Chemosensitive Neurons of the Locus Coeruleus and the
Nucleus Tractus Solitarius: Three Dimensional Morphology and
Association with the Vasculature

A DISSERTATION IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

By

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2014
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MY SUPERVISION BY CATHY D. GRAHAM ENTITLED
Chemosensitive Neurons of the Locus Coeruleus and the Nucleus Tractus
Solitarius: Three Dimensional Morphology and Association with the Vasculature
BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
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ABSTRACT

Graham, Cathy D., Ph.D., Biomedical Sciences Program, Wright State University, 2014. Chemosensitive Neurons of the Locus Coeruleus and the Nucleus Tractus Solitarius: Three Dimensional Morphology and Association with the Vasculature.

We studied the 3 dimensional structure of LC and cNTS neurons from neonatal rats. Neurons were identified as either chemosensitive or nonchemosensitive based on their firing rate response to hypercapnia, using whole-cell patch clamping and were passively loaded with Lucifer Yellow. The identified, loaded neurons were reconstructed and analyzed using Neurolucida Neuron Tracing Software. Chemosensitive LC neurons have less rounded soma than nonchemosensitive neurons, and they have dendrites arising from two poles to produce a fusiform appearance. The dendritic branches extend a greater distance from the soma of chemosensitive than nonchemosensitive neurons. Conversely, chemosensitive cNTS neurons were found to have ovoid multipolar somas with compact, complex arborization while nonchemosensitive neurons had bipolar somas with projections arising from opposite poles separated by ~180°. Chemosensitive LC neurons and cNTS neurons have at least one projection that makes a closer approach to the floor of the 4th ventricle than do nonchemosensitive neurons from both regions, but on average this closest approach is still greater than 50 microns. These data indicate that projection to the surface of the brainstem is not necessary for the chemosensitive response in
the LC and the cNTS. To evaluate regional vascularity and neuronal populations, neurons and blood vessels were labeled using immunohistochemical techniques. Neurons were labeled with NeuN and blood vessels with Isolectin-GS-IB4 (a marker for endothelial cells). Confocal microscopy was used to collect images through 50 µm sections. Image J analysis of the confocal projections revealed that in the LC there is a higher density of blood vessels in the chemosensitive dorsal and intermediate regions compared to the nonchemosensitive ventral region of the LC. Interestingly, the dorsal and intermediate zones of the LC have a very high percentage of chemosensitive neurons while the ventral zone has mostly nonchemosensitive neurons, suggesting greater perfusion of chemosensitive neurons. In the cNTS, the densest neutrophil was observed in the medial area with less neuronal density in the commissural and lateral areas. The overall cNTS regional microvascular density was more homogenous and considerably less dense than that of the LC. Finally, we found that capillaries make direct contacts with the somata of chemosensitive neurons but not with the somata of nonchemosensitive neurons. In the cNTS, there is no pattern of association of blood vessels with individual neuronal structures though some contact was observed. Our findings demonstrate that there is not a single neuronal morphology typical of chemosensitive neurons but that there are morphological distinctions between chemosensitive vs. nonchemosensitive neurons which may be region specific. Our data indicate that association between the dendritic arbor and the surface of the floor of the fourth ventricle is not necessary for chemosensing in these two areas. The demonstrated close
association between the somas of chemosensitive neurons and capillaries in the LC is consistent with chemosensitive signaling being based in the soma of LC neurons. When compared to cNTS, our findings do not support the hypothesis that this is a neuronal/vascular arrangement requisite for chemosensitive areas of the brainstem. Our data provide anatomically accurate information for the construction of physiologically relevant, multi-compartment computational models of chemosensitive neurons.
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DEDICATION

To my parents, who taught me that anything is possible.

&

To my children, Skylar, Cody and Quinlan.

Thank you for your boundless love and your wonder and your faith.

Always, always be curious.

&

To Daniel M. Gray, Jr., for keeping the home fires burning.

“Sometimes I've believed as many as six impossible things before breakfast.”

Alice in Wonderland
I. INTRODUCTION

Breathing is a highly regulated physiological process. One of the major drives to breathe is elevated CO₂ in the blood (Haldane and Priestly, 1905). CO₂ is sensed in large part by neurons from several regions of the brainstem whose firing rate is increased by elevated CO₂ (hypercapnia) (Pineda & Aghajanian, 1997; Oyamada et al., 1998, 1999; Wang et al., 1998; Studden et al., 2001; Filosa & Putnam, 2003; Mulkey et al., 2004; Ritucci et al. 2005). These neurons are referred to as central chemosensitive neurons, or central chemoreceptors (Coates, E.L. 1993; Feldman, J.L. 2003; Putnam et al., 2004; Nattie, E. 2009). Ballantyne and Scheid (2001) stated, in reference to chemosensitive neurons, that “One of the things which we would like to know is whether the specializations which make these neurons chemosensitive are restricted to the molecular level or whether they also show up in cell morphology.” In other words, do chemosensitive neurons have a unique morphology relative to nonchemosensitive neurons?

Over the intervening 13 years this question has remained largely neglected. This lack of attention is remarkable since Ballantyne and Scheid (2001) described two good reasons to do such studies. The first is that “...It would be convenient if it turned out that chemosensitive neurons come with a morphological “tag” by which they might be identified.” Chemosensitive neurons are almost always within heterogeneous regions of the brainstem that also contain nonchemosensitive neurons (Putnam et al., 2004) and, thus, the ability to readily identify them based on an obvious structural element or regional location
would be helpful, especially for electrophysiological studies. The second reason, given by Ballantyne and Scheid (2001), to study the morphology of chemosensitive neurons “…is that cell structure may influence numerous details in the form of the response to a chemostimulus.” This was discussed particularly in terms of the dendritic processes of chemosensitive neurons and whether they exhibited some distinct structure, like sending projections to the brainstem surface or associating closely with blood vessels.

This thesis is focused on morphometric study of two dorsal regions of the brainstem known to contribute to central ventilatory control and to contain chemosensitive and nonchemosensitive neurons. The findings presented here represent the first detailed studies of 3 dimensional cytoarchitecture in the LC and the cNTS, documenting whether structural differences exist between nonchemosensitive and chemosensitive neurons from the same region and whether there are fundamental similarities of neuronal morphology between regions of chemosensitivity. My experiments characterize for the first time regional topographical subdivisions of preferential arrangement of chemosensory neurons in the LC. I have addressed the question whether dendritic projections to the surface is a common feature of central chemoreceptors from all areas of chemosensitivity, or whether that is a region specific morphological characteristic. This work is the only comparative study of the association between chemosensitive and nonchemosensitive neurons and the microvasculature from two regions. The work performed for this thesis will provide a foundation and precedent for thorough future morphological analysis of
chemosensitive neurons in the brainstem and will guide and stimulate inclusion of morphometrics in the experimental designs exploring the cellular locus of the chemosensitive response, possible chemosensory stimuli and chemosensing targets.
II. REVIEW OF THE LITERATURE

1. Breathing: An Overview

Breathing (or ventilation) is part of a system of complex physiological mechanisms by which mammalian organisms inspire oxygen (O₂) for transport to tissues to support cellular aerobic metabolism and remove carbon dioxide (CO₂), a byproduct of that metabolism. Dissolved CO₂ forms carbonic acid (H₂CO₃) catalyzed by the enzyme, carbonic anhydrase. H₂CO₃ rapidly dissociates to yield bicarbonate (HCO₃⁻) and free hydrogen ions (H⁺). The pH of body fluids depends on the ratio of CO₂ to the amount of HCO₃⁻. As levels of CO₂ rise, the concentration of free H⁺ increases, resulting in an acid state in the blood. Major factors that contribute to the control of ventilation are low O₂ levels in the blood (hypoxia) or high CO₂ levels in the blood (hypercapnia). In fact, rising levels of CO₂ and concomitant acidification of the blood has long been identified as a major ventilatory stimulus (Haldane & Priestley, 1905). Mechanisms therefore exist to sense blood O₂ and CO₂/H⁺ and these mechanisms allow for the adjustment of ventilation to enable the organism to maintain a constant supply of O₂ to the tissue and a constant and appropriate level of removal of CO₂ from the body, that also serves to help maintain a stable level of blood pH.

2. Central Chemosensitivity and Ventilatory Control

Extensive studies in a variety of organisms have demonstrated that the neurochemical regulation of ventilatory function, maintenance of blood gas (O₂, CO₂) concentrations and stabilization of pH to within appropriate physiological
ranges (pH=7.35-7.45; PaCO$_2$ = 35-45 mmHg; PaO$_2$=80-100 mmHg; HCO$_3$=22-26mmol/L (Fischbach & Dunning, 2009)) are maintained by a complex network that involves a central pattern generator and numerous feedback loops from the periphery and the central nervous system (Feldman et al., 2003; Smith et al., 2010; Feldman et al., 2013). The central pattern generator, which sets the basic rhythm for breathing, has been associated with the pre-Bötzinger Complex (PBC) in the medulla (Smith et al., 1991; Feldman et al., 2003). However, slow ablation of the PBC results in only temporary disruption of the breathing rhythm with re-establishment of a normal breathing pattern despite near complete ablation (Krause et al. 2009) suggesting that the PBC may not be the primary kernel of rhythm generation but may have an important modulatory role and that there must be other sites for rhythm generation in the brainstem (Richter et al. 1986; Rubin et al., 2011). Thus there must be functional plasticity, redundancy and compensatory mechanisms within the respiratory rhythm centers.

The breathing rhythm is modified by inputs from numerous sensory components, the most important of these being the O$_2$ and CO$_2$ sensors (referred to here as chemosensors or chemoreceptors) that are located both peripherally and centrally (Gonzalez et al., 1994; Nurse CA. 2010; Guyenet and Mulkey, 2010; Hodges and Richerson, 2008; Nattie, 2000). The peripheral chemoreceptors, located in the carotid and aortic bodies, respond to changes in arterial oxygen (O$_2$) and also to CO$_2$ /H+ and provide afferent input to the central nervous system (CNS) respiratory centers (Nurse and Piskuric, 2013; Prabhakar,
Either hypoxia or hypercapnia will activate these peripheral chemoreceptors and lead to increased ventilation.

Ventilatory responses to hypercapnia remain after denervation of peripheral chemoreceptor input to the CNS. Carotid body denervation eliminates excitatory afferent inputs from the carotid bodies to the NTS, which has numerous target nuclei, including the ventral respiratory group, retrotrapezoid nucleus (Mulkey et al., 2004; Takakura et al., 2006) and medullary raphe nuclei (Jean A., 1991; Nuding et al., 1991). The respiratory network excitability in response to hypercapnia though reduced (Pan et al., 1998) remains, indicating that regulation of respiration involves both central and peripheral chemosensitivity (Nattie, 1987; Rodman et al., 2001).

Chemosensory components that impact ventilation within the CNS have indeed been described, especially within the brainstem (Coates et al., 1993; Feldman et al., 2003; Putnam et al., 2004; Dean and Nattie, 2010; Nattie and Forster, 2010; Nattie and Li, 2010; Smith et al., 2010). Early research into CNS ventilatory control focused on ventral medullary neuronal groups (Mitchell et al., 1963, Schlaefke et al., 1970, Loeschcke, 1982). More recent work has shown that central chemoreception exists in several regions of the brainstem, including several ventral areas such as the ventrolateral medulla (Coates et al., 1993; Nattie and Li, 1996), the retrotrapezoid nucleus (RTN) (Li et al., 1999), the medullary raphé (Bernard et al., 1996; Nattie and Li, 2001), and the PBC (Solomon et al., 2000; Krause et al., 2009) as well as dorsal areas such as the nucleus of the solitary tract (NTS) (Nattie and Li, 2002; Nichols, N. et al.) and the
locus coeruleus (Coates et al., 1993; Ritucci et al., 2005). One of the first lines of evidence for the involvement of these multiple central areas in ventilatory control came from *in vivo* experiments in anesthetized whole animals. These experiments employed focal acidification at a number of widely distributed sites, including ventrolateral medulla, NTS, and LC, resulting in increased respiratory motor (phrenic nerve) output (Coates et al., 1993; Bernard et al., 1996; Nattie & Li, 1996; Li & Nattie, 1997). Further evidence for a role for these different areas in central ventilatory control came from experiments in which ablation of any one of these areas caused a depression of the ventilatory response to inspired CO$_2$ (Dias et al., 2007; Biancardi et al., 2008; Nattie and Li, 2009). It has also been found that the ventilatory response can be suppressed and even eliminated depending on the state of arousal/awareness of the organism (Li et al., 1999; Nattie, 2000; Nattie, 2001).

In addition to the *in vivo* work in CO$_2$ sensitivity has been identified using various *in vitro* (slice and cell culture) preparations from multiple brainstem regions: the rostroventral medulla (RVM), the retrotrapezoid nucleus (RTN) (Nattie and Li, 1990; Coates et al., 1993; Akilesh et al., 1997; Wellner-Kienitz and Shams, 1998; Hewitt et al., 2004; Mulkey et al., 2004, Ritucci et al., 2005), the medullary raphé (Richerson, 1995; Bernard et al., 1996, Wang et al. 1998; Hodges and Richerson., 2008), the locus coeruleus (LC), (Coates et al., 1993; Pineda & Aghajanian, 1997; Oyamada et al., 1998) and the nucleus tractus solitarius (NTS), (Berger and Cooney, 1982; Dean et al., 1989; Dean et al.,
The growing body of work supports the theory that multiple areas within the brainstem contribute to central ventilatory control. There has been some debate about the relative role of each area and why there are so many different regions for central ventilatory control (Ballantyne and Scheid, 2001; Dean and Nattie, 2010; Nattie and Forster, 2010; Nattie and Li, 2010). Some of the suggestions that have been proposed include that the multiple regions represent redundancy in control of a critical physiological function or that each region might be specialized to function under certain conditions, such as awake vs. sleep states, or in neonates vs. adults (Li et al., 1999; Gargaglioni et al., 2010). The current accepted hypothesis in the field is that central ventilatory control is mediated by a complex integrated network (Coates, E.L. 1993; Huang, R.-Q. 1997; Putnam, R.W. 2004; Nattie and Li, 2010).

3. Individual Chemosensitive Neurons

In the previous section some of the evidence was reviewed for the presence of several central chemosensitive regions within the brainstem. All of these regions have an additional feature in common: they all contain some percentage of their neurons whose firing rate is altered by changes of CO₂ (Putnam et al., 2004). These neurons are referred to as central chemosensitive neurons. Upon exposure to hypercapnia, the vast majority of these neurons increase their firing rate although in some regions, a smaller percentage may decrease their firing rate in response to hypercapnia (Putnam et al., 2004).
While both are considered to be chemosensitive, this thesis will focus on those neurons whose firing rate is increased by hypercapnia because they are far more common (~80%), especially in a region like the LC (Filosa et al., 2002). Thus, for purposes of the work in this thesis, a neuron is identified as chemosensitive whose firing rate is increased by hypercapnia.

One factor complicating our understanding of chemosensitivity is that not all neurons within these regions are chemosensitive and the chemosensitive response is not the same in chemosensitive neurons from different regions. For example, nearly all the neurons in the LC (~80-90%) respond to hypercapnia by increasing firing rate but the magnitude of the response is quite modest (Pineda and Aghajanian, 1997; Oyamada et al., 1998; Filosa et al., 2002). By contrast, very few raphé neurons (~18%) respond to hypercapnia, but the magnitude of the increased firing rate is large (Richerson et al., 2001). Neurons from the NTS fall in between with approximately 50% of the neurons responding to hypercapnia with a CO₂ induced increase in firing rate that is intermediate between the LC and raphé responses (Conrad et al., 2009). The variation in the hypercapnic response among these neurons can be used to help assess the characteristics of a neuron that make it chemosensitive. The specific characteristics of chemosensitive neurons likely vary from region to region. This is why study and comparison across regions using similar techniques is so important. The focus of this study involved both chemosensitive and nonchemosensitive neurons from two of those regions, the locus coeruleus (LC) and the caudal nucleus tractus solitarius (cNTS).
4. What Determines Chemosensitivity?

Specialized chemosensitive neurons, chemoreceptors, are conventionally understood to be neurons that exhibit an altered firing rate in response to increased CO\(_2\) (hypercapnia) or decreased pH (acidaemia). This response, independent of synaptic input or gap junction coupling, is considered intrinsic chemosensitivity. Identifying the mechanism or mechanisms involved in the cellular response to CO\(_2\)/H\(^+\) has been elusive. Even precisely determining the stimulus has proved challenging.

One difficulty with the idea that it is the blood pH that is the stimulus lies in the fact that the brain is protected from direct contact with arterial blood by the blood-brain barrier (BBB) (Laterra et al., 1999). Another hypothesis is that it is the pH of the CSF or extracellular fluid (ECF) that stimulates the chemosensitive response (Winterstein, 1956). It has been shown that chemosensitive neurons in several regions (NTS, ventrolateral medulla, and locus coeruleus) increase firing rate in response to intracellular pH (pHi) and will remain acidified under hypercapnic conditions (Ritucci et al., 1997, Ritucci et al., 1998, Filosa et al., 2002, Mulkey et al., 2004) and that exposure to hypercapnia results in acidification of both extracellular and intracellular compartments (Ritucci et al., 1997, Filosa et al., 2002, Putnam et al., 2004). Whether or not the change in pH is the proximal cause for chemosensitive neuronal activation is not clear. That CO\(_2\) itself, independent of pH, can act as the stimulus for the chemosensitive response in these neurons has been demonstrated as well (Huckstepp et al., 2010; Meigh et al., 2013). Studies to date lead to a multiple factors theory.
suggesting, not a single stimulus, but a combination of stimuli working in concert to activate neurons controlling ventilation (Putnam et al., 2004).

Mechanisms of the neuronal chemosensory response are not well understood. Though CO₂ concentration is the most common experimental variable, the precise site of action in chemosensitive neurons is still unknown (Ritucci et al., 1997; Voipio & Ballanyi, 1997). It has been proposed that an increase in CO₂ leads to extracellular (pH₀) and/or intracellular acidosis (pHᵢ) which in turn inhibits pH-sensitive channels, causing depolarization and subsequent increased firing rate in chemosensitive neurons (Filosa et al., 2002; Putnam et al., 2004; Nichols et al., 2008; Putnam, 2010;). Multiple K⁺ channels sensitive to hypercapnia-induced acidification have been identified in LC neurons, including A-current and a delayed-rectifying K⁺ channels, inwardly-rectifying K⁺ channels, TASK channels, (Pineda and Aghajanian, 1997; Gargaglioni et al., 2010; Li and Putnam, 2013). A role for Ca²⁺ channels in the chemosensitive response has been suggested by many studies (Erlichman and Leiter, 1997; Wellner-Kienitz et al., 1998; Summers et al., 2002; Filosa and Putnam, 2003; Denton et al., 2007; Imber and Putnam, 2012). The adaptive ventilatory response in rats has been shown to decrease in response to Ca²⁺ channel blockade in the ventral medulla (Kanazawa et al., 1998) and in cultured medullary neurons, Ca²⁺ channel inhibition reduced chemosensitivity (Wellner-Kienitz et al., 1998). Evidence in LC neurons suggests that Ca²⁺ may play a role in chemosensitive signaling as well (Williams et al., 1984; Christie et al., 1989; Oyamada et al., 1998; Filosa and Putnam, 2003; Imber and Putnam, 2012).
Studies in both caudal and medial NTS have identified multiple Ca\(^{2+}\) channel types in these neurons (Kunze, 1987; Rhim and Miller, 1994; Hille, 2001). There is a wide array of ion channels that are instrumental in chemosensing by specialized neurons in the central chemosensitive network.

5. Intrinsic Chemosensitivity

In order to study chemosensitive neurons it is critical to be certain that they are indeed chemosensitive and that the response is intrinsic to the individual neuron in question. Intrinsically CO\(_2\) sensitive neurons retain CO\(_2\)/pH sensitivity in the absence of synaptic activity and/or gap junction input. A typical method to identify a neuron as chemosensitive involves whole cell patch electrophysiological recording during hypercapnic stimulation. It is possible that a recorded neuron which appears to respond to hypercapnic stimuli may not be intrinsically chemosensitive, but be excited by chemical synaptic input from an adjacent neuron which is in fact the intrinsically chemosensitive neuron. This possibility can be overcome by blocking the synaptic input, allowing accurate identification of the chemosensitive properties of a single neuron. Another possible source of excitation is electrical coupling via gap junctions. The role of neuronal gap junctions is to provide a pathway of low resistance for the spread of ionic currents between neurons, thus synchronizing and possibly amplifying electrical activity in a group of coupled neurons. Electrical coupling complicates the attempts to dissect out the mechanism of CO\(_2\)/H\(^+\) sensitivity and to identify a single neuron as intrinsically chemosensitive. A chemosensitive neuron may share gap junctions with a nonchemosensitive neuron, whereby the
nonchemosensitive neuron responds indirectly to the changes in pH due to electrical input from the chemosensitive neuron. Many chemosensitive regions have been shown to contain neurons which are electrically coupled with other neurons (Alvarez-Maubecin et al., 2000) including the LC (Oyamada et al., 1999; Ballantyne and Scheid, 2000; Dean et al., 2001;) and the NTS (Dean et al, 1997, Huang et al., 1997) and with glia (Alvarez-Maubecin et al. 2000). Electrotonic coupling appears to be expressed in both LC neurons and NTS neurons in neonatal rat and functioning during hypercapnic acidosis (Huang et al., 1997; Oyamada et al., 1999; Ballantyne and Scheid, 2000; Dean et al., 2001). Electrical activity between neurons joined by gap junctions remained synchronized during hypercapnic acidosis, suggesting that intracellular acidosis caused by rising CO2 tested was insufficient to uncouple gap junctions in these regions. Thus, in both regions, gap junctions could play a role in increasing neuronal excitability of electrically coupled cells in these CO2-chemosensitive brainstem regions during hypercapnic acidosis (Dean et al., 2001). In order to insure that the CO2 response detected in our experimentally identified neurons is intrinsic to those neurons and not dependent on chemical synaptic input or gap junction electrical coupling, we studied neurons with chemical synaptic input and gap junctions blocked by using artificial cerebrospinal fluid (aCSF) with carbenoxolone, a gap junction inhibitor (Goldberg et al., 1996) and low Ca2+ and elevated Mg2+ to reduce Ca2+-dependent synaptic transmission (Dean et al, 2001; Conrad et al, 2009; Nichols et al, 2009). In the work presented here, patched neurons identified as chemosensitive or nonchemosensitive were simultaneously loaded
with fluorescent dye, Lucifer Yellow, which has been shown to be contained within the target neuron by the use of carbenoxolone (Goldberg et al., 1996).

6. The Locus Coeruleus

The locus coeruleus (LC), so named by the Wenzel brothers in 1812 (Swanson, LW. 2014), for its blue-black appearance, is a dorsal pontine nucleus proximal to the floor of the fourth ventricle and the pontomesencephalic junction (Dahlström et al., 1964a). A densely packed cluster of norepinephrine (NE) producing cells, known to be the most significant group of NE neurons in the central nervous system (Dahlström et al 1964b, Nieuwenhuys 1985, Kiernan, 2005), the LC supplies input to multiple areas of the brain and spinal cord via extensive efferent projections. (Klimek at al., 1997)

The dendritic arborization of the LC neurons is oriented in a horizontal, disc-like plane and the arbor from individual cells has been observed to extend throughout and beyond the nucleus (Groves and Wilson 1980, Shipley et al 1996). Retrograde labeling of the LC has revealed strong regional specificity to the arrangement of the LC neurons according to their functional roles. For example, retrograde labeling from hippocampal injections of horseradish peroxidase (HRP) was clustered in the dorsal LC, consistent with a role in regulating emotions (fear, anxiety) and long-term memory, while the ventral portion was entirely unlabeled. Neocortex injections resulted in central LC labeling but no ventral labeling, and hypothalamus injection resulted in the labeling of large, multipolar LC neurons (Loughlin et al., 1986).
Like the array of LC efferents, the inputs to this locus are widely varied, indicative of the complexity of this neuromodulatory nucleus in diverse inter-related functions (Loughlin et al., 1982, Foote et al. 1983, Loughlin et al., 1986). Significant to respiratory control, the large proportion of LC demonstrated to be chemosensitive (Pineda and Aghajanian, 1997; Oyamada et al., 1998; Filosa et al., 2002) have not to date been localized to a specific region of the LC. The combined neuroanatomy, neurochemistry and physiology of this group of neurons speak to the importance of its role in the integration and control of many autonomic systems. Indeed, the LC has been shown to be involved in a myriad of responses including arousal, attention and memory (Aston-Jones 1999; Berridge and Waterhouse, 2003; Lemon et al.; 2009), stress, fear and panic (Tanaka et al., 2000) and respiratory control (Biancardi et al., 2008; Patrone et al., 2012).

7. The Nucleus Tractus Solitarius

The nucleus tractus solitarius (NTS) is a region of the brainstem that contributes to regulation of cardiovascular, respiratory, gastrointestinal, and laryngoesophageal reflexes. (Bailey et al., 2006; Browning et al., 2006). The NTS lies lateral to the dorsal motor nucleus of the vagus nerve (DMV) and surrounds the tractus solitarius. The caudal nucleus tractus solitarius (cNTS) is located in the caudal portion of the solitary complex (SC). The NTS has been shown to contain CO\textsubscript{2}-sensitive neurons (Dean et al., 1989; Dean et al., 1990; Huang et al., 1997; Mulkey et al., 2004) and ventilation is altered when the caudal NTS is focally acidified (Coates et al., 1993; Nattie and Li, 2002). The dorsal border of the area of cNTS selected for this study is the area postrema.
(AP), the nucleus gracilis (NG) and the fourth ventricle. At this location the cNTS is contiguous across the midline, ventrally bordered by the DMV and the central canal.

A vast array of neurotransmitters and neuropeptides and a multitude of receptor populations discovered in the NTS make up a complex network of both excitatory and inhibitory neurons involved in multiple autonomic systems (Kawai and Senba, 1996, 1999; Chan and Sawchenko, 1998; Bailey et al., 2006). These neurons have been shown to release many different neurotransmitters including norepinephrine (Barraco et al., 1995), epinephrine, dopamine (Lanzinger et al., 1989), glutamate (Davis et al., 2004; Toth et al., 2013), serotonin (Huang and Pickel, 2002, Raul, 2003), GABA (Okada et al., 2008, Zubcevic and Potts, 2010), and substance P (Williams et al., 2002; Wilkinson et al., 2011).

NTS neurons respond to a host of neuropeptides and neuro-active agents. Glial cells in the NTS have been shown to release ATP, modulating glutamate release by NTS neurons projecting to VLM (Accorsi-Mendonca et al., 2013). NTS activation by ATP (Gourine et al., 2003, Jin et al., 2004, Gourine et al., 2008, Barraco et al., 1988, Barraco and Phillis, 1991) has also been demonstrated. Orexin immunoreactive axons have been identified in the NTS (Date et al., 1999) and hypoglycemic activation of orexin neurons of the hypothalamus coincides with Fos activation in the NTS (Cai 2001). Neuronal activity-regulated pentraxin (Narp) terminals from the hypothalamus have been demonstrated to heavily innervate the dorsal NTS (Reti et al., 2002). Other neurotransmitters reported within the NTS include oxytocin (Cruz et al., 2007), nitric oxide (Haxhiu et al.,
Glutamate and GABA predominate in the cNTS and glutamatergic and GABAergic neurons are found throughout the region. GABAergic neurons are found in the ventral aspect of the cNTS (Fong et al., 2005, Okada et al., 2008). NTS neurons projecting to the ventrolateral medulla are subject to tonic GABAergic input (Kawai and Senba, 2000) and postsynaptic inhibition within the NTS is mediated by GABAA receptors (Mei et al., 2003).

Glutamate blockade in the cNTS has been shown to increase firing in the phrenic nerve (Costa Silva et al., 2010). Functional interconnectedness of the cNTS and DMV is evidenced in NTS neurons that receive visceral afferents and communicate to the DMV via glutamatergic neurons. These produce both excitatory and inhibitory postsynaptic potentials (Davis et al., 2004). In addition to glutamate and GABA, NTS neurons associated with the DMV receive catecholaminergic input (Browning and Travagli, 2010).

Simply put, the NTS is an extremely complex, heterogeneous nucleus with a seemingly endless array of inputs and extensive systemic influence. In these studies, we attempt to dissect out one functional type of neuron in the cNTS and characterize its three dimensional morphology and association with the local microvasculature.
8. Advantages of 3 Dimensional Reconstruction

The morphological characteristics of the neuronal structures including the soma, axon and dendritic arbor comprise a indispensable facet of neuronal phenotype and determine neural network connectivity. There are no 3 dimensional studies to characterize the morphology of identified chemosensitive neurons. This information is critical to inform anatomically relevant mathematical models of chemosensitive neurons for simulation of electrophysiological behavior. There are many advantages to 3D volume reconstruction vs. 2D renderings. Three dimensional reconstructions provide accurate interpretation of axial images. 2D reconstruction limits the investigator to one perspective of the neuronal structures. 3D reconstruction provides access to infinite perspectives on the cytoarchitecture. In 2D studies, the anatomical observables can be obscured by overlapping structural elements or structures which lie in the same trajectory as the sightline of the observer. (Imagine a dendrite coming directly toward the observer with the soma behind it. The dendrite would be effectively invisible). Trajectory, depth and volume cannot be detected with 2 dimensional studies. Even a parameter as seemingly straightforward as dendritic length can be especially difficult to measure accurately in 2D reconstruction because of the foreshortening effect of structures that are positioned toward or away from the observer. Any trajectory aside from a perfect 90° angle from the sightline of the observer will result in skewed analysis of length. 3D reconstruction not only eliminates those errors but provides volumetric data as well, a parameter that is completely neglected in 2D analysis.
9. Morphology of Chemosensitive Neurons

To date, little is known about the morphology of the identified chemosensitive neurons in regions of chemosensitivity. Among those studies which have considered this aspect of chemosensitive neurons, none consider the neurons in three dimensions and there is no continuity in the experimental approach to give relevant comparison between different chemosensitive regions. Chemosensitive raphé neurons in culture have been characterized in two dimensions, where two subtypes of chemosensitive neurons were found to be phenotypically distinct (Richerson et al., 2001) CO₂-activated neurons were found to have pyramidal shaped somas with four or more projections arising from multiple poles on the soma, whereas CO₂–inhibited neurons were more fusiform in shape with one to three projections arising from two obvious poles of the soma (Fig. 1). Using Rapid Golgi and Nissl staining, Cintra et al. (1982) studied LC neurons in two dimensions. They observed fusiform or ovoid neurons and multipolar neurons in sliced tissue from adult Sprague-Dawley rats. Swanson (1976) described two classes of neurons in the adult rat LC, fusiform and multipolar, and found that the fusiform neurons were densely packed and predominately located in the dorsal aspect of the LC. In contrast, the multipolar neurons were the predominant type in the ventral aspect of the LC. In none of these studies was any functional significance ascribed based on the morphological differences.
FIGURE 1. Two dimensional morphology of cultured chemosensitive raphé neurons (from Richerson et al., 2001). Both of these morphologies are associated here with chemosensitive neurons.

A. Ten drawings in two dimensions of cultured chemosensitive-stimulated raphé neurons. Note the pyramidal appearance of the somas and the complexity of the proximal dendrites.

B. Thirteen drawings in two dimensions of cultured chemosensitive-inhibited raphé neurons. Note the fusiform somas with less complex proximal dendrites and though they are relatively bipolar there is variance in the number and position of the dendritic origins.

A limitation of the anatomical observables: perspective includes only one profile of the neurons. It may be that the perceived morphology would be described differently if the viewing perspective changed. Volumetrics cannot be quantified. Cultured neuronal morphology may have no relevance to neurons which develop in vivo.
FIGURE 1.

A - Stimulated

B - Inhibited

100 μm
The NTS contains neurons identified as the dorsal respiratory group (DRG). In a unique attempt to associate morphological observations with physiological data, Dekin et al. (1987) evaluated DRG NTS neurons in guinea pigs for repetitive firing properties and corresponding morphologies. Due to experimental limitations, the electrophysiological analyses were not achieved in the same neurons as the morphological characterization and bursting activity did not reveal reliable data by which to distinguish the different functional types of neurons. They were however able to identify three distinct morphologies. Investigators identified type I neurons as multipolar with pyramidal somas approximately 25-35 μm in diameter, type II neurons as fusiform multipolar neurons, with somas of approximately 20-30 μm in the long axis and 10-15μm in the short axis, and type III neurons as spherical neurons with somas approximately 25-40μm in diameter. They limited their descriptions to somal shape and size in two dimensions, sighting photobleaching as an impediment to more complete observation (Dekin et al., 1987). Vincent and Tell (1999) described two types of NTS neurons, multipolar neurons and elongated neurons, and their electrophysiological phenotypes but they did not identify either morphology relative to chemosensitivity (Fig. 2). Though the morphology of chemosensitive neurons may not be responsible for their function as chemosensors, distinct morphological differences in neurons with differing responses to changing pH could be a useful tool in identifying and targeting chemosensitive neurons for study with electrophysiological and immunohistochemical techniques.
FIGURE 2. Camera Lucida 2D renderings of multipolar and elongated NTS neurons from P0 to adult. Sprague-Dawley rats (from Vincent and Tell, 1999). Once again the fusiform vs. multipolar theme can be observed here.

Another limitation of the 2D anatomical observables: Where one dendritic branch crosses paths with another, it becomes impossible to distinguish whether one is observing bifurcations and branching or overlap. Because these are 2D renderings, the trajectory of individual branches is difficult to trace and impossible to quantify. If a dendrite is coming toward the observer in 3D space it would be foreshortened or obscured by other structures.
FIGURE 2.

P0  Multipolar  Elongate

P5

P8

P12

P15

P20

Adult

Cells: 20 \( \mu \text{m} \)
10. Proximity to the Surface

It has been suggested that the position of chemosensitive neurons relative to the surface of the brain may provide them with functionally significant access to changes in pH in the cerebrospinal fluid (Kawai et al., 1996, Mulkey et al., 2004). Chemosensitive medullary neurons have been shown to send at least one projection to within 50 µm of the ventral surface (Kawai et al., 1996) (Fig. 3). Mulkey et al (2004) demonstrated a more dramatic instance of projections in close proximity to the surface (Fig. 4). This study showed CO$_2$-activated neurons in the RTN with the soma located in and/or projections running for nearly a millimeter within the marginal layer of the RTN. This proximity to the surface of the brain and resultant access to the CO$_2$ fluctuations in cerebrospinal fluid led to the hypothesis that perhaps the CO$_2$ sensory mechanism lies, at least in part, in the processes of chemosensitive neurons. Mulkey et al (2004) however, clearly state that this morphological proximity to the surface does not prove that the processes are a major site of chemosensitivity.

If the chemosensing machinery is indeed located in distal portions of the dendritic arbor and requires proximity to the surface, as these studies suggest, then this arrangement has implications for the location of ion channels and/or neuorpeptide receptors involved in the chemosensing pathways of chemosensitive neurons. If these processes are the locus of chemosensing, then the mechanisms that allow the neuron to respond to CO$_2$/H$^+$ must be localized to these processes.
FIGURE 3. Camera Lucida drawings of three Lucifer-Yellow filled chemosensitive VLM neurons with projections to the ventral surface. Thin arrows indicate axons and axon collaterals; broad arrows indicate projections to the surface (from Kawai et al., 1996). Note the location of the somas relative to the length of the dendritic tree which reaches and terminates at the ventral surface of the brainstem.
FIGURE 3.
FIGURE 4. Chemosensitive RTN neurons with soma (upper right) and dendritic projections (lower left) in the marginal layer (ML) (from Mulkey et al., 2004). This is a dramatic instance of dendritic arborization in close proximity to the ventral surface of the brainstem and has not been observed elsewhere in a region of chemosensitivity. Note that unlike in the VLM (Fig. 3), there are several dendritic terminals that lie in the marginal layer and one process running for a considerable distance along the surface. In the upper right there is a soma which is located in the marginal layer, with two polar processes traveling in opposite directions in the near the surface. The functional relevance is unknown.
FIGURE 4.
There have been no electrophysiological studies exploring the locus of the chemosensitive response except in one region of the brainstem, the locus coeruleus (Ritucci et al., 2005). This study explored the electrical response to focal acidification of the dendritic arbor vs. the soma and proximal dendrites of LC neurons. It was shown that the maximal chemosensitive response could be elicited from these neurons only if the soma was acidified with no change in firing rate when the distal dendrites were acidified. These results reveal that in the LC the chemosensitive response of the neurons depends on changing pH in close proximity to the soma and not on pH changes near the distal dendritic field. This strongly suggests that the chemosensing mechanism in LC neurons resides within or near the soma. However, Ritucci et al. (2005) did not specifically target any particular region of the arborization. It is not known whether or not acidification of a specific area of the dendritic arbor, such as a region that had directed growth toward and terminated near the floor of the fourth ventricle, would elicit a chemosensitive response. It may be that there are specialized synapses, gap junction coupling, or ion channels which heavily populate dendritic termini clustered near the surface. Similar and more complete studies in other areas of chemosensitivity are necessary to establish whether this is true for chemosensitive neurons from other chemosensitive brainstem regions.

11. Vasculature

An area of study that may shed more light on the regulation and function of chemosensitive regions of the brain is the relationship of the neurons of those regions to the vasculature. Chemosensitive neurons in the ventrolateral medulla
(VLM) and raphé have been shown to associate closely with blood vessels. (Bradley et al, 2002) (Fig. 5). Bradley et al. (2002) show processes of chemosensitive neurons in close association with the medullary blood vessels using immunocytochemistry and electron microscopy (Fig. 6). Studies of the VLM, a known chemosensitive region, showed significantly greater capillary density than in a nonchemosensitive area (gigantocellular nucleus) (Gobel et al, 1990). Falk and Rekling (2009) showed that respiratory neurons of two other chemosensitive regions, the pre-Bötzinger complex (PBC) and ventral respiratory group (VRG) are located in a region with a higher density of arterioles compared to nonrespiratory regions in preweanling mice. Studies of LC neurons have revealed dendritic branches in close contact with capillaries (Kalaria et al, 1989, Shimizu et al, 1979). Electron microscopy studies of LC neurons in squirrel and rhesus monkey brain slices showed direct apposition of the soma, somatic appendages and dendrites to capillaries and arterioles. These studies revealed that the neuronal membrane is in direct contact with capillaries, with no intervening glial layer (Felten and Crutcher, 1979) (Figs. 7 and 8). Early study of brainstem vasculature showed that each neuron of the LC lies in close apposition to two or more capillaries and that the density of the capillary bed of the human LC was found to be extraordinary, among the most highly vascularized nuclei of the brain and comparable only to two other regions, the paraventricular nucleus and the supra-optic nucleus (Finley and Cobb, 1940).
FIGURE 5. Biocytin labeled, chemosensitive, serotonergic medullary raphé neuron with processes closely associated with large medullary blood vessels (From Bradley et al., 2002). Though it appears that the processes and the blood vessel are colocalized, it is impossible to ascertain whether or not there is distance between the structures from a 2D image. Due to the limitations of 2D representations and in order to establish that there is contact between these structures, 3D morphological analysis or electron microscopy (EM) is necessary. See Fig. 6
FIGURE 6. (a) Electron microscopy of TpOH-IR neurons and a medullary artery. A neuronal process following along the outside of the arterial endothelium. (b) Large artery with smooth muscle and leptomeningial space (LS).
(c) A small blood vessel with TpOH-IR processes associated with it (arrowheads). L, vessel lumen; *, endothelial cells (from Bradley et al., 2002).

EM is a high resolution 2D imaging technique. It can provide information about microstructural associations; however, it is limited to one plane. We can see here that there is contact between the neuronal and vascular structures but we cannot see how the two intact structures interact in 3D space.
FIGURE 7.  Electron microscopy of the soma (nucleus indicated) of a monoaminergic LC neuron shown wrapping around half of the circumference of a small blood vessel (bv) (diameter > 10µm) and a dendrite (d) in close apposition to the same capillary. Note the lack of glial cells between the neuron and the endothelial layer.  Rhesus monkey. (from Felton and Crutcher, 1976).  This image provides a striking example of the unique structural associations between the soma of locus coeruleus neurons and the basement membrane of small capillaries.  Scale bar= 5.0µm.
FIGURE 7.
**FIGURE 8.** Magnification of box in figure 7. Electron micrograph of dendrite (d) which abuts the basement membrane (bm with yellow arrow) of a blood vessel (lumen indicate, lu). An axon terminal (red “a”) near a dendrite (d with yellow arrow) just beneath the junction (j with yellow arrow) between two endothelial cells (e). Upper left is the soma with cytosol indicated (red “cyt”). Rhesus monkey (from Felton and Crutcher, 1976). High Resolution of structural proximity.
FIGURE 8.
In contrast, studies of the distribution of blood vessels in the dorsal medullary complex showed that the NTS was significantly less vascularized than other areas examined (Koda and Bloom, 1983). Gross et al. (1990) provides evidence that the surface area of the NTS microvasculature is not “favorably large” considering its role in autonomic control. Porzionato et al. (2005) showed that the microvascular density in the cNTS is significantly less than the dorsal motor vagal nucleus (DMV). The functional specificity of chemosensitive neurons demands ready access to changes in blood pH. The observed associations between blood vessels and chemosensitive neurons from some regions paired with the apparent heterogeneity of microvascular density across different medullary nuclei suggest that, rather than increased vascularization to chemosensitive regions, there may be unique relationships between individual neurons and blood vessels within different chemosensitive regions.

The proximity of processes of chemosensitive neurons to the ventral medullary surface has been demonstrated in two regions of chemosensitivity (VLM and RTN) but has not been explored in dorsal chemosensitive areas. This study will address that question in the LC and the cNTS. Special association of chemosensitive neurons with the vasculature has been observed in two ventral regions of chemosensitivity. An examination of regional vascularity vs neuronal density and evaluation of the interaction of individual chemosensitive neurons with blood vessels for two dorsal regions, the LC and the cNTS will also be addressed in this study. Knowledge of the regional distribution of
chemosensitive neurons vs. nonchemosensitive neurons will prove a valuable tool to guide electrophysiological studies in these areas.
CHAPTER III.

HYPOTHESIS

The main hypothesis of this thesis is that in two dorsal chemosensitive brainstem regions, the LC and the cNTS, neurons identified as chemosensitive, based on electrophysiological responses to hypercapnia, will have a distinct 3 dimensional morphology compared to nonchemosensitive neurons. Further, these chemosensitive neurons will send at least one projection towards the surface of the brain, which for these regions is the floor of the 4th ventricle. Finally, the soma and/or the projections of chemosensitive neurons will show specialized association with blood vessels compared to nonchemosensitive neurons.

SPECIFIC AIMS

Aim 1: Determine and quantify the 3 dimensional structure of chemosensitive and nonchemosensitive neurons from the LC and the cNTS. Individual neurons within a 300 µm brainstem slice will be patched, identified as chemosensitive or nonchemosensitive based on their firing rate response to hypercapnia (high CO2) and loaded with Lucifer Yellow. The slices will then be fixed and the structure of the soma and dendrites will be assessed using confocal imaging, 3 dimensional reconstruction and Sholl analysis. 3D morphometric analysis will eliminate anatomical-observable limitations of 2D neuronal reconstruction and provide relevant morphological data for compartmental modeling. We hypothesize that there are morphological
characteristics of chemosensitive neurons in the LC and cNTS that are
distinct from nonchemosensitive neurons in the LC and cNTS.

Aim 2: Identify and quantify the nearest approach of any process of
chemosensitive vs. nonchemosensitive LC and cNTS neurons to the floor
of the 4th ventricle and determine the distribution of chemosensitive and
nonchemosensitive neurons. Reconstructed 3 dimensional models of
chemosensitive vs. nonchemosensitive LC and cNTS neurons will be localized to
their position within the digital optical slice and the shortest distance between the
surface of the floor of the 4th ventricle and a process from the neuron will be
determined. The localization of somas of identified neurons will be mapped within
the LC and the cNTS to determine distribution of chemosensitive vs.
nonchemosensitive neurons. We hypothesize that the terminus of one or
more of the dendrites of chemosensitive but not nonchemosensitive
neurons will be in close proximity to the anatomical surface of the
brainstem slice, specifically the floor of the 4th ventricle. We also
hypothesize that chemosensitive and nonchemosensitive neurons are
homogeneously distributed across both the LC and cNTS.
Aim 3: **Characterize the regional vascularity and the relationship between the vasculature and chemosensitive vs. nonchemosensitive neurons in the LC and cNTS.** Brainstem slices will be stained for neurons (with NeuN, a neuronal marker) and blood vessels (with Isolectin GS-IB4, a marker for endothelium) and the percent of the area in the LC and cNTS composed of neurons vs. blood vessels will be determined as a measure of regional vascularity. We will also stain fixed slices with identified neurons in them to study the relationship between chemosensitive and nonchemosensitive neurons and blood vessels. **We hypothesize that the soma and dendrites of LC and cNTS chemosensitive neurons lie in closer proximity to the microvasculature than do nonchemosensitive.**
CHAPTER IV.

CHEMOSENSITIVE NEURONS FROM THE LOCUS COERULEUS (LC):
THREE DIMENSIONAL STRUCTURE AND ASSOCIATION WITH BLOOD VESSELS

Rationale

As discussed previously, in the Review of the Literature, there are no studies of the 3 dimensional structure of chemosensitive vs. nonchemosensitive neurons. The 2 dimensional morphology of chemosensitive raphé neurons in tissue culture has been studied (Richerson et al., 2001) and the structure differed between raphé neurons whose firing rate increased in response to hypercapnia vs. those whose firing rate decreased in response to hypercapnia. Further, studies of chemosensitive neurons from the ventrolateral medulla (Kawai et al., 1996) and the RTN (Mulkey et al., 2004) show that some chemosensitive neurons send at least one projection to the surface of the slice and that this suggests that chemosensing by these neurons is based in these projections. Finally, chemosensitive neurons from the raphé have been shown to send projections that associate closely with nearby blood vessels (Bradley et al., 2002), which is an ideal arrangement for a neurons sensing changes in blood chemistry. None of the existing studies have been done in identified chemosensitive LC neurons nor have they generated 3 dimensional reconstructions of chemosensitive vs. nonchemosensitive neurons.
The main goals of the current study were to derive 3 dimensional reconstructions of known chemosensitive and nonchemosensitive LC neurons and determine if chemosensitive LC neurons send projections to the surface of the slice or form associations with nearby blood vessels.
METHODS AND MATERIALS

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University in accordance with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and is covered by NIH Assurance (no. A3632-01). All experiments were conducted on pre-weanling Sprague-Dawley rats of mixed sex and of postnatal ages (P) day P7-P16.

Solutions

Brain slices were superfused with artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 1.3 MgSO$_4$, 2.4 CaCl$_2$, 26 NaHCO$_3$, and 10 glucose, equilibrated with 5% CO$_2$-95%O$_2$, pH~7.45 at 37° C. Hypercapnic aCSF solution was identical but was equilibrated with 15% CO$_2$ /85% O$_2$, pH 7.0 at 37° C. To assure responses were intrinsic to the patched neuron, we used a solution that blocks both chemical and electrical synaptic transmission. This synaptic block solution (SNB/C) is identical to aCSF except that NaCl is reduced to 117 mM, MgSO$_4$ is elevated to 11.4, CaCl$_2$ is reduced to 0.2 and 100 μM carbenoxolone, a gap junction inhibitor, is added to the solution (Conrad et al, 2009). The pipette filling solution contained (in mM) 130 K$^+$-
gluconate, 1 MgCl₂, 10 HEPES, 0.4 EGTA, 2 Na₂ATP and 0.3 Na₂GTP with 0.5% Lucifer Yellow.

**Slice Preparation**

Transverse brain slices were prepared from preweanling Sprague-Dawley rats (age P7-P16) as is commonly done in the laboratory (e.g. Filosa *et al.*, 2002; Ritucci *et al.* 2005; Nichols *et al.*, 2009; Conrad *et al.*, 2009). Animals were rendered unconscious via hypothermia (age <P7) or high CO₂ exposure (age> P7) and then rapidly decapitated. The brainstem was dissected and sliced as follows: Two 300 μm slices from the dorsal pontine region containing the LC were cut (using a Pelco 100 series 1000 vibratome) into ice-cold aCSF equilibrated with 5% CO₂ / 85% O₂ for electrophysiological analysis and patched within 4 hours after removal from the animal. Individual slices were placed in a superfusion chamber (1.0 ml volume), immobilized with a grid of nylon fibers, and superfused with control solution at ~4ml/min at 37°C.

**Electrophysiology**

The whole cell patch clamp technique was used to measure voltage changes across the membranes of LC neurons. The whole cell pipette filling solution was modified from the solution previously described (lowered EGTA and no added CaCl₂) to prevent washout of the chemosensitive response (Filosa and Putnam, 2003). Neuronal identification was achieved by measuring the firing rate of neurons in response to an acidic challenge induced by hypercapnia. Membrane potential (Vₘ) was measured and current injected via a Dagan BVC-
700 amplifier. Integrated firing rate was determined (10s bins) using a window discriminator/integrator (Winston Electronics). In current clamp mode, a stable firing rate was established (1 Hz). All patched neurons, in the presence of SNB/C, were exposed to hypercapnic acidosis in SNB/C equilibrated with 15% CO₂/85% O₂, pH 7.0. We determined that the response was reversible by returning the bathing solution to the control SNB/C in aCSF equilibrated with 5% CO₂ and verifying that the firing rate returned to baseline. Neurons that responded with a 20% or greater increase in firing rate were identified as chemosensitive, while neurons which responded with less than 20% change in firing rate were considered nonchemosensitive. Inhibited neurons were not considered in this study. The question arises whether the increased firing rate seen in going from 5% CO₂/95% O₂ to 15% CO₂/85% O₂ is due to the hypercapnia or due to the reduction in O₂. We believe that the increased firing rate is due to the hypercapnia based on previous studies that have shown that increasing O₂ levels (from 40% to 95%) stimulates firing rate and decreasing O₂ levels decreases firing rate in cNTS neurons (Matott et al., 2014) and in LC neurons (Putnam laboratory, unpublished data). Further, studies of intracellular pH in cNTS neurons at decreasing levels of O₂ from 95% O₂ has no significant effect on pHᵢ until levels of about 60% are reached (Potter, 2005), indicating that a decrease in O₂ from 95% to 85% is not leading an acidification, which could lead to an increased firing rate. It is most likely then that the hypercapnia leads to the increased firing rate in chemosensitive cNTS and LC neurons and if anything, the small drop in O₂ from 95% to 85% should reduce firing rate.
The patched identified neuron was loaded with Lucifer Yellow from the whole cell pipette. Mulkey et al. (2004) showed that under such conditions the soma loads fluorescent dye in ~10 minutes. To optimize the loading of Lucifer Yellow into the cell and maximize the diffusion into the dendritic processes, we maintained the patch for ~30 minutes. This is important to achieve the best possible distribution of dye throughout the structures of the identified neuron, especially the distal dendrites. After identifying a neuron electrophysiologically and loading it with Lucifer Yellow, the slice (300 μm) was removed from the recording chamber and fixed for three days in 4% paraformaldehyde at 4°C. Slices were removed from fix and washed 5 times for 15 minutes each in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH = 7.3) then mounted on slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA).

**Confocal Imaging**

Identified labeled neurons were imaged using an Olympus FV300 laser-scanning confocal microscope with a PlanAPO N 60x N.A.1.35 oil immersion objective, oil refraction index 1.5, with Coherent Sapphire 568 and Nec gas 488 lasers or FV1000 laser-scanning confocal microscope with a PlanAPO 60x N.A.1.4 oil immersion objective (Olympus Corp. of the Americas, Center Valley, PA) with oil refraction index 1.5, and Melles Griot lasers 488 and 568. Multi-panel serial optical sections (z-axis: motorized z step=0.5 μm) were obtained to include the full depth of the soma and the arborization. From the soma, each process was followed as a series of panels until the end of the process. In each
panel, a series of optical sections were obtained at 60x from below to above the process. When a series of panels containing one process was completed, the imaging procedure was repeated for the next process, and so on until all processes had been imaged. After scanning, images were taken using the 10x objective to document anatomical localization of Lucifer Yellow labeled neurons. During confocal imaging, loaded identified neurons were evaluated for pruning of dendrites that may have projected out of the plane of the slice. Neurons with discontinuous loading of Lucifer Yellow, dendrites ending in visible blebs of Lucifer Yellow or with dendritic endings of >1–2 μm were not analyzed. A neuron was selected for further analysis only if the entire soma and dendritic arborization were contained within the 300 μm slice. (<10% of identified loaded neurons were rejected for reconstruction)

**Neuronal Reconstruction**

A single data file for each neuron was constructed using Neurolucida software (MBF Bioscience). Z-step calibration was customized in the reconstruction software by assigning the appropriate objective and oil refraction index prior to reconstruction. Z-Step voxel assignment is automated once the z-step is calibrated within Neurolucida software. Each soma was first reconstructed from the confocal images then each confocal image stack containing segments of the processes was linked to the somal reconstruction and reconstructed individually until the soma and all projections were included in a single data file, a complete digital 3-D reconstruction of the neuron. These 3D reconstructions provided the basis for the subsequent morphological analysis. Neurolucida
software was used to create black and white, multi-perspective images of 3D reconstructions (Figs 10, 11, 12, 21, 22, and 23) and space-filling models, a type of three-dimensional volume rendering useful for visualizing the effective shape and dimensions of and region of space occupied by an object, distinguished from other 3D representations such as wire-frame, skeleton, or ball and stick. (Figs. 19, 24).

Neurons were reconstructed and analyzed by an observer who was blinded to whether the neuron was chemosensitive or nonchemosensitive. For morphological data analysis NEUROEXPLORER® software (MicroBrightField) was used to conduct various morphological measurements to analyze the specific characteristics of soma and dendritic structures. Neuronal somas were analyzed and the following parameters quantified: volume; diameters in the X, Y and Z planes (smallest diameter assigned to X plane and largest diameter assigned to Z plane); and aspect ratio. The aspect ratio is a measure of the “roundness” of the soma, which is derived as the ratio of the largest diameter of a soma and its smallest diameter. A value of 1 indicates a sphere.

**Branch Structure Analysis**

Neuronal processes were quantified by branch structure analysis based on branch order. Branch order was determined using centrifugal branch ordering (Uylings et al., 1975). For each order of processes, the number of segments, and the average radius, surface area, length and volume of segments were calculated. Thus, first order processes are those that are directly attached to the soma. When that process bifurcates, both branches become second order, and
so on. We also quantified the total number of segments for all orders and the
total length, surface area and volume for the entire neuronal arborization. Somal
surface area was calculated as the sum of all somal perimeter contours per
neuron x z-step= (0.5µm). Somal volume was calculated by summing the
volumes of all somal perimeter contours. Perimeter contour volume was
calculated by extrapolating the radius and using the equation for volume of a
cylinder: Vol=π r² h, h=0.05 µm. These values were compared between
chemosensitive and nonchemosensitive LC neurons. NeuroExplorer software
applies branch taper rate ((final +initial diameter)/(initial diameter at each
branching node)) to quantification of individual dendritic order branches for S.A.
and Vol. calculations. Average taper rate ((final diameter-initial diameter)/branch
length) was applied to calculations of branch total S.A. and Vol. calculations.

**Sholl Analysis**

Neurons were also characterized using a Sholl analysis (Sholl, D.A., 1953)
to evaluate spatial distribution and extension of the arbor. Using a 3-D
reconstructed neuron, the center of mass of the soma was placed at point 0 in
the Sholl plot. The Sholl plot consists of concentric spheres whose radii increase
by 50 µm for each sphere. The neuronal processes were quantified for the
percent of total for process length, surface area and volume in each Sholl
sphere. This was done for both chemosensitive and nonchemosensitive LC
neurons. This analysis reveals the distribution of the mass of neuronal structures
moving outward from the soma for each type of neuron.
Closest Approach and Neuron Mapping

We superimposed a reconstruction of a neuron in its location on a low power magnification z-stack of the slice. We were then able to measure the closest approach of any neuronal process to the floor of the 4th ventricle for each neuron. We also made a simple plot locating identified LC neurons that were characterized electrophysiologically within the LC. We subdivided the LC into 3 roughly equivalent regions, dorsal, intermediate and ventral, based on staining for horseradish peroxidase that had been injected into different brain regions to which LC neurons project (Mason and Fibiger, 1979). This retrograde labeling study clearly indicated that there are regional topographical differences within the LC that were characterized by labeling in distinct areas. We were able to localize chemosensitive and nonchemosensitive neuronal somas within the LC to determine the distribution of chemosensitive and nonchemosensitive neurons within three regions.

The Association of Identified Neurons with Blood Vessels

As above, neurons were identified and fixed then incubated with Isolectin-GS-IB4 (specifically labels endothelial cells; Grossmann et al., 2002) conjugated to Alexa-568 at 1:200 concentration for 24 hours. The slices were washed in PBS 4 times for 20 minutes each and mounted on slides with Vectashield. Z-stack images were collected using confocal microscopy at 568 nm for the blood vessels and 488 nm for the Lucifer Yellow-loaded cell. A low magnification (10x) image was first obtained to orient the location of the loaded neuron in the slice. Images were obtained at 20x in order to collect a complete image of the neuron.
and projections. Finally, a detailed, multi-panel, 60x series of z stacks were obtained in order to evaluate the apposition of the cells to the blood vessels and to be used for reconstruction of the loaded neuron and closest blood vessels. Somal and dendritic reconstructions and blood vessels were modeled using the neuron tracing tools and contour modeling tool in Neurolucida. Where possible, interactions between blood vessels and projections were observed in either confocal images or in reconstructions. Measurements were made using Fluoview (for confocal images) or NeuroExplorer (for reconstructions) to determine the proximity of blood vessels to either soma or dendrites.

**Regional Vasculature Characterization**

Pre-weanling Sprague-Dawley rats were transcardially perfused with 500 ml of 4% paraformaldehyde (Sigma) in 0.1 M sodium phosphate buffer (pH 7.4). Brains were dissected free and tissue blocks containing the pontine region were stored overnight in 15% sucrose solution for cryoprotection. Tissue blocks from the dorsal pontine region containing the LC were mounted in Tissue-Tek O.C.T. (optimum cutting temperature compound) and 50 μm slices were cut (using a Pelco 100 series 1000 vibratome) and floated in PBS. The slices were washed 3 times for 15 minutes in PBS+ 0.1% Triton, then incubated with 10% Normal Donkey Serum (Sigma Aldrich) for at least 30 minutes. The blocking solution was pipetted off and the slices were incubated with primary antibody (mouse anti-NeuN at 1:500 concentration for neurons) and with Isolectin-GS-IB4 conjugated to Alexa-568 at 1:200 concentration for blood vessels overnight at 4° C. The slices were then washed 4 times for 15 minutes each and incubated with
secondary antibody (FITC anti-mouse) for 4 hours. The slices were then washed 3 times for 15 minutes each and mounted on slides with Vectashield. Z-stack images were collected using confocal microscopy at 568 nm for the Isolectin and 488nm for the neuronal marker.

These methods were developed in our laboratory for purposes of these studies.

**Analysis**

The vasculature was analyzed using IMAGE J, Fluoview and Neurolucida software. Fluoview was used to create a two channel projection of 0.5 µm optical sections with a total depth of ~45 µm. In Image J, the confocal projections were color channel separated, converted to eight bit, then to black and white binary images. The black and white images were analyzed as histograms reflecting the total of black and white pixels. The percent of the area that is blood vessels and the percent of the area that is neurons in a selected region were evaluated using the number of pixels for each structure divided by the total number of pixels in the selected region. The ratio of the percent of the area that is neurons to the percent that is blood vessels was calculated using the respective percentages. Fluoview and Neurolucida were used for counts of neurons and blood vessels over a given surface area and measurements of proximity between neuronal structures and blood vessels.

**Statistics**

Significant differences between two means were determined by two-tailed Student t-tests. When multiple t-tests were done with the same data set a
Bonferroni correction was applied to the P value. Comparisons of more than two means were conducted using multiple t-tests (with a Bonferroni correction). For the dendrite data, multiple t-tests (chemosensitive vs. nonchemosensitive and order number; with a Bonferroni correction) was employed. Linear regressions were done as least squares linear regressions. For all tests, P<0.05 was the criterion for statistical significance unless adjusted by a Bonferroni correction. Fisher’s exact test and χ²-distribution for neuronal distribution maps. All values are shown as means ± 1 Standard Error. Statistical analyses were performed by Bev Grunden, SAS software, Statistics Consulting Center, Wright State University.
Dye Loading of Electrically-identified Neuron

LC neurons were identified as either chemosensitive or nonchemosensitive by studying their firing rate response to hypercapnia using whole cell patch clamping. The patch electrode contained the fluorescent dye Lucifer Yellow which allowed the neuron to be loaded with the dye during the experiment for later analysis of its three dimensional structure. Two identified, dye-loaded LC neurons are shown in Fig. 9. Confocal image projections of a dye-loaded chemosensitive neuron reveal a neuron with a fusiform soma and projections arising from two distinct poles (Fig. 9A). This neuron was identified as chemosensitive based on its reversible increase in firing rate upon exposure to hypercapnia (Fig. 9B). A dye-loaded nonchemosensitive neuron appears to have a more pyramidal-shaped soma with dendrites arising from multiple poles (Fig. 9C). This neuron did not change its firing rate in response to hypercapnia and was thus identified as nonchemosensitive (Fig. 9D).

General Morphology of Reconstructed Chemosensitive and Nonchemosensitive LC neurons

Two chemosensitive LC neurons that were reconstructed using Neurolucida are presented in views from three different angles (Figs. 10A and B). The morphology of these reconstructed neurons show a generally fusiform appearance with elongated somas and dendrites arising from two poles separated by about 180 degrees. The dendritic fields were found to occupy
FIGURE 9.  A: A 300 μm brainstem slice containing an LC neuron which has been patch clamped and loaded with fluorescent dye, Lucifer Yellow. Note the dye has diffused along the primary dendrites, revealing the branched structures in several planes extending from the soma. The neuron is presented as a projection which is made by the overlap of all the Z stack optical images for the neuron. This neuron has a fusiform appearance, with an elongated nucleus and processes that arise from two poles that are separated by 180°.  

B: A representative electrophysiological recording from a chemosensitive neuron as in panel A. The top panel shows integrated firing rate (10 s bins) expressed as the number of action potentials per 10 s vs. time. Note the stable initial firing rate of about 0.5 Hz in. The aCSF was equilibrated with 5% CO₂ (normocapnic) (1) which then reversibly increases to about 1.5 Hz in response to aCSF equilibrated with 15% CO₂ (hypercapnia) (2). Firing rate returns to its initial value upon return to normocapnia (3). The lower panel shows individual action potentials at an expanded time scale for (1), (2) and (3). These findings identify a neuron as chemosensitive.  

C: Another LC neuron loaded as for panel A. Note that this neuron has a more pyramidal shaped soma with processes arising from multiple poles.  

D: A representative electrophysiological recording from a nonchemosensitive neuron as in panel C. The top panel shows integrated firing rate. Note that unlike the neuron in panel B, this neuron does not increase its firing rate in response to hypercapnia which is maintained throughout the transient hypercapnia exposure between 0.5-1 Hz. These findings identify a neuron as nonchemosensitive. (Electrophysiology, Dr. Ke-Yong Li, 2013).
FIGURE 10. A: An identified chemosensitive LC neuron reconstructed in Neurolucida shown from 3 angles. This is an example of a classic fusiform soma with bipolar dendritic arrangement. Note in the bottom perspective, the soma does not appear fusiform, indicating how 2 dimensional images can present a misleading picture of the true structure of the neuron.

B: A second identified chemosensitive LC neuron reconstructed in Neurolucida and shown from 3 angles. An example of the variability within the fusiform morphology. Note the characteristic elongated nucleus and processes that arise from two opposite poles, very similar to the appearance of the neuron in panel A. However there is one process arising from a deviant position demonstrating the variance of the bipolar arrangement of these neurons and underscoring the need for 3D reconstruction. Also note how in the bottom angle, the soma of this neuron appears more round than fusiform.
FIGURE 10.
elliptical planes of less than 30µm thickness that extended over 250µm from the soma (Figs. 10A and B). In contrast, two nonchemosensitive LC neurons that were reconstructed using Neurolucida had a very different general morphology (Figs. 11A and B). These nonchemosensitive neurons had somas that were pyramidal in shape and dendritic fields that were more compact, arising from multiple poles and extending for a shorter distance (less than 150 µm) in many directions from the soma.

**Somal Reconstructions for Chemosensitive and Nonchemosensitive LC Neurons**

The somas of identified LC neurons were reconstructed with Neurolucida using confocal images at 0.5µm resolution and the perimeter of each optical section was modeled using an average of 1 point/µm (Fig. 12). An example of each type of soma shows the more fusiform appearance of the soma from chemosensitive LC neurons (Fig. 12A) compared to the more pyramidal shape of the soma from nonchemosensitive neurons (Fig. 12B). The reconstructed somas were analyzed using NeuroExplorer and the surface area, volume, diameters (in three dimensions) and the aspect ratio were determined (Table 1). We found no significant differences in the surface area and the volume between chemosensitive and nonchemosensitive LC neurons (Table 1). The diameters in each plane of each soma were calculated using the widest point for each axis. We found that chemosensitive neurons had a significantly larger diameter (45% longer) in the longest plane compared to nonchemosensitive neurons (Table 1). For both types of somas, the greatest diameter (in the Z plane) was significantly
FIGURE 11. **A:** An identified nonchemosensitive LC neuron reconstructed in Neurolucida shown from 3 angles. Note the pyramidal appearance of the soma of this neuron and the processes that arise from multiple poles. Thus, the structure of this nonchemosensitive neuron is quite distinct compared to the structure of chemosensitive neurons (Fig. 10). **B:** A second identified nonchemosensitive LC neuron reconstructed in Neurolucida and shown from 3 angles. Note the pyramidal appearance of the soma and the processes that arise from multiple poles, very similar to the appearance of the nonchemosensitive neuron in panel A.
FIGURE 11.
FIGURE 12. The perimeters of adjacent optical sections (z=0.5 µm) are shown for the soma of a chemosensitive neuron (left figure) and for the soma of a nonchemosensitive neuron (right figure). The longest diameter for each soma was defined as the Z axis and the shortest diameter for each soma was defined as the X axis. Note that the chemosensitive neuron looks more elongated in the Z axis than the nonchemosensitive soma, which appears to be more pyramidal. This is a good illustration of the advantage of 3D reconstruction. The perimeters in this figure are presented to the reader with the z plane oriented vertically. During contour modeling in Neurolucida the investigator approached these neurons looking at the xy orientation. The differences in somal morphology are easily perceived when viewed from the "side" in the perspective presented in this image. However, from “above” these two neurons both appear oval. Without 3D reconstruction they may have been miscategorized as having the same or similar somal morphologies.
FIGURE 12.
<table>
<thead>
<tr>
<th></th>
<th>Surface Area (μm²)</th>
<th>Volume (μm³)</th>
<th>Diameters (μm)</th>
<th>Aspect Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>Y</td>
</tr>
<tr>
<td>CHEMOSENSITIVE</td>
<td>2050.1 ± 260.8</td>
<td>7522.4 ± 1166.3</td>
<td>17.0 ± 0.8</td>
<td>29.5 ± 3.1</td>
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<tr>
<td>(n=8)</td>
<td></td>
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</tr>
<tr>
<td>NONCHEMOSENSITIVE</td>
<td>1484.3 ± 128.6</td>
<td>6382.5 ± 799.1</td>
<td>20.1 ± 1.4</td>
<td>24.1 ± 0.7</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
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</table>

Multiple t-test with Bonferroni correction

* P<0.05 compared to the same value for nonchemosensitive neurons.
** P<0.01 compared to the same value for nonchemosensitive neurons.

a The diameter of the Y axis was significantly different than the diameter for the X axis.
b The diameter of the Z axis was significantly different than the diameters for the X and Y axes.
c The diameter of the Z axis was significantly different than the diameter of the X axis.
larger than the smallest diameter (in the X plane) (Table 1), suggesting that both somas deviated from spherical. We calculated the aspect ratios using the largest average diameter (the Z plane) divided by the smallest average diameter (the X plane). An aspect ratio of 1 would indicate a perfect sphere. This parameter provides a mathematical reference of the relative roundness of the soma. The aspect ratio of the chemosensitive somas was significantly greater than that of the nonchemosensitive somas (Table 1), consistent with the more fusiform or elliptical morphology of chemosensitive neurons (Figs. 8A and 9). Although the aspect ratio of nonchemosensitive neuronal somas does not indicate a perfect sphere (aspect ratio of 1), the morphology of the chemosensitive soma is significantly less spherical than a nonchemosensitive soma.

**Dendritic Reconstructions for Chemosensitive and Nonchemosensitive LC Neurons**

The dendritic field was evaluated by order of dendrite. Centrifugal branch ordering was used. First order dendrites are those that originate at the soma. Second order dendrites are those resulting from the bifurcation of the first order dendrites. Each consecutive bifurcation results in a new order of dendrite for each tree (Uylings et al., 1975). Each order was analyzed for quantity (number of dendrites), average radius, average length, average surface area and average volume of the dendritic segments in that order (Table 2). We found no differences in the quantity of dendrites for any order. It was clear from the order analysis that some processes did not bifurcate while other processes bifurcated
numerous times. The average radii of each order of dendrites were compared and were found to be between 0.2 – 0.8 µm, with no significant differences between chemosensitive and nonchemosensitive neurons (Table 2). However, the radius of the dendrite decreases steadily the further away from the soma it is (Table 2). The average length of second order chemosensitive dendrites was found to be significantly longer than second order nonchemosensitive dendrites (CS 96.9 µm ± 12.5 vs. NCS 47.2 µm ± 8.4, p<0.05). This is consistent with the overall morphology of the chemosensitive neurons extending further out from the soma as compared to the more compact overall arrangement of nonchemosensitive neurons. Corresponding to the increased length of second order chemosensitive neurons is a concomitant increase in the surface area of this order. The chemosensitive second order dendrites were found to have significantly more surface area than the nonchemosensitive second order dendrites (254.3 µm ± 25.2 vs. NCS 136.5 µm ± 39.4, p<0.05) (Table 2). No significant differences were found between the average volumes of second order dendrites. This reflects the tapering tendency of the individual dendrites (see materials and methods for tapering rate calculations). The extent of the dendritic field in overall length was remarkably different between groups. Some of the chemosensitive neurons extended to six orders from the soma, while only two of the nonchemosensitive neurons studied extended even as far as fourth order from the soma. There were no significant differences between chemosensitive vs. nonchemosensitive neurons in parameter totals (Table 2).
TABLE 2.
Dimensions of Dendrites from Chemosensitive vs. Nonchemosensitive LC Neurons

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Average Radius (μm)</th>
<th>Average Length (μm)</th>
<th>Average Surface Area (μm²)</th>
<th>Average Volume (μm³)</th>
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</thead>
<tbody>
<tr>
<td><strong>CHEMOSENSITIVE (N=9)</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>First Order</td>
<td>4.9 ± 0.5*</td>
<td>0.82 ± 0.18</td>
<td>66.8 ± 13.6</td>
<td>356.3 ± 35.0</td>
<td>246.0 ± 39.4</td>
</tr>
<tr>
<td>Second Order</td>
<td>6.3 ± 1.5</td>
<td>0.54 ± 0.04</td>
<td>96.9 ± 12.5*</td>
<td>254.3 ± 25.2*</td>
<td>78.6 ± 12.7</td>
</tr>
<tr>
<td>Third Order</td>
<td>4.0 ± 0.8</td>
<td>0.34 ± 0.06</td>
<td>78.5 ± 10.4</td>
<td>159.5 ± 28.5</td>
<td>43.3 ± 15.2</td>
</tr>
<tr>
<td>Fourth Order</td>
<td>2.3 ± 0.3</td>
<td>0.26 ± 0.02</td>
<td>59.7 ± 16.0</td>
<td>92.9 ± 25.9</td>
<td>15.7 ± 5.9</td>
</tr>
<tr>
<td>Fifth Order</td>
<td>2.3 ± 1.2</td>
<td>0.20 ± 0.06</td>
<td>59.5 ± 29.4</td>
<td>53.4 ± 25.0</td>
<td>6.3 ± 3.4</td>
</tr>
<tr>
<td>Sixth Order</td>
<td>3</td>
<td>0.30</td>
<td>11.6</td>
<td>17.6</td>
<td>12.8</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>21.3 ± 2.4</td>
<td></td>
<td>1645.3 ± 326.2</td>
<td>4849.7 ± 916.3</td>
<td>2096.4 ± 416.6</td>
</tr>
<tr>
<td><strong>NONCHEMOSENSITIVE (N=6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Order</td>
<td>6.2 ± 0.2</td>
<td>0.75 ± 0.16</td>
<td>70.1 ± 8.4</td>
<td>234.7 ± 52.4</td>
<td>117.1 ± 38.4</td>
</tr>
<tr>
<td>Second Order</td>
<td>5.2 ± 0.8</td>
<td>0.49 ± 0.10</td>
<td>47.2 ± 8.4</td>
<td>136.5 ± 39.4</td>
<td>55.5 ± 17.1</td>
</tr>
<tr>
<td>Third Order</td>
<td>4.5 ± 0.4</td>
<td>0.25 ± 0.08</td>
<td>109.6 ± 35.0</td>
<td>112.8 ± 58.1</td>
<td>22.9 ± 9.3</td>
</tr>
<tr>
<td>Fourth Order</td>
<td>3.5</td>
<td>0.31</td>
<td>7.4</td>
<td>13.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Fifth Order</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sixth Order</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>16.4 ± 2.1</td>
<td></td>
<td>993.9 ± 269.5</td>
<td>2928.1 ± 881.3</td>
<td>1381.1 ± 459.1</td>
</tr>
</tbody>
</table>

* P<0.05 compared to the same values for nonchemosensitive neurons. Multiple t-tests with Bonferroni correction.
1 Only two of the chemosensitive neurons had sixth order dendrites and these are the means for those two neurons.
2 Only two of the nonchemosensitive neurons had fourth order dendrites and these are the means for those two neurons.
The dendritic fields of chemosensitive and nonchemosensitive LC neurons were further analyzed using Sholl analysis. Sholl analysis was achieved by creating a series of concentric 50 µm spheres with the center of the mass of the soma of the identified neuron as the origin. Within each concentric sphere various metrics were evaluated including dendritic length, surface area, and volume. The silhouettes of reconstructed neurons, superimposed on a 2D representation of Sholl spheres out to 200 µm, suggest that chemosensitive neurons have dendrites that extend farther from the soma and the dendritic field is less compact than nonchemosensitive neurons (Fig. 13). Note that the chemosensitive neuron has a limited number of projections extending well beyond the 150-200 µm sphere while the nonchemosensitive neuron has a more compact appearance and most of the mass of the neuron remains within the first 100 µm from the soma.

An analysis of the distribution of the dendritic field using the Sholl spheres shows that the percentage of the length of dendrites in the first sphere (0-50 µm from the soma) is significantly greater in nonchemosensitive vs. chemosensitive neurons (Fig. 14A). This most likely is due to the somewhat larger average number of first order dendrites arising from the soma in nonchemosensitive neurons (Table 2). There are no other significant differences in the percentage of the length of dendrites between chemosensitive and nonchemosensitive neurons for any other Sholl sphere, although the length of chemosensitive neurons appear to be somewhat greater in all spheres and extends considerably farther out than in nonchemosensitive neurons (Fig. 14A).
**FIGURE 13.** The 2 dimensional representation of a chemosensitive (top figure) and a nonchemosensitive (bottom figure) LC neuron superimposed on a representation of the Sholl spheres. Note that the chemosensitive neuron has fewer projections but they extend for a greater distance than nonchemosensitive neurons. For comparison the figure only shows Sholl spheres out to 200 µm in radius but the entire chemosensitive neuron was analyzed which involved additional Sholl spheres.
FIGURE 13.

Chemosensitive LC Neuron

Nonchemosensitive LC Neuron
**FIGURE 14.** The results of the Sholl analysis for chemosensitive neurons (black lines and symbols) (n=8) and for nonchemosensitive neurons (gray lines and symbols) (n=5). In general, more of the length, surface area and volume of processes for nonchemosensitive neurons lie closer to the soma than do the processes for chemosensitive neurons. Asterisks indicate that the values for chemosensitive and nonchemosensitive neurons differ significantly: Multiple t-tests with Bonferroni correction ** P<0.01.

A. Y-axis is the percent of the total dendritic length of all trees in one group. The data point represent the % contribution of the portion of the tree that resides in the first Sholl sphere, within 50µm of the soma, then between 50 µm and 100µm from the soma, and so on (x-axis). Nonchemosensitive LC neurons have a greater proportion of their length close to the soma than do chemosensitive neurons. ** P<0.01.

B. Y-axis is the percent of the total dendritic surface area that is found in consecutive Sholl spheres and again we see that the nonchemosensitive neurons have a greater proportion of their surface area within the first 50 µm of the soma. ** P<0.01.

C. Y-axis is the percent of the total dendritic volume that is found in consecutive Sholl spheres. There is no significant difference between the chemosensitive and nonchemosensitive neurons. This difference is probably due in part to the disc-like arrangement of the dendritic field of the chemosensitive neurons with much of the overall length observed to wrap part way around the soma and run
FIGURE 14.

LENGTH

% of TOTAL LENGTH

Nonchemosensitive
Chemosensitive

Distance From Center Mass of Soma (μm)

SURFACE AREA

% of TOTAL SURFACE AREA

Distance From Center Mass of Soma (μm)

VOLUME

% of TOTAL VOLUME

Distance From Center Mass of Soma (μm)
for considerable length within one Sholl sphere.

The arrangement of the nonchemosensitive dendritic field is more complex, with dendrites extending outward from the center, entering and leaving each sphere with relatively straight trajectories. The dendritic surface area appears to distribute much like the length, with a higher percentage of the surface area in the first Sholl sphere in nonchemosensitive neurons but no other significant differences for any other Sholl spheres (Fig. 14B). However, as with length, chemosensitive neurons have dendrites which extend farther from the soma than nonchemosensitive neurons so the surface area of dendrites for chemosensitive neurons extends to considerably more distant Sholl spheres than those for nonchemosensitive neurons. A similar pattern is also seen for the percentage of the dendritic volume with distance from the soma (Fig. 14C). The volume of dendrites is not different between chemosensitive and nonchemosensitive neurons for any Sholl sphere (Fig. 14C) but the volume is somewhat larger for nonchemosensitive neurons in the first 50 µm Sholl sphere but dendrites from chemosensitive neurons extend volume to considerably more distant Sholl spheres (Fig. 14C). Together, these findings point to a more compact dendritic field in nonchemosensitive vs. chemosensitive LC neurons.

**Distribution of Chemosensitive and Nonchemosensitive Neurons Within the LC**

Based on patterns of efferent projections and neuronal morphology, the LC has been sub-divided into regions in the dorsal-ventral plane (Swanson, 1976; Cintra *et al.*, 1982; Loughlin *et al.*, 1986a, 1986b; Mason and Fibiger,
Here we sub-divide the region into 3 zones, dorsal, intermediate, and ventral, each constituting roughly one third of the LC (Fig. 15). Of 27 neurons studied in the dorsal portion of the LC, all were chemosensitive. Similarly, 13 of 15 (87%) neurons studied from the intermediate region of the LC were chemosensitive (Fig. 15). In contrast, only 3 of 18 (17% of neurons studied from the ventral region of the LC was chemosensitive, the remainder being nonchemosensitive (Fig. 15). Thus the chemosensitive responsive neurons in the LC seem to be largely restricted to the top 2/3 of the LC, closest to the floor of the 4th ventricle.

**Closest Dendritic Approach to the 4th Ventricle**

It has been suggested that chemosensitive neurons may send processes to the surface of the brain and that these processes are involved in chemoreception (Kawai *et al.*, 1996; Mulkey *et al.*, 2004). We studied the closest approach of any process to the floor of the 4th ventricle in chemosensitive and nonchemosensitive LC neurons. Chemosensitive neurons seem to send at least one process towards the floor of the 4th ventricle (Fig. 16A) while nonchemosensitive neurons do not (Fig. 16B). Indeed, chemosensitive neurons on average have a process that approaches the 4th ventricle floor significantly closer than the nearest process from a nonchemosensitive neuron (Fig. 16C).
FIGURE 15. An image of the LC region at 10x and with neurons stained with NeuN in the dorsal pons with the 4th ventricle indicated. The image was cropped to show the LC region, which is clearly identified by the dense packing of neurons, and the 4th ventricle. We have subdivided the LC into 3 roughly equal regions in the dorsal-ventral plane: Dorsal – roughly the top third of the LC; Intermediate – roughly the middle third of the LC; and Ventral – roughly the bottom third of the LC. We have superimposed on these regions the location of LC somas that we have characterized as either chemosensitive (green triangles) or nonchemosensitive (red triangles) using whole cell patch clamp techniques. White outlines indicate neurons which were reconstructed for morphometric analysis. Note that chemosensitive LC neurons are largely localized to the upper 2/3 of the LC while the nonchemosensitive neurons are largely localized to the ventral 1/3 of the LC. Fischer’s Exact Test P<0.00001. To determine where the significance lay, we examined the contribution of each cell type and each region to the Chi-Square Statistic. Chi-square contributions: Dorsal NCS=8 CS=3.37; Intermediate NCS=1.0, CS=0.36; Ventral NCS=20, CS=7.69 compared to the Chi-square statistic value of 3.84. The dorsal region had significantly less nonchemosensitive neurons than predicted by the Chi-square analysis and the ventral region had significantly more nonchemosensitive neurons and significantly less chemosensitive neurons than predicted.
Further, on average the closest approach is about $66 \pm 13 \mu m$ and ranges from 25 to 110 $\mu m$ (Fig. 16C). Nonchemosensitive neurons have processes that approach no closer on average than $199 \pm 32 \mu m$ and their range of nearest approach is 95 to 330 $\mu m$ (Fig. 16C). As suggested by Figure 15, this difference may reflect that the somas of chemosensitive neurons lie closer to the floor of the 4$^{th}$ ventricle than the somas of nonchemosensitive neurons. A linear regression of the distance of the soma from the surface vs. the closest approach of a process to the surface was significant for nonchemosensitive neurons ($r^2 = 0.817; P<0.05$). A linear regression of the distance of the soma from the surface vs. the closest approach of a process to the surface for chemosensitive neurons was not significant ($r^2 = 0.043, P>0.05$). This suggests that for nonchemosensitive LC neurons the proximity of a dendritic structure to the surface is a function of its somal location and for chemosensitive LC neurons it is not. The fact that physiological testing which strongly suggests that the chemosensing properties of LC neurons are localized to the soma (Ritucci et al., 2005), casts doubt on the hypothesis that chemosensitive neurons depend on dendritic proximity to the surface for excitatory input. In the LC there is no convincing morphological evidence that surface/dendritic proximity provides any chemosensing advantage. Nonetheless, chemosensitive LC neurons send but a single process towards the floor of the 4$^{th}$ ventricle and this ending is a small tapered ending (Fig. 16A).
**FIGURE 16.** **A:** A reconstructed chemosensitive neuron oriented to the tissue for analysis of proximity to the surface. The tissue image (10x) appears green due to auto fluorescence of the catecholaminergic LC neurons and the filter used. The 4th ventricle is the dark region at the top of the image. Note that the chemosensitive neuron has one process that approaches the floor of the 4th ventricle (white arrow). **B:** A reconstructed nonchemosensitive neuron oriented to the tissue for analysis of proximity to the surface. The 4th ventricle is the dark region at the upper right of the image. Note that the nonchemosensitive neuron does not have any process that approaches the floor of the 4th ventricle. **C:** A box plot that shows the closest approach for chemosensitive (n=6) and nonchemosensitive (n=6) neurons to the floor of the 4th ventricle. The mean closest approach was significantly smaller for chemosensitive neurons (around 75 µm) than for nonchemosensitive neurons (around 200 µm). Two-tailed unpaired t-test **P<0.01.
FIGURE 16.

A  CHEMOSENSITIVE LC NEURON

B  NONCHEMOSENSITIVE LC NEURON

C  CLOSEST APPROACH TO FOURTH VENTRICLE (µm)

0  50  100  150  200  250  300  350

CS

NCS

***
**Relationship of Blood Vessels to LC Neurons**

We stained coronal slices containing the LC with NeuN to mark neurons and Isolectin to mark blood vessels (n=9). Using Image J analysis, we were able to compare the percentage of the area that was either neurons or blood vessels in our three defined regions of the LC (Fig. 17, upper panels). We found that the percentage of the area that was blood vessels in the dorsal and intermediate region were similar (about 20-25%) and were significantly higher than the percentage of the area that was blood vessels in the ventral region (about 5-10%). In contrast, the percentage of the area that was neurons was the same in all three LC regions (Fig. 17, lower left panel) and was about 40-45%.

Expressing these as a ratio of blood vessels to neurons (i.e. the ratio of blood vessels divided by the ratio of neurons) showed that the intermediate and dorsal regions had significantly higher blood vessel to neuron ratios (0.5-0.6) than the ventral region (0.15) (Fig. 17, lower right panel) thus, greater perfusion of the regions of the LC that contain a higher proportion of chemosensitive neurons.

We next sought to determine if there were any specialized connections between dendritic processes of chemosensitive LC neurons and blood vessels, as suggested for chemosensitive raphé neurons (Bradley et al., 2002). An example of a possible area of interaction is shown in Fig. 18. Here an identified chemosensitive LC neuron (Fig. 18A lower white box) sends a process that lies over a blood vessel (Fig. 18A upper white box).
FIGURE 17. A cropped 10x confocal image of the LC stained for neurons (NeuN—green) and blood vessels (isolectin—red) (upper left panel A). The image was cropped and analyzed using Image J software. A binary image of the NeuN channel where dark regions indicate neurons (upper middle panel B). A binary image of the Isolectin channel where dark regions indicate blood vessels (upper right panel C). A selected region of the dorsal, the intermediate and the ventral areas were analyzed for pixel count corresponding to blood vessels vs. total pixel count and the percent of the area consisting of blood vessels was calculated for each area (lower left panel D). It can be seen that the percent of the area that was blood vessels was significantly lower in the ventral area compared to the dorsal and intermediate areas. The same selected regions were analyzed in the neuron channel for the pixel count corresponding to neurons vs. total pixel count and the percent of the area that was neurons was calculated. It can be seen that there was no significant difference in the percent of the area that was neurons among the three regions of the LC (lower middle panel E). Finally, we calculated the ratio of blood vessels to neurons by dividing the percent of the area that was blood vessels by the percent of the area that was neurons. It can be seen that the ratio of blood vessels to neurons is significantly higher in the dorsal and intermediate areas, which contain high proportions of chemosensitive neurons, compared to the ventral area, which contains a high proportion of nonchemosensitive neurons (lower right panel F).

Of 10 animals, 9 were selected for analysis. One was rejected due to tissue damage through the LC. n=9. *** P<0.001.
FIGURE 17.
FIGURE 18.  A: A 60x cropped image of an LC slice stained for isolectin (red) and containing an identified chemosensitive neuron (loaded with Lucifer Yellow). The soma and several processes of this chemosensitive neuron are apparent in the lower white box. In the upper white box is seen a process from this neuron that appears to associate with a blood vessel (most likely a capillary based on its size). B: A view of the content of the upper white box magnified 2-2.5x. A process from the neuron can be seen running along a blood vessel (aqua arrow), although in 3 dimensions it can be clearly seen that the process does not make direct contact with the blood vessel, but runs above it for 25-50 µm. Further, just past the blood vessel, the process seems to move away from the blood vessel with an apparent growth cone (purple arrow). C: A view of the content of the lower white box magnified 2-2.5x. Here it can be seen that there is a blood vessel very close to the soma and to a proximal dendrite (white arrows). This neuron and its associated blood vessel were reconstructed and can be viewed in panel C of Fig. 19.
FIGURE 18.
An expanded view of this potential region of interaction shows that the dendritic process runs over the blood vessel but confocal images revealed no direct contact between the two (Fig. 18B, blue arrow). Just beyond the blood vessel there appears to be a growth cone at the end of the dendritic process (Fig. 18B, purple arrow), suggesting that the dendritic process grew up to and then around the blood vessel without forming any special region of interaction. An expanded view of the soma and proximal dendrites of this chemosensitive neuron revealed parts of blood vessels lying very close to the soma and to a proximal dendrite (Fig. 18C, white arrows). The diameter of these blood vessels is under 10 µm, suggesting that they are capillaries. Thus, we studied a number of identified chemosensitive and nonchemosensitive neurons to examine the association between blood vessels and the soma.

To our surprise, we found in all chemosensitive neurons studies, a blood vessel (most likely a capillary based on diameter) lying in close apposition to the soma (Figs. 19A, B and C). Based on confocal images, no perceptible space separated the membranes of the blood vessel and the soma. In contrast, no blood vessels were found touching the soma of nonchemosensitive neurons, with a clear separation between the two (Figs. 19C, D and E). Based on analysis of confocal sections, the closest approach of a capillary to the soma of a nonchemosensitive neuron is between 3-8 µm. Thus, the soma of chemosensitive LC neurons should have very accurate measurement of diffusing CO₂ and falling pH in the blood because of the close association of capillaries with their somas.
FIGURE 19. **A, B and C:** Space filling representations of reconstructed somas from 3 chemosensitive neurons (green) and associated blood vessels (red). Note the very close association between the blood vessel and the soma for these chemosensitive neurons. In fact, no apparent separation could be discerned between the blood vessel and soma. These 3 chemosensitive neurons are representative of 8 chemosensitive neurons studied. **D, E and F:** Space filling representations of reconstructed somas from 3 nonchemosensitive neurons (green) and associated blood vessels (red). Note that, unlike chemosensitive neurons, there is a clearly discernible separation between nonchemosensitive neurons and blood vessels. These 3 nonchemosensitive neurons are representative of 5 nonchemosensitive neurons studied. Average distance between CS LC soma and a blood vessel =0, for NCS soma =10±2.04. Two tailed unpaired t-test, **P=0.0012.
FIGURE 19.
DISCUSSION

We present here for the first time 3 dimensional reconstructions of identified chemosensitive and nonchemosensitive neurons from the LC of neonatal rats. We have addressed three main hypotheses: 1) chemosensitive neurons will have a 3 dimensional morphology that is distinct from that of nonchemosensitive LC neurons; 2) chemosensitive neurons from the LC will have dendritic projections that approach the surface of the slice (the floor of the 4th ventricle) more closely than do projections of nonchemosensitive neurons; and 3) either the soma and/or the dendrites of chemosensitive LC neurons will be more closely associated with blood vessels than those of nonchemosensitive neurons.

3 Dimensional Morphology of Chemosensitive vs. Nonchemosensitive LC Neurons

Our findings support our initial hypothesis that chemosensitive LC neurons have a distinct 3 dimensional morphology compared to nonchemosensitive LC neurons. We found two distinct morphological patterns for LC neurons: a fusiform shape with an elliptical soma and processes that arise from opposite poles (Fig. 10); and a multipolar shape with a more pyramidal soma and processes that arise from multiple poles (Fig. 11). The former, the fusiform shaped neurons, increase their firing rate in response to hypercapnia and are thus chemosensitive, while the latter multipolar neurons are nonchemosensitive. Further, we discovered the previously unreported finding that the chemosensitive
LC neurons predominate in the dorsal and intermediate regions of the LC while the nonchemosensitive neurons are largely present in the ventral region of the LC (Fig. 15). It is not clear how such a morphology could facilitate the sensing of CO$_2$/H$^+$ by chemosensitive neurons, but our Sholl analysis suggests that chemosensitive neurons have a somewhat less compact dendritic field than nonchemosensitive neurons (Fig. 14) and present in 3 dimensions as flattened disc-shaped neurons, which would be optimal for sensing diffusible substances like CO$_2$ over a broad area. The more fusiform somal shape would also optimize surface area to volume ratio, further promoting the sensing of diffusible agents. Thus it may be that the fusiform 3 dimensional structure of chemosensitive LC neurons is a generalized feature of chemosensitive neurons from all brainstem regions.

The few previous studies of the morphology of chemosensitive brainstem neurons all employed 2 dimensional analysis. Richerson et al. (2001), studying cultured medullary raphé neurons, found two distinct morphological types that were similar to our morphological types in LC neurons: one fusiform with processes arising from opposite poles and an elliptical soma; and the other with multipolar processes and a more pyramidal soma. However, the fusiform medullary raphé neurons had firing rates that were inhibited by hypercapnia, rather than excited as with LC neurons, and the multipolar medullary raphé neurons had firing rates that were activated by hypercapnia, rather than being nonchemosensitive like with LC neuron (Richerson et al., 2001). Further, Mulkey et al. (2004) described retrotrapezoid nucleus (RTN) neurons whose firing rates
were increased by hypercapnia. These neurons were clearly not fusiform and appeared, if anything, more multipolar. Based on these comparisons of the structures of chemosensitive LC, RTN and medullary raphé neurons, it seems unlikely that there is a single morphology that characterizes chemosensitive neurons from all brainstem regions. It is more likely that if chemosensitive neurons have a distinct morphology, that morphology will be specific for the chemosensitive region of the brainstem from which the neurons derive.

It is interesting to compare our studies of the morphology of LC neurons with earlier, 2 dimensional studies, of LC neurons. There is agreement between our findings and those of earlier studies. Swanson (1976) studied LC neurons from adult albino rats and found two distinct classes of neurons, fusiform and multipolar, much as we report here, however his studies were carried out in two dimensions and did not relate morphology to chemosensitivity. Swanson (1976) examined the presence of these neurons in the dorsal 2/3 of the LC region and in the ventral 1/3 of the LC region. He described both types of neurons in both regions but that fusiform neurons predominated in the dorsal region while multipolar neurons predominated in the ventral region, just as we find in our study for chemosensitive (fusiform) and nonchemosensitive (pyramidal) LC neurons (Fig. 15). Finally, Swanson (1976) proposed that the fusiform neurons would appear lens-shaped in 3 dimensions, just as we observed for our fusiform neurons.

Very similar findings were made by Cintra et al. (1982) using adult Sprague-Dawley rats. They reported three morphological types: fusiform;
multipolar; and ovoid-shaped cells. The fusiform cells had elongated soma, primary dendrites that originated from opposite poles and were largely localized within the dorsal area of the LC. Multipolar neurons had more pyramidal shaped soma, a greater number of primary dendrites that arose from random locations on the soma, and were largely localized to the ventral region of the LC (Cintra et al., 1982). The ovoid-shaped cells were the smallest, had nearly round soma and were the least common neuronal type found. The fact that we did not observe ovoid cells may be because of their smaller size, the fewer numbers of such cells in the LC or these type of neurons may only be present in adult LC and not in LC from neonates. It is also very possible, that it is the inherent limitation of 2D study which caused a fusiform neuron to be perceived as ovoid or nearly round when viewed from one end of the long axis. Finally, Loughlin et al. (1986a,b) studying adult male Sprague-Dawley rats also found fusiform and multipolar neurons as well as neurons with round somas. Again, the appearance of a fusiform neuron viewed from one end of the long axis or in cross-section, would appear round. The multipolar neurons seemed to be most prevalent in the ventral region of the LC. Thus, there is agreement between the morphological findings of these early 2 dimensional studies and our current 3 dimensional studies of LC neurons.

Our findings add to our understanding of LC neuron morphology in a number of ways. Given that the LC is involved in the regulation of numerous functions (Gargaglioni et al., 2010), the fact that we and others find only 2, or perhaps 3 general morphological forms to LC neurons suggest that there is not a
direct link between neuronal morphology and function. In other words, the fact that all chemosensitive LC neurons within the LC have a similar morphology does not preclude an LC neuron with a different function from having the same morphology. Thus, the morphology of chemosensitive LC neurons probably does not give us great insight into how these neurons sense changes of CO₂/H⁺. This is further supported by the fact that chemosensitive neurons from other regions (e.g. the RTN and medullary raphé) have a different morphology, suggesting that there does not appear to be a single morphology for a chemosensitive neuron. Our work also shows for the first time that in LC it is the fusiform neurons that exhibit chemosensitivity and these neurons are largely restricted to the dorsal 2/3 of the region. Finally, we have shown a similar morphological pattern of LC neurons and a similar distribution pattern in the dorsal-ventral plane in LC neurons from neonatal rats (present study) compared to LC neurons from adult rats (Swanson, 1976; Cintra et al., 1982; Loughlin et al., 1986a,b;). This suggests that the morphology and distribution of LC neurons arise early in development and seem to remain intact into adulthood. Interestingly, the L.C. shows developmental changes in the regional chemosensitive response. 70% of the neurons tested were activated by hypercapnic acidosis from young animals (<P10) while in animals >P10, only 20 percent were activated. It may be then that the neuronal morphology is established early in development but there is connectivity that has yet to be established that is important to chemosensitivity. Or alternatively, it is the signal-target mechanism of chemosensitivity that changes with development.
Expression of pH sensitive ion channels could be very high in young neonates and down regulated by P10. It may be that rather than a reduction in chemosensing ability, the neurons have developed a modulatory mechanism like the activation of BK channels in order to act as a brake (Imber et al., 2012).

**Neuronal Processes Approaching the Floor of the 4th Ventricle**

Another theory that has been proposed is that chemosensitive neurons send processes to the surface of the brainstem. Kawai et al. (1996) showed that in chemosensitive neurons from the ventrolateral medulla, even though cell bodies were 100-700 µm from the ventral medullary surface, all these neurons sent at least one dendritic projection to within 50 µm of the ventral surface. This suggests that these neurons may be sensing the chemistry of the surface fluid, perhaps associated with pial blood vessels (intracranial vessels on the surface of the brain within the glia limitans), and that the sensing is based on the dendritic endings (Kawai et al, 1996). However, there are two problems with this idea. Respiratory input from the neurons in this study is inhibited by tetrodotoxin (TTX, a sodium channel inhibitor) or synaptic block medium (↑Mg²⁺, ↓Ca²⁺), suggesting that these neurons are not intrinsically chemosensitive, and yet they still send at least one projection to the ventral surface (Kawai et al., 1996 Figure 8). The other problem is that this theory implies that a small portion of a dendrite, possibly as much as 600 µm from the soma, is fully responsible for the membrane potential response of the neuron to hypercapnia. While this is certainly possible, it would require either a very large response to pH changes at the dendritic ending or some special properties of the dendrites in these neurons.
A more compelling example of this same principle of chemosensitive sensing occurring in dendritic processes has been suggested with RTN neurons (Mulkey et al., 2004). In this study, chemosensitive RTN neurons were found that either had a soma in the marginal layer of the ventral medulla or had at least one dendritic process that runs for as much as 1 mm in the marginal layer. This anatomical arrangement suggests that chemosensitive sensing is localized to these portions of RTN neurons that reside within the marginal layers, but as these authors point out “…Proximity to the ventral surface does not necessarily in itself confer CO₂ sensitivity…” (Mulkey et al., 2004).

Both of the studies mentioned above examined chemosensitive neurons from the ventral medulla. Ours is the first study to address surface projections of chemosensitive neurons form the dorsal brainstem. We found that one process from chemosensitive neurons approached the floor of the 4th ventricle significantly closer than from nonchemosensitive neurons (Fig. 15). Further, we found that there is a significant correlation between the distance of the soma from and the closest approach of a process to the floor of the 4th ventricle for nonchemosensitive neurons. This is what we would expect for nonchemosensitive neurons since it suggests that projections extend for a certain distance from the soma and are not directed toward the surface. Thus, the distance from the surface of a projection is more a function of how close the soma is to the surface. On the other hand, we found no significant correlation of the distance of the soma from and the closest approach of a process to the floor of the 4th ventricle for chemosensitive neurons. Once again, this is what we
would expect if the chemosensitive neurons are sending projections to the surface, since the projection would continue to grow towards the surface regardless of how far the soma was from the surface. In fact, Kawai et al. (1996) found a significant correlation between cell body depth and the nearest approach of a dendrite projection to the surface for nonchemosensitive but not for chemosensitive neurons from the ventrolateral medulla. While these findings are consistent with the hypothesis that projections to the surface are characteristic of chemosensitive neurons, it is unlikely that this is the locus of chemosensing in LC neurons. First, only a single process approaches the surface, it approaches to only about 50 um with a range of 25 to 110 µm, and it tapers to a small ending.

Even more convincing are physiological studies that involved puffing acid solutions on either an extensive portion of LC neuron dendrites or on the soma and proximal dendrites of LC neurons (Ritucci et al., 2005). Only when the soma and proximal dendrites were exposed to acid did the firing rate of the neuron increase, strongly indicating that chemosensitive signaling resides in the soma and/or the proximal dendrites of LC neurons, and not on distant portions of the dendrites. Ritucci et al., provided compelling evidence that it is the soma, not the dendrites that must be acidified in order to elicit the chemosensitive response in the LC. This raises the question, why look for surface dendrites in the LC? Though he clearly shows that acidification of the soma was necessary and the acidification of the dendrite that he exposed to acid was not, there was no test of a portion of the arbor that might be specialized to gather information from the floor of the fourth ventricle. The focal acidification study was not designed to test
any particular aspect of the arborization. In order to rule out “surface seeking”
dendrites as a locus for chemosensing, more specific arboreal acidification
experiments should be done. Our findings are consistent with processes
approaching the surface of the slice not being involved in chemosensitive
signaling in LC neurons. It remains to be determined, with physiological studies,
whether they are involved in chemosensitive signaling in ventral medullary
neurons and the cNTS.

**Association of LC Neurons with Blood Vessels**

A final theory regarding the morphology of chemosensitive neurons, and a
variation on the theme of sending processes to the surface, is that
chemosensitive neurons have processes that associate closely with blood
vessels. Bradley *et al.* (2002) showed evidence that chemosensitive
serotonergic neurons of the medulla have processes that associate with nearby
large arteries. Electron microscopy demonstrated that serotonergic nerve
endings actually penetrated the artery wall and made close contact with the
endothelial layer of the artery (Bradley *et al.*, 2002). It was proposed that this
made an ideal arrangement for these neurons to sense blood CO₂ and be involve
in chemosensing. Interestingly, Cintra *et al.* (1982) reported that the dendrites of
fusiform LC neurons, which we show to be the chemosensitive neurons, run
along nearby blood vessels, but unlike Bradley *et al.* (2002), they did not observe
any special structural association between these dendrites and the blood
vessels.
We have observed a similar proximity of a chemosensitive LC dendrite to a blood vessel that lacked any apparent direct association (Fig. 18B). Close contacts between the dendrites of LC neurons and capillaries have previously been reported and it was suggested this represented a possible influence of catecholamine release on blood vessels (Shimizu et al., 1979). In the present study, we did observe a very close contact between small blood vessels (probably capillaries) and the soma of chemosensitive neurons (Fig. 19). In fact, we could not detect any separation between the two. An old electron microscopic study of LC neurons and capillaries revealed a similar very close association of blood vessels with the soma of LC neurons from primates (Felten and Crutcher, 1979). Interestingly, at the area of contact the soma of an LC neuron directly contacted the endothelium of the capillary but the neuron made contact with only about 50% of the capillary surface. The remainder of the capillary surface made contact with astrocytic processes, which may allow for the maintenance of the blood brain barrier.

Felten and Crutcher (1979) observed a similar close association between capillaries and the soma of other monoaminergic neurons, leading them to suggest that this close association was related to blood born agents affecting these monoaminergic neurons. We note that such a close relationship of capillaries with the soma of chemosensitive LC neurons is an ideal arrangement for these soma to sense and respond to changes in blood chemistry, especially changes in \( \text{CO}_2/\text{H}^+ \). This anatomical arrangement, combined with previous physiological studies indicating that acid sensing is most likely localized to the
soma and proximal dendrites of LC neurons, suggest that the cellular chemosensing machinery in LC neurons is localized to the soma.
SUMMARY

Our work offers some important insights into the various theories associated with the structure of chemosensitive neurons as related to chemosensitive sensing. We have focused on neurons from the locus coeruleus.

1) As hypothesized, chemosensitive LC neurons have a distinct 3 dimensional structure from nonchemosensitive LC neurons. While this will be of help in identifying neurons that are either chemosensitive or nonchemosensitive for electrophysiological studies, we believe that this structure is more determined by the nature of the nucleus (the LC is densely packed with neurons) and by the nature of neural inputs and outputs. Knowledge of the 3 dimensional structure of chemosensitive LC neurons will also be of use for the development of multicompartment computational models of chemosensitive neurons (e.g. Chernov et al., 2008). Finally, the agreement of our differences in LC neuron structures and distribution in neonatal rats with that found by others in adult rats suggests that these structural differences arise early in development.

2) As hypothesized, chemosensitive LC neurons have at least one process that approaches the floor of the 4th ventricle more closely than the processes of nonchemosensitive LC neurons. While this is consistent with other studies suggesting that processes of chemosensitive neurons approach the brain surface we believe that this structural feature is most likely unrelated to the chemosensing function of LC neurons.
3) The most striking feature of chemosensitive LC neurons is their very close association with capillaries. This is an ideal structural feature for a neuron that senses blood chemistry. It could be argued, given the highly diffusible nature of CO$_2$, that capillaries that are separated from neuronal soma by only 3-8 μm will still be exposed rapidly to changes in blood CO$_2$. However, given the strong physiological data that acidification of LC soma, but not dendrites, results in increased firing rate and the close association of chemosensitive LC neuron soma with capillaries, we believe that we have strong evidence that the machinery for chemosensitive signaling most likely resides on the soma of chemosensitive LC neurons.
CHAPTER V.

CHEMOSENSITIVE NEURONS FROM THE CAUDAL REGION OF THE NUCLEUS TRACTUS SOLITARIUS (cNTS): THREE DIMENSIONAL STRUCTURE AND ASSOCIATION WITH BLOOD VESSELS

Rationale

In the previous study (Graham et al., 2014) we examined the 3 dimensional structure of and the relationship of identified chemosensitive and nonchemosensitive neurons with the slice surface and with blood vessels in LC neurons. This was the first such study of chemosensitive neurons from dorsal chemosensitive regions and the first 3 dimensional study of chemosensitive neurons. Our findings suggested that chemosensitive neurons from the LC have a distinct morphology compared to nonchemosensitive neurons from the LC, but that these distinct morphological forms were not the same for chemosensitive neurons from another region, the raphé (Richerson, 2001). Further, we found that the soma of chemosensitive LC neurons have a very close association to capillaries. In the current study, we wanted to determine the 3 dimensional morphology of identified chemosensitive vs. nonchemosensitive neurons from another chemosensitive brainstem region to see if, based on our techniques, there is a unique morphology for chemosensitive neurons and to what degree the morphology of chemosensitive neurons differs based on the brainstem region from which they derive. Further, we wanted to see if a close somal association with blood vessels is a characteristic of all chemosensitive neurons (Bradley et al., 2002; Graham et al., 2014), or only those from the medulla and the LC.
In the current study we examined the 3 dimensional structure, the association of neuronal projections with the surface and the association of neuronal soma and projections with blood vessels in chemosensitive and nonchemosensitive neurons from another dorsal chemosensitive brainstem region, the nucleus tractus solitarius (NTS). We use neurons from the caudal portion of the NTS (cNTS) since this region has been associated with the response of ventilation to hypercapnia and the control of breathing (Nattie and Li, 2002).
METHODS AND MATERIALS

The Methods and Materials are as described in Chapter IV except for the following differences:

Slice Preparation

Transverse brain sections were prepared from preweanling Sprague-Dawley rats (age P7-P16) as was done for studies in the locus coeruleus, however the location of the region of interest is contained in a dorsal medullary region which is caudal to the LC. Therefore the brain was dissected and sectioned differently as follows: One to two 300 μm slices from the dorsal medullary region containing the cNTS including the area postrema (AP), at stereotaxic coordinates approximately from Bregma -13.68mm-Interaural -4.68mm to Bregma -14.08mm –Interaural -5.08mm, were cut (using a Pelco 100 series 1000 vibratome) from each animal into ice-cold aCSF equilibrated with 5% CO₂ / 95% O₂ for electrophysiological analysis within 4 hours after removal from the animal. Individual slices were placed in a superfusion chamber (1.0 ml volume), immobilized with a grid of nylon fibers, and superfused with control solution at ~4ml/min at 37° C. The composition of the aCSF for cNTS slices was (in mM): 124 NaCl, 5 KCl, 1.24 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, 26 NaHCO₃, and 10 glucose, equilibrated with 5% CO₂-95%O₂, pH~7.45 at 37° C.

Electrophysiology

As in the methods for the LC, the whole cell patch clamp technique was used to measure voltage changes across the membranes of cNTS neurons. The
whole cell pipette filling solution was modified (lowered EGTA from 10 mM to 0.4 mM and from 1mM to 0.0 added CaCl$_2$) to prevent washout of the chemosensitive response (Filosa and Putnam, 2003; Conrad et al., 2009).

**The Association of Identified Neurons with Blood Vessels**

The cNTS and the LC are quite different with regard to the density of neurons and blood vessels. Both are much more diffuse in the cNTS so that both blood vessels and neuronal projections are much more visible. This easier visibility resulted in a greater number of instances where blood vessels and neuronal processes appeared to interact. Using confocal images, neuronal processes and nearby blood vessels that did not interact were easily discernible and the distance separating them was easily measurable. However, the fluorescence of some blood vessels and neuronal processes did appear to overlap. In these instances, confocal z-stack images (0.5 µm thick optical images) were obtained and the degree of separation could be determined by the number of images between the fluorescence for the blood vessels (red) and the fluorescence for the neuronal process (green). If the blood vessel and neuronal process appeared to be touching, the contact region was reconstructed in Neurolucida and analyzed in Neurolucida Explorer.
RESULTS

Dye Loading of Electrically-identified Neuron

We differentiated chemosensitive from nonchemosensitive cNTS neurons based on their firing rate response to hypercapnia. As with LC neurons, during these electrophysiological studies the neuron was loaded with Lucifer Yellow (from the patch pipette) which enabled the 3 dimensional structure of the loaded neuron to be studied. A dye-loaded cNTS neuron is shown in Fig. 20A that, based on its increased firing rate in response to hypercapnia (Fig. 20B), is a chemosensitive neuron. This chemosensitive cNTS neuron has a somewhat pyramidal-shaped soma and is multipolar, with dendrites arising from several regions of the soma (Fig. 20A). The dye-loaded cNTS neuron shown in Fig. 20C is nonchemosensitive, based on its lack of an increased firing rate in response to hypercapnia (Fig. 20D). Unlike the chemosensitive neuron (Fig. 20A), the nonchemosensitive cNTS neuron (Fig. 20C) has a soma that appears more fusiform and has dendrites that derive from only 2 poles that are about 180° apart (Fig. 20C).

General Morphology of Reconstructed Chemosensitive and Non-Chemosensitive cNTS Neurons

Neurons selected for reconstruction met the same criteria as those for the LC study: the entire loaded neuron and dendritic arborization was contained within the section. Neurons were evaluated for pruning of dendrites that may
FIGURE 20.  A: A 300 μm brainstem slice containing a cNTS neuron which has been patch clamped and loaded with fluorescent dye, Lucifer Yellow. Note that the dye has diffused along the primary dendrites, revealing the branched structures in several planes extending from the soma. The neuron is presented as a projection which is made by the overlap of all the Z stack optical images for the neuron. Note that this neuron appears to have a more pyramidal shaped soma with processes arising from multiple poles.  

B: An electrophysiological recording from the same neuron as in panel A. The top panel shows integrated firing rate (10 s bins) expressed as the number of action potentials per 10 s vs. time. Note the stable initial firing rate of about 0.5 Hz in aCSF equilibrated with 5% CO₂ (normocapnia) (1) which then reversibly increases to about 1.5 Hz in response to aCSF equilibrated with 15% CO₂ (hypercapnia) (2). Firing rate returns to its initial value upon return to normocapnia (3). The lower panel shows individual action potentials at an expanded time scale for (1), (2). These findings identify this neuron as a chemosensitive neuron.  

C: A cNTS neuron loaded as for panel A. Note that this neuron has a more fusiform appearance, with an elongated nucleus and processes that arise from two poles that are separated by 180°.  

D: An electrophysiological recording from the same neuron as in panel C. The top panel shows integrated firing rate. Note that unlike the neuron in panels A and B, this neuron does not increase its firing rate in response to hypercapnia which is maintained throughout the transient hypercapnia exposure between 0.5-1 Hz. These findings identify this neuron as a nonchemosensitive neuron. (Electrophysiology, Dr. Ke-Yong Li, 2013 and Joseph Santin).
FIGURE 20.
have projected out of the plane of the section. Neurons with poor loading or suspected pruning were not included in the analysis.

Selected identified neurons were analyzed for variables in their somal and dendritic morphology in the following parameters: somal surface area, somal volume, somal linear dimensions in the X, Y and Z plane, somal aspect ratio (relative roundness), and dendritic radius, length, surface area and volume.

Two reconstructed chemosensitive cNTS neurons are shown from three different angles that give a better sense of their 3 dimensional structure (Figs. 21A and B). Both neurons had small somas that appeared to be pyramidal in shape with extensive and complex dendritic fields, arising from multiple poles and projecting in many directions from the soma. In contrast, reconstructed nonchemosensitive cNTS neurons had a different morphology (Figs. 22A and B). These nonchemosensitive neurons appeared to have small somas that were fusiform with elongated somas and dendrites arising from two poles separated by about 180 degrees. Interestingly, this is the reverse of the findings from identified neurons in the LC (Figs. 10 and 11). The dendritic fields of both nonchemosensitive and chemosensitive cNTS neurons were found to occupy up to 250 µm in the Z plane and extended approximately 300 µm in the X and Y planes (Figs. 21 and 22). (Electrophysiology, Dr. Ke-Yong Li and Joe Santin, 2014).
**FIGURE 21.**  **A:** An identified chemosensitive cNTS neuron reconstructed in Neurolucida shown from 3 angles. Note the pyramidal appearance of the soma of this neuron and the processes that arise from multiple poles.  **B:** A second identified chemosensitive cNTS neuron reconstructed in Neurolucida and shown from 3 angles. Note the pyramidal appearance of the soma and the processes that arise from multiple poles, very similar to the appearance of the neuron in panel A. The neuron in panel B provides an example of the depth of the dendritic field that is achievable via 3D reconstruction that is impossible with 2D. In the top view the neuron reaches over a large surface area with an extensive arborization, then when it is viewed from the “side’ it becomes apparent that it also occupies over 400μm in depth.
FIGURE 21.
FIGURE 22. **A:** An identified nonchemosensitive cNTS neuron reconstructed in Neurolucida shown from 3 perspectives. Note the fusiform appearance of this neuron, with an elongated nucleus and processes that arise from two opposite poles. Also note, as in the LC, in the third (bottom left) perspective, the soma does not appear fusiform, underscoring the advantage of three dimensional characterization of a neuron and pointing out one of many limitations of two dimensional studies. **B:** A second identified nonchemosensitive cNTS neuron reconstructed in Neurolucida and shown from 3 angles. Note the fusiform appearance of this neuron, with an elongated nucleus and processes that arise from two opposite poles, very similar to the appearance of the neuron in panel A.
Somal Reconstructions for Chemosensitive and Nonchemosensitive cNTS Neurons

Confocal z-stacks of the soma from identified cNTS neurons were reconstructed (using Neurolucida) and the perimeter of each optical slice was modeled using an average of 1 point/µm (Fig. 23). Examples of each type of soma reveal that both nonchemosensitive and chemosensitive neurons had a somewhat fusiform shape with comparable dimensions in all three planes (Fig. 23). The reconstructed somas were then analyzed using NeuroExplorer and the surface area, volume, diameters (in three dimensions) and the aspect ratio were determined (Table 3). We found no significant differences in the surface area and the volume between chemosensitive and nonchemosensitive cNTS neurons (Table 3). The diameters in each plane of each soma were calculated and averaged for the group. We found that chemosensitive neurons had no significantly different diameter in any plane when compared to nonchemosensitive neurons (Table 3).

We calculated the aspect ratios using the longest average diameter in the Z plane divided by the shortest average diameter in the X plane. If the orientation of the neuron in the tissue was such that the longest somal axis was not oriented to the confocal Z plane, the data were arranged so that all longest axes were considered “Z” and all shortest axes considered “X” for this calculation. An aspect ratio of 1 would indicate a perfect sphere. This parameter provides a mathematical reference of the relative roundness of the soma. The
FIGURE 23. The modeled perimeters of adjacent optical slices are shown for the soma of a chemosensitive neuron (left figure) and for the soma of a nonchemosensitive neuron (right figure). For calculation of aspect ratio, the longest diameter for each soma was defined as the Z axis and the shortest diameter for each soma was defined as the X axis. Note that both the chemosensitive and nonchemosensitive somas have an overall elongated appearance, and in this instance the chemosensitive neuron actually appears more elongated than the nonchemosensitive soma. A limitation of 2D morphological representation is apparent in this image. In the bottom perspective of neuron A the soma appears stellate and the dendritic arbor appears complex and bushy near the soma. This is an illusion created by the vantage point of the viewer and if analysis was based on this perspective, would result in faulty interpretation of the morphology of this neuron.
TABLE 3.

Dimensions of Somas from Chemosensitive vs. Nonchemosensitive cNTS Neurons

<table>
<thead>
<tr>
<th></th>
<th>Surface Area (µm²)</th>
<th>Volume (µm³)</th>
<th>Diameters (µm) X</th>
<th>Y</th>
<th>Z</th>
<th>Aspect Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHEMOSENSITIVE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average:</td>
<td>305.7 ± 36.3</td>
<td>571.8 ± 59.0</td>
<td>8.6 ± 0.5</td>
<td>11.5 ± 1.2</td>
<td>15.4 ± 0.7</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td><strong>NONCHEMOSENSITIVE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average:</td>
<td>312.3 ± 24.5</td>
<td>591.0 ± 85.0</td>
<td>8.6 ± 0.4</td>
<td>11.6 ± 0.5</td>
<td>15.4 ± 1.3</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Chemosensitive (n=7); Nonchemosensitive (n=7)
aspect ratio of the somas from chemosensitive cNTS neurons was almost identical to the aspect ratio of the somas from nonchemosensitive cNTS neurons (1.8±0.1) (Table 3), consistent with a somewhat fusiform morphology for both the multipolar chemosensitive neurons (Figs. 20 and 21) and the bipolar nonchemosensitive neurons (Figs. 20 and 22). Unlike the findings in the locus coeruleus, there is no significant quantitative difference in the overall somal morphology of nonchemosensitive cNTS neurons and chemosensitive cNTS neurons.

Dendritic Reconstructions for Chemosensitive and Nonchemosensitive cNTS Neurons

As in the LC, the cNTS dendritic field was evaluated by order of dendrite (Table 4). Dendritic analysis included number of dendritic orders per neuron, quantity of dendrites in each order, average radius within each order, overall average radius, linear extent within each order, overall linear extent per tree, total dendritic extent, (summed length of all segments of dendrites per soma), volume within each order, overall average volume per tree, and total volume (sum volume of all dendrites per soma). Surprisingly, and contradicting the bipolar appearance of the nonchemosensitive neurons, there was no significant difference found between the number of first order dendrites arising from the somas of nonchemosensitive and chemosensitive neurons. No statistical difference was calculated in the quantity of first order dendrites which also was surprising due to the distinct bipolar appearance of the projections from the nonchemosensitive neurons. There is greater deviation within the
chemosensitive group, reflective of the multipolar arrangement of the first order dendrites. Both nonchemosensitive and chemosensitive neurons were found to include up to ninth order dendrites, extending as far as 450µm from the soma, though in limited numbers. The average radii of each order of dendrites were compared and were found to be between 0.45±0.09 µm and 0.09 µm, first order and ninth order respectively, with no significant differences between chemosensitive and nonchemosensitive neurons (Table 4). Similar to the dendritic morphology in the LC, the radii of the dendrites decrease as they extend further from the soma (Table 4), consistent with the tapering, elongated, conical morphology typical of dendrites. The branching of the bipolar first order dendrites appears further from the soma in chemosensitive neurons than in nonchemosensitive neurons. The total extent of the dendritic field in quantity, length, surface area and volume were not found to be statistically different between groups (Table 4).

Three dimensional Sholl analysis was conducted on the dendritic fields of the cNTS chemosensitive and nonchemosensitive neurons. Reconstructed neurons, both chemosensitive (Fig 24A) and nonchemosensitive (Fig 24B) are shown here superimposed on representative 2D concentric rings out to 350 µm. (Fig 24). Note here the polar arrangement of the primary dendrites arising from the nonchemosensitive neuron and the comparatively bushy appearance of the chemosensitive neuron. Both neurons have at least one projection extending several hundred micrometers from the soma, found to be typical of all neurons considered in this study.
<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Average Radius (µm)</th>
<th>Average Length (µm)</th>
<th>Average Surface Area (µm²)</th>
<th>Average Volume (µm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHEMOSENSITIVE (n=7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Order</td>
<td>5.3 ± 1.0</td>
<td>0.45 ± 0.09</td>
<td>454.5 ± 111.9</td>
<td>816.4 ± 158.2</td>
<td>180.6 ± 47.6</td>
</tr>
<tr>
<td>Second Order</td>
<td>6.9 ± 1.2</td>
<td>0.28 ± 0.03</td>
<td>452.1 ± 88</td>
<td>737.6 ± 202.1</td>
<td>148.1 ± 34.7</td>
</tr>
<tr>
<td>Third Order</td>
<td>7.7 ± 1.1</td>
<td>0.19 ± 0.02</td>
<td>551.3 ± 129.1</td>
<td>742.9 ± 236.1</td>
<td>119.9 ± 24.7</td>
</tr>
<tr>
<td>Fourth Order</td>
<td>5.6 ± 1.0</td>
<td>0.21 ± 0.03</td>
<td>325.7 ± 85.2</td>
<td>439.3 ± 115.2</td>
<td>72.5 ± 22.3</td>
</tr>
<tr>
<td>Fifth Order</td>
<td>4.4 ± 1.1</td>
<td>0.19 ± 0.01</td>
<td>270.3 ± 94.0</td>
<td>364.0 ± 153.3</td>
<td>51.7 ± 8.9</td>
</tr>
<tr>
<td>Sixth Order</td>
<td>3.4 ± 0.5</td>
<td>0.21 ± 0.03</td>
<td>140.6 ± 38.3</td>
<td>155.4 ± 56.5</td>
<td>19.7 ± 3.2</td>
</tr>
<tr>
<td>Seventh Order</td>
<td>3.0 ± 1.4</td>
<td>0.24 ± 0.10</td>
<td>79.3 ± 23.0</td>
<td>72.7 ± 19.1</td>
<td>8.4 ± 2.2</td>
</tr>
<tr>
<td>Eighth Order</td>
<td>2.0¹</td>
<td>0.2¹</td>
<td>163.4¹</td>
<td>109.8¹</td>
<td>9.9¹</td>
</tr>
<tr>
<td>Ninth Order</td>
<td>2.0¹</td>
<td>0.1¹</td>
<td>129.9¹</td>
<td>89.0¹</td>
<td>7.6¹</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>33.6 ± 5.0</td>
<td></td>
<td>2187.6 ± 305.9</td>
<td>3185.7 ± 623.3</td>
<td>580.9 ± 145.7</td>
</tr>
<tr>
<td><strong>NONCHEMOSENSITIVE (n=7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Order</td>
<td>4.3 ± 0.5</td>
<td>0.45 ± 0.05</td>
<td>270.8 ± 80.9</td>
<td>468.0 ± 121.0</td>
<td>117.8 ± 23.2</td>
</tr>
<tr>
<td>Second Order</td>
<td>6.6 ± 0.8</td>
<td>0.33 ± 0.03</td>
<td>272.2 ± 72.8</td>
<td>410.4 ± 85.7</td>
<td>82.4 ± 16.2</td>
</tr>
<tr>
<td>Third Order</td>
<td>8.3 ± 1.1</td>
<td>0.25 ± 0.01</td>
<td>497.1 ± 184.4</td>
<td>630.1 ± 224.2</td>
<td>99.7 ± 38.1</td>
</tr>
<tr>
<td>Fourth Order</td>
<td>9.6 ± 1.7</td>
<td>0.20 ± 0.01</td>
<td>409.9 ± 69.3</td>
<td>476.6 ± 91.4</td>
<td>69.0 ± 16.3</td>
</tr>
<tr>
<td>Fifth Order</td>
<td>7.4 ± 2.3</td>
<td>0.16 ± 0.01</td>
<td>387.8 ± 93.0</td>
<td>379.6 ± 88.7</td>
<td>46.2 ± 11.9</td>
</tr>
<tr>
<td>Sixth Order</td>
<td>4.1 ± 0.9</td>
<td>0.17 ± 0.02</td>
<td>221.0 ± 50.8</td>
<td>214.0 ± 39.7</td>
<td>25.3 ± 4.3</td>
</tr>
<tr>
<td>Seventh Order</td>
<td>2.3 ± 0.8</td>
<td>0.19 ± 0.01</td>
<td>232.2 ± 79.8</td>
<td>251.7 ± 96.9</td>
<td>29.1 ± 12.8</td>
</tr>
<tr>
<td>Eighth Order</td>
<td>2.7 ± 0.4</td>
<td>0.13 ± 0.01</td>
<td>68.2 ± 17.9</td>
<td>58.0 ± 12.5</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td>Ninth Order</td>
<td>2.0²</td>
<td>0.09²</td>
<td>14.0²</td>
<td>4.4²</td>
<td>0.2²</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>44.3 ± 5.8</td>
<td></td>
<td>2171.0 ± 305.0</td>
<td>2705.9 ± 401.4</td>
<td>453.4 ± 60.3</td>
</tr>
</tbody>
</table>

¹ Only two of the chemosensitive neurons had eighth or ninth order dendrites and these are the means for those neurons.
² Only two of the nonchemosensitive neurons had ninth order dendrites and these are the means for those neurons.
**FIGURE 24.** The 2 dimensional representation of chemosensitive (top panel) and nonchemosensitive (bottom panel) cNTS neurons superimposed on a representation of the Sholl spheres (50 µm each). Note the compact arrangement of the arbor around the soma of the chemosensitive neuron vs. the relatively simple arrangement of the nonchemosensitive neuron at the origins. The nonchemosensitive neuron has greater complexity at 150 µm from the soma and the chemosensitive neuronal structures become more simplified at approximately the same point.

Multiple t-tests with Bonferroni correction. No significant differences were found.
FIGURE 24.
Analysis of the distribution of the dendritic field occupying each Sholl sphere revealed that the percentage of total length in all spheres is remarkably homogeneous except for the sixth sphere, 250 to 300 µm from the soma (25A). This difference reflects the comparatively simple arrangement of the primary dendrites of the nonchemosensitive neurons closer to the soma. Greater complexity in the form of branching appears in Sholl spheres 250-400 µm from the somas of nonchemosensitive neurons while chemosensitive neurons exhibit greater complexity in Sholl spheres 50-200 µm from the soma. The nonchemosensitive neurons send primary projections in essentially two directions, separated by 180°, which then branch, predominantly in the third and fourth order. The arrangement of the chemosensitive dendritic field is more bush-like in chemosensitive neurons, with dendrites extending outward in all directions from the center.

The dendritic surface area echoes the distribution of length with a higher percentage of the surface area in Sholl spheres 50-200 µm in chemosensitive neurons and a slightly higher percentage in nonchemosensitive neurons in sphere 250 µm (Fig. 25B). None of these differences were statistically significant, most likely due to a large variance in the surface areas of dendrites from both chemosensitive and nonchemosensitive neurons. This pattern diminishes almost completely for dendritic volume, as seen in the graph of percent volume (Fig 25C). The volume of the dendrites in each Sholl sphere is similar in both
FIGURE 25. The results of the Sholl analysis for chemosensitive neurons (black lines and symbols) (n=7) and for nonchemosensitive neurons (gray lines and symbols) (n=7). There is a trend for greater length, surface area and volume of processes for chemosensitive neurons in the 3rd, 4th, and 5th Sholl sphere than for nonchemosensitive neurons. Note that in the 7th sphere this trend reverses. Asterisk indicates that the values for chemosensitive and nonchemosensitive neurons differ significantly: Multiple t-tests with Bonferroni correction * P<0.05
FIGURE 25.
types of neurons, gradually and proportionately decreasing with distance from the soma. While quantitatively the dendritic fields of both chemosensitive and nonchemosensitive cNTS neurons appear quite similar, there is still a difference in overall morphology in that chemosensitive cNTS neurons have dendrites that arise from multiple poles and branch closer to the soma, creating the more complex multipolar appearance of these neurons. In contrast, dendrites arise from two distinct poles in nonchemosensitive cNTS neurons and to branch somewhat more distant from the soma and thus create a more bipolar appearance.

**Distribution of Chemosensitive and Nonchemosensitive Neurons in the cNTS**

The anatomical locations of 31 identified neurons were mapped in the tissue sections. The distribution of chemosensitive (green diamonds) and nonchemosensitive (red triangles) neurons is indicated in Fig. 26. Also included are three inhibited neurons (yellow ovals) studied electrophysiologically but not reconstructed. Both the chemosensitive and nonchemosensitive neurons in the cNTS were evenly distributed over the Commissural, Medial and Lateral areas. There was no significance found in the anatomical location of chemosensitive vs. nonchemosensitive neurons in the caudal portion of the NTS (Fig. 26). This is in marked distinction to the highly regionalized localization of chemosensitive neurons within the LC (Fig. 15).
FIGURE 26. A 10x confocal montage of a caudal slice from the dorsal medulla of a neonatal rat at the level of the area postrema. Superimposed on this image are colored markers indicating the location within the slice of individual neuronal somas that were studied electrophysiologically. cNTS neurons that were chemosensitive are indicated by green-filled diamonds, neurons that were nonchemosensitive are indicated by red-filled triangles and neurons whose firing rate was inhibited by hypercapnia are indicated by yellow-filled ovals. White outlines indicate neurons which were included in the reconstructions. Note that all three types of neurons appear to be interspersed throughout the cNTS without being associated with any particular part of the cNTS. Abbreviations: 4th ventricle: the location of the 4th ventricle above the dorsal surface of the slice; XII: the hypoglossal region, containing cell bodies of the hypoglossal nerve; AP: area postrema; CC: the central canal; cNTS: the caudal region of the nucleus tractus solitarius; DMV: the dorsal motor nucleus of the vagus. Fisher's Exact Test, No significance was found.
Distribution of Chemosensitive and Nonchemosensitive neurons in the cNTS

FIGURE 26.
**Closest Dendritic Approach to the 4th Ventricle in Chemosensitive and Nonchemosensitive cNTS Neurons**

The observation that some chemosensitive neurons from other regions of the brainstem known to be involved in the control of ventilation send processes to the brain surface has led to the suggestion that this may be a contributing factor to the chemosensitive response of those neurons (Kawai *et al.*, 1996; Mulkey *et al.*, 2004). To determine if the cNTS had dendritic projections that reached the surface, we measured the distance from the floor of the 4th ventricle to the nearest neuronal structure in chemosensitive and nonchemosensitive neurons (Fig. 27). Of those studied, only two chemosensitive neurons sent projections to within 50 µm of the floor of the 4th ventricle (Figs. 27A and C). On average, the closest approach was 157 ± 49 µm which ranged from 20 to 325 µm (Fig. 27C). The nonchemosensitive neurons had processes that approached the floor of the 4th ventricle on average to 475 ± 146 µm which ranged widely from 200 to 725 µm (Figs. 27B and C).

Though there is a significant difference in the closest approach of projections from the chemosensitive neurons vs. the nonchemosensitive neurons, neither group appears to come consistently close enough to suggest that the mechanism of chemosensitivity relies on the proximity of neuronal processes to the floor of the 4th ventricle. Not surprisingly, the proximity of the projections to the 4th ventricle corresponded to the relative proximity of the somas to the 4th ventricle. The somas of the chemosensitive neurons studied ranged in distance from 150 to 525 µm and the somas of nonchemosensitive neurons...
FIGURE 27.  **A:** A reconstructed chemo-sensitive cNTS neuron superimposed on a low magnification (10x) confocal image of the tissue for analysis of proximity to the surface of dendritic projections. The 4th ventricle is located immediately above the area postrema (AP).  **B:** A reconstructed nonchemosensitive cNTS neuron superimposed on a low magnification (10x) confocal image of the tissue for analysis of proximity to the surface of dendritic projections. Note that neither the chemo-sensitive nor the nonchemosensitive neuron has any process that approaches the floor of the 4th ventricle.  **C:** A box plot that shows the range of closest approach for chemo-sensitive (n=6) and nonchemosensitive (n=6) cNTS neurons to the floor of the 4th ventricle. The mean closest approach was significantly closer for chemo-sensitive neurons (157 µm) than for nonchemosensitive (475 µm) cNTS neurons.

Two tailed unpaired t-test ** P<0.02.
FIGURE 27.
ranged from 300 to 1025 µm from the 4th ventricle. The average distance from the 4th ventricle overall for somas of chemosensitive neurons was 348 ± 60 µm and for somas of nonchemosensitive neurons was 612 ± 121 µm. Thus, it does not appear that chemosensitive cNTS neurons direct projections towards the floor of the 4th ventricle as part of their chemosensitive phenotype.

**Relationship of Blood Vessels to cNTS Neurons**

We studied blood vessels and neurons from three regions of the cNTS: the Commissural; the Medial; and the Lateral (Fig. 28A). We stained slices with NeuN to mark neurons and with Isolectin to mark blood vessels. An example of such a double labeled slice from the Commissural (Fig. 28B), Medial (Fig. 28E) and the Lateral (Fig. 28H) regions are shown (Fig. 28). Using Image J analysis, we were able to compare the percentage of the area that was either neurons (Figs. 28C, F and I) or blood vessels (Figs. 28D, G and J). We found that the percentage of the area that was neurons was significantly higher in the Medial region than both the Commissural and Lateral cNTS regions (Fig. 29). We found the percentage of the area that was blood vessels was low across the entire cNTS and was not significantly different from region to region within the cNTS (Fig. 30). Expressing these values as a ratio of blood vessels to neurons showed that the Lateral and Commissural regions of the cNTS had significantly higher blood vessel to neuron ratios (0.5-0.6) than did the Medial region (0.13) (Fig. 31). Thus, despite having a much lower density of neurons than the LC,
FIGURE 28.  A. A representative image of the cNTS (10x) including the AP and the 4\textsuperscript{th} ventricle with the three regions of consideration outlined in green. The dorsal most edge of the central canal is visible center bottom of the figure. Bar 100 µm.  
B, E and H: Cropped images of the Commissural region (B), the Medial region (E) and the Lateral region (H) stained for neurons (NeuN) and blood vessels (Isolectin-64). Bar 50µm.  
C, F and I: Channel-separated binary images of the NeuN labeled neurons in each of the corresponding areas.  
D, G and J: Channel-separated binary images of the isolectin labeled blood vessels in each of the corresponding areas. The binary images were used to evaluate the area occupied by neurons and blood vessels in each region.
FIGURE 28.
FIGURE 29. Percent surface area of neurons in the Commissural, Medial and Lateral regions of the cNTS and comparison with the same data from the LC (see Chapter IV Fig. 9). Percent surface area was calculated as pixels corresponding to neurons vs. total pixel count. It can be seen that the neuropil in the cNTS Medial region was significantly greater than that of both the Commissural cNTS and the Lateral cNTS regions. Total neuronal percent surface area in the cNTS regions (~20-35%) is less than that of the LC regions (45-55%).

Multiple t-tests with Bonferroni correction *P<0.05, ** P<0.01.
FIGURE 29.
FIGURE 30. Percent surface area of blood vessels in the Commissural, Medial and Lateral regions of the cNTS and comparison with the same data from the LC (see Chapter IV, Fig. 17). As for neurons, percent surface area was calculated as pixels corresponding to blood vessels vs. total pixel count. It can be seen that the blood vessels in the cNTS were approximately equivalent across the Commissural, Medial and Lateral regions. When compared to the LC, the vasculature of the cNTS is much less dense, representing approximately half that of the dorsal and intermediate LC regions. Note that the area occupied by blood vessels in the ventral region of the LC, which we found to not contain chemosensitive neurons, is very similar to that in the cNTS.

Multiple t-tests with Bonferroni correction ***P<0.001.
FIGURE 30.
FIGURE 31.

Ratio of blood vessels to neurons in the cNTS was calculated by dividing the percent of the area that consisted of blood vessels by the percent of the area that consisted of neurons. It can be seen that the ratio of blood vessels to neurons is significantly higher in the Commissural and Lateral regions compared to the Medial region, reflecting the denser neuropil in the Medial region of the cNTS. The mean ratio of blood vessels to neurons is similar in both the LC and in the cNTS (~ 0.5). Multiple t-tests with Bonferroni correction **P<0.01, ***P<0.001.
FIGURE 31.
cNTS neurons seem to have a similar ratio of blood vessel area to neuronal area, at least in the Commissural and Lateral regions.

We next sought to determine if there were any specialized connections between dendritic processes of chemosensitive cNTS neurons and blood vessels, as suggested for chemosensitive raphé neurons (Bradley et al., 2002). As in the LC, we identified a given neuron as either a chemosensitive or nonchemosensitive neuron and then loaded it with Lucifer Yellow. Fixed slices were incubated with Isolectin-GS-IB4 conjugated to Alexa-568 as described in the Methods and Materials. A detailed, multi-paned, 60x series of confocal images were obtained in order to evaluate the apposition of the blood vessels to dendrites and somas and to use for reconstruction of the loaded neuron and closest blood vessels. Somal and dendritic reconstructions and blood vessels were modeled using the neuron tracing tools and contour modeling tool in Neurolucida (Fig. 32). Where possible interactions between blood vessels and projections were observed, in either confocal images or in reconstructions and measurements were made to determine the proximity of the structures in Fluoview and in Neurolucida Explorer. Two chemosensitive neurons are shown in Fig. 32. The neuron in Fig. 32A appears to have a close contact between the soma and a blood vessel (Fig. 32A, white arrow) but at higher magnification a clear separation of about 5-8µm is evident (Fig. 32B, white arrow). However, a close association appears to be present between the blood vessel and a dendrite (Fig. 32B, purple arrow); once again at greater magnification a separation of some 5-10µm is clear (Fig. 32C, purple arrow).
FIGURE 32. Space filling models of 2 reconstructed chemosensitive (A and D) and 2 nonchemosensitive (G and J) cNTS neurons (green) and associate blood vessels (BV) (red). Each neuron-blood vessel frame is shown from different perspectives (A, B and C is one chemosensitive (CS) neuron while D, E and F is another chemosensitive neuron; G, H and I is one nonchemosensitive (NCS) neuron while J, K and L is another nonchemosensitive neuron). Close associations between somal structures and blood vessels (white arrows) and between dendritic structures and blood vessels (purple arrows) were evaluated for contact. Neurolucida modeling allows manipulation of the reconstruction in 3-dimensional space. B and C show enlargements and different perspectives of blood vessels and chemosensitive neuron A. E and F show enlargements and different perspectives of blood vessels and chemosensitive neuron D. H and I show enlargements and different perspectives of blood vessels and nonchemosensitive neuron G. Finally, K and L show enlargements and different perspectives of blood vessels and nonchemosensitive neuron J.

Average distance between CS soma and nearest BV = 9.66±2.7 µm
Average distance between NCS soma and nearest BV =16.66±5.6 µm

The two-tailed t-test, P = 0.2445.
FIGURE 32.
The chemosensitive neuron in Fig. 32D also appears to have a region of close association between a blood vessel and the soma (white arrow) and a dendrite (purple arrow). At higher magnification and from a perspective where the detail of the structures in question can be viewed, it can be seen that there is no apparent separation between a larger blood vessel and the soma and a proximal dendrite (Fig. 32E, white arrow) or from another dendrite (Fig. 32E, purple arrow). Upon enlargement, there is a clear separation visible between the blood vessel and this more distal dendrite (Fig. 32F, purple arrow).

A similar degree of association is seen in nonchemosensitive neurons. The nonchemosensitive neuron in Fig. 32G (white arrow) appears to have a close association between a blood vessel and the soma and a proximal dendrite. This intimate contact is confirmed by a higher magnification image showing the very close association between the soma/proximal dendrite and the blood vessel (Fig. 32H, white arrow). In Fig. 32I, a separation of 5-10µm can be seen between the blood vessel and a distal dendrite (purple arrow) although in a lower magnification image they appeared to come in direct contact (image not shown). Finally, another nonchemosensitive neuron is shown in Fig. 32J with a different pattern of association between neuron and blood vessels. The soma can be seen to be widely separated (by as much as 20µm) from nearby blood vessels (Fig. 32J, white arrow). However, a distal dendrite appears to be in close association with the blood vessel (Fig. 32K, purple arrow). In a high magnification image, it appears in fact that the dendrite fits into a depression within the blood vessel, making direct contact (Fig. 32L, purple arrow). It would
require electron microscopy to determine if, in fact, this represents a specialized region of contact between blood vessel and dendrite but the lack of an actual separation is consistent with some type of interaction between the two.

The overall impression from these studies is that the morphological associations between blood vessels and cNTS neurons are highly diverse and variable. The average separation between a blood vessel and the soma of chemosensitive cNTS neurons is 9.66± 2.6 µm away. For nonchemosensitive neurons the average is 16.67±5.6 µm. Sometimes the separation is as great as 40µm between nonchemosensitive neurons and the nearest blood vessel. There are occasional examples of nearly direct contact of blood vessels both on the soma and on dendrites, especially distal dendrites. A separation of 10 or even 20 µm between blood vessels and neurons probably has little consequence for chemosensitive (CO₂-sensing) neurons since CO₂ is highly diffusible through most biological tissue. It is noteworthy that, unlike in the LC, in the cNTS the pattern of association between blood vessels and neurons does not show any significant difference between chemosensitive and nonchemosensitive neurons. Thus, a difference in the interaction between blood vessels and neurons does not appear to be a part of what defines a chemosensitive from a nonchemosensitive neuron in the cNTS.
DISCUSSION

In this study we undertook a detailed cytostructural analysis of chemosensitive and nonchemosensitive cNTS neurons and compared these results to chemosensitive and nonchemosensitive neurons from our previous study of the LC.

Morphology of Chemosensitive vs. Nonchemosensitive cNTS Neurons

Initial inspection of images of identified neurons in the cNTS revealed two general morphological types: a small ellipsoidal soma with bipolar arrangement of primary projections and a small ellipsoidal soma with multipolar arrangement of primary dendrites. The neurons with the more bipolar arrangement were electrophysiologically identified as nonchemosensitive and those with the more multipolar arrangement were electrophysiologically identified as chemosensitive neurons, giving support to the hypothesis that chemosensitive cNTS neurons are morphologically distinct from nonchemosensitive cNTS neurons. This finding was interesting given that it was contrary to the morphological associations found in the LC (Figs. 9, 10, 11 and 13)(Graham et al., 2014). Chemosensitive LC neurons were found to have a bipolar arrangement of dendrites while nonchemosensitive LC neurons were multipolar. In a morphological study of cultured chemosensitive raphé neurons, the stimulated neurons were found to be pyramidal and multipolar, similar to ours in the cNTS, while inhibited neurons were found to be bipolar (Richerson et al, 2001). Together these results from several chemosensitive regions suggest that there is not a unique morphology for
chemosensitive neurons in the brainstem. Though there may not be a single
morphology typical of chemosensitive neurons, there may be morphology
specific to chemosensitive neurons within a given region of chemosensitivity
which is distinct from nonchemosensitive neurons from the same region.

To further assess the structure of cNTS neurons, somal reconstruction
and subsequent analysis of surface area, volume and aspect ratio were
performed (Fig. 23, Table 3). Though there are multiple studies which include
consideration of cell morphology in the NTS, nothing is known about the
comparative morphology of chemosensitive and nonchemosensitive neurons in
the cNTS. It has been shown that the NTS neuronal population is
morphologically and electrophysiologically heterogeneous (Paton et al., 1993;
dimensional study in the adult mouse cNTS showed neurons of soma size that
were described as either large or small, ranging in area from 59.2 µm² to 345.7
µm² (Okada et al., 2008). The smallest of these neurons were located in the part
of the cNTS near the area postrema and both large and small neurons were
found throughout the rest of the cNTS. Another study, in neurons from weaned
Sprague Dawley rat cNTS, showed a majority of the neurons studied to have a
mean diameter falling between 10.6 and 11.2 µm (Kawai and Senba, 1999). If
considered in two dimensions, the areas of the somas of both
nonchemosensitive and chemosensitive neurons in our study would be
approximately 104.0-139.0 µm², corresponding to the aforementioned small
neurons (107.1–134.0 µm²) with mean diameters of approximately 11.6 µm. We
found these small nonchemosensitive and chemosensitive neurons to be distributed homogenously within the cNTS with no regional prejudice for one type or the other (Fig. 26). We did not identify or study any cNTS neurons which fell into a larger category (though larger neurons were observed in NeuN-stained slices used for our regional vascularity analysis). The overall shape of the somas of both types of neurons in this study was ellipsoidal. The calculated aspect ratio (major/minor diameter) for both chemosensitive and nonchemosensitive showed no significant differences between the multipolar and bipolar neurons. This is similar to the findings of a developmental study of NTS neuronal morphology though their studies did not include volumetrics (Vincent and Tell, 1999). Our analysis of surface area, volume, and dimensions in three axes of identified neurons in the cNTS revealed that there were no significant differences between the nonchemosensitive and chemosensitive cNTS somas. On the contrary, the somas of these neurons were nearly identical in all parameters, providing evidence that the somal macrostructure is not significantly different between chemosensitive and nonchemosensitive neurons (Table 3).

The neuronal processes arising from the identified cNTS neurons were analyzed by branch order according to quantity, radius, linear extent, surface area and volume. The projections arising from the nonchemosensitive ellipsoid somas were typified by two larger (diameter at origin, 2.6 ± 0.3 µm) projections arising from opposite poles of the soma separated by approximately 180° (Fig. 22). There were frequently one to several smaller projections (diameter at origin, 1.3 ± 0.3 µm) arising from the soma close to the thicker polar structures. The
orientation of the polarity varied cell-to-cell from vertical (dorsoventral) to horizontal (mediolateral).

The arrangement of arborization for the chemosensitive cNTS neurons was markedly different. Dendritic sprouting showed no polarity and base diameters were notably smaller (1.4 ± 0.1 µm) with no thick projections from the soma as seen in the nonchemosensitive cNTS neurons. These multipolar neurons were highly variable in the quantity of primary projections arising from the soma (Fig. 21, Table 4). Though fewer primary projections were found on nonchemosensitive neurons, the variability in the chemosensitive population reduced the comparative significance. The average radii of the projections compared by dendritic order did not reveal any significant differences between the two groups. Average length, surface area and volume were greater in the first, second and third orders of the chemosensitive group which corresponds to the higher number of primary dendrites arising from the soma (Fig. 24). Greater branching complexity in the nonchemosensitive arbor further from the soma is reflected in a higher quantity of fourth and fifth order dendrites and a corresponding increase in dendritic length; however the surface area and volume parameters indicate that these are small branches and do not bear out any significant difference when compared to the chemosensitive neurons (Table 4).

Both of these arborization patterns have previously been described in the cNTS (Champagnat et al 1986; Kalia et al, 1993, Vincent and Tell, 1999). These studies were done in 2 dimensions. The limitations of 2D morphometric studies include limit to one perspective of a given neuron and it’s arbor, artificially
influencing the viewer’s estimation of overall shape and from where the primary dendrites arise. Accurate length of the dendritic arbor cannot be collected due to lack of depth, and perception of trajectory in the reconstruction. The morphologies reflected in 2D reconstructions cannot account for structural elements that lie below other elements. Dendritic pass (or the distance between overlapping dendrites) cannot be calculated nor included in 2D reconstructions. Complex, bushy arborizations cannot be reconstructed due to the extreme amount of overlap of structures. When dendritic structures overlap, it is impossible to distinguish with certainty whether one is looking at a bifurcation with branches or a process that is passing below another process. 3D reconstructions eliminate all of these issues and also provide for volumetric analysis. In addition, previous morphometric studies in these regions did not attempt to associate the morphological distinction with an electrophysiological phenotype. This is the first study to define and compare the morphologies with the chemosensing abilities of nonchemosensitive and chemosensitive neurons from two different regions.

Vincent and Tell (1999) describe these two types of neurons in the developing rat (P0-P20). They categorize two types of somas in NTS neurons, elongated and multipolar. Though they report somal diameters as slightly larger than those reported here, image comparisons and calculated aspect ratios show that the relative dimensions of the NTS neurons in our study are quite similar to their values (Vincent and Tell, 1999). The arboreal arrangements were described in very similar terms, noting thick bipolar projections in the elongated
neurons and a greater number of smaller diameter projections arising from the multipolar neurons. They also reported that the multipolar neurons were more numerous during all developmental stages. The area of influence (area of the polygon formed by the perimeter of a reconstructed neuron) of the neurons in this study (data not shown) is very similar to that of the same age group as reported by Vincent and Tell (1999). They show that elongated cNTS neurons from animals between ages P8 and P15 occupy areas of between approximately 8,000 and 20,000 µm$^2$. Our elongated or bipolar (nonchemosensitive) neurons (P7-P15) have a mean area of influence of 11,250 µm$^2$. Our multipolar (chemosensitive) neurons (P7-P15) have a mean area of influence of 39,475 µm$^2$, similar to their observed multipolar cNTS neurons, which fell between 10,000 and 45,000 µm$^2$. This suggests that the chemosensitive neurons in our study reach a greater area of the NTS than do the nonchemosensitive neurons.

Kalia et al. (1993) conducted electrophysiological and morphological studies in cNTS neurons from newborn rats (P0-P6). The morphologically polar neurons were noted during the early immature phase of development and morphologically bushy neurons were noted later. The authors limited their study to very young rats and interpreted the bipolar morphology as developmentally transient. Our studies were conducted in preweanling rats (p7-p15) and there was no dearth of cells with polar arborization, indicating that the polar morphology persists throughout development. Vincent and Tell (1999) also demonstrate the presence of elongated, bipolar cells throughout development.
from P0-P20, with 60-80% neurons studied being multipolar vs 20-40% found to be elongated.

In a study of NTS afferent pathways Bailey et al. (2007) reported that tracer-identified paraventricular nucleus (PVN)-projecting NTS neurons had more elaborate dendritic morphology than did caudal ventrolateral medulla (CVLM)-projecting neurons. They also observed that CVLM- and PVN-projecting NTS neurons express different K+ currents, with the A-type K+ current largely absent in the CVLM-projecting neurons but substantially expressed in the PVN-projecting NTS neurons. Their observations of morphology were limited to 2D which introduces perspective error in their quantification of somal size and dendritic length; however, their observation of greater dendritic complexity near the soma of PVN-projecting neurons coincides with our observations of dendritic complexity near the soma of chemosensitive cNTS neurons.

Kalia et al. (1993) also suggest that there is a close link between morphological development and electrophysiological behavior of the neurons in the cNTS. They describe an absence of the A current in early development (P0-P3) in neurons with a polar morphology. They go on to report that in bushy multipolar neurons an A current is present in slightly older animals (P3-P6). In more recent developmental electrophysiological studies of the NTS, the chemosensitive response has been shown to be completely abolished by application of 4-aminopyridine (4-AP), an A current inhibitor (Martino and Putnam, 2007; Li and Putnam, 2010). This is interesting in light of our current study correlating morphology with chemosensitivity. Our nonchemosensitive
cNTS neurons are bipolar in morphology and the chemosensitive cNTS neurons are multipolar. This would suggest that the bushy, multipolar cells, shown to be positive for A current (Kalia et al., 1993), may indeed be responsible for the chemosensitive response in the cNTS.

Developmental electrophysiological studies in the cNTS have shown no alteration in the chemosensitive response of cNTS neurons from rats of postnatal age P1-P19 (Conrad et al, 2009). Comparison of these data from cNTS neurons from neonatal rats with similar values from studies conducted in cNTS neurons in adult Sprague-Dawley rats shows no appreciable differences in the chemosensitive response of neurons from the cNTS in rats of any age (Nichols et al, 2008). Vincent and Tell (1999) show the presence of the multipolar cells as early as P0 with no change in the proportion of elongated to multipolar neurons through P20 (60-80% multipolar vs 20-40% elongated), though they did not find a correlation between the morphology and the firing patterns of the cNTS neurons. Together these data suggest that in the NTS, intrinsic chemosensitivity is established early in development and that in the cNTS, chemosensitive neurons may have a distinctive multipolar appearance with a bushy arboreal organization and nonchemosensitive neurons have a bipolar arboreal organization. It should be underscored however that the mechanism of the chemosensitive response likely does not depend upon the cytoarchitecture but rather on the ion channel population residing on these neurons.

In the LC, the somal phenotypes are likely to be in place early in neonatal development. The chemosensitivity of the area, however, changes with
development, dropping sharply after P10 (Gargaglioni et al., 2010). It is likely that the arborization patterns are also in place but still developing connectivity during this period. (note growth cone, Fig. 18). Integration with the network and development of the full adult complement of ion channels is likely the culprit for changing chemosensitivity associated with development in the LC.

**Neuronal Processes Approaching the Floor of the Fourth Ventricle**

Evidence that chemosensitive neurons may send projections to the surface of the brainstem has been reported in two studies, one in the ventral lateral medulla (Kawai et al, 1996) and one in the RTN (Mulkey et al, 2004). Chemosensitive neurons of the VLM were shown to send at least one projection to the ventral medullary surface (Kawai et al, 1996; (Fig. 3)) and chemosensitive RTN neurons showed projections running for some distance (> 1 mm) in the marginal layer. In the RTN the somas of some chemosensitive neurons were also observed to lie in very close proximity to the surface (Mulkey et al., 2004; Fig 4 from this thesis). Our study in the LC showed that chemosensitive neurons send at least one projection toward the 4\(^{th}\) ventricle, terminating significantly closer to this surface than did nonchemosensitive LC neuronal projections (Fig. 16). Though the difference between the approach of chemosensitive vs. nonchemosensitive dendrites was statistically significant, the average approach was still greater than 50\(\mu\)m away from the 4\(^{th}\) ventricle and there was great variance between the data. We show a strong correlation between the location of the some of the nonchemosensitive neurons and the reach of their dendrites toward the 4\(^{th}\) ventricle. Because the majority of the nonchemosensitive neurons
studied lay in the ventral most area of the LC, it may be the more dorsal regional
distribution of chemosensitive neurons which accounts for the quantitative
results.

In the cNTS we find similarly that the chemosensitive neurons have at
least one projection that seems to be directed toward the 4th ventricle (Fig. 27).
The average closest approach for chemosensitive neurons (157 µm) is
significantly closer than for nonchemosensitive cNTS neurons (475 µm). Even
so, the proximity of processes of chemosensitive neurons to the floor of the 4th
ventricle is rather distant with considerable variation. Further, there was no
observed pattern or obvious orientation of processes of chemosensitive neurons
towards the floor of the 4th ventricle. Given the fact that these neurons were
multipolar it is intuitive that at least one of the dendrites might project in that
direction. Additionally, the polar orientation of the nonchemosensitive neuronal
arbor varied from nearly vertical (dorsoventral) to horizontal (mediolateral).
Those which were oriented more vertically came closer to the ventricular surface
while those oriented more horizontally had no projections which grew toward the
4th ventricle. This arrangement, with some neurons projecting towards the
ventricle while others did not, probably accounts for the very large variation in
values for closest approach in nonchemosensitive neurons from the cNTS, which
varied from 200 to about 750 µm (Fig. 27). Thus the proximity of the
nonchemosensitive dendrites to the dorsal surface is most likely a function of the
polar orientation of the dendritic arbor. We consider both of these findings
negative for proximity to the surface and conclude that this is not a common trait.
of chemosensitive neurons in these dorsal regions. These negative findings in
two dorsal areas of chemosensitivity require reevaluation of the interpretation of
the findings in the VLM and RTN. Further study with larger “n” and accompanying
physiological studies segregating somal vs dendritic response in the VLM, RTN
and NTS would help clarify the input, if any, the “surface-seeking” dendrites have
on the chemosensitive response in these regions.

The observation that neuronal projections of chemosensitive neurons are
directed to the brainstem surface has given rise to a persistent hypothesis in the
field that these projections are involved in chemosensing. This hypothesis has
only been directly tested physiologically in LC neurons (Ritucci et al., 2005).
Focal acidification on LC neuronal somas and proximal dendrites resulted in
increased firing rate in chemosensitive neurons but acidification of distal
dendrites did not, strongly suggesting a somal or at least proximal site for cellular
chemosensing in these neurons. To date there have been no similar
physiological studies to determine the locus of chemosensing in cNTS neurons,
but it would be of interest to see if focal acidosis of the soma of cNTS neurons
was sufficient to elicit a chemosensitive response or whether the sensing
mechanism resides in the dendritic arbor. Chemosensitive cNTS neurons clearly
do not show a distinct pattern of dendritic projections to the brainstem surface as
do VLM (Kawai et al., 1996) and RTN (Mulkey et al., 2004) neurons and in this
regard they are more similar to LC neurons. The results of these two studies
require reevaluation of the hypothesis and new studies to further explore the
morphology of the chemosensitive phenotype.
Association of cNTS Neurons with Blood Vessels

Though the vasculature of the cNTS has been well characterized (Koda and Bloom, 1983; Gross et al. 1990, 1991; Gross, 1991; Shaver et al. 1991; Fodor et al., 2007), there have been no studies of the neuronal association with the blood vessels in this region. Neurons from other chemosensitive regions have been shown to have unique associations with blood vessels including medullary raphé neurons (Bradley et al., 2002) and chemosensitive neurons in the LC from our own work. In this study we have analyzed the association of identified chemosensitive and nonchemosensitive neurons in the cNTS with nearest blood vessels. The density of the neuropil in this region is significantly less than that of the densely packed LC. We have demonstrated that the neuronal density varies across the cNTS, occupying approximately 20% of the area in the central Commissural area, greater in the Medial area, ~35-40% (immediately lateral to our selected Commissural region) and somewhat less dense in the Lateral area (~15%) (Fig. 29). We find that the regional vasculature is also diffuse compared to the LC, occupying only from approximately 10-15% of the total area (Fig. 30) compared to ~30% in the dorsal and intermediate areas of the LC. The density of blood vessels in the ventral LC is more comparable to that in the cNTS at ~ 10%. Interestingly, the ratio of blood vessels to neurons in two areas of the cNTS (Commissural and Lateral ~ 60%) is similar to that of the two regions in the LC (dorsal and intermediate ~60%) found to correspond to the greatest proportion of chemosensitive neurons (Fig. 31). It could be that this ratio
provides not only for the metabolic needs of the neuronal population but also the optimum access to blood-derived pH changes.

Close contacts with individual blood vessels were observed in neurons from the cNTS (Fig. 32) but, unlike the LC, there were no strikingly consistent associations of either somas or dendrites with the microvasculature. The multipolar chemosensitive neurons were not statistically more likely than nonchemosensitive neurons to establish close contact with a capillary in any of the three cNTS areas studied.

It is of considerable interest that specialized vascular properties have been reported for the NTS (Roth and Yamomato, 1968; Gross et al., 1991; Porzionato et al., 2005; Fodor et al., 2007). Roth and Yamomato (1968) describe a portal system linking the dense capillary bed of the AP with the capillaries of the NTS. Fodor et al. (2007) also observed unique arrangements of the microvasculature in the AP (which lies outside the blood-brain barrier) and the area subpostrema and NTS in rats. In a study of human NTS in both infants and adults, it was reported that the microvascular density of the NTS vs other nuclei of the dorsal medulla is related to functional properties of the nuclei and that the high microvascular density in the AP and the NTS favors local neuropeptide infiltration and blood-gas monitoring. It was also suggest that specialized endothelial development arise depending on the microenvironments in different medullary nuclei (Porzionato et al., 2005). Gross et al. (1991) observed unique microvascular density with fenestrated capillaries with high permeability, surrounded by Virchow-Robin spaces, serving the dorsomedial and dorsolateral
commissural NTS at the AP in adult male Sprague-Dawley rats. The area they describe corresponds to the Commissural and Medial cNTS regions of our study. They determined that these specializations provide for rapid solute dispersion (such as monoamines, SP, angiotensin II, atriopeptin, glucocorticoids, etc.) and blood-sensing capability within the cNTS, facilitating viscerosensory and autonomic regulation.

We did not find a pattern of intimate proximity between chemosensitive cNTS neurons and the vasculature as we did for LC neurons. Given the peculiarities of the microvasculature in the subpostremal cNTS, the extensive capillary fenestration and potential confluence of the Virchow-Robin spaces between the AP and the NTS described by Gross et al. (1991) and the unique endothelial properties noted by Porzionato et al. (2005) would provide the ideal environment for accurate, rapid chemosensing within the cNTS and provide for a rich microenvironment in a region known to be the major relay center for autonomic integration and control.

Thus, the unique feature of the vasculature in the cNTS may be in the unusual properties of the capillaries and not in a uniquely different association between blood vessels and chemosensitive neurons vs. blood vessels and nonchemosensitive neurons.
SUMMARY

We have performed a study and comparison of the three dimensional morphology of identified chemosensitive and nonchemosensitive neurons in two known dorsal areas of chemosensitivity, the LC and the cNTS. This study focused on neurons from the cNTS.

1) Chemosensitive cNTS neurons have a visually apparent 3 dimensional morphology that is distinct from nonchemosensitive cNTS neurons. Specifically, the arboreal organization of the dendritic tree of chemosensitive cNTS neurons is multipolar with greater complexity closer to the soma while nonchemosensitive neurons are bipolar and have greater complexity further away from the soma.

2) Chemosensitive neurons of the cNTS do not appear to direct dendritic growth toward the 4th ventricle, rather their complex arboreal morphology suggests that they project in multiple directions in a wide field around each soma. Likewise, nonchemosensitive neurons do not have intentional growth toward the 4th ventricle but have bipolar dendritic arrangement with extensive linear reach in two directions from the soma.

3) Chemosensitive neurons of the cNTS do not have unique somal association with individual blood vessels though some neurons were within 2-5 micrometers of either large (~20µm) vessels or small (~5µm) capillaries. Chemosensitive cNTS neurons show no pattern of dendritic association with blood vessels though close contact (<5µm) was observed.
in several of the neurons studied. Nonchemosensitive cNTS somas and proximal dendrites may also have proximity to blood vessels. Further studies are necessary.

The importance of 3 dimensional reconstructions to our understanding of neuronal morphology is demonstrated here. 3D modeling provides volumetrics, a parameter completely neglected by 2D considerations. One advantage of 3D modeling is most apparent when comparing images of a single neuron from various perspectives (Figs 10, 11, 21 and 22). Miscalculations of soma dimensions, length, primary dendrite count and misinterpretation of axial images are eliminated by the methods employed here. Depending on the approach, a 2D image can make a distinctly bipolar neuron appear bushy or even multipolar and can make an ovoid soma appear round, stellate or pyramidal. Also, visually apparent characteristics observed in 2D are not borne out in statistical analysis of 3D surface area and volume, underscoring the importance of incorporating 3D modeling in the development of mathematical models of neuronal function. The 3D quantification from these two studies of dorsal chemosensitive areas will inform computational models for developing algorithmic descriptions of neurons and simulation of electrophysiological behavior.

We have demonstrated here for the first time that both the LC and the cNTS contain chemosensitive neurons which have a 3D morphology distinct from nonchemosensitive neurons in the same region. Both regions contain neurons with very different dendritic arrangements, multipolar and bipolar. Interestingly, the multipolar morphology corresponds to nonchemosensitive neurons in the LC
and to chemosensitive neurons in the cNTS, whereas the bipolar arrangement corresponds to chemosensitive neurons in the LC and to nonchemosensitive neurons in the cNTS. This reversal of the functional association with morphological type in these two regions suggests that, though there may be different morphologies attributable to functional differences within a given region, there is not a single morphology that is specific to chemosensitive neurons in all regions. This becomes even more apparent in consideration of previous 2D work which attributed similar morphologies to chemosensitive raphé neurons with different functional responses to CO₂ (Richerson et al., 2001).

Another insight that we have gained through these studies is that, at least in these two dorsal regions of the brainstem chemosensitive network, it is not requisite that chemosensitive neurons lie in proximity to or send projections to the surface of the brainstem. It is more likely that specializations in the vasculature and neuronal/vascular organization provide optimization for sensing changes in blood chemistry and extracellular pH or other neuroactive substances.

In both the LC and the cNTS there seem to be specializations of the vasculature that allow these regions to circumvent the limitations generally imposed by the blood-brain-barrier (BBB). Contact between chemosensitive LC neurons and capillaries which lack glial intervention (Felton and Crutcher, 1979) and highly permeable, fenestrated capillaries combined with penetrating Virchow-Robin spaces in the cNTS (Gross et al., 1990) create ideal environments for perfusion of CO₂ and dispersal of signaling molecules. Our reconstructions of
identified neurons in the LC provide evidence that the chemosensitive LC neuron has almost direct contact with circulating CO$_2$ and the comparative relative lack of contact between chemosensitive cNTS neurons and the microvasculature compared to chemosensitive LC neurons is of little consequence in light of vascular modifications (fenestrated capillaries, Virchow Robins spaces) which reduce the boundaries levied by the BBB in this region.

My doctoral thesis presented here has addressed many issues in our current understanding of morphological features in two regions of chemosensitivity and provided answers to several questions within the field. Also, I present here, two dramatic unexpected findings, not previously reported.

These studies were conducted in thick tissue slices rather than thin slice or culture; therefore the cytoarchitecture of the neurons studied was intact and had developed \textit{in vivo}.

These are the first studies using these methods for comparison between regions and between identified chemosensitive and nonchemosensitive neurons.

This is a novel approach using 3D modeling to demonstrate close anatomical association of physiologically identified neuronal structures with blood vessels in the locus coeruleus.

We report for first time that in the LC there is a pronounced regional distribution of chemosensitive and nonchemosensitive neurons.

My analysis of 3D structure of chemosensitive and nonchemosensitive neurons in the LC and the cNTS eliminates error inherent in 2D reconstructions and provides comprehensive morphometric data of neuronal structure in two
regions of chemosensitivity. These data can be used for computational models of chemosensitive electrophysiological behavior. Combined with emerging information about ion channel populations residing on neurons in these regions, they will inform development of physiologically relevant mathematical models.
VI. CONCLUSIONS AND FUTURE EXPERIMENTS

Conclusions

We present here for the first time 3 dimensional reconstructions of chemosensitive and nonchemosensitive neurons of the locus coeruleus and the caudal nucleus tractus solitarius. These reconstructions provide previously unknown insights into the morphologies of these neurons in two dorsal regions of chemosensitivity and emphasize the advantages to 3 dimensional modeling. We have shown that there are morphological distinctions between chemosensitive and nonchemosensitive neurons within a given region but that the observable differences in macrostructure do not correspond to like function from other regions. We show that there is not one definable structure which is typical of all chemosensitive neurons, leading us to the conclusion that structure does not in the LC and cNTS dictate function but is likely due to the arrangement of the regional anatomy and the projection patterns for each nucleus. It may be more correct to say that chemosensitive function does not in fact depend on cellular structure.

Our study also reveals for the first time that chemosensitive neurons from these two dorsal regions do not have projections which lie in proximity to the surface of the floor of the fourth ventricle. It may be that the dramatic observations of this phenomenon in ventral chemosensitive regions are significant due to their orientation to the ventral surface. Perhaps the fluid environment is such in the ventral medulla that it is more closely tied to the
chemosensory response of the neurons in the VLM and the RTN. It is possible that differences in the fluid environment of the 4th ventricle are not a contributing factor to the input to chemosensitive neurons of the LC and NTS. It may also be the case that specializations in the vasculature obviate the need for such proximity to the surface in these dorsal regions.

Lastly, we have found that unique associations between neurons and blood vessels exist in the LC but not in the cNTS. The dense vascularization and rich, closely packed neuropil of the LC provide optimal conditions for accurate sensing of changes in blood chemistry. That and the close apposition between capillaries and the soma of the chemosensitive neurons lead us to conclude that in the LC this vascular/neuronal relationship is important to the chemosensory function of the region. In fact, previous work in our laboratory by Ritucci et al. (2005) provided strong physiological evidence which, combined with our new anatomical evidence, supports the idea that the locus of chemosensitivity resides on the soma of LC neurons. Though the findings in the cNTS are not the same as those in the LC, others have shown very compelling evidence that the cNTS is likely bathed in a fluid environment which, due to capillary permeability and confluence with AP fluids, provides almost immediate access to changing blood gas, pH and neuropeptide levels. Physiological studies to test the response of chemosensitive neurons in the cNTS to acid puffs on the soma and dendrites would inform the field of research as to the likely location of chemosensing targets on the cell and guide experimental design for future studies.
Our findings support the hypothesis that there may be significant modifications of the vasculature in chemosensitive regions which support the metabolic needs of these neurons and provide rapid communication of changing physiological conditions to the chemosensitive network in order to dictate autonomic response and maintain homeostasis.

In summary, we conclude that there are differences in the 3 dimensional morphology of chemosensitive vs. nonchemosensitive neurons but that these differences are region specific and not consistent from one chemosensitive region to the next. We find no evidence in neurons from dorsal chemosensitive regions that neuronal processes grow toward the floor of the 4th ventricle. Finally, our evidence suggests that vascular adaptations in the LC and the cNTS potentially play a major role in the chemosensing of the neurons within these regions.

Future Experiments

While our work has provided answers to some questions related to the role of 3 dimensional structure and the associated blood vessels in neuronal chemosensitivity, there are a number of unanswered questions and future directions that require exploration. Future studies should certainly include 3 dimensional studies of chemosensitive and nonchemosensitive neurons in other regions of chemosensitivity (such as the RTN, the raphe, the VLM, the PBC, etc.) to see if one morphological type is more commonly associated with chemosensitive neurons or if each region is different from the other regions.
Our understanding of the association between morphology and chemosensitivity would also be enhanced by doing 3 dimensional reconstructions of another type of chemosensitive neuron, the inhibited neuron. The focus of the current study has been on neurons that are either activated or not activated by hypercapnia, but in several chemosensitive regions some neurons have been identified as inhibited by raising CO$_2$ (Conrad et al., 2009; Dean et al., 1989; Huang et al., 1997; Nichols et al., 2009; Richerson, 1995; Wang et al., 1998, Wellner-Kienitz and Shams, 1998). In the NTS, inhibited chemosensitive neurons may make up as much as 30% of the neuronal population (Nichols et al., 2009). Richerson et al. (2001) showed in cultured raphé neurons that inhibited neurons have a distinct morphology from activated neurons (see Fig. 1 from the thesis). The mechanism by which neurons are inhibited by hypercapnia has received virtually no study. If in most regions they have a morphology similar to activated neurons then it may indicate that inhibited neurons could transform into activated neurons and vice versa while a more physiological transition would be indicated if they have a morphology similar to nonchemosensitive neurons. Nichols et al. (2009) found that exposure of rats to chronic hypoxia resulted in a form of plasticity that increased the percentage of hypercapnia-inhibited neurons in cNTS neurons but this greater percentage of inhibited neurons arose from a decrease in nonchemosensitive neurons and not activated neurons, suggesting that activated and inhibited neurons are not necessarily easily interchangeable.

The degree to which the chemosensitive response of a neuron is interchangeable addresses the issue of plasticity. It would be of interest to do
experiments that result in a change in the chemosensitive response of neurons (a change in function) and follow those studies up with morphological studies to see if a change in neuronal function is accompanied by a change in neuronal morphology. Kalia et al., suggested that the change in the electrophysiological properties of the bipolar neurons in the neonatal NTS coincided with a change in the neuronal morphology, simple elongated arborization giving way to a more complex, bushy morphology. Rapid, dramatic seasonal morphological changes in the synapses of hibernating animals have been reported. Decrease in complexity, size and number of dendritic branches and synaptic profiles during torpor was reported with rapid regeneration upon arousal. These changes coincide with metabolic depression and, lowered respiratory rates and decreased body temperature (Kunjan et al., 2012).

It is possible that conditions that remodel the physiological responses of chemosensitive areas of the brainstem may also have a coinciding impact on the morphology as well. It is known that chemosensitivity, at least at the whole animal level, is state dependent, changing with sleep/wake states or with anesthesia, but these are rapid and short term changes, lasting only as long as the state is changed and are unlikely to involve neuronal morphological changes. Longer term plasticity could be induced by exposing animals to conditions known to affect ventilatory control, such as exposure to chronic hypoxia, chronic intermittent hypoxia, chronic hypercapnia or chronic hyperoxia, to see if such conditions alter both the physiological response of chemosensitive neurons and their morphology or just their physiology, suggesting that their morphology is
fixed and determined by other aspects of their environment beside being chemosensitive.

In this regard, it would be interesting to look at natural “plasticity” by looking at conditions that naturally may lead to a change in chemosensitivity. One type of example of this would be to study fossorial animals, which due to their burrowing activity are likely to have reduced responsiveness to elevated CO$_2$ (Ramirez et al., 2007). By comparing the morphology of neurons within chemosensitive regions of closely related species, one of which is fossorial, would indicate the degree to which neuronal morphology in given areas is independent of the chemosensitive response of that region. Another way to look for morphological changes associated with “natural” changes in chemosensitivity would be to study morphology in neurons from chemosensitive regions as a function of development. There is considerable evidence that chemosensitivity changes with development (e.g. Studden et al., 2001; Wong-Riley and Lui, 2005) and neuronal chemosensitivity has a different developmental pattern with different brainstem region, increasing with development in raphé neurons (Hodges et al., 2009), decreasing with development in LC neurons (Gargaglioni et al., 2010) and not changing with development in cNTS neurons (Conrad et al., 2009). These physiological studies have not been paralleled by morphological studies, which would prove interesting.

There have been extensive and on-going studies to determine the ion channel(s) that serve as the basis for neuronal chemosensitivity (see Putnam et al., 2004). While these studies are important in and of themselves (for instance
to suggest ion channel targets for drugs that could modify ventilatory control), if we could know which channel(s) are critical for chemosensitivity, we could use immunohistochemistry to localize these channels on the neuronal surface. For instance, based on studies that have suggested an important role for A currents in LC neuron chemosensitivity and other physiological studies that suggest that chemosensing is localized to the LC soma, we would predict that the proteins that make up A currents would be localized to the soma of LC neurons. Conversely, studying the distribution of ion channels within LC neurons, we could predict that channels that are strongly localized to or near the soma may play an important role in chemosensing. Thus, studies of the distribution of various ion channels in chemosensitive neurons from various regions could be combined with morphological and physiological studies of chemosensitivity to yield a rather complete picture of cellular chemosensing. Of course such studies will require physiological studies involving focal acidosis of neuronal soma vs. dendrites, as done in LC by Ritucci et al. (2005), to fully interpret the patterns of distribution of ion channels.

These studies are important for the interpretation of studies where neuronal processes appear to be targeted to the brain surface. It would be especially desirable to repeat the Ritucci et al. (2005) studies in neurons from the cNTS, the raphe and the RTN. If morphological and physiological studies of ion channels suggest a somal origin for chemosensitive signaling, then these processes are unlikely to play a critical role in chemosensing.
Finally, it will be most instructive to study the microvasculature in other chemosensitive areas and the association of individual chemosensitive neurons with the blood vessels. The techniques employed in the current study provide a fairly straightforward means to determine regional vascularity vs. neuronal population in multiple regions. However, in order to observe the more intimate relationships between minute structures of both neurons and blood vessels, electron microscopy studies would provide the necessary detail and resolution to describe functional associations between blood vessels and dendritic processes or the soma. Detailed studies of capillary endothelium and neuronal contacts, glial intervention and vascular specialization are needed in regions of chemosensitivity.
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Ph.D., Biomedical Sciences: Integrative Physiology & Neuroscience, Boonshoft School of Medicine and College of Science & Mathematics, Wright State University, Dayton, Ohio.

Graduate Research:

Neuroscience: Morphological Characterization of chemosensitive neurons in the locus coeruleus and the nucleus tractus solitarius of the rat brainstem, confocal microscopy study and 3D reconstruction of identified chemosensitive neurons, and their association with the vasculature. Kv1,4 channel distribution in the LC.

Cardiovascular and Diabetes: Nutritional study of the effects of dietary high fructose on circulating Angiotensin, renal Angiotensin receptors, circulating leptin, ghrelin and cortisol. Spectral analysis of cardiovascular parameters, analysis of glucose tolerance, activity, weight gain, and modification of diurnal behaviors in mice.

Graduate Certificate: Chemical, Biological, Radiological, and Nuclear Defense, Boonshoft School of Medicine, Department of Pharmacology and Toxicology, Wright State University, Dayton, Ohio 2013

Diplôme National D’Arts Plastiques: National French Ministry of Culture and Communication, Poitiers, France

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Bachelors of Fine Arts: Visual Arts and Education, Graceland University, Lamoni, IA
Certifications:

- **Laser Safety**: 2009-present
- **Radiation Safety**: 2004-present
- **Hazardous Waste**: 2004-present
- **Blood Bourne Pathogens**: 2006-present
- **Biological Safety**: 1998-present
- **Animal Occupational Health and Safety**: 2004-present

Work Experience:

- **Adjunct Faculty Pathophysiology** Blended Online and Live Internet Lecture Course Spring 2014; 100% Online Instruction Summer 2014, Missouri Valley College, Marshall, MO

- **Adjunct Faculty Biology, Anatomy and Physiology, Microbiology** 2012-2014, Clark State Community College, Springfield, OH

- **Research Assistant and Laboratory Management**: 2006-Present, Wright State University, Boonshoft School of Medicine, Dayton, Ohio

- **Mentor/Instructor: Horizons in Medicine and STREAMS programs**, 2006-2008, Wright State University, Boonshoft School of Medicine, Dayton, Ohio


- **Adjunct Faculty Microbiology**, Fall 1997, Wright State University, Dayton, OH

- **Lab Technician/Manager Microbiology**, Summer 1998, Organized laboratory, ordered supplies, prepared cell cultures. Advisor: James Amon, Ph.D.

- **Science/Math Teacher**: 1999-2003, Algebra, Biology, Physical Science, Hillel Academy, Dayton, OH, Samuel Feld, Ph.D., Principal

- **Visual Arts Teacher**: 1988-1993, Studio Arts, Art History and Art Appreciation, St. Henry High School, Erlanger, Ky, Dave Otte, Ph.D., Principal

- **Professional Artist and Muralist**: 1983 to present. Commissioned paintings/murals/faux finishes
Research Training:

Undergraduate Research

**Environmental Science:** Wetlands restoration. Identified and removed invasive alien flora, planted indigenous species. Beavercreek, OH, Advisor: James Amon, Ph.D., Wright State University, Dayton, Ohio

**Molecular Genetics:** Combination of molecular techniques and various computer platforms to analyze DNA sequences. Edited 2nd edition of Fundamental Concepts of Bioinformatics. Advisor: Dan Krane, Ph.D. Wright State University, Dayton, Ohio

**Neuroscience:** Employed immunohistochemistry techniques to study arrangement of motor interneurons in rat central nervous system. Advisor: Francisco Alvarez, Ph.D., Wright State University, Dayton, Ohio

Graduate Research Training and Laboratory Techniques:

*In vivo techniques and surgeries*

**Plethysmography** Measurement of respiratory response to increased CO2 in mice using Columbus Instruments Pegas 4000 MF Gas Mixer, Tidal Volume Meter and Respiromax/Tidal Volume Monitor

**Anesthesiology** Delivered and maintained anesthesia, injected and inhaled

**Radiotelemetry** Implantation surgery: Data Sciences radiotelemetry device measured blood pressure, heart rate and activity during long-term nutrition studies.

**Microinfusion pump** Implanted microinfusion pumps in mice to study the effects of chronic high Angiotensin II levels in mice

**Brain microinjection** Targeted injection of rhodamine for retrograde labeling in rats

**Thoracotomy** Performed thoracotomy on rabbit and dog in preparation for study of Myo-Vad ventricular assist device

**Laminectomy** Exposed spinal column in preparation for motor neuron recording in rats

**Electrophysiology** Whole cell patch clamp technique
Glucose Tolerance Testing Mice

Cheek Blood Collection Mice
Tail-clip technique Mice
Injection I.P., I.M., Sub.Q. in mice

Bench Techniques
  Extensive dissection and tissue collection
  Tissue Micropunch
  Tissue Homogenization
  Tissue Sectioning- Cryostat and Vibratome
  Histological Staining
  In situ Hybridization
  Western Blot Analysis
  Radioimmuneassay
  Mass Spectroscopy
  Immunohistochemistry
  Confocal Microscopy
  Neuronal Reconstruction
  Tissue Culture

DNA/ RNA Isolation of DNA/RNA from phage, bacterial, and mammalian tissue by chemical extraction

Real Time Amplification of DNA products for genotyping (BioRad I-cycler)
Polymerase Chain Reaction

Electrophoresis Agarose gel to determine DNA fragment size

SELDI Mass Spectrometry Ciphergen analytical technique that identifies the protein composition of a compound or sample on the basis of the mass-to-charge ratio of charged particles.

MALDI-TOF Mass Spectrometry

DNA Sequencing Sanger dideoxy method and dye-terminator methods

Spectrophotometry Determining DNA concentration and purity
Computer Skills:

Platforms: Macintosh, P.C. (XP, Vista and Windows 7, Blackboard 6)

BioInformatics: Gen Bank, Medline, and Pubmed

Database search for homologous sequences (BLAST)

Dot Plots (DottyPlot)

Multiple Alignment of related sequences (Clustal W)

Phylogenetic reconstruction (PAUP, Phylip, and McClade)


Data Analysis: Statistica, SigmaPlot, Ky-Plot, Autoregressive Spectral Analysis, Fluoview, NeuroLucida, NeuroLucida Explorer

Web Page: Adobe CS5, Acrobat, Illustrator, MS

Brochure Design: Front page & Publisher, DreamWeaver

Publications:

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Presentations:

“Chemosensitive Neurons of the Locus Coeruleus and the Nucleus Tractus Solitarius: Three Dimensional Morphology and Association with the Vasculature”
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“Nocturnal binge drinking of fructose disrupts diurnal behaviors in mice” Boonshoft School of Medicine Medical Student Research Symposium, Invited Poster Presenter 2009

“The Impact of Fructose Diet on Blood Pressure Angiotensin AT1 System” Wright State University, March 2008

“The Non-traditional Student in Graduate Education”, Platform Presentation, STREAMS, Wright State University, Summer 2008


“Circadian Rhythms in Autonomic Cardiac Function in Mice” Biomedical Sciences PhD Program Student Seminar Series, Wright State University, October 2006

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Membership in Professional Societies:

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