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The Role of Ca\textsuperscript{2+} in Central Respiratory Control Neurons of the Locus Coeruleus: Development of the Chemosensitive Brake

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THE ROLE OF Ca²⁺ IN CENTRAL RESPIRATORY CONTROL NEURONS OF THE LOCUS COERULEUS: DEVELOPMENT OF THE CHEMOSENSITIVE BRAKE

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

ANN NICOLE IMBER
M.S., Florida State University, 2006

2012
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY ANN NICOLE IMBER ENTITLED THE ROLE OF Ca\textsuperscript{2+} IN CENTRAL RESPIRATORY CONTROL NEURONS OF THE LOCUS COERULEUS: DEVELOPMENT OF THE CHEMOSENSITIVE BRAKE BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT


Chemosensitive LC neurons increase their firing rate in response to increased CO$_2$ (hypercapnia) in part via inhibition of K$^+$ channels. This increase gets smaller during the first two postnatal weeks (neonatal rats aged P3-P16). Alterations of this “accelerating” pathway may account for the developmental changes in the magnitude of the chemosensitive response in LC neurons. Alternatively, Ca$^{2+}$ and Ca$^{2+}$ channels may play a role in the response to hypercapnia, but little is known about the role of Ca$^{2+}$ in central chemosensitivity. Whole cell patch clamp and fluorescence imaging microscopy were used to study a different basis for the developmental changes in the chemosensitive response of LC neurons, a “braking” pathway. In the presence of tetrodotoxin (TTX-inhibitor of Na$^+$ channels), currents composed of both a rhythmic cycling (SRO-subthreshold rhythmic oscillation) and voltage-sensitive spikes were observed. These currents developed over the first 10 postnatal days. Since both currents were abolished by the L-type Ca$^{2+}$ channel inhibitor nifedipine, both were assumed to be due to the activity of L-type Ca$^{2+}$ channels. Hypercapnia increased the frequency of oscillations and the accompanying spikes in a HCO$_3^-$-dependent but pH-independent fashion. Voltage clamp studies supported the presence of L-type Ca$^{2+}$ currents in LC neurons that increased in postnatal rats aged P3 to P12 and were enhanced by hypercapnia, resulting in increased intracellular Ca$^{2+}$ (Ca$^{2+}_i$). Hypercapnia activated Ca$^{2+}$ channels in LC neurons via a HCO$_3^-$-dependent pathway involving soluble adenylate cyclase-cAMP-channel.
phosphorylation. The hypercapnic increase in \( \text{Ca}^{2+} \) activated hyperpolarizing \( \text{Ca}^{2+} \) activated \( K^+ \) currents (\( K_{Ca} \)). The BK (a large conductance \( K_{Ca} \)) channel inhibitor paxilline and voltage clamp were used to study this \( K_{Ca} \) current. Inhibition of the BK current removed the “brake” and increased the firing rate response to hypercapnia in LC neurons. Notably, the “braking” pathway increased during neonatal development in a fashion that paralleled the decrease in the chemosensitive firing rate response to hypercapnia. These findings suggest a unique role for \( \text{Ca}^{2+} \) in controlling the magnitude of the firing rate response to hypercapnia. Abnormalities in this pathway could be associated with disorders involving elevated central sensitivity to changes in CO\(_2\), such as sleep apnea and panic disorders.
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DEDICATION

This work is dedicated to my father, Dr. Peter C. Imber, with fond memories of your insatiable curiosity and ready kindness. Thank you for trying to answer all of my questions.
CHAPTER I

INTRODUCTION
Neurons in the central nervous system that respond to CO$_2$/H$^+$, termed central chemoreception, are thought to contribute significantly to the chemical drive to breathe (Coates et al., 1993; Nattie & Li, 2009). In addition to maintenance of normal breathing rhythm, areas within the medulla and pons contain neurons that increase their firing rate in response to elevations in CO$_2$ even in the absence of synaptic activity (Chapman et al., 1988; Putnam, 2010). Much is known about the individual CO$_2$-sensitive neurons from these areas with respects to respiratory chemosensitivity, including detailed studies of their cellular biology and electrophysiology (Coates et al., 1993; Dean & Nattie, 2010; Feldman et al., 2003; Nattie & Li, 2009; St-John, 1998). However, less is known about how neurons from each area contribute to the whole animal respiratory response.

Possible mechanisms for the depolarization of chemosensitive neurons in response to changes in CO$_2$ have mostly focused on the H$^+$-sensitive modulation of ‘accelerators’, because they describe how CO$_2$/H$^+$ may work to increase firing rate in chemosensitive neurons. Thus, a possible explanation for the control of the response of CO$_2$/H$^+$ sensitive neurons to hypercapnic acidosis could be the presence of varying numbers and types of H$^+$-sensitive K$^+$ channels. However, inhibitors for these channels fail to completely eliminate the chemosensitive response in neurons from the LC (Martino & Putnam, 2007). Thus, it is likely that several chemosensitive channel types work to modify the response of LC neurons to hypercapnia (Li & Putnam, 2009; Putnam, 2010).

There is not much known about the role of Ca$^{2+}$ channels in central chemosensitivity. One possibility is that the activation of an additional cation current adds to the chemosensitive ‘accelerator’ in LC neurons. However decreasing intracellular
Ca$^{2+}$ ($Ca_{i}^{2+}$) in LC neurons increases the spontaneous firing rate, possibly through the actions of Ca$^{2+}$-activated K$^{+}$ channels ($K_{Ca}$) (Aghajanian et al., 1983). This suggests that Ca$^{2+}$ channels and $K_{Ca}$ channels work in concert to decrease the firing rate response to CO$_2$ in LC neurons, possibly indicating the presence of a chemosensitive ‘brake’. In this thesis, I will be studying the role of Ca$^{2+}$ in chemosensitive neurons of the LC.

Particularly, I will focus on the mechanism of activation of Ca$^{2+}$ channels by CO$_2$ and the effect of this activation 1) on $K_{Ca}$ channels and 2) on the magnitude of the chemosensitive response of LC neurons during the development of young neonatal rats. Neurons of the LC are suited for this study both because of the developmental change in their sensitivity to hypercapnia and because of the extensive implications of their response to CO$_2$. LC neurons have been studied in fear and anxiety behavior (M. W. Coryell et al., 2008; Martinez et al., 2001; Nardi et al., 2009; Papp et al., 1993; Sullivan et al., 2003), sleep/wake cycles (Hagan et al., 1999), and attention and stress (Curtis et al., 1997; Reyes et al., 2006; Sullivan et al., 2003; Van Bockstaele et al., 1996; Van Bockstaele et al., 2001; Walling et al., 2004; Williams et al., 1984) in addition to ventilatory control (Coates et al., 1993; Gargaglioni et al., 2010). Abnormalities in pathways for regulating the response to CO$_2$ may underlie pathologies such as sleep apnea and panic disorder (Lousberg et al., 1988; Ryan & Bradley, 2005; Stein et al., 1995; Valentino & Van Bockstaele, 2008; Verbraecken et al., 1995; D. Wang et al., 2007).
CHAPTER II

LITERATURE REVIEW
Control of Ventilation

The rat LC is a group of noradrenergic neurons near the ventrolateral surface of the fourth ventricle that sends out projections to many regions of the CNS (Williams et al., 1984). Collectively, these cells account for a significant amount of the brain’s content of noradrenaline (Gaspar et al., 1989; Swanson & Hartman, 1975). Thus, the physiology of these neurons has important implications for the control and development of many autonomic functions, including respiratory control (Biancardi et al., 2008; Patrone et al., 2012) and the control of fear (Nardi et al., 2009), stress (C. W. Berridge & Waterhouse, 2003; Jedema & Grace, 2004), and attention (Hagan et al., 1999; Van Bockstaele et al., 2001). The vast majority of these neurons have been found to be chemosensitive (Filosa et al., 2002; Oyamada et al., 1998; Pineda & Aghajanian, 1997), increasing their firing rate in response to hypercapnia (elevated CO$_2$).

Studies on central chemoreception support a complex, integrated network for the detection of CO$_2$/H$^+$ and the stimulation of breathing (Coates et al., 1993; Huang et al., 1997; Nattie & Li, 2009; Putnam et al., 2004) (Fig 1A). Neurons involved in sensing increased CO$_2$ (hypercapnia) share the common trait of a maintained intracellular acidification and altered firing rate in response to elevated CO$_2$/H$^+$. This trait, when present irrespective of synaptic activity, is termed intrinsic chemosensitivity (Conrad et al., 2009; Filosa & Putnam, 2003; Leiter, 2009; Nichols et al., 2008; Ritucci et al., 2005a). In anesthetized whole animals, chemosensitive areas involved in respiratory control have been identified by measuring increased phrenic nerve output in response to focal acidification of various brainstem regions (Coates et al., 1993). Lesioning of these areas result in a significant loss of the ventilatory response to inspired CO$_2$ in the whole
Figure 1:  A. A model of the integration of the hypercapnic response of multiple areas in the medulla and pons. This model emphasizes the magnitude of the firing rate response to CO$_2$ from individual neurons within each area, represented as a chemosensitivity index (%). The connections between the individual chemosensitive neurons from each area are currently unknown. LC: locus coeruleus, NTS: nucleus of the solitary tract, RTN: retrotrapezoid nucleus. B. Sagittal view of the rat brainstem indicating the locations of multiple areas thought to be involved in central chemosensitive respiratory control, including the LC, locus coeruleus; NTS, nucleus of the solitary tract; cVRG, caudal respiratory group; rVRG, rostral ventral respiratory group; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; VII, facial nucleus; Amb, nucleus ambiguous; BC, Bötzinger complex; CVLM, caudal ventrolateral medulla; DVMN, dorsal vagal motor nucleus; K-F, Kölliker-Fuse nucleus; LRt, lateral reticular nucleus; Mo5, motor trigeminal nucleus; PB, parabrachial nucleus; preBötC, pre-Bötzinger complex; RVLM, rostral ventrolateral medulla.
Figure 1

A.

B.

animal, further supporting the hypothesis that multiple areas within the brainstem contribute to respiratory control (Biancardi et al., 2008; Dias et al., 2007; Nattie & Li, 2009). Areas of the brainstem identified by the above methods contain intrinsically chemosensitive neurons and include the retrotrapezoid nucleus (RTN) (Coates et al., 1993; Mulkey et al., 2004), serotonergic neurons within the medullary raphe (Dias et al., 2007; Richerson, 1995; W. Wang et al., 1998), neurons of the solitary complex including the nucleus tractus solitarius (NTS) (Coates et al., 1993; Conrad et al., 2009; N. L. Nichols et al., 2008; N. L. Nichols et al., 2009), and the noradrenergic neurons of the locus coeruleus (LC) (Fig 1B) (Biancardi et al., 2008; Coates et al., 1993; Filosa & Putnam, 2003). Significantly, chemosensitive neurons in each of these areas have a different magnitude of response to hypercapnia as determined by calculations of the chemosensitivity index (CI) (100 x 10^{\log [FR_{15} - \log FR_{5}] / (pH_{5} - pH_{15})^{0.2}}) (Wang & Richerson, 1999) (Fig 1A). By this scale, neurons of the RTN have the highest chemosensitive response with a value of around 300% (Mulkey et al., 2004; Ritucci et al., 2005a), NTS have a more moderate response with an average value of ~180% (Conrad et al., 2009; Nichols et al., 2009), and neurons of the LC have the lowest response with a value of ~125% (Gargagliani et al., 2010; Nichols et al., 2008; Putnam, 2010). In some chemosensitive areas these values were found to change with development. Raphe neurons have very little response in the young neonate, but some two weeks after birth, the chemosensitive response of these neurons becomes significantly larger (Hodges et al., 2009). Conversely, neurons of the LC have the smallest average CI of the identified chemosensitive regions in the adult animal. However when LC neurons from neonate rats younger than P10 were studied, the average CI was found to be around 240%
This suggests a possible developmental shift in respiratory control during development, and may emphasize the importance of the chemosensitive response of individual neurons in the medulla and pons to respiratory control.

The control of breathing is a complex topic, due in part to the multiple brainstem areas capable of responding to \( \text{CO}_2/\text{H}^+ \) and thereby altering respiration (Feldman et al., 2003; St-John, 1998; St-John & Leiter, 2008). For example, the maintenance of normal breathing (eupnea) was noted to rely on activity from both the pons and medulla, further emphasizing a highly integrated system for breathing control (Li & Nattie, 2006; St-John, 1998). With respect to respiratory chemosensitivity, much is known about individual \( \text{CO}_2 \)-sensitive neurons from a variety of areas in the medulla and pons, including detailed studies on their cellular biology and electrophysiology. However, less is known about how neurons from each area contribute to the whole animal chemosensitive respiratory response. It has been suggested that the degree of neuron chemosensitivity (i.e. the magnitude of the firing rate response for a given \( \text{CO}_2/\text{H}^+ \) change) is an important factor in the relative contribution of these various areas to the overall response of the animal to \( \text{CO}_2 \) (Putnam et al., 2004). For example, chemosensitive neurons of the RTN have the highest increase in firing rate per 0.1 pH unit decrease in external pH (pH_e), and hence have been suggested as the key chemosensitive neurons in the brainstem for respiratory control (Mulkey et al., 2004). This may indicate that the cellular mechanics behind the chemosensitivity of individual neurons is of singular importance to respiratory physiology.

There is not yet a full understanding of the cellular signaling mechanisms involved in the response of neurons to increased \( \text{CO}_2/\text{H}^+ \). Possible cellular mechanisms
for neuronal chemosensitivity include changes in both intra- (pH$_i$) and extra- (pH$_o$) cellular pH that could affect channels via charges on titratable groups (Filosa et al., 2002; Hartzler et al., 2008; Ritucci et al., 1997; Ritucci et al., 1997; Ritucci et al., 1998) (Fig 2) and CO$_2$/H$^+$-sensitive cytoplasmic modulatory agents capable of influencing channel activity (Putnam, 2010; Summers et al., 2002). To date, studies have focused primarily on H$^+$-sensitive K$^+$ channels inhibited by decreases in pH, such as TWIK-related acid-sensitive K$^+$ (TASK) channels (Bayliss et al., 2001; Washburn et al., 2002), inward rectifier potassium channels studied in LC neurons (Pineda & Aghajanian, 1997), and A-type and delayed-rectifier K$^+$ channels (Denton et al., 2001; Denton et al., 2007; Li & Putnam, 2009). These K$^+$ channels are inhibited by increases in CO$_2$/H$^+$, resulting in a depolarization of neurons and an increased firing rate (Pineda & Aghajanian, 1997; Putnam, 2010; Wenker et al., 2010) (Fig 2). Studies have also demonstrated the involvement of non-selective cation channels such as TRP channels (Cui et al., 2011) and pH regulating transporters such as Na$^+$-H$^+$ exchangers (NHE) (Kersh et al., 2009). TRP channels are activated by a decrease in pH to depolarize and increase the firing rate of LC neurons (Cui et al., 2011), while pH-regulating transporters are inhibited by hypercapnia to maintain an intracellular acidification (Fig 2). These studies emphasize the potential importance of pH to chemosensitive signaling (Kersh et al., 2009; Putnam et al., 2004). However, it is still unknown how pH-sensitive channels relate to the magnitude of the chemosensitive response.

Figure 3 shows the relationship of changes in firing rate in LC neurons to various changes in intracellular pH (pH$_i$). Although a correlation exists between decreases in pH$_i$
**Figure 2:** A model of current studies on the response of individual neurons to hypercapnia. CO$_2$ diffuses readily across the cell membrane, hydrates and disassociates into HCO$_3^-$ and a proton to decrease pH$_i$ and inhibit K$^+$ channels. Decreases in pH$_o$ also cause an inhibition of the Na$^+$-H$^+$ exchanger (NHE) to maintain the acidification of pH$_i$. This causes the neuron to depolarize and increases firing rate. This can be thought of as an “accelerator” pathway because it describes how neurons increase their firing rate in response to hypercapnia.
Figure 2.
and increases in firing rate in LC neurons, it was unable to completely account for the magnitude of the response under various levels of CO$_2$/HCO$_3^-$/H$^+$. For example, isohydric hypercapnic solution (no change in extracellular pH ($p$H$_e$), a small, transient decrease in $p$H$_i$, increased CO$_2$/HCO$_3^-$) had a similar increase in firing rate compared to hypercapnic acidosis (decreases in both $p$H$_e$ and $p$H$_i$, increased CO$_2$/HCO$_3^-$) (Ritucci et al., 1997; Filosa et al., 2002). Decreasing only $p$H$_i$ with propionate resulted in no change in firing rate, while acidified HEPES solution (decreased $p$H$_e$ and $p$H$_i$, nominal absence of CO$_2$/HCO$_3^-$) resulted in a significantly higher increase in firing rate than either hypercapnic solution or isohydric hypercapnic solution (Fig 3). This suggests that pH is not the only signaling mechanism involved in determining the magnitude of the chemosensitive response in LC neurons.

While the magnitude of the chemosensitive response of NTS neurons from both neonates and adults appear the same (Conrad et al., 2009; Nichols et al.; 2009), the chemosensitive response of raphe and LC neurons were appear to change with development. Raphe neurons have very little response in the young neonate, but some two weeks after birth, the chemosensitive gain of these neurons becomes significantly larger (Hodges et al., 2009). Conversely, neurons of the LC from older neonates have the smallest average CI of the identified chemosensitive regions. However when LC neurons from neonate rats younger than P10 were studied, the average CI was found to be around 240\% (Gargaglioni et al., 2010). This suggests a possible developmental shift in respiratory control during development.

A possible explanation for the change in the chemosensitive response of CO$_2$/H$^+$ sensitive neurons could be the presence of varying amounts of H$^+$-sensitive K$^+$ channels.
Figure 3: A comparison of the magnitude of the change in pH\textsubscript{i} to acid challenges versus the resulting change in the firing rate of LC neurons from neonatal rats aged P1 to P9. The labels are: HA, hypercapnic acidosis (decreased pH\textsubscript{i}/pH\textsubscript{o} & increased CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}); IH, isohydric hypercapnia (decreased pH\textsubscript{i} only & increased CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}); AcHepes, acidified HEPES (decreased pH\textsubscript{i}/pH\textsubscript{o} only); and IA, isocapnic acidosis (decreased pH\textsubscript{i}/pH\textsubscript{o} & decreased HCO\textsubscript{3}\textsuperscript{-}).
Figure 3.

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However, closer study of both chemosensitive NTS neurons and LC neurons suggest that this is not the case. By applying inhibitors of K$^+$ channels to neurons from each area, it was found that 4-AP was sufficient to completely eliminate the chemosensitive response of NTS neurons (Martino & Putnam, 2007). However, the addition of either 4AP or TEA (tetraethylammonium) fails to completely eliminate the chemosensitive response in neurons from the LC. These data further support the suggestion that multiple chemosensitive channels and possibly other regulatory mechanisms influence the chemosensitive response in LC neurons (Li & Putnam, 2009; Putnam, 2010).

Studies of Ca$^{2+}$ in central chemosensitive neurons have not been extensive. This is surprising given multiple studies that indicate the importance of Ca$^{2+}$ to the chemosensitive response of neurons from the snail *Helix aspersa* and in mammalian central chemosensors from the LC and ventral medulla and peripherally located carotid body (Denton et al., 2007; Erlichman & Leiter, 1997a; Erlichman & Leiter, 1997b; Filosa & Putnam, 2003; Imber & Putnam, 2012; Summers et al., 2002; Wellner-Kienitz et al., 1998). A question remains as to the role of Ca$^{2+}$ in central chemosensitivity and whether Ca$^{2+}$ works to increase or decrease the firing rate response to hypercapnia in LC neurons.

**LC Chemosensitivity and Ca$^{2+}$**

Two main types of voltage-activated Ca$^{2+}$ channels described in vertebrates are: 1) slow, persistent high-voltage activated channels (HVA) designated L-type; and 2) fast, rapidly inactivating low-voltage activated channels (LVA) labeled T-type (Hille, 2001). L-type HVA channels (Ca, 1.1-1.4) have a large single-channel conductance (25 pA) and have been shown to activate when depolarized to approximately -30 to -40 mV (Hille, 2001; Lipscombe et al., 2004). Once open, these channels inactivate slowly, with a $\tau$
>500 ms (Hille, 2001). In contrast, T-type LVA channels (CaV 3.1-3.3) open when depolarized to -70 mV and then inactivate rapidly (τ >50 ms) (Hille, 2001). L-type and T-type channels can be differentiated using voltage clamp electrophysiology by their characteristic voltage activation profiles and inactivation rates. They can also be differentiated by the high sensitivity of L-type channels, but not T-type, to dihydropyridines (such as nifedipine) (Hille, 2001). Further types of voltage-activated Ca\(^{2+}\) channel include P/Q, N, and R channels (CaV 2.1-2.3). These HVA channels rapidly inactivate and can be distinguished by their sensitivity to pharmacological blockade using ω-CTX (conotoxin) GVIA (Hille, 2001). In LC neurons, the Ca\(^{2+}\) current that is affected by changes in \(\text{CO}_2/\text{H}^+\) was sensitive to dihydropyridines, identifying the channels most likely to be involved in the chemosensitive response as L-type Ca\(^{2+}\) channels (Filosa & Putnam, 2003).

The role that L-type Ca\(^{2+}\) currents play in the response of LC neurons to hypercapnia is currently not known. The inward flux of positively charged Ca\(^{2+}\) ions due to the activation of L-type Ca\(^{2+}\) channels by hypercapnia could result in neuronal depolarization, increasing the firing rate in response to hypercapnia. Accordingly, there should be an increase in hypercapnic firing rate when the TTX-insensitive current is active and a reduced firing rate response when this current is blocked. Alternatively, the activation of Ca\(^{2+}\) channels could be increasing intracellular Ca\(^{2+}\), which could thereby activate Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{\text{Ca}}\)). This would result in neuronal hyperpolarization and blunting of the CO\(_2\)-increase in firing rate. To date, a hypercapnia-induced elevation of intracellular Ca\(^{2+}\) has not been shown for LC neurons. However, the presence of K\(_{\text{Ca}}\) channels on LC neurons has been demonstrated. Immunohistochemical
analysis of the mouse brain for BK (“big-K”, large-conductance $K_{Ca}$ channels) channels showed that the LC had the largest antibody staining in the pons (Sausbier et al., 2006). This leads to our hypothesis that $Ca^{2+}$-activated $K^+$ channels are present on the membrane of LC neurons and are activated by increased $CO_2/H^+$, resulting in a hyperpolarizing effect that could limit the firing rate response of LC neurons to hypercapnia.

Prior studies suggest that $K_{Ca}$ channels are playing a role in the electrophysiology of LC neurons by causing hyperpolarizations following either bursts of action potentials or the application of $Ca^{2+}$ channel agonists that raise $Ca^{2+}$ levels (Murai et al., 1997; Osmanovic & Shefner, 1993). In the latter case, these agonists activated outward currents that were specifically sensitive to BK channel inhibition (Murai et al., 1997). Combined with evidence for the development of a chemosensitive $Ca^{2+}$ current in LC neurons (Imber & Putnam, 2012), this suggests a role for $Ca^{2+}$ as a chemosensitive brake that may be substantially altering the magnitude of the chemosensitive response of LC neurons during postnatal development. Here, I assess $Ca^{2+}$ currents in LC neurons using whole cell voltage clamp studies during ages P3 to P16. Further, I examine the effects of hypercapnia on intracellular $Ca^{2+}$ levels in neonatal LC neurons, the effect of $Ca^{2+}$ currents on BK currents, and the effect of BK channel inhibition on the chemosensitive response.

**How are $Ca^{2+}$ channels affected by $CO_2/ H^+$?**

There has been ample evidence for the presence of $Ca^{2+}$ channels in chemosensitive neurons. Electrophysiology studies in both caudal (cNTS) and medial (mNTS) NTS neurons have identified high-voltage activated L, N, and P/Q and low-voltage activated T-type $Ca^{2+}$ channel types (Hille, 2001; Kunze, 1987; Rhim & Miller,
1994). The accumulation of intracellular Ca\(^{2+}\) (Ca\(^{2+}\)_i) during depolarization and the activity of calcium-activated K\(^+\) channels (K\(_{Ca}\)) also suggest that Ca\(^{2+}\) plays an integral part of NTS neuron physiology (Bissonnette, 2002; Kunze, 1987; Rhim & Miller, 1994).

In cultured medullary neurons, the addition of Ca\(^{2+}\) channel inhibitors Cd\(^{2+}\) or Ni\(^{2+}\) resulted in a loss of chemosensitivity (Wellner-Kienitz et al., 1998). In LC neurons, multiple studies have described the contribution of a dihydropyridine-sensitive Ca\(^{2+}\) current to the chemosensitive response (Filosa & Putnam, 2003; Imber et al., 2011; Imber & Putnam, 2012). However, less evidence is available for the mechanisms by which changes in CO\(_2\)/H\(^+\) modifies Ca\(^{2+}\) currents.

In the snails *Helix aspersa* and *Helix pomatia*, a discrete chemosensitive area was discovered capable of stimulating gas exchange when focally acidified by CO\(_2\) (Erlichman & Leiter, 1997b). Significantly, removal of extracellular Ca\(^{2+}\) inhibited the spontaneous generation of action potentials and the subsequent increase in firing rate to hypercapnia in neurons from this area (Erlichman & Leiter, 1997a). The chemosensitive response of these neurons could be restored by substitution of the Ca\(^{2+}\) with Ba\(^{2+}\), suggesting that the influx of depolarizing Ca\(^{2+}\) current was responsible for the increase in firing rate to CO\(_2\) (Erlichman & Leiter, 1997a; Erlichman & Leiter, 1997b). Based on these initial data, it seemed possible that the chemosensitive response of these neurons might be solely based on Ca\(^{2+}\) currents, similar to the H\(^+\)-sensitive activation of Ca\(^{2+}\) currents suggested in rat sensory neurons (Erlichman & Leiter, 1997a; Kovalchuk et al., 1990). Later studies on *Helix aspersa* chemosensitive neurons showed that H\(^+\)-sensitive K\(^+\) currents also contributed to the increase in firing rate during hypercapnia (Denton et al., 2001; Denton et al., 2007). Thus, a second emerging pathway for the chemosensitive
activation of Ca\(^{2+}\) currents by CO\(_2\)/H\(^+\) is the voltage-sensitive activation of Ca\(^{2+}\) channels following an initial depolarization by H\(^+\)-sensitive K\(^+\) channels.

There is yet another mechanism by which Ca\(^{2+}\) can alter the chemosensitive response. Accumulation of intracellular Ca\(^{2+}\) can activate Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{Ca}\)) in neurons from mammalian chemosensitive areas (Wellner-Kienitz et al., 1998). This has been shown in cultures of medullary chemoreceptive neurons, both Ba\(^{2+}\) (no change in Ca\(^{2+}\)) and Ca\(^{2+}\) channel blockade (no depolarizing current or change in Ca\(^{2+}\)) resulted in the complete loss of chemosensitivity (Wellner-Kienitz et al., 1998). It was concluded that a K\(_{Ca}\) channel must undergo H\(^+\)-sensitive inhibition to decrease outward current, depolarize the neuron and increase firing rate in response to hypercapnia (Wellner-Kienitz et al., 1998). This theory suggests a mechanism whereby a CO\(_2\)/H\(^+\)-sensitive K\(_{Ca}\) channel contributes significantly to a chemosensitive increase in firing rate (Wellner-Kienitz et al., 1998). Another hypothesis is that H\(^+\)-sensitive inhibition of Ca\(^{2+}\) channels and not K\(_{Ca}\) channels was responsible for the loss of medullary neuronal chemosensitivity. Such inhibition of Ca\(^{2+}\) channels would reduce Ca\(^{2+}\) and therefore K\(_{Ca}\) channel activity. Here, a small loss of depolarizing Ca\(^{2+}\) channel activity is opposed by an even greater loss of hyperpolarizing K\(_{Ca}\) conductance. This may be more likely, as acidification is commonly expected to inhibit Ca\(^{2+}\) channels (Shah et al., 2001; Tombaugh & Somjen, 1997). Ba\(^{2+}\) is also known to inhibit other types of K\(^+\) channel, including the H\(^+\)-sensitive K\(_{ir}\) 4.1 and 5.1 (Xu et al., 2000). Thus, in cultured medullary neurons, chemosensitivity could be due to a combination of the H\(^+\)-sensitive inhibition of both K\(^+\) channels and Ca\(^{2+}\) channels, the latter of which results in a loss of K\(_{Ca}\) activity.
Studies in peripheral chemoreceptors in the carotid body indicate that CO$_2$/H$^+$ may work to activate rather than inhibit Ca$^{2+}$ channels (Summers et al., 2002). An inhibitor of protein kinase A (PKA) blocked this activation, and increased CO$_2$/H$^+$ resulted in elevated cAMP in carotid body cells (Summers et al., 2002). This led to a model of CO$_2$/H$^+$ activation of L-type Ca$^{2+}$ channels: elevation in CO$_2$ increases intracellular HCO$_3^-$, which activates soluble adenylate cyclase (sAC), increasing cAMP and causing an activation of PKA, resulting in phosphorylation and activation of L-type Ca$^{2+}$ channels. Thus, in glomus cells the chemosensitive activation of Ca$^{2+}$ channels occurs via phosphorylation that results in an increase in current amplitude (Summers et al., 2002). However, it is currently unknown if such a mechanism exists in central chemosensitive neurons. Voltage clamp in LC neurons also showed an increase in the amplitude and voltage-sensitivity of the Ca$^{2+}$ current during hypercapnia (Imber et al., 2011), while additional work suggests that the chemosensitive activation of Ca$^{2+}$ channels in LC neurons is dependent upon intracellular HCO$_3^-$ concentrations and not pH (Imber & Putnam, 2012). Of further significance is that both cGMP and cAMP were shown to influence high-voltage activated Ca$^{2+}$ currents in Helix pomatia neurons from the same areas thought to be involved in respiratory control (Vorontsov, 2002; Zsombok et al., 2005). Collectively, these data suggest that CO$_2$-sensitive phosphorylation of Ca$^{2+}$ channels may be a common mechanism in chemosensitive neurons.

Given a mixture of data for the effects of both pH and HCO$_3^-$ on Ca$^{2+}$ channels, more work needs to be done to establish the mechanisms by which hypercapnia alters Ca$^{2+}$ currents in neurons involved in respiratory control. It is also unclear whether acidification predominantly inhibits Ca$^{2+}$ channels as reported in neurons from non-
chemosensitive areas (Shah et al., 2001; Tombaugh & Somjen, 1997), or whether the hypercapnic depolarization and/or channel phosphorylation works to activate Ca\(^{2+}\) channels in response to hypercapnia. It may be that neurons from different chemosensitive regions have unique pathways with respect to Ca\(^{2+}\) channel activation and chemosensitivity.

**What are the effects of opening Ca\(^{2+}\) channels?**

Once Ca\(^{2+}\) channels are opened, either due to an acid-sensitive K\(^{+}\) channel-induced membrane depolarization or due to changes to the Ca\(^{2+}\) channel itself (e.g. phosphorylation), many effects are possible (Berridge et al., 2000). As discussed previously in *Helix aspersa* chemosensitive neurons, the influx of positively charged ions could enhance the depolarization of a neuron to hypercapnia. In neurons of the LC, inhibition of L-type Ca\(^{2+}\) channels reduced the increase in firing rate in response to hypercapnia, again suggesting that the activity of L-type channels increases the chemosensitive response (Filosa & Putnam, 2003). The depolarizing effect of increased Ca\(^{2+}\) influx is a mechanism attractive for its simplicity; however, often the increased activation of Ca\(^{2+}\) channels results in increased intracellular Ca\(^{2+}\) (Ca\(^{2+}\)\(_{i}\)) levels as well. For example, Ca\(^{2+}\) imaging studies in LC neurons have shown an increase in somal Ca\(^{2+}\)\(_{i}\) levels both during activation of an L-type Ca\(^{2+}\) current and during exposure to hypercapnia (Imber & Putnam, 2010; Imber & Putnam, 2012). Rapid (~5 sec) depolarizations of caudal NTS neurons using high extracellular K\(^{+}\) show transient ~500 nM increases in Ca\(^{2+}\)\(_{i}\) levels (Rhim & Miller, 1994), and RTN neurons show increased Ca\(^{2+}\)\(_{i}\) with acidification (Gourine et al., 2010). Changes in Ca\(^{2+}\)\(_{i}\) levels could mean the activation of Ca\(^{2+}\)-sensitive intracellular pathways, including second messenger signaling.
such as calmodulin binding (Biber et al., 1984; Means, 2000; Miura et al., 1998; Shimokawa et al., 1998; Shimokawa et al., 2005), Ca$^{2+}$-induced exocytosis as described in peripheral chemoreceptors (Summers et al., 2002), Ca$^{2+}$-induced Ca$^{2+}$ release (Osmanovic & Shefner, 1993), and activation of K$_{Ca}$ channels (Berkefeld et al., 2006; Fakler & Adelman, 2008; Imber et al., 2012; Marcantoni et al., 2010).

Second messenger studies involving Ca$^{2+}$ have been extensive. In CO$_2$/H$^+$ signaling, most work has centered on the ubiquitous cytoplasmic Ca$^{2+}$ binding protein calmodulin in H$^+$-sensitive cultured pheochromocytoma cells (PC12), which express catecholaminergic traits similar to some central chemosensitive neurons (Means, 2000; Shimokawa et al., 2005). Once bound to Ca$^{2+}$, calmodulin binds to target proteins with a high affinity that is readily reversible upon a decline in Ca$^{2+}$, suited to rapid, transient changes in Ca$^{2+}$ (Means, 2000). Ca$^{2+}$/calmodulin targets include protein kinases and phosphatases, adenylyl cyclases and ion channel proteins, potentially affecting a wide range of cellular signaling (Means 2000). In PC12 cells, one Ca$^{2+}$/calmodulin target is c-Jun NH$_2$-terminal kinase (JNK) (Kuo et al., 1998; Shimokawa et al., 2004).

Phosphorylated JNK was shown to translocate to the nucleus and increase the activity of the transcription factor c-Jun (Shimokawa et al., 2004). When extracellular H$^+$ increased, JNK phosphorylation and c-Jun expression also increased in a dose-dependent manner (Shimokawa et al., 2004). Significantly, this increase could be inhibited by the L-type Ca$^{2+}$ channel inhibitor nimodipine (Shimokawa et al., 2004). Trifluoperazine, a Ca$^{2+}$/calmodulin inhibitor, also inhibited the extracellular H$^+$-induced expression of c-Jun in PC12 cells (Shimokawa et al., 1998). Thus, a possible downstream mechanism for the
hypercapnic activation of Ca\(^{2+}\) channels in chemosensitive neurons is calmodulin signaling and the regulation of transcriptional factors like c-Jun (Shimokawa et al., 2005).

In chemoreceptive areas of the brainstem, the LC and NTS were both reported to contain calmodulin protein far in excess of that needed to produce a biochemical response (Biber et al., 1984). During in vivo studies of the ventral medulla near the region of the RTN and raphe, significant immunohistochemical staining for the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin was found in individual neurons following exposure of the whole animal to 7\% CO\(_2\) (Miura et al., 1998). When this same ventral medullary area was exposed to the intracellular Ca\(^{2+}\) chelator BAPTA-AM in the anesthetized rat, a reduction in both the hypercapnic tidal volume and frequency was observed (Kanazawa et al., 1998). Although far from conclusive regarding possible Ca\(^{2+}\) signaling mechanisms for in vivo central chemosensitive neurons, significant indications do exist for the involvement of Ca\(^{2+}\) second messengers in chemosensitive control.

The mechanism of chemosensitive Ca\(^{2+}\) excitation and ATP release in VLM astrocytes is currently being investigated and depicts several possible pathways for Ca\(^{2+}\) as a second messenger in chemosensitive signaling. It has been understood that astrocytes are electrically non-excitable but display “Ca\(^{2+}\) excitability”, whereby a change of intracellular Ca\(^{2+}\) can result in paracrine signaling to adjacent neurons (Gourine et al., 2010; Verkhratsky et al., 2011). Recent studies of astrocytes in chemoreception have determined that astrocytes can influence chemosensitive neurons in the ventral medulla via the release of ATP into the extracellular matrix (Gourine et al., 2003; Gourine et al., 2005; Wenker et al., 2010). However, there has been discussion whether this is by a Ca\(^{2+}\)-
dependent or Ca$^{2+}$-independent mechanism (Gourine et al., 2010; Huckstepp et al., 2010; Mulkey & Wenker, 2011; Wenker et al., 2010). In 2010, two studies were conducted on the ATP release from VLM astrocytes. In the first, 0 mM Ca$^{2+}$ plus EGTA in the extracellular solution did not inhibit the release of ATP from VLM astrocytes exposed to hypercapnia, while exposure to gap junction blockade via 100 μM carbenoxolone inhibited the release of ATP by > 60% (Huckstepp et al., 2010). In the second, exposure to 0 mM Ca$^{2+}$ solution completely abolished the increase in Ca$^{2+}$ in response to an extracellular acidification, while 100 μM carbenoxolone inhibited the same response by some ~40% (Gourine et al., 2010). In the first mechanism, chemosensitive connexin hemichannels were proposed to underlie the ATP release form astrocytes and subsequent signaling to chemosensitive RTN neurons, and, in support of this interpretation, the application of hemichannel blockers in vivo reduced the adaptive respiratory response to CO$_2$ (Huckstepp et al., 2010). Although the second mechanism also suggests the importance of gap junctions to the H$^+$-sensitive increase in Ca$^{2+}$ in VLM astrocytes, a discrepancy remains on the importance of Ca$^{2+}$ to the astrocytic release of ATP in response to increases in CO$_2$/H$^+$.  

One possible explanation for the current studies of chemosensitivity and Ca$^{2+}$ signaling in VLM astrocytes is that there are two separate but related pathways. The first pathway is based on extracellular pH and relies of the influx of extracellular Ca$^{2+}$ to initiate Ca$^{2+}$-induced exocytosis of ATP from astrocytes. Consistent with this mechanism are data from Wenker et al. (2010) demonstrating an H$^+$-sensitive K$_{ir}$-like current and electrogenic Na$^+$/HCO$_3^-$ cotransporter in VLM astrocytes, suggesting that these astrocytes are excitable by pH and variations in HCO$_3^-$. Data from Gourine et al. (2010) showed the
Ca\textsuperscript{2+} excitability of VLM astrocytes was abolished by the inhibition of vesicular trafficking and significantly inhibited by blockade of both ATP receptors and gap junctions/hemichannels. Here, pH-sensitive astrocytes spread Ca\textsuperscript{2+} excitation to adjacent pH-insensitive astrocytes and RTN neurons via the Ca\textsuperscript{2+}-dependent release of ATP vesicles and gap junctions (Gourine et al., 2010; Wenker et al., 2010). An influx of extracellular Ca\textsuperscript{2+} through functional canonical type transient receptor potential (TRPC) channels seem likely, as these channels have been previously identified in astrocytes, are permeable to Ca\textsuperscript{2+}, and are associated with activation via ATP receptors (Alvarez et al., 2008; Mamenko et al., 2011; Turvey et al., 2010; Verkhratsky et al., 2011; Xie et al., 2005). However, in the case of astrocytes containing H\textsuperscript{+}-excitable K\textsuperscript{+} channels, the possibility of voltage-activated Ca\textsuperscript{2+} channels cannot be excluded, as the expression and activity of these channels have also been observed in astrocytes (D'Ascenzo et al., 2004; Duffy & MacVicar, 1994; Latour et al., 2003). The second pathway regarding chemosensitivity and Ca\textsuperscript{2+} signaling in VLM astrocytes involves the release of intracellular Ca\textsuperscript{2+} stores, potentially propagated via the CO\textsubscript{2}-sensitive opening of connexin hemichannels (Huckstepp et al., 2010). Verkhratsky et al. (2011) state that when Ca\textsuperscript{2+} excitations in astrocytes persist in the absence of extracellular Ca\textsuperscript{2+}, commonly metabotropic receptors (such as those activated by ATP) and an inositol phosphate/endoplasmic reticulum mechanism are involved. Although Huckstepp et al. (2010) measures the activation of ventral medullary astrocytes by the release of ATP and not by their Ca\textsuperscript{2+} excitability, it seems likely that additional chemosensitive mechanisms must be involved beyond the opening of intercellular channels by CO\textsubscript{2}. For example, a chemosensitive increase in Ca\textsuperscript{2+}-based currents passing through gap junctions has also
been noted in neonatal LC neurons (Andrzejewski et al., 2001; Ballantyne et al., 2004; Christie et al., 1989; Imber & Putnam, 2012; Ishimatsu & Williams, 1996; Maubecin & Williams, 1999). However, uncoupling these neurons with carbenoxolone did not eliminate the chemosensitive response (Andrzejewski et al., 2001). This collection of mechanisms suggests that CO₂, HCO₃⁻, and H⁺ ions support a dynamic interplay between extracellular and intracellular Ca²⁺ in ventral medullary astrocytes to affect second messengers and chemosensitive control.

The influx of Ca²⁺ via membrane Ca²⁺ channels need not be exceptionally large to significantly increase Ca²⁺i. One possibility is a localized increase of Ca²⁺i, such as the Ca²⁺-induced exocytosis of neurotransmitters that enhance glomus cell output to the central nervous system in response to CO₂ (Summers et al., 2002). A second possibility is that small influxes of extracellular Ca²⁺ activate intracellular ryanodine receptors to provoke Ca²⁺-induced Ca²⁺ release. In 1993, Osmanovic and Shefner (1993) described post-stimulus hyperpolarizations (afterhyperpolarization, AHP) in LC neurons whereby the V_m was shown to decrease in a slow (>5 sec) and fast (<1 sec) component following a depolarizing train of action potentials. Both slow and fast hyperpolarizing components were sensitive to extracellular Ca²⁺ concentrations, and thus were theorized to arise from K_Ca channels (Osmanovic & Shefner, 1993). The ryanodine receptor inhibitor dantrolene blocked the slow but not the fast component of the hyperpolarization, suggesting that initial Ca²⁺i increases in LC neurons from membrane channels were capable of being supplemented by Ca²⁺-induced Ca²⁺ release, perhaps as a mechanism to prolong Ca²⁺ transients following action potential bursts (Osmanovic & Shefner, 1993; Oyamada et al., 1999). Notably in rat brainstem preparations, LC neurons demonstrate action potential
bursts that are synchronized with discharges of the phrenic nerve. These bursts are followed by >5 sec hyperpolarizations of the $V_m$, similar to the Ca$^{2+}$-sensitive post-stimulus hyperpolarizations studied by Osmanovic and Shefner (Osmanovic & Shefner, 1993; Oyamada et al., 1998; Oyamada et al., 1999). This respiratory-related influx of Ca$^{2+}$ may participate in the regulation of intracellular pH (pH$_i$), as L-type channel inhibitors were shown to inhibit pH$_i$ recovery in *Helix apersa* chemosensitive neurons (Leiter et al., 2002). Alternatively, the influx in Ca$^{2+}$ may contribute to cellular signaling via Ca$^{2+}$/calmodulin pathways previously described. Other pathways may also contribute to the H$^+$-sensitive release of intracellular Ca$^{2+}$ stores, such as an H$^+$-sensing G-protein coupled receptor capable of increasing inositol phosphate (IP) formation (Ludwig et al., 2003). The full extent of these Ca$^{2+}$ influxes in central chemosensitivity remains poorly understood.

An additional target for a chemosensitive increase in Ca$^{2+}_i$ is the activation of K$_{Ca}$ channels. There have been a number of studies documenting the close association between K$_{Ca}$ channels and voltage-activated Ca$^{2+}$ channels (Berkefeld et al., 2006; Fakler & Adelman, 2008; Marcantoni et al., 2010). This work stresses the control of K$_{Ca}$ channels by localized Ca$^{2+}$ micro/nanodomains such that the binding rate constants of the Ca$^{2+}$ chelators BAPTA and EGTA have differing effects on K$_{Ca}$ currents (Berkefeld et al., 2006; Fakler & Adelman, 2008; Marcantoni et al., 2010). Although both BAPTA and EGTA have similar steady-state binding affinity for Ca$^{2+}$ ions, BAPTA is approximately 150 times faster than EGTA (Naraghi & Neher, 1997). Accordingly, BAPTA is capable of inhibiting K$_{Ca}$ currents in close (<100 nanometer) proximity to Ca$^{2+}$ channels, whereas EGTA inhibits “slower” currents from K$_{Ca}$ channels at a greater distance from a Ca$^{2+}$...
source (Augustine et al., 2003; Berkefeld et al., 2006; Marcantoni et al., 2010). Indeed, macromolecular complexes of the large conductance \( \text{K}_{\text{Ca}} \) channel BK (“Big”-K, Maxi-K, or \( \text{K}_{\text{Ca}}1.1 \) ) show co-precipitation with voltage-gated \( \text{Ca}^{2+} \) channels Cav1.2 (L-type), Cav2.1 (P/Q-type), and Cav2.2 (N-type) (Berkefeld et al., 2006). The kinetics and voltage activation of outward currents from BK channels were shown to differ depending upon the \( \text{Ca}^{2+} \) channel type(s) present, further emphasizing the dynamic interaction between \( \text{Ca}^{2+} \) channels and the activity of \( \text{K}_{\text{Ca}} \) channels (Berkefeld et al., 2006; Marcantoni et al., 2010). The localization of \( \text{K}_{\text{Ca}} \) channels near a \( \text{Ca}^{2+} \) source may mean that the peak \( \text{Ca}^{2+}_i \) near some \( \text{K}_{\text{Ca}} \) channels is several times greater than whole-cell cytoplasmic \( \text{Ca}^{2+} \) transients, with the rise and decay of the \( \text{Ca}^{2+} \) signal occurring more rapidly (Berkefeld et al., 2006; Neher & Almers, 1986). Thus, voltage-gated \( \text{Ca}^{2+}/\text{K}_{\text{Ca}} \) channels may represent mechanisms in chemosensitive control distinct from the effects of \( \text{Ca}^{2+} \)-based cytoplasmic signaling.

During studies on Helix aspersa neurons, a strong spike adaptation was observed during both normo- and hypercapnia, suggesting the activity of a \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) (\( \text{K}_{\text{Ca}} \) ) current (Denton et al., 2007). Because this \( \text{K}_{\text{Ca}} \) current was observed to “antagonize” the excitatory response to hypercapnia and because the authors suggest hypercapnia reduced the effects of the \( \text{K}_{\text{Ca}} \) current, it was reported that \( \text{K}_{\text{Ca}} \) channels also contributed to the Helix aspersa chemosensitive response. Here, the chemosensitive depolarization and accompanying influx of \( \text{Ca}^{2+} \) through voltage-sensitive channels is at least partially opposed by the efflux of \( \text{K}^+ \) through \( \text{K}_{\text{Ca}} \) channels. It was also suggested that \( \text{K}_{\text{Ca}} \) channels in chemosensitive Helix aspersa neurons are inhibited by \( \text{CO}_2/\text{H}^+ \), similar to the pathway described above for cultured mammalian chemosensitive neurons from the
ventral medulla (Denton et al., 2007; Wellner-Kienitz et al., 1998). However, there is a need for further studies to definitively determine the possible H⁺-sensitivity of K\textsubscript{Ca} channels in chemosensitive neurons.

Work on K\textsubscript{Ca} channels has not been done in NTS neurons with respect to respiratory control. However, studies on the cardiovascular effects of NTS neurons have demonstrated the presence of K\textsubscript{Ca} channels (Bissonnette, 2002; Butcher et al., 1999; Moak & Kunze, 1993). Microinjection of the small-conductance K\textsubscript{Ca} channel (S\textsubscript{K}) inhibitor apamin and the K\textsubscript{Ca} channel inhibitor charybdotoxin both caused changes in the excitability of NTS neurons (Butcher et al., 1999; Moak & Kunze, 1993). In some cell types, charybdotoxin is reported to inhibit smaller K\textsubscript{Ca} currents in addition to BK channels, possibly due to intermediate Ca\textsuperscript{2+}-activated K\textsuperscript{+} (I\textsubscript{K}) channels since these currents were not shown to be sensitive to apamin (Smith et al., 1986; Sollini et al., 2002). However, voltage-clamp studies of the charybdotoxin-sensitive currents in NTS neurons closely resemble the paxilline-sensitive BK currents studied in LC neurons (Imber et al., 2012; Moak & Kunze, 1993). In LC neurons, inhibition of these BK channels was shown to modify the magnitude of the chemosensitive response (Imber et al., 2011; Imber et al., 2012). It is possible that the K\textsubscript{Ca} channels present in NTS neurons also participate in chemosensitive respiratory control.

There has been ample evidence for K\textsubscript{Ca} channel activity in LC neurons. Aghajanian et al. (1983) showed that loading EGTA intracellularly into LC neurons caused an increase in the spontaneous firing rate, suggesting the presence of K\textsubscript{Ca} channels. Osmanovic and Shefner (1983) demonstrated the partial apamin-sensitivity of post-stimulus hyperpolarizations of the V\textsubscript{m} in LC neurons. Immunohistochemistry work
in the mouse brain noted that the LC contained the largest density for BK channel staining in the pons (Sausbier et al., 2006). Hypoxia studies have demonstrated charybdotoxin-sensitive outward currents in LC neurons (Koyama et al., 1999; Murai et al., 1997), and recent work with the BK channel inhibitor paxilline in LC neurons has linked BK channels to the hypercapnic activation of L-type Ca\textsuperscript{2+} channels (Imber et al., 2012). Considering this evidence for the widespread activity of Ca\textsuperscript{2+} channels and K\textsubscript{Ca} channels in central chemosensitive neurons, it seems likely that Ca\textsuperscript{2+} is involved in pathways impacting CO\textsubscript{2}-sensitive respiratory control.

**Role for Ca\textsuperscript{2+} in Central Chemosensitivity**

Since current evidence suggests that the chemosensitive gain is an important factor in the overall response of the animal to CO\textsubscript{2} (Putnam et al., 2004), an essential question becomes whether the activation of voltage-gated Ca\textsuperscript{2+} channels or increase in Ca\textsuperscript{2+\textsubscript{i}} augments or decreases the firing rate response of chemosensitive neurons to CO\textsubscript{2}. It could be that the activation of Ca\textsuperscript{2+} channels in response to acidification or hypercapnia-induced depolarization results in an increased influx of depolarizing current, resulting in an excitatory role for Ca\textsuperscript{2+} in central chemosensitivity. It is also possible that the influx of Ca\textsuperscript{2+} is minor compared with the secondary effects of Ca\textsuperscript{2+}, such as calmodulin binding, the activation of outward current via K\textsubscript{Ca} channels, or the release of signaling factors into the extracellular environment. These secondary effects could be either excitatory or inhibitory, and the mechanisms could vary depending upon the chemosensitive region. First, evidence for an excitatory role of Ca\textsuperscript{2+} in chemosensitive neurons will be discussed.

In chemosensitive neurons of the LC, 4-AP, tetraethylammonium (TEA), and nifedipine reduce but do not completely eliminate the chemosensitive response,
indicating multiple chemosensitive currents (Filosa & Putnam, 2003; Martino & Putnam, 2007; Putnam, 2010). This suggests that the net result of the activity of L-type Ca\textsuperscript{2+} channels (inhibited by nifedipine) present in the soma and the synapse is excitatory, and also emphasizes the potential complexity of the chemosensitive response. For example, since neighboring neurons can contribute to the chemosensitive response in the LC via synaptic junctions (Gargaglioni et al., 2010), it is possible that the inhibition of L-type Ca\textsuperscript{2+} influx also interrupts this synaptic input. A similar observation was made in earlier studies of chemosensitive neurons from Helix aspersa. Here, the removal of extracellular Ca\textsuperscript{2+} resulted in the complete loss of chemosensitivity, even though later studies indicated that both an A-type and a delayed-rectifier type K\textsuperscript{+} current also contributed to the CO\textsubscript{2}-sensitive response (Denton et al., 2007). Chemosensitive glomus cells are an example of the excitatory role of Ca\textsuperscript{2+} via synaptic signaling (Summers et al., 2002). Here, the increase in Ca\textsuperscript{2+}i in response to a HCO\textsubscript{3}-sensitive mechanism increased the exocytosis of neurotransmitters and enhanced carotid body output to the central nervous system (Summers et al., 2002). Thus, hypercapnia-induced Ca\textsuperscript{2+} entry into glomus cells is part of the activation pathway by which these cells respond to increased CO\textsubscript{2}/H\textsuperscript{+}. In contrast to the multiple H\textsuperscript{+}-sensitive channels in mammalian LC and Helix neurons, cultures of medullary chemoreceptive neurons could be completely inhibited by the application of the Ca\textsuperscript{2+} channel inhibitors Cd\textsuperscript{2+} and Ni\textsuperscript{2+} (Wellner-Kienitz et al., 1998)(see previous discussion). However, these experiments have not been repeated using in situ neurons. This collection of studies indicates the possible excitatory role of Ca\textsuperscript{2+} in chemosensitive neurons from multiple areas, however, more work needs to be done to establish whether
Ca$^{2+}$ predominantly contributes to the chemosensitive response via intercellular or intracellular mechanisms.

With respect to the excitatory role of Ca$^{2+}$ in augmenting the response to hypercapnia via intercellular signaling, recent studies have suggested that astrocytes may play a significant part (Gourine et al., 2010; Gourine & Kasparov, 2011; Huckstepp et al., 2010; Wenker et al., 2010). Astrocytes in the region of the VLM were examined for their sensitivity to CO$_2$/H$^+$ and their effects on adjacent chemosensitive neurons of the RTN (Gourine et al., 2003; Gourine et al., 2005; Gourine et al., 2010; Huckstepp et al., 2010). Significantly, pH sensitivity was found to be a unique feature of these astroglia, whereas astrocytes from the cerebral cortex were found to be pH-insensitive (Gourine et al., 2010). When the extracellular pH was decreased by a small amount (7.4 to 7.2), imaging studies using expression of an intracellular Ca$^{2+}$-sensitive dye showed the propagation of transient Ca$^{2+}$ increases in VLM astrocytes (Gourine et al., 2010). Adjacent chemosensitive RTN neurons also responded to the release of ATP from astrocytes with an increase in Ca$^{2+}$ and marked increase in firing rate, both of which could be inhibited by the application of ATP receptor antagonists (Gourine et al., 2010; Wenker et al., 2010). These results were the same whether VLM astrocytes were subjected to optogenetic stimulation using expressed channelrhodopsin-2 or selectively stimulated using fluorocitrate (Gourine et al., 2010; Nagel et al., 2003; Wenker et al., 2010). In addition, blockade of ATP receptors 1) completely abolished the chemosensitive response and increase in Ca$^{2+}$ of RTN neurons in response to a decrease in extracellular pH (Gourine et al., 2010); and 2) significantly inhibited the increase in firing rate to hypercapnia (Wenker et al., 2010). Optogenetic stimulation of VLM astrocytes in
anesthetized rats also resulted in an increase in phrenic nerve output that was inhibited by ATP receptor antagonists (Gourine et al., 2010). Collectively, these data suggest that the role of Ca\(^{2+}\) in astrocytes of the VLM is excitatory, and also that Ca\(^{2+}\) contributes significantly to the chemosensitive response of RTN neurons (Gourine et al., 2010; Wenker et al., 2010) (see mechanism discussion). However, further studies are needed to determine the role of chemosensitive Ca\(^{2+}\) changes in RTN neurons.

A possible role for Ca\(^{2+}\) in chemosensitive intercellular communication via gap junctions has also been demonstrated in LC neurons. However, it is currently unknown if this form of signaling contributes to chemosensitive gain. Two distinct L-type Ca\(^{2+}\) currents were evaluated in LC neurons. The first appeared as voltage sensitive spikes typical of TTX-insensitive somal voltage-activated Ca\(^{2+}\) channels, the second as rhythmic, voltage-insensitive oscillations. These oscillations were sensitive to carbenoxolone, and hence are likely due to the activity of Ca\(^{2+}\) channel currents conducted through gap junctions (Ballantyne et al., 2004; Filosa & Putnam, 2003; Imber & Putnam, 2012). Significantly, these Ca\(^{2+}\)-based oscillations also appeared to regulate Na\(^{+}\) action potentials and increased in both amplitude and frequency upon exposure to hypercapnia (Filosa & Putnam, 2003; Imber & Putnam, 2012; Oyamada et al., 1998). Thus, an amplification role has been proposed for the L-type Ca\(^{2+}\) oscillations, whereby an increase in the depolarizing rhythmic currents passing through gap junctions in individual neurons may serve to increase the number of neurons capable of responding to hypercapnia (Christie et al., 1989; Imber & Putnam, 2012). In the case of intrinsically chemosensitive neurons of the LC, this may mean synchronization of the gap-junction
coupled network to the collective stimulations of single neurons that respond to CO₂ and amplification of the chemosensitive response of the LC to hypercapnia.

A further role for Ca²⁺ in long-term adaptive changes in chemosensitive neurons has not yet been addressed. However, a possibility for Ca²⁺-dependent adaptations in chemosensitive gain involves a convergence in respiratory-related and stress-related pathways. It has been found that neurons from regions of the brain implicated in stress-related disorders with marked respiratory sensitization to CO₂ targeted LC dendrites (Coryell et al., 2008; Martinez et al., 2001; Papp et al., 1993; Van Bockstaele et al., 1998; Van Bockstaele et al., 2001; Ziemann et al., 2009). These axons contained a unique neurotransmitter, corticotropin releasing factor (CRF). When CRF was applied in slice preparations, LC neurons showed an increase in the frequency of TTX-insensitive oscillations (Jedema & Grace, 2004). As described above, these Ca²⁺-based oscillations in LC neurons also increase in frequency when exposed to CO₂ (Filosa & Putnam, 2003; Imber & Putnam, 2012; Oyamada et al., 1998). Given the extensive role of CRF signaling in adaptive responses and gene expression, it is intriguing to speculate that Ca²⁺ may be involved in the adaptation of the respiratory chemoreceptive network in response to stress and behavioral states (Hauger et al., 2009; Nardi et al., 2009; Papp et al., 1993; Risbrough & Stein, 2006; Rossant et al., 1999).

Evidence for the role of Ca²⁺ in intercellular signaling and second messenger chemosensitive pathways suggests that the role of Ca²⁺ is largely excitatory, increasing the firing rate response to increases in CO₂. In contrast, the role of Ca²⁺ in voltage-activated Ca²⁺/K⁺Ca channels appears to be inhibitory. Thus, it is possible that Ca²⁺ may contribute to both excitatory and inhibitory functions depending upon the source of Ca²⁺.
influx, localization of the changes of Ca\(^{2+}\), and the relative density of channels (see mechanism discussion above). For example, chelating Ca\(^{2+}\) in LC neurons results in an increase in the spontaneous firing rate and decreases the inactivation of firing, suggesting the presence of K\(_{Ca}\) channels and their potential to limit an increase in the firing rate of LC neurons, such as an increase caused by hypercapnia (Aghajanian et al., 1983; Li & Putnam, 2009; Osmanovic & Shefner, 1993). These observations suggest an additional role for Ca\(^{2+}\) in the neuronal chemosensitive response that involves K\(_{Ca}\) channels. The activity of this Ca\(^{2+}\)-dependent pathway may be to act as a ‘brake’, or limit on the firing rate response of LC neurons to hypercapnia (Imber et al., 2011).

In one example, the role of increasing a K\(_{Ca}\) current appeared to be excitatory. A point mutation in the human KCNMA1 gene for the pore-forming subunit of the BK channel was identified in a familial form of epilepsy. This mutation resulted in a gain of function whereby the BK affinity for Ca\(^{2+}\) increased, and the channels opened faster and at more hyperpolarized voltages. Thus, the mutation effectively increased BK currents under physiologic conditions (Du et al., 2005). Consistent with this, a possible role suggested for BK channels is to increase the rate of repolarization of Na\(^{+}\) action potentials, thus allowing for faster re-activation of Na\(^{+}\) channels and greater firing frequencies (Du et al., 2005). Although possible that under burst conditions a more rapid repolarization could allow for a greater firing frequency, it does not seem likely that increasing K\(^{+}\) conductance would increase firing rate unless the limiting factor for firing frequency was the time needed for single action potentials. Increasing BK channel activity may also result in abnormal down regulation of a neuronal network, hyperpolarizing inhibitory neurons to cause uncontrolled bursting in others. Consistent
with this, burst firing rates in LC neurons via injections of depolarizing current pulses were found to be much greater in the presence of Ca\(^{2+}\) channel blockade, supporting an inhibitory function for BK channels (Osmanovic & Shefner, 1993).

There have been several examples of Ca\(^{2+}/\text{K}_{\text{Ca}}\) mechanisms fulfilling inhibitory regulating roles in neurons from the CNS (Fakler & Adelman, 2008; Hosy et al., 2011). Perhaps most significant to central chemoreception are studies in cardiovascular control neurons from the caudal NTS near the area postrema (Butcher et al., 1999). As mentioned above, this area contains neurons involved in central chemoreception, and previous studies have noted the presence of $\text{K}_{\text{Ca}}$ channels (Bissonnette, 2002). When baroreceptive NTS neurons were studied using whole cell patch clamp, an “evoked hyperpolarization” was observed following excitatory synaptic responses (Paton et al., 2001). Microinjections of either charybdotoxin or apamin into the NTS resulted in an increase in the baroreceptor reflex gain (Paton et al., 2001). These data suggest that $\text{K}_{\text{Ca}}$ channels are capable of modulating excitatory input to NTS neurons, similar to the role suggested for BK channels in chemoreceptive LC neurons (Imber et al., 2012). However, significant to the caudal NTS area is that individual neurons respond differently to the inhibition of $\text{K}_{\text{Ca}}$ channels. For example, only some ~46% of patched baroreceptor NTS neurons demonstrated evoked hyperpolarizations to synaptic input (Paton et al., 2001). In a second study in transverse brainstem slices, patch clamp recordings in the caudal NTS revealed that a percentage of neurons were inhibited by the application of apamin, suggesting the increased input from adjacent GABAergic neurons expressing $\text{K}_{\text{Ca}}$ channels (Butcher et al., 1999). Although additional studies are needed on the possible role of these pathways in chemosensitive neurons of the NTS, it seems likely that the role
of Ca\(^{2+}/K_{Ca}\) channels in cardiorespiratory neurons of the brainstem is to modulate neuron responsiveness, either by excitatory or inhibitory effects.

Recently, a developmental role has been proposed for Ca\(^{2+}\) in chemosensitive neurons of the LC involving \(K_{Ca}\) channels (Imber et al., 2012; Imber & Putnam, 2012). Voltage-clamp studies showed that the average amplitude of Ca\(^{2+}\) currents in neonatal rat LC neurons increased by a factor of 3 between the postnatal (P) ages P3 to P14. This Ca\(^{2+}\) current was significantly inhibited by L-type channel inhibition (Imber et al., 2011; Imber et al., 2012). Across this age range, the development of a paxilline-sensitive BK current was also observed that increased in amplitude by a factor of 3. Decreasing Ca\(^{2+}\) levels with BAPTA or the exposure to L-type Ca\(^{2+}\) channel inhibition both markedly decreased the observed BK current (Imber et al., 2011; A. N. Imber et al., 2012). These data are consistent with increased expression of BK channels and/or L-type Ca\(^{2+}\) channels during postnatal development in LC neurons, resulting in the activation of a large (~2 nA) BK current by L-type Ca\(^{2+}\) channels by age P14 (Imber et al., 2011; Imber et al., 2012). When paxilline was applied to whole cell patched LC neurons in current clamp, there was an increase in the magnitude of the firing rate response to hypercapnia (Imber et al., 2011; Imber et al., 2012). This increase in the chemosensitive response to BK channel inhibition grew significantly larger over postnatal age ~P5 to ~P16, and coincided with a decrease in the average chemosensitivity of LC neurons (Gargaglioni et al., 2010; Imber et al., 2011; Imber et al., 2012). These results strongly suggest that the role of Ca\(^{2+}\) through somal L-type Ca\(^{2+}\) channels is to act as a brake on the chemosensitive response of LC neurons via the activation of \(K_{Ca}\) channels, modulating the increase in firing rate to CO\(_2\) as neonatal animals age (Imber et al., 2011; Imber et al., 2012).
Significance

This review emphasizes the potential importance of the role of Ca\textsuperscript{2+} in respiratory control neurons. For example, the implications that Ca\textsuperscript{2+} can modulate the magnitude of the chemosensitive response are significant to studies on human pathology. In cases of obstructive sleep apnea (OSA), multiple clinical studies have documented an association between OSA and an increase in the loop gain, or the ratio of ventilatory increase to a disturbance that initiated ventilation (Ryan & Bradley, 2005; Verbraecken et al., 1995; Wang et al., 2007; Younes et al., 2001). In other words, an increase in respiratory sensitivity was associated with the onset of irregular breathing such that medications designed to decrease loop gain were shown to be effective treatments (Kiwull-Schone et al., 2008). Thus, abnormalities in a braking pathway, such as the ones suggested above for Ca\textsuperscript{2+}/K\textsubscript{Ca} channels, may contribute to breathing pathology. It is also possible that the braking mechanism is unique to chemosensitive neurons of the LC. More specific to neurons of the LC is the profound body of evidence linking the pathology of panic disorder to respiratory abnormalities, including an enhanced sensitivity to hypercapnia (Abelson et al., 2001; Coryell et al., 2001; Lousberg et al., 1988; Nardi et al., 2009; Papp et al., 1993; Stein et al., 1995). Accordingly, abnormalities in a chemosensitive braking pathway in LC neurons may have direct implications to the sensitivity of panic disorder patients to CO\textsubscript{2}.

Studies in the NTS indicate that K\textsubscript{Ca} channels on GABAergic neurons may mean that the Ca\textsuperscript{2+}/K\textsubscript{Ca} mechanism is excitatory in some chemosensitive neurons and regions. In the area of the RTN, studies in astrocytes indicate a strong contribution of Ca\textsuperscript{2+}-based mechanisms to the magnitude of the chemosensitive response. There is a thus a great
need to better characterize pathways involved in Ca\(^{2+}\) and central chemoreceptive control. My hypothesis is that Ca\(^{2+}\) plays a significant role in the LC chemosensitive response of LC neurons. Specifically, I propose that hypercapnia activates Ca\(^{2+}\) channels, increasing intracellular Ca\(^{2+}\), and that this acts as a brake on the chemosensitive response of LC neurons via the activation of K\(_{Ca}\) channels. In accordance with the observation that chemosensitivity of the LC decreases over the first two weeks of postnatal development, I also expect this braking pathway to develop as the neonate matures. Similar to peripheral chemoreceptors, I suggest that the activation of LC Ca\(^{2+}\) channels by hypercapnic acidosis is based on changes of CO\(_2\)/HCO\(_3\) and not pH\(_o\)/pH\(_i\). Thus, I use electrophysiology and intracellular fluorescent indicators of pH and Ca\(^{2+}\) to study the effect of Ca\(^{2+}\) on chemosensitive LC neurons.
CHAPTER III

HYPOTHESIS & SPECIFIC AIMS
Study Hypothesis:

I hypothesize that the role of Ca\(^{2+}\) in the LC chemosensitive response is to decrease the firing rate response of LC neurons to hypercapnia. I further hypothesize that this braking phenomenon exhibits postnatal development. Finally, I hypothesize that this chemosensitive ‘brake’ activates as a function of CO\(_2\)/HCO\(_3^-\) levels and not due to changes in pH\(_i\) or pH\(_o\).

Specific Aims:

**Specific Aim 1: Nature and Development of the TTX-insensitive Current.**

1a) **I will characterize the TTX-insensitive current in chemosensitive LC neurons.** I hypothesize that the chemosensitive TTX-insensitive current in LC neurons is from L-type Ca\(^{2+}\) channels. I hypothesize that the L-type Ca\(^{2+}\) current in LC neurons takes two distinct forms: spikes and oscillations. I will use current and voltage clamp with whole cell patches and pharmacologic agonists and inhibitors to evaluate the TTX-insensitive current in LC neurons as to their activation/inactivation parameters, inhibitor profile and ionic basis.

1b) **I will evaluate any developmental trends in the TTX-insensitive current in LC neurons.** I hypothesize that the TTX-insensitive current in LC neurons develops gradually over the first two postnatal weeks. I will use whole cell patch clamp (current and voltage clamp) to evaluate the activity of the L-type Ca\(^{2+}\) current over postnatal (P) days P3-P16 in LC neurons.

**Specific Aim 2: Effect of Hypercapnia on the TTX-insensitive Currents and Ca\(^{2+}\).**

2a) **I will determine whether CO\(_2\) activates the TTX-insensitive current in LC neurons.** I hypothesize that CO\(_2\) activates the TTX-insensitive current in LC neurons. I will use whole cell patch clamp (current and voltage clamp) to study changes in the TTX-insensitive current in response to hypercapnia in LC neurons from neonatal rats of different ages.
2b) I will determine whether the activation of TTX-insensitive current raises intracellular Ca²⁺ in LC neurons. I hypothesize that the TTX-insensitive current is a Ca²⁺ current and that its activation by CO₂ will result in increased intracellular Ca²⁺ in LC neurons. I will use pharmacological blockade and activation of L-type Ca²⁺ channels to evaluate intracellular Ca²⁺ levels in patched LC neurons using whole cell patch clamp and Fura-2 imaging to determine if elevated CO₂ results in increased intracellular Ca²⁺.

**Specific Aim 3: Effect of Ca²⁺ Channel Activation on Firing Rate Response of LC neurons to Hypercapnia.**

3) I will study the role of activation of this putative Ca²⁺ current on CO₂-induced increased firing rate in LC neurons. I hypothesize that activation of this Ca²⁺ current by hypercapnia will increase the activation of KᵥCa channels thereby serving as a ‘brake’ on the hypercapnia-induced increase in the firing rate of LC neurons. I will use current clamp to study neuronal firing rate (without TTX) and voltage clamp to evaluate KᵥCa channels. Using various inhibitors and agonists I will study the role of L-type Ca²⁺ and KᵥCa channels in modulating the LC firing rate response to elevated CO₂/H⁺.

**Specific Aim 4: Signaling Pathway by which Hypercapnia Activates Ca²⁺ Channels.**

4) I will characterize the mechanism of activation of the putative L-type Ca²⁺ channel by hypercapnia in LC neurons. My hypothesis is that soluble adenylate cyclase (sAC) mediates the activation of L-type Ca²⁺ channels by hypercapnia in LC neurons. I will use whole cell patch clamping of LC neurons and inhibitors and/or activators of sAC and its downstream effectors to evaluate their effects on the activation of L-type Ca²⁺ channels by elevated CO₂.
CHAPTER IV
GENERAL METHODS
Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01). All experiments were conducted on mixed sex neonatal Sprague-Dawley rats postnatal (P) age P3-P16.

Solutions

Unless otherwise specified, all brain slices were immersed in artificial cerebrospinal fluid (aCSF) solution. This solution consisted of (in mM): 5 KCl, 124 NaCl, 1.3 MgSO₄, 26 NaHCO₃, 1.24 KH₂PO₄, 10 glucose, and 2.4 CaCl₂ and were equilibrated with 5% CO₂/95% O₂ gas, pH ~7.45 (at 35°C). Hypercapnic solutions were identical except for equilibration with either 15% CO₂/85% O₂ gas, pH ~7.0 or 10% CO₂/90% O₂, pH ~7.15. 15% CO₂ was chosen to maximize cellular effects to hypercapnia and was used for hypercapnic aCSF unless otherwise specified (Hartzler et al., 2008; Ritucci et al., 2005b). Isohydric hypercapnic solutions were identical to hypercapnic solutions except that NaHCO₃ was increased to 77 mM replacing NaCl isosmotically to bring the pH back to ~7.45. In solutions without CO₂/HCO₃⁻, the HCO₃⁻ in aCSF was replaced isosmotically with HEPES buffer and the solution equilibrated with 100% O₂. HCl and NaOH were used to pH the HEPES aCSF solution to 7.45 and 7.0, resembling the normal aCSF and hypercapnic acidotic solutions, respectively. The whole cell pipette filling solution consisted of (in mM): 130 K-gluconate, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, 2 ATP, and 10 HEPES. The pipette filling solution pH was buffered to ~7.35
using KOH. Whole cell pipette filling solution for voltage clamp studies of the Ca$^{2+}$ current consisted of (in mM): 130 CsCl, 10 EGTA, 1 MgCl$_2$, 0.3 GTP, 2 ATP, 10 HEPES, and 10 tetraethylammonium (TEA), buffered to pH ~7.45 using CsOH. For voltage clamp studies of BK currents, the intracellular filling solution was identical to those used for current clamp studies. For immunohistochemistry studies, the phosphate buffered saline (PBS) solution contained (in mM): 137 NaCl, 2.7 KCl, 4.3 Na$_2$HPO$_4$, and 1.47 KH$_2$PO$_4$. The filling solution was designed with low EGTA and no added Ca$^{2+}$ to minimize washout of the chemosensitive response (Filosa & Putnam, 2003).

**Brain Slice Preparation**

Mixed sex neonatal Sprague-Dawley rats postnatal (P) age P3-P16 were anesthetized using high CO$_2$ for animals P10 to P16 or hypothermia for animals P3 to P9 and rapidly decapitated. The brainstem was removed in ice-cold (4-6°C) aCSF solution and then transferred to a vibratome (PelcoVibratome 1000). 300 μm slices of the medulla and pons were obtained out in 4-6°C aCSF. Slicing started at the obex and continued through the pons, as previously described (Kersh et al., 2009). Pontine slices containing the LC region were incubated in room temperature aCSF equilibrated with 5% CO$_2$ / 95% O$_2$ for at least 1 hr after slicing and then placed individually into a superfusion chamber on the stage of an upright Nikon Eclipse 6600 microscope for study. During experiments, slices were immobilized using a nylon grid and superfused continuously by gravity flow (~4 ml/min) using solutions held at 37°C.

**Fluorescence: Intracellular Ca$^{2+}$**

The Ca$^{2+}$ sensitive dye Fura-2 (250 μM) was loaded into LC neurons using whole cell patch pipettes (Fig. 4A). Fura-2-loaded neurons were excited by light from a 75W
Figure 4:  

A. Individual LC neuron loaded with intracellular solution plus Fura-2 through a whole cell patch.  

B. Individual LC neuron loaded with intracellular solution plus pyranine through a whole cell patch. Scale bars represent 50 μm.
Figure 4.

A.

B.
xenon arc lamp alternately at 340 nm and 380 nm using a Sutter Lambda 10-2 filter wheel. Emitted fluorescence at 505 nm was directed to the Nikon multi-image port module and then to a GenII Sys Image intensifier and a CCD camera. Subsequent fluorescence images were acquired using a Gateway 2000 E-3100 computer and collected/processed using the software MetaFluor 4.6r. Image acquisition could be achieved within ~2 seconds and was repeated every 15 seconds. Light was blocked between acquisitions to reduce photobleaching. The Fura-2 fluorescence was not calibrated and arbitrary fluorescence units were used instead to monitor increases or decreases in the ratio of fluorescence ($R_\text{f}$) ($R_\text{f} = F_{340}/F_{380}$). $R_\text{f}$ is directly proportional to changes in intracellular $\text{Ca}^{2+}$.

**Fluorescence: Intracellular H$^+$**

The H$^+$ sensitive dye pyranine (50 μM) was loaded into LC neurons using whole cell patch pipettes (Fig 4B). Pyranine-loaded neurons were excited alternately at 450 nm and 415 nm by light from a 75W xenon arc lamp for pH-sensitive and pH-insensitive recordings, respectively, using a Sutter Lambda 10-2 filter wheel. Emitted fluorescence at 515 nm was directed to the Nikon multi-image port module and then to a GenII Sys Image intensifier and a CCD camera. Subsequent fluorescence images were acquired using a Gateway 2000 E-3100 computer and collected/processed using the software MetaFluor 4.6r. Image acquisition could be achieved within ~2 seconds and was repeated every minute. Light was blocked between acquisitions to reduce photobleaching. The 450/415 fluorescence ratio ($R_\text{f}$) was determined and the following calibration curve used to convert to intracellular pH (pHi): $pHi = 7.5561 + \log[(N_\text{f} – 0.1459)/(2.0798 – N_\text{f})]$, where
the experimental $R_{fl}$ values were divided by a calibration $R_{fl}$ value at pH 7.4 to yield $N_{fl}$ (Ritucci et al. 2005b).

**Electrophysiological Recordings**

Whole cell recordings were used throughout this study. Pipettes were made from thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm) pulled to a tip resistance of ~5 MΩ and filled with intracellular solution plus fluorescent dye (pyranine or Fura-2, see above). Positive pressure was maintained on the pipette. LC neurons were visualized using an upright microscope (Nikon Eclipse 6600) with an x60 water-immersion objective. Negative pressure was applied to the pipette to form a gigaohm seal with the cell membrane, followed by brief suction to rupture the membrane and form the whole cell patch. Typical series resistance during experiments was 25-40 MΩ. Membrane potential ($V_m$) was measured in current and voltage clamp mode and current was injected via an Axopatch 200B amplifier. All data were filtered at 5 kHz with a Bessel filter and sampling rate was at 10 kHz (voltage clamp) or at 2 kHz (current clamp). Integrated firing rate (FR) was measured in 10 s bins using a slope/height window discriminator (FHC Model 700B, Bowdoinham, ME). Both $V_m$ and FR were analyzed using pCLAMP software version 10.0. Recordings began when a stable resting $V_m$ was established. Criteria for healthy neurons were a stable resting $V_m$ of -45 to -60 mV and a spontaneous firing rate of < 4 Hz. The reversibility of all electrophysiological responses under investigation was verified by a return to baseline values upon change of the solution back to normal aCSF. Patched recordings in current clamp using the above techniques lasted > 45 minutes without any evidence of washout of the response (Filosa & Putnam, 2003), and effects to most solution changes were observed in < 2 minutes.
When two sequential hypercapnic effects were studied, current was injected to ensure that all normocapnic (resting) firing rates remained within 0.5 Hz of initial recorded values in order to accurately compare both chemosensitive responses.

For Ca\(^{2+}\) and BK voltage clamp experiments, LC neurons were clamped at -70 mV in aCSF and equilibrated with either 5% CO\(_2\)/95% O\(_2\) or 15% CO\(_2\)/95% O\(_2\) as indicated. 1 μM TTX was added to block Na\(^+\) currents. For Ca\(^{2+}\) currents, a separate intracellular solution was used to block K\(^+\) currents (see solutions) and 3 mM BaCl\(_2\) was added to the aCSF in order to enhance the Ca\(^{2+}\) current, replacing NaCl isosmotically. Step-wise depolarizations of some 600 ms duration in 10 mV increments from -60 mV to +50 mV were applied and the resulting peak current recorded. All currents are reported as nanoamps (nA) (see capacitance section below). For voltage-clamp experiments of BK channels, neurons were clamped at -70 mV, followed by a step depolarization to +10 mV for 40 ms to achieve Ca\(^{2+}\) loading in the neuron (pre-pulse), and immediately followed by a step depolarization to +80 mV to record the K\(_{Ca}\) and voltage-activated K\(^+\)/Ca\(^{2+}\) current (see BK voltage clamp section). This double-pulse protocol was repeated following a 3-minute exposure to 1 μM paxilline, and the resulting currents subtracted to obtain the BK difference current (ΔI). For BAPTA-AM voltage clamp experiments, the brain slice containing the LC was incubated in room-temperature aCSF containing 40 μM BAPTA-AM for 25 minutes prior to use.

Capacitance values for LC neurons were measured in current clamp mode according to the method detailed in Golowasch et al. (2009). In brief, a negative current pulse of 50-200 pA was applied for 800 ms and the resulting voltage decrease (between 10 and 20 mV) was recorded. The current pulse was repeated four times per neuron for
accuracy and the $V_m$ curve was fit using the Clampfit Analysis software in the pClamp package. Neurons demonstrated a multiphasic capacitance transient that was consistent with the presence of multiple electric compartments (see capacitance section below).

**Immunohistochemistry**

Brainstem slices from neonatal rats age P11 or P3 were fixed in freshly prepared 4% paraformaldehyde in PBS buffer, pH 7.4, for 72 hours. Afterwards, tissues were washed three times in PBS plus 0.1% TritonX for 15 minutes per wash, and then incubated in PBS/TritonX buffer plus 1% bovine serum albumin (BSA) for 30 minutes. Prepared slices were incubated with either 1:200 dilutions of reconstituted anti-KCa 1.1 (BK1) antibodies (Alomone Labs) and 1:500 dilutions of the neuronal marker antibody NeuN or 1:100 dilutions of anti-SAC-101-AP (SAC) (FabGennix) in PBS/TritonX buffer for 72 hours at 4°C. Afterwards, slices were washed three times in PBS/TritonX buffer for 15 minutes per wash and incubated overnight with anti-rabbit cyanine 3 (CY3)-conjugated donkey anti-rabbit (1:50; Jackson ImmunoResearch) antibody to identify either BK1 or SAC and fluoroscein isothiocyanate (FITC) donkey anti-mouse (1:50; Millipore) to identify NeuN. Tissues were washed three times with PBS buffer without detergent for 15 minutes per wash prior to imaging. Control tissues were treated identically except for no addition of primary antibody. Stained tissues and controls were visualized with a Fluoview 300 microscope (Olympus Optical, Tokyo Japan) using Argon (FITC) and Krytpon (CY3) lasers and Fluoview software. Excitation was set to 560 nm for CY3 fluorescence and emission collected between 580-680 nm. For FITC, excitation was set to 488 nm and emitted fluorescence was collected between 500-555 nm.
nm. A series of confocal optical sections (Z stack, resolution 0.5 μm) were used to assess the extent of channel labeling.

**Drugs**

TTX, BAPTA-AM, paxilline, nifedipine, BSA, 2-hydroxyestradiol (2HE), db-cAMP, H89, and Fura-2 were purchased from Sigma-Aldrich (St Louis, MO). Final concentrations for all experiments were as follows: TTX, 1 μM; BAPTA-AM, 40 μM; paxilline, 1 μM; nifedipine, 50 μM; BSA, 1%; 2HE, 10 μM; db-cAMP, 250 μM; H89, 10 μM; and Fura-2, 250 μM. Nifedipine, 2HE and paxilline was prepared as stock solutions at 50 mM, 20 mM, and 10 mM, respectively, in EtOH. BAPTA-AM stocks were prepared in DMSO at 10 mM. TTX, H89, db-cAMP and Fura-2 stocks were prepared in dH2O at 1 mM, 10 mM, 10 mM and 10 mM, respectively.

**Data Analysis and Statistics**

Where applicable, analysis for changes in firing rate (ΔFR) is calculated by the following: $\Delta FR = \left( \frac{\text{hypercapnic average firing rate} - \text{control average firing rate}}{\text{control average firing rate}} \right) \times 100\%$. Lines for IV plots were fit in Excel using a scatter plot with smooth lines. The chemosensitivity index (CI) was calculated using an equation of Wang et al. (1998) in Excel (Microsoft Office, 2003). CI is the percentage of the control firing rate upon a 0.2 pH unit change in extracellular pH. By this scale, 100% represents no firing rate change to hypercapnia. All values are expressed as mean ± SEM. Significant differences between two means were determined by student t-tests or paired t-tests. Comparisons of more than two means were assessed using ANOVA with multiple paired comparisons.
Oscillations (see chapter V) and pyranine and Fura-2 studies (see chapters V-VII) were analyzed using a custom JAVA-based program designed using NetBeans IDE 6.9.1 and Netbeans platform from netbeans.org. All classes and methods used by this program represent original code. Samplings of both \( V_m \) measurements and fluorescence values were synchronized by the program and averaged based on user-defined sections (usually in 3-5 minute periods of time). The program analyzed oscillations by computing an average membrane potential for each event and then counting the number of times the \( V_m \) crossed a threshold value and maintained the depolarization for >0.1 s. Threshold values were user-defined and were typically between 2-5 mV. \( \text{HCO}_3^- \), \( \text{pH}_i \) values were calculated by the program for each section using the Henderson-Hasselbalch equation (\( \text{pH}_i = \text{pKa} + \log([\text{HCO}_3^-]/\alpha\text{P}_\text{CO}_2); \text{pKa}=6.1 \)) and the calibration curve described above. Figure 5 shows typical screen shots for the program interface.

**Measuring Membrane Properties:**

One explanation for changes in both \( \text{Ca}^{2+} \) and BK currents with development could be that LC neurons increase their size over ages P3 to P16 (see chapters V and VI), and the relative current density remains constant. To address this possibility we evaluated the membrane properties from 17 neurons aged P3 to P16. 11 neurons from 3 slices ages P3 to P7 gave an average resistance of 136.8 MΩ (std error 22.16) and 6 neurons from two slices ages P12 to P16 gave an average resistance of 189.6 (std error 49.36). The capacitance values for these neurons ages P3 to P7 and P13 to P16 were 65.3 pF (std error 4.62) and 69.96 pF (std error 6.95), respectively. Neither resistance not capacitance values were significantly different from one another. These values were also not significantly different from capacitance values reported previously for LC neurons from neonatal rats (Lena et al., 1999). Thus, it is likely that the increase in both BK currents
Figure 5: Screenshots from the user interface of a JAVA-based program used to analyze and chart concurrent fluorescence and electrophysiological data. Data were loaded into the program in text format and all time points synchronized. Upper panel depicts \( \text{Ca}^{2+} \) record analysis, while lower panel was used to plot changes in intracellular \( \text{HCO}_3^- \). Colored brackets were used to define regions of interest within records.
Figure 5.
and Ca$^{2+}$ currents in LC neurons is due to an increase in channel density (Ca$^{2+}$ and BK) or possibly the increased activation of the same number of channels (BK) in LC neurons.

**BK Channel Voltage Clamp**

Voltage clamp experiments of BK channels is complicated by the sensitivity of the channel to both voltage and Ca$^{2+}$ concentrations. In order to increase intracellular Ca$^{2+}$ levels prior to test voltage steps, a pre-pulse step is used (see electrophysiology recordings) (Fig 6A). Figure 6B shows the large increase in intracellular Ca$^{2+}$ levels upon applying the pre-pulse and test voltage step protocol. The pre-pulse voltage step is used to increase intracellular Ca$^{2+}$ concentrations prior to the onset of the test voltage step (Marcantoni et al., 2010). In chromaffin cells, the increase in intracellular Ca$^{2+}$ concentrations due to this pre-pulse is necessary to activate BK currents (Marcantoni et al., 2010). In LC neurons, voltage steps above ~-20 mV appear sufficient to activate BK currents with or without a pre-pulse (see results for chapter VI). Thus, a paxilline-difference current is used to study the characteristics of the BK current in LC neurons, as discussed in chapter VI. However, initial Ca$^{2+}$ loading into LC neurons appears significant if the test voltage steps are not depolarized enough to activate voltage-activated Ca$^{2+}$ channels, supporting a Ca$^{2+}$ dependency for the paxilline difference current.

At voltage steps below -20 mV, the activation of the paxilline difference current is very small and no longer sustained (Fig 7). -30 mV is below the activation voltage for Ca$^{2+}$ currents (see chapter VI). At this voltage, the pre-pulse achieved an initial Ca$^{2+}$ loading that decreased rapidly, resulting in the closure of BK channels (Fig 7). At -10 mV, the paxilline difference current becomes larger and non-inactivating; possibly
**Figure 6:**  

*A.* The voltage-step protocol used to evaluate BK currents. Protocol consisted of a pre-pulse to +10 mV, followed by a voltage step to +80 mV. The pre-pulse step was designed to increase $\text{Ca}^{2+}_i$ prior to the voltage step.  

*B.* Fura-2 measurements during the voltage-step protocol used in A. In all neurons tested, the BK voltage-step protocol significantly increased $\text{Ca}^{2+}$ levels versus control measurements (N=3). Control measurements were taken ~2 sec prior to running the voltage step protocol. $R_f$ measurements were recorded at a rate of just under 1 per second. Notice the rapid rise in $\text{Ca}^{2+}_i$. $\text{Ca}^{2+}_i$ returned to resting levels in less than 5 seconds (data not shown).
Figure 6.

A.

B.
Figure 7: Whole cell voltage clamp recordings in the presence of TTX. Record from a >P10 animal showing the subtraction of the two separate records with and without the presence of paxilline. These data represent the paxilline-sensitive current, or Pax ΔI. These traces were recorded using voltage-steps to -10 mV and -40 mV following Ca\(^{2+}\) loading using a pre-pulse step. Notice the smaller, rapidly inactivating Pax ΔI at -40 mV.
Figure 7.
indicating a more sustained increase in intracellular \( \text{Ca}^{2+} \) (Fig. 7). Significantly, these effects on the BK current can also be demonstrated using the pre-pulse. Figure 8A shows a voltage clamp trace in the presence of TTX at voltage steps of -50 mV, -40 mV, and -30 mV, which are not observed to activate the \( \text{Ca}^{2+} \) current (see chapter VI). In the absence of a pre-pulse voltage step, the transient A-current is observed (lower trace) (Li & Putnam, 2009). With the addition of a pre-pulse, a rapidly inactivating current similar to the paxilline-sensitive current in figure 7 is seen (upper trace).

Figure 8B summarizes the difference between a pre-pulse step (control) and a test pulse only protocol (no pre-pulse) for measuring BK currents in LC neurons at depolarizations to \(-10\) mV. The absence of the pre-pulse shows a slower initial activation of BK currents, presumably as intracellular \( \text{Ca}^{2+} \) accumulates (see Fig. 6B). This effect is absent under control conditions where paxilline is used to block BK currents (lower trace, Fig 8B). Thus, the pre-pulse is a necessary addition to the protocols used to study BK currents in LC neurons.

**Measuring Intracellular Ca\(^{2+}\)**

Our research design involves the use of electrophysiology in combination with intracellular fluorescent indicators for \( \text{Ca}^{2+} \) ions to study the effects of \( \text{Ca}^{2+} \) on the chemosensitive response of LC neurons. In chapter VI, we show that intracellular \( \text{Ca}^{2+} \) levels are increased by hypercapnia and decreased by the inhibition of L-type \( \text{Ca}^{2+} \) channels. It is significant to our experimental design that these observations are not due to the effects of the whole cell patch on LC neurons. Figure 9A shows LC neurons individually patched and loaded with Fura-2, according to the methods described above. The whole-cell patch was then removed and the individual neuron remained loaded with
**Figure 8:** Whole cell voltage clamp recordings in the presence of TTX. 

*Figure A:* Record from a >P10 animal showing the subtraction of the two separate records with and without a pre-pulse preceding voltage steps between -50 and -30 mV. Notice the appearance of a Pax ΔI-like current with the presence of a pre-pulse. 

*Figure B:* Voltage-activated currents evoked by a pre-pulse at voltage steps to -10 mV. Note the appearance of a rapidly inactivating Pax ΔI-like current with the addition of increased Ca^{2+} prior to the voltage step. This same current is inhibited by either a loss of the pre-pulse or paxilline. 

These data indicate the Ca^{2+} sensitivity of the Pax ΔI, consistent with BK channels.
Figure 8.

A.

B.
**Figure 9:** *A.* Three adjacent LC neurons whole-cell patched and allowed to load with Fura-2. After loading, the patch was removed and the membrane allowed to re-seal. Scale bar represents 50 μm. *B.* Hypercapnia caused a reversible increase in Ca$^{2+}$i levels that was inhibited by nifedipine (see chapter VII). These imaging results are consistent with those recorded during the whole cell patch.
Figure 9.

A.

B.
Fura-2. When the slice was exposed to hypercapnia and nifedipine, the observed changes in intracellular Ca\(^{2+}\) were the same for the un-patched neuron as those observed in patched LC neurons (see chapter VI) (Fig 9B). Thus, the whole cell patch conditions do not interfere with measuring changes in intracellular Ca\(^{2+}\).
CHAPTER V

Postnatal development and activation of L-type Ca$^{2+}$ currents in locus coeruleus neurons: implications for a role for Ca$^{2+}$ in central chemosensitivity

Abstract

Little is known about the role of Ca\(^{2+}\) in central chemosensitive signaling. We use electrophysiology to examine the chemosensitive responses of TTX-insensitive oscillations and spikes in neurons of the locus coeruleus (LC), a chemosensitive region involved in respiratory control. We show that both TTX-insensitive spikes and oscillations in LC neurons are sensitive to L-type Ca\(^{2+}\) channel inhibition and are activated by increased CO\(_2\)/H\(^+\). Spikes appear to arise from L-type Ca\(^{2+}\) channels on the soma while oscillations arise from L-type Ca\(^{2+}\) channels that are distal to the soma. In HEPES-buffered solution (nominal absence of CO\(_2\)/HCO\(_3\)^-) acidification does not activate either oscillations or spikes. When CO\(_2\) is increased while extracellular pH is held constant by elevated HCO\(_3\)^-, both oscillation and spike frequency increase. Further, plots of both oscillation and spike frequency versus intracellular [HCO\(_3\)] show a strong linear correlation. Increased frequency of TTX-insensitive spikes is associated with increases in intracellular Ca\(^{2+}\) concentrations. Finally, both the appearance and frequency of TTX-insensitive spikes and oscillations increase over postnatal ages P3-P16. Our data suggest that 1) L-type Ca\(^{2+}\) currents in LC neurons arise from channel populations that reside in different regions of the neuron, 2) these L-type Ca\(^{2+}\) currents undergo significant postnatal development and 3) the activity of these L-type Ca\(^{2+}\) currents is activated by increased CO\(_2\) through a HCO\(_3\)-dependent mechanism. Thus, the activity of L-type Ca\(^{2+}\) channels is likely to play a role in the chemosensitive response of LC neurons and may underlie significant changes in LC neuron chemosensitivity during neonatal development.
Introduction

Central respiratory control has been shown to involve multiple locations within the brainstem. These areas contain neurons whose firing rates are altered in response to changes in CO₂/H⁺, referred to as chemosensitive neurons (Feldman et al., 2003; Nattie & Li, 2009; Putnam et al., 2004). One area identified as being involved in central chemoreception is the locus coeruleus (LC) (Biancardi et al., 2008; Coates et al., 1993; Filosa & Putnam, 2003). Most research on the chemosensitivity of LC neurons as well as other chemosensitive areas of the brainstem have focused on the role of pH-sensitive ion channels, especially K⁺ channels, as the basis for neuronal chemosensitive signaling (Pineda & Aghajanian, 1997; Putnam, 2010). Little is known, however, about the potential role of Ca²⁺ ions in central chemosensitive signaling.

The cellular basis for the firing rate response to hypercapnia of chemosensitive neurons is not fully known. It is believed to be due to CO₂-induced changes of pH inhibiting acid-sensitive channels (Putnam et al., 2004). Thus, studies of CO₂-sensitive cellular mechanisms by necessity include H⁺-sensitive mechanisms, and both intra- (pHᵢ) and extra- (pHₑ) cellular pH changes have been considered as possible chemosensitive stimuli (Filosa et al., 2002; Nichols et al., 2008; Putnam, 2010). For example, numerous acid-sensitive K⁺ channel targets for hypercapnia-induced acidification have been demonstrated in LC neurons, including inwardly-rectifying K⁺ channels, TASK channels, an A current and a delayed-rectifying K⁺ channel (Gargaglioni et al., 2010; Li & Putnam, 2009; Pineda & Aghajanian, 1997). It has been proposed that the magnitude of the firing rate increase in response to hypercapnia is the result of acid-induced inhibition of these
multiple K⁺ channels, which would decrease the outward K⁺ conductance, leading to depolarization and increased firing rate in response to CO₂ (Gargaglioni et al., 2010).

Evidence in LC neurons suggests that Ca²⁺ may also play a role in chemosensitive signaling. When the fast Na⁺-channel blocker tetrodotoxin (TTX) is applied to block Na⁺ action potentials in LC neurons, either TTX-insensitive action potentials (spikes) (Williams et al., 1984), smaller rhythmic membrane potential (Vₘ) oscillations (Christie et al., 1989; Ishimatsu & Williams, 1996; Maubecin & Williams, 1999), or both can be observed. TTX-insensitive oscillations are inhibited by cobalt, cadmium, high Mg²⁺ (11.5 mM), or the L-type Ca²⁺-channel inhibitor nifedipine, suggesting they arise from Ca²⁺ channels (Christie et al., 1989; Filosa & Putnam, 2003; Oyamada et al., 1998; Williams et al., 1984). TTX-insensitive spikes are not as frequently reported, but are also inhibited by Ca²⁺-free solutions (Williams et al., 1984). These findings strongly suggest the presence of Ca²⁺ channels in LC neurons.

The possible significance of Ca²⁺ channels in the neuronal chemosensitive response is profound (Berridge et al., 2000; Fakler & Adelman, 2008; Means, 2000). Multiple studies have documented the importance of extracellular Ca²⁺ to intracellular signaling in cultured H⁺-sensitive PC12 pheochromocytoma cells (Shimokawa et al., 1998; Shimokawa et al., 2004; Shimokawa et al., 2005). The injection of an intracellular Ca²⁺ chelating agent into chemosensitive areas of the ventral medullary surface decreased the adaptive ventilatory response to hypercapnia in rats (Kanazawa et al., 1998). In peripheral chemoreceptors, increases in [HCO₃⁻] i associated with hypercapnia result in the phosphorylation and activation of membrane Ca²⁺ channels, thereby resulting in an increase in intracellular Ca²⁺ levels (Ca²⁺ i) and enhanced exocytosis of neurotransmitters.
(Summers et al., 2002). Similarly in LC neurons, TTX-insensitive oscillations can be activated by high CO$_2$/HCO$_3^-$ in the absence of a change in pH$_o$ (Filosa & Putnam, 2003). These studies strongly suggest a role for Ca$^{2+}$ in the chemosensitive response of LC neurons.

Despite the apparent role of these Ca$^{2+}$-based currents in chemoreception, variations in preparations among different studies have prevented a clear, direct assessment of the role of observed oscillations and spikes in the chemosensitive response of LC neurons. Much of the previous work on TTX-insensitive oscillations and spikes in LC neurons has focused on their general characteristics and their relationship to cell-cell signaling (Ishimatsu & Williams, 1996; Williams et al., 1984). In several studies, not all LC neurons show oscillations or spikes in the presence of TTX (Christie et al., 1989; Oyamada et al., 1998). In others, oscillations appear to be due to Na$^+$ channels and can be completely inhibited by TTX (Ishimatsu & Williams, 1996; Oyamada et al., 1999). When TTX-insensitive oscillations or spikes were not observed, Ba$^{2+}$ or Ba$^{2+}$ and tetraethylammonium (TEA) were used to induce both oscillation and spike activity (Andrzejewski et al., 2001; Ishimatsu & Williams, 1996). Thus, further work needs to be done to characterize the nature of and the conditions that promote the appearance of Ca$^{2+}$ currents and the role of those Ca$^{2+}$ currents in the chemosensitive response of LC neurons.

Interestingly, recent evidence suggests that the firing rate response of LC neurons to hypercapnia changes during early neonatal development, decreasing markedly in LC neurons from rats older than P10 (Gargaglioni et al., 2010). Immunohistochemical studies in mice and rats have noted marked quantities of Ca$^{2+}$-sensitive proteins including
large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (Biber et al., 1984; Sausbier et al., 2006). We hypothesize that Ca\(^{2+}\) may be the main factor in this developmental transition. Consistent with this, we suggest that either TTX-insensitive spikes or oscillations arise from Ca\(^{2+}\) channels on the cell membrane of chemosensitive LC neurons. We hypothesize that these Ca\(^{2+}\) channels are activated by a HCO\(_3^-\)-dependent mechanism, and not by changes in pH\(_i\)/pH\(_o\). Activation of these channels is expected to elevate Ca\(^{2+}\_i\), potentially affecting intracellular mechanisms. We speculate that this elevated Ca\(^{2+}\_i\) serves to activate Ca\(^{2+}\)-dependent K\(^{+}\) channels and thereby reduce the firing rate response of LC neurons to hypercapnia. This could be the basis for the decreased firing rate response of LC neurons during development if Ca\(^{2+}\) oscillations/spikes show a corresponding increased activation after age P10.

The purpose of the current study was therefore to examine the presence of natively occurring oscillations and spikes in LC neurons from neonatal rats ages P3-P16 in order to: 1) determine whether observed oscillations and spikes are Ca\(^{2+}\)-based using both TTX and nifedipine; 2) study the role of changes of pH and HCO\(_3^-\) in hypercapnia-induced activation of oscillations/spikes; 3) show that activation of these Ca\(^{2+}\) currents by hypercapnia results in increased intracellular Ca\(^{2+}\) levels; and 4) determine whether these Ca\(^{2+}\) currents exhibit marked increases during early postnatal development in LC neurons.

A preliminary account of some of this work has previously been published (Imber & Putnam, 2010).
Methods

Slice Preparation.

Mixed sex neonatal Sprague-Dawley rats postnatal (P) age P3-P16 were anesthetized using a CO₂ overdose or hypothermia and rapidly decapitated. Removal of the brainstem and subsequent coronal brain slicing using a vibratome (PelcoVibratome 1000) were carried out in ice-cold (4-6°C) artificial cerebrospinal fluid (aCSF) solution as previously described (Filosa & Putnam, 2003; Ritucci et al., 2005b). Slices containing the LC region were then incubated in room temperature aCSF equilibrated with 5% CO₂/95% O₂ until use 1-4 hours after slicing. During experiments, slices were superfused continuously by gravity flow (~4 ml/min) using solutions held at 35°C. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01).

Solutions.

Unless otherwise specified, all brain slices were immersed in aCSF solution. This solution consisted of (in mM): 124 NaCl, 1.3 MgSO₄, 5 KCl, 1.24 KH₂PO₄, 10 glucose, 2.4 CaCl₂, 26 NaHCO₃, and was equilibrated with 5% CO₂/95% O₂, pH ~7.45 (at 35°C). Hypercapnic acidotic solutions were identical except for being equilibrated with 15% CO₂/85% CO₂, pH ~6.9 or in one experiment 10% CO₂/90% CO₂, pH ~7.10. This percentage of CO₂ was chosen to maximize cellular effects of hypercapnic acidosis (Nichols et al., 2008; Ritucci et al., 2005b). Isohydric hypercapnic solutions were identical to hypercapnic acidotic solutions except that NaHCO₃ was raised to 77 mM.
replacing NaCl isosmotically, bringing the pH back to ~7.45. During experiments, slices containing LC neurons were superfused with aCSF containing 1μM tetrodotoxin (TTX) to study the TTX-insensitive current. In solutions without CO₂/NaHCO₃, the NaHCO₃ in aCSF was replaced isosmotically with HEPES buffer and the solution equilibrated with 100% O₂. HCl and NaOH were used to pH the HEPES aCSF solution to 7.45 and 6.9, resembling the normal aCSF and hypercapnic acidotic solutions, respectively. The whole cell pipette filling solution consisted of (in mM): 130 K-gluconate, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, 2 ATP, and 10 HEPES, plus either 50 μM pyranine or 250 μM Fura-2. The pipette filling solution pH was buffered to ~7.35 using KOH. The filling solution was designed with low EGTA and no added Ca²⁺ to minimize washout of the chemosensitive response (Filosa et al., 2003).

All of our external solutions were equilibrated with the standard high levels of O₂ (95%-100%). It has been pointed out that this is hyperoxic to in vivo levels of O₂ within the brain and often results in hyperexcitability of neurons (Dean et al., 2004). While we do not know what impact this may have on the response of Ca²⁺ channels to hyperoxia, we chose to use this level of O₂ in order to compare our results to earlier studies and because no generally agreed upon lower level of O₂ is currently available.

Measurement of Intracellular pH/Ca²⁺.

The pH-sensitive fluorescent dye pyranine (50 μM) or the Ca²⁺-sensitive dye Fura-2 (250 μM) was added to the pipette filling solution and loaded into LC neurons using whole cell patch pipettes as previously described (Ritucci et al., 2005b). Pyranine-loaded neurons were excited alternately at 450 nm and 415 nm by light from a 75W xenon arc lamp for pH-sensitive and -insensitive recordings, respectively, using a Sutter
Lambda 10-2 filter wheel. Fura-2-loaded neurons were alternately excited at 340 nm and 380 nm. Emitted fluorescence at 515 nm (pyranine) or 505 nm (Fura-2) was directed to the Nikon multi-image port module and then to a GenIIISys Image intensifier and a CCD camera. Subsequent fluorescence images were acquired using a Gateway 2000 E-3100 computer and collected/processed using the software MetaFluor 4.6r. Image acquisition could be achieved within ~2 seconds and was repeated every 60 seconds for pyranine and every 30 seconds for Fura-2. Light was blocked between acquisitions to reduce photobleaching. For pyranine, the 450/415 fluorescence ratio ($R_{fi}$) was determined and the following calibration curve used to convert to intracellular pH ($pH_i$): $pH_i = 7.5561 + \log[(N_{fl} – 0.1459)/(2.0798 – N_{fl})]$, where the experimental $R_{fi}$ values were divided by a calibration $R_{fi}$ value at pH 7.4 to yield $N_{fi}$ (Ritucci et al., 2005b). The Fura-2 fluorescence was not calibrated and arbitrary fluorescence units were used instead to monitor increases or decreases in $R_{fi}$ and thus increases or decreases in $Ca^{2+}_i$.

*Electrophysiological Recordings.*

Whole cell recordings were used throughout this study. Pipettes were made from thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm) pulled to a tip resistance of ~5 MΩ as previously described (Filosa et al., 2003; Nichols et al., 2009; Ritucci et al., 2005b). LC neurons were visualized using an upright microscope (Nikon Eclipse 6600) with an x60 water-immersion objective and subsequently patched via formation of a gigaohm seal with the cell membrane. Membrane potential ($V_m$) was measured in current clamp mode and current injected via an Axopatch 200B amplifier. Firing rate (FR) was measured using a slope/height window discriminator (FHC Model 700B, Bowdoinham, ME). Both $V_m$ and FR were analyzed using pCLAMP software.
version 8.2. Recordings began when a stable resting $V_m$ was established. Criteria for healthy neurons were a stable resting $V_m$ of -45 to -60 mV and a spontaneous firing rate of < 4 Hz. The reversibility of all electrophysiological responses to altered superfusate solutions was verified by a return to baseline values upon change of the solution back to normal aCSF. Patched recordings of TTX-insensitive currents using the above techniques lasted > 45 minutes without any evidence of washout of the response (Filosa et al., 2003), and electrophysiological responses to most solution changes were observed in < 2 minutes. Multiple hypercapnic pulses caused the same activation of the chemosensitive $\text{Ca}^{2+}$ current under investigation without a decrease in response.

**Drugs.**

TTX, carbenoxolone, pyranine, nifedipine, and Fura-2 were purchased from Sigma-Aldrich (St Louis, MO). Nifedipine was prepared as a stock solution of 50 mM in EtOH prior to use. TTX, pyranine and Fura-2 stocks were prepared in dH$_2$O.

**Data Analysis and Statistics.**

Where applicable, analysis for changes in frequency (Δfrequency) was calculated by the following: $\Delta$frequency $= \left[\frac{((\text{hypercapnic average frequency} - \text{control average frequency})}{\text{control average frequency}}\right] \times 100\%$. All values are expressed as mean ± SEM. Significant differences between two means were determined by student t-tests or paired t-tests. Comparisons of more than two means were assessed using ANOVA with multiple paired comparisons. In all cases, means were considered significantly different if $P \leq 0.05$.  

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Results

Ca$^{2+}$ oscillations and spikes in locus coeruleus neurons.

When TTX is applied to block Na$^+$ action potentials in a whole cell-patched LC neuron, a rhythmic, TTX-insensitive current can be observed as either a small amplitude oscillation or larger amplitude spikes. Figure 10A shows the typical appearance for both TTX-insensitive oscillations and spikes from neonatal LC neurons in 5% CO$_2$-aCSF plus TTX. Oscillations observed in 30 neurons from 12 slices from neonatal rats aged P8-P13 occur at a frequency of 0.3-1.0 Hz and are approximately 10 mV in amplitude. Rapid depolarizing TTX-insensitive spikes with amplitudes between 20-40 mV often synchronize with the depolarizing rise of oscillations. In agreement with previous studies (Filosa & Putnam, 2003), our data demonstrate that the addition of the L-type Ca$^{2+}$ inhibitor nifedipine completely and reversibly eliminates both TTX-insensitive oscillations as well as spikes (N=5, neurons from 5 slices) (Fig. 10 B and C). This suggests that both oscillations and spikes arise from the activity of L-type Ca$^{2+}$ channels.

Despite the similar pharmacological characteristics of oscillations and spikes, they show differing voltage responses under whole cell patch conditions. Figure 11A shows the sensitivity of spikes to changes in $V_m$ due to injected current through a whole cell patch on the LC soma. These results were repeated in 23 neurons from 14 slices from neonates aged P5-P14. Typically, TTX-insensitive spikes appear upon depolarizations to approximately -35 mV or greater. Depolarizations from the average resting membrane potential of ~ -45 mV to ~ -35 mV can be observed upon exposure to TTX. When additional depolarizing current is applied, large increases in spike frequency can be observed, whereas hyperpolarizing current can completely eliminate the appearance of
Figure 10:  
A. Typical appearance of both TTX-insensitive oscillations and spikes (from a P10 animal). The arrow marks injection of depolarizing current to induce spikes.

B. (top panel) The addition of nifedipine results in the complete elimination of both oscillations and spikes. The addition of depolarizing current (arrow) in the presence of nifedipine fails to elicit spikes. (bottom panel) Washing the nifedipine-exposed neuron with normocapnic aCSF + TTX restores the appearance of both oscillations and spikes.

C. Summary of the effects of nifedipine on oscillations and spikes. Note that nifedipine completely abolishes both TTX-insensitive oscillations and spikes. Bars represent means ± SEM. N=5.
**Figure 11:** The effects of membrane potential on TTX-insensitive spikes and oscillations.  

**A.** Arrows mark the injection of either hyperpolarizing or depolarizing current into the soma through the whole cell patch pipette. Small hyperpolarizing or depolarizing injections (sufficient for a < 5 mV change in membrane potential) result in either decreases or increases in spike frequency, respectively.  

**B.** Arrows mark the injection of hyperpolarizing current into TTX-exposed LC neurons. Large hyperpolarizing current injections cause no change in either oscillation amplitude or frequency.  

**C.** The addition of 100 μM carbenoxolone (in 5% CO₂) inhibits TTX-insensitive oscillations (right panels) but does not affect TTX-insensitive spikes (left panels).
Figure 11.
spikes, indicating that TTX-insensitive spikes are voltage sensitive (Fig. 11A). In contrast, TTX-insensitive oscillations appear to be largely insensitive to changes of $V_m$ in the LC neuron soma. Figure 11B shows a step-wise hyperpolarization of the LC soma resting $V_m$ to ~ -70 mV with no observed change in either the amplitude or frequency of oscillations. These observations were repeated in 14 LC neurons from 10 slices from neonates aged P7-P16. As oscillations still demonstrate sensitivity to the L-type Ca$^{2+}$ channel inhibitor nifedipine (Fig. 10B), it is possible that the oscillations arise from channels located at some distance from the soma. In these distal regions, $V_m$ changes observed in the soma would be largely attenuated. Thus, TTX-insensitive spikes and oscillations may arise from channels with similar electrophysiological and pharmacological characteristics, but respond differently to changes of $V_m$ due to being located in different regions of an LC neuron. Consistent with this hypothesis, the addition of 100 μM carbenoxolone was shown to inhibit TTX-insensitive oscillations, but not spikes, in 3 LC neurons from 3 slices aged P10-P16 (Fig. 11C). This suggests that TTX-insensitive oscillations are dependent upon communication via gap junctions while spikes are not (Fig. 11C).

The conclusion that channels causing the rhythmic, TTX-insensitive oscillations are located some distance from the LC soma is also supported by intracellular Ca$^{2+}$ studies. Figure 12A and 12B show the measurement of intracellular Ca$^{2+}$ in the soma of two different LC neurons in the presence of TTX. In figure 12A, the neuron demonstrated TTX-insensitive spikes but not oscillations. Simultaneous electrophysiological and imaging studies of this neuron showed that increases in spike frequency correlated with a
Figure 12: Simultaneous whole cell patch and Fura-2 imaging, showing TTX-insensitive spikes (A, C) or oscillations (B) plus changes in intracellular (somal) Ca\(^{2+}\). 

**A.** An increase in spike frequency causes a concurrent increase in somal Ca\(^{2+}\) levels. Arrow marks the injection of hyperpolarizing current which eliminates spikes and results in a return to baseline Ca\(^{2+}\) levels. 

**B.** The presence or absence of TTX-insensitive oscillations has no effect on soma Ca\(^{2+}\) levels. 

**C.** Large (> 0.1 R\(_{n}\)) and small (< 0.1 R\(_{n}\)) relative increases in intracellular Ca\(^{2+}\) levels compared to the corresponding increases in spike frequency. Larger changes in spike frequency are significantly correlated with larger changes in somal Ca\(^{2+}\). Bars represent means ± SEM.
concurrent increase in somal Ca\textsuperscript{2+} concentrations (increase in R\textsubscript{h}) (Fig. 12A). When hyperpolarizing current was injected to inhibit the TTX-insensitive spikes, intracellular Ca\textsuperscript{2+} rapidly returned to baseline levels. In Figure 12B, the neuron was hyperpolarized to remove the presence of spikes, and oscillations were transiently stimulated by the addition of 15% CO\textsubscript{2} (hypercapnic acidotic solution) (N=6 from 6 slices in rats aged P5-P7) (see Fig. 15). Increased oscillation frequency (typically from 0 to 0.5 Hz) was not associated with a change in the soma Ca\textsuperscript{2+} level (a typical resting R\textsubscript{h} value might be between 0.79 and 0.82, with no change between 5% and 15% CO\textsubscript{2} as in Fig. 12B). This is consistent with TTX-insensitive spikes arising from the activation of Ca\textsuperscript{2+} channels located in the soma, while oscillations arise from Ca\textsuperscript{2+} channels located distal to the soma. The magnitude of the increase in somal Ca\textsuperscript{2+} concentrations appears to be a function of TTX-insensitive spike frequency with smaller changes of somal intracellular Ca\textsuperscript{2+} (<0.1 R\textsubscript{h}) being associated with relatively small increases in spike frequency (N=10 neurons from 7 slices in rats aged P7-P13), while larger changes of intracellular Ca\textsuperscript{2+} (>0.1 R\textsubscript{h}) were associated with larger increases in spike frequency (N=7 neurons from 4 slices in rats aged P8-P13) (Fig. 12C). These data indicate the presence of L-type Ca\textsuperscript{2+} channels in the somal membrane capable of increasing somal Ca\textsuperscript{2+} in LC neurons in a fashion that is dependent on spike frequency.

Effects of CO\textsubscript{2} and pH on Ca\textsuperscript{2+} spikes and oscillations.

To test the CO\textsubscript{2} sensitivity of the L-type Ca\textsuperscript{2+} currents in the largely chemosensitive neurons from the LC, we exposed neurons from neonatal animals between P10 and P14 to HEPES-buffered aCSF at pH 7.45 (nominal absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}) (Fig. 13). In the absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}, the TTX-insensitive oscillations that
Figure 13: Effects of the removal of CO₂/HCO₃ on TTX-insensitive oscillations and spikes (from a P12 rat). A. Variable, typically absent appearance of oscillations in HEPES-buffered aCSF equilibrated with 100% O₂. Inset shows that a large injection of depolarizing current is necessary to induce TTX-insensitive spikes. B. Changing from HEPES solution to normocapnic, HCO₃-buffered aCSF results in the restoration of both TTX-insensitive oscillations and spikes. Notice the appearance of spikes without the addition of depolarizing current. C. Returning to HEPES-buffered aCSF solution once again results in the inhibition of both spikes and oscillations.
Figure 13.

A. N=7

B. 5% CO₂

C. No CO₂/HCO₃
were observed were very small, varied in appearance, and were sometimes completely
absent (N=7 from 4 slices from rats aged P11-P13; Fig. 13A). TTX-insensitive spikes
were also absent in the HEPES-buffered solutions, but could be induced by membrane
depolarizations to above -25mV (Fig. 13A, inset). Restoring normocapnic, HCO₃-
buffered aCSF to the same neuron restored the normal appearance of both spikes and
oscillations (Fig. 13B). Also of note was the activation of TTX-insensitive spikes
without the addition of strong depolarizing current (Fig. 13B). Both oscillations and
spikes were again lost if CO₂/HCO₃⁻ was replaced by a HEPES-buffered solution (N=4
neurons from 2 slices from rats aged P12-P13). These findings suggest that the threshold
for TTX-insensitive spikes is lowered by the presence of CO₂/HCO₃⁻.

Hypercapnic acidosis reversibly increased spike (Fig. 14A) and oscillation (Fig.
14B) frequency, as previously observed (Filosa & Putnam, 2003; Oyamada et al., 1999).
Since hypercapnic acidosis appears to activate L-type Ca²⁺ channels, we tested whether it
is a change of CO₂, HCO₃⁻ or pH that is the basis for activation. For these studies we
used isohydric hypercapnic solutions, in which the HCO₃⁻ concentration is increased to
maintain extracellular pH (pHₒ) at 7.45 when equilibrated with 15% CO₂. To monitor
pHᵢ changes, we loaded the neuron with the pH-sensitive dye pyranine through the whole
cell patch. Figure 15A shows the normal decrease in pHᵢ due to exposure to hypercapnic
acidosis (~0.15 pH unit), and the smaller decrease in pHᵢ due to exposure to isohydric
hypercapnia (~0.05 pH unit). Despite the marked larger changes in both pHₒ and pHᵢ in
hypercapnic acidotic versus isohydric hypercapnic solutions, both oscillations and spikes
demonstrated a similar increase in frequency when exposed to 15% CO₂ (Fig. 15B). This
is clearly shown in figure 15C, where spike frequency increases in a dose-dependent
**Figure 14:** Reversible increases in TTX-insensitive spike (A) and oscillation (B) frequency due to hypercapnia (15% CO$_2$), indicating that these spikes and oscillations can be stimulated by increased CO$_2$/H$^+$. 
Figure 14.
Figure 15: Simultaneous whole cell patch and loading with a pH sensitive dye, showing $pH_i$ changes and the effects on the TTX-insensitive current.  

A. Hypercapnic acidosis (HA) causes a large decrease in $pH_i$ while isohydric hypercapnia (IH) causes a smaller, more variable decrease in $pH_i$.  

B. HA and IH result in similar frequency and amplitude for both oscillations and spikes despite their different effects on $pH_i$.  

C. $CO_2$ causes dose-dependent increases in the TTX-insensitive spike frequency. Despite the diminished intracellular and extracellular acidification seen with IH solutions, no decrease in spike rate is observed in 15% $CO_2$ (HA vs. IH).  

HA and IH $\Delta Hz$ values (firing rate in 15% $CO_2$ – firing rate in 5% $CO_2$) are significantly increased from $\Delta Hz$ values for 10% $CO_2$ (firing rate in 10% $CO_2$ – firing rate in 5% $CO_2$), with $P<0.01$ and $P<0.001$, respectively. Bars represent means $\pm$ SEM.
Figure 15.

A. 

B. 

C. 

Δ Frequency (Hz)
fashion with respect to changes of CO₂ but not with respect to changes of pHᵢ or pHₒ. Here, the change in spike frequency was higher from exposure to 15% CO₂ than 10% CO₂ (N=12 neurons from 10 slices in rats aged P9-P14), whereas there was no difference between the increases caused by 15% CO₂ and isohydric hypercapnia (N=6 neurons from 5 slices in rats aged P8-P14). These data suggest that changes in pH are not necessary for the CO₂-dependent activation of the L-type Ca²⁺ current in LC neurons.

L-type Ca²⁺ channels from peripheral chemoreceptors were activated in hypercapnia by a pathway that involves increased intracellular HCO₃⁻ ([HCO₃⁻]ᵢ) (Summers et al., 2002). Interestingly, isohydric hypercapnia resulted in a somewhat higher increase in spike frequency than hypercapnic acidosis (Fig. 15C). In 6 of the neurons studied, spike frequency was measured in both isohydric hypercapnia and hypercapnic acidosis. In this subset of LC neurons, the increase in spike frequency in response to isohydric hypercapnia was found to be significantly higher than the response to hypercapnic acidosis (P<0.001). This correlates with higher calculated values of [HCO₃⁻]ᵢ in isohydric hypercapnia than in hypercapnic acidosis. We further studied whether increased [HCO₃⁻]ᵢ could be involved in the activation of L-type Ca²⁺ channels by CO₂ in LC neurons. Based on our measurements of pHᵢ and the level of CO₂, we calculated [HCO₃⁻]ᵢ using the Henderson-Hasselbalch equation. A plot of TTX-insensitive spike frequency versus [HCO₃⁻]ᵢ values shows a significant positive correlation with a best fit slope of: Frequency = 0.017 [HCO₃⁻]ᵢ – 0.039, R² = 0.494 (P<0.01) (Fig. 16A) (N=11 from 8 slices aged P8-P14). Figure 16B shows the same relationship for the frequency of TTX-insensitive oscillations (N=34 from 20 slices aged
Figure 16:  
A. Plot of intracellular HCO$_3^-$ (mM) vs. TTX-insensitive spike frequency. Values were taken in neurons with membrane potentials between -32 to -37 mV.  
B. Plot of intracellular HCO$_3^-$ (mM) vs. TTX-insensitive oscillation frequency. In this chart, a clear age-related development of oscillation frequency is observed. Inset represents data points from 3 individual neurons from rats aged P16 (slope = 0.0116 Hz/mM HCO$_3^-$), P10 (slope = 0.014 Hz/mM HCO$_3^-$), and P8 (slope = 0.0161 Hz/mM HCO$_3^-$).
Figure 16.
Both charts indicate a strong correlation between $[\text{HCO}_3^-]_i$ and the frequency of the L-type TTX-insensitive current, suggesting that the activation of L-type Ca$^{2+}$ channels by elevated CO$_2$ may be mediated by $[\text{HCO}_3^-]_i$.

*Neonatal Development of L-type Ca$^+$ channels in LC neurons.*

Consistent with our hypothesis and the developmental changes noted by Gargaglioni et al (2010), we noticed variation in the frequency of Ca$^{2+}$ oscillations among animals when studying the effects of $[\text{HCO}_3^-]_i$. This variation appeared to be related to the age of the rat from which a neuron was studied so we examined the effects of $[\text{HCO}_3^-]_i$ on Ca$^{2+}$ oscillation frequency as a function of age (Fig. 16B). For neurons from rats older than postnatal day 6 (P6), the frequency of oscillations was positively correlated with $[\text{HCO}_3^-]_i$. Further, the oscillation frequency increased as the neonatal animal aged. Fit values are as follows: P3-P5- $R^2 = 0.0574$ (N.S.) (N=7 from 6 slices); P7-P9- Frequency = 0.0173$[\text{HCO}_3^-]_i - 0.2398$, $R^2 = 0.69$ (P<0.001) (N=9 from 6 slices); P10-P12- Frequency = 0.0119$[\text{HCO}_3^-]_i + 0.438$, $R^2 = 0.52$ (P<0.001) (N=14 from 7 slices); and >P13- Frequency = 0.0105$[\text{HCO}_3^-]_i + 1.217$, $R^2 = 0.48$ (P<0.01) (N=4 from 2 slices). These results suggest that L-type Ca$^{2+}$ channels that are distal to the soma show considerable development during the early postnatal period.

In order to examine the effects of $[\text{HCO}_3^-]_i$ on spike frequency, it was necessary to control for voltage sensitivity. Neurons with spike frequencies from similar $V_m$ values only were included in the data set in figure 16A. As with oscillations, spikes showed a strong positive correlation with $[\text{HCO}_3^-]_i$ (N=11 from 8 slices aged P8-P14) (fig 16A). Due to the dependence of spike frequency on both $[\text{HCO}_3^-]_i$ and voltage, neurons were selected for this study based on the appearance of spikes between a control voltage of -32
to -37 mV. Neurons meeting these criteria were observed from rats aged P8-P14, and so developmental changes in spike frequency were not accurately represented. However, when spike frequencies were observed under normocapnic conditions as a control for $[\text{HCO}_3^-]_i$ in LC neurons, a strong dependence of spike frequency on age was seen (Fig 17). Thus, Ca$^{2+}$ spikes and oscillations in LC neurons appear to be dependent upon $[\text{HCO}_3^-]_i$ and to undergo developmental increases from ages P3 to P16.

We studied in detail the age-dependence of the appearance of Ca$^{2+}$ oscillations and spikes in LC neurons in the presence of 5% CO$_2$ and of 15% CO$_2$. A transition period existed (P4-P9) whereby the appearance of Ca$^{2+}$ spikes and oscillations were highly variable and their amplitudes were smaller. Prior to age ~P9 the Ca$^{2+}$ currents observed in TTX were usually only seen after activation by hypercapnia (N=15 in 8 slices) (Fig. 18A). Upon return to normocapnia, the Ca$^{2+}$ currents were no longer visible. During this transition period, TTX-insensitive oscillations activated by hypercapnic acidosis were often small in amplitude and frequency, and spikes were often absent without additional depolarizing current (Fig. 18B). After age ~P10, TTX insensitive oscillations and spikes were larger and both oscillations and spikes occurred spontaneously without activation by hypercapnia. Our findings are summarized in Figure 19. Of 35 neurons from 20 slices, 23 exhibited spikes. There were 23 neurons in this group younger than P9, 12 of which did not demonstrate spikes. The majority of neurons that did not show spikes (N=10) were from rats younger than P7. Thus, Ca$^{2+}$ spikes are either largely absent or occur only in the presence of 15% CO$_2$ in LC neurons from rats younger than P8-P9 (Fig. 19A). However, Ca$^{2+}$ spikes were observed in most LC neurons from rats age P8-P9 even in the absence of hypercapnia. Spikes were also
**Figure 17:** An age-related development in TTX-insensitive spike frequency is observed similar to that noted for TTX-insensitive oscillations (Fig. 16B). All values were taken without the addition of depolarizing current in normocapnic aCSF. Age group values were significantly different from one another with $P < 0.001$. P13: N=9 from 5 neurons, 5 slices; P12-P10 N=36 from 24 neurons, 16 slices; P7-P9 N=5 from 4 neurons, 4 slices. Bars represent means ± SEM.
Figure 17.
Figure 18: Observations of TTX-insensitive spikes and oscillations in LC neurons from rats ages P3-P10. Prior to age ~P9, a transition period exists whereby oscillations and spikes can only be observed in hypercapnic aCSF. A. Record from a P8 animal showing activation of both TTX-insensitive spikes and oscillations in 15% CO$_2$. When 5% CO$_2$ is restored, both spikes and oscillations are no longer present. B. A comparison of the appearance and amplitude of TTX-insensitive oscillations/spikes activated by CO$_2$ versus age of neonatal rat. All records were taken in hypercapnia (15% CO$_2$). Note that in a young neonate (P3), even in 15% CO$_2$, no oscillations or spikes are seen. In neonates aged P5-P7, oscillations but not spikes are apparent in 15% CO$_2$. In a neonate aged P10, spikes are clearly evident in 15% CO$_2$. 
Figure 18.
observed in all LC neurons from rats older than P10, both in 5% and 15% CO$_2$ (Fig. 19A). A similar pattern of development was seen for Ca$^{2+}$ oscillations (Fig. 19B). From 26 neurons aged younger than P9, 8 did not show oscillations, and the majority of these neurons were from animals younger than P7. In 57% (N=15) of neurons younger than P9, oscillations could be evoked by hypercapnia; while oscillations were observed in all 20 neurons from 11 slices aged P10 to P16. Once again, most LC neurons from rats younger than P10 showed no Ca$^{2+}$ oscillations or only showed oscillations in the presence of 15% CO$_2$, but both oscillations and spikes were omnipresent in LC neurons from rats older than ~P10 (Fig. 19B). From this data set, there were 8 neurons from animals younger than P7 that showed neither oscillations nor spikes under any conditions. These data strongly suggest that Ca$^{2+}$ channels develop markedly during the neonatal period in rat LC neurons.
Figure 19: Summary of the effects of age on the appearance of TTX-insensitive spikes (A) and oscillations (B). In these records, depolarizing current was injected to bring membrane potential to -20 mV. Any spikes observed under these conditions in either 15% CO₂ or 5% and 15% CO₂ were recorded.
Figure 19.
Discussion

In this study, we have systematically characterized the appearance and development of chemosensitive TTX-insensitive current in LC neurons from neonatal rats. We found that 1) TTX-insensitive currents expressed as spikes and oscillations; 2) both spikes and oscillations were inhibited by the L-type Ca\(^{2+}\) channel inhibitor nifedipine; 3) spikes but not oscillations are capable of increasing somal Ca\(^{2+}\); 4) both spikes and oscillations were dependent on the presence of CO\(_2\)/HCO\(_3^-\); 5) CO\(_2\)-induce activation of spikes and oscillations appeared to be mediated by increased [HCO\(_3^-\)]; and 6) both oscillations and spikes showed a marked increase during neonatal development.

*Ca\(^{2+}\) currents in LC neurons.*

A rhythmic, TTX-insensitive current in LC neurons, due to L-type Ca\(^{2+}\) channels, has been previously described (Filosa & Putnam, 2003; Oyamada et al., 1999). In our work, we have shown that TTX-insensitive oscillations and spikes in LC neurons are both reversibly inhibited by the L-type Ca\(^{2+}\) channel inhibitor nifedipine, but respond differently to voltage changes in the soma of the LC neuron (see Figs. 10 and 11). Furthermore, our findings show that changes in TTX-insensitive spikes are associated with changes in the somal Ca\(^{2+}\) levels whereas changes in the TTX-insensitive oscillations are not (see Fig. 12). Thus, the Ca\(^{2+}\) channel-based oscillations and spikes may represent isolated populations of L-type Ca\(^{2+}\) channels in the dendrites and soma, respectively. The distal population of Ca\(^{2+}\) channels would not be affected by V\(_m\) changes in the soma, as we observed (Fig. 12). Alternatively, it has been suggested that TTX-insensitive oscillations reflect the strong synchronized firing patterns of LC neurons as seen through gap junctions (Andrzejewski et al., 2001; Maubecin & Williams, 1999).
In this theory, distal Ca\textsuperscript{2+} channels actually reside in adjacent neurons and the oscillating currents travel through gap junctions. This theory is supported by the elimination of TTX-insensitive oscillations by the gap junction blocker carbenoxolone in LC neurons (Ballantyne et al., 2004)(Fig. 11C). However, in our current study, we show that TTX-insensitive spikes are not sensitive to carbenoxolone (Fig. 11C). Thus, although immunohistochemical studies will be required to determine the actual distribution of L-type Ca\textsuperscript{2+} channels in LC neurons, there can be little doubt that L-type Ca\textsuperscript{2+} channels reside in or near the soma of LC neurons.

Chemosensitivity of L-type Ca\textsuperscript{2+} oscillations and spikes: Mechanism of activation.

In agreement with previous studies, we have shown that both Ca\textsuperscript{2+}-based spikes and oscillations increase with CO\textsubscript{2} (Filosa & Putnam, 2003). This is unusual, since acidification is commonly expected to inhibit Ca\textsuperscript{2+} channels (Shah et al., 2001; Tombaugh & Somjen, 1997). In an attempt to determine the mechanism of hypercapnic activation, we exposed slices from P12 rats to HEPES-buffered aCSF with a nominal absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}. TTX-insensitive spikes could be seen, but only with depolarizing current bringing V\textsubscript{m} to at least -25 mV, while oscillations were transient and reduced in amplitude (see Fig. 13). Both of these observations were unusual since the neurons were from older (>P10) rats. Normocapnic aCSF restored the normal appearance and chemosensitivity of the TTX-insensitive oscillations and spikes, and returning the patched neuron to HEPES-buffered aCSF again inhibited TTX-insensitive oscillations and spikes (see Fig. 13). These data suggest that CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} is necessary for the normal function of the L-type Ca\textsuperscript{2+} channel oscillations and spikes in LC neurons. Moreover, CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} appears to shift the activation voltage for TTX-insensitive spikes to a more
hyperpolarized $V_m$. These data may indicate a mechanism for L-type $\text{Ca}^{2+}$ current activation that is $\text{HCO}_3^-$-based (Fig 20, pathway 1).

To examine a potential role for changes of pH in hypercapnic activation of the L-type $\text{Ca}^{2+}$ current in LC neurons, we used isohydric hypercapnic solutions. In isohydric hypercapnia the same increase in CO$_2$ is present as in hypercapnic acidic solutions, but the extracellular pH is unchanged and intracellular acidification is roughly one-third or less than the intracellular acidification observed with hypercapnic acidosis (see Fig. 15A). With a diminished acidification in both pH$_o$ and pH$_i$, it would be expected that the chemosensitive increase in frequency for both spikes and oscillations would be decreased if changes of pH played a role in their activation. This is especially true for $\text{Ca}^{2+}$ spikes, as reduced acidification in LC neurons would result in a smaller hypercapnia-induced depolarization from $\text{H}^+$-inhibited $\text{K}^+$ channels (Filosa & Putnam, 2003; Li & Putnam, 2009). However, no change in the hypercapnic response of either oscillations or spikes was observed in isohydric hypercapnic solutions compared to hypercapnic acidic solutions (Fig. 15B). Figure 15C summarizes the dose-dependent increase of $\text{Ca}^{2+}$ spikes to CO$_2$, demonstrating that while 15% CO$_2$ clearly increases spike frequency over 10% CO$_2$, there is no significant difference in spike frequency between the isohydric hypercapnic and hypercapnic acidic solutions. This further supports a CO$_2$/HCO$_3^-$-based rather than a pH-based hypercapnic activation of L-type $\text{Ca}^{2+}$ oscillations and spikes. In fact, a paired comparison of the spike frequency response to isohydric hypercapnia vs. hypercapnic acidosis showed that both spike frequency and $[\text{HCO}_3^-]_i$ were significantly higher in isohydric hypercapnia vs. hypercapnic acidotic solutions,
Figure 20: Model of the chemosensitive K⁺- and Ca²⁺-sensitive pathways in LC neurons. Numbered pathways are referred to in the text. Left side represents a summary of the proposed role of H⁺-sensitive K⁺ channels in the hypercapnic depolarization and increase in firing rate in chemosensitive LC neurons. Right side depicts a possible pathway for hypercapnic activation of Ca²⁺ channels and potential roles for Ca²⁺ in chemosensitive signaling in LC neurons.
Figure 20.

HYPERCAPNIA

1. $\uparrow [\text{HCO}_3^-]_i$

2. $\uparrow \text{sAC ?}$

$\uparrow \text{Ca}^{2+} \text{ channel}$

3. Depolarization

$\uparrow \text{Firing Rate}$

$\downarrow \text{K}^+ \text{ channel}$

$\uparrow \text{Firing Rate}$

$\uparrow \text{K}_\text{ca channel}$

$\downarrow \text{Firing Rate}$

$\uparrow [\text{Ca}^{2+}]_i$

4. Neuronal Death

5.
while CO₂ was the same. This suggests that activation of L-type Ca^{2+} oscillations and spikes is due to increased [HCO₃⁻]ᵢ.

A relationship between [HCO₃⁻]ᵢ and Ca^{2+} spike and oscillation frequency is shown in figure 16. Interestingly, the slopes of the best-fit lines for age groups above P6 are very similar for both Ca^{2+} spikes and oscillations (~0.01-0.02 Hz/mM HCO₃ᵢ). These data are consistent with oscillations and spikes arising from the same type of Ca^{2+} channel and with hypercapnic activation being similar for oscillations and spikes. A mechanism for [HCO₃⁻]ᵢ activation of L-type Ca^{2+} channels has been proposed for peripheral chemoreceptor glomus cells in the carotid body (Summers et al., 2002). In this mechanism, [HCO₃⁻]ᵢ activates L-type Ca^{2+} channels via a HCO₃⁻-sensitive soluble adenylate cyclase (sAC). Increased intracellular HCO₃⁻ activates sAC resulting in increased intracellular levels of cAMP, activation of protein kinase A, and activation of L-type Ca^{2+} channels due to their phosphorylation (Fig 20, pathway 2). In peripheral chemoreceptive glomus cells, therefore, elevated [HCO₃⁻]ᵢ resulted in an increase in the magnitude of Ca^{2+} current as determined by whole cell voltage clamp recordings (Summers et al., 2002). It is possible that in LC neurons elevated [HCO₃⁻]ᵢ works through a similar pathway and causes an increase in the magnitude of the L-type Ca^{2+} current, but we did not directly study this pathway nor did we directly measure Ca^{2+} current. However, our findings suggest a shift in the threshold for voltage activation of L-type Ca^{2+} channels in LC neurons to more negative voltages (see Figs. 13, 15). Thus, at any given voltage, increased [HCO₃⁻]ᵢ should result in greater opening of L-type Ca^{2+} channels. Finally, we directly showed that hypercapnic-activation of L-type Ca^{2+}
channels resulted in a measurable increase in Ca\(^{2+}\)_i in the soma of LC neurons (see Fig. 12C and 20).

Postnatal development of L-type Ca\(^{2+}\) oscillations and spikes, a role for Ca\(^{2+}\).

Previous studies have shown that strong, rhythmic oscillations that regulate Na\(^{+}\) action potentials can be demonstrated in LC neurons from neonatal rats P1-P6 by blocking synaptic junctions with low Ca\(^{2+}\)/high Mg\(^{2+}\) (Andrzejewski et al., 2001; Oyamada et al., 1999). These oscillations can be completely abolished by TTX. Moreover, these Na\(^{+}\)-based, TTX-sensitive oscillations in LC neurons were shown to increase in frequency over ages P2-P17 and were inhibited by carbenoxolone (Christie et al., 1989; Oyamada et al., 1999). The Ca\(^{2+}\) -based oscillations under observation in this study are sensitive to both carbenoxolone and nifedipine and thus appear to rely solely on L-type Ca\(^{2+}\) channels and gap junctions (Ballantyne et al., 2004; Filosa & Putnam, 2003). Further, Gargaglioni et al. (2010) reported a shift in the chemosensitive response of LC neurons around age P10. Our findings suggest that the L-type Ca\(^{2+}\) oscillations in LC neurons undergo postnatal development over the age P3 to P16 such that prior to age ~P10, Ca\(^{2+}\) oscillations are typically absent without activation by hypercapnia (see Fig. 18, 19). Thus, these oscillations are distinct from Na\(^{+}\)-based oscillations, and may also account for the shift in chemosensitivity suggested by Gargaglioni et al. (2010). Over postnatal age P3-P16, Ca\(^{2+}\) oscillations increase in both amplitude and frequency (see Figs. 16B, 18, 19). Prior to age ~P10, Ca\(^{2+}\) spikes are also typically absent without either exposure to hypercapnia or the addition of strong depolarizing current (see Fig. 18, 19). After age ~P10, Ca\(^{2+}\) spikes appear without the addition of depolarizing current, and show a steady increase in frequency under these conditions (see Fig. 17). Thus, our data
suggest the presence of an L-type Ca\(^{2+}\) current in the LC neuron soma that increases
during early postnatal development and activates during exposure to CO\(_2\)/HCO\(_3^-\) to
increase intrasomal Ca\(^{2+}\) levels. We do not know if this developmental change is due to
changes in the properties of or changes in the level of expression of L-type Ca\(^{2+}\) channels
during early neonatal development in LC neurons. Nevertheless, the development of
these Ca\(^{2+}\) currents suggests that the role for Ca\(^{2+}\) in the LC chemosensitive response may
change as neonatal rats age, and may account for shifts in the intrinsic chemosensitivity
of LC neurons (Gargaglioni et al., 2010).

Membrane properties, gap junctions, and voltage-activated L-type Ca\(^{2+}\) channels:
significance to TTX-insensitive oscillations and spikes.

A discussion of changes in the membrane properties of LC neurons and their
possible effects on oscillations and spikes is a complex topic. Our current data indicate
that the amplitude of oscillations increases as the animal ages. This could be due to a
change in the number of channels expressed that contribute to the rhythmical TTX-
insensitive current, or increases in the input resistance and changes in the gap junction
coupling of neonatal LC neurons.

Studies have shown that action potentials, oscillations, and small fluctuations of
the V\(_m\) are synchronized by gap junction coupling between LC neurons (Andrzejewski et
al., 2001; Christie et al., 1989; Ishimatsu & Williams, 1996). Although injecting current
into single LC neurons failed to induce action potentials in adjacent neurons, sustained
(>100ms) current injections could be passed between neonatal LC neurons (<P10) when:
1) input resistance was increased using TEA; and 2) natively occurring oscillations were
inhibited using TTX and high MgCl\(_2\) (Andrzejewski et al., 2001; Christie et al., 1989).
Collectively, these findings led researchers to propose that oscillations were the result of the synchronized summation of single action potentials across multiple neurons coupled by low-resistance electrical pathways, such as via gap junctions (Andrzejewski et al., 2001; Ishimatsu & Williams, 1996). In other words, the long electrotonic length and low resistance of the coupling allows multiple simultaneous fast depolarizations to combine as slower, smaller amplitude oscillations, while non-synchronized depolarizations in a single neuron are filtered out. Based on this hypothesis, the TTX-insensitive oscillations that we observe could be the result of the activity of Ca\textsuperscript{2+} spikes across multiple gap-junction coupled neurons. This is in agreement with our current data, as both spikes and oscillations appear to have similar properties and development. During the transition age, for example, we can speculate that the depolarization induced by hypercapnia enables the activation of spikes within multiple neurons in the network, and thereby evokes oscillations in the patched neuron in addition to spikes. Thus, increases in the amount of gap junction coupling would increase the amplitude of the oscillations observed in neonatal LC neurons. Frequency would be predominantly dependent on the average resting $V_m$ established by the network and the properties of the voltage-sensitive Ca\textsuperscript{2+} channels.

It has also been observed that in LC neurons from very young (<P6) or older (>24 days) neonatal rats where oscillations are not seen, application of either Ba\textsuperscript{2+} or TEA can restore rhythmic oscillations (Andrzejewski et al., 2001; Ishimatsu & Williams, 1996). While Ba\textsuperscript{2+} increases the conductivity through voltage activated Ca\textsuperscript{2+} channels, both drugs also increase the input resistance of neurons by blocking K\textsuperscript{+} channels. The combination of these two actions may facilitate the appearance of the synchronized
depolarizations through distant, low-resistance gap junction pathways (Maubecin & Williams, 1999). However, the input resistance of individual LC neurons from neonatal rats younger than P15 was measured to be 67 MΩ versus values from adult rat LC neurons measured at 213 MΩ using sharp-tip electrodes (Christie et al., 1989; Williams et al., 1984). Since the amplitude of oscillations was found to decrease in rats over 24 days of age, this suggests that input resistance of individual neurons does not significantly contribute to the development of oscillation amplitude. Thus, the loss of amplitude and synchronization of oscillations in LC neurons from animals >24 days old may support a loss of coupling between LC neurons as rats age (Andrzejewski et al., 2001; Christie et al., 1989; Maubecin & Williams, 1999).

A third possibility is that there is an increase in the number of Ca^{2+} channels expressed in LC neurons that contributes to the amplitude of spikes, and, hence, the amplitude of oscillations. Although our current study does not quantitatively examine the amplitude of spikes or oscillations, our data suggest that there is an increase in the total Ca^{2+} current in LC neurons during the first three postnatal weeks. If so, Ca^{2+} could play an increasingly important role in chemosensitive signaling with development.

Significance.

The presence of oscillations and spikes arising from L-type Ca^{2+} channels in LC neurons could be of significance in several ways. An amplification role has been proposed for the L-type Ca^{2+} oscillations, whereby an increase in the depolarizing current arising from Ca^{2+} channels (Fig. 20, pathway 3) and passing through gap junctions in individual neurons may serve to increase the number of neurons capable of responding to synaptic input (Christie et al., 1989). In the case of intrinsically chemosensitive neurons
of the LC, this may mean synchronization of the gap-junction coupled network to the collective stimulations of single neurons that respond to CO$_2$ and amplification of the chemosensitive response of the LC to hypercapnia.

A different role for Ca$^{2+}$ oscillations has been proposed in the substantia nigra pars compacta in association with Parkinson’s disease (Chan et al., 2007). Here, an increase in rhythmic oscillations due to Cav1.3 Ca$^{2+}$ channels were theorized to be associated with increased Ca$^{2+}$ influx and eventual damage to the dopaminergic neurons, resulting in disease (Fig 20, pathway 4) (Chan et al., 2007). A similar pathway may be at play in LC neurons in patients with post-traumatic stress disorder (PTSD). Evidence for abnormal LC neuron cell death was observed during postmortem exams of PTSD patients (Bracha et al., 2005). Given the data for increased LC neuron Ca$^{2+}$ channel activity during stress (i.e., increased TTX-insensitive oscillation frequency), it is possible that Ca$^{2+}$ currents in LC neurons play a potentially damaging role similar to that seen in substantia nigra (Berridge et al., 2000; Chan et al., 2007; Jedema & Grace, 2004).

Our findings show that hypercapnia activates L-type Ca$^{2+}$ channels and results in increased intracellular Ca$^{2+}$. This raises the interesting and relatively unexplored possibility that Ca$^{2+}$ plays a role in chemosensitivity (Putnam et al., 2004). One potential role for Ca$^{2+}$ would be that activation of L type Ca$^{2+}$ channels by hypercapnia augments CO$_2$-sensitive depolarization and increases the chemosensitive response (Fig 20, pathway 3). Filosa and Putnam (2003) showed that nifedipine resulted in a decrease in the firing rate response to hypercapnia, which is consistent with Ca$^{2+}$-channel activation enhancing the chemosensitive response of LC neurons. However, only LC neurons from animals younger than P9 were used in that study. In our current study, we show that an increase
of the L-type Ca\(^{2+}\) current occurs during postnatal development (Figs. 17-19), and may result in the role of Ca\(^{2+}\) in chemosensitive signaling varying with postnatal age in LC neurons (see Figs. 17-19).

Beyond the potential effect of hypercapnia-induced Ca\(^{2+}\) current on \(V_m\), the increase in Ca\(^{2+}\) opens several possibilities for effects on chemosensitive signaling. For example, hypercapnia-induced increases in Ca\(^{2+}\) could activate Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{Ca}\)) in LC neurons (Fakler & Adelman, 2008; Gargaglioni et al., 2010). Activation of K\(_{Ca}\) channels could then be acting as a “brake” to limit the firing rate response of LC neurons to hypercapnia (Fig 20, pathway 5). Aghajanian et al. (1983) made a similar conclusion when the activity of K\(_{Ca}\) channels resulted in a negative feedback function on the spontaneous firing rate in LC neurons. Our findings indicate that the LC Ca\(^{2+}\) current develops during the initial postnatal period, suggesting that the firing rate response of LC neurons to hypercapnia may be reduced during neonatal development due to increased activation of K\(_{Ca}\) channels. This is consistent with the observations by Gargaglioni et al. (2010) where a reduction of the chemosensitive response in LC neurons with neonatal development was reported. If the braking phenomenon does occur, it would suggest that LC neurons might play a reduced role in central chemosensitivity as neonatal development progresses. By extension, abnormalities with this braking pathway could lead to hypersensitivity of the respiratory response to hypercapnia, which has been found in pathological conditions such as panic disorder and sleep apnea (Lousberg et al., 1988; Nardi et al., 2009; Papp et al., 1993; Ryan & Bradley, 2005; Wang et al., 2007; Younes et al., 2001). Thus, it is likely that Ca\(^{2+}\) channels and intracellular Ca\(^{2+}\) concentration play important and perhaps varied
roles in central chemosensitivity, at least in LC neurons, and more detailed studies of these roles is warranted.
CHAPTER VI

The role of Ca$^{2+}$ and BK channels as a brake to the firing rate response of locus coeruleus (LC) neurons to CO$_2$
Abstract

Neurons from multiple brainstem regions including the locus coeruleus (LC) are thought to be involved in central chemoreception, or the ability of the central nervous system (CNS) to sense CO₂/H⁺ and regulate breathing. The cellular mechanisms by which this occurs are usually attributed to an “accelerator” whereby increases in CO₂/H⁺ cause an inhibition of K⁺ channels to depolarize CO₂-sensitive neurons. Recently, we have used whole cell patch clamping, the Ca²⁺-sensitive fluorescent dye fura-2, and immunohistochemical studies on Ca²⁺-activated K⁺ channels (K_{Ca}) to provide novel evidence for a chemosensitive Ca²⁺ current in neurons of the LC that may function as a “brake” for the chemosensitive response. We show that hypercapnia increases intracellular Ca²⁺ levels and that this leads to activation of K_{Ca} channels to enhance the K⁺ current and hyperpolarize the neuron, decreasing the firing rate response to hypercapnia. Coupled with our findings that the L-type Ca²⁺ current develops gradually over the first ~16 days of postnatal development in rat LC neurons, this indicates a role for Ca²⁺ in the maturation and control of the chemosensitive response in LC neurons. This “brake” pathway may also underlie the developmental decrease in the chemosensitivity of LC neurons. We show that the K_{Ca} channel inhibitor paxilline increases the chemosensitive response of neurons of the LC from postnatal rats aged P3 to P16. Ca²⁺ and BK currents concurrently increase over this age period. These findings represent a unique role for Ca²⁺ in controlling the chemosensitive gain of LC neurons.
Introduction

There are many acid-sensitive neurons in the brain that contribute to a number of biological processes, including the pathophysiology of ischemic stroke, learning and memory, psychiatric disorders such as panic disorder and depression, and central respiratory control (Bayliss et al., 2001; Coryell et al., 2009; Pineda & Aghajanian, 1997; Putnam et al., 2004; Wemmie et al., 2004; Ziemann et al., 2009). These neurons respond to a change in the chemical environment (i.e. altered $\text{CO}_2/\text{H}^+$), and are referred to as chemosensitive neurons.

Chemosensitive neurons have been reported in many regions of the brain, including the amygdala (Ziemann et al., 2009), the locus coeruleus (LC) (Elam et al., 1981; Filosa & Putnam, 2003; Oyamada et al., 1998; Pineda & Aghajanian, 1997), the medullary raphé (Richerson, 1995; Wang et al., 1998), the nucleus tractus solitarius (NTS) (Conrad et al., 2009; Dean et al., 1989; Nichols et al., 2009) and the retrotrapezoid nucleus (RTN) (Mulkey et al., 2004; Ritucci et al., 2005a). The magnitude of the firing rate response of a neuron to a given acid stimulus can be expressed as the chemosensitivity index (CI), which represents a percentage increase in control firing rate per 0.2 pH unit decrease in external pH ($\text{pH}_o$) (Wang et al., 1998; Wang & Richerson, 1999). Neurons from different chemosensitive regions have widely different magnitudes of response, based on CI, ranging from large responses (300%) in RTN neurons (Mulkey et al., 2004; Ritucci et al., 2005a) to very small responses (~125%, with 100% indicating a non-chemosensitive neuron) in LC neurons (Gargaglioni et al., 2010; Putnam et al., 2004).
The magnitude of the chemosensitive response is significant because a high degree of CO₂ sensitivity has been correlated with several pathological conditions. Increased CO₂ sensitivity has been observed in cases of sleep apnea (Verbraecken et al., 1995; Younes et al., 2001) and patients who experience periodic breathing during sleep (Chapman et al., 1988). Patients with panic disorder show an increased hypercapnic ventilatory response (Nardi et al., 2009; Papp et al., 1993), and several anti-anxiolytic drugs reduce the ventilatory response to hypercapnia (Gorman et al., 1997; Pols et al., 1993). Thus, understanding the cellular basis for the magnitude of the response of neurons to elevated CO₂/H⁺ is likely to be of significance to human pathology.

Studies of the magnitude of the chemosensitive response of medullary and pontine neurons from neonatal animals are complicated by various developmental changes. For example, neurons from the NTS exhibit virtually no developmental changes either in the percentage of neurons that respond to elevated CO₂/H⁺ or in the magnitude of that response (Conrad et al., 2009; Nichols et al., 2009). In contrast, neurons from the medullary raphé show a large increase in the magnitude of their response to acid stimuli after age postnatal day 12 (P12) (Hodges et al., 2009). A completely different pattern is exhibited by LC neurons, with a marked decrease in the magnitude of the chemosensitive response to acid stimuli in LC neurons from rats aged >P10 (Gargaglioni et al., 2010). These different development changes offer an interesting system for studying the cellular basis of the magnitude of the chemosensitive response.

The cellular basis for acid sensing in these chemosensitive neurons is not fully understood. Current studies have focused on various acid-sensitive ion channels (Putnam et al., 2004) such as inwardly-rectifying K⁺ (Kᵢₑ) channels (Pineda & Aghajanian, 1997;
Xu et al., 2000), delayed-rectifying K\(^+\) (K\(_{dr}\)) channels (Denton et al., 2007; Putnam, 2010), transient K\(^+\) channels (A current) (Denton et al., 2007; Putnam, 2010), TWIK-related acid sensitive K\(^+\) (TASK) channels (Bayliss et al., 2001), a calcium-activated non-selective cation (CAN) current (Putnam, 2010), acid-sensitive non-selective cation (ASIC) channels (Ziemann et al., 2009), transient receptor potential (TRP) channels (Cui et al., 2011), and L-type Ca\(^{2+}\) channels (Filosa & Putnam, 2003; Imber & Putnam, 2012). These channels are either inhibited (K\(_{ir}\), K\(_{dr}\), A current, and TASK) or activated (ASIC, CAN, TRP and L-type Ca\(^{2+}\) channels) by increased CO\(_2\)/H\(^+\) and this results in neuronal depolarization and increased neuron firing rate (Putnam et al., 2004; Putnam, 2010). Since these channels all result in increased neuronal firing rate in response to elevated CO\(_2\)/H\(^+\), they can be thought of as “accelerators” in the neuronal chemosensitive response.

The activation of Ca\(^{2+}\) channels by increased CO\(_2\)/H\(^+\) could be part of the accelerator pathway. Alternatively, we hypothesize that Ca\(^{2+}\) channels work to decrease the chemosensitive response to hypercapnia. Ca\(^{2+}\) channel activation results in increased [Ca\(^{2+}\)]\(_i\) (Imber & Putnam, 2012) and therefore could activate Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) channels. This would lead to a CO\(_2\)/H\(^+\)-induced membrane hyperpolarization that could be thought of as a “brake” in the neuronal chemosensitive response.

To study the possible presence of a braking phenomenon we have chosen to use LC neurons. These neurons have been shown to be involved in both respiratory control (Biancardi et al., 2008) and the expression of anxiety and panic disorders (Bailey et al., 2003). Previous studies have confirmed the presence of large-conductance K\(_{Ca}\) channels (“Big” K or BK channels) in the LC (Sausbier et al., 2006). Further, based on membrane
potential ($V_m$) oscillations that arise from the activity of Ca$^{2+}$ channels, we have observed that Ca$^{2+}$ channels show a developmental pattern with increased channel activity in neurons from rats older than P10 (Imber & Putnam, 2012). This leads us to further hypothesize that the proposed braking pathway develops during the neonatal period. Since the observed fall in the magnitude of the chemosensitive response in LC neurons occurs after day P10, we propose that the Ca$^{2+}$ current in LC neurons would show a concurrent increase during this age period.

The main purpose of the current study was to provide evidence for the presence of a hypercapnia-induced braking pathway in LC neurons and to study the role of that pathway in the changes of CI seen in LC neurons during the neonatal period. We show that a hypercapnia-induced braking pathway that involves the activation of BK channels does exist in LC neurons. Inhibition of the BK channel results in an increased chemosensitive response in LC neurons from older neonates. This braking pathway may represent a novel target for altering the gain of chemosensitive neurons that could have implications for pathological conditions such as respiratory and panic disorders.
Methods

Ethical approval

All procedures in which animals were involved were reviewed and approved by the Wright State University Institutional Animal Care and Use Committee and are in agreement with standards set out in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01).

Slice preparation

Mixed sex neonatal Sprague-Dawley rats postnatal (P) age P3-P16 were anesthetized using high CO₂ or hypothermia and then rapidly decapitated as previously described (Filosa et al., 2002; Ritucci et al., 2005b). Removal of the brainstem and subsequent coronal brain slicing using a vibratome (PelcoVibratome 1000) was carried out in ice-cold (4-6°C) artificial cerebrospinal fluid (aCSF). Slices containing the LC region were then incubated in room temperature aCSF equilibrated with 5% CO₂/95% O₂ until use 1-4 hours after slicing. During experiments, slices were superfused continuously by gravity flow (~4 ml/min) using solutions held at 35°C.

Solutions

Unless otherwise specified, all brain slices were immersed in aCSF. This solution consisted of (in mM): 124 NaCl, 5 KCl, 1.3 MgSO₄, 26 NaHCO₃, 1.24 KH₂PO₄, 10 glucose, and 2.4 CaCl₂ and were equilibrated with 5% CO₂/95% O₂, pH₀ ~7.45 (at 35°C). Hypercapnic solutions were identical except for equilibration with either 15% CO₂/85% O₂, pH₀ ~7.0, or 10% CO₂/90% O₂, pH₀ ~7.15. These percentages of CO₂ were chosen to
maximize cellular effects of hypercapnia (Pineda & Aghajanian, 1997; Ritucci et al. 2005b) and mimic CO₂ used to study panic disorder (Ziemann et al. 2009). The whole cell pipette filling solution for current clamp studies consisted of (in mM): 130 K-gluconate, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, 2 ATP, and 10 HEPES. The pipette filling solution pH was buffered to ~7.35 using KOH. For intracellular Ca^{2+} (Ca^{2+}_i) measurements, 250 μM Fura-2 was also added to the whole cell filling solution. Whole cell pipette filling solution for voltage clamp studies of the Ca^{2+} current consisted of (in mM): 130 CsCl, 10 EGTA, 1 MgCl₂, 0.3 GTP, 2 ATP, 10 HEPES, and 10 tetraethylammonium (TEA), buffered to pH ~7.45 using CsOH. For voltage clamp studies of BK currents, the intracellular filling solution was identical to those used for current clamp studies. For immunohistochemical studies of BK channels, the phosphate buffered saline (PBS) solution contained (in mM): 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, and 1.47 KH₂PO₄, pH 7.4.

**Measurement of intracellular Ca^{2+}**

The Ca^{2+} sensitive dye Fura-2 (250 μM) was loaded into LC neurons using whole cell patch pipettes. Fura-2-loaded neurons were excited by light from a 75W xenon arc lamp alternately at 340 nm and 380 nm using a Sutter Lambda 10-2 filter wheel. Emitted fluorescence at 505 nm was directed to the Nikon multi-image port module and then to a GenII Sys Image intensifier and a CCD camera. Subsequent fluorescence images were acquired using a Gateway 2000 E-3100 computer and collected/processed using the software MetaFluor 4.6r. Image acquisition could be achieved within ~2 seconds and was repeated every 15 seconds. Light was blocked between acquisitions to reduce photobleaching. The Fura-2 fluorescence was not calibrated and arbitrary fluorescence
units were used instead to monitor increases or decreases in the ratio of fluorescence ($R_{fl} = \frac{Fl_{340}}{Fl_{380}}$), and thereby increases or decreases in $Ca^{2+}_{i}$.

**Electrophysiological recordings**

Whole cell recordings were used throughout this study. Pipettes were made from thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm) pulled to a tip resistance of ~5 MΩ. LC neurons were visualized using an upright microscope (Nikon Eclipse 6600) with an x60 water-immersion objective and subsequently patched via formation of a gigaohm seal with the cell membrane. Membrane potential ($V_m$) was measured in current and voltage clamp mode. Current was injected via an Axopatch 200B amplifier. Firing rate (FR) was measured using a slope/height window discriminator (FHC Model 700B, Bowdoinham, ME). Both $V_m$ and FR were analyzed using pCLAMP software version 10.0. Recordings began when a stable resting $V_m$ was established. Criteria for healthy neurons were a stable resting $V_m$ of -45 to -60 mV and a spontaneous firing rate of < 4 Hz. The reversibility of all electrophysiological responses under investigation was verified by a return to baseline values upon change of the solution back to normal aCSF. Patched recordings in current clamp mode using the above techniques lasted > 45 minutes without any evidence of washout of the response (Filosa & Putnam, 2003), and the response to most solution changes were observed in < 2 minutes after solution change. When two sequential hypercapnic exposures were studied, current was injected to ensure that all normocapnic (resting) firing rates remained within 0.5 Hz of initial recorded values in order to accurately compare both chemosensitive responses.
For voltage clamp experiments, LC neurons were clamped at -70 mV in aCSF and equilibrated with either 5% CO₂/95% O₂ or 15% CO₂/85% O₂ as indicated. 1 μM TTX was added to block Na⁺ currents. For Ca²⁺ currents, 3 mM BaCl₂ was also added, replacing NaCl isosmotically. Step-wise depolarizations of some 600 ms duration in 10 mV increments from -60 mV to +50 mV were applied and the resulting peak current recorded. For voltage-clamp data of BK channels, neurons were clamped at -70 mV, followed by a step depolarization to +10 mV for 40 ms to achieve Ca²⁺ loading in the neuron (pre-pulse), and immediately followed by a step depolarization to +80 mV to record the KᵦCa and voltage-activated K⁺/Ca²⁺ current. This double-pulse protocol was repeated following a 3-minute exposure to 1 μM paxilline, and the resulting currents subtracted to obtain the BK difference current (Pax ΔI). For BAPTA experiments, the brain slice containing the LC was incubated in room-temperature aCSF containing 40 μM BAPTA-AM for 25 minutes prior to use.

Capacitance values for LC neurons were measured in current clamp mode according to the method of Golowasch et al. (2009). In brief, a negative current pulse (50-200 pA) was applied for 800 ms and the resulting membrane hyperpolarization (10-20 mV) was recorded. The current pulse was repeated four times per neuron, averaged, and the Vₘ curve was fit using the Clampfit Analysis software in the pClamp package. LC neurons demonstrated a multiphasic capacitance transient that was consistent with the presence of multiple electric compartments.

**Immunohistochemistry**

Pontine brainstem slices from neonatal rats age P11 or P3 were fixed in freshly prepared 4% paraformaldehyde in PBS buffer, pH 7.4, for 72 hours. Afterwards, tissues
were washed three times in PBS buffer plus 0.1% TritonX for 15 minutes per wash, and then incubated in PBS/TritonX buffer plus 1% bovine serum albumin (BSA) for 30 minutes. Fixed tissue was incubated in PBS/TritonX buffer containing 1:200 dilutions of reconstituted anti-KCa 1.1 (BK1) antibodies (Alomone Labs) plus the neuronal marker antibody NeuN (1:500 dilutions) (Millipore) for 72 hours at 4°C. Afterwards, slices were washed three times in PBS/TritonX buffer for 15 minutes per wash and incubated overnight with both CY3 (anti-rabbit) (Jackson Laboratories, Inc.) and FITC (anti-mouse) (Millipore) secondary antibodies (1:50 dilutions). Tissues were washed three times with PBS buffer without detergent for 15 minutes per wash prior to imaging. Stained tissues and controls were visualized with either an Olympus FV300 or FV1000 Confocal Microscope. A series of confocal optical sections (Z stack, resolution 0.5 µm) were used to assess the extent of channel labeling.

Drugs

TTX, BAPTA-AM, paxilline, nifedipine, BSA, and Fura-2 were purchased from Sigma-Aldrich (St Louis, MO). Nifedipine and paxilline were prepared as stock solutions in ethanol (50 mM and 10 mM, respectively). BAPTA-AM stocks (10 mM) were prepared in DMSO, and TTX and Fura-2 stocks (1 mM and 10 mM, respectively) were in dH2O.

Data analysis and statistics

Where applicable, analysis for the change in firing rate (ΔFR) was calculated by the following: \( \Delta FR = \left( \frac{\text{hypercapnic average firing rate} - \text{control average firing rate}}{\text{control average firing rate}} \right) \times 100\% \). The magnitude of the firing rate increase was also quantified as the chemosensitivity index (CI) using the equation of Wang and
Richerson (1999). The CI is basically the percentage change of the control firing rate induced by a 0.2 pH unit decrease in extracellular pH. Using this calculation, 100% represents a non-chemosensitive neuron. Neurons that responded to hypercapnia with a 20% or greater increase in firing rate were considered chemosensitive. All values are expressed as mean ± SEM. Significant differences between two means were determined by student t-tests or paired t-tests. Comparisons of more than two means were assessed using ANOVA with multiple paired comparisons. Differences were considered significant if \( P < 0.05 \).
Results

Development of L-type Ca\textsuperscript{2+} currents in LC neurons from neonates

Whole cell voltage clamp of LC neurons in the presence of Na\textsuperscript{+}/K\textsuperscript{+} channel blockade demonstrated inward currents that activated at approximately -30 mV and reversed at around +45 mV (Fig. 21), consistent with Ca\textsuperscript{2+} currents (Hille, 2001; Marcantoni et al., 2010). The long inactivation time (>200 ms) and high activation threshold of this current is consistent with the activity of L-type Ca\textsuperscript{2+} channels (Ji et al., 2002; Marcantoni et al., 2010). L-type Ca\textsuperscript{2+} channel currents can display an initial rapid decay over the first 100 ms which has been attributed to Ca\textsuperscript{2+}-dependent inactivation of L-type channels in previous studies (Eckert & Tillotson, 1981; Marcantoni et al., 2010). In the current study, this inactivation was minimal due to the presence of 10 mM EGTA in the intracellular pipette solution, and the majority of the Ca\textsuperscript{2+} current inactivated slowly if at all (Fig. 21A). In LC neurons from animals postnatal (P) age P10 and above, the average amplitude of this current was found to be comparatively large, usually between 2 and 3 nA. In contrast, neurons from animals younger than P5 were found to have a markedly smaller current, sometimes immeasurable and often less than ~800 pA (Fig. 21B). Figure 21B compares I-V plots from three LC neurons from rats aged P4, P7, and P10, showing the age-dependent increase of the Ca\textsuperscript{2+} currents in LC neurons. Averages of the maximum recorded Ca\textsuperscript{2+} current from three age groups, P3-P5, P6-P8, and >P10 are shown in figure 22. During the first two weeks of postnatal development, the increases in the peak amplitude of Ca\textsuperscript{2+} current in the LC neurons was found to be significant (p<0.001). These data are consistent with our prior findings of an
Figure 21.  

A. Voltage-sensitive currents activated by +10 mV steps from -70 mV to +50 mV in the presence of Na⁺ and K⁺ blockade and 3 mM BaCl₂. Top trace represents typical results for neurons from a P10 rat, while the middle trace is characteristic of neurons from younger (<P5) animals. Note the large difference in the amplitude of inward current. 

B. IV plot of three separate current recordings under the same conditions as in A. Resulting IV plots are characteristic for high voltage activated Ca²⁺ channels, such as L-type. Triangles represent points from a P4 rat, circles are points from a P7 rat, and squares are from a P10 rat. The current amplitude increases as neonatal rats age.
FIGURE 21.

A.

B.
Figure 22: The effects of postnatal rat age on the peak Ca$^{2+}$ current amplitude.

Average Ca$^{2+}$ current peak amplitudes from older, >P10 rats (N=10) were significantly larger than transition (P6-P8, n=17) age rats and younger (>P5, n=13) rats with p < 0.001. Peak Ca$^{2+}$ currents recorded from transition rats were not significantly larger than younger rats with a p = 0.06. In all age groups, the peak Ca$^{2+}$ currents became larger as neonatal rats age.
FIGURE 22.
increase in an L-type Ca\textsuperscript{2+} current in LC neurons over this age period (Imber & Putnam, 2012).

To evaluate the possible contribution of L-type channels to the Ca\textsuperscript{2+} current in LC neurons, we applied the L-type Ca\textsuperscript{2+} channel inhibitor nifedipine in the aCSF solution for at 0 min, 6 min, and 9 min, and repeated our voltage clamp recordings (Fig 23). In four neurons older than P8, the total Ca\textsuperscript{2+} current was reduced by over 60% after 9 minutes of exposure to nifedipine, supporting a significant contribution from L-type Ca\textsuperscript{2+} channels (Fig. 23, inset). The remaining current in the presence of nifedipine could be due to incomplete inhibition of L-type Ca\textsuperscript{2+} channels by nifedipine or due to the presence of another type of Ca\textsuperscript{2+} channel. Regardless, our results suggest that the bulk of Ca\textsuperscript{2+} channel current in postnatal LC neurons is due to L-type Ca\textsuperscript{2+} channels.

**Effect of increased CO\textsubscript{2}/H\textsuperscript{+} on Ca\textsuperscript{2+} currents and intracellular Ca\textsuperscript{2+}**

Our previous work has suggested that Ca\textsuperscript{2+} channels are activated by hypercapnia in LC neurons (Filosa & Putnam, 2003; Imber & Putnam, 2012). We directly tested this possibility by recording IV plots of Ca\textsuperscript{2+} currents in the presence of 5% and 15% CO\textsubscript{2} in LC neurons. Hypercapnia increased the inward current during a depolarizing pulse from a holding potential at -70 mV to -10 mV (Fig. 24A). In five LC neurons aged between P7-P10, hypercapnia caused marked changes in the activation voltage at which Ca\textsuperscript{2+} current reached a peak and in the amplitude of the current at that peak (Fig. 24B). The inset in figure 24B shows that increased CO\textsubscript{2}/H\textsuperscript{+} induced a significant increase in the peak Ca\textsuperscript{2+} current in LC neurons, as previously observed in glomus cell (Summers et al., 2002).
Figure 23: The effects of the L-type Ca\textsuperscript{2+} channel inhibitor nifedipine on voltage clamp recordings of the peak Ca\textsuperscript{2+} current from neonatal animals aged P8-P13. Recordings were taken prior to the addition of nifedipine (0 min, 1), 6 minutes after addition (2), and 9 minutes after addition (3). By 9 minutes, the peak current amplitude had been significantly reduced by over 60\% (inset, n=4). These results indicate that the majority of the Ca\textsuperscript{2+} currents in LC neurons are due to the activity of L-type channels.
FIGURE 23.
**Figure 24:** Effects of CO$_2$/H$^+$ on the Ca$^{2+}$ current from LC neurons. 

**A.** Note the large increase in current amplitude at -10 mV in the presence of hypercapnia. Data from a P7 rat. 

**B.** Comparison of two IV plots recorded from the same neuron during exposure to 5% and 15% CO$_2$. The peak activation shifts by -10 mV, and the peak amplitude increases by some 0.2 nA. Inset compares the average peak Ca$^{2+}$ current amplitude recorded at either 0 or -10 mV in both 5% and 15% CO$_2$ from 5 LC neurons from rats aged P6 to P10.
FIGURE 24.

A.

B.
Since hypercapnia activates Ca\(^{2+}\) channels in LC neurons, we determined whether increased CO\(_2\)/H\(^+\) may also increase Ca\(^{2+}\)\(_i\) levels. We measured the intracellular Ca\(^{2+}\) levels using the Ca\(^{2+}\)-sensitive fluorescent dye Fura-2. Current clamp of LC neurons under these conditions revealed normal resting \(V_m\) of -40 to -45 mV and firing rates of 1-4 Hz in 5% CO\(_2\), in agreement with previous studies (Filosa & Putnam, 2003; Ritucci et al., 2005b). Simultaneous imaging of the patched neuron showed stable resting Ca\(^{2+}\)\(_i\) levels as indicated by stable \(R_{fl}\) values for Fura-2 (Fig 25). When the neuron was exposed to 15% CO\(_2\) firing rate increased as expected. Concurrent with the increase in firing rate, the observed \(R_{fl}\) values also increased, indicating a rise in the average Ca\(^{2+}\)\(_i\) during hypercapnia (Fig. 25). When 5% CO\(_2\) was restored, Ca\(^{2+}\)\(_i\) (\(R_{fl}\)) returned to resting levels.

Fig 26 summarizes the results for the average increases in \(R_{fl}\) (Ca\(^{2+}\)\(_i\)) in LC neurons from rats younger (n=13) and older (n=6) than P10. Note that resting levels of Ca\(^{2+}\)\(_i\) (in 5% CO\(_2\)) are significantly larger in the older neonates (Fig. 26), which is in agreement with a larger resting Ca\(^{2+}\) current in older neonates (see Fig. 22). Hypercapnia induces an increase in intracellular Ca\(^{2+}\) in both younger and older neonates as well (Fig. 26). Since our data indicate that Ca\(^{2+}\) currents in LC neurons are predominantly L-type (see Fig. 23), the hypercapnic pulse was repeated in five neurons with the addition of nifedipine. In each case, neurons were exposed to nifedipine for at least 10 minutes, and within five minutes Ca\(^{2+}\)\(_i\) levels had begun to decrease (data not shown). In the presence of nifedipine, there was no increase in \(R_{fl}\) values during exposure to hypercapnia, despite a similar increase in firing rate (Fig. 26). Thus, our findings show that increased CO\(_2\)/H\(^+\) results in an increase in intracellular Ca\(^{2+}\) and that this higher level of Ca\(^{2+}\)\(_i\) is due to L-type Ca\(^{2+}\) channel activity, and not the release of Ca\(^{2+}\) from internal stores.
**Figure 25:** Exposure to hypercapnia causes a reversible increase in firing rate and a concurrent increase in Ca\(^{2+}\). (Upper) LC neuron loaded intracellularly with the Ca\(^{2+}\)-sensitive dye Fura-2. Images are the ratio of fluorescence from excitation at 340nm/380nm (R\(_{fl}\)). Circles plot the relative changes in fluorescence over time. (Lower) Whole-cell current clamp patch of the depicted LC neuron showing a typical hypercapnic response upon exposure to 15% CO\(_2\). R\(_{fl}\) values increase during the chemosensitive response, indicating an increase in Ca\(^{2+}\) levels during exposure to hypercapnia. Scale bars represent 20 \(\mu m\).
FIGURE 25.
Figure 26: Comparison of the relative changes in $R_{r\|}$ between normocapnic (5% CO$_2$) and hypercapnic (15% CO$_2$) aCSF. In LC neurons from both younger (< P10, n=13) and older (> P10, n=6) postnatal rats, there was a significant increase in Ca$^{2+}$$_i$ during hypercapnia. Repeating these experiments in 5 LC neurons aged P7-P13 in the presence of nifedipine prevented the chemosensitive increase in Ca$^{2+}$$_i$. Average normocapnic $R_{r\|}$ values were also found to increase between neurons from younger and older rats (p<0.005).
FIGURE 26.
Presence, activation and development of $K_{Ca}$ channels in LC neurons

Given that hypercapnia induces an increase in intracellular $Ca^{2+}$ we next explored the possibility that elevated $Ca^{2+}$ activates $K_{Ca}$ channels. We first sought to determine if a $K_{Ca}$ channel is expressed in neonatal LC neurons. Figure 27 shows the results of immunohistochemical staining for large conductance $Ca^{2+}$- activated $K^{+}$ channels, “Big-K” or BK channels. Similar to previous results for mouse LC neurons (Sausbier et al., 2006), we found a distinct density of staining for BK channels in the neonatal rat LC (Fig. 27, left). Confocal microscopy of individual LC neurons from older (>P10) rats showed colocalization of the staining for the BK channel subunit BK1 with the marker for neuronal cell bodies, NeuN (Fig. 27, top right panel). These data indicate that BK channels are present on the soma of LC neurons. When the staining was repeated in LC neurons from younger rats (<P5), the channel subunit BK1 was again evident. However, the BK1 staining appeared less dense (Fig. 27, lower right panel) when compared to results from older rats (Fig. 27, top right panel). This suggests that BK channels may show a developmental increase during early development similar to the increase in $Ca^{2+}$ currents (Fig. 22).

To study BK currents, a two-step voltage clamp protocol, similar to a method used to study BK currents in chromaffin cells (Marcantoni et al., 2010; Prakriya & Lingle, 1999), was used (Fig. 28). First, a “pre-pulse” step to +10 mV initiated $Ca^{2+}$ loading into the neuron. Then a second, prolonged pulse to +80 mV activated voltage-sensitive currents (in the presence of $Na^{+}$ channel blockade) (Fig. 28A). The neuron was exposed to the BK channel inhibitor paxilline for 3 minutes, and the two-step protocol repeated. The resulting currents in the absence and presence of paxilline were subtracted
**Figure 27:** Immunohistochemical studies on LC neurons from a P11 rat incubated with the neuronal cell body marker NeuN (green) show the presence of the large-conductance Ca$^{2+}$-activated K$^+$ channel subunit KCa 1.1 (BK1) (red). (Left) distinct density of staining for BK1 in the LC area. (Upper right) BK1 subunits are shown to colocalize with the neuronal cell body membrane, indicating the presence of B$k$ channels on the soma. (Lower right) Identical staining protocol in LC neurons from a P3 rat also indicates the presence of B$k$ channels, although there appears to be less staining. Scale bars represent 100 μm (left) and 10 μm (upper and lower right).
FIGURE 27.
Figure 28: Whole cell voltage clamp recordings in the presence of TTX. A. Record from a >P10 rat showing activation of voltage sensitive currents with and without the presence of the BK channel inhibitor, paxilline. Middle trace is the subtraction of the two upper traces, representing the paxilline-sensitive current, or PaxΔI. Voltage steps to +80 mV were preceded by brief steps to +10 mV to initiate an increase in Ca\(^{2+}\) (pre-pulse). At this activation voltage, the Pax ΔI was largely inactivating. B. Pax ΔI evoked by 10 mV step-wise voltages from -70 mV to +50 mV. Inset shows the resulting IV plot of the peak Pax ΔI current.
FIGURE 28.

A.

B.
to give the paxilline-sensitive current, or paxilline difference current (Pax ΔI) (Fig. 28A). This putative BK current was found to be non-inactivating (Fig. 28B), similar to the “slowly inactivating” BK current type noted in chromaffin cells (Marcantoni et al., 2010).

An IV plot of the Pax ΔI showed an outwardly-rectified current that has a threshold for voltage activation at around -10 mV (Fig. 28B). Therefore, electrophysiological measurements confirm the immunohistochemical measurements in showing that neonatal LC neurons express a BK channel.

In order to study the development of the BK current in LC neurons, the two-step protocol shown in figure 28A was used to compare the maximum BK current across neonatal age. When the Pax ΔI was recorded in LC neurons from rats aged P4-P5, P9-P11, and P12-P14, a steady and significant increase in BK current with age was observed (Fig. 29). The average BK current from age ~P4 to ~P12 increased by some three fold. This developmental increase in the BK current was comparable to the development of Ca\(^{2+}\) current (Fig. 22). Thus, both Ca\(^{2+}\) and BK channels appear to develop in LC neurons during the first two neonatal weeks.

The development of both Ca\(^{2+}\) and BK channels with development could be due to either an increase in the size of LC neurons with development or an increase in the channel expression. To assess whether LC neurons increased with size during early neonatal development, we measured the membrane capacitance as an indicator of somal size. LC neurons from rats aged P3-P7 had an average resistance of 136.8 ± 22.2 MΩ (n=11) and neurons from rats aged P12-P16 had an average resistance of 189.6 ± 49.4 MΩ (n=6). The capacitance values calculated for these neurons age P3-P7 and P12-P16 were
**Figure 29:** Plot of average peak Pax ΔI over three age groups: P4-P5 (n=7), P9-P11 (n=12), and P12-P14 (n=10), as measured in voltage clamp with a pre-pulse to +10 mV and a voltage step to +80 mV. The Pax ΔI (BK current) increases significantly across all age groups.
65.3 ± 4.6 pF and 70.0 ± 7.0 pF, respectively. Neither the resistance nor capacitance values were significantly different for young (P3-P7) vs. older (P12-P16) neonates. Thus, the increased Ca\(^{2+}\) and BK currents with neonatal development in LC neurons appear to be due to increased channel expression.

We have assumed that the BK channels are being activated by increased Ca\(^{2+}\)\(_i\) resulting from CO\(_2\)/H\(^+\)-activation of L-type Ca\(^{2+}\) channels. We tested this directly. The large Pax ΔI seen in an LC neuron from an older neonatal rat (P12) was significantly inhibited by nifedipine (10 minute exposure) (Fig. 30A and 30B), indicating an important role for L-type Ca\(^{2+}\) channels in the activation of BK channels. Further, loading LC neurons with the Ca\(^{2+}\) chelator, BAPTA, also resulted in a substantial inhibition of Pax ΔI (Fig. 30B). This indicates that elevated Ca\(^{2+}\)\(_i\) is also involved in the activation of BK channels in LC neurons. Interestingly, decreasing Ca\(^{2+}\)\(_i\) levels with BAPTA and the inhibition of L-type channels with nifedipine diminished BK currents by similar amounts (Fig. 30B). This suggests a close functional relationship between L-type Ca\(^{2+}\) channels and BK channels in LC neurons, and supports the development of a significant (>1 nA) CO\(_2\)/H\(^+\)-activated Ca\(^{2+}\)-sensitive hyperpolarizing current in LC neurons from rats aged ~P10 or older.

**The role of BK channels as a chemosensitive brake**

Our results indicate that hypercapnia leads to increased hyperpolarizing BK current in LC neurons through the activation of L-type Ca\(^{2+}\) channels. This pathway should increase during the first two neonatal weeks in rat LC neurons. This activated hyperpolarizing BK current should serve as a brake to the increased firing rate response of LC neurons to hypercapnia. To test whether this braking phenomenon actually occurs
**Figure 30:** Summary of the effects of lowering intracellular Ca\(^{2+}\) (BAPTA) and the inhibition of L-type Ca\(^{2+}\) channels (nifedipine) on the BK current (Pax \(\Delta I\)). All data represents the peak Pax \(\Delta I\) as measured with a pre-pulse to +10 mV and voltage step to +80 mV. **A.** (Upper trace) Pax \(\Delta I\) recorded from a LC neuron from a P12 rat with and without the presence of nifedipine. Lower trace represents voltage steps. Nifedipine caused a significant decrease in amplitude and increase in the inactivation rate of the Pax \(\Delta I\). **B.** Comparison of the average peak Pax \(\Delta I\) (n=17) following either incubation with 40 \(\mu\)M BAPTA-AM (n=10) or in the presence of nifedipine (n=11). Both BAPTA and L-type Ca\(^{2+}\) channel inhibition decreased the Pax \(\Delta I\) amplitude by the same approximate amount (p<0.001). All data were from neonatal rats aged P10-P14.
FIGURE 30.

A.

B.
we added paxilline to the superfusate during current clamp studies to remove the braking effect of the BK channel. A patched LC neuron was first exposed to hypercapnia and the resulting change in firing rate (ΔFR) recorded. Next, neurons were exposed to paxilline for at least five minutes and the hypercapnic exposure repeated to compare the change in firing rate in the presence of BK channel inhibition (ΔFR_pax). Sample records of such experiments in LC neurons from rats of different age are shown in Fig. 31. The upper left panel of Figure 31 shows typical results for the effects of paxilline on the chemosensitive response from an LC neuron from a young rat (P6). Exposure to hypercapnia resulted in a chemosensitive ΔFR of ~0.6 and a CI of ~150%, and this response was not much changed in the presence of BK channel inhibition. When the experiment was repeated in an LC neuron from a P10 rat, a chemosensitive response typical of adult animals (CI ~125%) was observed, and the addition of paxilline increased the CI to ~170% (Fig. 31, upper right panel). This is consistent with the development of the chemosensitive hyperpolarizing function of the BK current in LC neurons. The effect of BK channel inhibition on the chemosensitive response of an LC neuron became more pronounced in older neonatal rats (P16) (Fig. 31, lower panel). Here, the neuron showed a hypercapnic response of ~120%, which just meets our criterion for chemosensitivity. When the neuron was exposed to paxilline, the hypercapnic response became much greater, with a CI of ~240%. Notably, the addition of paxilline to LC neurons from rats of all ages did not alter the basal firing rate in 5% CO₂, suggesting that BK channels are not active in resting LC neurons.

Figure 32 compares the effect of the chemosensitive braking function by BK channels across postnatal age. This effect was measured by subtracting the hypercapnic
Figure 31: The inhibition of BK channels by paxilline causes an increase in the chemosensitive response that is dependent on postnatal age. Upper panels represent typical results for whole cell current clamp experiments from younger (P6) and older (P10) neonatal rats, comparing the firing rate response to hypercapnia with and without paxilline. Notice the lack of firing rate response to the addition of paxilline during normocapnia. Lower panel shows the same experiment on an LC neuron from a P16 neonatal rat. Here, the chemosensitive response is characteristically low, and was increased significantly when exposed to paxilline. The ‘braking’ effect caused by BK channels was observed to increase over the same postnatal period that the Ca\(^{2+}\) and BK currents were observed to increase in voltage clamp studies.
FIGURE 31.
**Figure 32:** Summary of the results from comparisons of sequential hypercapnic responses in LC neurons with (ΔFR_{pax}) and without (ΔFR) the presence of paxilline. ΔFR represents the difference in average firing rate (Hz) in the presence of 15% CO$_2$ and 5% CO$_2$. ΔFR$_{pax}$ – ΔFR, then, measures the increase in the firing rate response to hypercapnia caused by inhibition of the braking pathway (see text). Control values represent sequential hypercapnic responses without the addition of paxilline (ΔFR$_2$- ΔFR$_1$). All values were significant from one another, and the results indicate a development of the braking pathway (control n=13, P5-P9 n=20, P10-P16 n=14). * P < 0.05; *** P < 0.001.
FIGURE 32.

[Bar chart showing the comparison of ΔFR, ΔFR_{paxilline}, ΔFR_{P5-P9}, and ΔFR_{P10-P16} with control. Significant differences are indicated by asterisks: *** for ΔFR_{P10-P16} compared to control and ΔFR_{P5-P9}, * for ΔFR_{P10-P16} compared to ΔFR_{P5-P9}.]
increase in firing rate in aCSF, FR, from the hypercapnic increase in firing rate in the presence of paxilline, \( \Delta FR_{pax} \) (\( \Delta FR_{pax} - \Delta FR \)). In addition, thirteen neurons were exposed to sequential hypercapnic events without the addition of paxilline, and the resulting \( \Delta FR \) values were subtracted as a control (\( \Delta FR_2 - \Delta FR_1 \)). The \( \Delta FR \) was not significantly different when two control exposures to hypercapnia were given in the same neuron indicating that our measurements of the firing rate response of LC neurons to hypercapnia are repeatable and stable measures. Paxilline significantly increased the hypercapnia-induced elevated firing rate in LC neurons from young neonates (P5-P9), but the paxilline-induced increase was more than twice as large in neurons from older neonates (P10-P16) (Fig. 32). This is consistent with the gradual increase in Ca\(^{2+}\) and BK currents over this age range (Figs. 22 and 29). These findings clearly indicate that BK channels operate in a braking pathway that develops during the first two neonatal weeks and that limits the firing rate response of LC neurons to hypercapnia.

The development of the braking function should result in a decrease in the magnitude of the chemosensitive response of LC neurons to increased CO\(_2\)/H\(^+\). In fact, the chemosensitivity of LC neurons (as measured by CI) from postnatal animals ages P3-P5, P6-P9, and older than P10 shows a dramatic and significant decrease with increased age (Fig. 33A). The average CI values from younger neonates P3-P5 were greater than 200\%, while animals older than P10 were significantly smaller, typically between 125 and 130\%, as previously shown (Gargaglioni et al., 2010). In addition, a transition period was evident from postnatal age P6–P9 whereby the recorded CI values were usually between 150 and 170\%. These values indicate a gradual decrease in chemosensitivity during the same age range as the development of Ca\(^{2+}\) and BK currents in LC neurons.
Figure 33:  

A. Comparison of the chemosensitivity of LC neurons across postnatal age P3-P5 (n=12), P6-P9 (n=18), and >P10 (n=36) measured by whole cell current clamp studies. Results show a steady decrease in the firing rate response to hypercapnia of individual neurons over the first ~2 postnatal weeks. These findings support the development of a chemosensitive “brake” over this period (see text). ** P < 0.01; *** P < 0.001. 

B. When the CI is plotted versus the average Ca$^{2+}$ current in LC neurons from rats from ages P3-P16, an inverse relationship is observed. These results suggest that the development of the Ca$^{2+}$ current in LC neurons may also be limiting the firing rate response to hypercapnia. (CI = -46.059I_{Ca} + 240.4, R^2 = 0.8685).
FIGURE 33.

A.

B.
To study the relationship between the fall of CI and the increase of the Ca\(^{2+}\) current in LC neurons during the neonatal period, we showed that the two parameters are highly correlated (\(R^2 = 0.88\)), with CI falling in inverse relationship to the increase in Ca\(^{2+}\) current (Fig. 33B). The linear fit suggests a relationship between the development of the Ca\(^{2+}\) current in LC neurons and the decrease in the average firing rate response to hypercapnia. That this most likely involves the activation of the BK channel is shown by the fact that the fall of CI in older neonates can be largely reversed by inhibition of the BK channel with paxilline (Fig. 34A). Hence, the development of L-type Ca\(^{2+}\) and BK channels in LC neurons form the basis for the appearance of a chemosensitive brake that is responsible for the decrease of the firing rate response to hypercapnia during the early postnatal period in LC neurons.

**The effect of 10% CO\(_2\) in activating the chemosensitive brake in LC neurons from neonatal rats**

While 15% CO\(_2\) is useful for maximizing the effects of hypercapnia, we were concerned that it represents a very high level of CO\(_2\). We thus did studies using 10% CO\(_2\) as a more modest hypercapnic challenge to see if the braking phenomenon was still activated under these conditions. Since our control aCSF was equilibrated with 5% CO\(_2\), we assume that the control CO\(_2\) in these solutions is about 40 mm Hg. We further assume that 10% CO\(_2\) would then result in CO\(_2\) levels of about 80 mm Hg. This level is still high, but it is lower than the value of ~87 mm Hg found in the blood of mice breathing 10% CO\(_2\) for the purposes of studying panic disorders (see Fig. 2A from Ziemann *et al.*, 2009).
**Figure 34:**  

**A.** When BK channels are inhibited by paxilline, the magnitude of the chemosensitive response of LC neurons from neonatal animals P10-P16 is restored to that of LC neurons from younger neonatal rats (<P10). This experiment was repeated for hypercapnic responses using both 10% CO$_2$ and 15% CO$_2$. *** P < 0.001.  

**B.** When the ΔFR values for LC neurons exposed to 15% CO$_2$ and 10% CO$_2$ with and without paxilline are plotted, a ~3.5 fold increase in the chemosensitive gain is observed with the inhibition of BK channels (aCSF: ΔFR = 0.0362ΔCO$_2$ + 0.0066, R$^2$ = 0.99; Pax: ΔFR = 0.1277ΔCO$_2$ + 0.0473, R$^2$ = 0.98).
FIGURE 34.

A.

![Bar graph showing CI (%) for different conditions and groups.

B.

![Graph showing the relationship between ΔFR and Δ % CO₂ for aCSF and Pax groups.]}
We found that the CI of neonatal LC neurons decreased from younger (<P10) to older (>P10) neonates in response to 10% CO₂, just as it did in response to 15% CO₂ (Fig. 34A). Further, paxilline was able to increase the CI in LC neurons from older neonates to values similar to the high values from younger neonates in response to 10% CO₂, just as we found in response to 15% CO₂ (Fig. 34A). These data indicate that the brake is activated by both 10% and 15% CO₂.

We plotted the increased firing rate of LC neurons from older (>P10) neonates in response to 10% and 15% CO₂ both in the absence and the presence of paxilline. Under control conditions (5% CO₂), both groups (without and with paxilline) had similar firing rates, 0.56 ± 0.09 Hz (n=16) and 0.59 ± 0.10 Hz (n=21), respectively. Note that hypercapnia resulted in a ~3.5 fold increase in the firing rate response to hypercapnia in the presence, versus the absence, of paxilline (Fig. 34B). This shows that inhibition of the braking pathway results in a substantial increase in the chemosensitive gain of LC neurons.
Discussion

The major findings of this study are that: 1) hypercapnia activates L-type Ca$^{2+}$ channels in LC neurons; 2) the activation of these channels leads to increased intracellular Ca$^{2+}$; 3) BK channels are activated under these conditions; 4) this pathway constitutes a brake on the firing rate response of chemosensitive LC neurons to hypercapnia; and 5) the development of this braking pathway accounts for the developmental decrease in the magnitude of the firing rate response of LC neurons to hypercapnia during early development. This braking pathway may represent a novel mechanism for controlling the gain of chemosensitive neurons to elevated CO$_2$/H$^+$. 

Activation of Ca$^{2+}$ currents by increased CO$_2$/H$^+$

The inward current that we observe in LC neurons in the absence of fast Na$^+$ currents has a characteristic Ca$^{2+}$ channel I-V plot (Figs. 21 and 24). The current shows fast activation, very slow inactivation and substantial inhibition (over 60%) by nifedipine (Fig. 23) suggesting that the bulk of the current is due to L-type Ca$^{2+}$ channels. The current remaining in the presence of nifedipine also had very slow inactivation, suggesting it was also an L-type Ca$^{2+}$ current. We do not know if this represents incomplete inhibition of the current by nifedipine, possible due to slow penetration of the inhibitor into the slice, or if it represents heterogeneity among L-type Ca$^{2+}$ channels (Lipscombe et al., 2004). Based on the increasing inhibition of Ca$^{2+}$ current by nifedipine with exposure time (Fig. 23), penetration of the inhibitor into the slice is clearly an issue. However, L-type Ca$^{2+}$ channels consisting of Ca$_v$1.3 subunits are only partially inhibited by dihydropyridines, although they are activated at voltages near -55 mV in many cells (Lipscombe et al., 2004). It is clear that LC neurons express L-type
Ca$^{2+}$ channels but the molecular identity of these channels must await future studies, most likely involving the use of single cell RT PCR.

Exposure to increased CO$_2$/H$^+$ resulted in an increase in the Ca$^{2+}$ current (Fig. 24). This agrees with previous work from our laboratory that showed that hypercapnia activates nifedipine-sensitive, TTX-insensitive Ca$^{2+}$ currents in LC neurons, clearly indicating a pH-insensitive activation of Ca$^{2+}$ currents (Filosa & Putnam, 2003; Imber & Putnam, 2012). However, this is in contrast to the H$^+$-sensitive inhibition of Ca$^{2+}$ currents observed in other cell types, particularly high-voltage activated (HVA) currents such as L-type Ca$^{2+}$ currents (Shah et al., 2001; Tombaugh & Somjen, 1997).

Hypercapnia activated L-type Ca$^{2+}$ currents in LC neurons by increasing the peak Ca$^{2+}$ current and also by shifting the peak voltage-sensitive activation by some 10 mV toward more negative potentials (Fig. 24B). We hypothesize that the activation of Ca$^{2+}$ currents by hypercapnia in LC neurons is mediated by an increase in intracellular HCO$_3^-$ (Imber & Putnam, 2012) and the activation of soluble adenylate cyclase (sAC), which has been characterized as a bicarbonate-dependent means of producing cAMP (Buck et al., 1999).

Such a mechanism has previously been demonstrated in peripheral chemosensitive glomus cells (Summers et al., 2002). In these cells, Ca$^{2+}$ channels were activated by protein kinase A (PKA)-mediated phosphorylation, although this phosphorylation resulted in only an increase in maximum current and not a shift in the voltage dependence of activation (Summers et al., 2002). Phosphorylation of skeletal muscle L-type Ca$^{2+}$ channels by protein kinase C (PKC), which was not dependent on increased cAMP, demonstrated both an increase in the maximum current and a shift in the peak voltage-sensitive activation by some 10 mV in the hyperpolarizing direction (Delbono et al.,
1997), similar to our results for hypercapnia-activated L-type Ca\(^{2+}\) current in LC neurons (Fig. 24B). We do not know whether hypercapnia activates L-type Ca\(^{2+}\) channels in LC neurons by a PKA or a PKC mediated pathway, but we have recently found evidence for a PKA pathway in LC neurons (Imber and Putnam, unpublished observations) similar to that shown in glomus cells (Summers et al., 2002). The presence of this pathway in LC neurons would indicate that intracellular HCO\(_3^-\) is another signal in chemosensitive neurons, like changes of intracellular pH (pH\(_i\)) and pH\(_o\) (Hartzler et al., 2008; Putnam et al., 2004).

**Activation of Ca\(^{2+}\) currents in LC neurons results in increased Ca\(^{2+}\)\(_i\)**

We previously found that Ca\(^{2+}\) spikes in LC neurons were capable of elevating intracellular Ca\(^{2+}\) in the soma (Imber & Putnam, 2012). The ability of hypercapnia to activate L-type Ca\(^{2+}\) channels raises the possibility that hypercapnia could lead to increased Ca\(^{2+}\)\(_i\) as well. The L-type Ca\(^{2+}\) channels in LC neurons are activated rapidly (Fig. 24A). Given the elevated magnitude of Ca\(^{2+}\) current, the hyperpolarized activation voltages and the rapid activation, it is likely that hypercapnia can result in an elevation of Ca\(^{2+}\)\(_i\) in LC neuron somas. Indeed, we found a measurable increase in intracellular Ca\(^{2+}\) in LC soma induced by hypercapnia (Fig. 25). This elevation of internal Ca\(^{2+}\) was blocked by the addition of nifedipine, suggesting that it arises from Ca\(^{2+}\) entry through surface channels and not by release from internal stores (Fig. 26). We did not quantify the amount of hypercapnia-induced increase of Ca\(^{2+}\)\(_i\) in the soma of LC neurons but it appears to be small. However, it is likely that the increase of Ca\(^{2+}\) is much larger in a restricted space, probably sub-membranous or within nanodomains immediately surrounding Ca\(^{2+}\) channels, where it could affect ion channels within the surface
membrane. An increase in intracellular Ca\(^{2+}\) induced by hypercapnia raises numerous possibilities for novel Ca-dependent pathways of regulating the chemosensitive response of LC neurons.

**Activation of BK channels by hypercapnia-induced elevated Ca\(^{2+}\): a chemosensitive brake**

The accumulation of intracellular Ca\(^{2+}\) in response to hypercapnia in LC neuron somas raises the possibility that exposure to hypercapnia results in the activation of K\(_{Ca}\) channels. Sausbier et al. (2006) have shown with immunohistochemistry the marked presence of large-conductance K\(_{Ca}\) (BK) channels in the LC of mice. We have confirmed here positive staining for BK channels in LC neurons from neonatal rats (Fig. 27). Thus, BK channels are present in LC neurons. We were able to assess the activity of these channels using the BK channel inhibitor paxilline and a voltage-clamp protocol that yielded a paxilline difference current (Pax \(\Delta I\)), which we attribute to BK channel activity (Fig. 28). That these channels are Ca\(^{2+}\)-activated K\(^+\) channels is further indicated by the marked reduction of the peak Pax \(\Delta I\) by nifedipine or by loading the neuron with the Ca\(^{2+}\) chelator BAPTA (Fig. 30). That hypercapnia-induced increased Ca\(^{2+}\) activates BK channels is best demonstrated by the effect of paxilline on the firing rate of LC neurons to hypercapnia.

LC neurons from neonatal rats older than P10 have a markedly increased firing rate response to hypercapnia in the presence compared to the absence of paxilline (Figs. 31, 32). These findings suggest that hypercapnia activates BK channels by increasing Ca\(^{2+}\), the BK channels hyperpolarize LC neurons and this hyperpolarization decreases the neuronal firing rate response to hypercapnia. We therefore propose that this pathway
of activation of BK channels serves as a brake to the chemosensitive response of LC neurons. Notably, when paxilline is applied to spontaneously firing LC neurons in control solutions (equilibrated with 5% CO₂), it has no effect on firing rate (Fig. 31), indicating that BK channels are only active in the presence of hypercapnia. This suggests that intracellular Ca^{2+} is too low in resting LC neurons to activate BK channels.

BK channels have been suggested to control firing rate in neurons under a variety of conditions. Recently, a point mutation in the human KCNMA1 gene for the pore-forming subunit of the BK channel was identified in a familial form of epilepsy. This mutation resulted in a gain of function whereby the BK channel affinity for Ca^{2+} increased, and the channels opened both more rapidly and at more hyperpolarized voltages. Thus, the mutation effectively increased BK currents under physiologic conditions (Du et al., 2005). Considering these data, a possible role suggested for BK channels was to increase the rate of repolarization of Na^{+} action potentials, allowing for faster re-activation of Na^{+} channels and greater firing frequencies (Du et al., 2005). Although it is possible that under burst conditions a more rapid repolarization and therefore faster action potentials could allow for a greater firing frequency, it does not seem likely that increasing K^{+} conductance at sub-maximum firing rates would increase firing rate. A plausible alternative to explain these findings may be that increasing BK channel activity causes abnormal regulation of a neuronal network, hyperpolarizing inhibitory neurons to cause uncontrolled bursting in others.

In LC neurons, burst firing rates, induced by injections of depolarizing current pulses, were found to be much greater in the presence of Ca^{2+} channel blockade (Osmanovic & Shefner, 1993). These results are consistent with a hyperpolarizing
function for BK channels that are sensitive to Ca$^{2+}$ influx via voltage-activated channels. Lowering Ca$^{2+}$ levels in LC neurons by loading with EGTA was also found to increase the spontaneous firing rate, as would be expected with the decreased activity of a hyperpolarizing K$_{Ca}$ channel (Aghajanian et al., 1983). Thus, it seems likely that the role for BK channels in LC neurons is to limit the increase in firing rate via a negative-feedback mechanism through increases in Ca$^{2+}$ (Fakler & Adelman, 2008). Our findings in this study are consistent with such a role for BK channels. In our case, however, the negative feedback control is related to the chemosensitive gain of LC neurons, with activation of BK channels by hypercapnia serving as a brake to the firing rate response of these neurons to hypercapnia.

Wellner-Kienitz et al (1998) examined the role of Ca$^{2+}$ and K$_{Ca}$ channels in the chemosensitive response of cultured neurons from the medulla. In these neurons, Ca$^{2+}$ channel blockade, which indirectly decreases K$_{Ca}$ channel activity, resulted in neuronal depolarization and an increase in spontaneous firing rate. Since Ca$^{2+}$ channel blockade resulted in the complete loss of chemosensitivity in these cultured neurons, it was concluded that a K$_{Ca}$ channel must undergo H$^+$-sensitive inhibition to decrease outward current and increase firing rate in response to CO$_2$ (Wellner-Kienitz et al., 1998). Thus, the role of Ca$^{2+}$ in these neurons may be to contribute to the magnitude of the chemosensitive response by providing a baseline activity of K$_{Ca}$ channels, allowing H$^+$-sensitive inhibition of K$_{Ca}$ channels to depolarize the neuron in response to CO$_2$. However, a second interpretation could be that direct, H$^+$-sensitive inhibition of Ca$^{2+}$ channels was responsible for the loss of medullary neuronal chemosensitivity via the accompanying loss of K$_{Ca}$ conductance. This may be more likely, as acidification is
commonly expected to inhibit Ca$^{2+}$ channels (Shah et al., 2001; Tombaugh & Somjen, 1997). Nevertheless, these data suggest that the role of BK channels differ in chemosensitive neurons from the medulla compared to LC neurons in that medullary BK channels are active even at rest, they are inhibited by hypercapnia-induced acidification and they result in a greater firing rate response to hypercapnia. This is in contrast to LC neurons, which have quiescent BK channels at rest that are activated by hypercapnia and that result in a reduced firing rate response to hypercapnia. It will be of interest to study the presence of and role of BK channels in chemosensitive neurons from other areas such as the NTS and the RTN.

**Development of the chemosensitive brake in LC neurons**

We have found that both Ca$^{2+}$ and BK channels, and thus the braking pathway, develop greatly during the early postnatal period in LC neurons from rats. The postnatal development of Ca$^{2+}$ channels has been reported in many cell types in the CNS (Iwasaki et al., 2000; Jiang et al., 1999; Imber & Putnam, 2012). In the predominantly chemosensitive neurons of the LC (Filosa et al., 2002), we report a significant development of the Ca$^{2+}$ currents as measured via whole cell patch in voltage clamp mode (Fig. 21). This current was found to increase by 308% during the first two weeks of postnatal life (Fig. 22). Although the amplitude of this current increases significantly, the IV plots and inactivation kinetics are not much changed (Fig. 21). This suggests that unlike cell types where development of Ca$^{2+}$ currents involve shifts in high and low-voltage activated channels or changes in the percentage of P/Q and N-type channels, the Ca$^{2+}$ channel development in LC neurons appears most consistent with an increase in L-type channels.
Our results indicate that the average paxilline-sensitive BK current increases by 320% between young (P4-P5) and older (>P12) postnatal rats. This value is very close to the average increase in peak Ca\(^{2+}\) current amplitude over approximately the same age range. As such, it is possible that the apparent development of the BK channel is actually due to the increase in Ca\(^{2+}\) channels and the accompanying increase in Ca\(^{2+}\)\(_i\) levels as postnatal rats age (Fig. 22, 25). This possibility is supported by the findings that decreasing Ca\(^{2+}\)\(_i\) levels with BAPTA and inhibiting L-type Ca\(^{2+}\) channels with nifedipine results in peak paxilline ΔI in neurons from older neonates being reduced to low values similar to those found in neurons from young neonatal animals (P4-P5). This suggests that BK channel expression may not change with development, only the Ca\(^{2+}\) channels. However, it is possible that BK channels also undergo postnatal development, as suggested by the immunohistochemistry results (Fig. 27).

**The role of Ca\(^{2+}\) in chemosensitivity of LC neurons**

Although several types of H\(^+\)-sensitive K\(^+\) currents and cation channels have been identified in LC neurons, little is currently known about the role of Ca\(^{2+}\) in the chemosensitivity of the LC (Cui et al., 2011; Li & Putnam, 2009; Putnam, 2010). Filosa and Putnam (2003) reported that the addition of an L-type Ca\(^{2+}\) channel inhibitor reduced the chemosensitive response of LC neurons from animals younger than P9. This age group is possibly too young for the development of the braking effect examined in our current study, which would have predicted an increase in the chemosensitive response upon L-type channel inhibition. It may be that activation of Ca\(^{2+}\) channels is excitatory in LC neurons from young neonates, at a time before BK channels have fully developed. However, the possible effects of nifedipine on synaptic input to chemosensitive neurons
may complicate the nifedipine exposure experiments, since blockade of L-type Ca\(^{2+}\) channels would impair neurotransmitter vesicle release at synaptic junctions. Thus, measured effects of nifedipine on chemosensitive LC neuronal output may be due to effects on synaptic input and not necessarily due to Ca\(^{2+}\) channels.

Filosa and Putnam (2003) suggested that an increase in the L-type Ca\(^{2+}\) current could be contributing to the chemosensitive increase in firing rate in LC neurons from rats younger than P9, which agrees with our results showing that a chemosensitive increase in Ca\(^{2+}\) currents occurs during exposure to hypercapnia (Fig. 24). However, the effect of BK channel inhibition was an increase in hypercapnia-induced firing rate in LC neurons from neonatal rats of all ages (Fig. 32). Thus, it is likely that the net effect of the activation of Ca\(^{2+}\) and BK channels is hyperpolarization of LC neurons and thus a braking phenomenon.

Apamin-sensitive, small conductance (S\(_k\)) K\(_{Ca}\) channels have also been identified in LC neurons (Osmanovic & Shefner, 1993; Williams et al., 1984). Unlike BK channels, S\(_k\) channels have no voltage dependence and are gated solely by Ca\(^{2+}\)\(_{i}\) (Fakler & Adelman, 2008). Thus, S\(_k\) channels in LC neurons should also be activated by the chemosensitive increase in Ca\(^{2+}\)\(_{i}\) and may contribute to the braking mechanism along with BK channels (Fig. 25).

**Summary and Significance**

Our findings are of significance to the understanding of chemosensitive neurons in several ways. The use of whole cell patch pipettes have often resulted in a phenomenon called “washout” in chemosensitive neurons, where the neuron remains viable but its firing rate response to hypercapnia is lost (Richerson, 1995). We have
previously shown that washout of the hypercapnic response in LC neurons can be prevented by adding very little EGTA (0.4 mM) and no \( \text{Ca}^{2+} \) to the whole cell patch pipette (Filosa & Putnam, 2003). In light of our current findings, we believe that the original loading solution (containing 10 mM EGTA and 1 mM \( \text{Ca}^{2+} \)) probably set intracellular \( \text{Ca}^{2+} \) level sufficiently high to activate the braking pathway and prevent any hypercapnia-induced increase in firing rate. Thus, our findings help explain why our modified patch pipette solution (Filosa & Putnam 2003) prevents washout of the chemosensitive response.

Studies in neurons from various cell types have documented the importance of \( \text{Ca}^{2+} \) and \( K_{\text{Ca}} \) channels during postnatal development (Bringmann et al., 2000; Chan et al., 2007). In retinal glial cells, changes in the types of \( \text{Ca}^{2+} \) channels expressed during the first postnatal week were thought to play a role in the development of BK currents (Bringmann et al., 1999). In the substantia nigra, the activity of the L-type \( \text{Ca}^{2+} \) channel \( \text{Ca}_{v}1.3 \) was found to increase significantly over the first four postnatal weeks, and contributed to adult pacemaking mechanisms in conjunction with small-conductance \( K_{\text{Ca}} \) channels (Chan et al., 2007; Shepard & Stump, 1999). Previous studies have also indicated a regulatory role for \( K_{\text{Ca}} \) channels (Fakler & Adelman, 2008). In Purkinje cells, \( K_{\text{Ca}} \) channels were thought to “act as a brake” on dendritic \( \text{Ca}^{2+} \) transients, thereby modulating neuron excitability and synaptic gain (Hosy et al., 2011). Our results in the chemosensitive neurons in the LC further suggest a regulatory role for BK currents in modulating chemosensitive gain.

The results of our current study support the postnatal development of a \( \text{CO}_2 \)-sensitive, largely L-type \( \text{Ca}^{2+} \) current in LC neurons. Consistent with this development,
Ca\textsuperscript{2+} levels were shown to increase both in older neonatal animals and in the presence of increased CO\textsubscript{2}. Over the same age period, large conductance K\textsubscript{Ca} currents underwent an increase in amplitude. Collectively, this supports the development of a Ca\textsuperscript{2+}-sensitive hyperpolarizing current in LC neurons that is activated by CO\textsubscript{2} during the first two weeks of postnatal life. Thus, the role of BK channels would be to respond to the activation of Ca\textsuperscript{2+} channels by increasing K\textsuperscript{+} conductance and hyperpolarizing the neuron in response to CO\textsubscript{2}. These findings support our previously proposed model of a CO\textsubscript{2}-induced hyperpolarization in LC neurons (Fig 20). This can be thought of as a braking mechanism to the chemosensitive response of LC neurons, which reduces chemosensitive gain.

Our data also confirm that there is a decrease in the magnitude of the response of chemosensitive LC neurons to hypercapnia during the first two postnatal weeks (Gargaglioni et al., 2010), from a large firing rate response in neurons from young (~P4) neonates to a very small response in neurons from older (>P10) neonates (Fig 33A). Chemosensitive neurons from other brainstem regions either display no development changes (Conrad et al., 2009; Nichols et al., 2009) or an increase in the chemosensitive response with age (Wang & Richerson, 1999). The unusual developmental decrease in chemosensitivity in LC neurons suggests that LC neurons may play a role in the control of breathing early in development but this role may shift to more of an alarm response to elevated levels of CO\textsubscript{2} in older neonates.

Our data strongly suggest that the decrease in the chemosensitive response in LC neurons from older neonates is due to the development of the brake. First, the fall of the firing rate response to hypercapnia shows a strong, negative correlation to the development of the L-type Ca\textsuperscript{2+} channel (Fig. 33B) and inhibition of the BK current in
older neonates nearly completely restores the large firing rate response to hypercapnia seen in young neonates (Fig. 34A). This role for Ca\(^{2+}\) in the chemosensitive neurons of the LC represents a paradigm shift for the approach on the magnitude of the response to hypercapnia in chemosensitive neurons. It is known that chemosensitive neurons from various brainstem regions show marked differences in the magnitude of their firing rate response to hypercapnia and this is generally ascribed to differences in H\(^+\)-sensitive channels that promote neuronal depolarization (Putnam et al., 2004). It was thus not clear why LC neurons, with numerous types of H\(^+\)-sensitive channels (Cui et al., 2011; Filosa & Putnam, 2003; Pineda & Aghajanian, 1997), should have such a small firing rate response to hypercapnia. Our results show that LC neurons do indeed have a large firing rate response to hypercapnia but that this response becomes markedly reduced not by the loss of these channels but by the development of the braking pathway. It will be of interest to see to what extent the magnitude of the response to hypercapnia in other chemosensitive neurons is modulated by a braking pathway.

The implications of this chemosensitive braking pathway are significant to studies on human pathology. In cases of obstructive sleep apnea (OSA), multiple clinical studies have documented an association between OSA and an increase in the loop gain, or the ratio of ventilatory increase to a disturbance that initiates ventilation (Ryan & Bradley, 2005; Verbraecken et al., 1995; Wang et al., 2007; Younes et al., 2001). In other words, an increase in respiratory gain was associated with the onset of irregular breathing, and medications designed to decrease loop gain were demonstrated to be effective treatments (Kiwull-Schone et al., 2008). Other studies have more directly associated an increase in the ventilatory sensitivity to CO\(_2\) with respiratory pathology (Chapman et al., 1988;
Wang et al., 2007). Thus, alterations in the development of a CO₂-sensitive braking pathway designed to limit the chemosensitive gain may have implications toward respiratory disease. More specific to neurons of the LC is the profound body of evidence linking the pathology of panic disorder to respiratory abnormalities, including an enhanced sensitivity to CO₂ (Abelson et al., 2001; Lousberg et al., 1988; Nardi et al., 2009; Papp et al., 1993; Stein et al., 1995). Since the noradrenergic neurons of the LC are: 1) already implicated via electrophysiological and immunohistochemical studies with the fear response of the amygdala, and 2) chemosensitive with over 80% of LC neurons sensitive to CO₂, it seems likely that the LC contributes to such a CO₂-induced fear response (Buffalari & Grace, 2007; Filosa et al., 2002). Accordingly, abnormalities in a chemosensitive braking pathway in LC neurons may have direct implications for the sensitivity to CO₂ of panic disorder patients and targeting the braking pathway may represent a novel approach for treatment of this disorder.
CHAPTER VII

A HCO$_3^-$-dependent mechanism for the activation of Ca$^{2+}$ currents in locus coeruleus neurons
Abstract

Hypercapnic acidosis activates Ca$^{2+}$ channels and increases intracellular Ca$^{2+}$ levels in neurons of the locus coeruleus (LC), a known chemosensitive region involved in respiratory control. We have also shown that large conductance Ca$^{2+}$-activated K$^+$ channels (BK), in conjunction with this pathway, limits the hypercapnic-induced increase in firing rate in LC neurons. Here, we present evidence that the Ca$^{2+}$ current is activated by a HCO$_3^-$-sensitive pathway. The increase in HCO$_3^-$ associated with hypercapnia activates HCO$_3^-$-sensitive adenylate cyclase (sAC). This results in an increase in cAMP levels and phosphorylation and activation of Ca$^{2+}$ channels via cAMP-activated protein kinase A (PKA). We also show the presence of sAC in the cytoplasm of LC neurons, and that the cAMP analogue db-cAMP increases Ca$^{2+}_i$. Disrupting this pathway by decreasing HCO$_3^-$ levels during acidification or inhibiting either sAC or PKA can increase the magnitude of the chemosensitive response in LC neurons to the same extent as inhibition of BK channels.
Introduction

Neurons that are sensitive to CO\textsubscript{2}/H\textsuperscript{+} exist in numerous brain regions and contribute to various functions and disorders including the control of breathing, learning and memory, depression and panic disorders (Coryell et al., 2009; Putnam, 2010; Wemmie et al., 2004; Ziemann et al., 2009). These CO\textsubscript{2}/H\textsuperscript{+}-sensitive neurons are referred to as chemosensitive and the ability of a neuron to respond in this way is generally attributed to the presence of acid-sensitive ion channels on its surface membrane (Putnam et al., 2004; Putnam, 2010). We have focused on chemosensitive neurons within one brain stem area, the locus coeruleus (LC). A high percentage of LC neurons are chemosensitive (Elam et al., 1981; Filosa et al., 2002; Pineda & Aghajanian, 1997) and they have been shown to contain a variety of pH-sensitive channels, including inward rectifying K\textsuperscript{+} channels (Pineda & Aghajanian, 1997), transient A currents and delayed rectifying K\textsuperscript{+} currents (Li & Putnam, 2009; Putnam & Li, 2009), TASK channels (Bayliss et al., 2001) and TRP channels (Cui et al., 2011). Acidification alters these channels in such a way that LC neurons depolarize and increase their firing rate. Our work has focused on the possible effects of Ca\textsuperscript{2+} channels on the chemosensitive response of LC neurons.

We have previously reported that hypercapnia activates L-type Ca\textsuperscript{2+} channels in LC neurons (Filosa & Putnam, 2003; Imber et al., 2012; Imber & Putnam, 2012). In LC neurons from young neonates, this activation seems to contribute to the increased firing rate response induced by elevated CO\textsubscript{2}/H\textsuperscript{+} (Filosa & Putnam, 2003), but in LC neurons from older neonates, hypercapnia-activated Ca\textsuperscript{2+} channels seem to stimulate K\textsubscript{Ca} channels and produce a braking effect on hypercapnia-induced increased firing rate (Imber et al.,
This effect of hypercapnia on Ca$^{2+}$ channels is unexpected since acidification is commonly expected to inhibit Ca$^{2+}$ channels (Shah et al., 2001; Tombaugh & Somjen, 1997). Recent findings provide evidence that elevated intracellular HCO$_3^-$ is involved in the pathway by which hypercapnia activates L-type Ca$^{2+}$ current in LC neurons (Imber & Putnam, 2012).

Little is known about a HCO$_3^-$-sensitive mechanism involved in the chemosensitive response of brainstem neurons, but a role for HCO$_3^-$ in the chemosensitive response of peripheral chemoreceptors has been described (Summers et al., 2002). This study determined that the CO$_2$/H$^+$-activation of L-type Ca$^{2+}$ channels in glomus cells was blocked by an inhibitor of protein kinase A (PKA) and occurred in association with an intracellular elevation of cAMP (Summers et al., 2002). These findings were consistent with a HCO$_3^-$-activated form of adenylyl cyclase termed soluble adenylate cyclase (sAC) (Summers et al., 2002). It was proposed that an elevation in CO$_2$ resulted in an increase in intracellular HCO$_3^-$, thereby increasing cAMP production via sAC, causing an activation of PKA, and subsequently phosphorylation and activation of L-type Ca$^{2+}$ channels.

Soluble adenylate cyclase (sAC) has been characterized as an intracellular HCO$_3^-$-dependent means of producing cAMP (Buck et al., 1999; Chen et al., 2000; Li et al., 2011; Zippin et al., 2001). Since intrinsic chemosensitivity requires that cells respond to CO$_2$, and that the diffusion of increased CO$_2$ across cell membranes results in elevated HCO$_3^-$, it follows that chemosensitive cells expressing sAC could increase their cAMP levels in response to raised CO$_2$. The presence of sAC in LC neurons has been shown in a preliminary report (Nunes et al., 2008), which raises the possibility that chemosensitive
cells of the LC may utilize a sAC-dependent pathway for the activation of their L-type calcium channels in response to hypercapnia.

In the current study, we hypothesize that a sAC-dependent pathway is responsible for the CO₂/H⁺ activation of the L-type Ca²⁺ current in LC neurons. If so, the addition of dibutyryl-cAMP should mimic the hypercapnia-induced increase in Ca²⁺ current (Imber et al., 2012). Conversely, we expect that the nominal absence of CO₂/HCO₃⁻ from the superfusion solution will decrease the sensitivity of this Ca²⁺ current to hypercapnia. In addition, we expect to find the presence of the HCO₃⁻-dependent sAC enzyme in the cytoplasm of neonatal LC neurons using immunohistochemistry. We have shown that the activation of Ca²⁺ currents in LC neurons from rats older than ~P10 decreases the firing rate response via the subsequent activation of large-conductance calcium-activated BK channels (Imber et al., 2012). If Ca²⁺ channels are activated by hypercapnia through a sAC-mediated mechanism, we further expect that the sAC inhibitor (Li et al., 2011; Schlicker et al., 2008) 2-hydroxyestradiol (2HE) or the PKA inhibitor H89, will increase the firing rate response to hypercapnia of LC neurons from older neonates to a similar extent as does the BK channel inhibitor paxilline (Imber et al., 2012). Our findings strongly support our hypothesis that a sAC-mediated pathway leads to the activation of L-type Ca²⁺ channels by hypercapnia in LC neurons.

A preliminary report of our findings has previously been published (Imber et al., 2012).
Methods

Slice Preparation: All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University, which is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01). All procedures are in agreement with the standards established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Neonatal Sprague-Dawley rats postnatal (P) age P3-P16 of mixed sex were used in these studies. Depending on the age of the neonate, they were anesthetized using either 100% CO₂ or hypothermia and then decapitated. The brainstem was removed and a vibratome (Pelco Vibratome 1000) was used to make coronal brain slices. Slicing was done in ice-cold (4-6°C) artificial cerebrospinal fluid (aCSF) solution. Slices of the pons (containing the LC) were maintained in aCSF equilibrated with 5% CO₂/95% O₂ at room temperature until used (1-4 hours after slicing). For all experiments, slices were continuously superfused at a rate of ~4 ml/min by gravity flow using solutions held at 35°C.

Solutions: All brain slices were immersed in aCSF solution unless indicated otherwise. This solution consisted of (in mM): 124 NaCl, 5 KCl, 1.3 MgSO₄, 26 NaHCO₃, 1.24 KH₂PO₄, 10 glucose, and 2.4 CaCl₂ and was equilibrated with 5% CO₂/95% O₂, pH ~7.45 (at 35°C). Hypercapnic solutions had the same composition but were equilibrated with 15% CO₂/85% CO₂, pH ~7.0. This level of CO₂ was chosen to maximize the activation of the cellular signaling pathway being studied (Hartzler et al., 2008; Ritucci et al., 2005b). In nominally CO₂/HCO₃⁻ free solutions, HEPES buffer isosmotically replaced
the HCO$_3^-$ in aCSF and the solution was equilibrated with 100% O$_2$. The pH of the HEPES aCSF solution was adjusted to 7.45 and 7.0 (similar to the normal aCSF and hypercapnic solutions, respectively) using HCl and NaOH. The whole cell pipette filling solution consisted of (in mM): 130 K-gluconate, 0.4 EGTA, 1 MgCl$_2$, 0.3 GTP, 2 ATP, and 10 HEPES, and was buffered to a pH of ~7.35 using KOH. For intracellular Ca$^{2+}$ (Ca$^{2+}_i$) measurements, 250 μM of the Ca$^{2+}$-sensitive fluorescent dye Fura-2 was also added to the pipette solution. The whole cell pipette filling solution for voltage clamp studies of the Ca$^{2+}$ current consisted of (in mM): 130 CsCl, 10 EGTA, 1 MgCl$_2$, 0.3 GTP, 2 ATP, 10 HEPES, and 10 tetraethylammonium (TEA), buffered to pH ~7.45 using CsOH. For immunohistochemistry studies of sAC, the phosphate buffered saline (PBS) solution contained (in mM): 137 NaCl, 2.7 KCl, 4.3 Na$_2$HPO$_4$, and 1.47 KH$_2$PO$_4$.

**Measurement of Intracellular Ca$^{2+}$**: We loaded LC neurons with the Ca$^{2+}$-sensitive dye Fura-2 (250 μM) from the whole cell patch pipettes. Dye-loaded neurons were alternately excited at 340 nm and 380 nm using a Sutter Lamda 10-2 filter wheel (light from a 75W xenon arc lamp). Emitted fluorescence (505 nm) was intensified by a GenIISys Image intensifier and captured by a CCD camera. Fluorescence images were acquired using a Gateway 2000 E-3100 computer and analyzed with MetaFluor 4.6r software. Images were acquired every 15 seconds (~2 seconds acquisition time). Photobleaching was reduced by blocking excitation light between acquisitions. We did not calibrate the Fura-2 fluorescence and instead used arbitrary fluorescence units to monitor increases or decreases in R$_f$. 
Electrophysiological Recordings: All electrophysiological recordings used in this study were whole cell recordings. Whole cell pipettes were pulled to a tip resistance of ~5 MΩ using thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm). We visualized LC neurons with an upright microscope (Nikon Eclipse 6600) using an x60 water-immersion objective. A visualized neuron was patched, forming a gigaohm seal. Membrane potential ($V_m$) was measured in both current and voltage clamp mode. An Axopatch 200B amplifier was used for the injection of either current or voltage. A slope/height window discriminator (FHC Model 700B, Bowdoinham, ME) was used to determine the integrated firing rate (FR). pCLAMP software version 10.0 was used to analyze $V_m$ and FR. Recordings were started when $V_m$ reached a stable resting value. A healthy neuron was defined as one with a stable resting $V_m$ of -45 to -60 mV and a spontaneous firing rate of < 4 Hz. All electrophysiological responses were shown to be reversible by returning to baseline values when the solution was changed back to the initial aCSF. We were able to successfully patch LC neurons for longer than 45 minutes in current clamp without washout of the chemosensitive response (Filosa & Putnam, 2003). Most drugs/solutions exhibited their effects within less than 2 minutes. When more than one hypercapnic pulse was used in an experiment, we returned resting FR between pulsed to within 0.5 Hz of the original FR by injecting current so that we could compare the chemosensitive responses to the two different pulses.

LC neurons were clamped at a holding potential of -70 mV in aCSF for voltage clamp experiments. TTX (1 µM) was added to block $Na^+$ currents and 3 mM BaCl$_2$, to maximize $Ca^{2+}$ currents, replaced NaCl isosmotically. Depolarizations (600 ms duration) were applied in 10 mV steps from -60 mV to +50 mV and the resulting peak current
determined. These measurements were made in either aCSF equilibrated with 5% CO₂/95% O₂ or in HEPES-buffered aCSF (nominal absence of CO₂/HCO₃⁻).

**Immunohistochemistry:** Brainstem slices (300 µm) from neonatal rats aged above P10 were fixed in freshly prepared 4% paraformaldehyde in PBS buffer, pH 7.4, for 72 hours. Slices were then washed three times in PBS plus 0.1% TritonX for 15 minutes per wash, and then incubated in PBS/TritonX buffer plus 1% bovine serum albumin (BSA) for 30 minutes. 1:200 to 1:100 dilutions of FabGennix (SAC-101-AP) anti-sAC antibodies in PBS/TritonX buffer were prepared. Fixed slices were incubated in primary antibody for 72 hours at 4°C. After incubation, slices were again washed three times in PBS/TritonX buffer for 15 minutes for each wash and incubated overnight with CY3 secondary antibody. Secondary antibody was then washed off the slice using three 15 minute washes in PBS buffer without detergent. Control slices were treated identically except that they were not incubated in primary antibody (SAC-101-AP). Stained and control slices were visualized with either an Olympus FV300 or FV1000 Confocal Microscope. A series of confocal optical sections (Z stack, resolution 0.5 µm) were used to assess the extent of neuronal labeling.

**Drugs:** TTX, BSA, 2HE, db-cAMP, H89, and Fura-2 were purchased from Sigma-Aldrich (St Louis, MO). 2HE (20 mM) was prepared as a stock solution in EtOH while TTX (1 mM), db-cAMP (10 mM), H89 (10 mM), and Fura-2 (10 mM) stocks were made in dH₂O.
Data Analysis and Statistics: Changes in firing rate (ΔFR) were quantified using the following equation: \[ \Delta FR = \frac{((\text{hypercapnic average firing rate} - \text{control average firing rate}) \times 100\%)}{\text{control average firing rate}} \]. The firing rate response to hypercapnia was quantified by calculating the chemosensitivity index (CI) according to the equation of Wang and Richerson (1999). CI represents the % change of firing rate for a 0.2 pH unit change in external pH. Thus, a nonchemosensitive neuron would have a CI of 100%. Neurons that responded to hypercapnia with a 20% or greater increase in firing rate were considered chemosensitive. All values are expressed as mean ± SEM. Significant differences between two means were determined by student t-tests or paired t-tests. We used ANOVA with multiple paired comparisons to compare more than two means. Differences were considered significant if P<0.05.
**Results**

Whole cell voltage clamp of LC neurons in the presence of blockers of Na\(^+\) and K\(^+\) channels demonstrated slowly inactivating inward currents that activated at approximately -30 mV and reversed at around +45 mV, consistent with L-type Ca\(^{2+}\) currents previously reported for LC neurons (Imber et al., 2012) (Fig. 35). Since the peak Ca\(^{2+}\) current for LC neurons was observed after day P10 (Imber et al., 2012), only rats P10 and older were used for this study. Figure 35A shows a typical appearance for the peak Ca\(^{2+}\) current at -10 mV, including the long (>200 ms) inactivation time consistent with L-type Ca\(^{2+}\) channels (Hille, 2001; Imber et al., 2012). When 250 μM db-cAMP was added to the perfusion solution, a marked increase in the amplitude of the peak Ca\(^{2+}\) current was observed (Fig. 35A). The increase in current amplitude could be reversed when the slice was washed in aCSF for ~5 minutes (Fig. 35A). These data suggest that increased intracellular cAMP levels can increase the L-type Ca\(^{2+}\) current in LC neurons. Figure 35B shows the average Ca\(^{2+}\) IV plots for 5 neurons from 3 slices aged P10-P13. The addition of db-cAMP to the perfusion solution results in both an increase in current amplitude and voltage sensitivity, activation being shifted in the hyperpolarizing direction (Fig. 35B). These results mirror the activation of the Ca\(^{2+}\) current by CO\(_2\) noted previously, and are consistent with an activation of an L-type Ca\(^{2+}\) channel by phosphorylation (Dai et al., 2009; Hille, 2001; Imber et al., 2012; Sculptoreanu et al., 1993; Sculptoreanu et al., 1995; Summers et al., 2002).

The activation of L-type Ca\(^{2+}\) currents by db-cAMP also increases resting intracellular Ca\(^{2+}\) levels in LC neurons, similar to the effects of 1) hypercapnia and 2) the voltage activation of nifedipine-sensitive Ca\(^{2+}\) currents previously reported (Imber et al.,
**Figure 35:**  
A. Peak voltage-sensitive currents activated by a step from -70 mV to -10 mV in the presence of Na$^+$ and K$^+$ blockade and 3 mM BaCl$_2$. Top trace is in aCSF, while middle trace is in the presence of db-cAMP. Note the large difference in the amplitude of inward current in the presence of the cAMP analogue and the long inactivation time, typical of L-type currents. Bottom trace is after 5 minutes of wash in aCSF, and reverses the increase in current amplitude.  
B. Average IV plot of current recordings under the same conditions as in A. Resulting IV plots are characteristic for high voltage activated Ca$^{2+}$ channels. Black trace is control in aCSF, while gray trace is in db-cAMP.
Figure 35.
2012; Imber & Putnam, 2012). Figure 36A shows the results from an LC neuron loaded with the Ca\(^{2+}\)-sensitive dye Fura-2. When db-cAMP is added to the superfusate, an increase in intracellular Ca\(^{2+}\) levels was observed (Fig. 36A). This increase reversed when db-cAMP was washed from the slice and a second exposure to db-cAMP once again reversibly increased intracellular Ca\(^{2+}\) (Fig. 36A). Figure 36B shows the average increase in R\(_{\text{fl}}\) values caused by the addition of db-cAMP in 3 neurons from 2 slices. In all cases, the membrane potential remained at rest or hyperpolarized slightly, and did not show an increase in firing rate (data not shown). The increase in intracellular Ca\(^{2+}\) levels by the addition of a cAMP analogue supports the enhanced activation of L-type Ca\(^{2+}\) channels by a cAMP-dependent pathway, similar to those reported previously (Dai et al., 2009; Summers et al., 2002).

The nominal absence of CO\(_2\)/HCO\(_3^-\) decreased both the current amplitude and voltage sensitivity of L-type Ca\(^{2+}\) channels in LC neurons. Replacing CO\(_2\)/ HCO\(_3^-\)-buffered aCSF solution with HEPES solution (equilibrated with 100% O\(_2\)) reduced the amplitude of the peak Ca\(^{2+}\) current, and was reversed by restoring CO\(_2\)/ HCO\(_3^-\)-buffered aCSF (Fig 37A). An IV plot for 4 neurons from 3 slices aged P10-P13. In the nominal absence of CO\(_2\)/ HCO\(_3^-\), an IV plot (4 neurons from 3 slices) showed that the amplitude and voltage sensitivity of the Ca\(^{2+}\) current in LC neurons was decreased compared to CO\(_2\)/HCO\(_3^-\) -buffered aCSF (Fig. 37B). These data suggest a loss of activation of L-type Ca\(^{2+}\) channels in LC neurons in the nominal absence of intracellular HCO\(_3^-\), consistent with our previous findings of an HCO\(_3^-\)-dependence of L-type Ca\(^{2+}\) activity in LC neurons (Imber & Putnam, 2012).
Figure 36:  

A. LC neuron loaded intracellularly with the Ca^{2+}-sensitive dye Fura-2. $R_{fl}$ is the ratio of fluorescence from excitation at 340nm/ 380nm. Exposure to the membrane permeable c-AMP analogue db-cAMP causes a reversible increase in intracellular Ca^{2+}.

B. Average increase in $R_{fl}$ values after adding db-cAMP to the superfusate (N=3). Increases in $R_{fl}$ values were significant with a P<0.005.
Figure 37: A. Peak voltage-sensitive currents activated by a step from -70 mV to -10 mV. Top trace is in aCSF, while middle trace is in HEPES-buffered aCSF equilibrated with 100% O$_2$. Note the decrease in the amplitude of inward current in the nominal absence of CO$_2$/HCO$_3^-$. Bottom trace is after 5 minutes of wash in aCSF, and reverses the decrease in current amplitude. B. Average IV plot of current recordings under the same conditions as in A. Black trace is control in aCSF, while gray trace is in HEPES-buffered aCSF.
Figure 37.
One way by which L-type Ca\textsuperscript{2+} channel activity can be HCO\textsubscript{3}\textsuperscript{-}-dependent is if LC neurons contain sAC. We performed immunohistochemistry experiments to look for the presence of sAC in LC neurons from neonatal rats. We observed positive staining for sAC (red puncta, Fig. 38A) in the cytoplasm of LC neurons from a P10 rat. The green is autofluorescence from the catecholamines in the largely catecholaminergic LC neurons, and readily identifies the cytoplasm of individual neurons (Fig. 38). No sAC staining was observed in LC neurons (rat aged P12) in a control slice treated with secondary antibody only (Fig. 38B). These immunohistochemical findings indicate the presence of sAC in the cytoplasm of LC neurons from older neonatal rats.

The possible role of sAC in a pathway involving HCO\textsubscript{3}\textsuperscript{-}-activated L-type Ca\textsuperscript{2+} channels can best be determined by studying the firing rate response of LC neurons to hypercapnia in the presence of an inhibitor of sAC, 2-hydroxyestradiol (2HE) (Li et al., 2011; Schlicker et al., 2008). The activation of an L-type Ca\textsuperscript{2+} current has previously been shown to significantly decrease the firing rate response of LC neurons to hypercapnia via the activation of BK channels in neonatal rats older than P10 (Imber et al., 2012). Since we hypothesize that the activation of sAC is the mechanism behind the hypercapnic activation of the Ca\textsuperscript{2+} current, it follows that inhibition of the sAC enzyme should increase the firing rate response of LC neurons to CO\textsubscript{2} in a similar fashion, i.e. inhibition of the braking pathway should increase the firing rate response. Figure 39A shows a typical chemosensitive response of increased firing rate in response to hypercapnia for an LC neuron from a P10 rat (Gargaglioni et al., 2010; Imber et al., 2012). When the same neuron was exposed to the sAC inhibitor 2HE, the firing rate response to hypercapnia was increased (ΔFR value of approximately 0.5 Hz in aCSF to
Figure 38:  A. Immunohistochemical studies of LC neurons from a P10 rat incubated with an antibody for sAC and secondary antibody CY3 show the presence of the sAC enzyme in the cytoplasm (red puncta). Green fluorescence is autofluorescence from the catecholamines in the largely catecholaminergic LC neurons. B. LC neurons from a P12 rat incubated with CY3 only. Notice the absence of red puncta. Scale bars represent 50 μm.
Figure 38.
Figure 39: The inhibition of sAC enzyme by 2-hydroxyestradiol (2HE) causes an increase in the chemosensitive response. A. A typical chemosensitive response for whole cell current clamp experiments from neonatal rats older than P10. B. The same neuron as in A in the presence of 2HE. Notice the significant increase in the firing rate response to hypercapnia in the presence of 2HE. C. A complete current clamp record of a whole cell patch from a P14 LC neuron. Notice the identical response to hypercapnia in the presence of aCSF that is greatly increased by the inhibition of sAC. Washing the slice in aCSF reverses the large apparent hyperpolarization due to a change in junction potential, caused by the addition of 2HE to the superfusate; however, the inhibition of sAC does not appear to reverse.
Figure 39.
1.5 Hz in the presence of 2HE) (Fig. 39B). Another example of the effects of 2HE are shown in Fig. 39C, where repeated pulses of hypercapnia yield a similar increase in firing rate but the firing rate is markedly increased in the presence of 2HE from an older (P14) neonate. Note that the effects of 2HE do not rapidly wash off (Fig. 39C). Note also that the addition of 2HE during normocapnia (5% CO₂) causes no change in the firing rate (Fig. 39C), suggesting that sAC has a very low activity under normal physiological conditions. These data are summarized in Figure 40 where it is clear that there is a significantly increased firing rate response to hypercapnia in the presence, compared to the absence, of 2HE. These data support our hypothesis that sAC is involved in the activation of L-type Ca²⁺ channels in a HCO₃⁻-dependent braking pathway that results in elevated intracellular Ca²⁺ and activation of BK channels.

If sAC/cAMP is activating the Ca²⁺ currents in LC neurons via PKA phosphorylation, then the inhibition of PKA should also increase the firing rate response of LC neurons to hypercapnia. When the PKA inhibitor H89 was added to the superfusate during the whole cell patch of a LC neuron from a P12 rat, there was no increase in firing rate under control conditions (5% CO₂) but an increase in the firing rate response to hypercapnia (Fig. 41A). This effect reversed when the slice was restored to normocapnic aCSF (Fig. 41A). Thus, it appears that PKA has very low activity in control LC neurons but that hypercapnia activates PKA.

We also hypothesized that acidified HEPES, in the nominal absence of CO₂/HCO₃⁻, would not activate the braking pathway (no activation of sAC) and would thus result in a large firing rate response of LC neurons to hypercapnia. Indeed, a large increase in the firing rate response to acidification was seen when a LC neuron (from a
Figure 40: Summary of the results from comparisons of sequential hypercapnic responses in LC neurons with (ΔFR\textsubscript{2HE}) and without (ΔFR) the presence of 2HE. ΔFR represents the difference in average firing rate (Hz) in the presence of 15% CO\textsubscript{2} and 5% CO\textsubscript{2}. ΔFR\textsubscript{2HE} – ΔFR measures the increase in hypercapnic response caused by inhibition of the braking pathway (N=5) (see text). Control values represent sequential hypercapnic responses without the addition of inhibitor (ΔFR\textsubscript{2}– ΔFR\textsubscript{1}). The heights of the bars represent means ± 1 SEM. Values were significant from one another with a P<0.001.
Figure 40.
**Figure 41:**  
A. The inhibition of PKA enzyme by H89 causes an increase in the chemosensitive response. The hypercapnic increase in firing rate from a LC neuron from a neonatal rat age P12 is increased by the incubation of the slice with H89 for ~ 5 minutes. This increase in the chemosensitive response is reversed by washing the slice in aCSF. 

B. A typical pH-sensitive response for a P12 LC neuron in HEPES-buffered aCSF equilibrated with 100% O₂ at pH 7.4. Exposure to HEPES-buffered aCSF at pH 6.4 causes a reversible increase in firing rate.
Figure 41.
neonatal rat older than P10) in HEPES-buffered aCSF (pH 7.4) was exposed to HEPESbuffered aCSF acidified to pH 6.9 (Fig. 41B). These data are consistent with the proposed HCO$_3^-$ dependence of the braking pathway in LC neurons from older neonatal rats.

We compared the magnitude of the firing rate response to hypercapnia under these various conditions by calculating the chemosensitivity index (CI) (Fig. 42). We found, in agreement with previous studies (Gargaglioni et al., 2010; Imber et al., 2012), that the CI of LC neurons decreased during early neonatal development, falling from an average value of 201.1% in neurons from rats P3-P6 to 127.6% in rats P10-P14 (Fig. 42). Interestingly, three treatments aimed at inhibiting the HCO$_3^-$-dependent activation of Ca$^{2+}$ channels in older neonates resulted in a complete reversal of the age-dependent decrease in CI. Thus, in LC neurons from rats P10-P14, in the presence of HEPES-buffered aCSF (nominal absence of CO$_2$/HCO$_3^-$) the CI was 191.9%, in the presence of the sAC-inhibitor 2HE it was 193.6%, and in the presence of the PKA-inhibitor H89 it was 186.9% (P<0.001 when compared to the normal value of CI in older neonates) (Fig. 42). These values are not significantly different from the value reported for LC neurons from neonatal rats aged P10 to P16 in the presence of an inhibitor of BK channels (CI of 196.1%) (Imber et al., 2012). Also, CI recorded from neonatal rats aged P4-P6 was likewise not significantly different than CI values recorded in the presence of the inhibition of sAC, PKA, or in the nominal absence of CO$_2$/HCO$_3^-$ (Fig 42). These results support our hypothesis that a HCO$_3^-$/sAC/PKA-dependent pathway leads to the chemosensitive activation of Ca$^{2+}$ channels, resulting in a BK channel-dependent braking mechanism on the firing rate response of LC neurons to hypercapnia.
Figure 42: Average CI values from LC neurons recorded in either aCSF (P3-P6, N=21; P10-P14, N = 18), HEPES-buffered aCSF (N = 5), or aCSF plus 2HE (N=5) or H89 (N=5). Heights of the bars represent means ± 1 SEM. Differences were significant at P< 0.001.
Figure 42.
Discussion

In this study we report on our findings of the presence of a HCO$_3^-$-dependent pathway, involving sAC and PKA, which mediates the activation of L-type Ca$^{2+}$ channels by hypercapnia in LC neurons. This pathway is similar to one previously observed in peripheral chemosensitive glomus cells (Summers et al., 2002) and is consistent with our previous demonstration of a HCO$_3^-$ dependence to Ca$^{2+}$ channel activation in LC neurons (Imber & Putnam, 2012). This is the first demonstration of such a pathway being active in central chemosensitive neurons, adds a novel pathway by which the magnitude of the chemosensitive response is regulated in LC neurons, and emphasizes the fact that Ca$^{2+}$ plays a role in central chemosensitivity.

We have demonstrated the presence of sAC in the cytoplasm of neonatal LC neurons (Fig. 38). sAC differs from the transmembrane adenylyl cyclase by being insensitive to G-proteins and to forskolin and by its regulation by HCO$_3^-$ (Summers et al., 2002; Zippin et al., 2001). In LC neurons, it is likely that sAC is activated by the increase in intracellular HCO$_3^-$ induced by hypercapnia. Most studies of the cellular signaling pathways in chemosensitive neurons emphasize changes in pH and the role of pH-sensitive ion channels (Bayliss et al., 2001; Bradley et al., 2002; Cui et al., 2011; Filosa et al., 2002; Li & Putnam, 2009; Pineda & Aghajanian, 1997; Putnam et al., 2004; Wiemann & Bingmann, 2001; Xu et al., 2000). Our findings strongly suggest that changes of intracellular HCO$_3^-$ are an additional important signal associated with the chemosensitive response to hypercapnia.

We have previously demonstrated that hypercapnia activates an L-type Ca$^{2+}$ current in LC neurons (Filosa et al., 2002) and that this activation is pH-independent and
develops over the ages P3-P16 (Imber et al., 2012; Imber & Putnam, 2012). The current study helps to define the precise mechanism of this activation. When db-cAMP was added to the superfusate, the IV plot for Ca\textsuperscript{2+} currents was enhanced in LC neurons from neonatal rats older than P10 (Fig. 35), resulting in increased levels of intracellular Ca\textsuperscript{2+} (Fig. 36). The ability of db-cAMP to increase intracellular Ca\textsuperscript{2+} levels and the Ca\textsuperscript{2+} current amplitude and voltage sensitivity in the presence of normocapnia (5% CO\textsubscript{2}) is analogous to the effects of hypercapnia on Ca\textsuperscript{2+} currents and intracellular Ca\textsuperscript{2+} levels in LC neurons (Imber et al., 2012). These findings clearly implicate increased cAMP in the hypercapnia-induced pathway of activation of Ca\textsuperscript{2+} channels in LC neurons. We have further shown that in the nominal absence of CO\textsubscript{2}/HCO\textsubscript{3}-, using a HEPES-buffered aCSF, Ca\textsuperscript{2+} currents recorded from LC neurons are decreased (Fig. 37). These results support the previous finding of a strong correlation between intracellular HCO\textsubscript{3}- and Ca\textsuperscript{2+} channel activity (Imber & Putnam, 2012).

Taken together, the above findings imply a HCO\textsubscript{3}-dependent pathway that leads to the production of intracellular cAMP. Given the previous findings in peripheral chemoreceptors (Summers et al., 2002), it seems likely that such a pathway should involve sAC. Our demonstration of the presence of sAC in LC neurons (Fig. 38) is consistent with its involvement in the activation of Ca\textsuperscript{2+} channels by hypercapnia in LC neurons. Further, the involvement of increased cAMP suggests activation of PKA in LC neurons by hypercapnia and activation of Ca\textsuperscript{2+} channels by PKA-mediated phosphorylation.

Our previous results have implicated hypercapnia-activated Ca\textsuperscript{2+} channels and increased intracellular Ca\textsuperscript{2+} in a braking pathway, mediated by activation of BK channels
(Imber et al., 2011; Imber et al., 2012). We have used this braking phenomenon to further investigate the pathway by which hypercapnia activates Ca\(^{2+}\) channels. We reasoned that if an inhibitor blocks part of the Ca\(^{2+}\) channel activation mechanism, it would also block the braking pathway in LC neurons from older neonates. Using such an approach, an inhibitor of sAC, 2HE, resulted in a highly significant increase in the firing rate response to hypercapnia of LC neurons from older neonates (Figs. 39, 40 and 42). The absolute increase in the firing rate response to hypercapnia in the presence of 2HE (ΔFR\(_{2\text{HE}}\)-ΔFR) and the CI values (Figs. 40 and 42) were similar to those recorded in the presence of an inhibitor of BK channels (Imber et al., 2011; Imber et al., 2012). We also found that the PKA inhibitor H\(_89\) resulted in a highly significant increase in the firing rate response to hypercapnia of LC neurons from older neonates (Figs. 41 and 42). Once again, PKA inhibition led to increases in firing rate and in CI (Figs. 41 and 42) that were similar to the increases resulting from inhibition of BK channels. These data are consistent with a role for a HCO\(_3^-\)-sAC-cAMP-PKA pathway in the activation of Ca\(^{2+}\) currents in LC neurons in response to hypercapnia causing a concurrent decrease of the chemosensitive response in neonatal rats older than P10 (Fig 42).

There are several significant findings to this study. Our findings indicate a significant role for HCO\(_3^-\) as a chemosensitive signal in LC neurons and describe the first role for an sAC-cAMP-PKA pathway in a central chemosensitive neuron. Further, that this pathway leads to the activation of Ca\(^{2+}\) channels and increased intracellular Ca\(^{2+}\) points to a previously nearly unexplored potential role of calcium in central chemosensitive signaling. There are several possible ways in which calcium could contribute to central chemosensitivity. The activation of Ca\(^{2+}\) channels should depolarize
and therefore activate chemosensitive neurons. In fact, the inhibition of L-type $\text{Ca}^{2+}$ channels by nifedipine decreased the chemosensitive response in LC neurons from young neonatal rats (P1-P9) (Filosa & Putnam, 2003). This could reflect a $\text{Ca}^{2+}$-dependent activation of chemosensitive LC neurons from young neonates. Alternatively, since L-type $\text{Ca}^{2+}$ channel inhibition can also diminish synaptic input, it is possible that the effects of nifedipine inhibition on LC neuron chemosensitivity is not due to depolarization of $V_m$ by activated $\text{Ca}^{2+}$ channels, but rather due to the inhibition of synaptic input. It is clear that in LC neurons from older neonates (>P10), increased intracellular $\text{Ca}^{2+}$ plays a role as a brake on the chemosensitive response due to activation of BK channels (Imber et al., 2011; Imber et al., 2012).

Our work raises some interesting unanswered questions as well. Elevated intracellular $\text{Ca}^{2+}$ could alter the activity of any number of channels or intracellular signaling pathways, all of which could affect the chemosensitive response of LC neurons, but such a possibility remains largely unexplored. The involvement of PKA and the pattern of changes in the IV plot of activated $\text{Ca}^{2+}$ channels suggest that these channels are being phosphorylated by hypercapnia. However, the seeming rapid reversal of this activation, as evidenced by the rapid fall of intracellular $\text{Ca}^{2+}$ when channels are no longer activated (Fig. 36), suggests that $\text{Ca}^{2+}$ channels are rapidly dephosphorylated upon return to normocapnic conditions. This implies that reversal of the hypercapnic effects on L-type $\text{Ca}^{2+}$ channels in LC neurons requires an as yet unknown phosphatase. In theory, alteration of the activity of this phosphatase could alter the chemosensitive response of LC neurons, a novel pathway for controlling the firing rate response of chemosensitive neurons. Finally, it is currently unclear to what extent sAC or the
activation of Ca\(^{2+}\) channels is involved in the hypercapnic response of chemosensitive neurons from other areas of the medulla and pons, although there has been evidence for hypercapnic Ca\(^{2+}\) signaling in astrocytes near the region of the retrotrapezoid nucleus (Gourine et al., 2010; Huckstepp et al., 2010; Wenker et al., 2010). In addition, pH-induced inhibition of tonically active \(K_{Ca}\) channels (possibly by inhibition of inhibition of Ca\(^{2+}\) channels) in cultured medullary neurons has been suggested to be part of the pathway by which hypercapnia activates these neurons (Wellner-Kienitz et al., 1998). It is clear, however, that there is a need to better characterize pathways involving Ca\(^{2+}\) and central chemoreceptive control.
CHAPTER VIII

CONCLUSION
My thesis has focused on the role of Ca\textsuperscript{2+} in the chemosensitive response of LC neurons. There has been a recent emphasis on the magnitude of the firing rate response to hypercapnia in central chemosensitive control (Mulkey et al., 2004). CO\textsubscript{2}-sensitive neurons of the LC are ideal candidates to study Ca\textsuperscript{2+} and the chemosensitive response because 1) there is existing evidence that a Ca\textsuperscript{2+} current in LC neurons is increased by hypercapnia (Filosa & Putnam, 2003; Oyamada et al., 1999); 2) neurons of the LC undergo a natural decrease in their firing rate response to hypercapnia around age P10 (Gargaglioni et al., 2010); and 3) the LC has been implicated in the pathology of multiple disease states including breathing, awareness, and panic and anxiety. Thus, this study has focused on pathways involving Ca\textsuperscript{2+} and the control of the hypercapnic response in chemosensitive LC neurons.

Previous studies have shown that rhythmic TTX-insensitive currents exist in LC neurons that are sensitive to L-type Ca\textsuperscript{2+} channel blockade (Filosa & Putnam, 2003). These currents increased with exposure to hypercapnia, and were observed only inconsistently in young neonatal rats aged P5 to P20 (Maubecin & Williams, 1999; Oyamada et al., 1999). Because of our emphasis on the role of Ca\textsuperscript{2+} currents in LC neurons, our current work has furthered these preliminary studies. First, we report that the TTX-insensitive current is actually a combination of voltage-insensitive oscillations and voltage-sensitive spikes. In agreement with previous work, our study found that both TTX-insensitive oscillations (also referred to as subthreshold rhythmic oscillations) and TTX-insensitive spikes increased in frequency when exposed to hypercapnia and were completely inhibited by exposure to the dihydropyridine nifedipine. Thus, we believe that the TTX-insensitive current in LC neurons represents the activity of L-type Ca\textsuperscript{2+} channels.
that are activated by hypercapnia. In addition, we examined the pH sensitivity of both

types of TTX-insensitive current using HEPES-buffered aCSF, which contains a nominal

amount of CO₂/HCO₃⁻, and isohydric hypercapnia, which contains increased HCO₃⁻ and

no change in extracellular pH (pHₒ). We found that decreasing CO₂/HCO₃⁻ levels

inhibited the appearance of both oscillations and spikes. Conversely, increasing

CO₂/HCO₃⁻ without an extracellular acidification while causing only a small intracellular

acidification resulted in an increased frequency of both oscillations and spikes (isohydric

hypercapnic solution). We also found that spike frequency increased during exposure to

isohydric hypercapnia. Since CO₂ levels are the same between hypercapnic acidotic and

isohydric hypercapnic solutions and HCO₃⁻ levels are changed, we believe that the

activation of the L-type Ca²⁺ current is sensitive to HCO₃⁻ levels. Consistent with this,

when we calculated intracellular HCO₃⁻ concentrations using the Henderson Hasselbalch

equation and intracellular pH (pHᵢ) (see Methods in chapter IV), we found that spike and

oscillation frequency increased in a linear fashion with intracellular HCO₃⁻ with a slope

of approximately 0.1.

Since LC neurons change the magnitude of their chemosensitive response and we

believe that this change is due to changes in Ca²⁺, we also studied the effects of age on

TTX-insensitive oscillations and spikes. Both oscillations and spikes appeared

completely absent in LC neurons from animals ages P3 to approximately P6. In LC

neurons from animals ages P8 to around P10, both oscillations and spikes could be

evoked by the addition of hypercapnic acidosis or isohydric acidotic solutions. After age

P10, both oscillations and spikes could be observed in LC neurons during normocapnia,

and increased in frequency when exposed to either hypercapnic acidosis or isohydric
hypercapnia. Significantly however, although the frequency of both oscillations and spikes increased over the first few weeks of postnatal development, the response to intracellular HCO$_3^-$ concentrations remained linear with a slope of approximately 0.1. Thus, although the Ca$^{2+}$ current appears to increase over ages P3 to P16 in LC neurons, we found the response to increases in CO$_2$/HCO$_3^-$ to be consistent.

Although TTX-insensitive spikes and oscillations differ in their voltage sensitivity and their waveform as recorded through whole cell patch clamp of single LC neurons, the remainder of the characteristics we observed for both oscillations and spikes appear very similar. Both oscillations and spikes appear to have similar sensitivity to pH, CO$_2$/HCO$_3^-$, dihydropyridines, and neonatal age. In addition, we studied the effects of oscillations and spikes on intracellular Ca$^{2+}$ levels. As previous studies have shown that $V_m$ oscillations in LC neurons are sensitive to gap junctions (Ballantyne et al., 2004), we also tested the effects of carbenoxolone on both oscillations and spikes. We found that spikes increased intracellular Ca$^{2+}$ levels in the whole-cell patched neuron in a frequency-dependent fashion. Oscillations, meanwhile, did not change resting intracellular Ca$^{2+}$. Conversely, gap junction blockade using carbenoxolone inhibited the appearance of oscillations, but did not change the appearance of spikes. Collectively, we believe that the above data are consistent with oscillations representing the appearance of spikes across multiple neurons as seen through the low-pass filtering effect of gap junctions. Thus, while spikes represent the activity of L-type Ca$^{2+}$ channels in a single LC neuron, oscillations represent the collective activity of the L-type Ca$^{2+}$ currents in other, un-patched LC neurons.
In order to address the CO$_2$/HCO$_3^-$ and age-dependency of the Ca$^{2+}$ current in LC neurons more directly, we used voltage-clamp studies. When Na$^{2+}$ and K$^+$ channels were blocked in voltage clamp mode and the Ca$^{2+}$ channels were enhanced using Ba$^{2+}$ as the partial charge carrier, we observed large (>1 nA) inward currents that activate at -30 to -20 mV and reverse at -35 to -45 mV, consistent with the activity of high-voltage activated Ca$^{2+}$ channels (Hille, 2001). Consistent with our previous observations on TTX-insensitive spikes, we found that the average Ca$^{2+}$ current increases from some 0.8 nA in LC neurons from neonatal rats ages P3 to P5 to some 2.4 nA in LC neurons from rats older than P10. Since we found that the average size of LC neurons does not increase over this age range (see chapter IV), we believe that the Ca$^{2+}$ current density is increasing during the first two weeks of postnatal development of LC neurons from neonatal rats.

We also found that increasing CO$_2$/HCO$_3^-$ can increase both the amplitude and the voltage sensitivity of these Ca$^{2+}$ currents, while decreasing CO$_2$/HCO$_3^-$ levels with HEPES-buffered aCSF decreased both the amplitude and voltage activation of the Ca$^{2+}$ current. These data are consistent with activation of the Ca$^{2+}$ channels in LC neurons by CO$_2$/HCO$_3^-$. Exposing the Ca$^{2+}$ current to nifedipine decreased the amplitude of these currents by over 60%, consistent with a significant presence of L-type Ca$^{2+}$ channels. It is not clear whether the remaining Ca$^{2+}$ current is due to another high voltage activated type of Ca$^{2+}$ channel such as R-type, or whether the remaining current represents the activity of additional L-type channels that were not completely inhibited by the superfusion of dihydropyridines (Lipscombe et al., 2004).

Our studies have presented evidence for large voltage-active Ca$^{2+}$ currents in LC neurons. In current clamp, these Ca$^{2+}$ currents also increased intracellular Ca$^{2+}$ levels, and
in both current and voltage clamp studies the Ca$^{2+}$ currents increased with hypercapnia. Thus, it follows that exposure to hypercapnia should increase intracellular Ca$^{2+}$ levels. We found that during hypercapnic exposure, intracellular Ca$^{2+}$ levels did increase. Furthermore, the resting intracellular Ca$^{2+}$ levels under normocapnia also increased when LC neurons were recorded from rats older than P10. These data are consistent with an increase in the Ca$^{2+}$ current over the first two postnatal weeks. However, since the R$_{fl}$ values for Fura-2 fluorescence were not calibrated, it is difficult to make comparisons between recordings in multiple LC neurons. Significantly, we also found that the hypercapnic increase in intracellular Ca$^{2+}$ levels could be abolished by the inhibition of L-type Ca$^{2+}$ channels by dihydropyridines.

Given the enhancement of the Ca$^{2+}$ current and the increase in intracellular Ca$^{2+}$ due to hypercapnia, we ask what role Ca$^{2+}$ might play in determining the magnitude of the chemosensitive response of LC neurons. First, we addressed the presence of large conductance Ca$^{2+}$-activated K$^+$ channels, or BK channels, with immunohistochemistry. Staining showed the presence of BK channels in the cell membrane of LC neurons from a P11 rat and a P3 rat. The BK channel staining in the P3 rat did not appear as robust as the staining from the P11 rat, however, it is difficult to quantify channel density using this technique. In order to study the magnitude of the BK current in LC neurons, we also used a voltage clamp protocol (see chapter IV). We found that the paxilline difference current (ΔI), which is a measure of the amount of total Ca$^{2+}$ and K$^+$ current present at a +80 mV voltage step that is sensitive to the BK channel inhibitor paxilline, increases in LC neurons from rats aged P4 to P14. This age range is the same we found previously for the developmental increase in the Ca$^{2+}$ current, using both voltage clamp and current clamp
techniques. Significantly, we also found that the proportional increase in total Ca\(^{2+}\) current and BK current as measured by voltage clamp was very similar. This may mean that the BK channel density remains constant over this age period and that the increase in BK current is due to the increase in Ca\(^{2+}\) current, or BK channels may increase in addition to Ca\(^{2+}\) channels. In support of the former conclusion, decreasing intracellular Ca\(^{2+}\) levels with the Ca\(^{2+}\) chelator BAPTA decreased the BK current from LC neurons from older (>P10) neonatal rats to values similar to the BK current recorded from LC neurons from younger (P4-P5) neonatal rats. This may indicate that the same number of BK channels are present, which are then activated by varying levels of intracellular Ca\(^{2+}\).

We found that L-type Ca\(^{2+}\) channel inhibition using dihydropyridines also decreased the BK channel current similarly to BAPTA. This is significant since dihydropyridines inhibited the total Ca\(^{2+}\) current by only some 60%, indicating that a substantial Ca\(^{2+}\) current should remain after exposure to nifedipine to increase intracellular Ca\(^{2+}\) levels and activate BK current. However, studies with Fura-2 showed that exposure to nifedipine decreased intracellular Ca\(^{2+}\) levels and prevented the hypercapnic increase in intracellular Ca\(^{2+}\). These data are consistent with a loss of BK current during exposure to dihydropyridines, similar to the chelation of intracellular Ca\(^{2+}\) using BAPTA. It may be that the activation of L-type Ca\(^{2+}\) channels is accompanied by other mechanisms designed to increase intracellular Ca\(^{2+}\). Alternatively, it may also be that L-type Ca\(^{2+}\) channels and BK channels are located in close proximity to one another on the cell membrane, in order to allow for efficient activation of the BK channel current (see discussion in chapter VI). In the latter case, whole neuron Ca\(^{2+}\) levels would not be
as relevant to the activation of BK channels as compartmentalized Ca\(^{2+}\) concentrations (micro- or nanodomains) surrounding the L-type and BK channels.

Given 1) the increase in Ca\(^{2+}\) currents in response to hypercapnia, 2) the increase in intracellular Ca\(^{2+}\) levels in response to increased Ca\(^{2+}\) currents, and 3) the developmental increase of both Ca\(^{2+}\) and BK currents, we believe that the role of Ca\(^{2+}\) in chemosensitive LC neurons is to activate hyperpolarizing BK currents during hypercapnia, decreasing the magnitude of the firing rate response to increased CO\(_2\).

Consistent with this, we found that we could increase the magnitude of the firing rate response to CO\(_2\) in LC neurons when BK channels were inhibited with paxilline. We further observed that inhibiting BK channels in LC neurons from rats aged P5 to P9 caused a markedly smaller increase in the firing rate response to hypercapnia than in neurons from rats aged P10 to P16. This is consistent with the developmental increase in the BK current during this age period. Thus, the BK current may function to relieve the chemosensitive respiratory response in LC neurons during early neonatal development. It may also be involved in the control of panic and anxiety in response to CO\(_2\) (see discussion in chapter III and IX).

Significantly, we found that the addition of paxilline does not alter firing rate during normocapnia. This suggests that the BK current is not substantially active during resting levels of CO\(_2\), but rather that the current becomes active during exposure to higher CO\(_2\) levels, perhaps to limit the firing rate response prior to the onset of a suffocation alarm (Papp et al., 1993). We found that the BK current becomes present at physiologically relevant V\(_m\) when intracellular Ca\(^{2+}\) levels are increased (see chapter IV). This may offer an explanation as to why the effects of paxilline are not observed in 5%
CO\textsubscript{2} during resting levels of intracellular Ca\textsuperscript{2+}: additional intracellular Ca\textsuperscript{2+} is necessary to activate BK currents at -50 mV to -40 mV.

Previous studies have noted a decrease in the chemosensitivity of LC neurons during the first two weeks of postnatal development (Gargaglioni et al., 2010). When we measured the magnitude of the firing rate response to CO\textsubscript{2} of LC neurons from rats age P3 to P16 in aCSF by calculating the chemosensitivity index (CI), we also found that the CI decreased significantly, from ~240\% (from rats aged P3 to P5) to some ~125\% (from rats aged P10 to P16). In addition, we found a transition period in LC neurons from rats aged P6 to P9 where the average CI was some ~160\%. Of note is that the gradual decrease in CI is proportional to the gradual increase in both Ca\textsuperscript{2+} and BK currents as measured by voltage clamp studies. These data suggest a correlation between an increase in the BK current and a decrease in the chemosensitive response to hypercapnia. In support of this, inhibiting BK channels increases the CI of LC neurons from neonatal rats aged P10 to P16 from ~125\% to some ~200\%, effectively restoring the chemosensitive response of LC neurons from older neonates to values like those from younger neonatal rats (ages P3 to P5). Thus, we believe that the decrease in the magnitude of the firing rate response to hypercapnia to the lower values typical of LC neurons from adult rats is due to the development of the BK current. The activity of Ca\textsuperscript{2+} and BK channels in LC neurons can thus be thought of as a ‘braking’ pathway.

Since the inhibition of BK channels during normocapnia does not affect the firing rate of LC neurons, the activation of Ca\textsuperscript{2+} channels appears to be of singular importance to the chemosensitive brake. During studies on the TTX-insensitive current, we had already noted that activation of the L-type Ca\textsuperscript{2+} current appeared sensitive to HCO\textsubscript{3}^{-}
levels and not changes in pH<sub>i</sub> or pH<sub:o</sub>. Next we asked if the HCO<sub>3</sub> sensitive enzyme soluble adenylate cyclase (sAC) was present in LC neurons from neonatal rats. Immunohistochemical studies with a commercial antibody for sAC plus CY3 secondary antibody resulted in cytoplasmic staining that was not present in tissue stained with CY3 alone. Since 1) sAC activated by increased HCO<sub>3</sub> produces cAMP, and 2) we had observed that lowering CO<sub>2</sub>/HCO<sub>3</sub> with HEPES buffer decreased the Ca<sup>2+</sup> current in voltage clamp studies, we examined the change in the Ca<sup>2+</sup> current when we increased cAMP levels with the membrane permeable cAMP analogue dibutyryl-cAMP (db-cAMP). We found that the Ca<sup>2+</sup> current was significantly enhanced during exposure to db-cAMP, increasing in both amplitude and voltage sensitivity, similar to activation by hypercapnia. Similar to the effects of hypercapnia, adding db-cAMP to the superfusate also increased intracellular Ca<sup>2+</sup> levels in LC neurons. These data are consistent with the activation of L-type Ca<sup>2+</sup> channels in LC neurons via a HCO<sub>3</sub> - sAC - cAMP mechanism. Thus, we would expect to inhibit the hypercapnic activation of L-type Ca<sup>2+</sup> channels if we interrupt this pathway. To test this, we inhibited the sAC enzyme with 2-hydroxyestradiol, we decreased CO<sub>2</sub>/HCO<sub>3</sub> with acidified HEPES buffered aCSF, and we inhibited phosphorylation by PKA with the inhibitor H89. In each case normocapnic firing rate was unaffected and we were able to increase the magnitude of the firing rate response to hypercapnia similarly to the inhibition of BK channels with paxilline (CI between 185% and 195% in LC neurons from neonatal rats aged P10 to P16 versus 125% in normocapnic aCSF alone). These data suggest that the activation of the Ca<sup>2+</sup> current by CO<sub>2</sub>/HCO<sub>3</sub> is necessary to activate the braking pathway via enhanced BK current.
The cellular mechanisms whereby neurons increase their firing rate in response to hypercapnia can be thought of as an “accelerator” pathway (see Fig 43). We have provided novel evidence for a chemosensitive Ca\(^{2+}\) current in neurons of the LC that may function as a “brake”. We show that increased CO\(_2\) increases intracellular Ca\(^{2+}\) levels via the activation of L-type Ca\(^{2+}\) channels. We believe that the activation of L-type Ca\(^{2+}\) channels occurs via increases in intracellular HCO\(_3^-\) that drives soluble adenylate cyclase to increase cAMP to phosphorylate L-type channels, possibly through PKA. The increased Ca\(^{2+}\) current then activates BK channels to enhance the K\(^+\) current and hyperpolarize the neuron, slowing the firing rate in response to hypercapnia. Coupled with our recent findings that the LC L-type Ca\(^{2+}\) current increases over the first two weeks of postnatal development in rats, this indicates a role for Ca\(^{2+}\) in the maturation and eventual adult regulation of the chemosensitive response by LC neurons. This developmental “brake” pathway may also underlie the developmental decrease in the chemosensitivity of LC neurons. Our findings represent a unique role for Ca\(^{2+}\) in controlling the magnitude of the chemosensitive response. Abnormalities of this pathway could be associated with disorders involving an altered sensitivity to CO\(_2\), such as sleep apnea and panic disorders.
Figure 43: Model for the hypercapnic activation of both an accelerator and brake pathway in central chemosensitive neurons. Left side represents the accelerator pathway involving the pH modification of various cation channels, including the inhibition of $K^+$ channels. Right side depicts a brake pathway involving the $HCO_3^-$ activation of $Ca^{2+}$ channels to increase BK current. The brake pathway may exist to control the firing rate response to hypercapnia.
Figure 43.
CHAPTER IX

FUTURE DIRECTIONS
The LC is a well-delineated cluster of approximately 1500 neurons per nucleus in the rat (Barnes & Pompeiano, 1991). Despite their modest number, it is noteworthy that LC neurons project widely throughout the CNS including the cortex, the brainstem and the spinal cord, and are the sole source of norepinephrine to both the hippocampus and neocortex (Foote et al., 1983; Mason & Fibiger, 1979; Swanson & Hartman, 1975). Afferents project to the LC core from the nucleus paragigantocellularis and the nucleus prepositus hypoglossus, related to sympathetic nervous system activation and control of eye movement, respectively (Aston-Jones et al., 1986). A dense plexus of LC neuronal dendrites extend beyond the borders of the nucleus core (pericoerulear region) (Shipley et al., 1996) and receives input from the amygdala and bed nucleus of the stria terminalis (BNST) (Van Bockstaele et al., 2001), the lateral hypothalamus (Hagan et al., 1999; Mignot, 2001; Peyron et al., 1998), the prefrontal cortex (Arnsten & Goldman-Rakic, 1984; Van Bockstaele et al., 1996) and other central chemosensitive areas in the medulla and pons including the contralateral LC, the serotonergic raphe, and the NTS (Lopes et al., 2012). These results suggest that LC neurons regulate a variety of functions important to attention (cortex), respiration (medulla and pons), emotional states (BNST, amygdala), and sleep/wake cycles (lateral hypothalamus). Thus, pathways involved in regulating the firing rate response to hypercapnia in LC neurons have implications toward several higher cognitive and affective processes in addition to ventilatory control. For future studies, I will review pathological conditions that may be related to abnormalities in LC neuron physiology, particularly in the context of chemosensitivity and the function of $\text{Ca}^{2+}$ and $K_{\text{Ca}}$ channels. In addition, I will discuss various experiments in the LC and other
chemosensitive areas of the medulla that may contribute to an understanding of the 
Ca\(^{2+}\)/BK channel braking pathway and the respiratory response.

Rett syndrome is a neurodevelopment disorder that is associated with mutations in 
the methyl-CpG binding protein 2 (MECP2) gene (Taneja et al., 2009). The mutation 
appears to be related to a reduction of biogenic amines, including dopamine, serotonin, 
and norepinephrine (Roux & Villard, 2010). Significantly, erratic breathing is one of the 
most common features observed in Rett syndrome patients. Prolonged exposure to a 
norepinephrine reuptake inhibitor improved the respiratory rhythm in MECP2-deficient 
mice, suggesting that norepinephrine-dependent systems were uniquely involved in 
abnormalities in the respiration (Roux et al., 2007). When the pathophysiology of LC 
neurons in MECP2-deficient mice was studied, it was found that neurons were smaller in 
size and deficient in the norepinephrine-synthesizing enzyme tyrosine hydroxylase (TH) 
in both the LC nucleus and the dendritic pericoerulear region (Roux et al., 2010; Taneja 
et al., 2009). A reduction in the number of LC neurons was not observed, suggesting that 
changes in the phenotype may be responsible for the abnormal physiology rather than cell 
death. Further studies in LC neurons from MECP2-deficient mice revealed an abnormal 
hyperexcitability related to an apamin-insensitive K\(_{Ca}\) current, which affected spike 
adaptation and the slow afterhyperpolarization (see discussion in chapter II). These data 
lead the authors to suggest that changes were occurring in either K\(^+\) or Ca\(^{2+}\) currents, or 
possibly in intracellular Ca\(^{2+}\) buffering mechanisms (Taneja et al., 2009). It is interesting 
to speculate that these observations could be related to the large CO\(_2\)-sensitive Ca\(^{2+}\)/BK 
currents observed in our current work. I would propose 1) to test if paxilline also 
increases the magnitude of the chemosensitive response in LC neurons in the mouse; and
2) to see if this increase is diminished in LC neurons from *MECP2*-deficient mice. This experiment would address whether the Ca\(^{2+}\)/BK braking pathway contributes to the dysregulation of LC function in a Rett syndrome model.

In 1993, it was first proposed that the respiratory abnormalities observed in patients with panic were due to a “hypersensitive brainstem autonomic control mechanism” (Papp et al., 1993). Panic disorder patients exhibit a significantly greater ratio of change in minute ventilation per PCO\(_2\) and have persistent, subclinical abnormalities in respiration during sleep and wakefulness, including an increased frequency of sighs (Abelson et al., 2001; Martinez et al., 2001; Stein et al., 1995). Wilhelm et al. (2001) noted that increased sighing was related to a lower PCO\(_2\) in PD patients versus healthy controls (34 vs. 48 mmHg end-tidal PCO\(_2\) in PD vs. controls). Consistent with this, PD patients also voluntarily hold their breath for lesser durations and experience more anxiety to elevated CO\(_2\) than healthy controls, patients with social phobias, or patients with generalized anxiety disorder (Nardi et al., 2009; Seddon et al., 2010; Stein et al., 1995). Collectively, these data strongly suggest that an increased sensitivity to CO\(_2\) contributes to panic disorder symptoms. Of the chemosensitive areas in the medulla and pons, the LC is perhaps the most likely area to be involved in both the chemosensitive respiratory drive and the stimulation of the limbic system. For one, there have been extensive studies linking the LC to the stress response via electrophysiological and neurochemical assessments of the activation of this system by known stressors, including footshock (Curtis et al., 1997; Reyes et al., 2006; Van Bockstaele et al., 2001). Second is that the central nucleus of the amygdala, which is thought to be heavily involved in the fear response, projects to the pericoerulear area surrounding the LC.
(Grillon, 2008; Van Bockstaele et al., 1998; Van Bockstaele et al., 2001; Walker et al., 2003). These projections contain a unique neurotransmitter of the central nucleus of the amygdala, corticotrophin releasing factor (CRF) (Van Bockstaele et al., 1998). When CRF is applied to the LC, the TTX-insensitive current increases in frequency (Jedema & Grace, 2004), similar to the effects of hypercapnia on the TTX-insensitive current discussed in chapter V.

It is currently unclear whether hypersensitivity of the brainstem chemoreceptors might be responsible for the overstimulation of the amygdala to result in panic disorder, or if abnormal regulation of the amygdala results in sensitizing the brainstem chemoreceptors to changes in CO2. Certainly the basolateral amygdala, a well-studied area of the limbic system also involved in the fear response, receives a dense norepinephrine innervation that originates primarily in the LC (Asan, 1998). Regardless, it seems likely that a limbic system-LC pathway that affects the Ca2+ currents in LC neurons would involve the Ca2+/BK channel braking mechanism to affect the magnitude of the firing rate response to hypercapnia. For example, increasing the frequency of the Ca2+ current in LC neurons by CRF neurotransmission may be designed to increase the BK current to decrease the sensitivity to CO2. Abnormalities in either the BK channels or the activation of the Ca2+ channels would then result in hypersensitivity to CO2, which may then feed back to the amygdala. To study this system, I first propose to test whether the increase in firing rate of LC neurons to the application of CRF (Jedema & Grace, 2004) can be increased by the inhibition of BK channels with paxilline, similar to the firing rate response of LC neurons to hypercapnia (see chapter VI).
In cases of obstructive sleep apnea (OSA), multiple clinical studies have documented an association between OSA and an increase in the loop gain, or the ratio of ventilatory increase to a disturbance that initiated ventilation (Verbraecken et al., 1995; Wang et al., 2007; Younes et al., 2001). Thus, an increase in respiratory sensitivity was associated with the onset of irregular breathing. Medications designed to decrease loop gain were demonstrated to be effective treatments for sleep apnea (Kiwull-Schone et al., 2008). Other studies have more directly associated an increase in the ventilatory sensitivity to CO$_2$ with respiratory pathology (Chapman et al., 1988; Wang et al., 2007). Thus, abnormalities in the development of a chemosensitive braking pathway designed to limit the magnitude of the firing rate response to hypercapnia may have implications for respiratory disease. In order to test the role of the Ca$^{2+}$/BK pathway to ventilation in the intact animal, I first propose to examine the potential role of $K_{Ca}$ channels in the firing rate response to hypercapnia in other chemosensitive areas of the brainstem. Next, I propose to combine microinjections of paxilline or 2-hydroxyestradiol in the unanaesthetized rat with plethysmography studies, similar to the experiments performed with carbenoxolone (Patrone et al., 2012). Because inhibiting the Ca$^{2+}$/BK channel braking pathway did not affect the resting firing rate of LC neurons in slices, I would expect to see little change in whole animal ventilation upon injecting paxilline or 2-hydroxyestradiol into the LC. However, when the animal is made to breathe increased CO$_2$, I would expect a greater increase in ventilation than with injection of vehicle.

A drug has recently been designed by Galleon Pharmaceuticals that is in phase-1 clinical trials. This drug (GAL-021) works as a respiratory stimulant that increases tidal volume and respiratory frequency and decreases PaCO$_2$ (Baby et al., 2012b; Baby et al.,
Interestingly, preliminary studies also indicate that GAL-021 inhibits BK channels by some 56% in cultured GH3 cells (Baby et al., 2012c). When a BK α subunit knockout (Slo1-/−) mouse is exposed to GAL-021, the respiratory stimulation effects were significantly attenuated compared to wild type mice (Baby et al., 2012c). Thus, it seems likely that the effect of inhibition of BK channels in the whole animal is to increase respiratory frequency, and perhaps even the ventilatory sensitivity to CO₂.

Preliminary studies show that GAL-021 affects peripheral chemoreceptors to increase ventilation, however, these respiratory effects were in the spontaneously breathing animal (Baby et al., 2012a). I would also propose to examine the effects of GAL-021 on the whole animal respiratory response to increased CO₂. In addition, I propose to study the effects of GAL-021 in slice preparations of LC neurons. I would expect that if GAL-021 inhibits BK channels, I would see an increase in the magnitude of the firing rate response similar to the effects of paxilline. In addition, since Slo1-/− knockout mouse showed an attenuated but not a loss of the respiratory stimulation caused by Gal-021, I propose to use Fura-2 and voltage clamp of Ca^{2+} currents to study whether Gal-021 effects BK channels or Ca^{2+} channels.

A current controversy in central chemoreception and respiratory control research will also be addressed in the context of future studies. A growing body of work has focused on establishing a single chemoreceptive area that contributes most strongly to the drive to breathe, in place of the theory that central CO₂ sensitivity involves multiple areas within the brainstem (Nattie & Li, 2009). In the current study, pathways are proposed whereby even small responses to hypercapnia by neurons of the LC are potentially carefully regulated, and may greatly affect cognitive processes. Thus, it cannot be argued
that the cellular mechanics behind these CO₂-sensitive pathways are insignificant to the integrated respiratory response or, possibly, to human pathology. Thus, if the above experiments are successful in identifying chemosensitive areas of the brainstem that contribute to the whole animal ventilatory response, future in vitro studies on the cellular chemosensitive mechanisms of these areas would be invaluable to an understanding of behavior and respiratory control.
CHAPTER X

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contribute to chemoreception by a purinergic mechanism. *Journal of Neurophysiology, 104*(6), 3042-3052.


CHAPTER XI

APPENDIX
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>2HE</td>
<td>2-hydroxyestradiol</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AHP</td>
<td>afterhyperpolarization</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>barium</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(2-aminophenoxy)ethane-N,N,N’&lt;sub&gt;i&lt;/sub&gt;,N’&lt;sub&gt;i&lt;/sub&gt;-tetraacetic acid</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-Bis(2-aminophenoxy)ethane-N,N,N’&lt;sub&gt;i&lt;/sub&gt;,N’&lt;sub&gt;i&lt;/sub&gt;-tetraacetic acid tetrakis(acetoxymethyl ester)</td>
</tr>
<tr>
<td>BK</td>
<td>large conductance Ca&lt;sup&gt;2+&lt;/sup&gt;-dependent K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>intracellular calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-cyclic monophosphate</td>
</tr>
<tr>
<td>Ca&lt;sub&gt;v&lt;/sub&gt;</td>
<td>voltage-activated calcium channel</td>
</tr>
<tr>
<td>CBX</td>
<td>carbenoxolone</td>
</tr>
<tr>
<td>Cd&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>cadmium</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>chemosensitivity index</td>
</tr>
<tr>
<td>cNTS</td>
<td>caudal nucleus of the solitary tract</td>
</tr>
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</table>
CO₂ carbon dioxide
CRF corticotropin releasing factor
CY3 cyanine 3-conjugated
Δ delta
ΔI difference current
db-cAMP dibutyryl adenosine 3’,5’-cyclic monophosphate
DMSO dimethyl sulfoxide
EGTA Ethylene glycoltetraacetic acid
EtOH ethanol
FITC fluorescein isothiocyanate
FR firing rate
H⁺ Hydrogen ion
H₈₉ N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
HCO₃⁻ bicarbonate
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA high-voltage activated
I current
IK intermediate conductance Ca^{2+}-dependent K⁺ channel
IP₃ inositol (1,4,5) trisphosphate
JNK c-Jun NH₂-terminal kinase
K₈ Ca^{2+}-dependent K⁺ channel
K_{ir} inward rectifying K⁺ channel
LC  locus coeruleus
LVA  low-voltage activated
mNTS  medial nucleus of the stria terminalis
MECP2  Methyl-CpG binding protein 2
μM  micromolar
mM  millimolar
ms  millisecond
mV  millivolt
min  minute
NE  norepinephrine
NHE  sodium-hydrogen exchangers
Ni^{2+}  nickel
NTS  nucleus of the solitary tract
O_{2}  oxygen
Ω-CTX-GVIA  omega-conotoxin-glycine-valine-isoleucine-alanine
OSA  obstructive sleep apnea
Pax  paxilline
PC12  pheochromocytoma cells
pH_{i}  intracellular pH
pH_{o}  extracellular pH
PBS  phosphate buffered saline
PKA  protein kinase A
PKC  protein kinase C
<table>
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<tbody>
<tr>
<td>RTN</td>
<td>retrotrapezoid nucleus</td>
</tr>
<tr>
<td>sAC</td>
<td>soluble adenylate cyclase</td>
</tr>
<tr>
<td>s/ sec</td>
<td>second</td>
</tr>
<tr>
<td>SIDS</td>
<td>sudden infant death syndrome</td>
</tr>
<tr>
<td>SK</td>
<td>small conductance Ca^{2+}-dependent K^+ channel</td>
</tr>
<tr>
<td>SRO</td>
<td>subthreshold rhythmic oscillation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TASK</td>
<td>acid sensitive K^+-channels of the tandem P-domain K^+-channel family</td>
</tr>
<tr>
<td>( \tau )</td>
<td>tau</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>VLM</td>
<td>ventrolateral medulla</td>
</tr>
<tr>
<td>( V_m )</td>
<td>membrane potential</td>
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CHAPTER XII

CURRICULUM VITAE
Ann Nicole Imber  
CURRICULUM VITAE

Wright State University  
Department of Neuroscience, Cell Biology and Physiology  
009 Medical Sciences Building  
3640 Colonel Glenn Highway  
Dayton, OH 45435  
imber.2@wright.edu

Education:

**Current Enrollment: M.D./Ph.D**  
Wright State University Boonshoft School of Medicine & Biomedical Sciences PhD Program, Dayton, OH 45435  
August 2007 - Present

**Masters in Biological Science: Molecular Biology**  
Florida State University, Tallahassee, FL  
Major Professor: Dr. Betty Gaffney  
January 2005 - May 2006

**Bachelors of Science: Biology**  
Florida State University, Tallahassee, FL  
August 2000 - May 2003

Research Experience:

July 2007-Present  
MD/PhD student, Wright State University. PhD studies under Dr. Robert Putnam. Neuron electrophysiology and immunohistochemistry, central respiratory control. JAVA programming for data analysis.

June 2008-July 2008  
Summer Rotation, Dr. Robert Putnam, Wright State University, Neuroscience. Whole cell patch clamp/ dissection of rat pons/medullary chemosensitive neurons.

May 2006-May 2007  
Laboratory Technician, Dr. Kenneth Taylor, Florida State University, Molecular Biophysics. Developing expression protocol and imaging samples of muscle proteins using electron microscopy; assisted in managing undergraduate researchers and
overseeing general lab projects.

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<tr>
<td>Sept 2003-Dec 2004</td>
<td>Laboratory Technician, Dr. Betty Gaffney, National High Field Magnetic Laboratory. Developed protein expression protocol, operated computer protein imaging and sequence alignment programs. Analyzed lipoxygenase EPR spectra, kinetic features, and enzyme metal binding. Development of java programming for amino acid sequence alignment and analysis.</td>
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**Teaching Experience:**

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<td>2011</td>
<td>Supervised graduate students in tissue preparation and whole cell patch electrophysiology</td>
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<tr>
<td>2008-2009</td>
<td>Boonshoft School of Medicine 1st year medical student tutor</td>
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<tr>
<td>2006</td>
<td>Supervised an undergraduate student in protein purification techniques</td>
</tr>
<tr>
<td>Fall semester Aug 2005- Dec 2005</td>
<td>Teaching Assistantship, Florida State University Department of Biological Science. Class: Experimental Biology/Protein Biotechnology Lab. Organized weekly lab materials, equipment, and reagents. Assisted with experimental design, weekly lectures, and directed students’ lab protocol.</td>
</tr>
<tr>
<td>August 2005</td>
<td>Florida State University Department of Biological Science Teaching Workshop</td>
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</table>
**Academic Honors:**


April 2012  Wright State University Academy of Medicine Student Excellence Award; Clinical Research

August 2011  Wright State University Biomedical Sciences Ph.D. Program Award; Superior Poster Presentation

May 2011  Invited Presentation at The Ohio Miami Valley Chapter of the Society for Neuroscience: 8th Annual Neuroscience Day

April 2011  Presentation at the Experimental Biology 2011 Neural Control of Breathing Symposium; Washington DC

Spring 2011  Boonshoft School of Medicine Medical Student Research Symposium Best Basic/Translational Science Award

May 2010  Invited Presentation at The Ohio Miami Valley Chapter of the Society for Neuroscience: 7th Annual Neuroscience Day

Spring 2010  Boonshoft School of Medicine Medical Student Research Symposium Best Basic/Translational Science Award

Spring 2009  Boonshoft School of Medicine Medical Student Research Symposium Best Basic/Translational Science Award


April 2005  Invited Presentation at the Florida State University Medical School Colloquium

**Peer Reviewed Publications:**


**Manuscripts in Preparation:**

Imber, A.N., Graham, C. and Putnam, R.W. The role of Ca\(^{2+}\) and BK channels as a brake to the chemosensitive firing rate response of locus coeruleus (LC) neurons. *J. Physiol.* IN PREPARATION.


**Published Abstracts:**

2012  
Imber, A.N., Graham, C. and Putnam, R.W. 2012. The role of Ca\(^{2+}\) and BK channels in the firing rate response of locus coeruleus (LC) neurons to CO\(_2\): controlling the chemosensitive gain. *FASEB J.* 26: Program # 984.8

2012  

2011  

2010  

**Presentations:**
1. “BK channel/capacitance measurements in locus coeruleus neurons: the chemosensitive brake” March 2012 Wright State University Patch Club Presentation; Dayton, OH.

2. “Ca\(^{2+}\)-activated K\(^{+}\) Channels Limit the Chemosensitive Response of Locus Coeruleus Neurons” Experimental Biology 2011 Neural Control of Breathing Symposium; Washington DC.

3. “The gain of chemosensitive neurons: Accelerators and brakes”. The Ohio Miami valley Chapter of the Society for Neuroscience: 8\(^{th}\) Annual Neuroscience Day; Wright State University May 20, 2011. Dayton OH.

4. “The role of calcium in the chemosensitive response of neurons from neonatal rat locus coeruleus (LC)” Neuroscience 40\(^{th}\) Annual Meeting; 2010 Nov 12-16; San Diego, CA.


**Graduate Studies (GPA 4.0):**

1. Ion Channels (Wright State University)
2. Quantitative Aspects of Membrane Transport (Wright State University)
3. Selected Topics in Physiology (Wright State University)
4. Programming Skills for Computational Biology and Bioinformatics (FSU)
5. 3D Electron Microscopy of Macromolecules (FSU)
6. Cell and Molecular Neuroscience (FSU)
7. Advanced Molecular Biology (FSU)

Community Service:

Spring 2012  Science Fair Judge at St. Peters School in Huber Heights, Ohio
Fall 2010    Gateway Shelter Tutoring Program, WSU/ St. Vincent de Paul
Summer 2010  Reach Out Chronic Care Clinic Volunteer
2007- 2011   Student to Student Medical Student Volunteer Program
July 2008    Dayton Air Show Medical Volunteer
Summer 2007  Life Guard/ Counselor Volunteer at Camp Boggy Creek (The Association of Hole in the Wall Camps) Tallahassee, FL