on the response to hypercapnia of NTS neurons from CHx-adapted rats since we observed significant changes in the percentage of neurons that respond to hypercapnia (Fig. 30A and B). Thus, we blocked chemical transmission using synaptic blockade medium (SNB). A sample recording of the basal firing rate response to SNB of NTS neurons can be seen in Fig. 31A and 31B for control and CHx animals, respectively. Basal firing rate increases in response to SNB (indicated by SNB in Fig. 31A and 31B). The average basal firing rate of NTS neurons from control rats was 1.38 ± 0.21 Hz (n = 22) (Fig. 31C) and for CHx rats was 0.96 ± 0.17 Hz respectively (n = 22) (Fig. 31C). NTS neuronal firing rate increased significantly in the presence of SNB to 2.76 ± 0.47 Hz for control and 2.96 ± 0.56 Hz for CHx-adapted rats. These data suggest that NTS neurons in slices from adult rats receive tonic inhibitory input.

SNB had no effect on basal pH, of NTS neurons from control animals (7.13 ± 0.03 pH units for control animals in aCSF versus 7.10 ± 0.03 pH units for control animals in the presence of SNB; n = 19) (data not shown). SNB slightly acidified basal pH, of NTS neurons from CHx animals (7.21 ± 0.02 pH units for CHx animals in aCSF versus 7.15 ± 0.02 pH units for CHx animals in the presence of SNB; n = 26; P=0.0004) (data not shown).

For SNB experiments, a neuron’s chemosensitive response was first studied in the absence of SNB, which was followed by the same experiment in the presence of SNB. Before the chemosensitive response was measured in the presence of SNB, the firing rate
**Figure 31:** A. Effect of SNB on basal firing rate of an NTS neuron from a control adult rat. Exposure to SNB solution is denoted by the arrow. Notice that SNB causes an increase in basal firing rate. B. Effect of SNB on basal firing rate of an NTS neuron from an adult rat adapted to CHx. Exposure to SNB solution is denoted by the arrow. Notice that SNB causes an increase in basal firing rate. C. The average basal firing rate for NTS neurons from control animals (white bar) significantly increases in the presence of SNB (gray bar) (* = P = 0.0035). The average basal firing rate for NTS neurons from adult rats adapted to CHx (black bar) also significantly increases in the presence of SNB (gray bar) (* = P = 0.0004). The height of each bar represents the mean firing rate for that group and the error bars represent 1 SEM.
Figure 31

A. 

B. 

C. 

Firing rate (Hz)

Control Chronic hypoxia

* *
was decreased back towards initial firing rate by injecting negative DC current. It was found that SNB did not affect the magnitude of acidification caused by hypercapnia in NTS neurons from either control animals or CHx animals (0.27 ± 0.03 pH units for control animals and 0.27 ± 0.02 pH units for CHx animals in the presence of aCSF and 0.32 ± 0.02 pH units for control animals and 0.31 ± 0.02 pH units for CHx animals in the presence of SNB; n = 19 for control and n = 26 for CHx). Additionally, there was a lack of pHᵢ recovery in NTS neurons from both control (-0.005 ± 0.002 pH unit/minute in aCSF and -0.011 ± 0.003 pH unit/minute in the presence of SNB) and CHx animals(-0.011 ± 0.002 pH unit/minute in aCSF and -0.009 ± 0.002 pH unit/minute in the presence of SNB). Upon return to normocapnia in aCSF and SNB, the pHᵢ of NTS neurons from both control and CHx animals returned towards initial values with no apparent overshoot (data not shown).

The integrated firing rate response to hypercapnic acidosis in NTS neurons from control and CHx adult rats did not change in the presence of SNB. We assessed neuronal responses to hypercapnic acidosis as described above (determining the percentage of neurons that respond to and the magnitude of their response to acute hypercapnia) (Fig. 32). We found that NTS neurons from control animals were 50 % activated (7/14) in the absence of SNB and 57% activated (8/14) in the presence of SNB, whereas NTS neurons from CHx animals were 30% activated (6/20) in the absence of SNB and 30 % activated (6/20) in the presence of SNB (Fig. 32A). NTS neurons from control animals were 14 % inhibited (2/14) in the absence of SNB and 14% inhibited (2/14) in the presence of SNB, whereas NTS neurons from CHx animals were 25% inhibited (5/20) in the absence of SNB and 30 % inhibited (6/20) in the presence of SNB (Fig. 32B). Notice that SNB does
**Figure 32:** A: The percentage of NTS neurons from control adult rats that were activated by hypercapnic acidosis in the absence (white bar) and presence of SNB (gray bar) and NTS neurons from adult rats adapted to CHx that were activated by hypercapnic acidosis in the absence (black bar) and the presence (gray bar) of SNB. B: The percentage of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in the absence (white bar) and presence of SNB (gray bar) and of NTS neurons from adult rats adapted to CHx that were inhibited by hypercapnic acidosis in the absence (black bar) and the presence (gray bar) of SNB. Notice that SNB does not affect the percentage of neurons that respond within control or CHx animals. C: The chemosensitivity index of NTS neurons from control adult rats that were activated by hypercapnic acidosis in the absence (white bar) and presence (gray bar) of SNB and of NTS neurons from adult rats adapted to CHx that were activated by hypercapnic acidosis in the absence (black bar) and presence (gray bar) of SNB. D: The chemosensitivity index of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in the absence (white bar) and presence (gray bar) of SNB and of NTS neurons from adult rats adapted to CHx that were inhibited by hypercapnic acidosis in the absence (black bar) and presence (gray bar) of SNB. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM. There are no error bars for the CI of inhibited neurons from control animals in the absence and presence of SNB because the two inhibited neurons in the absence and presence of SNB had the same CI. Notice that the CHx does not affect the chemosensitivity index of NTS neurons activated or inhibited by hypercapnic acidosis.
not affect the percentage of neurons that respond to hypercapnic acidosis in either control or CHx animals (Fig. 32A and 32B). The magnitude of the response of NTS neurons to hypercapnia was again quantified by calculating the CI. The CI for activated NTS neurons was 164 ± 17% in the absence of SNB and 204 ± 21% in the presence of SNB from control animals and 155 ± 13% in the absence of SNB and 178 ± 20% in the presence of SNB from CHx animals (Fig. 32C). Inhibited NTS neurons from control animals had a CI of 51% in the absence of SNB and 69% in the presence of SNB, whereas those from CHx animals had a CI of 55 ± 3% in the absence of SNB and 52 ± 5% in the presence of SNB (Fig. 32D). Thus, we found that SNB had no effect on the chemosensitive response so we conclude that chemical synaptic transmission is not required for the NTS neuronal chemosensitive response in control and CHx animals. Further, the CHx-induced changes in percentage neurons that are activated or inhibited by hypercapnic acidosis are not due to changes in chemical synaptic transmission. Therefore, the responses to hypercapnic acidosis of NTS neurons and the changes in these responses induced by CHx seem to involve changes of the intrinsic properties of these neurons.
Discussion

The main findings of this study of the effect of CHx on NTS neurons from adult rats include: 1) CHx induces a decrease in the percentage of activated NTS neurons, 2) CHx results in an increase in the percentage of inhibited NTS neurons, and 3) CHx does not alter the firing rate response of NTS neurons to SNB. Thus, CHx suppresses the adult chemosensitive response of NTS neurons and this suppression does not depend on chemical synaptic transmission.

It is known that CHx causes a decrease in arterial $P_{CO_2}$ ($Pa_{CO_2}$) (which normally inhibits breathing), however the mechanism of how $Pa_{CO_2}$ is controlled after chronic hypoxia is not well understood (Powell et al., 1998; Hansen and Sander, 2003). Further, CHx causes an increase in normoxic ventilatory drive, an increased hypoxic ventilatory response, and an increased hypercapnic ventilatory response (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998; Reid and Powell, 2005). The role of the peripheral chemoreceptors, found in the carotid body, in this response to CHx has been studied and CHx results in an increase in O$_2$ sensitivity of these cells (Smith et al., 1986; Bisgard, 2000). It is known that caudal NTS neurons receive afferent input from the carotid bodies (Mifflin, 1992; Donoghue et al., 1984) and therefore these NTS neurons will receive increased afferent input in response to CHx (Powell, 2007). However, it is not known if CHx causes a change in the chemosensitive response of caudal NTS neurons. Thus, this is the first study to quantify the chemosensitive response of NTS neurons after chronic hypoxia.
Possible change in network properties

We found that CHx resulted in fewer CO₂-activated and more CO₂-inhibited NTS neurons. This finding seems at odds with the whole animal ventilatory response to CHx, which involves an increase in both the HVR and HCVR (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998; Reid and Powell, 2005). A correlation of cellular chemosensitive responses of neurons from one brainstem region with whole animal ventilatory responses is complicated by the likelihood that there are many chemosensitive brainstem regions and the role of each in ventilatory control is not known (Nattie and Li, 2006). We have only studied one chemosensitive region and its response to hypercapnia after CHx and our results suggest that the NTS neuronal response to hypercapnia is reduced (Fig. 30A and 30B). Thus, it is possible that CHx could alter the response of another chemosensitive region or alter the properties of the whole respiratory network, which could lead to the increase in the HCVR and/or HVR.

Hypoxic ventilatory response

It is known that the whole animal response to hypoxia is increased after CHx and this is mainly sensed by peripheral chemoreceptors in the carotid bodies (Smith et al., 1986; Bisgard, 2000). Also, it is known that CHx increases the O₂ sensitivity of the carotid bodies (Smith et al., 1986; Bisgard, 2000), thus it has been suggested that afferent input will be tonically increased (Powell, 2007), which could cause central ventilatory plasticity. Peripheral afferent input terminates first in the caudal NTS (Donoghue et al., 1984; Finley and Katz, 1992; Finley et al., 1992; Mifflin, 1992). Thus, it may be that the NTS is involved in central ventilatory plasticity associated with CHx as well. It is difficult to relate our findings to plasticity of the HVR since it is not known if the NTS
neurons that are receiving input from the carotid body are CO2-activated, CO2-inhibited or non-chemosensitive NTS neurons. One interesting possibility is if the carotid afferents terminate on CO2-inhibited neurons. Hypoxia-induced hyperventilation causes a lowered PaCO\textsubscript{2} during CHx. It is possible that CO2-inhibited NTS neurons could be “activated” by this hypocapnia (Richerson et al., 2001) and could contribute to increased central gain during the HVR. It is also possible that NTS neurons from CHx-adapted rats have altered intrinsic properties unrelated to their CO\textsubscript{2} responsiveness that contributes to increased central gain. In this regard, it is noteworthy that CO2-sensitive NTS neurons have been shown to be sensitive also to hyperoxia (Mulkey et al., 2003) and to hypoxia (Dean et al., 1991; Pascual et al., 2002), and that O\textsubscript{2} sensitivity involves a distinct cellular mechanism from CO\textsubscript{2} sensitivity (Mulkey et al., 2003). Thus, it might be that CHx alters the O\textsubscript{2} sensitivity of NTS neurons, a possibility that we did not study. It has previously been shown in carotid body cells that CHx reduces K\textsubscript{ca} channel expression (Wyatt et al., 1994). It is clear that studies of the O\textsubscript{2} sensitivity of NTS neurons from CHx-adapted rats and of the NTS neuronal targets of carotid afferent input will go far in delineating the role of NTS neurons in central plasticity associated with CHx and altered HVR responses.

**Hypercapnic ventilatory response**

At this time, it is not clear how our results correlate with the whole animal response to hypercapnia after CHx, since it has been shown that CHx increases the ventilatory response to hypercapnia (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998). Whereas our data suggest that the output of CO2-sensitive NTS neurons in response to hypercapnia is reduced by CHx. It is possible that the increased HCVR after CHx is mediated by cells located in another CO2-sensitive region.
One possible region that could regulate the hypercapnic ventilatory response are the carotid bodies. Classically, the carotid bodies were believed to mainly sense changes in O₂, but recently they have been found to also contribute substantially to CO₂ chemoreception (Fatemian et al., 2003; Dempsey, 2004; Nattie, 2006b; Smith et al., 2006). For instance, when looking at the overall sensitivity to hypercapnia, it has been found that central chemoreceptors sense about 63% and peripheral chemoreceptors sense the other 37% (Smith et al., 2006). While it is known that CHx increases the O₂ sensitivity of the carotid bodies (Smith et al., 1986; Bisgard, 2000), it is not known how their CO₂-sensitivity is changed. Thus, it seems reasonable to postulate that the carotid bodies themselves may be, at least in part, responsible for the increased HCVR after CHx.

Another CO₂-sensitive region that may be responsible for increasing the HCVR after CHx is the retrotrapezoid nucleus (RTN) (Mulkey et al., 2004b; Ritucci et al., 2005b). It is known that RTN neurons receive input from peripheral chemoreceptors, although afferent output first gets relayed through the caudal NTS (Stornetta et al., 2006; Takakura et al., 2006). It would thus be of interest to know how CHx alters the CO₂-responsiveness of RTN neurons.

**Normoxic ventilatory response and decreased PaCO₂**

An interesting question that has been raised is how a decrease in PaCO₂ (which normally inhibits breathing) is compatible with an increased normoxic ventilatory response? One possible explanation for the increased ventilatory response is that neurons normally inhibited by hypercapnia would be activated by hypocapnia. Previously, it has been found that medullary raphé neurons that were inhibited by hypercapnia (9% CO₂)
were activated by hypocapnia (3% CO₂) (Richerson et al., 2001). Thus, we suggest that the increase in the percentage of NTS neurons that are inhibited by hypercapnia that occurs after CHx may be in response to the prolonged exposure to a decrease in PaCO₂ caused by CHx. Further, we hypothesize that NTS neurons that are inhibited in response to hypercapnic acidosis will be activated in response to hypocapnic alkalosis and could help to drive breathing during periods of reduced PaCO₂.

**CHx-adapted animals: ideal system to study inhibited neurons**

Although it is not clear at this time the role that increased CO₂-inhibited neurons play in the ventilatory response after CHx, it is clear that we have identified a model system in which to study inhibited neurons. In most chemosensitive regions, inhibited neurons are few in neonates. For instance, CO₂-inhibited neurons in slices comprise about 10-15% of NTS neurons (Conrad et al., 2008; Nichols et al., 2008b), about 15% of medullary raphé neurons (Wang and Richerson, 1999) and 5-15% of LC neurons (Hartzler et al., 2007). Here we showed that the percentage of NTS neurons inhibited by hypercapnia increased to 30% after exposure to CHx. It has been previously been difficult to study the mechanism of the response of inhibited neurons to hypercapnia because they were so few in number. Employing rats adapted to CHx, the NTS contains a sufficiently high proportion of CO₂-inhibited neurons to allow the study of the previously unexplored mechanism of how hypercapnia results in decreased neuronal firing rate. Additionally, we found that the shift in the percentage of activated and inhibited neurons was not due to chemical synaptic input, so the response we observe in NTS neurons after CHx must result from a shift in intrinsic neuronal properties. We
suggest that the shift observed with the cellular properties of activated and inhibited neurons is probably due to a change in the expression of CO$_2$/H$^+$-sensitive ion channels.

In summary, we have shown that in rats adapted to CHx there is a shift in the proportions of hypercapnia-activated and hypercapnia-inhibited NTS neurons. It is unclear what role this shift of NTS neuronal properties plays in the changes of ventilatory responses after CHx, although an increased proportion of hypercapnia-inhibited NTS neurons may facilitate normal breathing during hypocapnic conditions (as seen with CHx) as hypocapnia will activate these neurons. This study also broadens our understanding of the chemosensitive response and adds further complexity to understanding what role chemosensitive regions have on the ventilatory response after CHx. Finally, this study offers a model system for studying the mechanism of how neurons are inhibited by elevated CO$_2$/H$^+$. 
CHAPTER VII

The effect of substance P on the chemosensitive response of individual nucleus tractus solitarius (NTS) neurons from control and chronic hypoxia adapted adult rats
**Introduction**

The neuropeptide substance P is involved in several physiological processes including cardiovascular, respiratory, gastrointestinal, and nociceptive processes and even modulates the immune response (Pernow, 1983; Lagercrantz et al., 1991; Otsuka and Yoshioka, 1993; Hökfelt et al., 2001). Substance P has been shown to play a role in ventilation in that it can accentuate respiratory rhythm via activation of the pre-Bötzinger region as well as depolarize all pre-Bötzinger neurons recorded *in vitro* (Gray et al., 1999; Murakoshi et al., 1985, Pena and Ramirez, 2004). When substance P was applied to the caudal NTS respiratory frequency increased (Hedner et al., 1984; Morin-Surun et al., 1984; Yamamoto and Lagercrantz, 1985; Chen et al., 1990a). The role of substance P in breathing has also been studied by local injections of saporin conjugated to substance P, resulting in lesioning of NK1 expressing neurons (Gray et al., 1999, 2001; Wang et al., 2002; Nattie and Li, 2002b; McKay et al., 2005). When NK1 expressing pre-Bötzinger neurons were lesioned, it was found that breathing rhythm was completely disrupted *in vivo* (Gray et al., 2001; McKay et al., 2005). Additionally, it has been found that when saporin (complexed to substance P) was microinjected into a different chemoreceptor site, the RTN, of an adult rat, the HCVR (both frequency and tidal volume) was decreased (Nattie and Li, 2002b). Thus, it can be hypothesized that substance P plays a role in central chemosensitivity.

Substance P is known to be stored and released from carotid body afferent neurons located in the proximal petrosal ganglion and jugular ganglion (Finley et al. 1992). These peripheral afferents have their first primary synapse in the caudal portion of the NTS (Finley et al. 1992; Finley and Katz, 1992; Dobbins and Feldman, 1994;
Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003), a known site for central chemosensitivity (Nattie and Li, 2002a; Conrad et al., 2008; Nichols et al., 2008b). However, it is not known if the afferents synapse on caudal NTS neurons that are responsive to hypercapnia.

The primary receptor that substance P binds to is the NK1 receptor. NK1 receptors have been found in various regions involved in ventilation including an area involved in rhythm generation (pre-Bötzinger region) and regions involved in central chemosensitivity (Nakaya et al., 1994; Nattie and Li, 2002b). Thus, NK1 receptors have been proposed as markers of chemosensitive neurons and neurons involved in rhythm generation (Nakaya et al., 1994). NK1 receptors are metabotropic receptors which are G-protein linked (Wang and Marvizon, 2002; Roosterman et al., 2004). NK1 receptors are susceptible to desensitization due to receptor downregulation with prolonged exposure to substance P (Mazzone et al., 1998; Mazzone and Geraghty, 2000). One such exposure that could cause downregulation is a chronic exposure to hypoxia. Chronic hypoxia causes increased release of substance P from the peripheral afferents that leave the carotid body (Mifflin, 1997), a known site to contain cells sensitive to hypoxia, implying that there will be increased substance P binding on caudal NTS neurons. It has been shown that increased binding of substance P to NK1 receptors can cause desensitization both in vitro and in vivo (Grady et al., 1995; Mantyh et al., 1995; Mazzone et al., 1998). Furthermore, desensitization due to receptor downregulation could lead to decreased excitatory effects that substance P would normally have on respiration.

We have recently reported that chronic exposure to hypoxia causes a suppression of the chemosensitive response of NTS neurons from adult rats (Nichols et al., 2008c).
We wanted to know if desensitization of the response to substance P (Mazzone et al., 1998) played a role in the suppression of the chemosensitive response we observe in NTS neurons after chronic hypoxia. Therefore, the main goal of this study was to examine the role of substance P in the chemosensitive response of NTS neurons from control and CHx-adapted rats. We found that although substance P modulates basal firing rate of NTS neurons from control and CHx-adapted adult rats, substance P had no effect on the firing rate response to hypercapnic acidosis.

A preliminary report of these findings has previously been published (Nichols et al., 2007).
Methods

Chronic Adaptation to Hypoxia

Chronic adaptation to hypoxia was studied as described previously (Nichols et al., 2008c). Briefly, 5-6 Sprague-Dawley male adult rats was placed in a plexiglas chamber (cylinder 91.4 cm long and 27.9 cm in diameter) in which they were exposed to approximately 0.5 ATA of 21% O₂, which is approximately 0.1 ATA ((termed chronic hypoxia-CHx) starting at P50) for ≥ 7 days, until each animal was sacrificed and tested. This protocol had chamber controls, where 5-6 male adult rats were placed in the chamber with a flow through of room air (21% O₂). Then the adult male rats underwent the same procedure described above. To assess the effectiveness of CHx, hematocrit and body weight were both recorded as previously reported (Nichols et al., 2008c) for each animal prior to euthanasia. As previously observed (Villafuerte et al., 2007; Nichols et al., 2008c) hematocrit increased and body weight decreased in CHx-adapted rats. Temperature and relative humidity were recorded for all groups on HOBO HO8 RH recorders (Onset Computer Corporation, Bourne, MA) that were placed in the plexiglas chamber. The range of temperatures (17-23° C) and relative humidity (23-60%) in the chamber were similar to previous findings (Nichols et al., 2008c). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01).
Solutions

Synaptic blockade (SNB) solution (high Mg\textsuperscript{2+}, low Ca\textsuperscript{2+}) was modified from artificial cerebral spinal fluid in order to block chemical synapses (Dean et al., 1990) and contained the following (in mM): 124 NaCl, 5.0 KCl, 0.2 CaCl\textsubscript{2}, 11.4 MgSO\textsubscript{4}, 1.24 KH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 10 glucose, and was equilibrated with 95 % O\textsubscript{2}/5 % CO\textsubscript{2} (pH ~ 7.45 at 37°C). The whole cell patch intracellular solution contained (in mM): 130 K\textsuperscript+-gluconate, 10 KCl, 10 HEPES, 0.4 EGTA, 1 MgCl\textsubscript{2}, 0.3 GTP, and 2 ATP, (pH = 7.45 at room temperature) (Fillosa and Putnam, 2003). 1 mM of the pH-sensitive fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS, pyranine) (Invitrogen, Eugene, OR) was added to the whole cell patch intracellular solution. Substance P methyl ester (5 mg) (American Peptide, Sunnyvale, CA and Sigma, St. Louis, MO) was prepared in 3.7 mL of 100mM acetic acid and then aliquots of 50 \mu L were stored at -20°C until needed. 50 \mu L of substance P was directly added to 50 mL of SNB solution to give a final concentration of 1\mu M substance P for experiments. L-703,606 oxalate salt (5mg) (Alexis Biochemicals, San Diego, CA and Sigma, St. Louis, MO), an NK1 antagonist, was prepared in 8.35 mL of ddH\textsubscript{2}O and then aliquots of 50 \mu L were stored at 4°C until needed. 50 \mu L of L-703,606 was directly added to 50 mL of SNB solution to give a final concentration of 1\mu M L-703,606 for experiments. All chemicals were purchased from Sigma (St. Louis, MO) except where noted.

Slice preparation

Slices for study were prepared from control and CHx adult male rats (P57-P64) as previously described (Nichols et al., 2008c) Briefly, adult male rats were anesthetized with a brief exposure to CO\textsubscript{2} (100 %), and then rapidly decapitated. The brainstem was
removed and submerged in aCSF equilibrated with 5% CO₂ / 95% O₂ gas mixture. Transverse slices (300 μm) were prepared on a vibratome (Pelco 101, series 1000) beginning at the obex and extending rostrally for ~ 1mm and were allowed to recover for at least 1 hour at room temperature in aCSF equilibrated with a 5% CO₂ / 95% O₂ gas mixture. Individual slices for study from control and CHx adult male rats were placed in a superfusion chamber on the stage of an upright Nikon Optiphot-2 microscope. Slices were immobilized with a nylon grid and superfused at ~2.4 ml/min with aCSF equilibrated with a 5% CO₂ / 95% O₂ gas mixture (pH 7.45 at 37° C).

Individual neurons from the nucleus tractus solitarius, NTS, were then studied. The experimental protocol for studies consisted of a 5 minute exposure to SNB equilibrated with 5% CO₂ / 95% O₂, a 10-15 minute exposure to SNB equilibrated with 15% CO₂ / 85% O₂, and a 5-10 minute exposure to SNB equilibrated with 5% CO₂ / 95% O₂ (hypercapnic test of chemosensitive response). This protocol was then repeated in the same neuron with SNB containing 1μM substance P. For studies looking at the effect of the NK1 antagonist, we first looked at the effectiveness of the NK1 antagonist blocking the binding of substance P which was tested by giving a 5 minute exposure to SNB equilibrated with 5% CO₂ / 95% O₂, followed by a 5 minute exposure to the NK1 antagonist in SNB equilibrated with 5% CO₂ / 95% O₂, and lastly by 5 minute exposure to the NK1 antagonist plus substance P in SNB equilibrated with 5% CO₂ / 95% O₂. The chemosensitive response was then studied in the presence of the NK1 antagonist and in the presence of the NK1 antagonist plus substance P. The protocol for these experiments included a 5 minute exposure to SNB equilibrated with 5% CO₂ / 95% O₂, followed by a 5 minute exposure to the NK1 antagonist in SNB equilibrated with 5% CO₂ / 95% O₂.
The chemosensitive response was then tested by giving a 10-15 minute exposure to the NK1 antagonist in SNB equilibrated with 15% CO₂ / 85% O₂, and a 5-10 minute exposure to the NK1 antagonist in SNB equilibrated with 5% CO₂ / 95% O₂. This was followed by a 5 minute exposure to the NK1 antagonist plus substance P in SNB equilibrated with 5% CO₂ / 95% O₂. The chemosensitive response was then tested again in the presence of the NK1 antagonist plus substance P in SNB using the same protocol as above.

Imaging of Fluorescent Neurons

pHᵢ was measured as previously described (Ritucci et al. 2005a; 2005b; Nichols et al., 2008c). Individual NTS neurons were loaded with 1mM of the pH-sensitive dye, pyranine, through a whole cell patch pipette. NTS neurons were loaded with pyranine and images were collected every minute at 515 nm emission with alternating 450 and 410 nm excitation using a Sutter Lambda 10-2 filter wheel. Images were processed with MetaFluor 7.1.4.0 software (Meta Imaging) to yield Rₙ. We used the equation (pH = 7.4969 + log (Nₙ -0.2003)/(2.0194 - Nₙ); r² = 0.99), derived from a calibration curve that was generated previously for NTS neurons from adult rats for pyranine (Nichols et al., 2008b) to convert Nₙ into pHᵢ. A pHᵢ versus time plot was made in Microsoft Excel, from which the pHᵢ recovery rate from CO₂-induced acidification of an individual neuron was estimated by the slope of a linear fit to the pHᵢ versus time trace during acute hypercapnia (at least 5 points starting at minimum pHᵢ).

Electrophysiological Studies

The blind whole-cell patch clamp technique was used to measure neuronal membrane potential (Vₘ) and integrated firing rate as described previously (Blanton et al. 1989;
Nichols et al., 2008b; 2008c). The experimental setup that was used has been previously described (Dean et al. 1997; Huang et al. 1997; Filosa et al. 2002; Conrad et al., 2008; Nichols et al., 2008b; 2008c). Briefly, whole cell patch pipettes were used as previously described (Nichols et al., 2008b; 2008c) and then moved into the superfusion solution and down to the slice. Positive pressure was applied and then taken off once tip impedance resulted in a 1-2 mV downward deflection. Negative pressure was applied to the pipette to obtain a giga-ohm seal, brief suction was applied to the pipette to rupture the membrane, and then $V_m$ and integrated firing rate were measured throughout the experiment. Integrated firing rate (Hz) was determined as previously described (Nichols et al., 2008b; 2008c) and analyzed using pClamp 8.2 software. Viable neurons had a stable $V_m$ of between -40 and -60 mV and fired action potentials that crossed through zero. The firing rate response to hypercapnia was quantified as previously described using two measures: percentage of neurons activated by acute hypercapnia, and the magnitude of the firing rate response to acute hypercapnia, calculated as the chemosensitivity index or CI according to the equation of Wang and Richerson (1999). A neuron was designated as activated if its CI was greater than 120% or inhibited if its CI was less than 80%.

**Immunohistochemistry**

Adult rats were first anesthetized with pentobarbitol (100μg/g) followed by being perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH~7.4). The brainstem was isolated and fixed overnight in 4% paraformaldehyde, and then kept in 30% sucrose until sectioning (50 μm) on a cryostat (Microm, Walldorf, Germany). Sections were collected on slides (VWR Vistavision™ Histobon®). Tissue sections were
washed in PBST (0.01 M; Phosphate Buffered Saline and 0.1% Triton), incubated for 1 hour in normal donkey serum (1:10 in PBST) to reduce nonspecific staining, and incubated overnight (4°C) with rabbit polyclonal anti-NK1 antibody (1:750, Chemicon International) and mouse monoclonal anti-NeuN (a neuronal marker) (1:1000, Chemicon International) diluted in PBST. After washing in PBST, the sections were incubated for 2 hours with cyanine 3 (Cy3)-conjugated donkey anti-rabbit (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBST to identify anti-NK1 and fluorescein isothiocyanate (FITC)-donkey anti-mouse FITC (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBST to identify neurons (NeuN positive). Tissue sections were then washed in PBS, dried, and coverslipped after the application of Vectashield (H1000, Vector Laboratories, Inc., Burlingame, CA). Mounted sections were examined with a Fluoview 1000 microscope (Olympus Optical, Tokyo, Japan) (20X or 60X objective) using argon (FITC) and krypton (Cy3) lasers and Fluoview software. For Cy3 fluorescence, excitation was set to 560 nm, and emitted fluorescence was collected between 580-680 nm. For FITC fluorescence, excitation was set to 488 nm, and emitted fluorescence was collected between 500-555 nm. A series of confocal optical sections (z step 0.5 μm) were obtained to assess extent of NK1 receptor labeling and the degree to which it colocalized with NeuN immunolabeling.

Statistical Analysis

Values are reported as means ± S.E.M. χ² tests were used to compare differences between the percentage of neurons that respond to hypercapnia. Student’s t-tests were used to compare differences between two means with a level of significance at P<0.05. Differences between three or more means were determined by one-way ANOVA. If
significant differences existed, then multiple comparisons were done using Tukey's method with a level of significance of $P<0.05$. 
Results

Immunolabeling of NK1 receptors in control and CHx adapted adult rats

Since the goal of this study was to examine the role of substance P on the chemosensitive response of NTS neurons from both control and CHx adapted adult rats, we first wanted to verify that the receptor for substance P was present in the area of interest. It is known that substance P binds with high affinity to the NK1 receptor, so we chose to examine NK1 receptor presence on NTS neurons using immunohistochemistry. We found that indeed, the NK1 receptor is present in the NTS region from both types of rats (Fig. 33). It was observed that the amount of staining in the region just above the hypoglossal nucleus, which is the dorsal motor nucleus, was brighter and more compact compared to the diffuse staining observed in the NTS of both rats (Fig. 33). It appeared that most NK1 receptor immunolabeling colocalized with NeuN, although not all neurons were positive for NK1 receptors. In addition, we observed that NK1 receptors were localized to both the soma membrane and dendritic processes (Fig. 34A and B), including dendritic varicosities (Fig. 34B). We did not perform a quantitative analysis because the main question we wanted to address was if NK1 receptors were present or not. We did not observe any major differences in the pattern of NK1 receptor immunolabeling among all caudal NTS slices between control and CHx-adapted rats, e.g. heavier staining in dorsal motor nucleus, diffuse staining in NTS neurons and lack of staining in hypoglossal neurons. Further, we see both somal and dendritic staining in neurons under all conditions. There is a suggestion that NK1 receptor staining may be brighter on NTS neurons from control rats compared to CHx-adapted rats (Fig. 33A1 vs. A2 and B1 vs. B2), but confirmation of this possibility will require a detailed quantitative study.
Figure 33: Immunohistochemical reactivity for NK1 receptors (red) and NeuN (green) for a control (A) and a CHx-adapted rat (B). All scale bars are located in the bottom right corner of the picture. A1 and B1 are low magnification pictures from a control (A1) and a CHx-adapted (B1) rats at 20X1.0. A2, A3, B2, and B3 are higher magnification pictures from a control (A2, A3) and a CHx adapted rats (B2, B3) at 60X2.0. A1. NK1 immunoreactivity of a medullary slice from an adult rat at 20X1.0 with NK1 receptors shown in red (CY3) and NeuN (stain for neurons) shown in green (FITC). Notice the heavy staining just above the hypoglossal region (white *), which is mostly the dorsal motor nucleus, and the diffuse staining throughout the rest of the solitary complex. The asterisk is depicting a group of hypoglossal neurons to show the size difference compared to neurons from the solitary complex (defined as NTS plus the dorsal motor nucleus). The white and yellow boxes are shown at higher magnification in A2 and A3 respectively. A2. The white box shown in A1 is now shown at 60X2.0. The white arrows are pointing to NK1-immunoreactive neurons. A3. The yellow box shown in A1 is now shown at 60X2.0. The white arrows are pointing to NK1-immunoreactive neurons. B1. NK1 immunoreactivity of a medullary slice from a CHx-adapted rat at 20X1.0 with NK1 receptors shown in red (CY3) and NeuN (stain for neurons) shown in green (FITC). Notice the heavy staining just above the hypoglossal region (white *), which we believe is mostly the dorsal motor nucleus, and the diffuse staining throughout the rest of the solitary complex. The asterisk is depicting a hypoglossal neuron to show the size difference compared to neurons from the solitary complex. The white and yellow boxes are then shown at higher magnification in B2 and B3 respectively. B2. The white box shown in B1 is now shown at 60X2.0. The white arrows are pointing to NK1-
immunoreactive neurons. B3. The yellow box shown in B1 is now shown at 60X2.0.

The white arrows are pointing to NK1-immunoreactive neurons. Notice that there is no difference in the pattern of NK1 receptor staining between control and CHx-adapted rats, since NK1 receptor staining seems to be most intense right above the hypoglossal nucleus and diffuse throughout the rest of the solitary complex for both groups. Thus, NK1 receptors are present on NTS neurons from both control and CHx-adapted rats, although staining appears to be somewhat less for slices from CHx-adapted rats.
Figure 33

Control  | NK1-IR / NeuN-IR  | CHx


NK1-IR / NeuN-IR
Figure 34: Sample immunohistochemical reactivity for NK1 receptors to characterize the localization of NK1 receptors on NTS neurons (A, B). Scale bars are located in the bottom right corner of each picture. A. NK1 immunoreactivity at 60X2.5 with NK1 receptors shown in red (CY3). Notice that NK1 receptors are found both on the dendritic process (yellow arrow) as well as the soma (white arrow). B. NK1 immunoreactivity at 60X2.5) with NK1 receptors shown in red (CY3). Notice that NK1 receptors are found on the dendritic process (yellow arrow) and varicosities (green arrows), as well as the soma (white arrows) of both neurons. This pattern does not change when rats are adapted to CHx. Thus, NK1 receptors are present on the soma and processes (including varicosities) of NTS neurons from both control and CHx-adapted rats.
Figure 34
Effect of substance P on basal firing rate of NTS neurons from both control and CHx rats

Before we could study the effect of substance P on the chemosensitive response, we first wanted to see if substance P had any effect on the basal properties of NTS neurons from both control and CHx rats. We initially performed the experiments with substance P by only exposing the neuron to the neuropeptide for a short time (< 5 minutes). We then began to consider internalization since NK1 receptors are known metabotropic receptors (Regoli et al., 1994; Mazzone et al., 1998; Mazzone and Geraghty, 2000; Medhurst and Hay, 2002; Wang and Marvizon, 2002; Roosterman et al., 2004) and decided to expose the neuron for a longer period to substance P (≥8 -15 minutes). This longer exposure revealed two types of increased firing rate responses caused by substance P including a response that first peaked and then adapted (termed the adapted response) (Fig. 35) and a response that slowly plateaued (termed the plateau response) (Fig. 36). These two types of responses were seen in NTS neurons from both control (Fig. 35A for adapted and Fig. 36A for plateau) and CHx adapted rats (Fig. 35B for adapted and Fig. 36B for plateau). After the firing rate stabilized in the presence of substance P, we hyperpolarized the V_m back towards initial values preceding the addition of substance P (see Figs. 35 and 36 for example) in order to accurately measure the chemosensitive response in the presence of substance P compared to the absence of substance P.

To investigate if there were differences in the substance P response between control and CHx-adapted rats, we first quantified the maximal response induced by substance P. This took into account all NTS neurons that were exposed to substance P from both groups of rats (N=22 for control rats and N=23 for CHx adapted rats). In NTS
**Figure 35:** Sample adapted basal firing rate response to prolonged substance P (SP) exposure of NTS neurons from both a control (A) and a CHx-adapted rat (B). Green bar represents substance P exposure. **A.** Sample adapted response to substance P of an NTS neuron from a control rat. Notice that basal firing rate first peaked in response to substance P, but then firing rate adapted down to around 4Hz. Before we exposed the neuron to hypercapnia, we hyperpolarized the V_m by injecting negative DC current to return firing rate back to initial values to accurately compare the chemosensitive response in the absence and presence of substance P. **B.** Sample adapted response to substance P of an NTS neuron from a CHx-adapted rat. Notice that basal firing rate first peaked in response to substance P, but then firing rate adapted down to approximately 4Hz. Before we exposed the neuron to hypercapnia, we first hyperpolarized the V_m by injecting negative DC current to return firing rate back to initial values to accurately compare the chemosensitive response in the absence and presence of substance P.
Figure 35

A.

Firing rate (Hz)

1 μM SP

Hyperpolarized

2 min

B.

Firing rate (Hz)

1 μM SP

Hyperpolarized

2 min
**Figure 36:** Sample plateau basal firing rate response to prolonged substance P (SP) exposure of NTS neurons from both a control (A) and a CHx-adapted rat (B). Green bar represents substance P exposure. **A.** Sample plateau response to substance P of an NTS neuron from a control rat. Notice that basal firing rate slowly rose and plateaued in response to substance P around 4Hz. Before we exposed the neuron to hypercapnia, we first hyperpolarized the $V_m$ by injecting negative DC current to return firing rate back to initial values to accurately compare the chemosensitive response in the absence and presence of substance P. **B.** Sample plateau response to substance P of an NTS neuron from a CHx-adapted rat. Notice that basal firing rate plateaued in response to substance P to approximately 4Hz. Before we exposed the neuron to hypercapnia, we first hyperpolarized the $V_m$ by injecting negative DC current to return firing rate back to initial values to accurately compare the chemosensitive response in the absence and presence of substance P.
Figure 36

A.

B.

Firing rate (Hz)

1 μM SP

Hyperpolarized

2 min

Hyperpolarized

2 min
neurons from control rats, we found that substance P increased firing rate from $1.74 \pm 0.29$ Hz to $6.45 \pm 0.75$ Hz ($P<0.0001$). NTS neurons from CHx-adapted rats had a basal firing rate that increased from $2.11 \pm 0.28$ to $4.78 \pm 0.51$ Hz in response to substance P ($P=0.0001$). Thus, we found that substance P caused a significant increase in basal firing rate of NTS neurons from both groups (change of $4.71 \pm 0.59$ Hz for control rats and $2.67 \pm 0.59$ Hz for CHx-adapted rats), however, NTS neurons from CHx-adapted rats had a significantly smaller change in firing rate in response to substance P when compared to that seen in control rats ($P=0.0162$) (Fig. 37). Thus, in both cases we found that substance P significantly increased firing rate, although substance P did not affect the basal response of NTS neurons from CHx-adapted rats quite as robustly when compared to the response seen in NTS neurons from control rats. It is possible that the reason we are seeing less of a response to substance P in NTS neurons from CHx rats is because there are less NK1 receptors present, as suggested by the immunolabeling we observed in this study.

Another main question we wanted to address was if there were any differences in the response to substance P between NTS neurons that were activated, inhibited or not affected by hypercapnia. Thus, we further quantified the response of basal firing rate to substance P by classifying the response of NTS neurons to hypercapnic acidosis and then compared the maximal basal firing rate response induced by substance P between the three groups (activated, non-chemosensitive, and inhibited) (Fig. 38). This revealed that substance P significantly increased firing rate of NTS neurons that were classified as activated or non-chemosensitive in both control (Fig. 38A) and CHx-adapted rats (Fig. 38B). In activated NTS neurons from control and CHx adapted rats, we found that firing
Figure 37: The basal firing rate change that is induced in response to substance P of NTS neurons from both control and CHx-adapted rats. Notice that although substance P induces a significant increase in basal firing rate of NTS neurons from both types of rats, substance P induces a significantly smaller increase in basal firing rate in NTS neurons from CHx-adapted rats (P=0.0162) compared to the change induced by substance P in control rats.
Figure 37

Δ basal firing rate (Hz)

Control  Chronic hypoxia
**Figure 38:** The average basal firing rate response to substance P for NTS neurons that are activated, non-chemosensitive (non-CS), or inhibited in response to hypercapnia of NTS neurons from both control (A) and CHx-adapted rats (B).  

**A.** Average basal firing rate in the absence (white bar) and presence (black bar) of substance P for NTS neurons that are activated, non-CS, or inhibited in response to hypercapnia of NTS neurons from control rats. Substance P significantly increased the basal firing rate of activated (P=0.0023) and non-CS NTS neurons (P<0.0001). However, inhibited NTS neurons were not significantly affected by substance P.  

**B.** Average basal firing rate in the absence (white bar) and presence (black bar) of substance P for NTS neurons that are activated, non-CS, or inhibited in response to hypercapnia of NTS neurons from CHx-adapted rats. Substance P significantly increased the basal firing rate of activated (P=0.0026) and non-CS NTS neurons (P=0.0317). However, inhibited NTS neurons were not affected by substance P.
Figure 38

A. Activated Non-CS Inhibited

B. Activated Non-CS Inhibited
rate increased in response to substance P (1.31 ± 0.42 Hz to 6.05 ± 1.47 Hz for control; 
P=0.0023 and 1.71 ± 0.34 Hz to 4.90 ± 0.79 Hz for CHx adapted; P=0.0026) (Fig. 38).
Similarly, non-chemosensitive NTS neurons from control and CHx-adapted rats also had an increase in firing rate in response to substance P (2.44 ± 0.45 Hz to 7.28 ± 0.89 Hz for control; P<0.0001 and 2.24 ± 0.64 Hz to 5.03 ± 0.74 Hz; P=0.0317) (Fig. 38). However, substance P did not significantly affect the firing rate of inhibited NTS neurons from either control (Fig. 38A) or CHx-adapted rats (Fig. 38B). Inhibited NTS neurons from control rats had an average initial firing rate of 1.09 ± 0.72 Hz which was 5.25 ± 1.67 Hz in the presence of substance P, in which the change in firing rate was non-significant and may reflect the small numbers of inhibited neurons. NTS neurons from CHx-adapted rats that were classified inhibited went from 2.92 ± 0.70 Hz to 4.27 ± 1.10 Hz in the presence of substance P. Thus, we see no significant change in the basal firing rate response of inhibited NTS neurons to substance P from either control or CHx-adapted rats, so we conclude that inhibited NTS neurons do not have NK1 receptors since it is the receptor that substance P has been found to preferentially bind to on NTS neurons (Mazzone and Geraghty, 2000). If we then remove the values for inhibited NTS neurons that were averaged into the maximal response to substance P for NTS neurons from control and CHx-adapted rats (Fig. 37), there is no longer a significant difference between the two groups (change of 4.79 ± 0.66 Hz for control rats and 3.13 ± 0.68 Hz for CHx-adapted rats). It is possible that the reason we are seeing a significantly lower response to substance P of NTS neurons from CHx rats (Fig. 37) is because we are seeing no effect of substance P on the response of inhibited NTS neurons, which are significantly increased in CHx rats.
Given these results, we then decided to only compare NTS neurons that were either activated or non-chemosensitive to quantify the adapted and plateau responses to substance P from both groups of rats. As mentioned before, we observed that NTS neurons had two different responses to substance P. One response to substance P included a significant peak response (N = 8 for control rats and N = 6 for CHx-adapted rats) to substance P (1.95 ± 0.55 Hz to 7.43 ± 1.67 Hz for control rats; P=0.0018 and 1.45 ± 0.29 Hz to 6.80 ± 0.94 Hz for CHx-adapted rats; P=0.0025) that then adapted to a value that was significantly larger than the initial firing rate (4.75 ± 0.69 for controls; P<0.0001 and 4.20 ± 1.10 Hz for CHx-adapted rats; P=0.0453) (Fig. 39). In addition, we found that the peak response in firing rate of NTS neurons to substance P from both groups was significantly larger than the adapted response (P=0.0287 for controls and P=0.0160 for CHx-adapted rats). Notice that in both control and CHx adapted rats, firing rate adapted to around 4Hz (Fig. 39). Other NTS neurons responded to substance P by slowly increasing firing rate until it plateaued (N = 5 for control rats and N = 11 for CHx rats). We found that the plateau response to substance P significantly increased firing rate in NTS neurons from both groups (1.69 ± 0.59 Hz to 4.56 ± 1.22 Hz for control rats; P=0.0308 and 2.04 ± 0.42 Hz to 3.95 ± 0.63 Hz for CHx-adapted rats; P=0.0129) (Fig. 40). Again, notice that in both control and CHx adapted rats, firing rate plateaued around 4Hz (Fig. 40). Thus, the stable firing rate that was reached in the presence of substance P of all neurons (both activated and non-chemosensitive) and regardless of the type of response (adapted or plateau) from both control and CHx-adapted rats was around 4Hz.

Effect of substance P on steady-state pHᵢ of NTS neurons from both control and CHx rats

In addition to recording firing rate responses to substance P, we also
Figure 39: The average adapted basal firing rate response to substance P for NTS neurons from both control (A) and CHx-adapted rats (B). A. Average adapted basal firing rate (white bar) increased in response to substance P to a peak (gray bar) and then adapted to a steady value (black bar) for NTS neurons from control rats. Notice that basal firing rate initially peaked at around 7Hz (P=0.0018) and then adapted to around 4Hz (P<0.0001). The peak firing rate was significantly larger than the adapted firing rate (P=0.0287). B. Average adapted basal firing rate (white bar) increased in response to substance P to a peak (gray bar) and then adapted to a steady state value (black bar) for NTS neurons from CHx-adapted rats. Notice that basal firing rate initially peaked at around 6Hz (P=0.0025) and then adapted to around 4Hz (P=0.0453). The peak firing rate was significantly larger than the adapted firing rate (P=0.0160). Thus, the adapted response to substance P of NTS neurons is not changed by CHx. In all cases, the height of a bar represents the mean firing rate with an error of 1 SEM.
Figure 39

A. SNB
B. peak
final

Firing rate (Hz)

*
Figure 40: The average plateau basal firing rate response to substance P for NTS neurons from both control (A) and CHx-adapted rats (B). A. Average basal firing rate (white bar) increased to a plateau value (black bar) in response to substance P for NTS neurons from control rats. Notice that basal firing rate plateaued around 4Hz (P=0.0308).

B. Average basal firing rate (white bar) increased to a plateau value (black bar) in response to substance P for NTS neurons from CHx-adapted rats. Notice that basal firing rate plateaued around 4Hz (P=0.0129). Thus, the plateau response to substance P of NTS neurons is not changed by CHx. The height of a bar represents the mean firing rate with an error of 1 SEM.
Figure 40

A. 

B. 

Firing rate (Hz)
simultaneously measured the pH$_i$ response to substance P. Since we found that firing rate significantly increased in NTS neurons that were activated and non-chemosensitive but did not change in NTS neurons that were inhibited, we wanted to quantify the pH$_i$ response in the same manner. We found that substance P did not induce any effects on steady state pH$_i$ in any of the three groups (activated, inhibited or non-chemosensitive NTS neurons) from control or CHx-adapted rats, so we decided to lump the effect of substance P on pH$_i$ of all NTS neurons for both groups of rats. After grouping all NTS neurons together, we found that substance P induced a small significant acidification (7.24 ± 0.01 in the absence of substance P and 7.21 ± 0.02 in the presence of substance P; P=0.0165) in NTS neurons from control rats and CHx rats (7.23 ± 0.02 in the absence of substance P and 7.17 ± 0.03 in the presence of substance; P=0.0013). There were no significant differences in the effect of substance P on pH$_i$ found between control and CHx-adapted rats. This small substance P-induced acidification may be related to the increased firing rate, which has been shown in other neurons to induce a small acidification (Ritucci et al., 2005a).

Effect of substance P on the chemosensitive response of NTS neurons from both control and CHx-adapted rats

It has been found that substance P has a role in other areas involved in breathing (Gray et al., 1999, 2001; Murakoshi et al., 1985, Pena and Ramirez, 2004; Wang et al., 2002; Nattie and Li, 2002b; McKay et al., 2005), and it has been found that NK1 receptors exist in the NTS (Mazzone and Geraghty, 2000). However, the chemosensitive response of substance P responding NTS neurons has not been studied. Thus, one of the main goals of this study was to examine the effect of substance P on the chemosensitive
response of NTS neurons from both control and CHx-adapted rats. We also wanted to study the effect of substance P on the chemosensitive response of NTS neurons from CHx-adapted rats since it has been shown that hypoxia causes increased release of substance P from the peripheral chemoreceptors (Mifflin, 1997), which are known to synapse in the caudal NTS (Finley et al. 1992; Finley and Katz, 1992; Dobbins and Feldman, 1994; Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003).

We quantified the chemosensitive response to substance P by looking at the percentage of neurons that respond and the magnitude of the response or the CI in neurons isolated from their synaptic inputs in SNB medium. Substance P did not change the percentage of activated NTS neurons in either control rats (N= 10/22 in the absence of substance P and N= 12/22 in the presence of substance P) (Fig. 41A) or CHx-adapted rats (N= 12/22 in the absence of substance P and N= 10/22 in the presence of substance P) (Fig. 41B). Similarly, the CI of activated NTS neurons from control rats (165 ± 13% in the absence of substance P and 200 ± 29% in the presence of substance P) (Fig. 41C) and from CHx-adapted rats (CI of 142 ± 7% in the absence of substance P and 161 ± 11% in the presence of substance P) (Fig. 41D) were not significantly different. Thus, the addition of substance P did not affect the response of NTS neurons activated by hypercapnic acidosis.

A similar lack of substance P effect was seen in NTS neurons that were inhibited by hypercapnic acidosis. Both the percentage and CI of inhibited NTS neurons from control rats were unaffected by substance P (14% or 3/22 inhibited with a CI of 55 ± 2% in the absence and 14% or 3/22 inhibited with a CI of 63 ± 8% in the presence of substance P (Fig. 42A and C). The same lack of effect was seen with CHx-adapted rats,
**Figure 41:** A and B shows the percentage of NTS neurons from control rats (in synaptic blockade medium, SNB) (A) and rats adapted to CHx (B) that are activated by hypercapnic acidosis in the absence (white bar) and presence of substance P (SP) (black bar). C and D shows the chemosensitivity index of NTS neurons from control rats (C) and rats adapted to CHx (D) that are activated by hypercapnic acidosis in the absence (white bar) and presence of substance P (black bar). A: The percentage of NTS neurons from control adult rats that were activated by hypercapnic acidosis in the absence and presence of substance P. Notice that the percentage activated by hypercapnic acidosis was not affected by substance P in NTS neurons from control rats. B: The percentage of NTS neurons from CHx-adapted adult rats that were activated by hypercapnic acidosis in the absence and presence of substance P. Notice that the percentage activated by hypercapnic acidosis was not affected by substance P in NTS neurons from CHx-adapted rats. C: The chemosensitivity index (CI) of NTS neurons from control adult rats that were activated by hypercapnic acidosis in the absence (white bar) and presence (black bar) of substance P. Notice that the CI of NTS neurons activated by hypercapnic acidosis was not affected by substance P in NTS neurons from control rats. D: The chemosensitivity index of NTS neurons from CHx-adapted adult rats that were activated by hypercapnic acidosis in the absence (white bar) and presence (black bar) of substance P. Notice that the CI of NTS neurons activated by hypercapnic acidosis was not affected by substance P in NTS neurons from CHx-adapted rats. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM.
Figure 41

A. Percentage (%)

B. Percentage (%)

C. Chemosensitivity Index (%)

D. Chemosensitivity Index (%)
**Figure 42:** A and B shows the percentage of NTS neurons from control rats (in synaptic blockade medium, SNB) (A) and rats adapted to CHx (B) that are inhibited by hypercapnic acidosis in the absence (white bar) and presence of substance P (SP) (black bar). C and D show the chemosensitivity index of NTS neurons from control rats (C) and rats adapted to CHx (D) that are inhibited by hypercapnic acidosis in the absence (white bar) and presence of substance P (black bar). A: The percentage of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in the absence and presence of substance P. Notice that the percentage inhibited by hypercapnic acidosis was not affected by substance P in NTS neurons from control rats. B: The percentage of NTS neurons from CHx-adapted adult rats that were inhibited by hypercapnic acidosis in the absence and presence of substance P. Notice that the percentage inhibited by hypercapnic acidosis was not affected by substance P in NTS neurons from CHx-adapted rats. C: The chemosensitivity index of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in the absence and presence of substance P. Notice that the CI of NTS neurons inhibited by hypercapnic acidosis was not affected by substance P in NTS neurons from control rats. D: The chemosensitivity index of NTS neurons from CHx-adapted adult rats that were inhibited by hypercapnic acidosis in the absence and presence of substance P. Notice that the CI of NTS neurons inhibited by hypercapnic acidosis was not affected by substance P in NTS neurons from CHx-adapted rats. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM.
Figure 42

A. Percentage (%)

B. Percentage (%)

C. Chemosensitivity Index (%)

D. Chemosensitivity Index (%)
where neurons were 27% (6/22) inhibited with a CI of 66 ± 9% (Fig. 42B and D) in the absence of substance P and 14% (3/22) inhibited with a CI of 51 ± 10% (Fig. 42B and D) in the presence of substance P. Thus, substance P modulates basal firing rate, but has no effect on the chemosensitive response of NTS neurons from both control and CHx-adapted rats. It should be noted here that we have found previously that CHx decreases the percentage of NTS neurons activated and increases the percentage inhibited by hypercapnic acidosis (Nichols et al., 2008c). However, in this study we found that CHx did not affect the percentage activated either in the absence or presence of substance P when compared to the percentage activated in control rats. Thus, we conclude that CHx either does not affect the percentage activated or decreases the percentage activated, but we have never observed an increase in the percentage activated after CHx in any case. In all studies done with CHx, it has been found that the percentage of inhibited NTS neurons always increases.

Effect of substance P on pHi of NTS neurons from both control and CHx rats during acute hypercapnia

In addition to quantifying the firing rate response to substance P during the exposure to acute hypercapnia, we also simultaneously measured the effect of substance P on the pHi response to acute hypercapnia. We first measured the effect of substance P on pHi during acute hypercapnia as we did for examining the effect of substance P on steady state pHi, in that we grouped all NTS neurons together for control and CHx-adapted rats. The magnitude of acidification induced in response to acute hypercapnia in the presence of substance P (0.24 ± 0.01 pH units) was significantly larger (P=0.0009) than the change induced in the absence of substance P for NTS neurons from control rats.
(0.19 ± 0.01 pH units), although this difference is quite small. On the other hand, in NTS neurons from CHx rats we found that substance P did not affect the pH$_i$ response to acute hypercapnia (0.23 ± 0.02 pH units in the absence of substance P to 0.27 ± 0.02 pH units in the presence of substance P). Thus, substance P does not appear to have much of an effect on hypercapnia-induced acidification in adult rat NTS neurons.

**Effect of NK1 antagonist on the chemosensitive response of NTS neurons from both control and CHx-adapted rats**

In order to verify that substance P was not working through a different receptor other than the NK1 receptor, we used an NK1 antagonist to block the effect of substance P on NTS neurons from both control and CHx rats. We first wanted to check the specificity of the NK1 antagonist to ensure that it was blocking the substance P response. Although the NK1 antagonist did not completely inhibit the effect of substance P, we found that it significantly blunted the firing rate response to substance P of NTS neurons from both control (4.71 ± 0.59 Hz in the presence of substance P to 0.61 ± 0.42 Hz in the presence of the NK1 antagonist and substance P; P<0.0001) (Fig. 43A) and CHx-adapted rats (2.67 ± 0.59 Hz in the presence of substance P to 0.85 ± 0.66 Hz in the presence of the NK1 antagonist and substance P; P=0.0431)(Fig. 43B). In addition, we measured the effect of the NK1 antagonist alone on steady state pH$_i$ and found that it did not have an effect on pH$_i$ of NTS neurons from control and CHx-adapted rats. From this we conclude that substance P is predominately working through the NK1 receptor.

We then quantified the chemosensitive response of NTS neurons from control and CHx rats in the presence of the NK1 antagonist and in the presence of the NK1 antagonist
Figure 43: **A** and **B** show the basal firing rate changes of NTS neurons from both control (**A** and CHx-adapted (**B**) rats that is induced in response to substance P (SP) (white bar) and when an NK1 antagonist is added with substance P (NK1 A + SP) (black bar). **A.** Effect of the NK1 antagonist on basal firing rate of NTS neurons from control rats. Substance P induces a significant increase in basal firing rate that is significantly blunted in the presence of the NK1 antagonist (P<0.0001). **B.** Effect of the NK1 antagonist on basal firing rate of NTS neurons from CHx-adapted rats. Substance P induces a significant increase in basal firing rate that is significantly blunted in the presence of the NK1 antagonist (P=0.0431). The height of each bar represents the mean firing rate for that group and the error bars represent 1 SEM.
Figure 43

A.

\[ \Delta \text{Firing rate (Hz)} \]

\[ \begin{array}{c|c}
 SP & NK1 A + SP \\
 \hline
 0 & 0 \\
 4 & * \\
 6 & * \\
\end{array} \]

B.

\[ \Delta \text{Firing rate (Hz)} \]

\[ \begin{array}{c|c}
 SP & NK1 A + SP \\
 \hline
 0 & 0 \\
 4 & * \\
\end{array} \]
plus substance P. We quantified the percentage of neurons that responded to hypercapnia and their CI. In the presence of the NK1 antagonist or NK1 antagonist plus substance P, it was found that the percentage of activated NTS neurons and their CI did not change in control rats (45% or 10/22 with a CI of 165 ± 13% in SNB, 54% or 7/13 with a CI of 163 ± 17% in the presence of SNB plus the NK1 antagonist, and 46% or 6/13 with a CI of 146 ± 11% in the presence of SNB plus the NK1 antagonist and substance P) (Fig. 44A,C). For activated NTS neurons from CHx-adapted rats, it was found that the percentage activated (55% or 12/22 with a CI of 142 ± 7% in SNB) significantly decreased with no change in CI in the presence of the NK1 antagonist alone (27% or 3/11 with a CI of 187 ± 20%), but when substance P was combined with the NK1 antagonist, no significant differences were observed in percentage or CI (36% or 4/11 with a CI of 207 ± 76%) (Fig. 44B,D). In the presence of the NK1 antagonist or NK1 antagonist plus substance P, it was found that the percentage of inhibited NTS neurons and their CI did not change in control rats (14% or 3/22 with a CI of 55 ± 2% in SNB, 0% in the presence of the NK1 antagonist, and 8% or 1/13 with a CI of 48% in the presence of the NK1 antagonist and substance P) (Fig. 45A,C). Similarly, the NK1 antagonist or the combination of the NK1 antagonist and substance P did not affect the percentage or CI of NTS neurons that were inhibited by hypercapnic acidosis in CHx-adapted rats (27% or 6/22 with a CI of 66 ± 9% in SNB, 27% or 3/11 with a CI of 78 ± 2% in the presence of the NK1 antagonist, and 27% or 3/11 with a CI of 57 ± 12% in the presence of the NK1 antagonist and substance P) (Fig. 45B,D). In addition, we measured the pH, effect of the NK1 antagonist alone and in the presence of substance P during acute hypercapnia and found that a significantly larger acidification was induced in the presence of both the
Figure 44: A: The percentage of NTS neurons from control adult rats that were activated by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (NK1 A) (gray bar) or the NK1 antagonist and substance P (NK1 A + SP) (black bar). Notice that the percentage activated by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from control rats. B: The percentage of NTS neurons from CHx-adapted adult rats that were activated by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). Notice that the percentage of NTS neurons activated by hypercapnic acidosis was significantly (*) decreased by the NK1 antagonist alone (P<0.05), but had no effect on the percentage of NTS neurons activated when combined with substance P from CHx-adapted rats. C: The chemosensitivity index of NTS neurons from control adult rats that were activated by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). Notice that the CI of NTS neurons activated by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from control rats. D: The chemosensitivity index of NTS neurons from CHx-adapted adult rats that were activated by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). Notice that the CI of NTS neurons activated by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from CHx-adapted rats. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM.
**Figure 45:** A: The percentage of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (NK1 A) (gray bar) or the NK1 antagonist and substance P (NK1 A + SP) (black bar). Notice that the percentage inhibited by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from control rats. B: The percentage of NTS neurons from CHx-adapted adult rats that were inhibited by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). Notice that the percentage of NTS neurons inhibited by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from CHx-adapted rats. C: The chemosensitivity index of NTS neurons from control adult rats that were inhibited by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). There is no error bar for the NK1 A + SP experiment since both neurons had the same CI. Notice that the CI of NTS neurons inhibited by hypercapnic acidosis was not significantly affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from control rats. D: The chemosensitivity index of NTS neurons from CHx-adapted adult rats that were inhibited by hypercapnic acidosis in SNB (white bar), the NK1 antagonist (gray bar) or the NK1 antagonist and substance P (black bar). Notice that the CI of NTS neurons inhibited by hypercapnic acidosis was not affected by the NK1 antagonist alone or when combined with substance P in NTS neurons from CHx-adapted rats. The height of each bar represents the mean chemosensitivity index for that group and the error bars represent 1 SEM.
Figure 45

A. 

Percentage (%)

SNB NK1 A NK1 A + SP

B. 

Percentage (%)

SNB NK1 A NK1 A + SP

C. 

Chemosensitivity Index (%)

SNB NK1 A NK1 A + SP

D. 

Chemosensitivity Index (%)

SNB NK1 A NK1 A + SP
NK1 antagonist and substance P (0.36 ± 0.28 pH units) compared to when the NK1 antagonist was present alone (0.22 ± 0.02 pH units) in NTS neurons from control rats (P=0.0009). There was no difference in the acidification induced in the presence of the NK1 antagonist alone versus the presence of the NK1 antagonist in addition to substance P in NTS neurons from CHx-adapted rats (0.19 ± 0.04 pH units vs. 0.20 ± 0.04 respectively). Thus, the NK1 antagonist blunts the firing rate response of NTS neurons to substance P, but when this antagonist is applied alone or along with substance P, the firing rate response to hypercapnic acidosis of NTS neurons from both control and CHx-adapted rats is unchanged. This implies that neither the endogenous nor the exogenous release of substance P plays a role in the firing rate response to hypercapnic acidosis of NTS neurons from either control or CHx-adapted rats.
Discussion

It has been suggested that NK1 receptors can be used for markers of neurons that are involved in breathing, including ones participating in rhythm generation and central chemosensitivity (Nakaya et al., 1994). This implies that substance P must play a role in ventilatory control. Further, it has been found that substance P release from the peripheral afferents increases in response to hypoxia (Mifflin et al., 1997). These peripheral afferents are known to synapse on caudal NTS neurons (Finley et al. 1992; Finley and Katz, 1992; Dobbins and Feldman, 1994; Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003), where NK1 receptors are known to exist (Mazzone and Geraghty, 2000). However, neither the cellular response of NTS neurons to substance P or the chemosensitive response of these neurons have been studied in control rats or rats adapted to chronic hypoxia.

The main question of this study was to examine the chemosensitive response of NTS neurons in the presence of substance P. Here we studied the effects of substance P on the chemosensitive response (both pH; and firing rate measurements) of individual NTS neurons from control and CHx-adapted adult rats. We quantified the chemosensitive response by examining the percentage of neurons that respond and the magnitude to which they respond to acute hypercapnia in the presence of substance P. The main findings of this study include: 1) NK1 receptors are present on the soma and dendritic processes of NTS neurons as well as those of the dorsal motor nucleus, 2) substance P increases the basal firing rate of nearly all solitary tract neurons, 3) substance P has no effect on the firing rate response to acute hypercapnia of solitary tract neurons, and 4) when NK1 receptors are blocked we see no effect on the firing rate response of
solitary tract neurons to acute hypercapnia. Thus, substance P has a role in modulating basal firing rate but does not appear to be required for the firing rate response to acute hypercapnia in NTS neurons from adult rats.

*Characterization and localization of NK1 receptors on NTS neurons from control and CHx-adapted rats*

Recent studies have shown that NTS neurons have NK1 receptor positive immunolabeling (Solomon et al., 2001). In addition, peripheral afferents deriving from the carotid body are known to have their first primary synapse in the caudal NTS (Finley et al., 1992; Finley and Katz, 1992; Dobbins and Feldman, 1994; Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003). When rats are subjected to chronic or sustained hypoxia, it has been shown that substance P release from the carotid body increases (Mifflin, 1997). It has been further suggested that the CNS gain after chronic hypoxia is increased (Powell et al., 1998; Dwinell and Powell, 1999). Thus, we hypothesized that changes occur in the caudal NTS after chronic hypoxia and that these changes are mediated by substance P. In order to study the effect of substance P, we first wanted to verify that the main receptor for substance P (NK1 receptor) is present on NTS neurons from control and CHx-adapted rats. To characterize and localize the NK1 receptor, we used immunohistochemistry. We first determined the overall staining pattern in the NTS and surrounding areas and found that there were some distinct differences. For instance, NK1 receptors seem to have an increased presence in the dorsal motor nucleus (known chemosensitive region (Huang et al., 1997) found just above the hypoglossal nucleus and below the NTS) (Dean and Mulkey, 2000) compared to the diffuse staining throughout the NTS in both control and CHx-adapted rats (Fig. 33). Additionally, in slices from
control and CHx-adapted rats, we saw a lack of staining in the hypoglossal nucleus, which is an area known to contain non-chemosensitive motor neurons. Without careful quantification, we do not know whether CHx results in a change of expression of NK1 receptors.

We further wanted to localize the presence of NK1 receptors on NTS neurons. We compared high magnification pictures of neurons in the dorsal motor nucleus to high magnification pictures taken in the NTS of both control and CHx-adapted rats (Fig. 33). In general, the pattern of NK1 receptor immunolabeling was not different when comparing the two areas because we found NK1 receptors on the membrane of both the soma and dendritic processes of neurons from both control and CHx-adapted rats. NK1 receptor localization is clearly seen in Fig. 34, where we took high magnification pictures of NK1 receptor-positive staining on NTS neurons, which shows NK1 receptors on the soma and dendritic processes (including varicosities). Overall, we find that NK1 receptors are present on the soma and dendritic processes in the NTS of control and CHx-adapted rats, although they may be decreased in the NTS of CHx-adapted rats.

A potential role for substance P on basal levels of NTS neurons from control and CHx-adapted rats

Previously, it has been found that when substance P was either injected or applied into the NTS, respiratory frequency increased (Hedner et al., 1984; Morin-Surun et al., 1984; Yamamoto and Lagercrantz, 1985; Chen et al., 1990a). Given these results, it seemed reasonable to hypothesize that substance P would have excitatory effects on caudal NTS neurons in vitro. Here, we simultaneously recorded both firing rate and pH\textsubscript{i} and studied the effect of substance P on basal firing rate and steady-state pH\textsubscript{i}. We found
that substance P significantly increased firing rate of NTS neurons (Figs. 35 and 36) as well as inducing a small but significant acidification in both groups of rats. However, the firing rate response to substance P was significantly smaller for NTS neurons from CHx-adapted rats. This suggests that either the neuronal response to substance P is becoming desensitized or that there is a decrease in receptor number. The results from the immunolabeling presented in this study suggest the latter. Thus, it can be hypothesized that a downregulation of NK1 receptors leads to a smaller effect of substance P in NTS neurons from CHx-adapted rats.

In response to prolonged substance P, we observed two different firing rate responses of NTS neurons from both groups of rats including an adapted and a plateau response. Both responses caused a significant increase in firing rate which stabilized around 4 Hz. To quantify the response, we examined the effect of substance P on classified NTS neurons (activated, non-chemosensitive, and inhibited). Substance P caused a significant increase in firing rate of activated and non-chemosensitive NTS neurons, but had no significant effect on inhibited NTS neurons from control and CHx-adapted rats. This suggests that inhibited NTS neurons from both groups do not express NK1 receptors. Further, it can be hypothesized that substance P may not have as large of an effect on firing rate of NTS neurons from CHx-adapted rats because of the increase in inhibited NTS neurons, which do not express NK1 receptors. This hypothesis is further suggested because when inhibited NTS neurons are removed from the maximal response to substance P, there is no longer a significant difference between control and CHx-adapted rats when comparing the effect of substance P on firing rate.
We found that NTS neurons have two different responses to substance P. However, regardless of the type of response to substance P, firing rate seemed to stabilize at around 4 Hz. We are not sure why there are different patterns of responses to substance P nor why the firing rate ultimately stabilizes at 4 Hz.

Substance P does not affect the firing rate response to acute hypercapnia of NTS neurons from control and CHx-adapted rats

It has been found that substance P has a role in breathing. Specifically, NK1 receptors have been found in all brainstem chemoreceptor sites and the pre-Bötzinger complex (Nakaya et al., 1994), which is important for rhythm generation in breathing (Gray et al., 2001; Guyenet and Wang, 2001). In preparations in which various chemosensitive regions were exposed to substance P, respiratory output increased (Hedner et al., 1984; Morin-Surun et al., 1984; Murakoshi et al., 1985; Yamamoto and Lagercrantz, 1985; Chen et al., 1990a; Gray et al., 1999; Pena and Ramirez, 2004). Furthermore, when substance P was focally injected into the NTS and ventral medulla, breathing increased (Chen et al., 1990a; 1990b). When a substance P antagonist was focally applied to the ventral medulla, it was found that breathing was inhibited (Chen et al., 1990a; 1990b). Substance P seems to be involved in the respiratory response to hypercapnia and possibly hypoxia. Focal lesions (using saporin conjugated to substance P) induced in the pre-Bötzinger complex, RTN, and the medullary raphé resulted in a decreased ventilatory response to increased CO₂ (Gray et al., 2001; Nattie and Li, 2002b; Nattie et al., 2004). Interestingly, NK1 knockout adult mice have a reduced ventilatory response to hypoxia (Ptak et al., 2002). These reductions may involve changes within the NTS, since it is known that peripheral afferents arising from the carotid body synapse on
to NTS neurons (Finley et al. 1992; Finley and Katz, 1992; Dobbins and Feldman, 1994; Donoghue et al., 1984; Mifflin, 1992; Lopez-Barneo 2003).

We found that both the percentage and CI were unchanged by substance P in NTS neurons from control and CHx-adapted rats. Substance P did cause a significantly larger acidification in response to hypercapnic acidosis in NTS neurons from control rats, but had no effect on the pH response to hypercapnic acidosis in CHx-adapted rats. We are not sure of the exact mechanism by which substance P is causing a larger acidification in control rats but not CHx-adapted rats.

It has been found that neurons within regions playing a role in breathing also can respond to changes in oxygen, including the neurons from the NTS (Dean et al., 1991; Nolan and Waldrop, 1993; Sun and Reis, 1994; Ramirez et al., 1998; Solomon et al., 2000b; Mazza et al., 2000; Pascual et al., 2002). It has also been found that NTS neurons that were activated in response to hypercapnic acidosis were also activated in response to hyperoxia, although the pathways of response to acidosis and to hyperoxia are distinct (Mulkey et al., 2003). However, it is not clear at this time if NTS neurons that sense hypercapnia also sense hypoxia, although this has been observed in some NTS neurons (Dean et al., 1991; Pascual et al., 2002). Thus, although we are seeing a lack of an effect of substance P on the response of NTS neurons to hypercapnia, it is possible that substance P may play a role in the response of these neurons to acute hypoxia, especially on NTS neurons from CHx-adapted rats.

There is evidence that substance P is released endogenously in the NTS (Mazzone and Geraghty, 2000; Wickström et al., 2004) as well as exogenously from peripheral afferents arising from the carotid body (Mifflin, 1997). Thus, we used a NK1 antagonist
to study possible effects of both endogenous release of substance P as well as exogenous effects of substance P. In our slice preparation, we have removed the main exogenous input of substance P deriving from the peripheral afferents of carotid bodies. To study the effect of exogenous substance P on NTS neurons, we added substance P alone and with the NK1 antagonist in SNB solution. We found that exogenous substance P significantly increased basal firing rate of NTS neurons from both control and CHx-adapted rats. We also wanted to verify that substance P was working through the NK1 receptor on NTS neurons. The NK1 antagonist blunted the substance P response on basal firing rate in NTS neurons from control and CHx-adapted rats. However, it should be noted that the substance P response was not completely inhibited, so it is possible that substance P was able to bind to another neurokinin receptor. Specifically, low amounts of NK3 have been found in the NTS (Monteau et al., 1996; Mazzone and Geraghty, 2000).

We quantified the chemosensitive response of NTS neurons to acute hypercapnia in the presence of the NK1 antagonist alone and in the presence of substance P in both control and CHx-adapted rats. We found that NK1 antagonist alone only significantly decreased the percentage of activated NTS neurons from CHx-adapted rats, but neither the NK1 antagonist alone nor the combination of the NK1 antagonist with substance P affected the firing rate response to acute hypercapnia in NTS neurons from control and CHx-adapted rats. In addition, the combination of NK1 antagonist and substance P caused a larger acidification compared to the NK1 antagonist alone in control rats, but had no effect in CHx-adapted rats. We have previously found that the percentage of activated NTS neurons significantly decreases in rats adapted to CHx (Nichols et al.,
Here, we show that in the presence of SNB, the percentage of activated NTS neurons does not change compared to controls. Thus, we conclude that the NTS neurons that are activated in response to acute hypercapnia either do not change or they are decreased, but in no case are they increased in response to CHx. In addition, in all studies looking at the percentage of inhibited NTS neurons after rats were adapted to CHx, we found that the percentage of NTS neurons that were inhibited in response to acute hypercapnia increased. These findings strongly indicate that the increased inhibited NTS neurons arise from non-chemosensitive neurons and not by transition of activated to inhibited neurons. Overall, these experiments suggest that substance P does not play a major role in determining the firing rate response of NTS neurons to acute hypercapnia, but may accentuate the magnitude of acidification that is induced in response to hypercapnic acidosis.

Significance

It has been found that substance P affects the whole animal ventilatory response (Gray et al., 1999; Murakoshi et al., 1985, Pena and Ramirez, 2004; Hedner et al., 1984; Morin-Surun et al., 1984; Yamamoto and Lagercrantz, 1985; Chen et al., 1990a). Specifically, it has been found that substance P can increase breathing and when substance P binding neurons are lesioned breathing is disrupted (Gray et al., 2001; McKay et al., 2005; Nattie and Li, 2002b). The findings reported in this study represent the first time substance P has been shown to directly modulate basal firing rate of NTS neurons, but it had no effect on the firing rate response of NTS neurons to hypercapnic acidosis. It is likely that substance P released from the carotid bodies leads to an increase in basal activity of NTS neurons, as we have shown here. However, in response to
hypercapnic acidosis, the NTS neuronal response to substance P is unchanged. Thus, we suggest that substance P only acts to modulate the basal level activity of and not the chemosensitive response of NTS neurons. If our presumptions are correct, then caudal NTS neurons from CHx rats are a good system to study the mechanism of how neurons are inhibited by hypercapnic acidosis.
CHAPTER VIII

Conclusion
The chemosensitive firing rate response of NTS neurons from adult rats is similar to that of neonatal rats. This response is intrinsic after P10 throughout adulthood and is not mediated by substance P. Further, plasticity is induced by chronic hypoxia in NTS neurons from adult rats, consisting of an increase in CO₂-inhibited neurons and a decrease in CO₂-activated neurons. This plasticity is not mediated by substance P.

Central chemosensitivity has been studied in neonatal rats. It has been found that the cellular response to hypercapnia of NTS neurons from neonates is partly dependent on electrical synaptic transmission (gap junctions) until P10, but after that the response to hypercapnia of NTS neurons from neonates is intrinsic and does not depend on either chemical or electrical synaptic transmission (Conrad et al., 2008). In this study we found that like NTS neurons from neonatal rats older than P10, NTS neurons from adult rats are also intrinsically chemosensitive and their response to hypercapnia does not depend on either chemical or electrical synaptic transmission. Thus, intrinsic cellular chemosensitivity in NTS neurons is quite similar in adults and in neonates. It is likely, however, that the chemosensitive response of NTS neurons in vivo is modulated both by gap junction activity and chemical synaptic input.

This study represents the first to quantitatively study pHᵢ of NTS neurons from adult rats. We found that NTS neurons from adult rats have a significantly less alkaline pHᵢ compared to NTS neurons from neonatal rats. We hypothesize that this difference in steady state pHᵢ of NTS neurons from adult rats is due to the expression of Cl⁻/HCO₃⁻ exchange, which is missing in NTS neurons from neonatal rats (Ritucci et al., 1998). While ΔpHᵢ induced by hypercapnic acidosis is the same in neonates and adults, these changes occur over a different range of pHᵢ’s due to the differences in initial pHᵢ.
(neonates: 7.49-7.23; adults: 7.30-7.05). This indicates that there must be a shift in the pH$_i$-responsiveness of adult NTS neurons.

Tonic input to NTS neurons in brainstem slices appears to change between neonatal and adult rats. In neonates, it has been found that SNB decreases basal firing rate, suggesting that NTS neurons in slices receive tonic excitatory input (Conrad et al., 2008). On the other hand, in this study we found that SNB increases firing rate in NTS neurons from adult rats, suggesting that there is inhibitory input to NTS neurons in adult slices. This is different than a previous study looking at the effect of SNB, where it was found that tonic input was excitatory in adult NTS neurons (Dean et al., 1990). We propose that this difference is due to the difference in slice preparations. In the previous study using slices from adults, the slices were transected with the ventral portion of the slice removed (Dean et al., 1990). This suggests that adult NTS neurons receive inhibitory input from neurons in the ventral portion of the slice, which is present in our slices. Regardless, we found that tonic input in the slice preparation is different between neonates and adults. We believe that this is due to the local neuronal network within the slice changing developmentally from providing excitatory input in neonates to inhibitory input in adults. This type of pattern has been found in hippocampal neurons in rats, and it was proposed that this occurred because of a Cl$^-$ shift that causes GABA to have a depolarizing effect in neonates and hyperpolarizing in adults (Zhang et al., 1991). Thus, since GABA has been found to bind to NTS neurons in both neonates and adults (Kawai and Senba, 2000), we propose that this could in part cause the shift from excitatory to inhibitory tonic input in NTS neurons from neonates to adults, respectively.
Another major finding of this study was that CHx induces plasticity in NTS neurons from adult rats. Specifically, we found that CHx causes suppression of the response to hypercapnia in NTS neurons from adult rats in increasing the percentage of inhibited NTS neurons and either not changing or decreasing the percentage of activated NTS neurons. Our data suggest that the increase in the percentage of inhibited NTS neurons caused by CHx is not due to NTS neurons that were activated now becoming inhibited because in one study we found that the percentage of activated NTS neurons did not change while the percentage of inhibited NTS neurons increased from 10% to 30% (Fig. 44B). We propose that the increase in percentage of inhibited NTS neurons is due to non-chemosensitive neurons now becoming CO2-inhibited. This change most likely occurs by CHx inducing the expression of an ion channel or channels not present on non-chemosensitive neurons, thus rendering the neuron capable of being inhibited by hypercapnia. Candidate ion channels whose expression could be induced by CHx include TREK1 which is activated by decreased pHᵢ (Honoré, 2007), or KCₐ channels (Wellner-Kienitz et al., 1998).

Our data suggest that CHx suppresses the chemosensitive response of NTS neurons from adult rats, but it appears to increase ventilation, including the hypoxic ventilatory response, the hypercapnic ventilatory response, and the normoxic ventilatory response (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and Robbins, 1998; Reid and Powell, 2005). Thus, at first glance there seems to be a lack of correlation between the findings of this study on the cellular response after CHx and previous studies on the whole animal ventilatory response after CHx. Since we only studied one chemosensitive area, the NTS, and found plasticity induced, it is possible that plasticity is
induced in other regions involved in central chemosensitivity. However, in order to understand how this plasticity caused by CHx is translated to the whole animal level, we must first understand how the neuronal network for control of breathing operates and how it is altered by CHx.

NTS neurons may play a role in changing the normoxic ventilatory response after CHx. The increase in the percentage of NTS neurons that are inhibited by hypercapnia after CHx, would mean more NTS neurons are activated by hypocapnia, which occurs due to hyperventilation in hypoxia. In a previous study, it has been found that medullary raphé neurons that were inhibited by hypercapnia (9% CO₂) were activated by hypocapnia (3% CO₂) (Richerson et al., 2001). Thus, we suggest that the increase in the percentage of NTS neurons that are inhibited by hypercapnia could increase normoxic respiratory drive at the decreased levels of PaCO₂ seen in response to CHx.

NTS neurons may play a role in the HVR. We do not know if the NTS neurons that have increased inhibition in response to hypercapnia respond to hypoxia. Interestingly, it has been shown that NTS neurons are sensitive to changes in the level of O₂. Some NTS neurons respond to hypoxia (Pascual et al., 2002; Dean, unpublished observations) and respond to hyperoxia (Mulkey et al., 2003) with an increase in their firing rate. It is thus possible that the NTS plays a role in the HVR. In this regard, it would be interesting to determine the nature of the response to hypoxia of NTS neurons and whether this response changes with CHx.

It is not clear how or whether our results correlate with the whole animal response to hypercapnia after CHx, since it has been found that CHx increases the ventilatory response to hypercapnia (Bisgard et al., 1986; Engwall and Bisgard, 1990; Fatemian and
Robbins, 1998). We suggest three possibilities to explain how there is an increase in the HCVR (Fig. 46): 1) the carotid sinus nerve synapses on a subset of NTS neurons that remain activated by hypercapnia and whose response to hypercapnia is increased further after CHx, thereby amplifying the response to hypercapnia and increasing HCVR (green); 2) changes in post-synaptic non-chemosensitive NTS neuronal properties (e.g. increased input resistance) occur in response to CHx and increase the NTS neuronal response to afferent input from the carotid body, increasing HCVR (blue); or 3) the increased HCVR after CHx is mediated by neurons or cells located in other regions within the distributed network (Nattie and Li, 2008) such as the carotid bodies, the RTN (orange), the LC, or the medullary raphé. One possible region that could regulate the hypercapnic ventilatory response are the carotid bodies, since they also sense changes in CO₂ (Fatemian et al., 2003; Dempsey, 2004; Nattie, 2006b; Smith et al., 2006). Specifically, it was found that carotid bodies were responsible for the rapid response to hypercapnia, whereas the central chemoreceptors were responsible for the steady-state response to hypercapnia (Fatemian et al., 2003; Dempsey, 2004; Smith et al., 2006). Therefore, it is possible that after CHx the main response to hypercapnia is mainly mediated by an increase in the responsiveness of carotid bodies to hypercapnia. Another region that may be responsible for increasing the HCVR after CHx is the RTN. We propose this site because it is known that RTN neurons are highly sensitive to hypercapnia (Mulkey et al., 2004b; Ritucci et al., 2005b) and receive input directly from the NTS or receive input from the carotid bodies through the NTS (Stornetta et al., 2006; Takakura et al., 2006).

Regardless, we have reported that an altered environmental condition can affect the neuronal response to hypercapnia of NTS neurons. The increased percentage of
Figure 46:

Summary of proposed possibilities to explain an increase in HCVR in CHx-adapted rats. For the green and blue pathways, we propose that the carotid body is first activated by high CO$_2$ which then activates the carotid sinus nerve (CSN) to send peripheral afferent input to the NTS. 1) green pathway represents the CSN synapsing on a subset of NTS neurons that remain activated by hypercapnia and whose response to hypercapnia is proposed to increase further after CHx. 2) blue pathway represent the carotid sinus nerve synapsing on a non-chemosensitive NTS neuron whose neuronal properties (such as the conductance, (g)) are proposed to change in response to CHx resulting in an increased HCVR. 3) orange text represents how the increased HCVR after CHx may be mediated by neurons or cells located in other regions such as the carotid bodies or the RTN. The red pathway represents NTS neurons that are inhibited by hypercapnia, which we believe are not receiving input from the carotid bodies, and whose sites of projection we do not know.
Figure 46:

Type 1 cells or glomus cells in carotid bodies

High CO₂
hypercapnia-inhibited NTS neurons in CHx-adapted rats offers a good model system in which to study the cellular signaling mechanisms involved in inhibition of neurons by hypercapnia. Previously, we have been unable to study the mechanism of the response of inhibited neurons because they were so few in number. Thus, using rats exposed to CHx will allow us to identify the mechanism(s) of the response of hypercapnia-inhibited NTS neurons, since the percentage of the neurons increases from 10% up to 30%.

The last major finding of this study is that substance P modulates basal firing rate of NTS neurons that are hypercapnia-activated and non-chemosensitive in both control and CHx-adapted rats. Substance P does not significantly affect the firing rate of neurons that are hypercapnia-inhibited. Further, neither the addition of substance P nor the blocking of NK1 receptors altered the response (percentage or CI) of NTS neurons to hypercapnia. In whole animal studies, injection of substance P into the 4th ventricle or directly into the NTS increased ventilation or the firing of NTS neurons (Hedner et al., 1984; Morin-Surun et al., 1984; Yamamoto and Lagercrantz, 1985; Chen et al., 1990a). These findings are entirely consistent with our results, since substance P substantially increases the firing rate of non-chemosensitive and hypercapnia-activated NTS neurons. We presume that in the intact rat, the major source of exogenous substance P to the NTS will come from afferent terminals from the carotid bodies, a pathway which is lacking in the NTS slice. Our data indicate, however, that substance P does not appear to play a role in increased HVR or HCVR in CHx-adapted rats, at least not by modifying the sensitivity of NTS neurons. We considered the possibility that increased HVR or HCVR in CHx-adapted rats could have been due to NTS neurons being more sensitive to substance P, which we would have observed as a bigger firing rate response to exogenously applied
substance P in NTS neurons from CHx rats. In contrast, if anything, we saw a decrease in the firing rate response to substance P of NTS neurons from CHx rats. Further the response of NTS neurons to acute hypercapnia was unaltered by the presence of substance P, whether the neurons from control or CHx-adapted rats. Finally, the fact that an NK1 antagonist did not alter basal firing rate shows that in our slices there was no apparent tonic release of substance P. Thus, if substance P plays a role in altered HVR or HCVR in CHx-adapted rats it does so in a way that does not involve altered NTS neuronal sensitivity to either substance P or hypercapnia. It is possible that substance P could affect other properties of the respiratory network. This possibility adds further importance to the need for studies of the nature of the respiratory network and how the network properties are modulated by acute hypercapnia, CHx, and substance P.
CHAPTER IX

FUTURE DIRECTIONS
Our findings suggest several future experiments and research directions to further understand control of breathing and specifically central chemosensitivity. The following studies will further help address the mechanism of how neurons respond to a hypercapnic stimulus, aid understanding of the network more clearly, and contribute to our understanding of how the control of breathing changes in pathological conditions like sleep apnea or chronic obstructive pulmonary disease.

First, one result of this study was the decrease in steady state pH$_i$ in adult rats compared to neonatal rats. It is possible that the change in steady state pH$_i$ is due to the expression of Cl$^-$/HCO$_3^-$ exchangers on NTS neurons from adult rats, which are absent in NTS neurons from neonatal rats (Ritucci et al., 1998; Putnam, 2001). It was concluded in a previous study that the more alkaline steady state pH$_i$ of 7.49 in NTS neurons from neonatal rats, compared to neurons from other medullary regions, is due to the lack of expression of acidifying Cl$^-$/HCO$_3^-$ exchange and to the presence of alkalinizing Na$^+$/H$^+$ exchange (Ritucci et al., 1998). The steady state pH$_i$ was less alkaline (around 7.30) in neurons from other medullary regions of neonatal rats including the hypoglossal and ventrolateral medulla, which were found to express Cl$^-$/HCO$_3^-$ exchangers (Ritucci et al., 1998). If the change in steady state pH$_i$ of NTS neurons from adult rats is due to the expression of Cl$^-$/HCO$_3^-$ exchangers vs. NHE exchangers, then it is possible that other changes occur involving pH regulating transporters, such as alkalinizing HCO$_3^-$ dependent transporters in NTS neurons from adult rats. It would thus be interesting to conduct studies of pH$_i$ regulation in NTS neurons from adult rats using inhibitors of pH-regulating transporters including amiloride (sodium hydrogren exhanger inhibitor), 5-(N-
ethyl-N-isopropyl)-amiloride (EIPA: sodium hydrogen exchanger inhibitor), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS: HCO$_3$-Cl exchanger inhibitor).

In addition to conducting these studies of pH$_i$ regulation in NTS neurons from control adult rats, it would also be interesting to conduct these studies on NTS neurons from CHx-adapted rats. We found that the steady state pH$_i$ of NTS neurons from CHx-adapted rats was also less alkaline (~7.35), suggesting that NTS neurons from CHx-adapted rats also express Cl-/HCO$_3^-$ exchangers. Although we did not observe any significant differences in either steady state pH$_i$ or the magnitude of acidification induced by hypercapnia, exposure to CHx has been shown to alter the expression of HCO$_3^-$ dependent pH regulating transporters (Chen et al., 2007) indicating pH$_i$ regulating transporters can be altered by CHx. Thus, it would be interesting to examine whether or not pH$_i$ regulating mechanisms change in NTS neurons exposed to CHx using the same inhibitors mentioned above.

We found, surprisingly, that substance P induced a larger magnitude of acidification in response to hypercapnia when compared to the magnitude of acidification in response to hypercapnia in the absence of substance P. We are unsure of how substance P could cause a larger response of pH$_i$, but we suggest that substance P is acting on a pH$_i$ regulatory transporter. This could be studied by measuring pH$_i$ recovery from acidification induce by an NH$_4$Cl prepulse technique in the absence and presence of substance P, as well as using the inhibitors of pH regulating transporters mentioned above. In addition, substance P leads to an increase in firing rate and this could contribute to the enhanced hypercapnic-induced acidification. However, Ritucci et al. (2005a) showed that an increase in firing rate of 4 Hz resulted in a 0.06 pH unit change,
suggesting this mechanism plays at most a small part in the hypercapnia-induced acidification.

Another future area of research would be to pursue identified neurons electrophysiologically (distinguish the response to hypercapnia) and then studying differences/similarities between the three classes of NTS neurons (activated, inhibited, non-chemosensitive), specifically looking at morphology and the ion channels that they express. Since we believe that pyranine was causing the lack of visualizing immunolabeling, we suggest that neurons be identified using Lucifer yellow or neurobiotin. It has been observed previously that the morphology of neurons in cell culture conditions differed depending on how they responded to hypercapnia (Richerson et al., 2001). Specifically, it was found that raphé neurons activated by hypercapnia had more dendritic processes, whereas neurons inhibited by hypercapnia had less dendritic processes (Richerson et al., 2001). Given this information, it would interesting to study the morphology of NTS neurons and how they differ depending on their response to hypercapnia. In addition, after neurons are identified for their electrophysiological response to hypercapnia, these neurons could then be immunohistochemically processed for the presence of different pH-sensitive ion channel subunits. Specifically, activated NTS neurons could be stained for either $K_{ir}4.1$ (Schulte and Fakler, 2000; Xu et al., 2000), $K_{c}1.4$ (Berger et al., 1998), or TASK 1 and 3 (Bayliss et al., 2001; Duprat et al., 1997). Inhibited NTS neurons would be stained for either TREK1 (Honoré, 2007) or $K_{Ca}$ channels (Wellner-Kienitz et al., 1998). These channels are the ones that have been proposed to be involved in the mechanism for the increase (activated neurons) or decrease in firing rate (inhibited neurons) caused by hypercapnia.
Our findings suggest there is a decrease in the number of NK1 receptors on NTS neurons from CHx animals. We studied the response to substance P, which was found to increase basal firing rate of NTS neurons from control rats significantly more than it stimulated the basal firing rate of NTS neurons from CHx-adapted rats. In addition, we found that NK1 receptors were not present on inhibited neurons (in either group of rats), and that the percentage of inhibited NTS neurons were increased in CHx-adapted rats. Further, we observed less immunolabeling of NK1 receptors on NTS neurons from CHx-adapted rats, but we did not perform any quantification of NK1 receptor staining. Thus, our findings suggest that NK1 receptor immunofluorescence will be decreased in NTS neurons from CHx-adapted rats compared to control rats, and that a lack of NK1 receptor staining may be a marker for hypercapnia-inhibited neurons. Thus, a detailed, quantitative study of NK1 receptor staining and a correlation of that staining with firing rate response of NTS neurons seems warranted.

Another future area of research is to investigate the mechanism(s) by which neurons are inhibited by hypercapnia. Our findings have resulted in a model to study inhibited neurons. Specifically, we find that CHx increases the percentage of NTS neurons that are inhibited in response to hypercapnia, and we have found that inhibited NTS neurons do not respond to substance P. Therefore, NK1 receptors could be fluorescently labeled (using TMR-SP), and then neurons that lack staining could be electrophysiologically characterized by studying the firing rate response to hypocapnia and hypercapnia. Neurons which are inhibited by hypercapnia could be immunohistochemically processed for subunits of TREK1 and KCa channels. We assume that hypercapnia-inhibited NTS neurons must hyperpolarize in response to hypercapnic
acidosis, which could occur if these neurons possessed a pH$_i$-activated K$^+$ channel, such as TREK1 (Honoré, 2007) or activated K$_{Ca}$ channels through increased intracellular Ca$^{2+}$ (Wellner-Kienitz et al., 1998). These possibilities could be tested with our model system.

We only studied the effect of CHx on one chemosensitive area, the NTS. We found that CHx causes changes in the chemosensitive response of NTS neurons, however, these changes do not explain how either the hypoxic or hypercapnic ventilatory responses are increased in response to CHx. It is possible that the increased ventilatory response to either hypoxia and/or hypercapnia could be due to changes in other chemosensitive areas including the RTN, pre-Bötzinger complex or carotid bodies. We could directly test this using the approaches from the current study to examine cells from these 3 regions.

Another way to study the network would be to use retrograde labeling techniques. Areas that we propose to first retrogradely label include the phrenic motor nucleus, pre-Bötzinger complex, RTN, and LC. Then neuronal properties of NTS neurons that are retrogradely labeled could be studied. Specifically, we would study the nature of NTS neurons that project to the phrenic motor nucleus to directly modulate ventilation including whether NTS neurons are activated or inhibited by hypercapnia. We would also study the nature of NTS neurons (whether NTS neurons are activated or inhibited by hypercapnia) that project to other chemosensitive areas such as the RTN, LC, and pre-Bötzinger complex. Lastly, we could study what neurotransmitters are released from NTS neurons. This would then give us information about how the network for breathing works. In addition, these experiments could be repeated in animals adapted to CHx.
Again, it would be interesting to look at the nature of neurons and how their properties do / do not change after exposure to CHx.

In summary, many ideas for future research arise based on the results of this work. One of the most important questions, which is increasingly becoming important, is what role central chemosensitive areas play in the ventilatory response. However, in order to completely understand how ventilatory control works, we must understand all of the various levels that are known to have a role in breathing (Fig. 1). Although none of the future studies suggested here directly answer this question, they are all necessary experiments to be done to begin to understand how individual cells/neurons work as well as how areas work together to form a network to regulate the fundamental physiological process of breathing.


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CHAPTER XI

CURRICULUM VITAE
NICOLE L. NICHOLS
CURRICULUM VITAE

Wright State University
Department of Neuroscience, Cell Biology and Physiology
009 Medical Sciences Building
3640 Colonel Glenn Highway
Dayton, Ohio 45435 U.S.A.
Tel: (937)775-3543
Fax: (937)775-3391
email: nichols.36@wright.edu

EDUCATION:
2003-present Wright State University Boonshoft School of Medicine, Dayton, OH Ph.D. in Biomedical Sciences with concentration in Neuroscience and Physiology
2003 Otterbein College, Westerville, OH B.S. in Molecular Biology

RESEARCH EXPERIENCE:
2003-present Doctoral research advised by Dr. Robert Putnam
(robert.putnam@wright.edu). The effects of chronic hypoxia on the chemosensitive response of individual nucleus tractus solitarius (NTS) from adult rats using electrophysiology, fluorescence imaging, and immunohistochemistry. Department of Neuroscience, Cell Biology, and Physiology, Wright State University, OH.
2002-2003 Undergraduate research advised by Dr. Long-Sheng Chang (Long-Sheng.Chang@nationwidechildrens.org). The transcriptional start site for an isoform of the p73 gene using PCR, 5’RACE, sequencing, and cloning. The Research Institute at Nationwide Children’s Hospital in Columbus, Ohio.

ACADEMIC HONORS:
2003-present Pre-doctoral Fellowship – Biomedical Sciences Ph.D. Program, Wright State University
2008 Selected to participate in the 2008 St. Jude National Graduate Student Symposium
2008 Selected for the American Physiological Society Trainee Highlights in Respiration Physiology Poster Discussion Session
2008 American Physiological Society Carolyn tum Suden/Francis A. Hellebrandt Professional Opportunity Award
2007 Ohio Miami Valley Chapter for Neuroscience Travel Award for Neuroscience 2007
2006 Invited presentation at “pH, CO₂, and Brain Function” Symposium in Helsinki, Finland
2006 Selected to participate in the APS Professional Skills Training short course on "Writing and Reviewing for Scientific Journals," Englewood, Colorado
2002-2006 Licking County Foundation ~ Julius and Beulah Lenz Scholarship for Outstanding Science and Engineering Majors
2003 Graduated with Distinction in Molecular Biology, Department of Life Sciences, Otterbein College
1999-2000, 2002-2003 Deans List at Otterbein College
2000-2003 Alpha Epsilon Delta
2000-2003 Alpha Lambda Delta

TEACHING EXPERIENCE:
2007-present Tutor for undergraduate anatomy and physiology class for nursing students
2005-present Tutor for undergraduate human physiology class for nursing students
2007 Supervised undergraduate student Catalina Martinez (Characterization and identification of NK1 receptors on nucleus tractus solitarius neurons using immunohistochemistry)
2004-2005 Graduate Teaching Assistant for undergraduate human physiology class for nursing students

LIST OF PUBLICATIONS:
MANUSCRIPTS IN PREPARATION:


PROFESSIONAL MEMBERSHIPS

American Physiological Society
Society for Neuroscience
Ohio Miami Valley Chapter for Neuroscience
Ohio Physiological Society

COMMUNITY SERVICE

2008 Science Fair Judge at St. Peters School in Huber Heights, Ohio