Role of Dbx1-Derived Pre-Bötzinger Complex Interneurons in Breathing Behaviors of Adult Mice

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Role of Dbx1-derived pre-Bötzinger complex interneurons in breathing behaviors of adult mice

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A dissertation presented to the Graduate Faculty of The College of William and Mary in Candidacy for the Degree of Doctor of Philosophy

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ABSTRACT

Breathing is a rhythmic motor behavior essential to sustain homeostasis and life itself in humans and all terrestrial mammals. A specialized neuronal network is responsible for generating and controlling the rhythm and pattern for breathing. The core rhythm-generating microcircuit in particular is located within a site dubbed the preBötzinger complex (preBötC). The preBötC is a heterogeneous region containing neurons with both respiratory and non-respiratory activity that express excitatory and inhibitory transmitters, peptide transmitters, and peptide receptors. More recently, preBötC neurons have been characterized by molecular genetics. The excitatory transmitter phenotype, and peptide and peptide receptors, commonly used to define the respiratory core oscillator within the preBötC are properties associated with neurons whose precursors express the embryonic transcription factor, developing brain homeobox 1 (Dbx1). Our lab, and our French colleagues, hypothesized that neurons derived from the Dbx1-expressing precursor cells (Dbx1 neurons) form the core microcircuit for inspiration breathing rhythm, that is, the Dbx1 core hypothesis. Evidence from many labs supports the Dbx1 core hypothesis at embryonic and neonatal stages of development. However, the role of Dbx1 neurons in adult animals remains incompletely understood. Furthermore, contemporary data suggests the portfolio of functions for brainstem Dbx1 neurons includes premotor and arousal-related functions, which casts doubt on the veracity of the Dbx1 core hypothesis. Here I investigate the role of Dbx1 neurons in adult animals with intact sensorimotor integration systems using intersectional mouse genetics to express light-responsive membrane proteins to excite or depress Dbx1 neurons while simultaneously measuring breathing. Using these light-sensitive proteins to manipulate Dbx1 neuron function to depress or stop breathing, enhance breathing, and alter the precise timing of inspiratory breaths, I offer evidence that affirms the Dbx1 core hypothesis. I conclude that Dbx1 preBötC neurons are essential for breathing and form the respiratory core oscillator in adult mice. Knowing the cellular point of origin for breathing behavior gives us a target to study the cellular and synaptic mechanisms to this key physiological behavior and provides general insight into rhythmic networks and physiological brain function.
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PREFACE

In this dissertation provide evidence that identifies the molecular genetic neuron class at the point of origin of an essential behavior, breathing. I will show that these neurons comprise the circuit that provides its underlying rhythm. The core of the breathing neural generation and control system is located within a heterogenous, functionally and anatomically defined, region located in the ventral medulla oblongatta, the preBötzinger complex (preBötC). The preBötC is essential for providing the breathing rhythm and contains the core of the respiratory oscillator (Smith et al., 1991). Within this region a subset of interneurons derived from the homeobox gene Dbx1-expressing precursors (Dbx1 neurons) were proposed to form the respiratory core oscillator and are essential for breathing in neonates (Bouvier et al., 2010; Gray et al., 2010; Wang et al., 2014). However, at the outset of these studies (i.e., which collectively make up this dissertation) the role of Dbx1 neurons in adults was unknown. Given that neural development in perinatal and juvenile mammals can dramatically modify brain microcircuits and their attendant behavioral expressions, this lack of knowledge represented a major lacuna in the field of respiratory neurobiology, and one I endeavored to rectify.

Included in this dissertation are two chapters that aim to answer the same question: Do Dbx1 neurons comprise the core oscillator of the respiratory neural control system (i.e., the Dbx1 core hypothesis)?

In Chapter 1, I test the role of Dbx1 neurons by functionally diminishing their putative contribution to breathing rhythm by diminishing their activity in vivo. I use
the optogenetic construct Archaerhodopsin (Arch) to attempt to silence Dbx1 neurons in adult mice. I first demonstrate that Arch activation can hyperpolarize Dbx1 neurons \textit{in vitro} and that hyperpolarization is based on predominately on postsynaptic mechanisms, that is, my optogenetic perturbation acts on the neurons of interest and not on inputs to the preBötC microcircuits from other (remote) sites. Performing this experiment in adult mice in both sedated and awake states, I show significant decreases in breathing frequency, as well as standard metrics for respiratory physiology including minute ventilation and tidal volume in response to Arch-mediated silencing of Dbx1 neurons. Furthermore, in sedated animals, Arch activation \textit{in vivo} induced periods of apnea. However, in awake animals no periods of apnea were induced. These data support the Dbx1 core hypothesis and provide evidence that Dbx1 neurons are necessary for breathing in adult mice.

The findings and conclusions of Chapter 1 (published in 2016) were further supported by reports from colleagues and competitors, working concurrently on the same problem, and published during that year. For example, Koizumi and coauthors showed that light pulses applied with graded intensity, activated Arch and had a graded effect on fictive breathing using reduced breathing models, both \textit{in situ} and \textit{in vitro} (Koizumi et al., 2016). Additionally, using channelrhodopsin, Cui and coauthors showed that photostimulating Dbx1 neurons increased breathing in terms of amplitude and frequency of individual inspiratory breaths, and could induce inspiration before an endogenous breath was expected, i.e., a phase advance of the breathing cycle (Cui et al., 2016).
These three studies (including mine, Cui et al., 2016; Koizumi et al., 2016; Vann et al., 2016) provided compelling evidence for the role of Dbx1 neurons as the respiratory core oscillator, thus favoring the Dbx1 core hypothesis. Nevertheless, in Cui et al. and later Yackle et al. uncertainty regarding the role of Dbx1 neurons was also brought to the front. Cui and coauthors showed that neurons expressing somatostatin (Sst), which are predominately Dbx1 derived (~91-93%) within the preBötC (Bouvier et al., 2010; Gray et al., 2010) are related to motor pattern formation as opposed to rhythm generation per se (Cui et al., 2016). Although Sst neurons are predominately Dbx1 derived, they make up only a small fraction of the total Dbx1 population in the preBötC (~13-17%) (Bouvier et al., 2010; Gray et al., 2010). Therefore, premotor function of Sst-expressing, Dbx1-derived neurons alone would not preclude non-Sst-expressing, Dbx1-derived neurons from forming the respiratory core oscillator microcircuit, because by the statistics above some 83-87% of non-Sst, Dbx1 neurons could be dedicated to rhythm, whereas 13-17% of Sst-expressing Dbx1 neurons remain pattern-focused.

Quite apart from rhythm and pattern, a subset of Dbx1 neurons expressing cell adhesion molecule cadherin-9 (Dbx1/Cdh9 neurons) were shown to have a role in general arousal (Yackle et al., 2017). Of Dbx1 preBötC neurons, ~56% express Cdh9. The Dbx1/Cdh9 neurons do not generally discharge bursts in sync with respiratory rhythm, and when they do, those bursts are much weaker than typically associated with rhythmogenic Dbx1 preBötC neurons (Gray et al., 2010; Picardo et al., 2013). Furthermore, Dbx1- and Sst-coexpressing as well as
Dbx1- and Cdh9-coexpressing neurons have no overlap (Yackle et al., 2017). These two populations account for almost three-quarters of Dbx1 neurons within the preBötC. If a substantial majority of Dbx1 preBötC are dedicated to motor pattern or arousal functionality, then the Dbx1 core hypothesis would appear to be falsified. Therefore, I deemed it necessary to reinvestigate the role of Dbx1 neurons to definitively determine if they provided rhythmogenic function for respiration, or if my initial foray was somehow spurious.

In Chapter 2, I use improved optogenetic technologies to photoinhibit or photostimulate Dbx1 neurons to test the Dbx1 core hypothesis. I first show that photoinhibition can silence Dbx1 neurons in vitro based on predominately postsynaptic mechanisms. I then used graded long pulse (5s) photoinhibition and photostimulation to alter breathing in sedated and awake mice. Using adult mice in both sedated and awake states I show significant changes in breathing frequency. Photoinhibition induced apnea in an awake animal, unrestrained with fully intact sensorimotor systems. Furthermore, we used transient light pulses (100 ms) to reset the respiratory rhythm and assess-phase dependent changes in breathing due to perturbation of the putative Dbx1 core oscillator microcircuit.

Although, Dbx1 neurons have proposed roles in pattern forming and arousal circuits this does not preclude a rhythmogenic role in addition. In Chapters 1 and 2 I present evidence that Dbx1 neurons are respiratory rhythmogenic. Specifically, changes in frequency and respiratory resets that can only be attributed to manipulating the core microcircuit. If premotor, arousal, and
rhythmogenic circuits are unique populations is undetermined. However, it is clear that Dbx1 neurons have an essential role in respiratory rhythmogenesis.

References


CHAPTER 1. Transient suppression of Dbx1 preBötzinger interneurons disrupts breathing in adult mice

1.1 Abstract

Interneurons derived from Dbx1-expressing precursors located in the brainstem preBötzinger complex (preBötC) putatively form the core oscillator for inspiratory breathing movements. We tested this Dbx1 core hypothesis by expressing archaerhodopsin in Dbx1-derived interneurons and then transiently hyperpolarizing these neurons while measuring respiratory rhythm in vitro or breathing in vagus-intact adult mice. Transient illumination of the preBötC interrupted inspiratory rhythm in both slice preparations and sedated mice. In awake mice, light application reduced breathing frequency and prolonged the inspiratory duration. Support for the Dbx1 core hypothesis previously came from embryonic and perinatal mouse experiments, but these data suggest that Dbx1-derived preBötC interneurons are rhythmogenic in adult mice too. The neural origins of breathing behavior can be attributed to a localized and genetically well-defined interneuron population.

1.2 Introduction

Central pattern generator (CPG) networks produce neural activity that underlies rhythmic motor behaviors such as walking, swimming, chewing, and breathing. The CPG for inspiratory breathing movements resides in the preBötzinger Complex (preBötC) of the ventral medulla (Feldman et al., 2013; Smith et al., 1991), but its cellular composition in adult mammals remains incompletely understood.
Efforts to classify the cellular core of the preBötC have focused on peptide and peptide receptor-expressing, as well as glutamatergic, brainstem interneurons (Gray et al., 1999, 2001; Guyenet et al., 2002; Stornetta et al., 2003a; Tan et al., 2008; Wallen-Mackenzie et al., 2006; Wang et al., 2001). Silencing or killing peptide and peptide receptor-expressing neurons causes severe respiratory pathology as well as long-lasting apnea in adult rats (Gray et al., 2001; McKay et al., 2005; Tan et al., 2008). In addition, excitatory synaptic communication mediated by AMPA receptors is essential for rhythmogenesis and respiratory motor output in in vitro breathing models (Funk et al., 1993; Greer et al., 1991). Mice lacking the vesicular glutamate transporter VGLUT2 fail to breathe, even though the preBötC forms, because its constituent rhythmogenic neurons do not activate and synchronize (Wallen-Mackenzie et al., 2006).

These competing classification schemes may converge in one genetic class of brainstem interneurons whose precursors express the homeodomain transcription factor Dbx1 (hereafter referred to as Dbx1 neurons). When studied at perinatal stages of development, Dbx1 preBötC neurons express the same peptides and peptide receptors described above and are overwhelmingly glutamatergic. The commissural axons of Dbx1 preBötC neurons synchronize embryonic respiratory rhythms, and Dbx1 knock-out mice die at birth of asphyxia (Bouvier et al., 2010; Gray et al., 2010; Pierani et al., 2001). Moreover, the selective laser ablation of Dbx1 preBötC neurons in a neonatal slice model of breathing degrades and decelerates inspiratory-related motor output until irreversible rhythm cessation (Wang et al., 2014). Therefore, we, and others,
proposed the Dbx1 core hypothesis (Bouvier et al., 2010; Gray et al., 2010; Picardo et al., 2013), which posits that Dbx1 neurons comprise the core CPG for inspiratory breathing movements. As recounted above, accumulating evidence suggests that Dbx1 preBötC neurons are rhythmogenic at perinatal stages of development. Regarding their role in adults, Koizumi et al. (Koizumi et al., 2016) transiently inhibited Dbx1 preBötC neurons in rhythmically active in situ preparations from adult mice and reported changes in the frequency of respiratory motor output corresponding to the strength of optogenetic inhibition. Further, using vagus-intact adult mice in vivo, Cui et al. found that stimulating Dbx1 preBötC neurons via channelrhodopsin could evoke inspiratory motor bursts during the expiratory cycle (Cui et al., 2016). These in situ cell-silencing experiments (Koizumi et al., 2016) coupled with the in vivo stimulation experiments (Cui et al., 2016) further support the Dbx1 core hypothesis. Nevertheless, an important test yet to be performed is to silence or diminish the function of Dbx1 preBötC neurons in intact adult mice.

Here we test the Dbx1 core hypothesis by activating the proton pump archaeorhodopsin-3 (Arch) in Dbx1 interneurons while observing breathing behavior in vagus-intact adult mice as well as in vitro models of the behavior. Photoinhibition impedes fictive breathing, and breathing movements, up to and including complete cessation of the (fictive) behavior. Whereas Dbx1-derived interneurons were previously studied in the context of embryonic and early neonatal development, these results provide additional evidence that Dbx1 preBötC neurons are rhythmogenic in adult mice as well. Therefore, we now
understand both the site (preBöTC) for inspiratory rhythm generation and have further confidence regarding the neuron class (Dbx1-derived) responsible for rhythmogenesis in adult as well as perinatal rodents.

1.3 Materials and Methods

**Mice**

The Institutional Animal Care and Use Committee at the College of William and Mary approved these protocols. We used female mice that express Cre recombinase fused to a tamoxifen-sensitive mutant form of the human estrogen receptor (CreER\(^{T2}\)) in cells that express Dbx1, i.e., Dbx1\(^{CreERT2}\) (Hirata et al., 2009). These mice were mated with male Ai35D reporter mice whose Rosa26 locus was modified by targeted insertion of a LoxP-flanked STOP cassette followed by a fusion gene coding for Arch and enhanced green fluorescent protein (EGFP) (Madisen et al., 2012). Tamoxifen was administered (22.5 mg/kg) to pregnant dams at embryonic day 9.5 (i.e., E9.5), which resulted in Arch-EGFP expression in Dbx1 neurons of their Dbx1\(^{CreERT2}\);Ai35D offspring.

**Respiratory active transverse slice preparations**

Neonatal Dbx1\(^{CreERT2}\);Ai35D pups (postnatal days 0-4) were anesthetized by hypothermia and decerebrated. Mice were then dissected in 4\(^\circ\) C artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.5 CaCl\(_2\), 1 MgSO\(_4\), 25 NaHCO\(_3\), 0.5 NaH\(_2\)PO\(_4\), and 30 dextrose. The aCSF was aerated continuously with carbogen (95% O\(_2\) and 5% CO\(_2\), pH 7.4). We removed the neuraxis, glued it to an agar block, and then cut 500-µm-thick transverse slices whose rostral surface exposed the border of the preBöTC (Ruangkittisakul et al.,
Slices were anchored in a recording chamber on a fixed-stage microscope and perfused with aCSF at 27° C at 2 ml·min⁻¹. We recorded inspiratory-related motor output from hypoglossal (XII) nerve rootlets using a differential amplifier (gain 2000x) and a band-pass filter (300-1000 Hz). Nerve output was full-wave rectified and smoothed for display. Extracellular K⁺ in the aCSF was elevated to 9 mM to sustain robust rhythm and motor output (Funk and Greer, 2013; Ruangkittisakul et al., 2006).

We identified Dbx1 neurons by membrane-bound native EGFP expression (which does not fill the cytosol) and performed whole-cell patch-clamp recordings under visual control. Patch pipettes with tip resistance of 4-6 MΩ were fabricated from capillary glass (1.50 mm outer diameter, 0.86 mm inner diameter) and filled with solution containing (in mM): 140 potassium gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Na₃-GTP. Alexa 568 hydrazide dye was added to the patch-pipette solution (50 µM, Invitrogen, Carlsbad, CA). Membrane potential was amplified (100x) and low-pass filtered (1 kHz) using a current-clamp amplifier (Dagan IX2-700, Minneapolis, MN) before being digitally acquired at 4 kHz (PowerLab 4/30, AD Instruments, Colorado Springs, CO).

**Surgery for optical fiber implantation**

We anesthetized adult mice (aged 8-20 weeks) via intraperitoneal injection of ketamine (100 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹) and performed aseptic surgeries in a stereotaxic frame. After exposing the skull, we performed two 0.5-mm-diameter bilateral craniotomies in the range 6.95 to 7.07 mm posterior to bregma and 1.15 to 1.5 mm lateral to the midline suture. In control animals,
craniotomies were performed 0.25 to 1.0 mm rostral to preBöC targeted locations. We joined 1.27-mm-diameter ceramic ferrules (Precision Fiber Products, Milpitas, CA) with 105-µm-diameter 0.22 numerical aperture (NA) multimode fibers (Thorlabs, Newton, NJ) and implanted them 4.95 to 5.10 mm deep for preBöC experiments and 2.75 to 4.25 mm deep for control. Implants were secured using a cyanoacrylate adhesive (Loctite 3092, Westlake, OH) and anchored with a screw. Wounds were closed with a suture and tissue adhesive. The ferrule-fibers were connected to a 200-mW, 589-nm diode-pumped solid-state laser (Dragon Lasers, Chang Chun, China) using a line splitter and fiber coupler (OZ Optics, Ottawa, Canada). We injected ampicillin (4 mg·kg⁻¹) and ketoprofen (125 mg·kg⁻¹, s.c.) following surgery, and again 24 hr later, to manage pain and prevent infection. Mice recovered a minimum of ten days before further experimentation.

**Breathing measurements**

After anesthetizing mice using 2% isoflurane we connected their ferrules to the 589-nm laser. Mice recovered from anesthesia for ~1 hr before we measured breathing behavior. Awake mice were placed unrestrained in a whole-body plethysmograph (Emka Technologies, Falls Church, VA). In a separate session, these same mice were lightly sedated via intraperitoneal ketamine injections (25 mg·kg⁻¹ minimum dose) and titrated as needed to reduce limb movements but not abolish toe pinch and blink reflexes with a maximum aggregate dose of 50 mg·kg⁻¹. Mice were then fitted with a nose cone (SOMNO-0801, Kent Scientific, Torrington, CT) for breathing measurements.
We applied a circuit of positive pressure, with balanced vacuum, to continuously flush the plethysmograph or nose cone with breathing air. A 1-liter respiratory flow head and differential pressure transducer (Spirometer, AD Instruments) measured airflow in all cases. Analog breathing signals were digitized at 1 kHz (PowerLab 4/30).

Bouts of illumination (2 s in duration) were applied during periods of restful wake over a 1-hr recording period. If a subject moved during a 5-s time window preceding, succeeding, or during the 2-s illumination phase, then these data were not analyzed to exclude movement-related artifacts superimposed in the breathing pattern. Light intensity measured 15 mW at the tissue contact point. Given the wavelength of light (589 nm), the diameter (105 µm) and NA (0.22) of the optical fiber, as well as an estimated distance of 0.75 mm from the fiber tip to distal edge of the preBötC (determined from histological sections), we used light dispersal and tissue scattering formulae (Aravanis et al., 2007; Yizhar et al., 2011) to calculate that preBötC neurons experienced an irradiance of not less than 12 mW·mm⁻². Whole-cell recordings from hippocampal pyramidal neurons in mouse brain slices showed that 10 mW·mm⁻² activates 60% of the available Arch-mediated outward current and evokes 83% of the maximum Arch-inducible hyperpolarization (Madisen et al., 2012). Those data, in combination with measurements and calculations above, suggest that our protocols deliver sufficient light to Dbx1 preBötC neurons to evoke close to saturating levels of Arch-mediated outward current.
**Histology**

After the experiments we administered a lethal dose of pentobarbital (100 mg·kg⁻¹, i.p.) to adult mice, which were then transcardially perfused with 0.1 M phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The neuraxes were removed and post-fixed overnight in 4% paraformaldehyde, and later sliced in 50-µm contiguous transverse sections. Free-floating sections were stained using 1% thionin acetate solution for 1 min, rinsed in distilled water, and finally washed in successive ethanol baths (2 min in 50% EtOH, 1 min in 75% EtOH, and then 2 min in 50% EtOH twice). Slices were mounted on gelatin coated slides and dehydrated using four graded ethanol baths (3 min in 70% EtOH, 5 min in 90% EtOH, and 5 min in 100% EtOH twice), cleared using four xylenes immersions (one for 30 s followed by three for 5 min), and then cover-slipped using DPX (Sigma-Aldrich, St. Louis, MO). Tissue sections were visualized using bright-field microscopy. Images were arranged as mosaics and brightness and contrast were adjusted uniformly across the entire ensemble image using the public domain software package ImageJ (Schneider et al., 2012).

**Data analysis**

The airflow signal was band-pass filtered (0.1 to 20 Hz) and analyzed using LabChart 7 software (AD Instruments). From filtered airflow traces using the spirometry module in LabChart 7 we calculated tidal volume ($V_T$), inspiratory duration ($T_i$), and respiratory rate ($f_R$). Minute Ventilation (MV) was calculated automatically by LabChart 7 by multiplying the average $V_T$ and $f_R$ within a 0.5-s
rolling time window. We employed a 0.5-s time window centered on peak inspiratory airflow to compute cycle-triggered averages (CTA) prior to and during bouts of light presentation. Peak airflow was detected via a local maxima-detecting algorithm and CTAs were calculated using algorithms built into LabChart 7. We computed statistics using Graphpad Prism 6 (La Jolla, CA) and prepared figures using Adobe Illustrator (Adobe Systems Inc., San Jose, CA) as well as IGOR Pro 6 (Wavemetrics, Lake Oswego, OR). Group data are reported as mean ± standard deviation (SD). We employed the Friedman Test, which is a non-parametric statistical test comparable to a one-way ANOVA, for statistical hypothesis testing.

1.4 Results

Light-evoked hyperpolarization of Dbx1 preBötC neurons interrupts inspiratory rhythm and motor output in vitro

Transverse medullary slices from neonatal mice that retain the preBötC spontaneously generate inspiratory rhythm and XII motor output. Dbx1-CreERT2;Ai35D mouse slices show native EGFP expression continuously from the XII nucleus dorsomedially, through the intermediate reticular formation and preBötC, to the ventrolateral border (Fig. 1.1A). We recorded Dbx1 neurons ventral to the semi-compact division of the nucleus ambiguus and orthogonal to the dorsal boundary of the principal loop of the inferior olive (Fig. 1.1B), which coincides with the rostral face of the preBötC (Ruangkittisakul et al., 2014a). Exposure to 589-nm light hyperpolarized the baseline membrane potential of five
Dbx1 preBötC neurons by 5.6 ± 1.8 mV, which persisted in the presence of 1 µM tetrodotoxin (TTX) (Fig. 1.1C,D).

In contrast, light exposure had a negligible impact on the baseline membrane potential of six non-Dbx1 preBötC neurons that showed either expiratory (Fig. 1.1).

**Figure 1.1** Light activation of Arch-expressing Dbx1 preBötC neurons hyperpolarizes Dbx1 neurons and precludes respiratory rhythm. A, Rostral slice surface of a P2 Dbx1CreERT2;Ai35D mouse showing Arch-GFP expression. Dotted box marks the preBötC. B, Dodt image of the slice in A showing location of the preBötC relative to known anatomical markers, the principal loop of the inferior olive (IOP_loop) and semicompact division of the nucleus ambiguus (scNA). The scale bar represents 150 µm and applies to both A and B. C, Inspiratory Dbx1 neuron visually identified by membrane-delimited EGFP expression (top), Dodt contrast microscopy (middle), and by dialysis of Alexa Fluor 568 introduced via the patch pipette solution after the onset of whole-cell recording (bottom). Scale bar represents 10 µm. D, Membrane potential trajectory of the neuron in C with synchronous XII output. TTX was applied at 1 µM. Voltage and time calibrations are shown E, A non-Dbx1 neuron lacking EGFP expression (top), identified in Dodt contrast microscopy and via Alexa Fluor 568 dialysis (bottom). Scale bar represents 10 µm. F, Membrane potential trajectory of the non-Dbx1 preBötC neuron in E with synchronous XII output. G, Membrane potential trajectory of a non-Dbx1 neuron with inspiratory modulation. Voltage calibration in D applies to F and G; separate time calibrations are shown. Yellow bars in D, F, and G indicate 589-nm light applications.
1.1E,F) or non-respiratory firing patterns, as well as non-Dbx1 preBöC neurons with evidence of inspiratory modulation (Fig. 1.1G). The average change in baseline membrane potential for all non-Dbx1 neurons measured $-0.68 \pm 0.3$ mV.

Illumination of the preBöC in 30-s bouts generally stopped XII output (26 total bouts in four slices). However, a single attenuated XII burst occurred in the last 10 s of the 30-s bout in four instances across three slices (e.g., Fig. 1.1D). These results indicate that activation of Arch hyperpolarizes Dbx1 preBöC neurons not via network disfacilitation but rather direct postsynaptic effects.

In neonatal mouse slices, the majority of Dbx1 preBöC neurons are rhythmogenic, expect perhaps those that express the peptide transmitter somatostatin (Cui et al., 2016). A subset of Dbx1 neurons at the dorsal edge of the preBöC have premotor functionality (Revill et al., 2015; Song et al.), but far more XII premotor neurons are located in the intermediate reticular formation, adjacent dorsally to the preBöC (Koizumi et al., 2008; Revill et al., 2015). Therefore, in the present context, bilateral illumination of the preBöC most likely stops rhythmic XII output via suppression of rhythmogenesis rather than premotor blockade.

**Arch-mediated inhibition in Dbx1 preBöC neurons suppresses breathing in anesthetized and sedated mice**

We examined Arch-EGFP expression in adult $Dbx1^{CreERT2, AI35D}$ mice, which recapitulated the pattern characterized by Dbx1-reporter expression in developing embryos and neonates (Bouvier et al., 2010; Gray et al., 2010;
Ruangkittisakul et al., 2014a). Viewed in transverse sections, Dbx1-derived cells form an inverted V-shaped pattern extending from the XII nucleus dorsomedially to the ventrolateral border of the section, which incorporates the intermediate reticular formation and the ventral respiratory column (Fig. 1.2A). The position of the preBötC can be determined from anatomical markers including the principal loop of the inferior olive (IO\textsubscript{loop}), the semi-compact division of the nucleus ambiguus (scNA), and the shallow U-shape of the fourth ventricle, which indicates proximity to the obex (Fig. 1.2B, B') (Franklin and Paxinos, 2013).

**Figure 1.2** Arch-EGFP expression and histology of fiber-optic implants in adult \(D\text{bx}1\text{CreERT2};\text{Ai35D}\) mice. A, EGFP expression in 35 week-old \(D\text{bx}1\text{CreERT2};\text{Ai35D}\) mouse. Scale bar represents 500 µM. A\textquoteright, Inset of boxed region in A showing an expanded view of the ventral region of the slice, which includes the preBötC. Scale bar represents 250 µM. B, Bright field image of a thionin-stained section adjacent to A. Scale bar represents 500 µM and applies to B, C, and D. B\textquoteright, Inset of boxed region in B showing an expanded view of the ventral region of the slice, which shows visible markers that co-locate with the preBötC including the semicompact division of the nucleus ambiguus (scNA) and the principal loop of the inferior olive (IO\textsubscript{loop}). Scale bar represents 250 µM. C, Bright field images of adjacent thionin-stained sections from an experimental mouse whose fiber-optics and ferrules targeted the preBötC. D, Bright field image of thionin-stained section from an experimental mouse whose fiber-optics and ferrules targeted medullary circuitry dorsal and rostral to the preBötC.
We implanted fiber optics bilaterally to activate Arch in Dbx1 preBöC neurons in vagus-intact adult Dbx1^{CreERT2};Ai35D mice (positions confirmed post-hoc, e.g., Fig. 1.2C). Control littermates had fiber optics implanted dorsally in the medulla at a position rostral to the preBöC (e.g., Fig. 1.2D). Immediately after implantation we delivered 589-nm laser pulses (30 s) while visually monitoring breathing.

![Figure 1.3](image_url)  
**Figure 1.3** Activation of Arch in Dbx1 preBöC neurons in sedated mice. A, Airflow, V_t, MV, and f_R plotted continuously during two consecutive 2-s light pulses. Inspiratory airflow is plotted upward, which reflects nose-cone measurements. A_1, A_2, Expanded airflow traces from A. Arrowhead in A_2 indicates light-evoked interruption of the inspiratory phase. Yellow bars indicate 589-nm light application. B_1, B_2, Cycle-triggered averages of airflow from each bout prior to (not highlighted) and during illumination (highlighted). Time calibrations are shown for each panel.

Anesthetized mice whose ferrules were implanted in the preBöC transiently stopped ventilation for intervals of approximately 18 s (n=6), whereas breathing
remained unperturbed in control mice with ferrules in the dorsal medulla (n=7).

After ten days of recovery, we measured breathing via a nose cone in six lightly ketamine-sedated \( Dbx1^{CreERT2};Ai35D \) mice. Prior to preBötC illumination, these mice breathed at a \( f_R \) of 2.9 ± 0.5 Hz, with \( V_T \) of 0.12 ± 0.08 ml, MV of 21.8 ± 14.9 ml·min\(^{-1}\), and \( T_i \) of 130 ± 30 ms. During 2 s of preBötC illumination, \( f_R \) decreased to 1.5 ± 0.9 Hz (p=0.0001), \( V_T \) decreased to 0.07 ± 0.04 ml (p=0.0001), and MV decreased to 9.9 ± 5.6 ml·min\(^{-1}\) (p=0.0001). In contrast, \( T_i \) increased to 280 ± 90 ms (p=0.008) during preBötC illumination (Figs 3A and 7 left column, cyan symbols).

We examined the airflow fluctuations during bouts of illumination (Fig. 1.3A\(_{1,2}\)). Inspiratory movements discontinued at the onset of light application (e.g., Fig. 1.3A\(_2\) arrowhead) followed by damped airflow fluctuations. Cycle-triggered averaging showed these airflow fluctuations to be aperiodic and attenuated in amplitude, which indicates respiratory ataxia (Fig. 1.3B\(_{1,2}\)).

Illumination of the dorsal medulla in seven lightly ketamine-sedated \( Dbx1^{CreERT2};Ai35D \) controls (positions confirmed post-hoc, e.g., Fig. 1.2D) did not modify breathing. Prior to illumination of the dorsal medulla, \( f_R \) measured 3.3 ± 0.7 Hz, with \( V_T \) of 0.12 ± 0.12 ml, MV of 14.4 ± 14.1 ml·min\(^{-1}\), and \( T_i \) of 140 ± 20 ms. During bouts of illumination, \( f_R \) measured 3.3 ± 0.7 Hz (p=0.30), with \( V_T \) of 0.11 ± 0.12 ml (p=0.62), MV of 13.9 ± 13.2 ml·min\(^{-1}\) (p= 0.2), and \( T_i \) of 140 ± 20 ms (p=0.97) (Figs 4A and 7 left column, magenta symbols). Cycle-triggered averages of respiratory airflow prior to and during illumination were virtually superimposable (Fig. 1.4B).
Arch-mediated inhibition in Dbx1 preBötC neurons perturbs breathing in awake mice

In the same cohort of awake and unrestrained adult $\text{Dbx}^1{^{\text{CreERT2};\text{Ai35D}}}$ mice, we illuminated the preBötC and monitored breathing via whole-body plethysmography. Light delivery to the preBötC decreased $f_R$ (from $2.3 \pm 0.6$ in control to $2.1 \pm 0.5$ Hz during preBötC illumination, $p=0.0012$) without modifying $V_T$ ($0.05 \pm 0.008$ vs. $0.05 \pm 0.001$ ml, $p=0.24$) or MV ($0.87 \pm 0.1$ vs. $1.0 \pm 4.7$ ml·min$^{-1}$, $p=0.49$; because of two outliers in the ‘Light’ condition [see Fig. 1.7, right column, cyan symbols] we report MV in the text using the median ± SD

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**Figure 1.4** Light application to the dorsal medulla rostral to preBötC in sedated mice. A, Airflow, $V_T$, MV, and $f_R$ plotted continuously during two consecutive applications of 2-s light pulses. Inspiratory airflow is plotted upward, which reflects nose-cone measurements. A1, A2, Expanded airflow traces from A. Yellow bars indicate 589-nm light application. B1, B2, Cycle-triggered averages of airflow from each bout prior to (not highlighted) and during illumination (highlighted). Time calibrations are shown for each panel.
(rather than the mean ± SD). Illumination of the preBötC also increased $T_i$ from $100 ± 20$ ms to $120 ± 11$ ms ($p=0.0012$) and decreased inspiratory airflow with no concomitant effect on expiration (Figs 5A and 7 right column, cyan symbols). The decrease in $f_R$, reduced inspiratory amplitude, and longer $T_i$ during illuminated cycles are illustrated more clearly at faster sweep speed (Fig. 1.5A,2) and in cycle-triggered averages (Fig. 1.5B,2).

![Figure 1.5](image)

Figure 1.5 Activation of Arch in Dbx1 preBötC neurons in freely behaving awake mice. A, Airflow, $V_T$, MV, and $f_R$ plotted continuously during two consecutive applications of 2-s light pulses. Inspiratory airflow is plotted downward, which reflects whole-body plethysmography. A1, A2, Expanded airflow traces from A. Yellow bars indicate 589-nm light application. B1, B2, Cycle-triggered averages of airflow from each bout prior to (not highlighted) and during illumination (highlighted). Note that inspiratory airflow is attenuated, whereas expiratory airflow is not. Time calibrations are shown for each panel. Averages (Fig. 1.6B,2).

Illumination of the dorsal medulla in seven $Dbx1^{CreERT2};Ai35D$ mice had no notable effect on respiration. Prior to illumination of the dorsal medulla, $f_R$
measured $2.6 \pm 0.3$ Hz, with $V_T$ of $0.06 \pm 0.01$ ml, MV of $1.00 \pm 0.12$ ml·min$^{-1}$ and $T_i$ of $120 \pm 150$ ms. During bouts of illumination, $f_R$ measured $2.8 \pm 0.3$ Hz ($p=0.18$, $n=7$), with $V_T$ of $0.06 \pm 0.15$ ml ($p=0.25$), MV of $1.10 \pm 0.11$ ml·min$^{-1}$ ($p=0.429$), and $T_i$ of $120 \pm 15$ ms ($p=0.25$) (Figs 6A and 7 right column, magenta symbols). The breathing pattern remained unchanged during cycles of illumination (Fig. 1.6A), which was clear at faster sweep speed (Fig. 1.6A$_{1,2}$) and in cycle-triggered averages (Fig. 1.6B$_{1,2}$).

**Figure 1.6** Light application to the dorsal medulla rostral to preBötC in freely behaving awake mice. **A**, Airflow, $V_T$, MV, and $f_R$ plotted continuously during two consecutive applications of 2-s light pulses. Inspiratory airflow is plotted downward, which reflects whole-body plethysmography. **A**$_1$, **A**$_2$, Expanded airflow traces from **A**. Yellow bars indicate 589-nm light application. **B**$_1$, **B**$_2$, Cycle-triggered averages of airflow from each bout prior to (not highlighted) and during illumination (highlighted). Time calibrations are shown for each panel.
Figure 1.7 The respiratory effects of Arch-mediated photoinhibition. The left column shows effects in sedated animals. The right column shows effects in freely-behaving awake animals. Cyan symbols pertain to illumination of the preBötC whereas magenta symbols pertain to illumination of the dorsal medulla rostral to preBötC. Respiratory measurements include $T_i$ (first row), MV (second row), $V_T$ (third row), and $f_R$ (fourth row). Control, light application, and recovery data are shown for all experimental subjects. Double asterisks refer to the probability of a type I statistical error with alpha < 0.01. Triple asterisks refer to the probability of a type I statistical error with alpha < 0.001. “n.s.” (i.e., not significant) refers to the probability of a type I statistical error with alpha > 0.05.
1.5 Discussion

According to the Dbx1 core hypothesis, interneurons derived from Dbx1-expressing precursors comprise the CPG for inspiratory breathing movements. We investigated this hypothesis in vagus-intact adult mice using intersectional mouse genetics to express Arch in Dbx1 neurons, and then perform acute optogenetic silencing while monitoring its respiratory effects.

Evidence from studies in perinatal mice favored the Dbx1 core hypothesis at the outset of this investigation. Dbx1 neurons in the ventral medulla express glutamate and peptide neuromessengers, as well as peptide receptors, which are characteristics closely aligned with respiratory rhythmogenic function (Cui et al., 2016; Gray et al., 1999, 2001; Guyenet et al., 2002; Llona and Eugenín, 2005; McKay et al., 2005; Stornetta et al., 2003a; Tan et al., 2008; Wang et al., 2001). The commissural axons of Dbx1 neurons synchronize preBötC rhythms bilaterally and no recognizable preBötC forms in Dbx1 knock-out mice, which die at birth of asphyxia (Bouvier et al., 2010; Gray et al., 2010). Furthermore, laser ablation of Dbx1 preBötC interneurons in neonatal slices ultimately precludes respiratory rhythm and motor output (Wang et al., 2014). Therefore, it was not surprising that bilateral illumination of the preBötC in Dbx1CreERT2;Ai35D mouse slice preparations hyperpolarized Dbx1 neurons and arrested rhythmic XII output. Koizumi et al. reported similar data in reduced in situ preparations as well as mouse slices using a different Dbx1 Cre-driver strain but the same floxed-Arch reporter (Koizumi et al., 2016).
We interpret these results to mean that hyperpolarizing Dbx1 preBötC neurons directly impedes core rhythmogenic function, an interpretation equally advocated by Koizumi et al. (Koizumi et al., 2016). One potential caveat is that Arch-EGFP expression is not constrained to the cell bodies of Dbx1 neurons, so one must consider photoinhibition of axons and axon terminals. Dbx1 neurons are found throughout the respiratory medulla (Ruangkittisakul et al., 2014a) and some likely project to the preBötC. Axon terminals with remote origin that express optogenetic fusion proteins remain viable in transverse slices from the respiratory medulla (Holloway et al., 2013). Therefore, illumination of Arch-EGFP-expressing axon terminals could conceivably disfacilitate the preBötC to impede rhythmogenesis.

While we cannot rule out this interpretation, slice experiments provide negligible evidence of disfacilitation. Light application hyperpolarized Dbx1 neurons by ~6 mV in the context of network activity and in TTX, which is consistent with direct postsynaptic photoinhibition (Fig. 1.1). We presume that Dbx1 and non-Dbx1 neurons in the preBötC, both of which exhibit inspiratory rhythmic behavior and have similar membrane properties (Picardo et al., 2013), receive commensurate sources of tonic drive. That drive could originate (at least in part) from Dbx1 neurons with distant somata whose Arch-EGFP-expressing axon terminals synapse in the preBötC. However, illumination resulted in less than 1 mV of hyperpolarization in non-Dbx1 neurons (that do not express Arch-EGFP and thus cannot experience postsynaptic photoinhibition), which suggests that light-
evoked disfacilitation inappreciably influences baseline membrane potential and excitability in Dbx1 as well as non-Dbx1 preBötC neurons.

We cannot yet noninvasively monitor neural activity in the preBötC of awake intact adult mice to ascertain whether photoinhibition silences Dbx1 neurons or simply diminishes their activity. Nevertheless, if Dbx1 preBötC neurons are rhythmogenic in intact adult mice then straightforward predictions are that photoinhibition should either: i) cause apnea or ii) retard the progress of the inspiratory phase and thus prolong inspiratory duration (if breathing persists during bouts of preBötC illumination), as well as lengthen the interval between inspiratory phases (i.e., decrease $f_R$). Continuous laser pulses that affect the respiratory cycle in its entirety caused all of the predicted effects: apnea, prolonged $T_i$, and decreased $f_R$.

Anesthetized mice experienced 18-s apneas during 30-s bouts of preBötC illumination in accord with the first prediction. Sedated mice transiently increased $T_i$ and decreased $f_R, V_T$, and MV during 2-s bouts of preBötC illumination (Figs 3 and 7). The effects on $T_i$ and $f_R$ match the second prediction (Fig. 1.7). The decrease in $V_T$ and MV reflect the reduced $f_R$ as well as smaller amplitude inspiratory breaths, and remain consistent with the second prediction.

We analyzed the airflow signal during preBötC illumination in sedated mice as if it represented an attenuated preBötC respiratory rhythm; diminished $f_R, V_T$, and MV, combined with prolonged $T_i$ are consistent with this explanation. However, the reduced-amplitude airflow fluctuations during light application were aperiodic according to the CTAs. Therefore, it remains possible that illumination precludes
a preBötC-driven respiratory rhythm and that the attendant airflow fluctuations reflect a non-preBötC behavior that nonetheless affects airflow. In support of this idea, the onset of the laser pulse halted inspiratory efforts mid-cycle (e.g., Fig. 1.1A₂). Whisking or other orofacial behaviors could register airflow if the preBötC were offline. The whisking CPG is adjacent to the preBötC but employs disparate cellular and synaptic mechanisms (Deschênes et al.; Moore et al., 2013) that unlikely to be affected by photoinhibition of Dbx1 neurons.

Whether or not preBötC-generated, airflow fluctuations during bouts of preBötC illumination would not ventilate the mouse. V_T was attenuated by ~50% (Fig. 1.3A₁,₂). This volume is insufficient to clear the dead space associated with airways and trachea, which constitutes 30-45% of V_T in mammals ranging from rodents to horses (Kleinman and Radford, 1964; Stahl, 1967) and 19-30% of vital capacity in mice (Schulz et al., 2002).

Illuminating the preBötC in awake intact adult mice transiently decreased f_R and increased T_i combined with lowered amplitude inspiratory breaths. However, there was no change in V_T or MV. The effects on f_R, T_i, and inspiratory breath amplitude are consistent with suppressing the preBötC core oscillator, but the lack of effect on V_T and MV is at odds with that interpretation. If the Dbx1 core hypothesis is true, then why does ventilation persist in intact adult mice? Perhaps the light-evoked outward current in Dbx1 preBötC neurons is insufficient to suspend rhythmogenesis. According to our measurements and calculations, the light intensity at the preBötC was adequate to evoke near maximum Arch-mediated current (see Materials and Methods). However, if Arch-EGFP
expression were limited within the Dbx1 preBötC neuron population, then (regardless of light delivery), it could diminish the potential for optogenetic suppression of respiratory rhythmogenesis.

Dbx1 is expressed between E8.5 and E12.5 (Bouvier et al., 2010; Gray et al., 2010; Pierani et al., 2001). We activated CreER\textsuperscript{T2} at E9.5 when we presume Dbx1 expression peaks. Thus, Cre-Lox recombination will not occur in the fraction of Dbx1-expressing precursors that enter mitosis prior to E9.5. Furthermore, CreER\textsuperscript{T2} recombination is inherently fragmentary, so one expects Arch-EGFP underexpression in the target population.

Even if we stipulate ideal Arch-EGFP expression and light delivery, optogenetic suppression of respiration in awake intact mice may not be feasible because of excitatory drive and sensory feedback. Chemosensitive neurons in the retrotrapezoid nucleus (Guyenet et al., 2016) as well as excitatory inputs from the pons and raphé (Hilaire et al., 2004; Ptak et al., 2009; Viemari et al., 2003) tonically excite the preBötC. Furthermore, with the vagus nerve intact, lung inflation and deflation reflexes maintain high \( f_R \) and limit \( T_i \) (generally 2-4 Hz and \( \sim 100 \) ms, respectively, in mice). Vagotomy reduces respiratory frequency by 50-65\% and extends inspiratory duration two-fold in rodents (Smith et al., 1990; Song et al., 2015). Therefore, sources of tonic excitation and sensory feedback may override the \( \sim 6 \) mV of light-evoked hyperpolarization in some fraction of the Dbx1 preBötC neuron population such that photoinhibition impedes but does not stop rhythmogenesis nor inspiratory breathing movements. In support of this idea that tonic sources of drive can override Arch effects, optogenetic inhibition of
Dbx1 preBötC neurons was unable to stop fictive respiratory rhythms in a completely deafferented adult in situ preparation, except when the medulla was transversely transected at the medullary junction rostral to the preBötC, which would abolish all sources of tonic drive (Koizumi et al., 2016). Arch-mediated photoinhibition probably provides a stronger impediment to breathing in anesthetized and sedated mice because drugs, notably ketamine and ketamine-xylazine, generally suppress respiration (Erhardt et al., 1984; Flecknell, 2015; Green et al., 1981), which would act in concert with Arch. Alternatively, it is conceivable that the respiratory core oscillator in adults incorporates non-Dbx1-derived interneurons, which are not active perinatally and in adults would remain unperturbed by 589-nm light. One candidate population in the ventral medulla would be catecholamanergic C1 neurons, which also utilize glutamate as a fast transmitter (Guyenet et al., 2013; Stornetta et al., 2002). However, these neurons are associated with autonomic regulation, particularly circulation at the level of the rostral ventrolateral medulla. Although optogenetic excitation of C1 neurons modulates respiratory rhythm in conscious mice, it does so in a cardiorespiratory integrative context (Abbott et al., 2013). Furthermore, C1 neurons do not express neurokinin-1 receptors (Wang et al., 2001) nor do they form commissural projections onto preBötC neurons, which are hallmark features of respiratory rhythmogenic preBötC neurons. Therefore, we think it unlikely that C1 neurons contribute a heretofore unidentified respiratory rhythmogenic circuit.
Although we cannot rule out the existence of non-Dbx1 interneurons that sustain rhythmogenesis during photoinhibition of Dbx1 preBötC neurons, the most parsimonious explanation for persistent ventilation in intact adult Dbx1\textsuperscript{CreERT2};Ai35D mice during preBötC illumination is that excitatory drive from modulatory and chemosensitive inputs, as well as vagal sensory feedback, provide sufficient excitation to Arch-EGFP-expressing Dbx1 preBötC neurons to overcome light-mediated inhibition and sustain ventilation. Nevertheless, photoinhibition impedes core rhythmogenic function, resulting in inspiratory breaths at lower amplitude and frequency, with prolonged inspiratory duration. We can attribute rhythmogenic function to Dbx1-derived interneurons in the preBötC (Cui et al., 2016; Koizumi et al., 2016), but neurons from the same genetic class serve in other respiratory and non-respiratory functions as well. For example, Dbx1 neurons at the dorsal edge of the preBötC, and in the intermediate reticular formation have premotor function related to inspiratory movements of the tongue muscle genioglossus (Revill et al., 2015; Wang et al., 2014). Therefore, it may be possible to further subdivide Dbx1 preBötC neurons on the basis of genetic variation (Bikoff et al., 2016; Gabitto et al., 2016) to discern a specifically rhythmogenic subset.

1.6 References


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CHAPTER 2. Dbx1 pre-Bötzinger complex interneurons comprise the core oscillator for breathing in adult mice

2.1 Abstract

The brainstem pre-Bötzinger complex (preBötC) generates the rhythm for inspiratory breathing movements in mammals, but which neurons comprise its rhythmogenic core? Dbx1-expressing progenitor cells develop into neurons and glia that serve respiratory and non-respiratory functions; the preeminent neuronal role we posit to be inspiratory rhythm generation. Here we used intersectional mouse genetics to express archaerhodopsin (ArchT) or channelrhodopsin (CatCh) and thus manipulate Dbx1 preBötC neurons while measuring breathing in intact adult mice. Prolonged (5 s) photoinhibition of Dbx1 preBötC neurons slowed down or stopped breathing in a manner corresponding to light intensity. In contrast, prolonged photostimulation sped up breathing in a manner linked to light intensity. Brief (100 ms) photoinhibition of Dbx1 preBötC neurons late in the inspiratory phase evoked the next breath earlier than expected, whereas brief photoinhibition later in the cycle delayed the subsequent breath. Conversely, brief photostimulation late in the inspiratory phase augmented the magnitude and duration inspiration, whereas brief photostimulation during the expiratory phase evoked the next breath earlier than expected. Because optogenetic perturbations operate postsynaptically on Dbx1 preBötC neurons and modulate the phase and frequency of inspiration, we conclude that these neurons comprise the inspiratory core oscillator in adult mice.
2.2 Introduction

Inspiratory breathing movements in mammals originate from neural rhythms of the brainstem preBötzinger Complex (preBötC) (Feldman et al., 2013; Smith et al., 1991). Although the preBötC has been identified in a range of mammals including bats, moles, goats, cats, rabbits, rats, mice, and humans (Mutolo et al., 2002; Pantaleo et al., 2011; Ruangkittisakul et al., 2011; Schwarzacher et al., 1995, 2010; Smith et al., 1991; Tupal et al., 2014; Wenninger et al., 2004) its borders and neuronal constituents are incompletely understood. Competing classification schemes have emphasized peptide and peptide receptor expression (Gray et al., 1999, 2001; Stornetta et al., 2003a; Tan et al., 2008) as well as a glutamatergic transmitter phenotype (Funk et al., 1993; Stornetta et al., 2003b; Wallen-Mackenzie et al., 2006) as cellular markers that define the preBötC rhythmogenic core.

Interneurons derived from precursors that express the homeodomain transcription factor Dbx1 (i.e., Dbx1 neurons) also express peptides and peptide receptors associated with respiratory rhythmogenesis, and are predominantly glutamatergic. Dbx1 knock-out mice die at birth of asphyxia and the preBötC never forms (Bouvier et al., 2010; Gray et al., 2010). Laser ablation of Dbx1 preBötC neurons in rhythmically active slice preparations slows and then stops respiratory motor output (Wang et al., 2014). These results obtained from perinatal mice suggest that Dbx1 neurons comprise the rhythmogenic preBötC core, i.e., the Dbx1 core hypothesis.
Nevertheless, in addition to their putatively rhythmogenic role, Dbx1 preBötC neurons also influence motor pattern. Hypoglossal motoneurons that maintain airway patency receive rhythmic synaptic drive from Dbx1 neurons within the preBötC and adjacent intermediate reticular formation (Revill et al., 2015; Wang et al., 2014). Photostimulation of Dbx1 preBötC neurons in anesthetized vagotomised adult mice modulates motor pattern as well as inspiratory timing (Cui et al., 2016). That modulation of inspiratory motor output pattern is mediated in part by somatostatin-expressing preBötC neurons (Cui et al., 2016), a large fraction (78-91%) of which are derived from Dbx1-expressing progenitors (Bouvier et al., 2010; Cui et al., 2016; Gray et al., 2010; Koizumi et al., 2016). Further diversifying their potential roles, a subset of Dbx1-derived preBötC neurons that expresses Cadherin-9 (Cdh9) exhibits a weak respiratory phenotype and projects to the pontine locus coeruleus to influence arousal (Yackle et al., 2017). Collectively, the fractions of motor output-related (somatostatin-expressing) and arousal-related (Cdh9-expressing) Dbx1 neurons could account for up to 73% of Dbx1 neurons within the preBötC: 13-17% of Dbx1 preBötC neurons express somatostatin (Sst) and 56% express Cdh9 with no overlap between somatostatin and Cdh9 expression (Bouvier et al., 2010; Cui et al., 2016; Gray et al., 2010; Yackle et al., 2017). That accounting would leave 27% of Dbx1 preBötC neurons exclusively rhythmogenic, if one assumes that all remaining Dbx1 neurons are dedicated to respiration and that single Dbx1 preBötC neurons cannot fulfill multiple duties. The contemporary studies recapped above from adult mice imply that rhythm generation may not be the
principal function of Dbx1 preBötC neurons, in strong contrast to the pioneering studies in perinatal mice that solely identified their rhythmogenic role (Bouvier et al., 2010; Gray et al., 2010).

Here we reevaluate the respiratory rhythmogenic role of Dbx1 preBötC neurons in adult mice with intact sensorimotor feedback. Using genetic technologies to photoinhibit or photostimulate Dbx1 neurons, we show that their perturbation affects breathing frequency and the precise timing of individual breaths within the breathing cycle, which are key properties of a core oscillator microcircuit. Other respiratory and non-respiratory roles notwithstanding, these data indicate that Dbx1 preBötC neurons constitute the essential core oscillator for inspiration.

2.3 Materials and Methods

Mice

The Institutional Animal Care and Use Committee at The College of William and Mary approved these protocols. We maintain a colony of mice that express tamoxifen-sensitive Cre recombinase in Dbx1-derived progenitor cells, i.e., Dbx1CreERT2 (Ruangkittisakul et al., 2014a). Female Dbx1CreERT2 mice were mated with males from two different reporter strains. The first reporter strain expresses an Archaerhodopsin-3 and EGFP fusion protein (ArchT-EGFP) in a Cre-dependent manner from the endogenous Gt(ROSA)26Sor locus (Ai40D, Jax #021188, Jackson Laboratories, Bar Harbor, ME, USA). The second reporter strain features Frt- and LoxP-flanked STOP cassettes flowed by a fusion gene coding for calcium translocating channelrhodopsin and EYFP (CatCh-EYFP), which is expressed following Cre- and Flp-mediated recombination (Ai80D, Jax
We administered tamoxifen to pregnant dams (22.5 mg/kg) at embryonic day 9.5 to maximize neuronal expression (Kottick et al., 2017). This administration schedule resulted in Arch-EGFP or Frt-flanked CatCh-EYFP expression in Dbx1 neurons of $Dbx1^{CreERT2};Ai40D$ (hereafter referred to as Dbx1;ArchT) or $Dbx1^{CreERT2};Ai80D$ (hereafter referred to as Dbx1;CatCh) offspring, respectively. Dbx1;ArchT or Dbx1;CatCh mice were distinguished from wildtype (WT) littermates, which lack EGFP or EYFP, via post-hoc histology. Therefore, WT littermates formed a control group whose constituent members were unknown to the experimenter.

**Respiratory active transverse slice preparations**

Neonatal Dbx1;ArchT mice (0-4 days old) were anesthetized via hypothermia, decerebrated, and then dissected in 4º C aCSF containing (in mM): 124 NaCl, 3 KCl, 1.5 CaCl$_2$, 1 MgSO$_4$, 25 NaHCO$_3$, 0.5 NaH$_2$PO$_4$, and 30 dextrose aerated continually with carbogen (95% O$_2$ and 5% CO$_2$) at pH 7.4. The isolated neuraxes were glued to an agar block and mounted rostral side up in the vise of a vibratome. The mounted neuraxes were sequentially cut in the transverse plane until reaching at the rostral border of the preBötC, at which we point we acquired a single 500-µm-thick section containing the preBötC as well as the hypoglossal (XII) cranial motor nucleus and its rostral nerve rootlets. The anatomical criteria for isolating the preBötC in rhythmically active slices from neonatal Dbx1-reporter mice are detailed in a series of open access atlases (Ruangkittisakul et al., 2014a). Slices were anchored using a silver wire grid in a recording chamber on a fixed-stage upright physiology microscope. We perfused
the slice with aCSF at 27º C (2 ml/min) and elevated the K+ concentration to 9 mM. Inspiratory motor output was recorded from the XII nerve rootlets using a differential amplifier (gain 2000x) and a band-pass filter (300-1000 Hz). Nerve root output was full-wave rectified and smoothed for display.

We identified Dbx1 neurons under epifluorescence via EGFP expression and then performed whole-cell patch-clamp recordings under visual control via differential interference contrast microscopy. Patch pipettes with tip resistance of 4-6 MΩ were fabricated from capillary glass (1.50 mm outer diameter, 0.86 mm inner diameter) and filled with solution containing (in mM): 140 potassium gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Na3-GTP. Alexa 568 hydrazide dye was added to the patch-pipette solution (50 µM, Invitrogen, Carlsbad, CA, USA) as a color contrast to EGFP following whole-cell dialysis. Membrane potential was amplified (100x) and low-pass filtered (1 kHz) using a patch-clamp amplifier (EPC10, HEKA Elektronic, Holliston, MA, USA) and digitally acquired at 4 kHz (PowerLab 4/30, AD Instruments, Colorado Springs, CO, USA).

**Virus injection and fiber optic implantation**

We anesthetized adult Dbx1;ArchT and Dbx1;CatCh (aged 8-20 weeks) via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and performed aseptic surgeries in the prone position using a stereotaxic frame. After exposing the skull, we performed either one (Dbx1;CatCh mice) or two (Dbx1;ArchT mice) 0.5-mm-diameter craniotomies in the range 6.95 to 7.07 mm posterior to bregma and 1.1 to 1.3 mm lateral to the midline suture, which
allowed us to then install fiberoptic appliances in all mice for photoinhibition or photostimulation of Dbx1 preBötC neurons. In Dbx1;CatCh mice only, however, in preparation for photostimulation experiments, we unilaterally injected an adeno-associated virus (AAV) to induce Flp-mediated recombination. We loaded an ultrafine, microvolume syringe (Neuros series, Hamilton, Reno, NV) with 120 nl of AAV-eSyn-FLPo (titer $10^{13}$ vg/ml, Vector Biolabs, Malvern, PA, USA). The syringe was lowered at 10 µm/s through the cerebellum and the virus was injected at the target site at approximately 60 nl/min. The syringe remained in place for 10 min before being retracted at 10 µM/s. All animals (Dbx1;ArchT and Dbx1;CatCh) were equipped with fiber optic appliances constructed by joining 1.27-mm-diameter ceramic ferrules (Precision Fiber Products, Milpitas, CA, USA) with 105-µm-diameter 0.22 numerical aperture (NA) multimode fibers (Thorlabs, Newton, NJ, USA). We implanted fiber optic appliances bilaterally in Dbx1;ArchT mice. We implanted fiber optic appliances unilaterally in Dbx1;CatCh mice. These appliances were inserted at a depth of 5.5 to 5.9 mm from bregma and secured with a cyanoacrylate adhesive (Loctite 3092, Henkel Corp., Rocky Hill, CT, USA). Dbx1;ArchT animals recovered for a minimum of 10 days following fiber optic implantation before any further experimentation. Dbx1;CatCh mice recovered for a minimum of 21 days before further experimentation; the longer wait time allowed for protein expression.

**Breathing measurements**

After anesthetizing mice using 2% isoflurane we connected the ferrules of Dbx1;ArchT mice to a 589-nm laser (Dragon Lasers, Changchun, China). The
ferrule of Dbx1;CatCh mice was connected to a 473-nm laser (Dragon Lasers). Mice recovered from isofluorane anesthesia for ~1 hr, and then we measured breathing behavior in a plethysmograph (Emka Technologies, Falls Church, VA, USA) that allowed for fiberoptic illumination in a sealed chamber with constant airflow rate.

In a separate session, these same mice were lightly sedated via intraperitoneal ketamine injections (15 mg/kg minimum dose), which we titrated as needed to reduce limb movements but retain toe-pinchr and blink reflexes. The maximum aggregate dose was limited to 50 mg/kg. Mice were fitted with a modified anesthesia mask (Kent Scientific, Torrington, CT, USA) to measure breathing.

We applied a circuit of positive pressure, with balanced vacuum, to continuously flush the plethysmograph with breathing air. The plethysmograph and the mask were connected to a 1-liter respiratory flow head and differential pressure transducer that measured airflow; positive airflow reflects inspiration in all cases. Analog breathing signals were digitized at 1 kHz (PowerLab).

**Optogenetic protocols**

We applied 5-s bouts of light to Dbx1;ArchT and Dbx1;CatCh mice at graded intensities of 6.8, 8.6, and 10.2 mW. All ferrules were tested with a power meter prior to implantation to verify that illumination intensity did not vary more than 0.1 mW from the specified values (6.8, 8.6, and 10.2 mW). Bouts of light application were separated by a minimum interval of 30 s. We also applied 100-ms light pulses at a fixed intensity of 15 mW. Each mouse received 85-200 pulses spaced
at random intervals of 1-5 s. All of the 5-s and 100-ms stimuli were digitally acquired in sync with breathing measurements using logic pulses (PowerLab).

**Data analysis**

The airflow signal was band-pass filtered (0.1-20 Hz) and analyzed using LabChart 8 software (AD Instruments), which computes airflow (units of ml/s), respiratory rate ($f$, units of Hz), tidal volume ($V_T$, units of ml), and minute ventilation (MV, units of ml/min). We computed statistics using GraphPad Prism 6 (La Jolla, CA, USA) and R: The Project for Statistical Computing (R, The R Foundation, Vienna, Austria) and prepared figures using Adobe Illustrator (Adobe Systems Inc., San Jose, CA, USA), GraphPad Prism 6, and IGOR Pro 6 (Wavemetrics, Lake Oswego, OR, USA). We analyzed the experiments in which 5 s light pulses were applied to the preBötC using paired t-tests, specifically comparing mean $f$, $V_T$, and MV for control and illumination conditions at three different light intensity levels (i.e., at each laser strength tested, the pre-illumination ventilation serves as its own control). In the Results we report the mean change between control and illumination periods in $f$, $V_T$, and MV, while plotting all the data including the mean and SD for control and illumination conditions in the figures.

We analyzed phase-response properties of the breathing cycles perturbed by 100 ms-duration light pulses (photostimulation and photoinhibition) (see Fig. 2.4c inset). The expected cycle period was measured from the cycle immediately before the light pulse, peak inspiration to peak inspiration, which was defined as spanning 0-360° ($\Phi_{\text{Expected}}$). 100-ms light pulses were applied at time points
spanning the breathing cycle to test for phase shifts. $\Phi_{\text{Stim}}$ marks the time at which the light pulse occurred. The induced cycle period ($\Phi_{\text{Induced}}$) was measured from the perturbed cycle, peak inspiration prior to light application until the peak inspiration of the next breath. In perturbed cycles when a full expiratory phase before the onset of the stimulus, i.e., a return to functional residual capacity, did not occur, then the transition between breaths was defined as having occurred after the volume signal (i.e., the integrated airflow) reaches 30% of functional residual capacity. Two peaks in the inspiratory phase that did not meet the above criteria were considered one single (augmented) breath. The perturbation of breathing phase, $\Phi_{\text{Shift}}$, was defined as the difference between $\Phi_{\text{Induced}}$ and $\Phi_{\text{Expected}}$. We calculated normalized change in $V_T$ ($\Delta V_T$) and $T_i$ ($\Delta T_i$) in the perturbed breath compared to the expected breath. Further, we calculated the phase shift of the breath following the perturbed breath (i.e., the cycle after $\Phi_{\text{Induced}}$) with respect to $\Phi_{\text{Expected}}$ ($\Phi_{N+1}$). Values for each measurement (i.e., $\Phi_{\text{Shift}}$, $\Delta V_T$, $\Delta T_i$, and $\Phi_{N+1}$) for all trials across all mice were pooled together and then sorted in to 12 bins of equal size over the interval 0-360° (i.e., 30° per bin). We computed the mean and standard deviation (SD) for $\Phi_{\text{Shift}}$, $\Delta V_T$, $\Delta T_i$, and $\Phi_{N+1}$ within each bin, which we then plotted in phase-response curves along with values calculated from WT-littermates. A Tukey’s HSD to test was used to determine significance between $\text{Dbx1;ArchT}$ or $\text{Dbx1;CatCh}$ and WT littermates for each bin.
**Histology**

We administered a lethal dose of pentobarbital (100 mg/kg i.p.) and then transcardially perfused the mice with 0.1 M PBS followed by 4% PFA in 0.1 M PBS. The neuraxes were removed and post-fixed overnight in 4% PFA, and later sliced in 50-µm contiguous transverse sections using a vibratome. Free-floating sections were stained using NeuroTrace 530/615 red fluorescent nissl stain (Invitrogen) for 1 min, rinsed in distilled water and then mounted on slides and cover-slipped using Vectashield (Vector Labs, Burlingame, CA, USA). Tissue sections were visualized using bright-field and confocal microscopy. Images were arranged as mosaics and brightness and contrast were adjusted uniformly across the entire ensemble image using the public domain software package ImageJ. We employed no other image manipulations.

**2.4 Results**

*ArchT activation hyperpolarizes Dbx1 preBötC neurons postsynaptically*

We illuminated the preBötC in transverse medullary slices from neonatal Dbx1;ArchT mice that spontaneously generate inspiratory rhythm and XII motor output. Light application to the preBötC stopped rhythm and motor output at all light intensities (Fig. 2.1A and B). Dbx1 preBötC neurons recorded in whole-cell patch-clamp hyperpolarized 6.5 ± 1.0, 8.1 ± 1.1, and 11.0 ± 2.5 mV in response to 589 nm light pulses of increasing intensity: 6.8, 8.6, and 10.2 mW, respectively (Fig. 2.1A and D). To test whether light-induced hyperpolarization could be due to network disfacilitation we applied 1 µM TTX and reapplied 10.2 mW light pulses, which hyperpolarized Dbx1 preBötC neurons by 8.6 ± 1.4 mV (Fig. 2.1C.
and D). Hyperpolarization evoked by 10.2 mW light was commensurate before and after TTX application (Mann-Whitney U, \( p = 0.2, n_1 = 8, n_2 = 3 \)), which suggests that ArchT activation acts predominantly postsynaptically to hyperpolarize Dbx1 preB\(\ddot{o}C\) neurons.

**Figure 2.1 Photoinhibition of Dbx1 preB\(\ddot{o}C\) neurons and respiratory rhythm in vitro.** A, Patch-clamp recording of an ArchT-expressing Dbx1 preB\(\ddot{o}C\) neuron. B, Patch-clamp recording of non-Dbx1, non-ArchT-expressing preB\(\ddot{o}C\) neuron. Light pulses (30 s) were applied bilaterally to the preB\(\ddot{o}C\) at three intensities (units of mW) in A and B. Voltage and time calibrations apply to A and B, including baseline membrane potential of -60 mV. Action potentials have been truncated for display to emphasize the trajectory around the baseline membrane potential. C, Patch-clamp recordings of Dbx1 and non-Dbx1 preB\(\ddot{o}C\) neurons (same cells as in A and B) exposed to 30-s bouts of 10.2 mW illumination in the presence of 1 \(\mu\)M tetrodotoxin (TTX). Voltage and time calibrations apply only to C. D, Group data showing membrane hyperpolarization (\(\Delta V_M\)) evoked by light pulses at three intensities in Dbx1 and non-Dbx1 preB\(\ddot{o}C\) neurons from the experiments illustrated in A-C. Bars show mean and SD.
Baseline membrane potential in non-Dbx1 neurons responded negligibly to 589 nm light, hyperpolarizing 0.7 ± 0.3, 1.1 ± 0.5, and 1.1 ± 0.6, 3 mV in response to light of 6.8, 8.6, and 10.2 mW, respectively (Fig. 2.1B and D). In the presence of TTX, non-Dbx1 neurons hyperpolarized 0.3 ± 0.8 mV in response to 10.2 mW illumination (Fig. 2.1C and D), which was indistinguishable from light-evoked hyperpolarization before TTX application (Mann-Whitney U, p = 0.2, n₁ = 8, n₂ = 4). These results suggest that light-evoked effects on rhythm and motor output are largely attributable to direct postsynaptic effects on Dbx1 preBötC neurons.

ArchT and CatCh expression in Dbx1 preBötC neurons in adult mice

We implanted fiber optics in the preBötC of adult Dbx1;ArchT or Dbx1:CatCh mice to perform photoinhibition and photostimulation experiments, respectively. In post-hoc histology we verified that ferrule tip location was within 500 µm of dorsal border of the preBötC in all cases (Fig. 2.2A). Adult Dbx1;ArchT mice expressed fusion protein in all Dbx1-derived cells forming an inverted V-shape extending from the lateral border of the XII nucleus to the preBötC at the ventral border of transverse sections (Fig. 2.2B), as previously described in neonatal mice (Ruangkittisakul et al., 2014) and adult mice (Kottick et al., 2017; Vann et al., 2016).

To limit CatCh expression to Dbx1 neurons within the preBötC we unilaterally injected an adeno-associated virus that expresses Flp recombinase, which resulted in CatCh-EYFP expression focused to the preBötC (Fig. 2.2C). This limited expression of fusion protein, combined with ferrule placement, allowed for targeted excitation of Dbx1 neurons within the preBötC.
Figure 2.2 ArchT and CatCh fusion protein expression in the preBötC of adult mice. A, Bright field images of transverse sections from an adult Dbx1;ArchT mouse. The upper section was obtained -6.85 mm (caudal) from bregma and is approximately 50 µm rostral to the preBötC border. The compact division of the nucleus ambiguus (cNA), the fourth ventricle (4v), and the rostralmost portion of the inferior olive (IO) are visible, which are anatomical landmarks consistent with ventral respiratory medulla at the level of the Bötzinger complex. The lower section was obtained -6.90 mm (caudal) from bregma at the level of rostral border of the preBötC, as indicated by the fully developed loop of the inferior olive (IO_loop, also see B, inset), the smaller size of the fourth ventricle (thus closer to the obex), and the semi-compact division of the nucleus ambiguus (scNA). Parallel tracks of implanted fiberoptic appliances are visible from the dorsal border of the section to the dorsal preBötC border. B, Fluorescence image of the transverse slice from panel A (lower, -6.90) showing ArchT-EGFP fusion protein expression (pseudocolored cyan) in the Dbx1-derived cells of the preBötC and intermediate reticular formation. The selection box in B (upper) is expanded (lower) to show the preBötC in greater detail. Nissl stain (magenta) shows anatomical landmarks including the IO_loop (upper and lower) and XII nucleus (upper). Scale bar applies to both A and B. Note: the ferrule placement compresses surrounding tissue, which intensifies the ArchT-EGFP fluorescence in the reticular formation. C, Parasagittal sections from an adult Dbx1;CatCh mouse. Nissl (magenta) shows anatomical landmarks including the facial (VII) cranial nucleus, Bötzinger complex (BötC), and the preBötC. CatCh-EYFP (pseudocolored cyan) expression is limited to the preBötC.
Photoinhibition of Dbx1 preBötC neurons attenuates breathing and resets inspiration

We photoinhibited the preBötC bilaterally in sedated adult Dbx1;ArchT mice using long duration (5 s) bouts of 589 nm light at three laser intensities, which reduced breathing in all instances (Fig. 2.3A). Frequency, ~3.5 Hz in control, decreased by 0.3 Hz (t-test, p = 0.05, n = 6) in response to the lowest intensity light (6.8 mW). Tidal volume, ~0.1 ml in control, changed by 0.2 ml (t-test, p = 0.06, n = 6), which was not statistically significant. However, MV approximately 50 ml/min in control, decreased by 9 ml/min (t-test, p = 0.01, n = 6) (Fig. 2.3A,B). Frequency, \( V_T \), and MV decreased to a greater extent in response to 8.6 and 10.2 mW intensity light applications (Fig. 2.3A). Frequency decreased by 1.2 and 2.0 Hz, respectively (t-test, \( p = 0.001 \) and \( p = 0.0001 \), \( n = 6 \)) (Fig. 2.3B). Apnea, no inspiratory effort, resulted in 11 of 30 individual bouts (i.e., 5 bouts in 6 animals, Fig. 2.3A, bottom). Tidal volume decreased in both cases by 0.03 in response to 8.6 and 10.2 mW illumination (t-test, \( p = 0.04 \) and \( p = 0.02 \), \( n = 6 \)) (Fig. 2.3B). Minute ventilation decreased by 11 and 20 ml/min in response to 8.6 and 10.2 mW illumination, respectively (t-test, both \( p = 0.02 \), \( n = 6 \)) (Fig. 2.3B). In wild-type littermates, also sedated and subjected to the same light application protocol, we detected no changes in breathing (Fig. 2.S1). These results show that ArchT-mediated Dbx1 preBötC neuron hyperpolarization perturbs breathing up to and including inducing apnea in sedated mice.
We repeated these experiments in Dbx1;ArchT mice while awake and unrestrained. The lowest intensity light (6.8 mW) minimally affected breathing (Fig. 2.3C). Frequency and \( V_T \) decreased 0.01 Hz (t-test, \( p = 0.06 \), \( n = 5 \)) and 0.03 ml (t-test, \( p = 0.07 \), \( n = 5 \)), respectively, but those changes were not significant (Fig. 2.3D). Minute ventilation, however, decreased significantly by 7.4 ml/min (t-test, \( p = 0.04 \), \( n = 5 \)) (Fig. 2.3D).
Further, in awake animals $f$ and MV decreased significantly in response to 8.6 and 10.2 mW intensity light application (Fig. 2.3C). Frequency decreased by 1.1 and 1.2 Hz, respectively ($t$-test, $p = 0.002$ and $p = 0.02$, $n = 5$). Apnea resulted in one animal (Fig. 2.3C, bottom trace). MV Minute ventilation decreased by 22 and 32 ml/min in response to 8.6 and 10.2 mW illumination, respectively ($t$-test, $p = 0.04$ and $p = 0.03$, $n = 5$) (Fig. 2.3D). In contrast, $V_T$ decreased by 0.05 and 0.15 ml in response to 8.6 and 10.2 mW illumination. Conventional statistical hypothesis testing did not detect significant light-induced changes in $V_T$, probably due to the high variability of this parameter in awake animals ($t$-test, $p = 0.2$ and $p = 0.08$, $n = 5$) (Fig. 2.3D).

In wild-type littermates, also awake and intact, no light application at any intensity affected breathing (Fig. 2.S1). These data show that ArchT-mediated Dbx1 preBöC neuron hyperpolarization reduces breathing up to and including apnea in awake intact mice.

**Figure 2.S1 Light application to the preBöC does not affect breathing in wild-type Dbx1;ArchT littermates.** A, Airflow traces from a sedated wild type mouse (littermate of Dbx1;ArchT mice) exposed to 5-s bouts of bilateral preBöC illumination at three intensities (units of mW). B, Group data from experiments in A quantifying $f$, $V_T$ and MV in response to light application. Symbols show mean $f$, $V_T$, and MV in each mouse. Bars show the mean and SD for all animals tested ($n = 6$). C, Airflow traces from an awake unrestrained wild type mouse (littermate of Dbx1;ArchT mice) exposed to 5-s bouts of unilateral preBöC illumination at three intensities (units of mW). D, Group data from experiments in C quantifying $f$, $V_T$ and MV in response to light application. Symbols show mean $f$, $V_T$, and MV in each mouse. Bars show the mean and SD for all animals tested ($n = 6$).
Next we applied brief (100 ms) light pulses to assess phase dependent changes in breathing. Illumination of the preBötC during late inspiration triggered inspiration earlier than expected with a phase shift of -74± 10º ($\Phi_{\text{Stim}}$ of 0-30º, $p = 1e-6$, $n = 4$) (Fig. 2.4A1 and A3 top trace). For $\Phi_{\text{Stim}}$ from 30 to 120º, no further phase advance occurred, yet $\Phi_{\text{Shift}}$ showed a monotonic trend approaching a phase delay. During expiration (120-150º) the first significant phase delay occurred (35 ± 10º, $p = 0.04$, $n = 4$, Fig. 2.4A1 and A3 middle trace). Phase delays continue to be observed through the inspiratory phase (150 to 360º); the maximal phase delay of 130 ± 14º occurred for $\Phi_{\text{Stim}}$ of 270-300º ($p = 1e-6$, $n = 4$). During the periods just before peak inspiratory effort (i.e., 300-330º and 330-360º) the mean $\Phi_{\text{Shift}}$ was 93 ± 16 and 105 ± 21º respectively ($p = 1e-6$ and $p = 3e-5$, $n = 4$), but those shifts were less compared to the maximal advance (Fig. 2.4A3). This downward trend in $\Phi_{\text{Shift}}$ reflects a bifurcation of responses to the light where either a phase delay occurred, which contributes to the mean $\Phi_{\text{Shift}}$, or the light-induced breath occurred as expected, which diminishes the mean $\Phi_{\text{Shift}}$. Brief light pulses had no significant effect on $V_T$ throughout most of the respiratory cycle (i.e., 30 to 330º). However, during both the falling and rising edges of peak inspiration (i.e., 0-30 and 330-360º) significant changes in $V_T$ were observed. During the falling edge of peak inspiration $\Delta V_T$ of the perturbed breath was 0.3 ± 0.1 times larger than the previous breath ($p = 0.005$, $n = 4$). Conversely, during the rising edge $\Delta V_T$ was reduced by 0.3 ± 0.4 ($p = 0.05$, $n = 4$). We observed no relationship between the phase of the subsequent breath ($\Phi_{N+1}$) and inspiratory
time ($\Delta T_i$) (Fig. 2.4A and 2.S2A). These data indicate that brief photoinhibition of Dbx1 preBötC neurons alters breathing in a phase dependent manner.

We repeated these phase-response experiments in awake unrestrained Dbx1;ArchT mice. The plot of $\Phi_{\text{Shift}}$ vs $\Phi_{\text{Stim}}$ was qualitatively similar throughout the breathing cycle for the sedated and awake animal (compare Fig. 2.4A1 and 2.4B1). However, no trend was apparent in phase-diagrams for $\Delta V_T$ (Fig. 2.B2), $\Phi_{N+1}$, and $\Delta T_i$ (Fig. 2.S2B). Light application during late inspiration (0-30º) resulted in a $\Phi_{\text{Shift}}$ of -45 ± 12º, a phase advance ($p = 0.02$, $n = 4$) (Fig. 2.4B1 and B3 top trace). The first significant phase delay in the awake animal occurred during expiration (180-210º), where $\Phi_{\text{Shift}}$ measured 46 ± 15º ($p = 0.01$, $n = 4$) (Fig. 2.4B1). The upward trend in $\Phi_{\text{Shift}}$ continued and the maximal phase delay of 128 ± 21º occurred during inspiration (300-330º, $p = 1e-6$, $n = 4$). During the period just before peak inspiratory effort (i.e., 330-360º) $\Phi_{\text{Shift}}$ was still positive (87 ± 22º, $p = 0.002$, $n = 4$), but slightly less than $\Phi_{\text{Shift}}$ at its maximum (Fig. 2.4A3). This downward trend in $\Phi_{\text{Shift}}$ near peak inspiration can be attributed (like in the sedated animal) to a bifurcated response to preBötC illumination where the stimulus either causes a phase delay or has no effect. These data indicate that brief photoinhibition of Dbx1 preBötC neurons alters the timing of breathing in a phase-dependent manner, which suggests effects within the core oscillator microcircuit.
Figure 2.4 Effects of brief photoinhibition on the phase and magnitude of inspiratory breaths. All data were obtained from Dbx1;ArchT mice (A: n = 6, B: n=5, cyan) and their wild type littermates (n = 6, magenta). A1, Phase-response curve plotting $\Phi_{\text{Shift}}$ following 100-ms photoinhibition at $\Phi_{\text{Stim}}$ throughout the breathing cycle in sedated mice. $\Phi_{\text{Stim}}$ has been collected across all animal subjects in 12 equal size bins (30º per bin) in A and B. A2, Phase-response curve showing changes in $V_T$ following photoinhibition (i.e., the perturbed breath) in the same cohort of sedated mice. A3, Sample airflow traces from a representative sedated Dbx1;ArchT mouse ($\Phi_{\text{Stim}}$ is indicated). B1, Phase-response curve plotting $\Phi_{\text{Shift}}$ following 100-ms photoinhibition at $\Phi_{\text{Stim}}$ throughout the breathing cycle in awake unrestrained mice. B2, Phase-response curve showing changes in $V_T$ following photoinhibition (i.e., the perturbed breath) in the same cohort of awake unrestrained mice. The abscissa maps the breathing cycle (0-360º) to inspiratory (I) and expiratory (E) phases, which applies to all phase-response curves in A and B. B3, Sample airflow traces from a representative awake unrestrained Dbx1;ArchT mouse ($\Phi_{\text{Stim}}$ is indicated).
Figure 2.S2 Effects of brief photoinhibition and photostimulation on inspiratory time and the phase of subsequent breaths. All data in A and B were obtained in Dbx1;ArchT mice (A: n=5, B: n = 6, cyan) or their wild type littermates (n = 6, magenta). A1, Phase-response curve plotting the phase of the breath following the perturbed cycle ($\Phi_{N+1}$) in response to 100-ms photoinhibition at $\Phi_{stim}$ throughout the breathing cycle in sedated mice. A2, Phase-response curve for changes in $T_i$ following photoinhibition (i.e., the perturbed breath) in the same cohort of sedated mice. B1, Phase-response curve plotting the phase of the breath following the perturbed cycle ($\Phi_{N+1}$) following 100-ms photoinhibition at $\Phi_{stim}$ throughout the breathing cycle in awake unrestrained mice. B2, Phase-response curve for changes in $V_T$ following photostimulation (i.e., the perturbed breath) in the same cohort of awake unrestrained mice. All data in C and D were obtained in Dbx1;CatCh mice (n = 4, cyan) or their wild type littermates (n = 4, magenta). C1, Phase-response curve plotting the phase of the breath following the perturbed cycle ($\Phi_{N+1}$) following 100-ms photostimulation at $\Phi_{stim}$ throughout the breathing cycle in sedated mice. C2, Phase-response curve for changes in $T_i$ following photostimulation (i.e., the perturbed breath) in the same cohort of sedated mice (n = 4). D1, Phase-response curve plotting the phase of the breath following the perturbed cycle ($\Phi_{N+1}$) following 100-ms photostimulation at $\Phi_{stim}$ throughout the breathing cycle in awake unrestrained mice. D2, Phase-response curve for changes in $V_T$ following photostimulation (i.e., the perturbed breath) in the same cohort of awake unrestrained mice. The abscissae in C2 and D2 maps the breathing cycle (0-360º) to inspiratory (I) and expiratory (E) phases, which applies to all phase-response curves in A-D.
**CatCh mediated excitation of Dbx1 preBötzC neurons enhances breathing and modifies both the phase and amplitude of individual breaths**

We photostimulated Dbx1 preBötzC unilaterally in sedated adult Dbx1;CatCh mice using long duration (5 s) bouts of 473 nm light at three laser intensities, which increased breathing in all instances (Fig. 2.5A). Frequency, ~3 Hz in control, increased significantly by 0.8 Hz (t-test, p = 0.03, n = 4) in response to the lowest intensity light (6.8 mW). Tidal volume, ~0.1 ml in control, increased by 0.06 ml (t-test, p = 0.3, n = 4) and MV, ~50 ml/min in control, increased by 18 ml/min (t-test, p = 0.2, n = 4) but neither of these changes was statistically significant (Fig. 2.5A and B).

Frequency increased to a greater extent, by 1.1 and 1.3 Hz, respectively, in response to 8.6 and 10.2 mW intensity light applications (t-test, p = 0.05 and p = 0.03, n = 4) (Fig. 2.5A and B). Tidal volume changed by 0.03 and 0.01 ml in response to 8.6 and 10.2 mW illumination (t-test, p = 0.40 and p = 0.3, n = 4) but those changes were not significant (Fig. 2.5A and B). Minute ventilation decreased by 10.9 and 19.6 ml/min in response to 8.6 and 10.2 mW illumination, respectively (t-test, both p = 0.2 and p = 0.4, n = 4) neither passing the threshold of statistical significance (Fig. 2.5B). These results show that CatCh-mediated Dbx1 preBötzC neuron excitation enhances the frequency of breathing, but not tidal volume or minute ventilation, in sedated mice.
We repeated these photostimulation experiments in Dbx1;CatCh mice while awake and unrestrained. The lowest intensity light (6.8 mW) minimally affected breathing (Fig. 2.5C). Frequency increased by 0.6 Hz (t-test, p = 0.16, n = 4) (Fig. 2.5D) and MV increased by 2 ml/min (t-test, p = 0.07, n = 4) (Fig. 2.5D). Tidal volume, however, decreased by 0.04 ml (t-test, p = 0.27, n = 4) (Fig. 2.5D). None of these light-induced changes were significant.
In awake animals $f$ increased by 1.1 and 1.6 Hz, respectively, in response to 8.6 and 10.2 mW intensity light application ($t$-test, $p = 0.051$ and $p = 0.04$, $n = 4$), the latter effect reaching statistical significance (Fig. 2.5C and D). Minute ventilation increased by 2 ml/min at a light intensity of 8.6 mW ($t$-test, $p = 0.12$, $n = 4$), but decreased by 0.2 ml/min at the highest intensity of 10.2 mW ($p = 0.49$, $n = 5$) (Fig. 2.5D). Neither change reached statistical significance. Similarly, $V_T$ increased at the intermediate intensity (8.6 mW), by 0.04 ml ($t$-test, $p = 0.1$, $n = 4$), and decreased at the highest intensity (10.2), by 0.05 ml ($t$-test, $p = 0.1$, $n = 4$). Again, neither change reached statistical significance.

Conversely, in wild-type littermates no light application at any intensity modified breathing in either sedated or awake states (Fig. 2.S3). These data show that CatCh-mediated Dbx1 preBöC neuron photostimulation enhances the frequency of breathing, while not significantly altering other respiratory parameters.

**Figure 2.S3** Light application to the preBöC does not affect breathing in wild-type Dbx1;CatCh littermates. A, Airflow traces from a sedated wild type mouse (littermate of Dbx1;CatCh mice) exposed to 5-s bouts of unilateral preBöC illumination at three intensities (units of mW). B, Group data from experiments in A quantifying $f$, $V_T$, and MV in response to light application. Symbols show mean $f$, $V_T$, and MV in each mouse. Bars show the mean and SD for all animals tested ($n = 4$). C, Traces from an awake unrestrained wild type mouse (littermate of Dbx1;CatCh mice) exposed to 5-s bouts of unilateral preBöC illumination at three intensities (units of mW). D, Group data from experiments in C quantifying $f$, $V_T$, and MV in response to light application. Symbols show mean $f$, $V_T$, and MV in each mouse. Bars show the mean and SD for all animals tested ($n = 6$).
Next we applied brief (100 ms) light pulses to assess phase dependent changes in breathing in response to photostimulation. Illumination of the preBötC during late inspiration and early expiration (i.e., $\Phi_{\text{Stim}}$ of 0 to 60º) increased the time to the next breath (phase delay) with a maximal $\Phi_{\text{Shift}}$ of 249 ± 22º ($\Phi_{\text{Stim}}$ of 0-30º, $p = 1e^{-6}, n = 4$) (Fig. 2.6A₁ and A₃ top trace). As $\Phi_{\text{Stim}}$ progressed through the range 0 to 120º, we observed a monotonic trend approaching phase advances. $\Phi_{\text{Shift}}$ crossed from phase delay to phase advance at $\Phi_{\text{Stim}}$ of ~90º. The maximum phase advance occurred at $\Phi_{\text{Stim}}$ of 120-150º with a $\Phi_{\text{Shift}}$ of -118 ± 18º ($p = 1e^{-6}, n = 4$) (Fig. 2.6A₁). Phase delays continued to be observed throughout the expiratory phase and into the early inspiratory phase (90 to 300º). The last statistically significant phase delay occurred at $\Phi_{\text{Stim}}$ of 210-240º with $\Phi_{\text{Shift}}$ of -33 ± 6º ($p = 0.1, n = 4$). After $\Phi_{\text{Stim}}$ of 240º no significant changes in $\Phi_{\text{Shift}}$ were observed in response to brief photostimulation.

Brief light pulses had significant effects on $V_T$ during late inspiration and early expiration (0 to 150º) but no significant changes occurred after $\Phi_{\text{Stim}}$ of 150º. Maximal $\Delta V_T$ occurred at $\Phi_{\text{Stim}}$ of 0-30º with a change of 2.0 ± 0.6 ($p = 1e^{-5}, n = 4$) (Fig. 2.6A₂). At $\Phi_{\text{Stim}}$ of 120-150º the last statistically significant $\Delta V_T$ occurred ($\Phi_{\text{Shift}}$ of 0.44 ± .12, $p = 0.05, n = 4$) (Fig. 2.6A₁). The reason for large effects on VT can be gleaned from the sample traces (Fig. 2.6A₃). Because the animal does not return to near FRC the VT of the perturbed breath appears much larger than the cycle preceding, hence a large $\Delta V_T$. We observed no interpretable relationship between the phase of the subsequent breath ($\Phi_{N+1}$) and inspiratory
time ($\Delta T_i$) (Fig. 2.S2C). These data indicate that brief photostimulation of Dbx1 preBötC neurons alters breathing in a phase dependent manner. We repeated these phase-response experiments in awake intact Dbx1;CatCh mice. The plots of $\Phi_{\text{Shift}}$ and $\Delta V_T$ vs $\Phi_{\text{Stim}}$ were qualitatively similar throughout the breathing cycle for the sedated and awake animal (compare Fig. 2.6A1 and 6B1).

Light application during late inspiration (i.e., $\Phi_{\text{Stim}}$ of 0-30°) increased the time to the next breath with $\Phi_{\text{Shift}}$ of 150 ± 50 ($p = 6e^{-6}, n = 4$) (Fig. 2.6B1 and B3 top trace). As $\Phi_{\text{Stim}}$ progressed from 0 to 120° we observed a monotonic trend approaching the maximal phase delay. $\Phi_{\text{Shift}}$ crossed from phase delay to phase advance at $\Phi_{\text{Stim}}$ of ~45°. The maximal phase advance occurred at $\Phi_{\text{Stim}}$ of 90-120° with $\Phi_{\text{Shift}}$ of -129 ± 10° ($p = 3e^{-6}, n = 4$) (Fig. 2.6B1). The last statistically significant phase delay occurred at $\Phi_{\text{Stim}}$ of 180-210° with $\Phi_{\text{Shift}}$ of -52 ± 6° ($p = 6e^{-4}, n = 4$). After $\Phi_{\text{Stim}}$ of 210° no significant changes in $\Phi_{\text{Shift}}$ were observed.

Light application had significant effects on $V_T$ during late inspiration and early expiration (0 to 120°) but no significant changes occurred for $\Phi_{\text{Stim}}$ exceeding 120°. Maximal $\Delta V_T$ occurred at $\Phi_{\text{Stim}}$ of 0-30° with a change of 2.6 ± 0.6 ($p = 1e^{-6}, n = 4$) (Fig. 2.6B2). The effect on $V_T$ decreases until $\Phi_{\text{Stim}}$ of 90-120° where the last statistically significant $\Delta V_T$ is observed ($\Phi_{\text{Shift}}$ of 0.97 ± 0.18, $p = 0.02, n = 4$) (Fig. 2.6B1). We observed no interpretable relationship between the phase of the subsequent breath ($\Phi_{N+1}$) and inspiratory time ($\Delta T_i$) due to brief photostimulation (Fig. 2.S2C). These data indicate that brief photostimulation of Dbx1 preBötC alters breathing in a phase dependent manner, which suggests effects on core rhythmogenic microcircuits for breathing.
Figure 2.6 Effects of brief photostimulation on the phase and magnitude of inspiratory breaths. All data were obtained from Dbx1;CatCh mice (n = 4, cyan) and their wild type littermates (n = 4, magenta). A1, Phase-response curve plotting $\Phi_{\text{Shift}}$ following 100-ms photostimulation at $\Phi_{\text{Stim}}$ throughout the breathing cycle in sedated mice. $\Phi_{\text{Stim}}$ has been collected across all animal subjects in 12 equal size bins (30° per bin) for A and B. A2, Phase-response curve for changes in $V_T$ following photostimulation (i.e., the perturbed breath) in the same cohort of sedated mice. A3, Sample airflow traces from a representative sedated Dbx1;CatCh mouse ($\Phi_{\text{Stim}}$ is indicated). Integrated airflow is shown in black. B1, Phase-response curve plotting $\Phi_{\text{Shift}}$ following 100-ms photostimulation at $\Phi_{\text{Stim}}$ throughout the breathing cycle in awake unrestrained Dbx1;CatCh mice. B2, Phase-response curve for changes in $V_T$ following photostimulation (i.e., the perturbed breath) in the same cohort of awake unrestrained mice. The abscissa maps the breathing cycle (0–360°) to inspiratory (I) and expiratory (E) phases, which applies to all phase-response curves in A and B. B3, Sample airflow traces from a representative awake unrestrained Dbx1;ArchT mouse ($\Phi_{\text{Stim}}$ is indicated). Integrated airflow is shown in black.
2.5 Discussion

The preBötC of the ventral medulla contains the inspiratory core oscillator but it is a heterogeneous region. Determining its neuronal core constituents represents a significant milestone for understanding breathing. Dbx1 neurons in the preBötC, a predominately glutamatergic population that expresses markers commonly associated with respiratory rhythmogenesis, e.g., neuropeptides and peptide receptors (Gray et al., 2001, 2010, Stornetta et al., 2003a, 2003b; Tan et al., 2008), potentially defines the core oscillator and thus achieves this milestone. The formation and subsequent functioning of the preBötC in perinatal animals depends on Dbx1 preBötC neurons (Bouvier et al., 2010; Gray et al., 2010; Wang et al., 2014). Evidence supporting Dbx1 neurons as the constituents of the core oscillator in adult animals is limited by methodology where studies were conducted in reduced preparations or the results were inconclusive (Cui et al., 2016; Koizumi et al., 2016; Vann et al., 2016). Further, recent studies into subpopulations of Dbx1 neurons in adult animals suggests non-rhythmogenic functions are widespread in the preBötC (Cui et al., 2016; Yackle et al., 2017), which necessitates further evaluation of the Dbx1 core hypothesis.

We reasoned that if Dbx1 neurons form the respiratory core oscillator that prolonged silencing or stimulating them would depress or enhance breathing frequency, respectively, over multiple breathing cycles. We further hypothesized that transient silencing or stimulating Dbx1 preBötC neurons would alter breathing timing by inducing phase-dependent changes in breaths as expected
for an oscillator (note: phase-independent effects would be expected in the pattern-generating component of CPG circuits).

Here we show that photoinhibition and photostimulation of Dbx1 preBötC neurons profoundly affects breathing in both sedated and awake animals. Using prolonged applications of graded laser strengths, we show corresponding graded effects on breathing with greater intensities resulting in greater effects. Photoinhibition produced apnea in 11 of 30 bouts in sedated animals and in one bout in an awake animal at the greatest intensity (Fig. 2.3A and C, bottom traces). Photostimulation produced hyperventilation in sedated animals and awake animals at the highest intensity (Fig. 2.3A and C, bottom traces). We then used transient light applications to investigate phase-dependent changes in breathing timing. Using transient photoinhibition, we show that silencing Dbx1 neurons during late inspiration produces a breath earlier than anticipated and a delayed inspiratory effort when silencing occurred after peak expiration. Conversely, using transient photostimulation, we show that exciting Dbx1 neurons during late inspiration produced an augmented breath and delayed the next breath and photostimulation during expiration induced an inspiratory effort. Because of their ability to reset or otherwise influence the phase of breaths, and their ability to modulate breathing frequency, these experiments indicate that Dbx1 neurons comprise the inspiratory rhythmogenesic microcircuit.

We aimed to test the Dbx1 core hypothesis under normal physiological conditions. Breathing with sensorimotor feedback intact is a robust behavior. The vagus nerve, which provides mechanosensitive feedback, and the carotid sinus
nerve, which provides tonic drive from peripheral chemoreceptors, were left intact. Both the vagus and carotid sinus nerves are necessary for robust and normal eupneic breathing (Feldman et al., 1990; Sheikhbahaei et al., 2017) and leaving ascending feedback intact preserves the level of excitability within the preBöC. In an awake intact nonvagotomised unrestrained mouse we show the first example of apnea produced by silencing Dbx1 neurons. Apnea in a freely behaving animal from silencing the Dbx1 population indicates that Dbx1 preBöC neurons are essential for breathing and provides the strongest evidence to date supporting the Dbx1 core hypothesis.

**Effects are predominately rhythmogenic**

Both photoinhibition and photostimulation experiments produces predominantly breathing frequency changes, although tidal volume and minute ventilation effects are recorded as well. That photoinhibition and photostimulation evokes changes in frequency supports the hypothesis that Dbx1 neurons make up the core respiratory microcircuit.

Dbx1 neurons within the preBöC and intermediate reticular formation (IRT) have a premotor function (Revill et al., 2015; Wang et al., 2014). Could effects on Dbx1 premotor neurons explain the data, instead of effects on the core oscillator? Although V\textsubscript{T} is reduced in photoinhibition experiments, this decrease only reaches statistical significance in sedated animals at the highest laser strength, so the effect is not a general one. We aimed to limit photoinhibition of Dbx1 premotor neurons by using targeting illumination to the preBöC selectively, which precludes photoinhibition of Dbx1 IRT neurons. Furthermore,
photostimulation experiments, which were limited to the preBötC by viral
injection, had no significant effects on \( V_T \). Even if some Dbx1-derived premotor
neurons in the preBötC or neighboring regions of the IRT were affected by my
illumination protocols, such an effect would not contradict the conclusion that
Dbx1 neurons play the key rhythmogenic role. Frequency changes were the most
significant throughout this study and can only be attributed to manipulations that
impact the core oscillator.

**Neurons drive rhythmogenesis**

Neurons perform the vast majority of brain computations, including rhythm
generation in CPGs, breathing not withstanding (Kandel et al., 2000; Shepherd,
2015). However, the role of neuroglia in brain function (Bains, 2017), including
respiratory function, has become a topic of interest. For example glia have
proposed roles in modulating the respiratory circuit, particularly in the area of
chemosensation (Gourine et al., 2010; Huxtable et al., 2010), and one group has
even suggested a direct role in rhythm generation (Okada et al., 2012), but that
remains a highly debated, and at this time, dubious issue for most in the field.

Evidence from photostimulation experiments helps cement neurons as the
cellular population that forms the core respiratory microcircuit. CatCh
recombination was induced using a synapsin promoter and therefore it is safe to
assume that only neurons would be transfected and express CatCh protein.
Similarly, the expression of ArchT is limited in the preBötC by the timing of
tamoxifen administration, where studies from our lab have shown that properly
inducing Cre/lox recombination at E9.5 dramatically reduces fusion protein
expression in Dbx1 preBötC glia, but does not diminish fusion protein expression in Dbx1 preBötC neurons, which again, gives us a high degree of confidence that optogenetic perturbations act largely on neurons (not glia) and that neurons are the predominate rhythmogenic constituents and the most parsimonious explanation for the light-induced changes in breathing. These results do not rule out important supporting contributions from Dbx1-derived preBötC glia but those contributions remain to be identified.

**Breathing effects are attributable to postsynaptic mechanisms**

One potential caveat of the ArchT experiments is the possibility that illumination within the preBötC affects axon terminals of Dbx1 neurons whose cell bodies are outside of preBötC, and that photoinhibition actually disfacilitates the preBötC and thus causes the breathing phenotype (i.e., reduction in frequency up to apnea). Because expression of optogenetic proteins is not constrained to the cell bodies of Dbx1 neurons one must consider photoinhibition of axons and axon terminals of distant neurons. Given that Dbx1-derived neurons are present throughout medulla (Ruangkittisakul et al., 2014), not limited to the preBötC, it is likely that Dbx1 neurons outside the preBötC project to the preBötC. Axon terminals that express optogenetic fusion proteins remain viable in transverse slices and therefore, illumination of ArchT-EGFP-expressing axon terminals from neurons not located within the preBötC could conceivably remove excitation from the rhythmogenic core and slow down or stop breathing (Cui et al., 2016; Holloway et al., 2013). It has been previously shown that axons from the chemosensitive retrotrapezoid nucleus remain viable in transverse slices.
containing the preBötC and their optogenetic stimulation can modulate breathing rhythm (Holloway et al., 2013). Furthermore, axons from Sst-expressing neurons remote from the preBötC but which send input to it can be evoked optogenetically in vivo, exerting profoundly inhibitory effects on breathing (Cui 2016).

While we cannot rule out the interpretation that ArchT activation disfacilitates the preBötC via axons of remote origin, we argue that direct postsynaptic effects in Dbx1 preBötC neurons are predominate. Slice experiments provide negligible evidence of light-induced disfacilitation in Dbx1;ArchT mice. Light application hyperpolarized Dbx1 neurons by ~11 mV in the context of network activity and ~9 mV in TTX, which is consistent with direct postsynaptic photoinhibition (Fig. 2.1). Further, illumination resulted in ~1 mV of hyperpolarization in non-Dbx1 neurons at a laser strength of 10.2 mW and less than 1 mV at all other laser strengths in the context of network activity as well as at 10.2 mW in TTX. If disfacilitation were the proximal perturbation to the preBötC, then one would have expected TTX to diminish significantly the post-synaptic hyperpolarization of Dbx1 preBötC neurons. Furthermore, one would expect that non-ArchT-expressing non-Dbx1 preBötC neurons, which presumably also receive inputs from Dbx1-derived neurons that project to the preBötC in this scenario, would have exhibited robust light-mediated hyperpolarization, which would have also been significantly attenuated by TTX. The insignificant reduction in light-mediated $\Delta V_M$ of non-Dbx1 preBötC neurons, and the less than 2 mW reduction in light-mediated $\Delta V_M$ in TTX conditions for Dbx1 preBötC neurons suggest that postsynaptic effects, and not a
reduction of tonic excitatory exogenous drive to Dbx1 preBötC neurons is the predominant effect in the preBötC. Therefore, it would be unlikely for disfacilitation of the preBötC to be the source of Dbx1 neurons significant hyperpolarization and therefore rhythm cessation.

Additionally, breathing changes induced by preBötC illumination in Dbx1;CatCh mice cannot be explained by additional excitatory drive from axon terminals and axons of passage that originate outside of, but synapse within, the preBötC. By using both genetic and viral mediated recombination expression, CatCh expression is limited predominately to the preBötC; fusion protein expression outside of the preBötC does not occur, beyond the very limited range of tissue directly adjacent to the preBötC or tissues traversed by the microsyringe needle used to introduce the viral construct. Those sources of error cannot be quantitatively evaluated, but given the evidence of fusion protein expression throughout the preBötC, and the dorsal approach for microinjection, we argue that only adjacent circuits of IRT could possibly be affected. The dominant projections are directed from preBötC to IRT, not the other way around (Revill et al., 2015; Song et al., 2016; Wang et al., 2014) so even if some IRT Dbx1 neurons are affected, they are highly unlikely to influence the preBötC because the information flow in the microcircuit is largely unidirectional from preBötC to IRT, and then to the final common path, i.e., the XII motoneurons. Furthermore, the IRT projects to XII which is responsible for upper airway patency and not driving the diaphragm (we measured ventilation, not tongue protrusion and airway patency). Therefore, by using a "double stop" approach to limit fusion
protein expression in preBötC we ensure that breathing phenotypes in CatCh experiments are due to postsynaptic mechanisms only.

**Does a respiratory refractory period exist and influence breathing rhythm?**

Breathing is a phasic behavior, with alternating periods of inspiration and expiration. The maximum rate that breathing can achieve is limited by the inspiratory and expiratory times and, presumably, the minimum time for the network to reset and initiate another effort to breathe, a refractory period. Refractory periods have been observed in many preparations using a multitude of excitation methods (Alsahafi et al., 2015; Cui et al., 2016; Del Negro et al., 2009; Kam et al., 2013a; Kottick and Del Negro, 2015). *In vitro* refractory periods of 500 ms to 2 s have been observed (Kam et al., 2013a; Kottick and Del Negro, 2015). *In vivo* refractory periods of 100-200 ms have been reported (Alsahafi et al., 2015; Cui et al., 2016). The disparity of refractory periods *in vitro* and *in vivo* are probably attributable to differences in temperature and neural excitability. The presence of a refractory period in all the aforementioned studies suggests that a breath cannot be evoked immediately after a preceding breath. One problem with that framework for interpretation is that almost all studies (even those *in vivo*) are done in the context of deep anesthesia, which can obscure or otherwise modify the function of the preBötC or other parts of the respiratory CPG.

We show that in adult animals photostimulation during late inspiration and early expiration can induce a breath earlier than expected. Although, we induce inspiration in earlier phases than other studies (Alsahafi et al., 2015; Cui et al., 2016) there is a quiescent period before a secondary inspiratory effort can be
induced and therefore a refractory period was observed. However, the
calculation of an absolute refractory period was confounded by photostimulation
inducing an augmented inspiratory effort during early segments of late inspiration
(Fig. 2.6A, top trace). These data suggest that prior calculation of refractory
periods based on phase reset analyses that did not induce breaths during early
expiration may overestimate the time needed for the core-microcircuit to reset.
However, breathing refractory periods are conserved in awake intact adult mice.

**Excitatory neurons are responsible for respiratory timing**

Considerable differences between our results and previous investigations of
breathing *in vivo* using photostimulation exist with regards to phase-reset
experiments and long-pulse experiments. However, by interpreting those
disparities in the context of methodological differences, one can make sense of
the disparities and come to a better understanding of the preBötC core and its
operation.

Differences in phase-curves exist between the present study and previous
literature. Earlier studies were unable to induce inspiratory efforts during early
expiration (Alsahafi et al., 2015; Cui et al., 2016) whereas we were able to evoke
inspiratory breaths during late inspiration and early expiration. Differences with
Alsahafi et al. can be attributed to populations targeted by optogenetic fusion
proteins. We targeted Dbx1 (i.e., excitatory) preBötC neurons whereas Alsahafi
et al. targeted all preBötC neurons by using a syanspin promoter to virus-
transfect neurons with ChR2. Excluding the inhibitory neurons within the preBötC
from photostimulation would lead to an increase in network excitability and could explain earlier subsequent breath induction during phase-reset experiments.

However, Cui et al. conducted their study in Dbx1 neurons, as we did, using similar intersectional mouse genetic approaches. Two major differences in methodology exist between the present study and Cui et al. First, Cui et al. deeply anesthetized their animals using ketamine and xylazine. However, sedated animals used in the present study were only given low dose ketamine and awake animals were completely intact and unrestrained. Xylazine is a known respiratory depressant (Erhardt et al., 1984; Green et al., 1981) and a such would reduce the excitability of the preBötC, which could counteract photostimulation. Moreover, there were differences in laser strength: Cui et al. used 7 mW whereas we applied 10.2 mW. We show that breathing phase can be reset during early expiration and late inspiration probably because mice used in this study had a higher overall level of baseline neuronal excitability and were exposed to light at a higher intensity.

Previous long duration photostimulations of preBötC neurons in rats produced an increase in frequency with a decrease in flow and $V_T$ (Alsahafi et al., 2015). We show changes in frequency like Alsahafi et al. but no decrease in respiratory flow or $V_T$. The most parsimonious explanation for the disparity is that Alsahafi et al. targeted all preBötC neurons, both excitatory and inhibitory, while we targeted Dbx1 preBötC neurons, almost exclusively excitatory. Given that our experiments (stimulating purely excitatory neurons) caused no changes in VT or flow, then photostimulation of inhibitory and excitatory preBötC neurons by Alsahafi et al.
would have a net inhibitory or at minimum a diminished excitatory effect within the preBötC, which resulted in the reduced flow and $V_T$.

**Non-rhythmogenic roles of Dbx1 subpopulations in preBötC**

Genetically defined distinct subpopulations of Dbx1 neurons have been identified and are proposed to have non-rhythmogenic function. Cdh9 and Dbx1 coexpressing neurons, identified using Cdh9 protein, have a proposed role in arousal and comprise up to 56% of the Dbx1 preBötC population (Yackle et al., 2017). Additionally, Sst and Dbx1 coexpressing neurons have a proposed premotor functions and make up 13-17% of Dbx1 preBötC neurons (Cui et al., 2016). These populations together make up to 73% of the Dbx1 population within the preBötC. Given the large fraction of Dbx1 preBötC neurons recounted above, is it possible that the respiratory effects shown could be attributed to the Dbx1 and Cdh9 coexpressing population (Dbx1/Cdh9 neurons) or the Dbx1 and Sst coexpressing population (Dbx1/Sst neurons)?

Given that we could not exclude Dbx1/Cdh9 neurons from optogenetic targeting and large proportion of Dbx1 neuron express Cdh9 it is certain that we affected those neurons with optogenetic perturbations. However, it is unlikely that our results are attributable to photoinhibition or photostimulation of Dbx1/Cdh9 neurons because we show a direct effect on respiratory measures during eupnea. In Yackle et al., changes in breathing frequency due to experimental manipulation of Dbx1/Cdh9 neurons were attributed to the duration of time the animal subjects spent in different behavioral paradigms (i.e., eupnea, grooming, active, or sniffing). In contrast, our optogenetic perturbations (which modulate
frequency) were only applied, by experimental design, during eupnea. This contrast emphasizes that our protocols are directed to the core oscillator circuit whereas Yackle and colleagues perturbed a subpopulation with respiratory-related function but not its core oscillator. During different behaviors, respiratory needs increase or decrease, and therefore call for alterations in ventilation to accommodate metabolic need. The authors, Yackle et al., argue that Dbx1/Cdh9 influence basal levels of arousal in the brain, via direct projections to the locus coeruleus, rather than rhythmogenesis per se (Yackle et al., 2017) and we concur with that interpretation.

Differences in Cre/lox recombination may explain the high coexpression between Dbx1 and Cdh9. Yackle et al. used a Dbx1 constitutive Cre mouse (Dbx1-Cre) whereas we used an inducible Dbx1-Cre driver mouse (Dbx1\(^{CreERT2}\)) in which the estrogen receptor agonist tamoxifen limits Cre/lox recombination based administration timing during embryonic development. Offspring from the intersection of Dbx1-Cre and Cre-dependent reporter strains have a greater density of reporter expression in Dbx1-derived cells (neurons and glia) in regions more dorsal than the preBötC (Kottick et al., 2017). Additionally, with properly timed tamoxifen administration there is negligible difference in the number of Dbx1 neurons in offspring from the intersection of Dbx1-Cre and Dbx1\(^{CreERT2}\) mice, coupled with a suitable reporter strain, in analyses centered within the preBötC. However, expression outside the preBötC is limited in the offspring of Dbx1\(^{CreERT2}\) mice to smaller bands extending from the preBötC to XII (Kottick et al., 2017). Given we limited reporter expression predominately to the center of
the preBötC by virtue of using Dbx1\textsuperscript{CreERT2} driver mice, and that Dbx1/Cdh9 neuron ablation causes no deleterious respiratory phenotype, we contend that our results can be directly attributed altering the preBötC microcircuit and not higher brain centers.

Dbx1/Sst neurons, which are proposed to have premotor function, could be a source of some results shown, particularly the induction of apnea. Perturbations affecting Sst-expressing preBötC neurons have considerable effects on breathing (Cui et al., 2016; Koizumi et al., 2016); these effects are strong enough to completely stop all breathing movements in adult intact rats (Tan et al., 2008). However, Sst-expressing preBötC neurons have been shown to have limited effect on respiratory timing and strong effects on strength of output suggesting that Sst neurons are predominately premotor in nature, or interneurons in the larger pattern-generating system. Additionally, coexpression between Dbx1 and Sst limited; only 13-17% of Dbx1 preBötC neurons express Sst (Bouvier et al., 2010; Gray et al., 2010). Given that our results show an effect on breathing were predominantly attributable to changes in frequency and the few number of Dbx1/Sst neurons, it is unlikely that my results can be predominately attributed to perturbation of Dbx1/Sst neurons.

**Size of the Dbx1 core oscillator and burstlet theory**

If Cdh9 and Sst subpopulations of Dbx1 preBötC neurons, which are putatively non-rhythmogenic, are independent of the core respiratory oscillator, then only a small fraction (~27%) of Dbx1 neurons are available for rhythmogenesis. The total number of Dbx1 neurons within the preBötC has been estimated to be
approximately 600 neurons (Kottick et al., 2017; Wang et al., 2014) and if one excludes Cdh9 and Sst neurons from this estimation, then as few as only 160 Dbx1 preBötC neurons would remain for rhythmogenesis (assuming subpopulations cannot serve more than one function). Is it possible that a small number of neurons can comprise the respiratory core oscillator?

Cell-specific holographic glutamate uncaging studies have shown that stimulation of as few as 4-9 preBötC neurons can evoke fictive respiratory output in vitro (Kam et al., 2013a). Although stimulation was not limited to Dbx1 preBötC neurons (there was no Dbx1-reporter system in Kam et al.’s study) calcium imaging was used to select targets with strong correlation to respiratory output and therefore more likely to select neurons central to respiratory rhythmogenesis. This method would likely exclude Cdh9 neurons that were weakly respiratory active or had tonic or non-respiratory firing patterns (Kam et al., 2013a; Yackle et al., 2017). Sst-expressing and inhibitory preBötC populations (Kuwana et al., 2006; Morgado-Valle et al., 2010; Winter et al., 2009) would likely not be excluded from selection in holographic glutamate uncaging experiments and selection of Sst-expressing and inhibitory neurons may explain the variability in the number of targets needed to induce a burst. Nonetheless, small numbers of neurons are capable of inducing fictive breathing, which lends credence to the notion that a small subfraction of Dbx1 preBötC neurons could be rhythmogenic in the midst of a potentially larger population of non-rhythmogenic preBötC neurons.
Fortuitously, there is a new rhythmogenic paradigm that might explain small-population rhythmicity. Subthreshold rhythmic oscillations, or burstlets, have been recorded in the preBötC (Kam et al., 2013b) – note that is the same author, K. Kam, and same year, 2013, but a unique paper from the one referenced above, which could suggest that subsets of rhythmically active neurons provide the underlying drive for breathing. Burstlets were shown to be 10-30% smaller than field recordings of the preBötC (Kam et al., 2013b) which aligns with the relatively few number of Dbx1 neurons available for rhythmogenesis.

Given that breaths can be evoked by glutamate-mediated stimulation of small numbers of neurons, the close relationship between the small number of potential core respiratory rhythmogenic Dbx1 preBötC neurons, and the relative size of burstlets, I argue that it could be possible for only 27% of Dbx1 preBötC neurons to form the respiratory core microcircuit.

**Dbx1 neurons are the inspiratory core microcircuit.**

Dbx1 preBötC neurons are necessary for respiratory rhythmogenesis in perinatal mice (Bouvier et al., 2010; Gray et al., 2010; Wang et al., 2014). However, commensurate evidence in support of the Dbx1 core hypothesis in awake unrestrained adult mice was nonexistent prior to this study. By testing the Dbx1 core hypothesis in adults, we show that photoinhibition and photostimulation of Dbx1 neurons has a profound effect on breathing in both sedated and awake animals, both with fully intact sensorimotor integration. We show that photoinhibition of Dbx1 neurons produces apnea and that photostimulation produces hyper-ventilation. Further, transient photoinhibition and
photostimulation can phase shift and reset the breathing rhythm in ways indicative of effects on the core rhythmogenic circuits. Importantly, we show a prolonged apnea in an awake freely behaving animal by silencing Dbx1 neurons, which suggests that despite all levels of tonic drive from supramedullary brain centers, as well as the rhythm-promoting effects of mechanoreceptor and chemosensory feedback, taking Dbx1 preBötC neurons offline arrests breathing. Even considering the set of non-rhythmogenic Dbx1-derived neurons in preBötC, it is clear that there is a subset of Dbx1 preBötC neurons, not distinguished by Sst or Cdh9 expression, that is central to inspiratory rhythmogenesis. Identifying the core of the inspiratory breathing circuit will lead to a better understanding of the mechanisms that lead to rhythm formation and may reveal cellular and synaptic properties commonly applicable to rhythmic systems, such as CPGs, in the brain and central nervous system.

2.6 References


CONCLUSION

Central pattern generators (CPGs) are specialized networks that can produce rhythm in the absence of descending motor commands and sensory afferent feedback; that is, the ability to generate rhythmicity is intrinsic to the network itself (Grillner, 2003, 2006; Kiehn, 2016). CPGs are generally understood to have two components: one that produces the underlying rhythm and a second component that produces the spatiotemporal pattern of muscle activation, which is necessary to perform behaviors like walking, chewing, and breathing. While both rhythm and pattern are important to understand, this dissertation focuses principally on understanding rhythm, particularly the cellular composition of the rhythmogenic core of the CPG for breathing.

Breathing is an essential and unceasing behavior that must function perpetually from late embryonic stages of development to be ready at birth until death. By identifying the cellular and synaptic mechanisms that make its core oscillator rhythmic, we can deepen our understanding of neural mechanisms that regulate and thus maintain homeostasis as well as fundamental principles underlying rhythm generation, which is a ubiquitous brain function. However, we must first characterize the core microcircuit at the cellular level.

How the breathing rhythm is produced has long been a central topic in the neural control of respiration. The existence of an independent and central circuit for breathing in the lower brainstem was recognized as early as 1887 (for review see: Wyman, 1977), but its precise anatomical location and cellular constituency was unknown, and impossible to discern with the tools available at the time.
Because technological advances permit new and more powerful experiments, our collective ability to discover the underlying microcircuits that comprise the respiratory CPG has grown exponentially.

The state of knowledge regarding the neural plant for breathing remained largely unchanged from the late 1800’s until whole animal physiological investigations of the latter half of the 20th century showed that respiratory circuits were concentrated in the brainstem and pons. That early work into understanding the respiratory CPG was conducted predominately *in vivo*, using mammalian species such as cats, dogs, large farm animals, and ultimately rodents. However, discovery of the underlying sites and mechanisms was limited because the CPG was difficult to probe in whole animals, which remain refractory for most types of cellular recording and biophysical manipulations.

In 1984 a breakthrough study (Suzue, 1984), and a more detailed and thorough follow-up study in 1990 (Smith et al., 1990), it was demonstrated that the respiratory CPG could be isolated in *en bloc* brainstem-spinal cord or even 500-μm-thick transverse medullary slice preparations (Smith et al., 1991) that remained spontaneously rhythmically active, generating measurable rhythmic motor output from spinal and cranial nerves. These preparations contained all the necessary components to study the respiratory CPG *in vitro*: rhythm generator, premotor circuits, and motor output from cranial and spinal respiratory nerves (Smith et al., 1990, 1991). The reduced preparations provided investigators direct access to the newly discovered respiratory rhythmogenic site, the preBötzinger Complex (preBötC) under precisely controlled experimentally optimal conditions.
Over the next 20 years direct access to the rhythmogenic core resulted in a massive expansion of our knowledge of the breathing CPG. Early studies (1991 through approximately 2000) used direct electrical recordings of preBötC neurons to improve our understanding of the cellular and synaptic mechanism leading to rhythm generation (Connelly et al., 1992; Funk et al., 1993; Rekling and Feldman, 1998). Later studies focused on identification of the cellular composition of the core oscillator through peptide and peptide receptor expression, which appeared to mark the key rhythmogenic population (Gray et al., 1999, 2001; Guyenet et al., 2002; Hayes and Del Negro, 2007; Hayes et al., 2008; Stornetta et al., 2003a). These studies provided insights into preBötC function, but which cells (neurons) comprised its core remained elusive because many of the markers promiscuously labeled neighboring regions or were otherwise found lacking. Therefore, the cellular core constituents of the preBötC remained incompletely understood until at least a decade later.

Starting in the early 2000’s, a major paradigm shift in how neurons were classified occurred. Rather than emphasize electrical properties, this new scheme relied on developmental neurobiology and molecular genetics. According to developmental cues, governed by classes of transcription factors (i.e., genes that control other genes) specific classes of neurons could be identified using transcription factors, both pre- and post-mitotic. This developmental molecular genetic classification scheme was particularly effective in discovering structural and operational principles of the lumbar spinal cord in the study of locomotion (Alaynick et al., 2011; Goulding and Pfaff, 2005; Grillner and Jessell, 2009;
Jessell, 2000). The use of molecular genetics to identify neuronal subpopulations thus provided a new set of tools and a strategy for elucidating the cellular constituents of respiratory core oscillator.

Reports that appeared in 2010 reflected how the application of molecular genetics led to a major breakthrough to understanding the core oscillator. Two groups showed that a class of interneurons derived from Dbx1-expressing progenitors were essential for the development and function of the preBötC (Bouvier et al., 2010; Gray et al., 2010). Dbx1 knock-out mice died at birth and the preBötC, as recognized by conventional markers, did not exist (Bouvier et al., 2010; Gray et al., 2010). In rhythmically active slices from Dbx1 reporter mice, Dbx1 preBötC neurons were rhythmically active in sync with respiratory motor output (Picardo et al., 2013). Sequentially laser ablating Dbx1 preBötC neurons in vitro attenuated rhythmic motor output in slice preparations and slowed and then stopped the respiratory rhythm (Wang et al., 2014). These findings led directly to the Dbx1 core hypothesis, and most in the field accepted it as true shortly after the watershed 2010 studies. However, prior to the onset of my work the role of Dbx1 neurons in adult animals remained untested.

Given strong supporting evidence from experiments in perinatal animals, why would the Dbx1 core hypothesis need to be tested in adults? Transformation of brain microcircuits can occur at any stage of development, but particularly for motor circuits as animals grow and their biomechanics changes. For example, prior to P12, rodents receive nutrition primarily via suckling their mothers, but after that stage they chew and swallow, thus requiring transformation of feeding
CPG in early juvenile stages (Westneat and Hall, 1992). Likewise, locomotion changes from simple rudimentary patterns during embryonic and early postnatal stages where animals have limited control over posture and locomotive effort to full postural and gait control later postnatal stages (Vinay et al., 2002).

Therefore, the composition of the respiratory CPG might change too as animals develop chewing behavior, become ambulatory, and grow physically larger (thus respiratory demand increases) during postnatal and juvenile development. It cannot be taken for granted that respiratory CPG structure at birth remains fixed.

Regarding expiratory circuits (note, my focus is inspiratory), the oscillator microcircuit begins embryonically as a spontaneously and continuously active inspiratory-synchronized entity (Thoby-Brisson et al., 2009) but it becomes biphasically active around the inspiratory phase by neonatal stages (Onimaru et al., 2008), and by juvenile and adult stages it is a conditional oscillator entirely dependent on inspiratory rhythms and only active during late expiration (Feldman and Del Negro, 2006; Huckstepp et al., 2016, 2015; Janczewski and Feldman, 2006). Given the possibility for developmental changes that impact inspiration, testing the Dbx1 core hypothesis in adults was essential.

In this work, I show that photoinhibition of Dbx1 preBötC neurons precludes breathing. Similar photoinhibition experiments were performed in so-called in situ preparations, where the heart and ribcage is retained and the neuraxis (also maintained) is retrogradely perfused with artificial cerebrospinal fluid (Koizumi et al., 2016). Brief photoinhibition delayed or prematurely induced breaths. I show that photostimulation increases breathing frequency and brief pulses of
photostimulation can phase-shift individual inspiratory breaths. Additionally, my findings are commensurate with phase dependent shifts in breathing, where breaths can be induced earlier than expected during expiration, shown in other studies (Cui et al., 2016). Altering the frequency and timing of a behavior can be directly attributed altering to the core oscillator and not pattern forming circuits (Getting, 1989; Grillner, 2006; Kiehn, 2016). This leads me to conclude that Dbx1 neurons form the core of the respiratory microcircuit, which strongly supports the Dbx1 core hypothesis.

Furthermore, this present research on the cellular composition of the breathing CPG sets the stage for the future (next) advances in understanding respiratory rhythmogenesis. I show that Dbx1 preBötC neurons are essential for breathing and comprise the respiratory core oscillator in adult mice. However, Dbx1 is not a perfect marker because Dbx1 neurons are present throughout the ventral medulla and have potential roles in pattern formation and arousal circuits (Cui et al., 2016; Revill et al., 2015; Yackle et al., 2017). Therefore, the next horizon is to unravel a subset of Dbx1 neurons, on the basis of molecular genetic criteria, which are responsible solely for rhythmogenesis.

Using advanced technologies like next generation mRNA sequencing, including patch-seq, which can elucidate the transcriptome of single neurons, we may discover a uniquely identifiable subpopulation of Dbx1-derived neurons that comprises the core respiratory microcircuit. However, until Dbx1 neurons can reliably be divided into rhythmogenic, pattern-forming, and arousal-related subpopulations (and perhaps others?) Dbx1 remains to be the best molecular
genetic marker for the core oscillator. Therefore, it provides the best opportunity for understanding the cellular and synaptic mechanisms that underlie breathing.

Identifying and understanding the inspiratory core oscillator is paramount to understand breathing behavior overall because the preBötC coordinates all phases (inspiratory, post-inspiratory, and expiratory) of the respiratory cycle (Feldman et al., 2013; Ramirez et al., 2016) and will advance our understanding of the other aspects of respiratory control including those responsible for 'sigh' breaths, chemosensation, and motor pattern formation (Feldman et al., 2013; Guyenet et al., 2005; Koshiya et al., 2014; Li et al., 2016; Pagliardini et al., 2011).

Furthermore, the preBötC acts as a 'master clock' that coordinates the rhythms of other orofacial behaviors like whisking, licking, sniffing (Deschênes et al., 2016; Kleinfeld et al., 2014a, 2014b; Moore et al., 2013) and the more we comprehend the composition and operation of preBötC furthers our understanding orofacial behaviors that it coordinates.

The CPG circuit proposed to underlie mastication is not intrinsically rhythmically active; that would make no sense for an episodic behavior like chewing. Nevertheless, it can be studied in vivo and in vitro and its location has been proposed to be in the principal sensory trigeminal nucleus of the lower pons (Gossard et al., 2011; Westberg and Kolta, 2011). A novel rhythmogenic mechanism involving glia-neuron interactions in the principal sensory trigeminal nucleus has been offered to explain the cellular mechanisms for chewing, but no collective rhythms (only cellular) have been demonstrated, and there is still no direct link showing these neuron-glia rhythms pertain to jaw movements, real or
fictive (Kolta et al., 2015; Morquette et al., 2015). Pattern and motor microcircuits for locomotion are well understood however the location and cellular constituency of the core oscillator is unknown (Bouvier et al., 2015; Britz et al., 2015; Kiehn, 2016). In that regard, by identifying the cellular basis for inspiratory rhythm we can better understand underlying cellular and synaptic mechanisms, which may be commonly shared among a suite of rhythmic motor behaviors (walking, running, swimming, chewing, whisking [for rodents], and breathing) and therefore provide insights into the function of CPGs in general.

**References**


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