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Neuroanatomical and Morphological Properties of Neurons that Generate Inspiratory Related Breathing Rhythm and Influence Respiratory Motor Pattern in Mice

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Neuroanatomical and morphological properties of neurons that generate inspiratory related breathing rhythm and influence respiratory motor pattern in mice

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Doctor of Philosophy

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The relationship between neuron morphology and function is a perennial issue in neuroscience. Information about synaptic integration, network connectivity, and the specific roles of neuronal subpopulations can be obtained through morphological analysis of key neurons within any given microcircuit. Breathing is essential behavior for humans and all mammals, yet the neural microcircuit that governs respiration is not completely understood. The respiratory neural microcircuit resides within the ventral respiratory column located in the medulla. Within the respiratory column, the site of respiratory rhythm generation is the bilaterally distributed preBötzinger complex (preBötC). Rhythm-generating neurons in the preBötC are derived from a single genetic line, i.e., precursor cells expressing the transcription factor Developing brain homeobox-1 (Dbx1). An analysis of over 40 dendritic morphological features of rhythmogenic Dbx1 preBötC neurons and putatively premotor Dbx1 neurons in the intermediate reticular formation, revealed these two populations are similar except reticular neurons have a larger dendritic diameter, which may contribute to a greater passive transmembrane conductance. Both populations showed commissural axon projections and reticular formation neurons show premotor-like projections to the XII motor nucleus. These morphological data provide additional evidence supporting bilateral synchronization the preBötC through Dbx1 neurons, and demonstrate that Dbx1 preBötC neuron connectivity includes recurrent interconnections. On the molecular level, the ion channels that mediate rhythm-generating whole-cell ion currents have not been not identified, and were investigated using principally an anatomical approach. The nonspecific cation current, $I_{\text{CAN}}$, underlies robust inspiratory drive potentials in the preBötC and the persistent sodium current, $I_{\text{NaP}}$, may play a role in the production of robust bursts when respiration is challenged in such cases as anoxia or hypoxia. The leading candidate for ion channels that contribute to $I_{\text{CAN}}$ belong to the transient membrane receptor (Trp) ion channel superfamily and the leading ion channel candidate for $I_{\text{NaP}}$ is Na$_{\alpha}$1.6. I determined the presence of Trpc3 ion channels and Na$_{\alpha}$1.6 ion channels on Dbx1 preBötC neurons (as well as their expression in neighboring non-Dbx1 preBötC neurons). Finally, breathing behavior involves periodic sighs, which are slower than normal eupneic breathing but critical for lung function. I examined receptor expression for bomesbin-like peptides neuromedin B (NMB) and gastrin releasing peptide (GRP), which are important for sigh behavior. I show that NMB and GRP receptors are expressed in Dbx1 preBötC neurons and are not expressed by glia in the preBötC, as posited by some because of the low frequency of sigh breaths. These advances in morphological and anatomical knowledge can be used to design targeted in vitro and in vivo experiments to further explore their role in respiratory rhythm and pattern generation.
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I dedicate this dissertation to my mom, Marion Akins. She has always encouraged me and inspired me. My accomplishments in life would not be possible without her.
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LITERATURE REVIEW

Neural origins of breathing behavior are experimentally accessible

Neuroscience aims to understand how the brain and nervous system produce behaviors through molecular, cellular, and network levels of analysis. A behavior with obvious physiological importance is breathing, which refers to periodic movements of the diaphragm, airways, and thorax, that tidally ventilate the lungs to support pulmonary gas exchange and ultimately aerobic respiration. Breathing is a constant, yet dynamic behavior that changes in response to different levels of physiological demand during exercise, sleep, and emotional states. Breathing must function harmoniously with actions such as swallowing, speaking, laughing, and crying in humans, and whisking and sniffing in rodents. Breathing begins at birth (although mock breathing movements occur episodically during embryonic development) and breathing continues uninterruptedly to maintain life and homeostasis in all terrestrial mammals, including notably human beings. Therefore, the neural origin of breathing presents an important puzzle to solve. Nevertheless, the neural networks that generate and control breathing remain incompletely understood.

Understanding respiratory neural networks is a large problem; this dissertation will approach only limited aspects of it. By examining morphological properties of respiratory neurons including their dendritic structure and their axon projections, we gain insight into their synaptic integration and potential network...
connectivity. Through further characterization of intrinsic membrane properties such as ion channel and peptide receptor expression, one can develop a better understanding of the physiological mechanisms underlying respiratory rhythmogenesis and other respiratory-related behaviors such as sighing or gasping. This dissertation aims to use those anatomical methods to develop insights into breathing’s neural underpinnings.

The respiratory rhythm and the essential underlying motor pattern for breathing movements are generated by a network of neurons in the brainstem. Malfunctions in the respiratory neural circuit can lead to serious morbidity and mortality in humans. For example, premature infants can suffer apnea of prematurity due to an incomplete development of the respiratory network (Di Fiore et al., 2013; Martin and Wilson, 2012). Adults can also suffer from sleep-disordered breathing (Gaig and Iranzo, 2012) and obstructive sleep apnea (Tahmasian et al., 2016). Fatal respiratory failure is associated with neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and multiple systems atrophy because such disorders appear to cause collateral damage to medullary respiratory circuits that putatively disrupt their respiratory functionality (Benarroch, 2003; Benarroch et al., 2003; Brunnström and Englund, 2009; Tuppy et al., 2015). Failure to adequately respond to rising carbon dioxide dissolved in the blood and cerebrospinal fluid (hypercapnia) is linked to breathing disturbances including sleep disordered breathing and may be involved in sudden infant death syndrome through an insufficient response to hypoxia or hypercapnia (Darnall, 2013; Garcia III et al., 2013; Guyenet and Bayliss, 2015).
Genetic mutations can also lead to respiratory problems. For example, Rett’s syndrome is caused by a mutation in the \textit{MECP2} gene (Amir et al., 1999) and leads to breathing abnormalities including hypoventilation and desynchronization of the cardiac and respiratory systems (Julu et al., 2001; Weese-Mayer et al., 2008). Also, people who suffer from a condition known as congenital central hypoventilation syndrome (CCHS, also known as “Ondine’s curse”) fail to respond normally to hypercapnia, and can cause respiratory arrest while sleeping; CCHS is the result of poly-alanine mutations of the \textit{PHOX2B} gene which cause frameshifts that translate into mutant proteins and the disease phenotype, which is the destruction of chemosensitive neurons that provide the excitatory drive to breathe (Amiel et al., 2003; Dubreuil et al., 2008).

Understanding the neural origins of respiratory rhythm and pattern generation provides a baseline knowledge level that we can apply to understand respiratory pathology, which leads to enhanced therapies and prophylaxis of breathing disorders with etiologies related to brain and central nervous system (CNS) dysfunction.

Rhythmic behaviors, such as breathing, chewing, and walking, are driven by central pattern generators (CPGs) in the brainstem and spinal cord (Grillner, 2006; Kiehn, 2016; Westberg and Kolta, 2011). CPGs are networks of neurons that can generate periodic signals without any rhythmic external input (for example from other neural centers) or sensory feedback. CPGs produce rhythmic activity that drives premotor and motor neurons, which leads to motor output, i.e., movements (Frigon, 2012; Grillner, 2006; Guertin, 2013).
However, CPG rhythms are not rigid; sensory feedback and higher cortical commands must be accommodated and integrated so that behaviors in real environments respond to internal demands of the organism and demands imposed by the external world and environment. CPG operation and function is quite well understood in simpler invertebrate model systems (Marder et al., 2015; Nusbaum and Blitz, 2012), and in non-mammalian vertebrates considerable progress has been made in the past decade (Grillner and Manira, 2015; Kiehn, 2016; McLean and Dougherty, 2015). We aim to achieve the same level of understanding in a canonical rhythmic motor behavior in a mammalian species. This dissertation focuses on the CPG that drives breathing in mammals.

The breathing behaviors pertinent to this dissertation are eupneic breathing, or normal breathing, and sighs, which are necessary for proper lung function because they overinflate alveoli to reduce surface tension and prevent their collapse. The breathing movements involved with eupnea and sighs originate due to a neural rhythm, a periodic signal composed of burst discharges from interneurons, as well as a distinct motor pattern, which is mediated by premotor neurons and conveyed ultimately to motoneurons. The respiratory movements themselves involve airway resistance muscles and pump muscles responsible for tidal ventilation, consisting of inspiration (air in) and expiration (air out). A thorough understanding of respiratory rhythm and motor pattern generation mandates analyses ranging from a behavioral level, followed by more detailed neurophysiological examinations of the respiratory network (considered as a whole microcircuit), to an in-depth analysis of cellular and molecular
properties of individual neurons to determine their role in producing network activity.

This dissertation investigates three morphological and anatomical aspects of neurons that generate the respiratory rhythm and motor pattern. First, morphometric properties of respiratory rhythm-generating neurons are compared to neurons involved with respiratory motor pattern generation to ascertain potential distinctions and specializations pertaining to their synaptic integration properties. Next, the expression of ion channels that carry intrinsic membrane currents that may influence respiratory rhythm and pattern generation is examined on respiratory rhythm-generating neurons. Finally, the involvement of glia or neurons in sighing behavior will be investigated through the presence of peptide receptors shown to evoke sighs.

Respiratory system anatomy

The respiratory rhythm and motor pattern in mammals is governed by a neural network that resides in the medulla. The neurons that govern the respiratory rhythm and pattern generate the necessary periodic timing for breathing, integrate sensory information, such as CO₂ and O₂ levels, and send signals to motor neurons to generate respiratory motor output (Guyenet and Bayliss, 2015; Guyenet et al., 2009). Medullary neurons control muscles involved in respiratory motor output including the diaphragm which separates the thoracic cavity from the abdominal cavity and is the predominant pump for breathing, the tongue, and intercostal muscles which are in between the ribs. The thoracic
cavity consists of the ribs, vertebral column, and sternum encases the lungs, middle, and lower airways (Figure 1).

During the intake of breath, known as inspiration, air enters the upper airway through the mouth or nasal cavity. The tongue protrudes slightly to open the airway and maintain airway patency during the breath. The thoracic cavity expands as the diaphragm and external intercostal muscles contract, which drives air to enter the bronchi and the air-filled sacks of lungs (alveoli) due to the drop in the pressure gradient (Feldman and Del Negro, 2006). During expiration, breath is expelled from the chest cavity. At rest, the diaphragm relaxes and its passive recoil returns the lungs to their functional residual volume (i.e., normal resting volume). Expiration occurs in two sub-phases (Guyenet et al., 2005; Janczewski and Feldman, 2006). Post-inspiration is the first sub-phase, during which resistance muscles constrict the airways and can cause an eccentric contraction of the diaphragm if necessary, both of which increase time for gas exchange in the lungs. Second is the E2 phase (second stage expiration), which is not driven by any active muscle contraction at rest, but does involve active contraction of expiratory muscles such as abdominals and internal intercostal muscles during increased respiratory demand. This dissertation focuses on the neuroscience of the inspiratory rhythm and motor pattern, whereas the physical plant for breathing, involving muscles and organs, pertains more to pulmonary physiology.
Figure 1. Anatomy of the respiratory system (from Box 1, Feldman and Del Negro, 2006). The neural circuit that governs the respiratory rhythm and motor pattern resides in the brainstem. Signals are sent from neurons in the brainstem to organs and muscles required for taking a breath including the genioglossus tongue muscle and diaphragm.

**Respiratory and pattern rhythm generation**

The brain and CNS are extensively interconnected. However, the concept of *microcircuits* enables neuroscientists to analyze the brain and CNS in smaller more manageable constituent parts. Having an identified function and taking up a limited amount of brain real estate, a neuronal microcircuit is characterized by having specific inputs, containing interneurons whose axon projections and synaptic contacts remain within the microcircuit, and ultimately featuring principal neurons whose axons make up the output of the system and thus project to other
parts of the brain and CNS (Shepherd, 2004). Interneurons can facilitate communication between groups of neurons (e.g., between sensory neurons and motor neurons) or within populations of neurons. In the respiratory rhythm-generating microcircuit, the rhythm is generated by interneurons, but can be modulated by sensory input sources such as lung mechanoreceptors (Dutschmann et al., 2014) and central/peripheral chemoreceptors (Guyenet and Bayliss, 2015; Guyenet et al., 2009), and its principal neurons consist of cranial and spinal motor nuclei, whose output results in breathing movements. Normal breathing, or eupnea, involves motor output, coordination of breathing with orofacial behaviors (Kleinfeld et al., 2014; Moore et al., 2013), and integration of chemosensory inputs, all of which function based on a rhythm.

The site for rhythm generation, specifically the inspiratory rhythm, is the preBötzinger Complex (Feldman and Del Negro, 2006; Feldman et al., 2013; Ramirez et al., 2016; Smith et al., 1991), which is located within the ventral medulla. During a breath, the cranial hypoglossal nerve (CN XII) facilitates tongue protrusion to maintain an open airway (Fregosi and Ludlow, 2014), thoracic nerves T1-T11 facilitate the movement of the external intercostal muscles and the phrenic nerve controls the diaphragm. The output from these nerves that facilitate respiratory motor output is in phase with the neurons governing the breathing rhythm.

The preBötzinger Complex (preBötC), a functionally and anatomically specialized region of the ventrolateral medullary brainstem is the CPG for the inspiratory rhythm (Feldman et al., 2013; Gray, 1999; Gray et al., 2001; Moore et
Rhythmogenic neurons in the preBötC send signals to various neuron populations, demonstrated in Figure 2, including other respiratory regions and premotor neuron populations.

To generate respiratory related motor output such as tongue protrusion and diaphragm contraction, a subset of premotor neurons transmit signals to genioglossus and phrenic motoneuron populations respectively (Chamberlin et
The rostral ventral respiratory group (rVRG) contains glutamatergic premotor neurons that project to the phrenic nucleus and spinal cord, innervating the diaphragm and external intercostal motoneurons (Ellenberger and Feldman, 1990, 1994; Ellenberger et al., 1990).

To further classify respiratory neurons, we must be able to distinguish key neurons within the respiratory microcircuit from one another. Specific combinations of transcription factors, which control gene expression, are vital to neuronal development and can provide markers for neuron subpopulations (Alaynick et al., 2011; Garcia-Campmany et al., 2010; Gray, 2008, 2013). Using transcription factors as a marker allows us to classify populations of neurons within the respiratory neural network.

**Genetic classification of respiratory rhythm-generating neurons**

The temporal and spatial expression of transcription factors, genes that control other genes, give rise to the different types and classifications of neurons (and glia) in the brainstem and spinal cord (Garcia-Campmany et al., 2010; Grillner and Jessell, 2009). The formation of the respiratory central pattern generator may rely strongly on genetic components, evident through unstable respiratory rhythm and fatalities in mice with mutations in transcription factors involved in hindbrain interneuron specification (Blanchi et al., 2003; Gray et al., 2010; Jacquin et al., 1996; Pagliardini et al., 2008; Rose et al., 2009). Thirteen
different dorso-ventral domains of the brainstem can be distinguished by transcription factor expression (Alaynick et al., 2011). Dorsal domains pertain to sensory function and sensorimotor integration. The ventral domains are associated with CPGs and motor output functions. The ventral neural tube contains V0, V1, V2, VMN, and V3 domains. V0, V1, V2, and V3 make up interneurons, whereas VMN makes up motoneurons. In the hindbrain, the progenitor of the mature brainstem, the putative respiratory rhythm-generating interneurons reside within the V0 population (and the V3 population makes up visceral vagal motoneurons) (Briscoe and Ericson, 1999).

The V0 domain has two main subdivisions: dorsal and ventral. The dorsal V0 population are mainly GABAergic while the ventral V0 population are glutamatergic (Lanuza et al., 2004; Moran-Rivard et al., 2001; Pierani et al., 2001). Rhythmogenesis in vitro requires excitatory, glutamatergic transmission (Funk et al., 1993; Wallen-Mackenzie et al., 2006) and is modulated by peptidergic transmission (Rekling et al., 1996; Tupal et al., 2014; Li et al., 2016; Gray et al., 1999). Regular breathing depends upon rhythm-generating neurons that express receptors for neurokinin-1 (NK1R) demonstrated when in vivo saporin-mediated metabolic poisoning of NK1R-expressing neurons caused rats to develop ataxic breathing (Gray et al., 2001) as well as sleep-disordered breathing (McKay et al., 2005). Additionally, applying the NK1R agonist substance P, which depolarizes preBötc neurons and accelerates breathing rhythms, to the preBötc of adult rats in vitro increased the frequency of respiratory behavior (Gray et al., 1999), suggesting the depolarized preBötc
neurons are responsible for respiratory rhythm generation. However, NK1R-expressing preBötC neurons are not sufficient for respiratory rhythmogenesis. First of all, the NK1R-expressing cell-ablated rats (above) exhibited severe respiratory pathology, but did not die of respiratory failure. Moreover, silencing neurons that express the peptide somatostatin (SST) results in persistent apnea (Tan et al., 2008), which suggests that a class of SST-expressing neurons could define the core oscillator (although that depends to what extent NK1R and SST co-express in preBötC neurons, see below). In neonatal mice, NK1R expression extends (caudally) from the lateral reticular nucleus to the facial nucleus (rostrally) and includes the retrotrapezoid nucleus (RTN) and the parafacial respiratory group (pFRG). Also in the region of the preBötC and its rostrally sited neighbor the Bötzinger complex, NK1R labeling extends to pharyngeal and oesophygeal motoneurons of the compact and semicompact nucleus ambiguus (Bieger and Hopkins, 1987; Gray et al., 1999). so this marker alone cannot define the inspiratory rhythmogenic core (Gray et al., 1999; Stornetta et al., 2003; Thoby-Brisson et al., 2009). However, NK1R-expressing neurons can overlap with SST-expressing neurons. Glutamatergic, peptidergic, and peptide receptor-expressing interneurons in the preBötC develop from the V0 ventral post-mitotic domain, where the homeodomain transcription factor Dbx1 is expressed pre-mitotically. During embryonic development, Dbx1-expressing precursors are the sole source of glutamatergic interneurons in the preBötC (Bouvier et al., 2010; Gray et al., 2010).
Dbx1 expression is necessary for respiration; without Dbx1, mice die at birth without ever taking a breath because the preBötzC never forms (Bouvier et al., 2010; Gray et al., 2010). Dbx1-derived neurons (hereafter Dbx1 neurons) are the putative rhythmogenic neurons in the preBötzC. Laser ablation of Dbx1 neurons in the preBötzC slows and eventually abolishes the respiratory rhythm, indicating their requirement for respiratory rhythm generation (Wang et al., 2014). Also, transient optogenetic hyperpolarization of Dbx1 preBötzC neurons results in a disruption of the respiratory rhythm in vitro and in lightly sedated intact mice in vivo; when fully awake, optogenetic hyperpolarization of Dbx1 preBötzC neurons causes a reduction in breathing frequency (Vann et al., 2016). Optogenetic photostimulation in vivo of Dbx1 preBötzC neurons expressing channelrhodopsin (ChR2) resulted in inspiratory firing patterns and excitatory effects of burst timing and pattern (Cui et al., 2016). Also, selective photoinhibition of Archaerhodopsin-3 (Arch) expressing preBötzC neurons that were Dbx1-derived, SST-expressing, or glutamatergic neurons demonstrated that the three populations have similar respiratory rhythmogenic properties (Koizumi et al., 2016). These studies suggest the significant role of Dbx1 neurons in respiratory rhythm generation, but there is more to learn of their connectivity within the microcircuit, synaptic integration, ion channels that could give rise to rhythmogenic currents, and peptide receptors. This dissertation will elucidate morphological and anatomical information about respiratory Dbx1 neurons, giving insight into their potential network connectivity, synaptic integration properties, and properties contributing to respiratory rhythm and pattern generation.
Dbx1 neurons are found throughout the brain. Their role(s) have been studied in the amygdala, the hypothalamus (Hirata et al., 2009; Lischinsky et al.; Sokolowski et al., 2015, 2016), the brainstem respiratory microcircuit, and in lumbar spinal cord locomotor microcircuits (Bouvier et al., 2010; Gray et al., 2010; Hirata et al., 2009; Pierani et al., 2001). Dbx1 neurons are involved in a variety of behaviors. For instance, Dbx1 neurons in the lumbar spinal cord drive motor neurons and are vital for controlling motor neurons responsible for left-right alternation in hindlimb muscles (Lanuza et al., 2004; Talpalar et al., 2013). Dbx1 neurons in the hypothalamus may also regulate stress responses (Sokolowski et al., 2015). We are most interested in Dbx1 expression in the brainstem and spinal cord because these neurons are involved in CPGs. Dbx1 is expressed in respiratory regions of the brain outside of the preBötzC including the intermediate reticular formation. The intermediate reticular formation contains a mixture of premotor neurons, some of which govern oral-motor coordination (Gestreau et al., 2005; Kleinfeld et al., 2014; Moore et al., 2013). When precursor cells expressing Dbx1 are labeled with a fluorescent marker at embryonic day 10.5, transverse brain slices near the level of the preBötzinger complex and reticular formation exhibit an inverted U-shape pattern of fluorescent cells in neonatal mice from postnatal day 0 to 5 as demonstrated in Figure 3.
Figure 3. Visualization of Dbx1 neurons in transverse brainstem sections from Dbx1<sup>CreERT2</sup>; Rosa26<sup>tdTomato</sup> reporter mice. Dbx1 neurons have been labeled via induced expression of the red fluorescent protein tdTomato at embryonic day 10.5. Distances from the facial cranial nucleus (VII) are indicated to the right of each section. The preBötC is located at -0.40 µm from the facial nerve (Ruangkittisakul et al., 2014)

Respiratory modulated premotor neurons known to be located in the intermediate reticular formation include trigeminal premotor neurons, hypoglossal premotor neurons, and vibrissa premotor neurons (Kleinfeld et al., 2014; Revill et al., 2015; Stanek et al., 2014). The study by Revill et al. in particular showed that
hypoglossal respiratory premotor neurons are \textit{Dbx1}-derived, although the genetic 
origins of the other respiratory and orofacial reticular neurons remain 
uncharacterized. \textit{Dbx1}-derived hypoglossal premotor neurons exist in the 
preBötC as well as the reticular formation (Revill et al., 2015; Song et al., 2016; 
Wang et al., 2014). The role of respiratory Dbx1 neurons in the reticular 
formation is beginning to be unraveled through morphological and physiological 
analyses, which will be further discussed in this dissertation.

\textbf{Neurophysiology of respiratory rhythm generation}

In the last few decades, neuroscientists have exploited the experimental 
advantages offered by respiratory rhythms that can be studied in reduced 
breathing model systems \textit{in vitro}. In 1984, Suzue determined that rhythmic neural 
activity can be recorded from cranial and spinal respiratory nerves of a 
brainstem-spinal cord preparation from newborn rats and that neural activity 
matched the movements of the ribcage when left semi-intact, indicating rhythmic 
neural activity was respiratory (Suzue, 1984). In a more thorough analysis of 
respiratory nerve activities \textit{in vitro} and their cellular bases, Smith et al. confirmed 
Suzue’s initial conclusion that rhythmic activity in the isolated brainstem and 
spinal cord was breathing-related and amenable to investigate the cellular and 
synaptic mechanisms underlying respiratory rhythm and motor pattern (Smith et 
al., 1990).
Further reduction of the brainstem-spinal cord preparation led to the development of a medullary slice preparation that retains fictive rhythmic respiratory activity when the preBötC is included in the slice (Funk et al., 1993; Smith et al., 1991). From these transverse slices that are 350-550 µm thick, rhythmic fictive respiratory activity, depicted in Figure 4, can be recorded from nerves and individual cells (Smith et al., 1991; Funk et al., 1993; Ruangkittisakul et al., 2006; Funk and Greer, 2013).

![Figure 4](image)

**Figure 4.** Characteristic physiology of a Dbx1 neuron in the preBötC (Picardo et al., 2013). The top physiological trace shows a whole-cell recording of a respiratory Dbx1 neuron with a baseline activity level of -47 mV. The bottom physiological trace is the integrated output of the hypoglossal cranial nerve (XII). The respiratory Dbx1 neuron is active during the inter-inspiratory burst interval, which is the interval between peak XII activity. The respiratory Dbx1 neuron also displays voltage dependent spike inactivation known as depolarization block. Red arrows signify where the inspiratory drive begins. The horizontal scale bar is 1s and the vertical scale bar is 20 mV.

During the inspiratory phase of respiration, Dbx1 preBötC neurons exhibit synchronous bursts of 300-500 ms *in vitro* (Funk and Greer, 2013). Individually, Dbx1 preBôtC neurons produce bursts of 10 to 30 mV amplitudes; the underlying envelope of depolarization has been characterized as the *inspiratory drive potential*. This dissertation will analyze the morphological and anatomical properties of neurons governing this inspiratory drive.
Understanding rhythm generation in central pattern-generating networks presents a formidable challenge. Three prominent theories, explained in the following sections, emerged that describe rhythmogenesis in central pattern generating networks. The first theory involves inhibition, the second involves autorhythmic pacemaker neurons, and the third is based on emergent network properties.

Respiratory rhythm through phasic inhibition

The hypothesis of respiratory rhythm generation through cyclic inhibition consists of three neuron populations: early-inspiratory neurons in the preBötz and postinspiratory Bötz neurons and augmenting-expiratory Bötz neurons (Janczewski et al., 2013). Inhibition was thought to terminate inspiratory bursts and facilitate expiration. Inspiratory and expiratory patterns were thought to be kept as separate processes through reciprocal inhibition (Euler, 1983; Feldman, 2011).

Several studies have investigated the role of inhibitory neurons in respiratory rhythm generation, since inhibition has been hypothesized to guide rhythmic behaviors such as locomotion and chewing since the early 1900s (Brown, 1914). In 2013, the effect of postsynaptic inhibitory transmission in respiratory rhythm generation was analyzed by blocking GABA_A and glycine receptors in preBötz and Bötz neurons; but the breathing rhythm persisted indicating postsynaptic inhibition is not required for respiratory rhythm generation.
(Janczewski et al., 2013). Another study of inhibitory preBötC neurons in 2015 involved expressing either channelrhodopsin-2 or archaerodopsin in glycinergic (inhibitory) preBötC neurons in mice (Sherman et al., 2015). Photostimulation of glycinergic preBötC neurons in channelrhodopsin-2 transfected mice ends inspiration prematurely while photostimulation during the expiratory phase delays the start of the next inspiratory cycle (Sherman et al., 2015). In archaerodopsin-transfected mice, photoinhibition of glycinergic neurons causes an increase in tidal volume but does not change inspiratory duration (Sherman et al., 2015). These results indicate that inhibitory glycinergic preBötC neurons can modify inspiration, but are not required for rhythm generation since the rhythm persists after optogenetic dampening.

In 2016, to disrupt inhibition within the preBötC or BötC, GABA\textsubscript{A} and glycine receptors in the preBötC or BötC in \textit{in situ} (reduced) and anesthetized \textit{in vivo} rat preparations were blocked with antagonists gabazine and strychnine while suppressing preBötC or BötC neuronal activity with a muscimol microinjection. This disruption of inhibition perturbed the respiratory rhythm frequency and motor pattern; in some cases the motor output was terminated (Marchenko et al., 2016). In this case, changes in rhythmic frequency might be attributable to elevated excitability caused by disinhibition of the respiratory network creating an induced state of hyperexcitability such that motor activity is tonic rather than rhythmic. However, modulation of the rhythm, even inducing a tonic state, shows that chloride-mediated synaptic inhibition is not necessary for
rhythm generation. Phasic inhibition does not adequately explain respiratory rhythm generation, so other theories emerged.

**Pacemaker hypothesis**

Another theory of respiratory rhythm generation, which became widely accepted because of its precedents in invertebrate and cardiac systems, involves pacemaker neurons that are autorhythmic. Autorhythmicity refers to neurons that are intrinsically rhythmically active, generating brief bursts of spiking activity (~250 ms duration) separated by periods of quiescence (2-5 sec duration) when synaptically isolated. This theory evolved because the respiratory rhythm persists in en bloc and slice and preparations containing the preBötC after postsynaptic inhibition has been blocked (Ballanyi et al., 1999; Feldman and Smith, 1989). A subset of preBötC neurons that exhibit voltage-dependent bursting properties and depend on the persistent sodium current ($I_{NaP}$) were discovered in the preBötC and it was quickly suspected that these intrinsic bursting neurons might be rhythmogenic pacemakers for the network (Del Negro et al., 2002a; Smith et al., 1991). However, pacemaker activity is not required for respiratory rhythm generation because when voltage-dependent bursting behavior is terminated in preBötC neurons, rhythmogenesis still occurs (Del Negro et al., 2002b) and bath application of riluzole does not affect respiratory motor output (Del Negro et al., 2005). Experiments blocking $I_{NaP}$ with riluzole and tetrodotoxin (TTX) in thin neonatal rat slices (250 – 350 µm) and preBötC islands do stop the rhythm
(Koizumi and Smith, 2008), but only produce small disturbances in more intact preparations (Paton et al., 2006; Smith et al., 2007). Additionally, when similar experimental methods were applied to thicker slices (500-550 µm) in neonatal mice that contain the preBötC and surrounding network components, the respiratory rhythm was not disrupted (Pace et al., 2007a). In the embryonic preBötC, attenuation of $I_{NaP}$ at embryonic day 16.5 (E16.5) decreased respiratory rhythm frequency and blockade of $I_{NaP}$ and the nonspecific cation current $I_{CAN}$ blocked network activity, but at E18.5, after the embryo switched from a purely $I_{NaP}/I_{CAN}$ driven network to a combined network and pacemaker network the same blockade did not completely silence network activity (Chevalier et al.). Thus far, pharmacological experiments testing the pacemaker hypothesis either do not stop the respiratory rhythm, use drugs that could affect synaptic transmission or ion channels, or do not produce comparable results between experimental preparations, species, or developmental stages; therefore, autorhythmic neurons alone cannot explain respiratory rhythm generation (Del Negro et al., 2005; Pace et al., 2007a).

Neither the pacemaker hypothesis nor phasic inhibition can explain respiratory rhythmogenesis; each theory has been falsified by experimental data. Another hypothesis, the group-pacemaker, is based on emergent network properties, and provides an alternative to explain respiratory rhythmogenesis that is (at least at present) consistent with the existing data.
**Group-pacemaker hypothesis**

A group-pacemaker hypothesis is a proposed, alternative theory to intrinsic pacemakers and phasic inhibition that describes respiratory rhythm generation. Group-pacemaker behavior is an emergent network property; no individual neuron recaps network activity via its intrinsic bursting-pacemaker properties in the absence of synaptic transmission. According to the group pacemaker, the collective activity of the network’s constituent neurons produces the respiratory rhythm in the preBötC (Del Negro et al., 2002b, 2010). In a group-pacemaker system, a set of intrinsic currents is normally inactive or latent within the constituent neurons. These latent intrinsic currents are evoked through synaptic activity in the context of network activity. The evoked currents result in periodic inspiratory bursts (Del Negro et al., 2010; Rekling and Feldman, 1998; Rubin et al., 2009).

In the group-pacemaker hypothesis, recurrent synaptic excitation facilitates and propagates activity similar to the way a traditional network oscillator would behave (Grillner, 2006); yet there is an additional intrinsic property of the respiratory rhythm generating neurons that make them different from traditional central pattern generating neurons.

Current data suggest glutamatergic transmission induces post synaptic calcium transients that recruit a calcium-activated nonspecific cation current, $I_{CAN}$
which then amplifies the synaptic currents resulting in robust inspiratory drive potentials (Crowder et al., 2007; Pace and Del Negro, 2008; Pace et al., 2007b).

There are preBötzC neurons whose burst activity is reduced by Cd$^{2+}$, flufenamic acid, or intracellular dialysis by the Ca$^{2+}$ chelator BAPTA, indicating that rhythmic bursting relies on Ca$^{2+}$ and the Ca$^{2+}$-activated nonspecific cation current $I_{CAN}$ (Crowder et al., 2007; Del Negro et al., 2005; Pace et al., 2007b; Peña et al., 2004). When AMPA receptors, i.e., non-NMDA ionotropic glutamate receptors, and metabotropic glutamate receptors, i.e., mGluRs, are activated, then transient postsynaptic Ca$^{2+}$ currents or intracellular Ca$^{2+}$ release occurs, which in turn evoke $I_{CAN}$. The combination of these biophysical events evokes inward currents that facilitate robust inspiratory burst generation; the resulting inspiratory bursts can be of such high amplitude that depolarization block of spiking occurs during the inspiratory burst phase. Dendritic synaptic drive resulting in Ca$^{2+}$ influx or propagating Ca$^{2+}$ waves evoke $I_{CAN}$ (Del Negro et al., 2011; Mironov, 2008), while somatic Ca$^{2+}$ transients do not contribute (Morgado-Valle et al., 2008). The role of ion channels that can generate $I_{CAN}$ in rhythmogenic Dbx1 preBötzC neurons will be further investigated in this dissertation.

**Molecular and ion channel contributions to respiratory rhythmogenesis**

Ion channels belonging to the transient receptor potential (Trp) superfamily are likely candidates leading to the generation of $I_{CAN}$. The Trp family of ion
channels consists of several subtypes. The most promising candidates of the Trp family of ion channels for generating $I_{CAN}$ are Trpm4, Trpm5 (Crowder et al., 2007; Mironov, 2008; Mironov and Skorova, 2011), Trpc3, and Trpc7 (Ben-Mabrouk and Tryba, 2010). Trpm4 and Trpm5 channels exhibit biophysical properties associated with $I_{CAN}$ such as being Ca$^{2+}$ impermeable cation channels that are gated by intracellular Ca$^{2+}$ and sensitive to blockade by flufenamic acid (Hofmann et al., 2003; Launay et al., 2002; Ullrich et al., 2005). Trpc3 and Trpc7 ion channels are expressed in rhythmically active ventral respiratory group neurons and may play a role in respiratory rhythm regularity (Ben-Mabrouk and Tryba, 2010), although it is not quite clear whether the authors (Ben-Mabrouk and Tryba) advocate that Trpc3 and Trpc7 comprise a tonic inward current that maintains baseline excitability in preBötC neurons or if they putatively give rise to $I_{CAN}$. Regardless of the authors’ intent, none of their data indicate that Trpc3 or Trpc7 act phasically during burst generation, but those channels could underlie baseline excitability and could be the targets of neuromodulation.

Here, we are particularly concerned with the molecular origins of $I_{CAN}$, which acts physically in the service of inspiratory burst generation. While the expression of these Trp ion channels on Dbx1 neurons has yet to be determined, mRNA of several Trp channels have been detected via PCR experiments in preBötC neurons in previous studies (Crowder et al., 2007) and through RNA sequencing shown in Figure 5. In this dissertation, I examine the morphology of Dbx1 neurons to determine if ion channels are present on Dbx1 neurons that could putatively give rise to inspiratory burst-generating currents and if Dbx1
rhythm-generating neurons are structurally different from other respiratory-modulated Dbx1 neurons.

Figure 5. Heat map of Trp mRNA expression from single neurons analyzed through RNA sequencing (RNASeq). Expression levels are determined by the number of RNA copies present in terms of reads per kilobase of transcript per million mapped reads (RPKM), so high expression means a high number of RNA copies. In this heat map, low expression is designated as black and as expression levels increase the color transitions from black through teal and finally to white. In Dbx1 preBötC neurons, Tprc3 had the highest mRNA expression. Tprc3, Trpm4, and Tprc7 are likely candidates for the generation of the calcium activated non-specific cation current $I_{\text{CAN}}$. Supplementary figure from (Hayes et al., 2017).

Relationship between neuronal structure and physiology

Neural network information can be derived from anatomical studies; for example, axon projections indicate who is connected to whom in the network. Knowledge of potential neuron populations that are communicating with one another facilitates understanding network connectivity. For example, the connectivity of a neural network can influence its synchronization and robustness (Belykh et al., 2005). More specifically, we have shown the influence of network connectivity on synchronization and robustness in respiratory rhythm generation (Song et al., 2015, 2016; Wang et al., 2014). In this dissertation, we will analyze
the axon projections of respiratory-modulated neurons to determine likely recipients of their information. We will also analyze their dendritic structure to scrutinize relationships between structure and function.

The relationship between neuron morphology and function was proposed by Santiago Ramon y Cajal in the late 1800s concerning the functional organization of the nervous system. He proposed that the shape and synaptic connectivity of neurons would influence their function within a neural network (Llinás, 2003). Cajal proposed the neuron doctrine, which stipulates that neurons comprise the information-carrying units of the nervous system, which laid the foundation for neuroscience today (Guillery, 2007).

Wilfred Rall, a founder of computational neuroscience helped establish the integrative function of dendrites showing how dendritic properties can contribute to synaptic integration in different ways including the spatiotemporal summation of excitatory post synaptic potentials (EPSPs) (Rall, 1969; Segev and Rall, 1998). Dendritic trees influence neuronal firing patterns because dendritic size and topology govern the electrotonic properties, which in turn affect how signals propagate within single cells, particularly synaptic signals that predominantly arrive at remote sites in the dendrite (rather than near the soma or axon hillock). The impact of dendritic morphology on the behavior of neurons, especially the input vs output relationship, has been studied in the classic principal neurons of the neocortex and hippocampus, i.e., layer 3 or layer 5 cortical pyramidal neurons as well as CA1 and CA3 hippocampal pyramidal neurons (Ferrante et al., 2013). Multiple studies demonstrate the impact of dendritic morphology on
the physiology of a single neuron; one study found that the dendrites of bursting neurons have a larger diameter compared with spiking neurons (Halavi et al., 2012; Komendantov and Ascoli, 2009). Dendritic structure can be examined through a Sholl analysis, which examines the branch points, dendritic volume, length and area of a neuron’s dendritic arbor for specified distances from the soma to quantify dendritic geometry (Langhammer et al., 2010; Sholl, 1953). Therefore, Sholl analysis provides a way to quantify dendritic structure, which can then be applied to understand, from anatomical information alone, how a neuron might characteristically respond to its inputs to generate its output pattern. In terms of our neural microcircuit of interest, this type of Sholl analysis and dendritic electrotonic analysis could help understand how rhythmogenic (or premotor) interneurons would respond to synaptic drive in the context of rhythm- and motor-pattern generation.

A study analyzing how dendritic structure governs the firing pattern of neurons used computational neuron models to compare the ratio of the axon and somatic area to the dendritic membrane area (Mainen and Sejnowski, 1996). Neurons with the same ion channel types and ion channel densities demonstrated a range of firing patterns when only the dendritic morphology was varied. Neurons with the smallest dendritic arborizations, such as smooth stellates (Figure 6a), exhibited spike trains with monophasic afterhyperpolarization potentials and weak spike frequency adaptation. Slightly larger dendritic arbors, such as those in layer 4 spiny stellates (Figure 6b) produced adaptable spike trains. The second most extensive dendritic arbors
studied, layer 2 and 3 pyramidal neurons (Figure 6c) exhibited doublets or bursting and depolarization after spiking. The largest dendritic arbors studied, layer 5 pyramidal neurons (Figure 6d) exhibited burst spike trains (Mainen and Sejnowski, 1996).

![Image of firing patterns](image1)

Figure 6. Firing patterns from neurons with identical ion channel distributions but various dendritic morphologies. (A) Layer 3 aspiny stellate and response to somatic current injection of 50 pA; (B) Layer 4 spiny stellate and response to 70 pA somatic current injection; (C) Layer 3 pyramidal morphology and response to 100 pA somatic current injection; (D) Layer 5 pyramid morphology and response to 200 pA somatic current injection. Scale bars: 100 ms (horizontal) and 25 mV (vertical) Adapted from (Mainen and Sejnowski, 1996).

The study also varied the coupling resistance (i.e., conductance) between the axon and somatic area to its dendritic arbor. Ion channel densities were held constant while the two ratios (area of the compartments, and the conductance coupling them) were varied systematically. Various firing patterns (Figure 7d & e) including bursting behavior were possible when weak to moderate coupling strength was used whereas the uncoupled and fully coupled models produced
spiking without after spike depolarization (Mainen and Sejnowski, 1996). This study is one of many that emphasize the significance of dendritic structure on its physiology; this concept is the focus of the first chapter of this dissertation.

Figure 7. Effect of electrical coupling on neuronal firing pattern in a two-compartment model. A, two compartment model where $\kappa$ is the electrical resistance (coupling) between the axon and soma and $\rho$ is the ratio of dendritic to axo-somatic area; B, uncoupled dendritic (top) and axo-somatic compartments (bottom) are each capable of firing repetitively with current injected with the axo-somatic compartment firing at a much higher frequency; C, in fully coupled compartments, the spike frequency adaptation is modified by the size of the dendritic compartment indicated by $\rho$, but there is no bursting; D, partial coupling creates voltage gradients between axo-somatic and dendritic compartments and firing patterns change with regard to dendritic area or coupling strength (E). Adapted from (Mainen and Sejnowski, 1996).
Morphological characteristics of Dbx1 preBötC neurons

Structure-function relationships regarding the dendritic trees of neurons in the respiratory neural circuit are not yet fully understood. While important biophysical properties of Dbx1 preBötC rhythm generating neurons have been elucidated and continue to be examined, morphological and anatomical information about these neurons is necessary in order to obtain a more complete understanding of the role of these neurons in respiratory rhythmogenesis. The Del Negro lab began analyzing the morphology of Dbx1 preBötC neurons in 2011, through digital reconstructions which were presented in a master's thesis by Krishanthi Weragalaracchihi and then published in the Journal of Physiology (Picardo et al., 2013). Our findings, which include my reconstructions and morphometric data, were published within the Journal of Physiology (2013) paper are outlined below. My reconstructions of Dbx1 reticular formation neurons, which will be discussed in chapters 1 and 2, were published in eLife (Revill et al., 2015) and the Dbx1 preBötC neurons not discussed in the 2013 paper were included in the Scientific Data publication (Akins et al., 2017), which is the foundation for chapter 1.

We have learned that Dbx1 neurons in the preBötC have larger dendritic projection ranges than non-Dbx1 neighbors in the preBötC and neurons in the preBötC with commissural axon projections are mainly glutamatergic (Picardo et al., 2013). Commissural excitatory Dbx1 interneurons are of particular importance as the preBötC is a bilaterally synchronous population (Bouvier et al.,
When compared to respiratory neurons that are not Dbx1 derived (non-Dbx1 neurons or Dbx1'), Dbx1 neurons had commissural axons while non-Dbx1 neurons had more localized axon projections (Figure 8).

Figure 8. Respiratory Dbx1 preBötC neurons (A-C) tend to exhibit commissural axons, while respiratory non-Dbx1 preBötC neurons (D-F) have ipsilaterally projecting axons (Picardo et al., 2013).

Also, Dbx1 preBötC neurons are more confined to a transverse plane (Figure 9), meaning they have less of a span in the rostral to caudal direction (Picardo et al., 2013).
While some of the morphometric properties of respiratory preBötzC Dbx1 neurons have been elucidated, we have yet to determine the morphometric properties of respiratory modulated Dbx1 neurons in other parts of the respiratory microcircuit such as Dbx1 premotor neurons. In this dissertation, I will examine the morphometric properties of respiratory Dbx1 neurons in the reticular formation, a heterogeneous population dorsal to the preBötzC that includes...
respiratory modulated Dbx1 premotor neurons. Since reticular Dbx1 neurons are largely premotor rather than rhythmogenic, we expect Dbx1 reticular neurons and Dbx1 preBötC neurons will differ in some dendritic morphological features such as diameter. Also, given their premotor propensity, we suspect Dbx1 reticular neurons will project to the hypoglossal motor nucleus to facilitate respiratory related motor output.

**Sighs**

Sighs are long, deep breaths that most people would automatically associate with emotional states such as sadness, relief, frustration, or exhaustion. However, physiological sighs occur regularly during normal breathing (eupnea) to prevent collapsed alveoli in the lungs and to help preserve normal lung function (Ramirez, 2014; Reynolds, 1962). The increased inspiratory volume associated with sighs is reflected by a double-burst from the preBötC, the second part of the burst comes immediately at the peak (or slightly after) the eupneic preBötC burst (Lieske, 2000; Ruangkittisakul et al., 2008). Those data (and references) suggest that sighs and eupnea originate from the same neural population in the preBötC. Sighs are distinct from eupnea and gasping as demonstrated in an *in vitro* slice model of breathing when cadmium (Cd\(^{2+}\)) eliminated sighs without affecting eupneic bursts (Lieske, 2000; Toporikova et al., 2015). While the neural network that controls sighs has not been fully elucidated,
it is known that bombesin-like neuropeptides can evoke sighing behavior in rodents (Li et al., 2016; Ramirez, 2014).

Neuromedin B (Nmb), a gene that encodes a bombesin-like neuropeptide in mammals similar to the bombesin peptide found in frogs that influences sighing (Niewoehner et al., 1983), is expressed in the neurons of the rostral medulla around the RTN and pFRG in mice (Li et al., 2016). Neurons expressing Nmb project to the preBötC with some evidence that there may be contacts with somatostatin-positive neurons in the preBötC, but the data are ambiguous because the resolution of the neuroanatomy is insufficient to be sure the visualized cells are neurons or some other cell type (Li et al., 2016). Some preBötC neurons express mRNA for the receptor for the peptide NMB (Li et al., 2016), though the role of sigh receptor peptides in the preBötC needs to be further investigated. In anaesthetized rats that exhibited normal breathing patterns with regularly occurring sighs, bilateral injection of the peptide NMB into the preBötC increased the sigh frequency 6-17 times the normal frequency for several minutes (Li et al., 2016). In neonatal mouse slices containing the preBötC that exhibited rhythmic bursts and occasional doublet sigh bursts, NMB increased the frequency of doublets by changing inspiratory bursts to sighs, which indicates NMB influences the preBötC to increase sighing (Li et al., 2016). Bilateral injection of an NMB receptor antagonist reduced sighing but did not noticeably change eupneic respiration in wild type mice. In Nmbr⁻/⁻ knockout mice, sighing frequency was reduced overall compared to wild type mice (Li et al., 2016) suggesting NMBR signaling in the preBötC is important for basal sighing.
Pharmacological inhibition of *Nmbr* and knocking it out of the genome attenuates sighing behavior, but does not eliminate sighing, which indicates that NMB and NMB receptors do not comprise the sole pathway for generating and controlling sighs. Mammals also express the gastrin-releasing peptide (*Grp*) gene, another bombesin-like peptide gene (Jensen et al., 2008). In mice and rats, *Grp* was expressed in some cells in the dorsal part of the RTN and pFRG region as well as the nucleus tractus solitarius and the parabrachial nucleus, which are other regions involved in respiration (Guyenet et al., 2010; Li et al., 2016). Neurons expressing *Grp* project to the preBötC, some of which project onto somatostatin-positive neurons (Li et al., 2016). Bilateral injection of GRP into the preBötC of anaesthetized rats increased sighing 8-16 times the baseline sighing frequency and nearly doubled the amount of doublets in slices containing the preBötC (Li et al., 2016) indicating GRP also influences sighs through the preBötC. In *Grp<sup>-/-</sup>* mice, sighs were reduced by half though eupneia was unaffected. Bilateral injection of a GRPR antagonist into the preBötC of anaesthetized rats decreased sighing without changing normal respiration (Li et al., 2016). Basal sighing may be maintained by GRPR-expressing neurons in the preBötC, although the presence of GRP receptors on Dbx1 preBötC neurons has not yet been studied. Potentially, NMB or GRP receptors on glia play a role in sigh regulation because glial toxins inhibited sighs while eupneic breathing persisted (Dashevskiy et al., 2016). The current data suggest, but do not demonstrate, that NMR- and GRP-expressing neurons of the rostral medulla
directly innervate preBötC neurons. More investigation is needed to determine whether NMBRs and GRPRs are expressed in preBötC neurons or glia.

This dissertation will investigate morphological and anatomical properties of neurons that generate inspiratory behavior and influence respiratory motor pattern in mice. Morphological differences between Dbx1 preBötC rhythm generating neurons and Dbx1 premotor reticular formation neurons will also be addressed. The presence of Trpc3 ion channels, a candidate for facilitating $I_{CAN}$, and $NaV_{1.6}$ ion channels, a candidate for $I_{NaP}$, will be assessed for Dbx1 preBötC neurons. Finally, this dissertation will address whether bombesin-like receptors, which are involved in sigh behavior, are present on preBötC neurons and glia.
CHAPTER 1: Morphology of Dbx1 Respiratory Neurons in the preBötzinger Complex and Reticular Formation of Neonatal Mice

INTRODUCTION

Neuronal morphology, particularly the structure of the dendritic tree, influences how a neuron integrates synaptic inputs and generates physiological output patterns. Axon projections provide information about connectivity patterns in microcircuits. This study documents the morphology of brainstem interneurons that generate and control breathing.

Breathing is a rhythmic motor behavior that ventilates the lungs to support respiration and homeostasis in air-breathing vertebrates. For humans and all mammals, rodents serve as an advantageous model system to study the breathing’s neural origins. Key interneuron populations that generate inspiratory, expiratory, and (very recently) post-inspiratory related rhythms have been characterized in terms of physiology, genetic background, and transmitter phenotype (Feldman and Del Negro, 2006; Feldman et al., 2013; Ramirez et al., 2016; Richter and Smith, 2014). Premotor neurons that influence airway resistance have been similarly characterized (Chamberlin et al., 2007; Gestreau et al., 2005; Koizumi et al., 2008; Ono et al., 1998; Revill et al., 2015; Travers et al., 2005; Volgin et al., 2008). However, only a limited number of morphologies of constituent neurons in these populations have been documented and analyzed (Picardo et al., 2013; Revill et al., 2015; Wang et al., 2014). This chapter, which
corresponds to a published data descriptor: doi:10.1038/sdata.2017.97, aims to ameliorate that problem by providing annotated, high-quality digital reconstructions of the morphologies of rhythm-generating interneurons and motor pattern-related premotor neurons from neonatal mice.

The respiratory cycle is dominated by the rhythm underlying inspiration, which is generated within the preBötzinger complex (preBötC) of the ventral medulla (Feldman and Del Negro, 2006; Feldman et al., 2013; Moore et al., 2013; Ramirez et al., 2016; Richter and Smith, 2014; Smith et al., 1991). Rhythmogenic preBötC neurons are derived from precursor cells that express the homeobox transcription factor $Dbx1$ (Bouvier et al., 2010; Gray et al., 2010; Vann et al., 2016; Wang et al., 2014), hereafter referred to as Dbx1 neurons. The intermediate reticular formation, immediately adjacent (dorsal) to the preBötC, is a diverse region containing respiratory Dbx1 premotor interneurons that control inspiratory related muscles of the tongue and pharynx (Chamberlin et al., 2007; Peever et al., 2002; Stanek et al., 2014; Welzl and Bureš, 1977).

In this study we used intersectional mouse genetics to induce fluorescent protein expression in Dbx1 neurons of neonatal mice. Neuronal morphologies were acquired following patch-clamp recordings in transverse brainstem slices that retain the preBötC, the intermediate reticular formation, as well as the hypoglossal (XII) motor nucleus. These slices expose the preBötC and reticular formation at the rostral surface and spontaneously generate inspiratory rhythm and XII motor output, thus providing an experimentally advantageous breathing model in vitro (Funk and Greer, 2013; Ruangkittisakul et al., 2014).
We obtained three-dimensional morphologies of respiratory Dbx1 preBötC and Dbx1 intermediate reticular formation neurons by filling neurons with biocytin during whole-cell patch-clamp recordings (Halavi et al., 2012; Jacobs et al., 2010; Parekh and Ascoli, 2013, 2015). Compared to other reconstruction methods such as fluorescence microscopy of dye-filled neurons, biocytin reconstructions can be more time consuming but provide better visualization of thinner neuronal processes and axons (Blackman et al., 2014). Once labeled, we visualized the recorded neurons via confocal imaging and manually reconstructed their morphologies in a convenient digital format suitable for storage, display, and analysis.

Over the past four years, the Del Negro laboratory contributed 47 digital neuronal morphologies to the public open access database NeuroMorpho.org (this author, V.T. Akins, was either the lead morphologist or played a major role in managing the reconstructions for publication. Of those 47 digital reconstructions, 23 correspond to Dbx1 preBötC neurons (Picardo et al., 2013; Wang et al., 2014). Six have not been previously published; this report and the corresponding data descriptor publication (Akins et al., 2017) describes them for the first time. Twelve of the 47 correspond to preBötC neurons not derived from Dbx1-expressing precursors (Picardo et al., 2013) (i.e., non-Dbx1 preBötC neurons), and 12 correspond to Dbx1 reticular formation neurons (Revill et al., 2015).

Digital morphologies can be analyzed by software packages such as L-measure (Scorcioni et al., 2008), which computes more than 40 different
morphometric properties of dendritic trees and axons. Sholl analysis, which provides branching and dendritic density information in regular distance intervals from the soma (Langhammer et al., 2010; Sholl, 1953), can be performed with software such as NeuronStudio (Wearne et al., 2005). Digital morphologies can also be readily ported to simulation packages such as NEURON (Hines and Carnevale, 1997) and GENESIS (Bower et al., 2003) to form compartmental mathematical models that are high-fidelity representations of real neurons. We intend that these morphological data be meta-analyzed and incorporated into models of inspiratory rhythm- and pattern-generating circuits of the lower brainstem to better understand the neural mechanisms of breathing. That is why we published the data descriptor in addition to this dissertation chapter, and have released the data in the public domain on Neuromorpho.org.

METHODS

Mice

All of the animal protocols were approved by the Institutional Animal Care and Use Committee at The College of William and Mary, which follows the guidelines provided by the US National Institutes of Health Office of Laboratory Animal Welfare (NIH Office of Laboratory Animal Welfare, 2015).

Figure 10 recaps the workflow, which is detailed below. We crossed female mice that express Cre recombinase fused to a tamoxifen-sensitive
estrogen receptor (CreERT2) under the control of the Dbx1 promoter, i.e., 
$Dbx1^{CreERT2}$ (stock no. 028131, Jackson labs, Bar Harbor, ME) (Hirata et al., 
2009) with floxed male reporter mice that express red fluorescent protein variant 
tdTomato in a Cre-dependent manner ($Rosa26^{tdTomato}$, stock no. 007905, 
Jackson labs)(Madisen et al., 2010). Offspring with both alleles ($Dbx1^{CreERT2};$
$Rosa26^{tdTomato}$ mice), whose pregnant dams received tamoxifen during embryonic 
development, express the fluorescent reporter in Dbx1-derived cells (Hirata et al.,
2009; Picardo et al., 2013; Ruangkittisakul et al., 2014) (Fig. 10, step 1). 
$Dbx1^{CreERT2}$ mice were maintained on a CD-1 background strain. $Rosa26^{tdTomato}$
reporter mice were maintained using a C57BL/6J background strain.
Expression of the red fluorescent protein tdTomato is induced in Dbx1 neurons in mice (1). A transverse slice of the brainstem (indicated by the gray box) containing the preBötzinger complex is taken from a neonatal transgenic mouse (2). The slice is used for physiology recordings, during which respiratory modulated neurons are filled with biocytin (3). The slice is then preserved in 4% paraformaldehyde (4) and made transparent via incubation in Scale solution (5). The slice is treated with ExtrAvidin FITC (6) which binds to the biocytin allowing for visualization of the neuron through confocal microscopy (7). Confocal images in the x-, y-, and z- dimensions are taken of the entire neuron morphology and stitched together using FIJI (8). Using the 3D confocal images, neurons are digitally reconstructed using Neuromantic (9). XII, hypoglossal nucleus; NA, nucleus ambiguus, preBötC, preBötzinger complex; IO, inferior olive. (Akins et al., 2017)

Dbx1<sup>CreERT2</sup> mice were also mated with floxed reporter mice that express a channelrhodopsin-2/tdTomato fusion protein (<i>Rosa26<sup>ChR2-ttdTomato</sup></i>, stock no. 12567, Jackson labs) (Madisen et al., 2012). The <i>Dbx1<sup>CreERT2</sup>; Rosa26<sup>ChR2-ttdTomato</sup></i> mice were employed in separate electrophysiological experiments; here we recovered the morphology of the recorded neurons in the same way as <i>Dbx1<sup>CreERT2</sup>; Rosa26<sup>tdTomato</sup></i>, which was possible because both expressed native tdTomato in Dbx1-derived neurons. Channelrhodopsin, while important for
physiological tests, has no impact on morphological studies. Figure 10 only indicates \( \text{Dbx1}^{\text{CreERT2}}; \text{Rosa26}^{\text{tdTomato}} \) mice for simplicity.

Animal genotypes were verified using real-time PCR using primers for \( \text{Cre} \) and tandem dimer red fluorescent protein (Transnetyx, Cordova, TN). Timed matings were monitored such that embryonic day 0.5 (E0.5) was defined as 12 hours after the start of cohabitation. Cre recombination was then induced by administering tamoxifen (T5648; Sigma Aldrich, St Louis, MO) at E10.5 when \( \text{Dbx1} \) is at or near peak expression in the hindbrain (Bouvier et al., 2010; Gray et al., 2010; Hirata et al., 2009; Pierani et al., 2001). Tamoxifen was administered by oral gavage to pregnant dams at a concentration of 0.9 mg / 40 g body mass.

**Transverse slice preparations**

Neonatal \( \text{Dbx1}^{\text{CreERT2}}; \text{Rosa26}^{\text{tdTomato}} \) and \( \text{Dbx1}^{\text{CreERT2}}; \text{Rosa26}^{\text{ChR2-tdTomato}} \) mice were anesthetized then euthanized via decapitation at postnatal days 0-5 (P0-5), consistent with protocols outlined by the American Veterinary Medical Association Guidelines for euthanasia of animals (Leary et al., 2013). Transections were made at the bregma and the thorax. The neuraxis, from the pons to the lower thoracic spinal cord, was then removed within two minutes and further dissected in artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 3 KCl, 1.5 CaCl\(_2\), 1 MgSO\(_4\), 25 NaHCO\(_3\), 0.5 NaH\(_2\)PO\(_4\) and 30 dextrose, equilibrated with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4) (Fig. 10, step 2). The neuraxis was then glued to an agar block with the ventral surface facing out and placed in
the vise of a vibratome. We cut 550-µm-thick transverse brainstem slices that exposed the preBötC at the rostral face and retained the rostral XII nerve rootlets (Ruangkittisakul et al., 2014) (Fig. 10, step 3). Slices were perfused with ACSF at 28°C in a recording chamber on a fixed-stage upright microscope equipped with differential interference contrast optics and epifluorescence, which enables visual identification and selective recording of target neurons. The K⁺ concentration in the ACSF was elevated to 9 mM to maintain long-term stability of the preBötC rhythm (Funk and Greer, 2013; Ruangkittisakul et al., 2006, 2014). Rhythmic inspiratory-related motor output was recorded from the XII nerve rootlets using suction electrodes and a differential amplifier. Whole-cell patch-clamp recordings were acquired using capillary glass micro-pipettes and a current-clamp amplifier. Patch pipettes were positioned under visual control after fluorescent identification of Dbx1 neurons. The patch solution contained (mM): 140 potassium gluconate, 10 Hepes, 5 NaCl, 1 MgCl₂, 0.1 EGTA, 2 Mg-ATP, 0.3 Na₃-GTP and 2 mg/mL biocytin (B4261; Sigma Aldrich). All of the neurons in this data set were rhythmically active in sync with inspiratory XII motor output.

After the recordings, transverse slices containing biocytin-filled neurons were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for at least 16 h at 4°C (Fig. 10, step 4). Then, the slices were treated with Scale solution containing 4 M urea, 10% (mass/volume) glycerol and 0.1% (m/v) Triton X-100, for 10 days to clear the tissue and remove opaque background staining (Hama et al., 2011) (Fig. 10, step 5). Slices were washed three times for 15 minutes each in phosphate buffer solution (PBS) + 1% Triton X-100 (PBST) and then blocked
in PBST with 10% heat-inactivated fetal bovine sera (F4135; Sigma Aldrich) for 45 minutes. The biocytin was revealed by incubating the slices with fluorescein isothiocyanate-conjugated ExtrAvidin (E2761; Sigma Aldrich) overnight at 4°C with three-dimensional rotation on a nutator (Fig. 10, step 6). Next, the slices were rinsed with PBS five times for 15 minutes each and cover-slipped in Vectashield (H-1500; Vector Laboratories, Burlingame, CA).

**Confocal microscopy and digital neuronal reconstruction**

We visualized recorded neurons using a spinning-disk confocal microscope (Olympus BX51, Center Valley, PA) and a laser scanning confocal microscope (Zeiss LSM 510, Thornwood, NY) Three-dimensional (3D) confocal images of the individual neurons were obtained using a 20x objective (Olympus numerical aperture 0.5, Zeiss LSM numerical aperture 1.0) at increments of 1 µm in the z-axis (Fig. 10, step 7). The series of confocal images (i.e., z-stacks) were aligned in three-dimensions, merged or ‘stitched together’ at contiguous borders using ImageJ software (Schneider et al., 2012) and the Stitching plugin (Preibisch et al., 2009) (Fig. 10, step 8). This stitching process was iterated until the entire morphology of the neuron was contained within a single three-dimensional image file. Finally we digitized neuronal morphologies using the Neuromantic reconstruction tool, which is also free and in the public domain (Myatt et al., 2012). The digital reconstructions were scaled to the appropriate size based on the micron-to-pixel ratio for each microscope (Fig. 10, step 9).
Images acquired from the LSM microscope were scaled with a 0.41 micron-to-pixel ratio and images from the Olympus microscope were scaled using a 0.322 micron-to-pixel ratio. This chapter (and its associated data descriptor) pertain to 47 digital morphologies of inspiratory modulated Dbx1 preBötC neurons, six of which are previously unpublished (Data Citations 1-6) and 41 which are associated with previous publications (Data Citations 7-47). The morphologies are all publicly available via NeuroMorpho.org.

DATA RECORDS

Digital reconstructions of Dbx1 neurons are located in the Del Negro archive of the NeuroMorpho database (Data Citations 1-47). Digital reconstruction files are in SWC format, which is a commonly used format for neuron morphologies (Jacobs et al., 2010). The reconstruction files contain an x-coordinate, y-coordinate, and z-coordinate of each neuronal segment. The type of neuronal process, such as cell body, axon, or dendrite is also specified by type 1, 2, and 4, respectively. (Type 3 represents basal dendrites, but there is no such distinction in brainstem interneurons, so type 3 is omitted as a classifier in our dataset. Our dendrites were all designated type 4.) The radius in microns is given for each neuronal segment as well as the “parent” segment or the index number of the previous segment. Table 1 provides an example of an SWC file output for a neuron reconstruction. Physiological properties of Dbx1 preBötC and Dbx1
reticular neurons have been described (Picardo et al., 2013; Revill et al., 2015; Wang et al., 2014).

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Table 1. Sample SWC file data for digital neuron reconstruction. This table contains the first 17 rows of an SWC file generated for a digital reconstruction. For each reconstruction segment there is an index number, segment type (soma (1), axon (2), dendrite (4)), x-, y-, and z-coordinates, radius (in microns), and parent segment.

**TECHNICAL VALIDATION**

In newborn *Dbx1CreERT2*; *Rosa26tdTomato* and *Dbx1CreERT2*; *Rosa26ChR2*; *tdTomato* mice, *Dbx1* neurons form an inverted U-shape in the transverse (coronal) plane, which is visible in brainstem slices at the level of the preBötC. The inverted U-shape originates at the lateral border of the hypoglossal motor nucleus, located within the dorso-medial portion of the slice, and continues
ventrolaterally until the ventral border of the tissue slice (Ruangkittisakul et al., 2014). The dorsal border of the preBötC is identifiable because it is immediately ventral to the semi-compact division of the nucleus ambiguus, which does not express Dbx1 (Ruangkittisakul et al., 2014). Visual identification of the principal loop of the inferior olive and the flattening of the V-shape of the fourth ventricle are other indicators that the rostral surface of the transverse slice is at the level of the preBötC (Ruangkittisakul et al., 2014).

Slices remained in the recording chamber for at least 15 minutes after biocytin dialysis to maximize biocytin diffusion throughout the cytoplasm (Jacobs et al., 2010; Picardo et al., 2013). A clearing agent was used to facilitate visualization of the morphology; however clearing reagents can cause tissue shrinkage or expansion which could distort morphological features (Hama et al., 2011). The Scale solution used to clear the tissue in these experiments minimizes or completely precludes tissue expansion (compared to other methods) (Hama et al., 2011).

The quality of digital reconstructions depends on histology methods, image acquisition, as well as the digital reconstruction algorithms. To minimize disparities, we consistently used the same method of histological labeling. The software Neuromantic (Myatt et al., 2012), used for digitizing our image stacks, offers up to 16,000% magnification. This zoom feature enables the user to adhere to the most minute details captured in the image, which results in the most accurate reconstruction possible.
Two of the six new neurons and nine of the previously published neurons had no discernible axon, which might have indicated insufficient biocytin filling or that the axon was severed during tissue preparation. We recommend that the end user of the data draw no firm conclusions regarding connectivity from the lack of an axon in reconstructed digital morphology.

For those neurons whose axons were discernible, we distinguished the axons from the dendrites according to these criteria: 1) axons generally have a constant diameter whereas dendrites taper distal to the soma. 2) Axons exhibit fewer branches and never show spine-like protrusions. 3) Truncated axons near the slice surface exhibit a bleb or fluorescent circle from the cut end (Furness et al., 2003; Swietek et al., 2016).

Digital reconstructions were uploaded to NeuroMorpho.org, where they undergo a standardization process. The soma (type 1) should be the initial parent segment for all subsequent segments, whether dendritic or axonal. Neuronal processes should only connect to either the soma or to segments of the same type; for example, dendrite segments connect to dendrite segments and axon segments connect to axon segments. All processes should have a designated type and should not be undefined. A process can branch into no more than two processes at any given point.

Some irregularities can be fixed automatically during the standardization process (Halavi et al., 2008). If a neuronal segment is designated as a different type than its parent and daughter segments (e.g., a type 3 surrounded by type 2s) the erroneous segment type is automatically changed to match the type of its
parent and daughter segments. If the soma is not the initial segment in the file, the soma segment is automatically changed to the first segment in the file. If a segment has a radius of zero microns, then the radius is automatically changed to match the radius of its parent. Other digitization issues must be corrected by the submitting investigator (Halavi et al., 2008). For example, if a segment has not been designated with a process type, the correct type must be manually entered, rather than automatically assigned, which ensures that the proper type has been documented. Segments with a radius of zero (i.e., less than 0.05 µm), or larger than four standard deviations above the average radius of the cell are flagged as physiologically unrealistic during standardization and must be resolved by the submitting investigator. After the standardization process, digital reconstruction files and images are then reviewed and approved by the submitting investigator before being added to the public database (Halavi et al., 2008).

DATA CITATIONS

Del Negro, C. A. NeuroMorpho.org NMO_45917 (2017)
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Del Negro, C. A. NeuroMorpho.org NMO_45921 (2017)
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33. Del Negro, C. A. NeuroMorpho.org NMO_45919 (2016)
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CHAPTER 2. Analysis of dendritic arbor and axon projections of Dbx1 preBötC and reticular formation neurons

INTRODUCTION

Information about neural network structure as well as insights into the roles of neuron subpopulations can be obtained through morphological analysis of key neurons within the circuit (Parekh and Ascoli, 2014). Here, we seek to understand the neural mechanisms underlying breathing behavior via morphological analyses.

Breathing is a rhythmic behavior essential to humans and all mammalian life, yet the neural circuit(s) responsible for this vital behavior are incompletely understood. The putative populations of neurons that generate the respiratory rhythm are known, but the cellular and network properties that make this population unique are still being elucidated. In addition to their biophysical properties, the morphometric properties of respiratory neurons must be examined in order to obtain a more comprehensive picture of their role in the neural network. The specific objective of this study is to examine dendritic morphometric properties and axon trajectories of key respiratory neurons in order to better understand the neural origins of the rhythm underlying inspiratory breathing movements in mammals.

Inspiratory rhythm is generated within the preBötzinger complex (preBötC), a bilaterally distributed site in the ventral medulla (Feldman et al.,
Putatively rhythmogenic neurons in the preBöCtC are derived from a single genetic line, whose precursors express homeodomain transcription factor Dbx1, hereafter Dbx1 neurons (Bouvier et al., 2010; Gray et al., 2010). Dbx1 neurons are involved in important motor behaviors such as left-right alternation of limb movements during locomotion at any speed (Lanuza et al., 2004; Talpalar et al., 2013). Respiratory Dbx1 preBöCtC neurons project to various neuron populations including the intermediate reticular formation. The reticular formation contains neurons that drive whisking and sniffing rhythms, which must be coordinated with the breathing rhythm generated by the preBöCtC. (Gestreau et al., 2005; Kleinfeld et al., 2014; Moore et al., 2013, 2014).

Inspiratory breathing movements in terrestrial mammals must be coordinated with orofacial behaviors that include phonation, chewing, suckling, whisking, licking, and sniffing. While we know the preBöCtC generates the breathing rhythm and coordinates breathing with other orofacial behaviors (Kleinfeld et al., 2014; Moore et al., 2013, 2014), the cellular and morphological properties, as well as axon projection patterns, of respiratory rhythm and pattern-generating interneurons that may coordinate breathing with orofacial behaviors remain incompletely understood.

Respiratory modulated neurons, some of which express Dbx1, have been recognized in the region of the dorsal medulla near the preBöCtC. Respiratory modulated Dbx1 neurons may serve as premotor neurons for the airway and tongue that help maintain airway patency during respiration and may also coordinate respiration with other orofacial behaviors (Kleinfeld et al., 2014;
Koizumi et al., 2008; Moore et al., 2013, 2014). This chapter aims to characterize the morphologies of Dbx1 preBötC and reticular formation neurons to gain insight into mechanisms of respiratory premotor function or coordination of breathing with orofacial behaviors; we suspect respiratory Dbx1 reticular formation neurons will be premotor neurons that send signals to the hypoglossal motor nucleus (XII).

**METHODS**

Methods involving mouse genetics, dissection, labeling neurons via biocytin, tissue fixation, confocal microscopy, and digitization were covered in the previous chapter (See Figure 10). This chapter describes the analyses of the dendritic arbor that I performed on the digitized morphologies introduced in chapter 1. The 3D reconstructions functioned as digital objects that we analyzed further using L-Measure ([L-measure Link](#)) (Scorcioni et al., 2008) and NeuronStudio ([NeuronStudio Link](#)) (Wearne et al., 2005), which are additional tools that are freely available in the public domain. Eighty-five transverse slices containing biocytin-filled neurons were processed, resulting in a total of 23 digitized morphologies of Dbx1 preBötC neurons and 12 digitized morphologies of Dbx1 reticular formation neurons. As described in the literature review, our framework for analysis is that inspiratory-modulated Dbx1 preBötC interneurons are core rhythm-generators for respiration, whereas inspiratory-modulated Dbx1 neurons of the reticular formation may be premotor neurons for inspiratory
tongue movements (because motoneurons for the genioglossus muscle reside in the hypoglossal nucleus). We compared the morphometric properties of Dbx1 preBötC neurons (n=23) and inspiratory modulated Dbx1 reticular formation neurons (n=12) according to 40 different measurements computed by L-measure. The 40 measurements are defined in Table 2.

We also performed a Sholl analysis for each dendritic tree, which measures dendritic length, surface area, volume, and branching at concentric segments beginning at the soma, increasing in diameter throughout the dendritic tree and is used to provide a sense of dendritic complexity (Langhammer et al., 2010; Sholl, 1953). Sholl analysis was performed using NeuronStudio at 25 µm increments for reconstructed neurons in the preBötC and the reticular formation.

Statistical analysis of data was performed using the free and publicly available statistical software PAST, version 3.06, designed for analysis of scientific data in life sciences, earth science, engineering, and economics (Hammer et al., 2001). PAST, a creative acronym for paleontological statistics, originally developed to facilitate the use of quantitative methods in paleontology, is now a comprehensive software package offering, univariate statistics, multivariate statistics, curve fitting of data, time series analysis, and data plotting among other features. The Shapiro-Wilk test, the Anderson-Darling test, normal probability plots, and an analysis of the residuals were performed to determine whether the morphometric properties of Dbx1 preBötC neurons and Dbx1 reticular formation neurons were normally distributed. The Shapiro-Wilk test determines a statistic, $W$, which quantifies the extent to which sample data
depart from normality by generating a normal probability plot and testing the correlation between the predicted normal data and the actual data. $W$ values close to 0 suggest a small departure from normality and as $W$ approaches a value of 1, the departure from normality increases. (Liang et al., 2009; Shapiro and Wilk, 1965). The Anderson-Darling test determines the goodness of fit for the sample data to a normal distribution.

Based on the Shapiro-Wilk test results and the Anderson-Darling test results, some of the morphometric data have a normal distribution but not all. In order to be consistent in the comparison of these data, the same test should be used for each comparison. Parametric tests could not be used for each metric since not all of the metrics were normally distributed. Therefore, a nonparametric test was most appropriate for further data evaluation.

The Mann-Whitney U test is a non-parametric test to evaluate whether two independent samples are likely to be drawn from the same underlying population. Assumptions for the Mann-Whitney U test are that the data are randomly distributed, values are continuous, and values are ordinal meaning that they can be ranked higher or lower than one another. The collected data are in compliance with the assumptions of the Mann-Whitney test and thus was used to compare the morphometric data for respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons.

When comparing two independent samples, the null hypothesis for the Mann-Whitney U test is that a randomly chosen value from one group is equal to the value of the second group; thus, an alternative hypothesis would state that a
sample from the first group has a higher probability of having a larger value than a randomly chosen sample from the second group. The Mann-Whitney test is performed in the following manner for two independent samples. A value is randomly chosen from the first group, then a random value is chosen from the second group, and the larger of the two values is determined. This method is repeated for all of the values in both groups. The total number of times a value in each group was the largest is calculated. The smaller of these two totals is the test statistic $U$. The maximum number of comparisons made to compute the $U$ statistic is the product of the number of values in group A and group B. The 23 reconstructed preBötC neurons and 12 reconstructed reticular formation neurons result in 276 comparisons between the two groups for each data category. The null hypothesis for the Mann Whitney test is that the distributions of both populations are equal, suggesting that the two groups are drawn from the same underlying population.

RESULTS

Axon projections

We expect Dbx1 neurons in the preBötC to be commissural (i.e., to project axons across the midline) to synapse with their counterparts in the contralateral half of the preBötC and thus synchronize the bilaterally distributed neuron population (Bouvier et al., 2010). Ten of the twenty-six Dbx1 preBötC neurons
that we patch-recorded to verify their inspiratory burst-generating functionality showed axons that projected toward the midline, four of which definitively cross the midline. Six of the midline-projecting Dbx1 preBötC neurons have their morphologies published in a previous master’s thesis (Weragalaarachchi, 2012). The author of this work assisted with data collection and collaborative analyses pertaining to that work. Five of the preBötC neurons had axons with ipsilateral projections, all of which are shown in Figures 25-29.

We expect Dbx1 respiratory modulated premotor neurons in the reticular formation to project axons to the hypoglossal motor nucleus. Three of the Dbx1 reticular formation neurons had ipsilateral projecting neurons that approach the hypoglossal motor nucleus, which is consistent with the expected axon projection. However, seven axons in respiratory Dbx1 reticular formation neurons projected across the midline, which was a surprise. In the entire data set of 35 Dbx1 neurons, eleven had short axons or no discernible axon perhaps due to severance during the slice-cutting procedure or due to a fault in biocytin filling.

_Dendritic analysis of Dbx1 neurons with no discernible axon in PreBötC_

Respiratory Dbx1 preBötC neurons with no discernible axon from the newly reconstructed data set are shown in Figures 30-32. The neurons without discernible axons were viable for dendritic analysis since their morphometric data were not outliers among the entire Dbx1 preBötC population. In fact, some dendrites of neurons without discernible axons had a larger number of stems,
bifurcations and branches that their neighbors with axons as with neuron 130212, whose data are in Table 3 and whose morphology is shown in Figure 30. This particular neuron also has similar height and greater depth (Table 3) than its neighbor from the same slice preparation in which it was recorded and biocytin-filled. Therefore, the morphological data from this neuron was included and analyzed with Dbx1 preBötC neurons that showed discernible axon projections.

Figure 31 showing neuron 120621 had the lowest number of stems (Table 3), which are dendritic protrusions originating at the soma; however, the dendritic height and depth (Table 3) are comparable to the dendritic height and depth (Table 3) of neuron 140117, shown in Figure 23. Figure 32 showing neuron 120623 has the same number of stems as 4 other neurons from the new reconstructions as well as one of the largest values in depth (Table 3). While the neuron shown in Figure 32 has the smallest width (Table 3) of the analyzed neurons, it does not have the smallest height (Table 3). The smallest height belongs to the neuron in Figure 28, whose reconstruction exhibits an axon. While the neurons in Figures 30-32 do not have fluorescently labeled axons, their dendritic morphologies do not appear to be outliers and will be pooled with the rest of the data from the preBötC in the morphometric analysis between respiratory Dbx1 neurons in the preBötC and the reticular formation.
Somatic and dendritic morphometric analysis

Of the forty morphometric measurements, defined in Table 2, used to compare respiratory Dbx1 neurons in the reticular formation and the preBötC, 23 are significantly different based on Mann-Whitney analysis (Table 5). The morphometric properties are categorized by their type: area and volume, branching, path, proportion, angle, and terminal (defined below). Area and volume measurements relate to the surface area and volume of the soma and dendritic compartments. Branching measurements involve the subdivisions or bifurcations of the dendritic tree. Path measurements encompass the span of the individual neurites in all three dimensions. Proportional metrics are ratios between path metrics and branching measurements. Angle measurements pertain to the space between branches within the dendritic tree. Terminal measurements involve the region of the dendritic tree after the last bifurcation.

Area and volume measurements

Neuronal soma sizes have been analyzed in the nervous system to monitor proper development, define certain cell types, and may suggest a relationship between morphology and physiology in some neuronal classes (Oyster et al., 1981). Respiratory Dbx1 neurons in the preBötC and reticular formation have different tasks – Dbx1 preBötC neurons are putative rhythm generators while Dbx1 reticular formation neurons are thought to be premotor
neurons sending signals to motor nuclei. As they have different roles, we might expect their soma sizes to differ. We found that respiratory preBötzC neurons have a smaller average soma surface area compared to respiratory reticular formation neurons (Table 5). Mann-Whitney analysis revealed the difference was statistically significant.

Dendritic diameter will affect the length constant, which is a measure of how the electric potential propagates through the neurite passively, and is the basis for analyzing active dendritic properties (Stuart and Spruston, 2015). A larger length constant will mean that the electrical potential will propagate farther with less attenuation of the input signal, and can thus contribute to synaptic integration more effectively. Respiratory Dbx1 preBötzC neurons have a significantly smaller diameter than respiratory reticular formation neurons (Table 5). Dendritic surface area and volume are directly related to the dendritic diameter. There was a statistically significant difference in volume, but not surface area (Table 5).

**Branching**

Respiratory Dbx1 reticular formation and preBötzC neurons do not differ significantly in the number of protrusions from the soma, or stems, nor do they differ significantly in branch order, which is a measure progressive branching within the dendritic tree. The soma, which has no branch points, has a branch
order of 0, the first branch point has an order of 1, the next branch point has an order of two, and this process continues until the final branch point.

A significant difference was computed for respiratory Dbx1 reticular formation compared to Dbx1 preBötC neurons in the number of dendritic bifurcations, which are the branch points, and total number of branches (Table 5). Respiratory Dbx1 reticular formation neurons have significantly fewer bifurcations and branches than respiratory Dbx1 preBötC neurons. Branching patterns will be further analyzed via Sholl Analysis in the subsequent section of the results.

Path Measurements

Respiratory Dbx1 preBötC and reticular formation neurons differ significantly in height, which is the span of the dendrites in the vertical direction, width, which is the span of the dendrites in the horizontal plane, and depth, which is the span of the dendrites perpendicular to the horizontal and vertical planes. Respiratory Dbx1 reticular formation neurons have a smaller average height, width, and depth compared to respiratory Dbx1 preBötC neurons (Table 5).

Respiratory preBötC Dbx1 neurons have a significantly greater total dendritic length than respiratory Dbx1 reticular formation neurons (Table 5), yet they have less dendritic volume (Table 5). The lower average volume in respiratory Dbx1 preBötC neurons is directly related to the smaller diameter of preBötC neurons compared to respiratory Dbx1 reticular formation neurons.
Volume is calculated via the equation $\pi r^2 h$, where $r$ is the radius of the dendrite and $h$ is the height of the dendrite.

Respiratory Dbx1 reticular formation neurons are significantly smaller in average and maximum path distance as well as average and maximum Euclidean distance (Table 5).

**Proportional measurements**

Respiratory Dbx1 reticular formation neurons are significantly larger in Burke taper and maximum Hillman taper (Table 5), though similar in average Hillman taper (Table 5). Though respiratory Dbx1 preBötz and respiratory Dbx1 reticular formation neurons differ based on the Mann Whitney results for the contraction ratio, their averages and standard error are the same (Table 5). The average and standard error for the fractal diameter is also identical between respiratory Dbx1 preBötz and reticular formation neurons, yet their data differ significantly based on the Mann-Whitney analysis (Table 5). Respiratory Dbx1 preBötz and respiratory Dbx1 reticular formation neurons are similar in daughter ratio, parent-daughter ratio, partition asymmetry, and Rall power.

**Angle measurements**

Six metrics provide information about the relationships between bifurcation nodes, which are bifurcation points or terminating points, and bifurcation planes,
which is defined by the two daughter compartments. The first two bifurcation metrics are concerned with the bifurcation amplitude. Local amplitude determines the angle between the first two compartments after the bifurcation point. Remote amplitude is the angle between two consecutive bifurcation nodes or between two terminal points. Bifurcation tilt measures the angle between parent or “father” nodes and child or “daughter” compartments to make an outer angle between the father and daughter compartments. Local bifurcation tilt is determined by the angle between the father compartment of the current bifurcation point and the two daughter compartments of the current bifurcation. Remote bifurcation tilt is the angle between the previous father node of the current bifurcation and the two daughter nodes. Bifurcation torque is a measure of the inner angle between the current bifurcation and the parent bifurcation. Local bifurcation torque is the angle between the bifurcation plane, of the previous bifurcation node and the current bifurcation plane. Remote bifurcation torque defines the angle between the current bifurcation plane and the previous bifurcation plane. Respiratory Dbx1 preBötC and respiratory Dbx1 reticular formation neurons are similar in all of these measurements.

Terminal measurements

Last parent diameter and diameter threshold both differ significantly between respiratory Dbx1 preBötC and respiratory Dbx1 reticular formation neurons with the reticular formation neurons having the largest in both cases.
(Table 5). The Hillman threshold is significantly larger in reticular formation neurons (Table 5). While the Mann-Whitney analysis indicates differences in fractal diameter among respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons, their average values and standard error are equivalent (Table 5). Respiratory Dbx1 reticular neurons have a significantly smaller number of terminal tips and terminal segments than respiratory Dbx1 preBötC neurons (Table 5). The smaller number of terminal segments, which are the compartments of the reconstructed neuron between a bifurcation and a terminal tip, suggests that the respiratory Dbx1 reticular formation neurons have more branch points further out in the more distal parts of the dendritic tree than preBötC neurons.
Figure 11. Dbx1 inspiratory cell 130624 in the reticular formation A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree.
Figure 12. Dbx1 inspiratory cell 140114 in the reticular formation. A, slice mosaic showing *Dbx1* expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree.
Figure 13. Dbx1 inspiratory cell 140120 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree
Figure 14. Dbx1 inspiratory cell 140124 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree
Figure 15. Dbx1 inspiratory cell 140207 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree
Figure 16. Dbx1 inspiratory cell 140221 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree.
Figure 17. Dbx1 inspiratory cell 140306 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows the axon crosses the midline; C, enlarged view of cell morphology showing dendritic tree
Figure 18. Dbx1 inspiratory cell 140208 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection toward the hypoglossal nucleus; C, enlarged view of cell morphology showing dendritic tree
Figure 19. Dbx1 inspiratory cell 140220 in the reticular formation A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection toward the hypoglossal motor nucleus; C, enlarged view of cell morphology showing dendritic tree
Figure 20. Dbx1 inspiratory cell 140301 in the reticular formation. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection toward the hypoglossal nucleus; C, enlarged view of cell morphology showing dendritic tree
Figure 21. Dbx1 inspiratory cell 140127 in the preBötC. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows a midline axon projection; C, enlarged view of cell morphology showing dendritic tree.
Figure 22. Dbx1 inspiratory cell 140109 in the preBötC. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows a midline axon projection; C, enlarged view of cell morphology showing dendritic tree
Figure 23. Dbx1 inspiratory cell 140117 in the preBötc A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows a midline axon projection; C, enlarged view of cell morphology showing dendritic tree
Figure 24. Dbx1 inspiratory cell 130910 in the preBötc. A, slice mosaic with reconstructed cell morphology on the slice mosaic shows a midline axon projection; tdTomato expression in Dbx1 neurons was not visible after histological processing but was present and visible during the experiment; C, enlarged view of cell morphology showing dendritic tree
Figure 25. Dbx1 inspiratory cell 1 130212_1 in the preBötC. The reconstructed neuron is shown as an overlay over the confocal microscopy image. Dorsal and medial direction arrows indicate the slice orientation.
Figure 26. Dbx1 inspiratory cell 130625 in the preBötC. A, slice mosaic showing Dbx1 expression via tdTomato transgenic labeling. Location of the cell body is highlighted with a yellow box; B, reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection; C, enlarged view of cell morphology showing dendritic tree
Figure 27. Dbx1 inspiratory cell 130819 in the preBötC. A, slice mosaic with reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection; tdTomato expression in Dbx1 neurons was not visible after histological processing but was present and visible during the experiment; C, enlarged view of cell morphology showing dendritic tree.
Figure 28. Dbx1 inspiratory cell 140110 in the preBötC. A, slice mosaic with reconstructed cell morphology on the slice mosaic shows an ipsilateral axon projection; tdTomato expression in Dbx1 neurons was not visible after histological processing but was present and visible during the experiment; C, enlarged view of cell morphology showing dendritic tree
Figure 29. Dbx1 inspiratory cell 120417 in the preBötC. The reconstructed neuron is shown as an overlay over the confocal microscopy image. Dorsal and medial direction arrows indicate the slice orientation.
Figure 30. Dbx1 inspiratory cell 130212_2 in the preBötz. The reconstructed neuron is shown as an overlay over the confocal microscopy image. Dorsal and medial direction arrows indicate the slice orientation.
Figure 31. Dbx1 inspiratory cell 120621 in the preBötC. The reconstructed neuron is shown as an overlay over the confocal microscopy image. Dorsal and medial direction arrows indicate the slice orientation.
Figure 32. Dbx1 inspiratory cell 120623 in the preBötC. The reconstructed neuron is shown as an overlay over the confocal microscopy image. Dorsal and medial direction arrows indicate the slice orientation.
<table>
<thead>
<tr>
<th>Morphometric property</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>Soma Surface</td>
<td>Surface area of the soma (area of a sphere) calculated with the equation $4\pi r^2$, where $r$ is the radius of the soma</td>
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<tr>
<td>Number of Stems</td>
<td>Number of protrusions attached to the soma</td>
</tr>
<tr>
<td>Number of Bifurcations</td>
<td>Branch points resulting in two daughter branches</td>
</tr>
<tr>
<td>Number of Branches</td>
<td>Branch consists of dendritic compartments that lie between 2 branching points or between one branching point and a termination point</td>
</tr>
<tr>
<td>Number of tips</td>
<td>Final compartment or endpoint of each dendritic branch</td>
</tr>
<tr>
<td>Width</td>
<td>Horizontal span of dendrites calculated by the difference between maximum and minimum of x-coordinates in the xyz plane</td>
</tr>
<tr>
<td>Height</td>
<td>Vertical span of dendrites calculated by the difference between maximum and minimum of y-coordinates in the xyz plane</td>
</tr>
<tr>
<td>Depth</td>
<td>Span of dendrites perpendicular to the surface of the slice calculated by the difference between maximum and minimum of z-coordinates in the xyz plane</td>
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<tr>
<td>Diameter</td>
<td>Diameter of each compartment of the neuron</td>
</tr>
<tr>
<td>Length</td>
<td>Sum of the length of the entire dendritic tree</td>
</tr>
<tr>
<td>Surface Area</td>
<td>Surface area of each compartment (area of a cylinder) calculated by the equation $2\pi rh$, where $r$ is the radius of the dendrite and $h$ is the height</td>
</tr>
<tr>
<td>Volume</td>
<td>Calculated with the equation $\pi r^2 h$, where $r$ is the radius of the dendrite and $h$ is the height</td>
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Table 2. Definitions of morphometric properties analyzed
<table>
<thead>
<tr>
<th>Morphometric property</th>
<th>Definition</th>
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<tr>
<td>Euclidean Distance</td>
<td>Straight line distance of the dendritic compartment from the soma</td>
</tr>
<tr>
<td>Path Distance</td>
<td>Sum of the lengths of the dendritic compartment from the soma</td>
</tr>
<tr>
<td>Branch Order</td>
<td>Hierarchy of branching where the soma = 0, the first bifurcation results in a branch order of 1, the second bifurcation the branch order = 2, etc.</td>
</tr>
<tr>
<td>Terminal Segment</td>
<td>Number of compartments in a branch that ends as a terminal branch (returns a 1 for all compartments in a terminal branch). This gives an idea of the length of the final branch</td>
</tr>
<tr>
<td>Burke Taper</td>
<td>Burke Taper which is measured between 2 bifurcation points (Diameter of bifurcation compartment minus previous bifurcation compartment diameter divided by total length of the branch)</td>
</tr>
<tr>
<td>Hillman Taper</td>
<td>Hillman Taper is measured by computing the ratio between the final diameter and initial diameter of a branch</td>
</tr>
<tr>
<td>Contraction</td>
<td>Ratio between Euclidean distance of a branch and its path length</td>
</tr>
<tr>
<td>Fragmentation</td>
<td>Total number of compartments that constitute a branch between 2 bifurcation points or between a bifurcation point and a terminal tip</td>
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<tr>
<td>Daughter Ratio</td>
<td>Ratio between 2 daughter compartments of a bifurcation (Daughter compartments are the two compartments that follow a bifurcation point)</td>
</tr>
<tr>
<td>Parent Daughter Ratio</td>
<td>Ratio between diameter of daughter and parent compartments of a bifurcation</td>
</tr>
<tr>
<td>Partition asymmetry</td>
<td>Calculated for each bifurcation via the equation $\frac{abs(n1-n2)}{(n1+n2-2)}$, where n1 is the number of terminal tips on the left side of the bifurcation and n2 is the number of tips on the right side of the bifurcation</td>
</tr>
<tr>
<td>Rall power</td>
<td>The diameter value that best fits Rall's three-halves power law. This is the idealistic dendritic diameter that can propagate signal transmission without loss from the starting point to the terminal point in a cable model.</td>
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</table>

Table 2 (continued). Definitions of morphometric properties analyzed.
<table>
<thead>
<tr>
<th>Morphometric property</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Bifurcation amplitude local</td>
<td>Returns the angle (in degrees) between the first two compartments in a bifurcation</td>
</tr>
<tr>
<td>Bifurcation amplitude remote</td>
<td>Angle between 2 bifurcation points, between bifurcation point and terminal point, or between 2 terminal points (computed at each bifurcation point)</td>
</tr>
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<td>Bifurcation tilt local</td>
<td>Angle between previous compartment of bifurcating parent and the 2 daughter compartments of the bifurcation; smaller of the 2 child angles is reported (consider as angle of deflection of the parent orientation compared to the orientation of the midline of the bifurcation amplitude angle)</td>
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<tr>
<td>Bifurcation tilt remote</td>
<td>Angle between previous parent node and two daughter nodes of a bifurcation (node is terminating point, bifurcation point, or root point) The smaller of the 2 angles is returned; not computed for the root node</td>
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<tr>
<td>Bifurcation torque local</td>
<td>Angle between the plane of previous bifurcation and the current bifurcation (bifurcation plane is defined by the two daughter compartments leaving the bifurcation)(torque is the inner angle measured between 2 planes of bifurcations)</td>
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<tr>
<td>Bifurcation torque remote</td>
<td>Angle between the current plane of bifurcation and previous plane of bifurcation</td>
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<tr>
<td>last-parent diameter</td>
<td>Diameter of last bifurcation before the terminal tips</td>
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<tr>
<td>Diameter threshold</td>
<td>Diameter of the first compartment after the last bifurcation leading to a terminal tip</td>
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<tr>
<td>Hilmann threshold</td>
<td>Weighted average between 50% of parent and 25% of daughter diameters of the terminal bifurcation</td>
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<tr>
<td>Fractal diameter</td>
<td>Slope of linear fit of regression line obtained from the log-log plot of path distance vs Euclidean distance</td>
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Table 2 (continued). Definitions of morphometric properties
Table 3. Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray were reported in a previous thesis (Weragalaarachchi, 2012), but used in the current analysis. Unshaded data are calculated from new digital reconstructions. The entire table spans 37 columns and 23 rows. Columns 1-6 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
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<th>Length (µm)</th>
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Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 7-12 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
<table>
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<th>Average Euclidean Distance (µm)</th>
<th>Maximum Euclidean Distance (µm)</th>
<th>Average Path Distance (µm)</th>
<th>Maximum Path Distance (µm)</th>
<th>Average Branch Order</th>
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Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 13-17 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 18-23 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
<table>
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<tr>
<th>Row Number</th>
<th>preBötC Neuron Index</th>
<th>Average Daughter Ratio</th>
<th>Average Parent-Daughter Ratio</th>
<th>Average partition asymmetry</th>
<th>Average Rall power</th>
<th>Average Bifurcation amplitude local</th>
<th>Average Bifurcation amplitude remote</th>
</tr>
</thead>
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<td>0.41</td>
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<td>84.0</td>
<td>81.4</td>
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<td>95.0</td>
<td>75.7</td>
</tr>
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<td>54.3</td>
<td>56.9</td>
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<td>1.3</td>
<td>68.7</td>
<td>54.3</td>
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<td>52.0</td>
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<td>65.6</td>
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<td>1.33</td>
<td>0.79</td>
<td>0.46</td>
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<td>86.6</td>
<td>66.5</td>
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<tr>
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<td>0.55</td>
<td>3.4</td>
<td>70.2</td>
<td>75.0</td>
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<td>0.85</td>
<td>0.63</td>
<td>2.0</td>
<td>61.7</td>
<td>59.2</td>
</tr>
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<td>1.13</td>
<td>0.97</td>
<td>0.59</td>
<td>0.0</td>
<td>59.9</td>
<td>70.3</td>
</tr>
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<td>0.90</td>
<td>0.25</td>
<td>3.9</td>
<td>68.6</td>
<td>57.8</td>
</tr>
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<td>0.53</td>
<td>1.7</td>
<td>96.9</td>
<td>82.8</td>
</tr>
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<td>0.79</td>
<td>0.33</td>
<td>0.0</td>
<td>62.8</td>
<td>63.5</td>
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<td>140117</td>
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<td>60.3</td>
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<td>140127</td>
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<td>0.81</td>
<td>0.57</td>
<td>2.3</td>
<td>56.9</td>
<td>60.9</td>
</tr>
</tbody>
</table>

Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 24-29 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
Row Number | preBötC Neuron Index | Average bifurcation tilt local | Average bifurcation tilt remote | Average bifurcation torque local | Average bifurcation torque remote | Average last-parent diameter |
---|---|---|---|---|---|---|
1 | 111101 | 111.4 | 95.6 | 96.4 | 81.9 | 1.2 |
2 | 111102_1 | 91.1 | 103.9 | 110.7 | 119.1 | 1.2 |
3 | 111102_2 | 121.3 | 127.4 | 108.9 | 97.3 | 0.9 |
4 | 111108 | 125.8 | 117.0 | 92.4 | 100.9 | 1.0 |
5 | 111109_1 | 122.5 | 135.4 | 96.4 | 78.9 | 1.5 |
6 | 111109_2 | 125.4 | 130.4 | 106.6 | 92.3 | 1.1 |
7 | 111208 | 91.4 | 93.8 | 73.1 | 50.0 | 1.0 |
8 | 111209 | 123.8 | 118.1 | 47.7 | 78.2 | 0.6 |
9 | 111212 | 129.7 | 138.5 | 108.9 | 97.7 | 0.8 |
10 | 111214 | 96.8 | 126.0 | 80.7 | 81.8 | 5.8 |
11 | 111219_S1 | 111.1 | 112.3 | 106.7 | 101.8 | 0.9 |
12 | 111219_S2 | 112.6 | 117.2 | 99.2 | 76.3 | 1.0 |
13 | 111220_1 | 108.9 | 128.5 | 87.4 | 84.4 | 0.6 |
14 | 111220_2 | 120.8 | 111.3 | 90.2 | 73.4 | 0.3 |
15 | 120621 | 127.0 | 120.4 | 166.7 | 116.9 | 1.1 |
16 | 120623 | 126.9 | 129.8 | 82.4 | 89.8 | 0.9 |
17 | 130819 | 118.9 | 135.3 | 110.2 | 81.5 | 1.9 |
18 | 130910 | 125.7 | 128.3 | 100.1 | 83.9 | 1.7 |
19 | 130212_1 | 120.1 | 131.8 | 70.6 | 70.5 | 2.0 |
20 | 130212_2 | 91.6 | 102.4 | 125.4 | 89.8 | 2.1 |
21 | 140110 | 119.7 | 121.8 | 82.1 | 81.4 | 4.4 |
22 | 140117 | 118.6 | 139.0 | 102.0 | 106.6 | 2.9 |
23 | 140127 | 135.6 | 133.1 | 75.4 | 51.6 | 3.7 |

Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 30-34 are shown in this portion of the table. The subsequent columns will be continued on the following pages.
<table>
<thead>
<tr>
<th>Row Number</th>
<th>preBötC Neuron Index</th>
<th>Average Diameter (mm)</th>
<th>Average Hilmann threshold</th>
<th>Average fractal Diameter</th>
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<tbody>
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<td>0.93</td>
<td>1.03</td>
</tr>
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<td>111102_1</td>
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<td>0.98</td>
<td>1.03</td>
</tr>
<tr>
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<td>111102_2</td>
<td>1.07</td>
<td>0.70</td>
<td>1.04</td>
</tr>
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<td>0.67</td>
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<td>1.03</td>
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<td>0.75</td>
<td>1.04</td>
</tr>
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<td>1.05</td>
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<td>1.05</td>
</tr>
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<td>1.04</td>
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<td>1.05</td>
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<td>3.60</td>
<td>1.03</td>
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<td>1.03</td>
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</table>

Table 3 (continued). Morphometric properties of Dbx1 neurons in the preBötC. Data shaded gray was reported in a previous thesis, but used in the current analysis. Unshaded data are calculated from new reconstructions. The entire table spans 37 columns and 23 rows. Columns 35-37 are shown in this portion of the table.
Table 4. Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 1-6 are shown in this portion of the table. The table will continue on the subsequent pages.
<table>
<thead>
<tr>
<th>Row Number</th>
<th>Reticular Formation Neuron Index</th>
<th>Height (µm)</th>
<th>Depth (µm)</th>
<th>Average Diameter (µm)</th>
<th>Length (µm)</th>
<th>Surface Area (µm²)</th>
<th>Volume (µm³)</th>
</tr>
</thead>
<tbody>
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</table>

Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 7-12 are shown in this portion of the table. The table will continue on the subsequent pages.
<table>
<thead>
<tr>
<th>Row Number</th>
<th>Reticular Formation Neuron Index</th>
<th>Average Euclidean Distance (µm)</th>
<th>Maximum Euclidean Distance (µm)</th>
<th>Average Path Distance (µm)</th>
<th>Maximum Path Distance (µm)</th>
<th>Average Branch Order</th>
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</thead>
<tbody>
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<td>39</td>
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<td>128</td>
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<td>1.7</td>
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<td>31.2</td>
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<td>78</td>
<td>0.7</td>
</tr>
<tr>
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<td>78.0</td>
<td>182</td>
<td>118</td>
<td>363</td>
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</tr>
<tr>
<td>9</td>
<td>140220</td>
<td>32.1</td>
<td>80</td>
<td>47</td>
<td>126</td>
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</tr>
<tr>
<td>10</td>
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<td>66.1</td>
<td>149</td>
<td>90</td>
<td>249</td>
<td>2.3</td>
</tr>
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<td>64</td>
<td>177</td>
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</tr>
<tr>
<td>12</td>
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<td>42.5</td>
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<td>116</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 13-17 are shown in this portion of the table. The table will continue on the subsequent pages.
Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 18-22 are shown in this portion of the table. The table will continue on the subsequent pages.
Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 23-27 are shown in this portion of the table. The table will continue on the subsequent pages.
<table>
<thead>
<tr>
<th>Row Number</th>
<th>Reticular Formation Neuron Index</th>
<th>Average Bifurcation amplitude local (°)</th>
<th>Average Bifurcation amplitude remote (°)</th>
<th>Average bifurcation tilt local (°)</th>
<th>Average bifurcation tilt remote (°)</th>
<th>Average bifurcation torque local (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>77.6</td>
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<td>126.3</td>
<td>138.8</td>
<td>180.0</td>
</tr>
<tr>
<td>2</td>
<td>130625</td>
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<td>55.9</td>
<td>131.5</td>
<td>130.5</td>
<td>92.7</td>
</tr>
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<td>57.7</td>
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<td>125.0</td>
<td>152.2</td>
<td>103.9</td>
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<td>63.7</td>
<td>118.4</td>
<td>129.8</td>
<td>115.1</td>
</tr>
<tr>
<td>5</td>
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<td>73.7</td>
<td>74.4</td>
<td>123.3</td>
<td>116.6</td>
<td>54.0</td>
</tr>
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<td>66.2</td>
<td>47.3</td>
<td>134.1</td>
<td>137.1</td>
<td>119.1</td>
</tr>
<tr>
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<td>140207</td>
<td>79.4</td>
<td>43.9</td>
<td>122.0</td>
<td>145.3</td>
<td>70.3</td>
</tr>
<tr>
<td>8</td>
<td>140208</td>
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<td>119.0</td>
<td>115.0</td>
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<td>97.4</td>
<td>95.0</td>
<td>106.4</td>
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<tr>
<td>10</td>
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<td>105.9</td>
<td>106.5</td>
<td>76.2</td>
</tr>
<tr>
<td>11</td>
<td>140301</td>
<td>71.2</td>
<td>61.8</td>
<td>126.4</td>
<td>132.8</td>
<td>78.8</td>
</tr>
<tr>
<td>12</td>
<td>140306</td>
<td>45.2</td>
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<td>145.1</td>
<td>134.2</td>
<td>140.9</td>
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</tbody>
</table>

Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 28-32 are shown in this portion of the table. The table will continue on the following page.
<table>
<thead>
<tr>
<th>Row Number</th>
<th>Reticular Formation Neuron Index</th>
<th>Average bifurcation torque remote (°)</th>
<th>Average last-parent diameter</th>
<th>Average Diameter threshold</th>
<th>Average Hilmann threshold</th>
<th>Average fractal Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>130624</td>
<td>125.9</td>
<td>2.6</td>
<td>5.3</td>
<td>5.6</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>130625</td>
<td>99.4</td>
<td>3.5</td>
<td>3.9</td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>140109</td>
<td>111.1</td>
<td>2.6</td>
<td>6.2</td>
<td>6.6</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>140114</td>
<td>106.4</td>
<td>4.9</td>
<td>4.6</td>
<td>5.70</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>140120</td>
<td>23.7</td>
<td>2.6</td>
<td>9.8</td>
<td>12.7</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>140124</td>
<td>116.4</td>
<td>3.2</td>
<td>5.8</td>
<td>5.9</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>140207</td>
<td>35.0</td>
<td>2.4</td>
<td>7.2</td>
<td>7.2</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>140208</td>
<td>90.2</td>
<td>4.4</td>
<td>8.2</td>
<td>9.1</td>
<td>1.0</td>
</tr>
<tr>
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<td>140220</td>
<td>94.9</td>
<td>3.2</td>
<td>6.8</td>
<td>7.2</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>140221</td>
<td>63.6</td>
<td>4.0</td>
<td>7.9</td>
<td>8.4</td>
<td>1.0</td>
</tr>
<tr>
<td>11</td>
<td>140301</td>
<td>59.0</td>
<td>3.7</td>
<td>6.5</td>
<td>6.6</td>
<td>1.0</td>
</tr>
<tr>
<td>12</td>
<td>140306</td>
<td>122.9</td>
<td>2.9</td>
<td>8.0</td>
<td>9.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 4 (continued). Morphometric properties of Dbx1 neurons in the reticular formation. The entire table consists of 37 columns and 12 rows. Columns 33-37 are shown in this portion of the table.
<table>
<thead>
<tr>
<th></th>
<th>PreBötC Average</th>
<th>Reticular Formation Average</th>
<th>U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma Surface (µm²)</td>
<td>544.2 ± 54</td>
<td>758.3 ± 82.5</td>
<td>77</td>
<td>0.02</td>
</tr>
<tr>
<td>Number of Stems</td>
<td>4.3 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>101</td>
<td>0.09</td>
</tr>
<tr>
<td>Number of Bifurcations</td>
<td>11.6 ± 1.3</td>
<td>6.7 ± 0.7</td>
<td>70.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Number of Branches</td>
<td>27.4 ± 2.8</td>
<td>17.3 ± 1.5</td>
<td>71.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Number of Tips</td>
<td>16.2 ± 1.4</td>
<td>11.6 ± 0.8</td>
<td>80</td>
<td>0.02</td>
</tr>
<tr>
<td>Width (µm)</td>
<td>470.8 ± 50.6</td>
<td>172.9 ± 27.1</td>
<td>21</td>
<td>4E-06</td>
</tr>
<tr>
<td>Height (µm)</td>
<td>366.0 ± 32.5</td>
<td>159.6 ± 18.7</td>
<td>24</td>
<td>9E-06</td>
</tr>
<tr>
<td>Depth (µm)</td>
<td>39.1 ± 3.1</td>
<td>22.3 ± 3.1</td>
<td>47.5</td>
<td>5E-04</td>
</tr>
<tr>
<td>Average Diameter (µm)</td>
<td>1.3 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>7.5</td>
<td>2E-07</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>1766.6 ± 166.6</td>
<td>628.5 ± 88.0</td>
<td>20</td>
<td>3E-06</td>
</tr>
<tr>
<td>Surface Area (µm²)</td>
<td>6849.5 ± 875.0</td>
<td>6759.6 ± 729.5</td>
<td>124</td>
<td>0.32</td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>5575.4 ± 1540.2</td>
<td>14124.3 ± 1840.6</td>
<td>38</td>
<td>1E-04</td>
</tr>
<tr>
<td>Average Euclidean</td>
<td>141.7 ± 15.2</td>
<td>57.7 ± 5.7</td>
<td>13.5</td>
<td>9E-07</td>
</tr>
<tr>
<td>Distance (µm)</td>
<td>392.6 ± 50.6</td>
<td>139.8 ± 13.4</td>
<td>35</td>
<td>3E-07</td>
</tr>
</tbody>
</table>

Table 5. Mann Whitney U test comparison of morphometric data obtained from L-measure for preBötC and reticular formation neurons. P values <0.05 values are highlighted in yellow.
<table>
<thead>
<tr>
<th></th>
<th>PreBötC Average</th>
<th>Reticular Formation Average</th>
<th>U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Path Distance (µm)</td>
<td>194.0 ± 23.7</td>
<td>70.3 ± 7.8</td>
<td>19</td>
<td>3E-06</td>
</tr>
<tr>
<td>Maximum Path Distance (µm)</td>
<td>537.6 ± 75.4</td>
<td>179.8 ± 22.1</td>
<td>16.5</td>
<td>2E-06</td>
</tr>
<tr>
<td>Average Branch Order</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>95.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Terminal segments</td>
<td>584.1 ± 73.7</td>
<td>301.5 ± 48.2</td>
<td>65</td>
<td>5E-03</td>
</tr>
<tr>
<td>Average Burke Taper (µm)</td>
<td>0.1 ± 0.03</td>
<td>0.3 ± 0.03</td>
<td>19</td>
<td>3E-05</td>
</tr>
<tr>
<td>Maximum Burke taper (µm)</td>
<td>0.9 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>39.5</td>
<td>2E-04</td>
</tr>
<tr>
<td>Average Hillman Taper (µm)</td>
<td>0.5 ± 0.04</td>
<td>0.4 ± 0.01</td>
<td>93</td>
<td>0.06</td>
</tr>
<tr>
<td>Maximum Hillman Taper (µm)</td>
<td>0.9 ± 0.01</td>
<td>0.9 ± 0.01</td>
<td>58.5</td>
<td>1E-03</td>
</tr>
<tr>
<td>Average Contraction</td>
<td>0.9 ± 0.01</td>
<td>0.9 ± 0.01</td>
<td>61.5</td>
<td>3E-03</td>
</tr>
<tr>
<td>Average Daughter Ratio</td>
<td>1.2 ± 0.02</td>
<td>1.1 ± 0.02</td>
<td>99</td>
<td>0.09</td>
</tr>
<tr>
<td>Average Parent-Daughter Ratio</td>
<td>0.8 ± 0.01</td>
<td>0.8 ± 0.02</td>
<td>122.5</td>
<td>0.30</td>
</tr>
<tr>
<td>Average partition asymmetry</td>
<td>0.5 ± 0.02</td>
<td>0.5 ± 0.02</td>
<td>110.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Average Rall power</td>
<td>2.4 ± 0.3</td>
<td>1.6 ± 0.5</td>
<td>95</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 5 (continued). Mann Whitney U test comparison of morphometric data obtained from L-measure for preBötC and reticular formation neurons. P values <0.05 values are highlighted in yellow.
<table>
<thead>
<tr>
<th></th>
<th>PreBötC Average</th>
<th>Reticular Formation Average</th>
<th>U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Bifurcation amplitude local (°)</td>
<td>75.6 ± 3.4</td>
<td>71.2 ± 4.6</td>
<td>123.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Average Bifurcation amplitude remote (°)</td>
<td>69.8 ± 3.7</td>
<td>61.8 ± 6.2</td>
<td>102</td>
<td>0.11</td>
</tr>
<tr>
<td>Average bifurcation tilt local (°)</td>
<td>116.4 ± 2.7</td>
<td>122.7 ± 3.7</td>
<td>101</td>
<td>0.10</td>
</tr>
<tr>
<td>Average bifurcation tilt remote (°)</td>
<td>121.6 ± 2.8</td>
<td>128.8 ± 4.2</td>
<td>97.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Average bifurcation torque local (°)</td>
<td>96.5 ± 4.8</td>
<td>108.0 ± 11.6</td>
<td>123</td>
<td>0.30</td>
</tr>
<tr>
<td>Average bifurcation torque remote (°)</td>
<td>86.3 ± 3.6</td>
<td>87.4 ± 9.9</td>
<td>115</td>
<td>0.21</td>
</tr>
<tr>
<td>Average last-parent diameter</td>
<td>1.7 ± 0.3</td>
<td>3.3 ± 0.2</td>
<td>35.5</td>
<td>8E-05</td>
</tr>
<tr>
<td>Average Diameter threshold</td>
<td>2.5 ± 0.4</td>
<td>6.7 ± 0.5</td>
<td>21</td>
<td>5E-06</td>
</tr>
<tr>
<td>Average Hilmann threshold</td>
<td>2.5 ± 0.5</td>
<td>7.4 ± 0.6</td>
<td>28</td>
<td>2E-05</td>
</tr>
<tr>
<td>Average fractal Diameter</td>
<td>1.0 ± 0.003</td>
<td>1.0 ± 0.003</td>
<td>32.5</td>
<td>3E-05</td>
</tr>
</tbody>
</table>

Table 5 (continued). Mann Whitney U test comparison of morphometric data obtained from L-measure for preBötC and reticular formation neurons. P values <0.05 values are highlighted in yellow.
**Sholl and branching pattern analysis**

Dendritic Sholl analysis results were compared from 0 µm to 500 µm distal from the cell soma in 50 µm increments. Only eight of the analyzed neuron morphologies in the preBötC and three neurons in the reticular formation have dendritic trees extending beyond 500 µm, so a comparison beyond 500 µm would not provide much value. Our Sholl analysis provided data about the number of branch points, total dendritic length, total dendritic volume, and total dendritic surface area for each 50 µm increment.

**Branch Points**

Branch points are similar for respiratory Dbx1 reticular formation neurons and respiratory Dbx1 preBötC neurons between 0 µm and 50 µm from the soma (Table 6). Between 50 µm and 200 µm from the soma, the Mann Whitney test (Table 6) indicates respiratory Dbx1 preBötC branch points and Dbx1 reticular formation branch points differ. After 300 µm, there were no differences between the respiratory Dbx1 neuron populations. Neither neuron group had branch points beyond 450 µm from the soma. Both groups branch most extensively between 0 µm and 50 µm from the soma; however, the branching in reticular formation neurons drops dramatically beyond 50 µm from the soma while the branching in the preBötC neurons decreases more gradually as the dendritic tree develops further away from the soma (Table 6).
**Dendritic Length**

Dendritic length is similar among respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons closest to the soma, between 0 µm and 50 µm. Dendritic length becomes disparate between 50 µm and 250 µm (Table 7). The respiratory Dbx1 preBötC neurons have greater average dendritic length than the Dbx1 reticular formation neurons from 50 µm to 400 µm, indicating Dbx1 preBötC neurons have more dendritic fiber in this range. Beyond 400 µm, the two neuron groups are nearly even in dendritic length (Table 7).

**Dendritic Volume**

Respiratory Dbx1 reticular formation neurons have greater average dendritic volume than respiratory Dbx1 preBötC neurons for each measured increment (Table 8). The two respiratory Dbx1 neuron populations differ significantly for the majority of the dendritic tree, based on the Mann Whitney analysis (Table 8).

**Dendritic Surface Area**

Respiratory Dbx1 reticular formation neurons have greater average dendritic surface area than respiratory Dbx1 preBötC neurons for all but one measured increment, between 150 µm and 200 µm from the soma (Table 9).
Differences between the two respiratory Dbx1 neuron populations differed significantly closest to the soma and beyond 250 µm from the soma (Table 9).

<table>
<thead>
<tr>
<th>Distance from the Soma</th>
<th>PreBötz Branch point average</th>
<th>Reticular Formation Branch point average</th>
<th>Mann Whitney U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µm - 50 µm</td>
<td>6.0 ± 0.8</td>
<td>6.8 ± 0.7</td>
<td>103</td>
<td>0.11</td>
</tr>
<tr>
<td>50 µm - 100 µm</td>
<td>2.7 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>52.5</td>
<td>8E-4</td>
</tr>
<tr>
<td>100 µm - 150 µm</td>
<td>1.6 ± 0.5</td>
<td>0.3 ± 0.1</td>
<td>88</td>
<td>0.03</td>
</tr>
<tr>
<td>150 µm - 200 µm</td>
<td>1 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>79.5</td>
<td>0.01</td>
</tr>
<tr>
<td>200 µm - 250 µm</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>112.5</td>
<td>0.10</td>
</tr>
<tr>
<td>250 µm - 300 µm</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>100.5</td>
<td>0.04</td>
</tr>
<tr>
<td>300 µm - 350 µm</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>130.5</td>
<td>0.32</td>
</tr>
<tr>
<td>350 µm - 400 µm</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
<td>126</td>
<td>0.15</td>
</tr>
<tr>
<td>400 µm - 450 µm</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
<td>132</td>
<td>0.42</td>
</tr>
<tr>
<td>450 µm - 500 µm</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>138</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 6. Sholl analysis branch points data and Mann Whitney U test. Average and standard error given for both respiratory Dbx1 preBötzC neurons and respiratory Dbx1 reticular formation neurons. Mann-Whitney U statistic and p value for comparison between respiratory Dbx1 preBötzC and respiratory reticular formation neurons. Statistically significant p values (p<0.05) are highlighted in yellow.
<table>
<thead>
<tr>
<th>Distance from the Soma</th>
<th>PreBötC Dendritic Length (µm)</th>
<th>Reticular Formation Dendritic Length (µm)</th>
<th>Mann Whitney U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µm - 50 µm</td>
<td>404 ± 35</td>
<td>393 ± 32</td>
<td>132</td>
<td>0.42</td>
</tr>
<tr>
<td>50 µm - 100 µm</td>
<td>437 ± 41</td>
<td>171 ± 44</td>
<td>35</td>
<td>7E-5</td>
</tr>
<tr>
<td>100 µm - 150 µm</td>
<td>337 ± 34</td>
<td>184 ± 30</td>
<td>56</td>
<td>2E-3</td>
</tr>
<tr>
<td>150 µm - 200 µm</td>
<td>253 ± 32</td>
<td>105 ± 18</td>
<td>49</td>
<td>6E-4</td>
</tr>
<tr>
<td>200 µm - 250 µm</td>
<td>150 ± 23</td>
<td>71 ± 11</td>
<td>69.5</td>
<td>8E-3</td>
</tr>
<tr>
<td>250 µm - 300 µm</td>
<td>94 ± 19</td>
<td>77 ± 13</td>
<td>132.5</td>
<td>0.43</td>
</tr>
<tr>
<td>300 µm - 350 µm</td>
<td>76 ± 17</td>
<td>57 ± 6</td>
<td>135.5</td>
<td>0.47</td>
</tr>
<tr>
<td>350 µm - 400 µm</td>
<td>53 ± 14</td>
<td>54 ± 7</td>
<td>114.5</td>
<td>0.21</td>
</tr>
<tr>
<td>400 µm - 450 µm</td>
<td>33 ± 7</td>
<td>45 ± 9</td>
<td>104</td>
<td>0.12</td>
</tr>
<tr>
<td>450 µm - 500 µm</td>
<td>19 ± 6</td>
<td>37 ± 9</td>
<td>93</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 7. Sholl analysis dendritic length data and Mann Whitney U test. Average and standard error given for both respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons. Mann-Whitney U statistic and p value for comparison between respiratory Dbx1 preBötC and respiratory reticular formation neurons. Statistically significant p values (p<0.05) are highlighted in yellow.
<table>
<thead>
<tr>
<th>Distance from the Soma</th>
<th>PreBötC Dendritic Volume (µm³)</th>
<th>Reticular Formation Dendritic Volume (µm³)</th>
<th>Mann Whitney U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µm - 50 µm</td>
<td>4666 ± 939</td>
<td>8447 ± 1120</td>
<td>51</td>
<td>8E-4</td>
</tr>
<tr>
<td>50 µm - 100 µm</td>
<td>651 ± 137</td>
<td>1108 ± 181</td>
<td>75</td>
<td>0.01</td>
</tr>
<tr>
<td>100 µm - 150 µm</td>
<td>443 ± 101</td>
<td>604 ± 110</td>
<td>96</td>
<td>0.07</td>
</tr>
<tr>
<td>150 µm - 200 µm</td>
<td>274 ± 71</td>
<td>307 ± 63</td>
<td>93</td>
<td>0.06</td>
</tr>
<tr>
<td>200 µm - 250 µm</td>
<td>152 ± 54</td>
<td>210 ± 35</td>
<td>71.5</td>
<td>0.01</td>
</tr>
<tr>
<td>250 µm - 300 µm</td>
<td>97 ± 36</td>
<td>230 ± 51</td>
<td>56.5</td>
<td>2E-3</td>
</tr>
<tr>
<td>300 µm - 350 µm</td>
<td>53 ± 17</td>
<td>155 ± 27</td>
<td>54.5</td>
<td>1E-3</td>
</tr>
<tr>
<td>350 µm - 400 µm</td>
<td>39 ± 17</td>
<td>143 ± 28</td>
<td>44.5</td>
<td>3E-4</td>
</tr>
<tr>
<td>400 µm - 450 µm</td>
<td>23 ± 10</td>
<td>135 ± 41</td>
<td>68</td>
<td>6E-3</td>
</tr>
<tr>
<td>450 µm - 500 µm</td>
<td>18 ± 8</td>
<td>101 ± 34</td>
<td>76</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 8. Sholl analysis dendritic volume data and Mann Whitney U test. Average and standard error given for both respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons. Mann-Whitney U statistic and p value for comparison between respiratory Dbx1 preBötC and respiratory reticular formation neurons. Statistically significant p values (p<0.05) are highlighted in yellow.
<table>
<thead>
<tr>
<th>Distance from the Soma</th>
<th>PreBötC Dendritic Surface Area (µm²)</th>
<th>Reticular Formation Dendritic Surface Area (µm²)</th>
<th>Mann Whitney U</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µm - 50 µm</td>
<td>3549 ± 542</td>
<td>5629 ± 525</td>
<td>47</td>
<td>4E-4</td>
</tr>
<tr>
<td>50 µm - 100 µm</td>
<td>1720 ± 254</td>
<td>1935 ± 242</td>
<td>104</td>
<td>0.12</td>
</tr>
<tr>
<td>100 µm - 150 µm</td>
<td>1202 ± 193</td>
<td>1150 ± 184</td>
<td>135</td>
<td>0.46</td>
</tr>
<tr>
<td>150 µm - 200 µm</td>
<td>788 ± 132</td>
<td>613 ± 102</td>
<td>130</td>
<td>0.39</td>
</tr>
<tr>
<td>200 µm - 250 µm</td>
<td>449 ± 112</td>
<td>425 ± 65</td>
<td>95.5</td>
<td>0.07</td>
</tr>
<tr>
<td>250 µm - 300 µm</td>
<td>285 ± 78</td>
<td>459 ± 86</td>
<td>73.5</td>
<td>0.01</td>
</tr>
<tr>
<td>300 µm - 350 µm</td>
<td>187 ± 47</td>
<td>321 ± 39</td>
<td>72.5</td>
<td>0.01</td>
</tr>
<tr>
<td>350 µm - 400 µm</td>
<td>123 ± 36</td>
<td>297 ± 41</td>
<td>50.5</td>
<td>8E-4</td>
</tr>
<tr>
<td>400 µm - 450 µm</td>
<td>75 ± 22</td>
<td>261 ± 64</td>
<td>69</td>
<td>7E-3</td>
</tr>
<tr>
<td>450 µm - 500 µm</td>
<td>57 ± 21</td>
<td>200 ± 55</td>
<td>78</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 9. Sholl analysis dendritic volume data and Mann Whitney U test. Average and standard error given for respiratory Dbx1 preBötC neurons and respiratory Dbx1 reticular formation neurons. Mann-Whitney U statistic and p value for comparison between respiratory Dbx1 preBötC and respiratory reticular formation neurons. Statistically significant p values (p<0.05) are highlighted in yellow.
DISCUSSION

The preBötC is the kernel of respiratory rhythm generation in mammals (Feldman and Del Negro, 2006; Feldman et al., 2013; Ramirez et al., 2016; Rekling and Feldman, 1998; Richter and Smith, 2014; Smith et al., 1991) and the preBötC has been definitively identified in humans (Schwarzacher et al., 2011). Glutamatergic (excitatory) neurons derived from precursors that express the transcription factor Dbx1 comprise the crucial rhythmogenic population in preBötC (Bouvier et al., 2010; Gray et al., 2010). The preBötC is responsible for generating the inspiratory phase of the respiratory rhythm and projecting to other centers of the brain, such as the reticular formation, to convey respiratory motor output (Wang et al., 2014) and facilitate breathing coordination with other orofacial behaviors (Kleinfeld et al., 2014; Koizumi et al., 2008; Moore et al., 2014).

Three-dimensional digital representations of Dbx1 preBötC and reticular formation neurons offer a considerable amount of geometric information, which we analyze to help unravel the rhythmogenic and premotor nature of these neurons. This morphological study showed that Dbx1 preBötC and reticular formation neurons are similar in a great many aspects of their dendritic trees that have physiological relevance, except for diameter which was significantly different from one population to the other. Also, this study further confirms the prior suggestion that Dbx1 preBötC neurons exhibit contralateral axonal projections (Bouvier et al., 2010; Gray et al., 2010; Pierani et al., 2001; Lanuza et
al., 2004), but surprisingly, only two of the twelve Dbx1 reticular formation neurons project toward the hypoglossal motor nucleus to generate respiratory related motor output, suggesting Dbx1 reticular neurons are involved in additional orofacial behaviors that must function harmoniously with respiration.

*Dbx1 reticular formation neurons may have larger passive conductance*

Input received via dendrites is attenuated before reaching the soma, which is the nature of cable properties. The way in which electrical signals from the dendrites are combined and influenced by the properties of dendrites is the basis of cable theory (Beierlein, 2014). Cable theory models dendrites as cylinders with separate conductances (resistances) governing flow of passive current across the plasma membrane (which is the radial boundary of the cylinder) and through the cylinder longitudinally. The plasma membrane also has intrinsic capacitance in parallel with membrane resistance.

Membrane resistance, $r_m$, will be proportional to the membrane area if the passive ion channels are uniformly distributed over that area of membrane; therefore the membrane resistance will be inversely proportional to the area. This relationship is quantified in the equation $r_m = \frac{R_m}{\pi r^2}$ (Beierlein, 2014) where $R_m$ is the specific resistance of the membrane (in units of $\Omega$-cm), $r$ is the radius (in units of cm). The denominator of the equation for input resistance is the area of the dendrite. Specific resistance is defined for a 1-cm$^2$ patch of membrane, while
membrane resistance varies based on the diameter and length of the segment of dendrite analyzed.

The data suggest Dbx1 reticular formation neurons have a larger mean diameter, thus a larger radius, than Dbx1 preBötC neurons. In the equation for membrane resistance, $r_m$, the term for the diameter is on the bottom of the fraction. Therefore, the larger diameter among respiratory Dbx1 reticular formation neurons will result in a lower membrane resistance. Since resistance and conductance have an inverse relationship, respiratory Dbx1 reticular formation neurons may have a greater conductance than respiratory Dbx1 preBötC neurons if their specific resistance is the same, which is a reasonable assumption since both neuron types are from the same genetic precursors, are located in the respiratory medulla, and are from the same age of mice.

Conductance, measured in Siemens (S), is a measure of how much electric current flows given an applied electromotive force. Thus, a larger conductance means that more current can flow across the membrane, making the neuron more leaky. Note that disparate specific membrane values or the presence of some active membrane properties may affect the conductance such that the difference in diameter does not lead to a distinct difference in conductance.

Six of the analyzed morphometric properties provide information about the relationships between bifurcation nodes and bifurcation planes within the dendritic arbor. Respiratory Dbx1 neurons in the preBötC and the reticular formation are similar in all of these measurements. Similarities in bifurcation amplitude suggest the dendritic trees among respiratory Dbx1 neurons in both
the preBötC and the reticular formation have a similar radial spread. Bifurcation tilt and torque give a sense of how the dendritic tree twists as it progresses away from the soma. Similarities in these bifurcation measurements suggest that the three-dimensional radial spread of the dendrites of respiratory Dbx1 neurons in the preBötC and reticular formation will be similar. The process through which dendrites develop and orient their dendritic trees is not yet completely understood, but neighboring electrical activity, nerve growth factors, and expression of specific genes that govern branching such as kak may play a role in dendritic growth and branching (Scott and Luo, 2001). Kak may govern microtubules that are involved in branch formation. (Scott and Luo, 2001). Since respiratory Dbx1 preBötC and respiratory Dbx1 reticular formation neurons have similar bifurcation or branching, they may experience similar microtubule activity during development resulting in similar branching properties.

Closest to the soma, between 0 µm and 50 µm, respiratory Dbx1 neurons in the preBötC and reticular formation are similar in number of branch points and dendritic length, but differ in dendritic volume and surface area. This difference is likely attributable to the difference in diameter between the two neuron groups. Reticular formation neurons have a larger dendritic diameter than preBötC neurons and so it follows that their dendritic volume and surface area would be larger. Between 50 µm and 450 µm away from the soma, there is a difference in at least one of the four categories evaluated in the Sholl analysis.

The morphometric data depend on analysis of the digital reconstructions and visual identification of the neuron’s location. The rostrocaudal distribution of
Dbx1 neurons is uniform throughout the ventral respiratory column and does not have a region that is visually distinct as the preBötz (Ruangkittisakul et al 2014). The dorsal border of the preBötz is identifiable as it is immediately ventral to the nucleus ambiguus. The dorsal-most Dbx1 preBötz neurons blend into the ventral-most region of the reticular formation. Respiratory Dbx1 neurons are aligned in a continuum in a dorsomedial to ventrolateral manner. Therefore, there may be a few neurons that are designated as being part of the reticular formation where they could in fact be in the dorsal region of the preBötz (Wang et al., 2014). There is not a clear border between preBötz and reticular formation, particularly given that Dbx1 neurons are continuous throughout.

Digital reconstructions are known to be different depending on the way the structure of the neuron is labeled, the type of imaging used to acquire the neuron image, the method of reconstruction, and even the person who transcribes confocal images into the digital reconstructions of morphology. We endeavored to minimize these differences in several ways. We used the same method of labeling for the morphology of all of the reconstructed neurons. While there were two people creating the reconstructions that were analyzed in this study (the vast majority done by me, the author of this dissertation), both used the same software to create the digital reconstructions and used the same software to assemble the microscopy images to use. Also, the reconstruction software that we used, Neuromantic, allows the user to zoom in to the microscopy images up to 16000%, as mentioned in the previous chapter. This considerable amount of zoom makes it easy for the user to determine the details of the microscopy image.
in order to make the most accurate reconstruction possible, up to the point of the resolution of the images and their underlying pixels. Respiratory Dbx1 neurons have a simpler morphology than other neuron types such as Purkinje neurons, which have extensively branched dendritic arbors. Some preBötC neurons that express bursting properties in neonatal rats may have spines and protrusions (Koizumi et al., 2013) but Dbx1 preBötC neurons in mice do not have spines or protrusions (Picardo et al., 2013). Spines and protrusions within the dendritic tree would provide another level of complexity in the reconstructions leaving room for greater discrepancy between Dbx1 preBötC and IRT neurons. Also, while respiratory Dbx1 neurons do have branched dendritic trees, their dendritic trees are not heavily branched compared to other common dendritic morphologies such as Purkinje neurons or basket neurons (Okhotin and Kalinichenko, 2002).

There were eleven analyzed neurons that did not have a discernible axon. The neurons with short axons or no axon at all may be making local connections within the preBötC or were not complete axon fills, or the axons were cut off during the slicing procedure. Axons were identified and distinguished from dendrites in several ways, described in the previous chapter. Only neurons with dendritic morphometric properties that had at least 100 µm in height and/or width were considered as part of this study. Neurons with less than 100 µm of dendrite were considered to be incomplete or unsuccessfully filled with biocytin and would not be appropriate to include. We recommend that the reader draw no firm conclusions from the lack of a reconstructed axon because one cannot determine for certain whether the axon was removed by virtue of being cut or it never filled.
Respiratory Dbx1 reticular formation neurons may coordinate breathing with orofacial behaviors

The intermediate reticular formation contains a mixture of premotor neurons including trigeminal premotor neurons, hypoglossal premotor neurons, and vibrissa premotor neurons (Kleinfeld et al., 2014; Stanek et al., 2014). We hypothesized that respiratory modulated Dbx1 neurons in the reticular formation would mainly be hypoglossal premotor neurons as the hypoglossal motor nucleus is a pool of motor neurons that innervates all of the intrinsic muscles of the tongue and four out of five of the extrinsic tongue (Ono et al., 1998). The hypoglossal motor nucleus helps maintain airway patency during respiration via tongue protrusion (Fregosi and Ludlow, 2014).

Only three respiratory modulated Dbx1 neurons in the reticular formation exhibited axon projections toward the hypoglossal nucleus. A larger proportion of Dbx1 reticular formation neurons were commissural than anticipated and did not project toward the hypoglossal motor nucleus. Commissural reticular formation neurons may have a role in coordinating respiration with orofacial behaviors that include phonation, chewing, suckling, licking, and sniffing (Kleinfeld et al., 2014; Moore et al., 2013, 2014). Since whisking or vibrissa premotor neurons are present in the reticular formation, it is possible that these commissural respiratory modulated reticular formation neurons are vibrissa premotor neurons. However, lesions of the vibrissa neurons in the intermediate reticular formation only
affected ipsilateral whisking (Moore et al., 2013) so the contralateral Dbx1 reticular formation neurons may not be vibrissa neurons, but could be involved in inspiratory drive for airway tongue movements or coordination of orofacial behaviors aside from whisking (Ono et al., 1998; Peever et al., 2002)

In summary, the morphometric properties and axon projections of respiratory Dbx1 neurons in the preBötC and reticular formation were evaluated in order to further ascertain their contribution to the respiratory neural network. Respiratory Dbx1 neurons in the preBötC and reticular formation are similar in many dendritic morphometric measurements. However, there was a difference in diameter which may contribute to a greater passive conductance in respiratory Dbx1 reticular formation neurons. Some respiratory Dbx1 reticular formation neurons projected to the ipsilateral hypoglossal motor nucleus, as was expected since we suspected respiratory Dbx1 reticular formation neurons were premotor neurons. More respiratory Dbx1 neurons in the reticular formation were commissural than expected and are thought to coordinate orofacial behaviors with breathing.
INTRODUCTION

Inspiratory breathing rhythm originates in the preBötC of the ventral medulla (Feldman and Del Negro, 2006; Feldman et al., 2013; Smith et al., 1991). Neuroscientists striving to elucidate the cellular-level origins of breathing have determined that Dbx1 preBötC neurons are crucial for respiratory rhythmogenesis (Bouvier et al., 2010; Cui et al., 2016; Gray et al., 2010; Koizumi et al., 2016; Picardo et al., 2013; Vann et al., 2016; Wang et al., 2014). Knowing the site, and that Dbx1-derived neurons are the rhythmogenic cellular substrate, I now turn my attention to the molecular (ion channel) level mechanisms of inspiratory burst- and rhythm generation.

The ion channels that give rise to rhythm-generating whole-cell ion currents have not been identified, and will be investigated in this chapter using principally an anatomical approach. These anatomical experiments are a key step in ultimately testing the roles of these ion channels in respiratory rhythm generation in *in vitro* models of breathing as well as real breathing *in vivo*.

For nearly the past three decades, investigators have suspected two ionic mechanisms may underlie rhythmic network bursts in the preBötC: a voltage sensitive mechanism depending on subthreshold activation of persistent sodium current, $I_{NP}$, and a less voltage-sensitive mechanism linked to a non-specific cation current $I_{CAN}$ (Feldman and Del Negro, 2006; Feldman et al., 2013; Ramirez...
et al., 2016). In respiratory rhythmogenesis, $I_{NaP}$ has been implicated but without any conclusive demonstration of its putative rhythmogenic role (Ben-Mabrouk and Tryba, 2010; Del Negro et al., 2005, 2010, Pace et al., 2007b, 2007a; Peña et al., 2004; Rubin et al., 2009; Thoby-Brisson and Ramirez, 2001), although for an alternative view of the role of $I_{NaP}$ in rhythmogenesis for inspiration see: (Koizumi and Smith, 2008). On the other hand, $I_{CAN}$ underlies robust inspiratory drive potentials in the preBötC, which are rhythmic bursts driven by network activity and $I_{CAN}$ may itself be rhythmogenic (Crowder et al., 2007; Mironov, 2008; Mironov and Skorova, 2011; Pace and Del Negro, 2008; Pace et al., 2007b; Rubin et al., 2009). Although its putative role in eupnea and inspiratory rhythms in vitro is equivocal, $I_{NaP}$ may play a role in the production of robust bursts when respiration is challenged in such cases as anoxia or hypoxia (Paton et al., 2006).

Physiology, simulations, and pharmacology have examined the potential roles of $I_{NaP}$ and $I_{CAN}$ in respiratory rhythm. However, immunohistochemistry is needed to verify the presence of specific ion channels that may give rise to these whole-cell currents. If we can unravel what specific ionophores underlie $I_{NaP}$ and $I_{CAN}$, then we can design genetic experiments to definitively test their roles in inspiratory rhythm generation and breathing.

Identifying ion channels via immunohistochemistry is notoriously difficult: antibodies react with epitopes found on multiple (genetically distinct) Na+ channel subunits that share similar protein structure. Moreover, Trp channels have few existing commercial or custom-built antibodies. A great fraction of the work presented in this dissertation was preceded by years of screening and testing
antibodies for effectiveness. That background work was necessary to finalize
protocols and thereby provide anatomical evidence for ion channel presence in
Dbx1 preBötC neurons to complement our recently published the mRNA
transcriptome (Hayes et al., 2017). Note: the presence of mRNA transcripts does
not guarantee translation as protein.

Intracellular Ca\(^{2+}\) transients have been measured during inspiratory drive
potentials, which is consistent with the ability to evoke \(I_{\text{CAN}}\) during the inspiratory
phase (Del Negro et al., 2011). Drive potentials are sensitive to flufenamic acid
(FFA), an \(I_{\text{CAN}}\) antagonist. Ion channels in the Trp family are the leading
candidates for mediating \(I_{\text{CAN}}\) in Dbx1 preBötC neurons because several
members of the Trpm and Trpc subfamilies are modulated (usually activated) by
intracellular Ca\(^{2+}\) and are sensitive to blockade by FFA (Del Negro et al., 2010;
Guinamard et al., 2014; Pace et al., 2007b). mRNA for several Trp channels are
present in Dbx1 preBötC neurons; in fact, transcriptome data (Hayes et al., 2017)
show that Trpc3 is a strong candidate because its mRNA expression is the
highest among Trp channels analyzed in Dbx1 preBötC neurons. Since mRNA
expression does not guarantee protein translation, I will determine whether Trpc3
ion channel proteins are expressed on Dbx1 preBötC neurons in neonatal and
adult mice through immunohistochemistry. Those data would provide direct
evidence for a potential ion channel candidate that may mediate \(I_{\text{CAN}}\). Trpc3 ion
channel expression in Dbx1 preBötC neurons has not been shown before – and
such data are scarcely available for any neuron class \textit{in situ} in the nervous
system (Trpc1 and Trpc5 expression in cultured neurons does not constitute the same criteria for in situ expression, see (Strübing et al., 2001).

Physiological data demonstrates that the $I_{NaP}$ is ubiquitous in preBötC inspiratory neurons (Del Negro et al., 2002a, 2002b, 2005; Paton et al., 2006; Ptak et al., 2005; Rybak et al., 2003). Despite its being expressed throughout the ventral medulla including, of course, the preBötC the role of $I_{NaP}$ in respiratory rhythm has never been documented convincingly, although a definitive test – by genetically knocking out the underlying ion channel specifically in respiratory rhythmogenic neurons – has yet to be performed. In mice that do not express ion channel Na$_{v}$1.6 (a genome-wide knockout, not specific to preBötC), $I_{NaP}$ is greatly reduced, although fast (Hodgkin-Huxley-like) and resurgent Na$^{+}$ currents are largely unaffected (Osorio et al., 2010). Our mRNA data from Dbx1 and non-Dbx1-derived preBötC neurons (Hayes et al., 2017) also suggests that Scn8a, which codes for Na$_{v}$1.6 channels, is present in Dbx1 and non-Dbx1 neurons, which implicates this channel type as a potential source of $I_{NaP}$ in the preBötC and thus a good target for my studies.

In this chapter, I will examine the expression of Na$_{v}$1.6 in Dbx1 preBötC neurons and verify the absence of Na$_{v}$1.6 expression in an intersectional mouse genetic model that enables us to target Scn8a knockout to Dbx1 preBötC neurons, the source of the inspiratory rhythm. The specific mouse model intersecting Dbx1$^{Cre}$ mice with Scn8a$^{LoxP}$ (floxed) reporter mice will be explained in the following methods section and will be used in further physiology experiments and behavioral experiments to examine the effects of Na$_{v}$1.6
deficiency in Dbx1 preBötC neurons. Verification of the knockout of Nav1.6 is important to validate that any changes, or lack thereof, of breathing behavior in knockout animals are in response to the lack of Nav1.6 ion channels.

**METHODS**

All of the animal protocols were approved by the Institutional Animal Care and Use Committee at The College of William and Mary, which follows the guidelines provided by the US National Institutes of Health Office of Laboratory Animal Welfare (NIH Office of Laboratory Animal Welfare, 2015).

*Trpc3 immunohistochemistry in neonatal mice*

Methods involving intersectional mouse genetics, dissection of neonatal tissue, and fixation of neonatal tissue covered in chapter 1 (See Figure 10, Steps 1, 2, and 4) will be the same for Trpc3 immunohistochemistry in neonatal mice. The brainstem and spinal cord were removed and placed in 4% paraformaldehyde (PFA) overnight on an orbital shaker. Brainstem-spinal cords were rinsed in 1X phosphate buffered solution (PBS) (BP399-1, Fisher Scientific, Hampton, NH) for at least 30 minutes after overnight fixation. The brainstem-spinal cords were then suspended in 4% low melting point agar for sectioning with a vibratome (Thermo Scientific Microm HM 650 V, Waltham, MA). Transverse 50-μm-thick sections were cut from the preBötC region of the
brainstem. Neonatal sections were incubated with 20% normal donkey serum (50-413-367, Fisher Scientific) overnight at 4°C on an orbital shaker. The following day, the slices were washed with 1X PBS 2 x 30 min and were incubated in 1:500 rabbit anti-Trpc3 antibody (ACC-016, Alomone labs, Jerusalem, Israel) solution with 0.4% TritonX-100 overnight at 4°C on an orbital shaker. Sections were then washed 4 x 30 minutes with 1X PBS and placed in 1:200 donkey anti-rabbit Alexa 405 (711-475-152 Jackson ImmunoResearch Labs, West Grove, PA) for one hour. After washing for a minimum of 6 x 30 min with 1X PBS, sections were mounted on glass slides and cover-slipped with Vectashield hardset mounting medium (H-1500; Vector Laboratories, Burlingame, CA). Images were acquired with a 10x air (NA = 0.45) and 60x oil immersion (NA = 1.49) objectives on a Nikon A1R confocal laser scanning microscope using NIS Elements AR imaging software (Nikon Instruments Inc., Melville, NY).

**Trpc3 immunohistochemistry in adult mice**

To perform Trpc3 immunohistochemistry experiments in adult mice, neonatal $\text{Dbx}^\text{CreERT2}; \text{Rosa}^{26\text{tdTomato}}$ mice were aged for a minimum of 6 weeks. These adult mice were anesthetized by pentobarbital (100 mg·kg$^{-1}$), and then transcardially perfused with 1X PBS followed by 4% PFA. The brainstem and spinal cord were removed and placed in 4% PFA overnight at 4°C to complete fixation. Brainstem-spinal cord preparations were rinsed in 1X PBS, placed in 4%
low melting point agar, and sectioned at 50 μm in the same way as the neonatal tissue. The adult medullary slices were incubated in 1:400 rabbit anti- Trpc3 antibody (ACC-016, Alomone labs) solution with 0.4% TritonX-100 overnight at 4°C on an orbital shaker. Sections were then washed 2 x 30 minutes with 0.4% TritonX-100 1X PBS and incubated in 20% normal donkey serum with 0.4% TritonX-100 for 60-90 minutes on an orbital shaker at room temperature. Next, the sections were washed 2 x 10 minutes with 1X PBS and placed in 1:100 donkey anti-rabbit Alexa 405 (711-475-152 Jackson ImmunoResearch Labs) with 0.4% TritonX-100 for one hour. After washing for a minimum of 6 x 30 min with 0.4% TritonX-100, sections were mounted on glass slides and cover-slipped with Vectashield hardset mounting medium. Images were acquired with a 10x air (NA = 0.45) and 60x oil immersion (NA = 1.49) objectives on a Nikon A1R confocal laser scanning microscope using NIS Elements AR imaging software (Nikon Instruments Inc.).

**Na\textsubscript{v}1.6 immunohistochemistry in neonatal mice**

To perform Na\textsubscript{v}1.6 immunohistochemistry, mice with the potential for a genetic knockout of the Scn8a gene were bred using the following strategy, which begins with three strains of mice. The first mouse strain is a homozygous Dbx1\textsuperscript{CreERT2} mouse as described in Chapter 1. Since there are no alterations to the Scn8a gene or the Rosa26 gene locus, this mouse can be denoted as Dbx1\textsuperscript{Cre/Cre};Scn8a\textsuperscript{+/+};Rosa26\textsuperscript{+/+}, where a + indicates a wild type allele. The
second mouse strain, also described in Chapter 1, is homozygous for floxed tdTomato and can be denoted as $Dbx1^{+/+};Scn8a^{+/+};Rosa26^{FtdT/FtdT}$, where FtdT indicates a tdTomato allele preceded by a LoxP-flanked STOP codon. The third mouse strain has LoxP sites flanking exon 1 of $Scn8a$ and is denoted as $Dbx1^{+/+};Scn8a^{Flx/Flx};Rosa26^{+/+}$, where Flx indicated the floxed allele. In the first generation of the breeding strategy $Dbx1^{Cre/Cre};Scn8a^{+/-};Rosa26^{+/-}$ are crossed with $Dbx1^{+/+};Scn8a^{+/+};Rosa26^{FtdT/FtdT}$ mice to produce $Dbx1^{Cre/+};Scn8a^{+/+};Rosa26^{FtdT/+}$ mice. In the second generation, $Dbx1^{Cre/+};Scn8a^{+/+};Rosa26^{FtdT/+}$ mice are crossed with $Dbx1^{+/+};Scn8a^{Flx/Flx};Rosa26^{+/+}$, which produces 4 types of offspring: 

$Dbx1^{Cre/+};Scn8a^{Flx/+};Rosa26^{FtdT/+}$, $Dbx1^{Cre/+};Scn8a^{Flx/+};Rosa26^{+/+}$,

$Dbx1^{+/+};Scn8a^{Flx/+};Rosa26^{FtdT/+}$, and $Dbx1^{+/+};Scn8a^{Flx/+};Rosa26^{+/+}$. The offspring of interest from this generation, $Dbx1^{Cre/+};Scn8a^{Flx/+};Rosa26^{FtdT/+}$, were mated and given tamoxifen at embryonic day 10.5 to produce 64 different combinations of genetic offspring resulting in 4 phenotypes. To simplify the genetic combinations, x will represent a mutant or wild type allele (e.g., $Dbx1^{Cre/x}$ would stand for both $Dbx1^{Cre/Cre}$ and $Dbx1^{Cre/+}$). 44% of the offspring are $Dbx1^{x/x};Scn8a^{x/x};Rosa26^{x/x}$ and would not be useful for analysis. 28% of the offspring are $Dbx1^{Cre/x};Scn8a^{+/+};Rosa26^{TdT/x}$, signifying a heterozygous knockout of the $Scn8a$ gene. 14% of the offspring are $Dbx1^{Cre/x};Scn8a^{+/+};Rosa26^{TdT/x}$, which will express tdTomato but do not have a genetic knockout of $Scn8a$. Finally, 14% of the offspring are $Dbx1^{Cre/x};Scn8a^{-/-};Rosa26^{TdT/x}$ which results in a genetic knockout of one exon (specifically exon 1) of the $Scn8a$ gene. Only
Dbx1\textsuperscript{Cre/\texttimes;Scn8a\textsuperscript{\texttimes;/}Rosa26\textsuperscript{TdT/\texttimes} mice were used for immunohistochemistry experiments. Tails from the neonates were genotyped and a section of the spinal cord was used to perform polymerase chain reaction (PCR) fragment analysis to further verify the knockout of the Scn8a gene. Immunohistochemistry was performed on third generation offspring dissected and prepared in the same manner described in Chapter 1 (See Figure 10, Steps 2 and 4).

Brainstem-spinal cords were removed and placed in 4% PFA overnight and rinsed in PBS for at least 30 minutes after overnight fixation. The brainstem-spinal cords were then placed in 4% low melting point agar for sectioning with a vibratome (Thermo Scientific Microm HM 650 V). Transverse 50 to 60-μm-thick sections were cut from the preBötC region of the brainstem. Two different protocols for antibody staining were used. In the first method, sections were incubated with 20% normal donkey serum (50-413-367, Fisher Scientific) overnight at 4°C on an orbital shaker. The slices were subsequently washed with PBS for one hour and were incubated in 1:5000 rabbit anti- Na\textsubscript{v}1.6 polyclonal antibody (ASC-009, Alomone labs) solution with 0.4% TritonX-100 overnight at 4°C on an orbital shaker. Sections were then washed 4 x 30 minutes with 1X PBS then placed in 1:1000 donkey anti-rabbit Alexa 405 (711-475-152 Jackson ImmunoResearch Labs) for one hour. After washing for a minimum of 6 x 30 min with 0.4% TritonX-100, sections were mounted on glass slides and cover-slipped with Vectashield hardset mounting medium (H-1500; Vector Laboratories).
For the second protocol, sections were incubated with 20% normal donkey serum (50-413-367, Fisher Scientific) and 10% donkey anti-mouse Fab fragment antibody (715-007-003) overnight at 4°C on an orbital shaker. In a separate tube, 1:1000 mouse anti- Naᵥ1.6 monoclonal antibody (K87A/10, Antibodies Incorporated, Davis, CA) was combined with 1:500 donkey anti-mouse Alexa 647 (711-607-003 Jackson ImmunoResearch Labs) for 90 minutes on an orbital shaker to make an antibody complex. Next, 200-400 µL of normal mouse serum was added to the tube and placed on the orbital shaker for an additional 2 hours to remove any secondary antibody that had not bound to the primary antibody. Slices previously incubated with donkey serum and mouse Fab fragments were then incubated in the antibody complex solution overnight. After washing for a minimum of 8 x 30 min with PBS, sections were mounted on glass slides and cover-slipped with Vectashield hardset mounting medium (H-1500; Vector Laboratories).

Images for all Naᵥ1.6 immunohistochemistry experiments were acquired with a 10x air (NA = 0.45) and 60x oil immersion (NA = 1.49) objectives on a Nikon A1R confocal laser scanning microscope using NIS Elements AR imaging software (Nikon Instruments Inc.). Note that Naᵥ1.6 immunohistochemistry was not performed in adult animals because we have no reason a priori to suspect that Na⁺ channel expression changes with development and because the triple transgenic intersectional mice are so difficult to breed in sufficient numbers that we cannot reasonably accomplish adult and neonatal anatomical experiments. We opted for neonatal experiments because these animals can be used for in
vivo and in vitro experiments and thus provide an advantageous complement to physiological experimentation on the role of $I_{NaP}$, i.e., $Na_v1.6$ (Hayes, 2017; Hayes, Dermer, Barnett, Hunt, Del Negro, 2017 (in preparation)).

**RESULTS**

*Trpc3 ion channel expression in neonatal mice*

To study Trpc3 ion channel expression in neurons of the preBötC, I used brainstem slices from neonatal and adult $Dbx1^{Cre/\times};Scn8a^{\alpha/\times};Rosa26^{TdT/\times}$ mice. I found that Trpc3 ion channels are expressed in Dbx1 and non-Dbx1 preBötC neurons in both adult and neonatal mice, demonstrated in Figures 33 and 34. Glial cells are also labeled with tdTomato during Cre-lox recombination and tamoxifen-induced expression of tdTomato to label Dbx1 preBötC neurons, but glia can be distinguished from neurons as they are smaller in size (Kottick et al., 2017). I observed, as expected, that glial cells do not express Trpc3 ion channels.
Figure 33. Trpc3 expression in Dbx1 and non-Dbx1 preBötC neurons of adult mice. A, image of the slice demonstrating the immunohistochemistry was performed at the level of the preBötC. B, immunohistochemistry against Trpc3. C, tdTomato labeling of Dbx1 neurons. D, composite of B and C. Filled yellow arrowheads indicate examples of co-localization of Dbx1 tdTomato preBötC neurons and Trpc3. White triangles indicate examples of non-Dbx1 neurons that express Trpc3. Scale bars in both C and D are 20 µm.
Figure 34. Trpc3 expression in Dbx1 and non-Dbx1 preBötC neurons of neonatal mice. A, image of the slice showing the immunohistochemistry was performed at the level of the preBötC. B, immunohistochemistry against Trpc3. C, tdTomato labeling of Dbx1 neurons. D, composite of B and C. Filled yellow arrowheads indicate examples of co-localization of Dbx1 tdTomato preBötC neurons and Trpc3. White triangles indicate examples of non-Dbx1 neurons that express Trpc3. Scale bars in both C and D are 20 µm.
Na\textsubscript{v}1.6 ion channel expression in neonatal mice

To study Na\textsubscript{v}1.6 ion channel expression in key neurons of the preBöC, we used brainstem slices from neonatal $Dbx1^{Cre/\times};Scn8a^{\times/\times};Rosa26^{TdT/\times}$ mice. We found that Na\textsubscript{v}1.6 ion channels are ubiquitously expressed in both Dbx1 and non-Dbx1 preBöC neurons in mice, demonstrated in Figures 35 and 36. Glial cells do not express Na\textsubscript{v}1.6 ion channels.

Figure 35. Na\textsubscript{v}1.6 monoclonal immunohistochemistry in neonatal mice. A, image of the slice showing the immunohistochemistry was performed at the level of the preBöC. B, immunohistochemistry against Na\textsubscript{v}1.6. C, tdTomato labeling of Dbx1 neurons. D, composite of B and C. Filled yellow arrowheads indicate examples of co-localization of Dbx1 tdTomato preBöC neurons and Na\textsubscript{v}1.6. White triangles indicate examples of non-Dbx1 neurons that express Na\textsubscript{v}1.6. Scale bars in both C and D are 20 µm.
Figure 36. Na\(_{v}1.6\) polyclonal immunohistochemistry in neonatal mice. A, image of the slice showing the immunohistochemistry was performed at the level of the preBötC. B, immunohistochemistry against Na\(_{v}1.6\). C, tdTomato labeling of Dbx1 neurons. D, composite of B and C. Filled yellow arrowheads indicate examples of co-localization of Dbx1 tdTomato preBötC neurons and Na\(_{v}1.6\). White triangles indicate examples of non-Dbx1 neurons that express Na\(_{v}1.6\). Scale bars in both C and D are 20 µm.
DISCUSSION

*Trpc3 ion channels may be involved in the generation of I_{CAN}*

Members of the Trp superfamily of ion channels likely contribute to $I_{CAN}$, as characterized in preBötC neurons (Crowder et al., 2007; Mironov, 2008; Mironov and Skorova, 2011; Pace and Del Negro, 2008; Thoby-Brisson and Ramirez, 2001). Among Trp channel mRNA analyzed in Dbx1 preBötC neurons, *Trpc3* mRNA had the highest expression among Trp mRNA family members in the preBötC, as demonstrated in Figure 5 of the literature review and (Hayes et al., 2017). Through immunohistochemistry, I revealed the presence of Trpc3 ion channels in Dbx1 neurons, exhibited in Figures 33 and 34. These figures also show the presence of Trpc3 ion channels in non-Dbx1 preBötC neurons, which indicates that this channel is expressed by rhythmogenic (Dbx1) neurons as well as non-rhythmogenic (non-Dbx1) neurons that are part of the pattern-generating respiratory circuitry.

Trpc3 has been proposed to contribute to $I_{CAN}$ in preBötC neurons (Ben-Mabrouk and Tryba, 2010), although the evidence in that study is relatively weak considering that the authors showed relatively low resolution western blot analyses and used promiscuous pharmacology. Nevertheless, Trpc3 channels are known to be sensitive to intracellular Ca^{2+} concentration and conduct monovalent cations (Trebak et al., 2003). If this is the case, we would expect Dbx1 neurons to express Trpc3. I found that Trpc3 is expressed ubiquitously in
both Dbx1 and non-Dbx1 preBötC neurons. Given this widespread expression, it is plausible that Trpc3 works in conjunction with other Trp ion channel subunits to mediate $I_{CAN}$ and contribute to the inspiratory bursts and rhythm.

*Trpc3 ion channels may play a role in mechanisms other than respiratory rhythm generation*

Ubiquitous expression of Trpc3 may indicate that Trpc3 ion channels are involved in other cellular mechanisms in addition to respiratory rhythm generation including simple cellular functions and cellular survival. Trpc channels may be important for signal transduction and axon guidance during brain development (Li et al., 2005). The development of functional neuronal circuits requires the finely tuned growth of nerve fibers toward their targets, a process called nerve pathfinding (Cui and Yuan, 2007; Goodman and Shatz, 1993). It is plausible that Trpc3, being a member of the Trpc family, is involved with the development of neuronal circuits and the growth of nerve fibers as well as having a role in the $I_{CAN}$ current; it is also possible that Trpc3 is mainly important for development and pathfinding and has a minimal role in respiratory rhythm generation.

Trpc3 is also involved in motor control, specifically coordination which is a function of the cerebellum. There, Trpc3 channels operate in conjunction with metabotropic glutamate receptor (mGluR) signaling in mouse Purkinje cells (Hartmann et al., 2008). Group 1 mGluRs regulate slow synaptic excitation; without Trpc3, mice had no slow excitatory post synaptic currents (EPSC) or
mGluR mediated currents. $I_{CAN}$ gated in part by group 1 mGluR signaling are important for respiratory burst generation and respiratory rhythm (Pace et al., 2007b). Group 1 mGluRs are coupled through phospholipase C to inositol triphosphate (IP$_3$) synthesis, which evokes intracellular Ca$^{2+}$ release, thus serving as a potential source of $I_{CAN}$ activation (Pace et al., 2007b). Trpc3 ion channels present on Dbx1 neurons in both neonatal and adult mice may mediate group 1 mGluR-mediated $I_{CAN}$ activation, thus contributing to inspiratory burst generation.

$Na_{v}1.6$ ion channels may be involved in $I_{NaP}$ generation

Between 5 and 25% of preBötc neurons in neonatal mice exhibit voltage-dependent conditional bursting properties in vitro, often called bursting-pacemaker activity (Del Negro et al., 2005; Koizumi and Smith, 2008; Peña et al., 2004). At the time the preBötc was discovered in 1991 (Smith et al. Science 1991) the voltage-dependent bursting-pacemaker neurons were hypothesized to be rhythmogenic. Most studies to test that idea (i.e., the pacemaker hypothesis) have approached it via pharmacology. Our lab, and our collaborators, have concluded that voltage-dependent pacemaker activity is not required for respiratory rhythm generation because rhythmogenesis still occurs when voltage-dependent bursting behavior is precluded in preBötc neurons (Del Negro et al., 2002b). Rhythm continued and indeed respiratory motor output frequency was unaffected by bath application of blockade of $I_{NaP}$ (Del Negro et al., 2005). Those experiments utilized the drug riluzole, which blocks $I_{NaP}$ (Doble, 1996; Urbani and
Belluzzi, 2000). However, not every group obtained the same results. In thin (250-350 µm) neonatal rat slices and preBötC explants, experiments blocking $I_{NaP}$ with riluzole and tetrodotoxin (TTX) do stop the rhythm (Koizumi and Smith, 2008), but not in in situ or in vivo preparations (Paton et al., 2006; Smith et al., 2007). Furthermore, when similar experimental methods were applied to thicker neonatal mouse slices (500-550 µm) containing the preBötC and surrounding network components such the rostral ventral respiratory group (rVRG), the respiratory rhythm was not disrupted (Pace et al., 2007a). $I_{NaP}$ increases spiking frequency, but has not been shown to contribute to respiratory drive potentials (Morgado-Valle et al., 2008; Pace et al., 2007a).

The disparities in respiratory rhythm cessation could be due to the pharmacological properties of riluzole and differences in experimental design. Riluzole depresses neuronal excitability, thus when added to a slice via direct injection in the preBötC, the force of the injection can cause a channel to form in the tissue allowing riluzole to travel to other nearby areas of the slice such as the raphe obscurus. Serotonergic raphe neurons the preBötC help maintain rhythmic preBötC function (Peña and Ramirez, 2002; Tryba et al., 2006), so a loss in excitability in the raphe would disrupt rhythmogenesis. The narrative above recounts more than a decade of disagreement and discord because of the promiscuous pharmacology of riluzole, which blocks $I_{NaP}$ but has attendant side effects that never lead to a ‘clean’ interpretation of experimental results. The convoluted nature of the experimental results is confusing and had led us to
search for a specific ion channel (and its gene) to perform an unambiguous test of the putative role for $I_{NaP}$ in respiratory rhythm generation and breathing.

We would expect Dbx1 preBötC neurons to express ion channels that give rise to $I_{NaP}$. Sodium channel Na$_{v}$1.6, derived from the gene Scn8a, is the leading candidate Na$_{v}$1.6 because blockade of Na$_{v}$1.6 ion channels abolishes $I_{NaP}$ while transient Na$^+$ currents are unaffected (Osorio et al., 2010; Raman and Bean, 1997). Through immunohistochemistry, I revealed the presence of Na$_{v}$1.6 ion channels in Dbx1 neurons, exhibited in Figures 35-36. These figures also show the presence of Na$_{v}$1.6 ion channels in non-Dbx1 preBötC neurons.

I performed numerous experiments to perfect immunohistochemistry techniques on neonatal mice and subsequently perform immunohistochemistry for Na$_{v}$1.6 ion channels on $Dbx1^{Cre/\times;Scn8a^{\times/}\times;Rosa26^{TdT/\times}}$ mice. Immunohistochemistry experiments on neonatal tissue are known to present more of a challenge than immunohistochemistry on adult tissue but necessary to understand breathing behaviors at early postnatal stages. I began working with a polyclonal antibody published in knockout experiments similar to our goals (Osorio et al., 2010). Polyclonal antibodies increase the likelihood of having a successful experiment because they recognize multiple epitopes of an antigen, thus offering more robust detection. However, when genotyping tails corresponding to mice used for immunohistochemistry experiments, I learned that the polyclonal antibody was continuing to label Na$_{v}$1.6 ion channels in the $Dbx1^{Cre/\times;Scn8a^{\times/};Rosa26^{TdT/\times}}$ knockout mice. This unexpected result can be attributed to the properties of polyclonal antibodies; polyclonal antibody serum
will contain a mixture of antibody subtypes with different affinities and the ability to detect multiple epitopes and fragments of epitopes (Pohanka, 2009). While polyclonal antibodies have their advantages, it was clear I needed to use a more specific monoclonal antibody to appropriately label $Dbx1^{Cre/x};Scn8a^{-/-}$.

Monoclonal antibodies recognize only one epitope of an antigen, thus making them more specific than polyclonal antibodies. I have since chosen an appropriate monoclonal antibody against Na$_v$1.6, but at the time of this writing I am still working to perform immunohistochemistry on a $Dbx1^{Cre/x};Scn8a^{-/-}$ ;$Rosa26^{TdT/x}$ mouse. The images in the results were from mice that did not have a complete Scn8a knockout. I expect I will be able to obtain these results as soon as possible. Nevertheless, the present lack of knockout data does not affect the goal of determining whether Na$_v$1.6 ion channels are expressed in Dbx1 preBötzC neurons and provide a viable target for future physiology experiments.

In this chapter, I show Dbx1 and non-Dbx1 preBötzC neurons express both Na$_v$1.6 and Trpc3 ion channels that mediate $I_{NaP}$ and $I_{CAN}$ respectively. This advance in knowledge can be used to help design targeted experiments to knock down these channels and further explore their role in respiratory rhythm generation and real breathing.
CHAPTER 4: Cellular mechanisms underlying sighs and the potential roles of preBötC neurons and glia

INTRODUCTION

Sighs were once thought to be a reflex caused by hypo-inflation of the lungs, but a study in 1993 suggested that sighs are produced by a centrally mediated mechanism because respiratory rhythmogenic neurons change discharge patterns during sighs (Orem and Trotter, 1993). A few years later, experimental data obtain in vitro, using mouse slice preparations, demonstrated that the preBötC can generate inspiratory breathing rhythms as well as other fictive breathing-related behaviors including sighs and gasps (Lieske, 2000; Ruangkittisakul et al., 2008). Note, that those data in vitro do not preclude the importance of other brainstem or higher brain centers in regulating breathing-related sighs and gasps. Rather, those in vitro data showed that an isolated preBötC can generate the rudiments of those behaviors in vitro. Furthermore, the rate of fictive sighs can be modulated by changes in blood gases, especially during hypoxia (Lieske et al., 2000). Sighs also act as a psychophysiological mechanism that resets eupneic breathing after behaviors characterized by periods of high variability such as speech and laughter (Li and Yackle, 2017; Li et al., 2016; Ramirez, 2014, 2017; Vlemincx et al., 2010).

Sighs are longer, deeper inspiratory breaths that are necessary for proper lung function (Cherniack et al., 1981; Ferris and Pollard, 1960; Koch et al., 2013). Sighs expand the lung beyond tidal volume (i.e., the normal volume traversed
during ordinary inspiratory breaths), which occurs periodically to diminish the surface tension within alveoli that can otherwise lead to alveolar collapse. Alveolar collapse must be avoided because it would preclude gas exchange, the essential function of lung ventilation. Therefore, periodic sighs are important to maintain normal lung function.

Sighs are coupled to emotional responses such as relief and sadness and are often generated when a mammal is transitioning between wakefulness to non-REM sleep and when transitioning between from sleep to arousal (Li and Yackle, 2017; Ramirez, 2014). Malfunctions associated with sighs may be drivers for some diseases including sudden infant death syndrome (SIDS) (Franco et al., 2003; Kahn et al., 1988) and panic disorders (Abelson et al., 2001). With SIDS, victims experience a decrease in spontaneous and induced arousals during sleep (Groswasser et al., 2001; Kahn et al., 1992) and as previously stated the sigh mechanism has a role in successfully transitioning between sleep and arousal. With panic disorders, the affected experience an increase in hyperventilation and breathing variability that persists even when panic itself is pharmacologically controlled (Abelson et al., 2001, 2008). Having a clearer picture of the sigh signaling pathways in the brain may lead to a better targeted approach for disease therapies as well as contribute to our overall understanding of how the brain and CNS generate and control the full range of breathing behaviors, which includes eupnea and sighs all the time in physiologically normal conditions, and sometimes gasps in non-physiological environments with low oxygen availability.
The neuropeptide bombesin, found in frogs, can influence sighs in a range of rodents too (Niewoehner et al., 1983; Ramirez, 2014). Neurons sending signals that evoke sighs are thought to be located in the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) (Li et al., 2016). A subset of neurons in the RTN/pFRG of mice express bombesin-like peptides neuromedin B (NMB) and gastrin releasing peptide (GRP). Microinjection of NMB into the preBötC of anaesthetized rats increased sighs from an average in vivo basal sigh rate of 44 sighs/hour up to 858 sighs/hour (Li et al., 2016). Analogously, microinjection of GRP into the preBötC of anaesthetized rats increased sighs from the basal sigh rate up to 643 sighs/hour (Li et al., 2016). Bilateral injection of NMB receptor (NMBR) antagonist BIM23042, or the GRP receptor (GRPR) antagonist RC3095, in anaesthetized wild type rats transiently reduced sighs by 50% in both cases, and in both cases eupnea remained unperturbed (Li et al., 2016).

In transgenic knockout mice lacking NMBRs, the sigh rate is reduced from the in vivo wild type average basal sigh rate of 40 sighs/hour to an average of 29 sighs/hour while eupnea is unaffected. Comparably, in transgenic knockout mice lacking GRPRs the sigh rate is reduced to half of the wild type basal sigh rate without any change in eupnea (Li et al., 2016).

In vitro application of NMB onto rhythmically active neonatal mouse brainstem slices containing the preBötC doubled the frequency of fictive sighs (~0.01 Hz); likewise, introducing GRP onto mouse brainstem slices containing the preBötC nearly doubles the rate of fictive sighs (1.7 fold increase) suggesting...
GRP and NMB receptors are contained within slices encompassing the preBötC (Li et al., 2016). Neither NMBR null mice nor pharmacological inhibition of NMBRs extinguishes sighs completely, which can be explained by the fact that two different peptidergic systems, NMB- and GRP-mediated, modulate the sigh rhythm (Li et al., 2016).

Additionally, tracing the projections of NMB-expressing and GRP-expressing RTN/pFRG neurons (through transgenic co-expression of NMB or GRP with GFP) in neonatal mice showed that these peptidergic neurons project axons to somatostatin-expressing cells within the preBötC. However, whether those cells in the preBötC were neurons or glia was not addressed directly; the published data and extended data in that study do not resolve their identity (Li et al., 2016). Collectively, these data suggest the presence of NMB and GRP receptors that potently modulate sighs – without affecting eupnea – within the preBötC, but the cellular mechanisms remain unidentified. If Nmb-expressing and Grp-expressing neurons do project to the preBötC, then neurons or glia in the preBötC should express NMB and GRP receptors; this hypothesis which will be tested in this dissertation chapter.

The reason several have speculated on the role of glia (e.g., (Dashevskiy et al., 2016; Ramirez, 2017)) is that sighs in intact animals, and sigh rhythms in vitro, occur at frequencies at least one order of magnitude slower than eupnea in intact animals, as well as inspiratory rhythms measured in breathing models in vitro. Recent evidence suggests that glia in the preBötC may play an instrumental role in sigh generation. Bath application of glial toxin eliminated
sighs without terminating eupnea (Dashevskiy et al., 2016). It is hypothesized that the release of astrocytic ATP is important for sigh generation (Dashevskiy et al., 2016). If this is the case, preBötC glial cells may express NMB or GRP receptors, a hypothesis which will also be tested in this dissertation chapter. Analogous to the motivation behind the research presented in the prior chapter, anatomical evidence for peptide receptors on preBötC neurons or glia will lead directly to physiological experiments to test their role(s) in sigh generation in living animals, which will enhance our understanding of respiratory physiology in humans and all terrestrial mammals.

METHODS

All of the animal protocols were approved by the Institutional Animal Care and Use Committee at The College of William and Mary, which follows the guidelines provided by the US National Institutes of Health Office of Laboratory Animal Welfare (NIH Office of Laboratory Animal Welfare, 2015).

**NMBR and GRPR immunohistochemistry**

*Dbx1^{CreERT2}; Rosa26^{tdTomato}* mice were used for both neonatal and adult experiments. Methods involving mouse genetics, and dissection of neonatal tissue, and fixation of neonatal tissue covered in chapter 1 (See Figure 10, Steps 1, 2, and 4) will be the same for NMBR and GRPR neonatal
immunohistochemistry. Additionally, fixation of neonatal brainstem-spinal cord preparations, transcardial perfusion of adult mice and subsequent fixation were performed in the same manner as described in chapter 3. Brainstem-spinal cord preparations were rinsed in 1X phosphate buffered solution (PBS) (BP399-1, Fisher Scientific, Hampton, NH) for at least 30 minutes after overnight fixation. The brainstem-spinal cord preparations were then placed in 4% low melting point agar for sectioning with a vibratome (Thermo Scientific Microm HM 650 V, Waltham, MA). Transverse 50-60 μm sections were cut from the preBötC region of the brainstem. Neonatal sections were incubated with 20% normal donkey serum (50-413-367, Fisher Scientific, Hampton, NH) overnight at 4°C on an orbital shaker. The following day, the slices were washed with 1X PBS 2 x 30 min and were incubated in 1:500 rabbit anti-Neuromedin B Receptor antibody (ABR-004, Alomone labs, Jerusalem) or 1:500 rabbit anti-Gastrin Releasing Peptide Receptor antibody (ABR-002, Alomone labs) and 1:250 goat anti-GFAP antibody (ab53554, Abcam) with 0.4% TritonX-100 overnight at 4°C on an orbital shaker. Sections were then washed 4 x 30 minutes with 1X PBS and placed in 1:200 donkey anti-rabbit Alexa 405 (711-475-152 Jackson ImmunoResearch Labs, West Grove, PA) and 1:500 donkey anti-goat Alexa 647 (705-605-147 Jackson ImmunoResearch Labs) for one hour. After washing for a minimum of 6 x 30 min with 1X PBS, sections were mounted on glass slides and cover-slipped with Vectashield hardset mounting medium (H-1500; Vector Laboratories, Burlingame, CA). Images were acquired with a 10x air and 60x oil immersion.

RESULTS

*preBötC neurons express GRP receptors*

Visualization of immunohistochemistry against GRP receptors and glial marker GFAP in both neonatal and adult *Dbx1\textsuperscript{CreERT2} \cdot \textit{Rosa26}\textsuperscript{tdTomato*} mouse brain slices revealed that GRP receptors are expressed in Dbx1 and non-Dbx1 preBötC neurons in neonatal mice and are not expressed in glia, as demonstrated in Figures 37 and 38.
Figure 37. GRP receptors on Dbx1 and non-Dbx1 preBötC neurons in neonatal mice. Dbx1 neurons are labeled with tdTomato (A). Immunohistochemistry for the glial marker GFAP (B) GRP receptors (C) are combined with tdTomato labeling (D). Filled yellow triangles designate examples of GRP receptors on tdTomato neurons. White triangles designate examples of GRP receptors on non-Dbx1 neurons. White diamonds designate examples of glia labeling, none of which overlap with GRP receptor labeling. Scale bar is 20 µm.
Figure 38. GRP receptors not present on glia in preBöC of neonatal mice. Dbx1 neurons are labeled with tdTomato (A). Immunohistochemistry for the glial marker GFAP (B) GRP receptors (C) are combined with tdTomato labeling (D). Filled yellow triangles designate examples of GRP receptors on tdTomato neurons. White triangles designate examples of GRP receptors on non-Dbx1 neurons. White diamonds designate examples of glia labeling, none of which overlap with GRP receptor labeling. Scale bar is 20 µm.
Figure 39 shows GRP receptor expression in preBötC neurons persists into adulthood while glia still do not express GRP receptors.

![Figure 39](image)

Figure 39. GRP receptors on Dbx1 and non-Dbx1 neurons in adult mice. Dbx1 neurons are labeled with tdTomato (A). Immunohistochemistry for the glial marker GFAP (B) GRP receptors (C) are combined with tdTomato labeling (D). Filled yellow triangles designate examples of GRP receptors on tdTomato neurons. White triangles designate examples of GRP receptors on non-Dbx1 neurons. White diamonds designate examples of glia labeling, none of which overlap with GRP receptor labeling. Scale bar is 20 µm.
preBötC neurons express NMB receptors

Immunohistochemistry against NMB receptors and glial marker GFAP in both neonatal and adult $Dbx1^{CreERT2}; Rosa26^{tdTomato}$ mouse brain slices revealed that NMB receptors are expressed in Dbx1 and non-Dbx1 preBötC neurons in neonatal mice and are not expressed in glia, as demonstrated in Figure 40. Figure 41 shows NMB receptor expression in preBötC neurons persists into adulthood while glia still do not express NMB receptors.
Figure 40. NMB receptors on Dbx1 and non-Dbx1 neurons in neonatal mice. Dbx1 neurons are labeled with tdTomato (A). Immunohistochemistry for the glial marker GFAP (B) NMB receptors (C) are combined with tdTomato labeling (D). Filled yellow triangles designate examples of NMB receptors on tdTomato neurons. White triangles designate examples of NMB receptors on non-Dbx1 neurons. White diamonds designate examples of glia labeling, none of which overlap with NMB receptor labeling. Scale bar is 20 µm.
Figure 41. NMB receptors on Dbx1 and non-Dbx1 neurons in adult mice. Dbx1 neurons are labeled with tdTomato (A). Immunohistochemistry for the glial marker GFAP (B) NMB receptors (C) are combined with tdTomato labeling (D). Filled yellow triangles designate examples of NMB receptors on tdTomato neurons. White triangles designate examples of NMB receptors on non-Dbx1 neurons. White diamonds designate examples of glia labeling, none of which overlap with NMB receptor labeling. Scale bar is 20 µm.
DISCUSSION

The preBötC has been established as the site of eupneic respiratory rhythm generation. Investigation of the circuits for other respiratory behaviors such as sighs and gasps suggest the preBötC is an integral part of these behaviors as well. In 2000, recordings from the preBötC generated eupnea, sighs, and gasps – three distinct respiratory activity patterns (Lieske et. al., 2000). Sigh bursts in vitro are physiologically distinct from eupneic bursts due to their larger amplitude and doublet peak. Both eupneic breaths and sighs must occur on a regular, periodic basis to sustain life (Cherniack et al., 1981; Ferris and Pollard, 1960; Koch et al., 2013); sighs prevent the collapse of alveoli, re-inflate alveoli that have collapsed, and help maintain normal lung function. Sighs are also proposed to reset eupneic breathing variability (Li and Yackle, 2017; Li et al., 2016; Ramirez, 2014, 2017; Vlemincx et al., 2010). Breathing variability is a normal and necessary occurrence, allowing the respiratory network to adapt to environmental changes and behaviors such as speech and laughter. Sighs terminate periods of irregular breathing and facilitate the return of breathing homeostasis. Mice that do not sigh due to genetic engineering develop fatal lung problems (Koch et al., 2013). Bombesin-like receptors NMBR and GRPR are important in the sigh signaling pathway (Li et al., 2016). We show that NMB and GRP receptors are present on Dbx1 and non-Dbx1 preBötC neurons and are not expressed on glia.
Sigh bursts have been recorded *in vitro* from the preBöC along with eupneic bursts (Lieske et. al., 2000), but the underlying mechanisms and possible microcircuits governing sighs are not yet clearly defined. One explanation for the generation of distinct breathing behaviors being generated by the same neural microcircuit is network reconfiguration; the preBöC can adopt different network states as a result of oxygen levels and neuromodulators present (Lieske et. al., 2000). Neural networks can generate several rhythmic neuronal activities with different timescales; this theory makes it possible for the preBöC to generate both eupnea and sighs. In fact, *in vitro* experimental data further suggests that the preBöC is involved in the generation of three distinct fictive breathing behaviors: eupnea, sighs, and gasps (Lieske et. al., 2000). The data provided in this chapter further corroborates this concept.

*In vivo*, eupnea occurs at a rate of 117 breaths/ne in rats and 218 breaths/hour in mice while sighs occur at an average rate of 44 sighs/hour in rats and 22 sighs/hour in mice (Li et al., 2016); thus, sighs occur at a relatively low frequency when compared to eupnea in both rats and mice. Glial modulation of breathing has been proposed as a theory to explain the different periodicities between eupneic bursts and sigh bursts because glia related behaviors, especially those involving Ca$^{2+}$ signals, operate on a slower timescale than neuronal behaviors (Parpura et al., 2012). Also, Ca$^{2+}$ signals in glial cells were observed to be synchronous with sigh bursts and glial toxins inhibited fictive sighs without altering eupneic bursts (Dashevskiy et al., 2016). Since NMB and GRP are shown to induce sigh bursts and neurons expressing NMB and GRP
project to the preBötC, one logical speculation was that glial cells would express NMB or GRP receptors. However, my anatomical data demonstrate that preBötC neurons, not glia, express NMB or GRP receptors. The presence of sigh NMB and GRP peptide receptors on preBötC neurons provide anatomical evidence that preBötC neurons, not glia, are part of the sigh signaling pathway. Those data definitively rule-out any sigh-generating mechanism that depends on peptidergic signaling to glia in the preBötC. This discovery also provides a basis for further experimentation though physiology to target NMB and GRP receptors in preBötC neurons to elucidate their functional role in sigh behavior.

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