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Francis D. Pham
College of William and Mary

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Optogenetic Investigations of the PreBötzinger Complex: Support for the Dbx1 Core Hypothesis

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Chemistry from The College of William and Mary

by

Francis Duc-Hung Pham

Accepted for __________________________
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______________________________
Christopher Del Negro, Director

______________________________
Randolph Coleman

______________________________
Dana Lashley

______________________________
Douglas Young

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Abstract

The preBötzinger complex (preBötC) is the central pattern generator for inspiratory behaviors. Previous studies on perinatal mice and in vitro suggest that Dbx1-derived neurons within the preBötC form the core oscillator. Here, we provide support for the Dbx1 core hypothesis and show that Dbx1-derived neurons are essential for respiratory rhythmogenesis in adult mice. Using optogenetic strategies, we transiently hyperpolarized Dbx1 neurons of the preBötC in vitro and in adult mice. In both cases, the inspiratory rhythm was disrupted. It is possible that axons from Dbx1 neurons projecting into the preBötC were also hyperpolarized, leading to disfacilitation. To determine if this is the case, we conducted a second optogenetic study targeting the preBötC in its entirety. Hyperpolarization of the preBötC in sedated mice resulted in results like those seen from only hyperpolarizing Dbx1 preBötC neurons. Thus, we conclude the disruption of inspiratory rhythm is attributed to the hyperpolarization of Dbx1 preBötC neurons and not disfacilitation.
I would like to thank my advisor Christopher Del Negro and my committee members Randy Coleman, Dana Lashley, and Douglas Young. Additionally, I would like to thank graduate student Nikolas Vann who, for all practical purposes, has served as my second advisor.
**General Introduction**

Breathing is a multisystem behavior that persists through one’s lifetime nearly uninterrupted. This seemingly simple behavior consisting of inspiration and expiration requires an ensemble of muscles and organs such as the diaphragm, abdominals, and lungs. The origins of the respiratory rhythm, however, lies in a region of the ventral medulla called the preBötzing complex.

Respiratory pathologies afflicting neural control represent some of the most severe breathing disorders. For instance, some cases of sudden infant death syndrome (SIDS) are preceded by apneas and hypoxic gasping. The failure of autoresuscitation suggests that the neural control of breathing could be a major contributor to these fatal outcomes [1]. Thus, it is imperative that we study and understand the underlying neural control of breathing. In this defense, we show that suppressing Dbx1-derived preBötC neurons perturbs the respiratory rhythm, suggesting these neurons form the core inspiratory rhythm generator.

**PreBötzing Complex**

Respiratory neurobiology is, by its nature, difficult to study. Breathing is closely tied to life, however studying neural processes can be quite invasive. In the late 1900s, this constraint was relieved with the viability of an in vitro model of respiration. Researchers determined that brain stem and spinal cord dissections of neonatal mice retained electrical activity representative of breathing. Measurements from the phrenic nerves displayed periodic activity. Furthermore, the preparation was responsive to chemosensory feedback, a key trait of respiration. Perfusing the preparation with low pH solution increased the frequency of activity and administering opioids decreased activity [2]. In a separate study, researchers demonstrated periodic ribcage
contractions of a brain stem-spinal cord preparation with intact intercostal musculature [3],
attesting to the viability of the in vitro model.

Using this paradigm, Smith et al. were able to determine the location of the respiratory
oscillator within the brainstem. They demonstrated that respiratory-related oscillations similar to
those in the whole brainstem persisted in medullary slices containing the preBötC. Furthermore,
whole-cell patch-clamp recordings of neurons in the preBötC revealed some with voltage-
dependent pacemaker-like properties [4]. These data together suggest that respiratory
rhythmogenesis occurs in the preBötC.

Figure 1. Schematic of a medullary slice preparation containing the preBötC. Electrodes
demonstrate how researchers recorded activity of preBötC neurons and hypoglossal nerve output.
Reproduced from Smith et al. 1990 [3].

Developing Brain Homeobox Protein 1 (Dbx1)

The preBötC is a functionally heterogeneous region. Previous studies have shown that
somatostatin (Sst) and neurokinin type 1 receptor (NK1R) expressing neurons are essential for
respiratory rhythm generation [5-6]. However, these peptides and peptide receptors can be
imperfect markers of the core preBötC population. The preBötC still develops and respiration
persists when Sst or NK1R genes are knocked-out [7]. Moving from peptidergic markers, we attempt to classify the preBötC genetically.

Brainstem interneurons derived from the embryonic transcription factor Dbx1 express these peptide receptors, peptides, and glutamate, all of which are characteristics that help distinguish the core of the preBötC. A previous study conducted by our lab showed laser ablation of Dbx1 neurons in an in vitro slice preparation irreversibly terminated inspiratory-related rhythmic motor output [8]. These results point to Dbx1-derived preBötC neurons as the rhythmogenic core of the preBötC.

**Optogenetic Technologies**

In vitro studies of respiration can explain neural circuitry in great detail. However, the ability to extrapolate data from a slice preparation to normal breathing remains controversial. For instance, the frequency of the inspiratory rhythm in slice preparations is significantly less than that of breathing in rodents. This is likely due to the absence of sensory feedback and highlights key differences between normal breathing conditions and breathing in vitro [3]. Innovations in biochemistry, however, have provided new techniques and paradigms for studying respiratory neurobiology in vivo. The method that our research utilizes is optogenetics.

Optogenetics, in its most literal form, is a tool that involves optics and genetics. Transgenic breeding or viral transfection strategies allow for the expression of light-activated ion channels in the membranes of neurons. These ion channels fall under the rhodopsin family of proteins and possess diverse functions. Channelrhodopsin, for instance, allows cations to passively flow when activated by blue light whereas archaerhodopsin pumps protons from the
cytoplasm in response to yellow light. The result of this ion movement in neurons is depolarization or hyperpolarization, which is effectively excitation or inhibition.

Applying these optogenetic strategies to in vitro slice preparations is simple as the neuronal populations of interest are exposed and can be readily illuminated. A greater challenge is applying this technology to intact model organisms to study behavior. In 2007, this challenge was resolved through the development of an optical neural interface. Fiber optics were surgically implanted dorsal to channelrhodopsin-2 expressing excitatory neurons of the motor cortex in mice. The fiber optics were coupled to a blue-laser diode and upon photostimulation of these neurons, the researchers noted a marked increase in whisker deflections [9]. This experiment laid the framework for optogenetic experiments in mammals.
Chapter 1

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 (Smith et al., 1991; Feldman et al., 2013), 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; Wang et al., 2001; Guyenet et al., 2002; Stornetta et al., 2003; Wallen-Mackenzie et al., 2006; Tan et al., 2008). 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 (Greer et al., 1991; Funk et al., 1993). 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 (Pierani et al., 2001; Bouvier et al., 2010; Gray et al., 2010). 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.

**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<sup>T2</sup>) in cells that express *Dbx1*, i.e., *Dbx1<sup>CreERT2</sup>* (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<sup>CreERT2</sup>*;Ai35D offspring.

**Respiratory active transverse slice preparations**

Neonatal *Dbx1<sup>CreERT2</sup>*;Ai35D pups (postnatal days 0-4) were anesthetized by hypothermia and decerebrated. Mice were then dissected in 4º C artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.5 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 0.5 NaH<sub>2</sub>PO<sub>4</sub>, and 30 dextrose. The aCSF was aerated continuously with carbogen (95% O<sub>2</sub> and 5% CO<sub>2</sub>, 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., 2014). 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 (Ruangkittisakul et al., 2006; Funk and Greer, 2013).

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 preformed 0.25 to 1.0 mm rostral to preBötC targeted locations. We joined 1.27-mm-diameter ceramic ferrules (Precision Fiber Products, Milptas, 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 preBötC 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 recorded 2 min videos that incorporated 30-s bouts of light application to assess whether visible breathing movements would be affected by fibers implanted in preBötC or rostral to preBötC (e.g. S1 Video 1 and S2 Video). 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 preBötzC (determined from histological sections), we used light dispersal and tissue scattering formulae (Aravanis et al., 2007; Yizhar et al., 2011) to calculate that preBötzC 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 preBötzC 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 neuraxaxes 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, 1 min in 95% EtOH, and then 2 min in 100% EtOH twice).
EtOH, and 5 min in 100% EtOH twice), cleared using four xylene 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.

**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*\textsuperscript{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. 2A). 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. 2B), which coincides with the rostral face of the preBötC (Ruangkittisakul et al., 2014). 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. 2C,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. 2E,F) or non-respiratory firing patterns, as well as non-Dbx1 preBötC neurons with evidence of inspiratory modulation (Fig. 2G). The average change in baseline membrane potential for all non-Dbx1 neurons measured −0.68 ± 0.3 mV.

Illumination of the preBötC 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. 2D).

These results indicate that activation of Arch hyperpolarizes Dbx1 preBötC neurons not via network disfacilitation but rather direct postsynaptic effects. In neonatal mouse slices, the majority of Dbx1 preBötC 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 preBötC have premotor functionality (Song et al., n.d.; Revill et al., 2015), 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.
Figure 2. 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 (IOPloop) 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.
Arch-mediated inhibition in Dbx1 preBötC 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., 2014). 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. 3A). The position of the preBötC can be determined from anatomical markers including the principal loop of the inferior olive ($IO_{loop}$), 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. 3B, B’) (Franklin and Paxinos, 2013).

We implanted fiber optics bilaterally to activate Arch in Dbx1 preBötC neurons in vagus-intact adult $Dbx1^{CreERT2};Ai35D$ mice (positions confirmed post-hoc, e.g., Fig. 3C). Control littermates had fiber optics implanted dorsally in the medulla at a position rostral to the preBötC (e.g., Fig. 3D). Immediately after implantation we delivered 589-nm laser pulses (30 s) while visually monitoring breathing. Anesthetized mice whose ferrules were implanted in the preBötC transiently stopped ventilation for intervals of approximately 18 s (n=6, S1 Video), whereas breathing remained unperturbed in control mice with ferrules in the dorsal medulla (n=7, S2 Video).
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 $2.9 \pm 0.5$ Hz, with $V_T$ of $0.12 \pm 0.08$ ml, MV of $21.8 \pm 14.9$ ml·min$^{-1}$, and $T_i$ of $130 \pm 30$ ms. During 2 s of preBötC illumination, $f_R$ decreased to $1.5 \pm 0.9$ Hz ($p=0.0001$), $V_T$ decreased to $0.07 \pm 0.04$ ml ($p=0.0001$), and MV decreased to $9.9 \pm 5.6$ ml·min$^{-1}$ ($p=0.0001$). In contrast, $T_i$ increased to $280 \pm 90$ ms ($p=0.008$) during preBötC illumination (Figs 4A and 8 left column, cyan symbols).

Figure 3. Arch-EGFP expression and histology of fiber-optic implants in adult $Dbx1^{CreERT2};Ai35D$ mice. A, EGFP expression in 35 week-old $Dbx1^{CreERT2};Ai35D$ mouse. Scale bar represents 500 µM. A’, 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’, 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_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 examined the airflow fluctuations during bouts of illumination (Fig. 4A1,2). Inspiratory movements discontinued at the onset of light application (e.g., Fig. 4A2 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. 4B1,2).

Illumination of the dorsal medulla in seven lightly ketamine-sedated Dbx1creERT2;Ai35D controls (positions confirmed post-hoc, e.g., Fig. 3D) 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 5A and 8 left column, magenta symbols). Cycle-triggered averages of respiratory airflow prior to and during illumination were virtually superimposable (Fig. 5B).

**Figure 5.** 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. 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.

**Arch-mediated inhibition in Dbx1 preBötC neurons perturbs breathing in awake mice**

In the same cohort of awake and unrestrained adult Dbx1^{CreERT2};Ai35D mice, we illuminated the preBötC and monitored breathing via whole-body plethysmography. Light delivery to the preBötC decreased f_R (from 2.3 ± 0.6 in control to 2.1 ± 0.5 Hz during preBötC illumination, p=0.0012) without modifying V_T (0.05 ± 0.008 vs. 0.05 ± 0.001 ml, p=0.24) or MV (0.87 ± 0.1 vs. 1.0 ± 4.7 ml·min⁻¹, p=0.49; because of two outliers in the ‘Light’ condition [see Fig. 8, right column, cyan symbols] we report MV in the text using the median ± SD (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 6A and 8 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. 5A,2) and in cycle-triggered averages (Fig. 5B,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 7A and 8 right column, magenta symbols). The breathing pattern remained unchanged during cycles of illumination (Fig. 7A), which was clear at faster sweep speed (Fig. 7A,2) and in cycle-triggered averages (Fig. 7B,2).

**Figure 6.** Activation of Arch in $Dbx1$ preBötC neurons in freely behaving awake mice. 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). Note that inspiratory airflow is attenuated, whereas expiratory airflow is not. Time calibrations are shown for each panel.
Figure 7. 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 8. 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 $T_1$ (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.
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 (Gray et al., 1999, 2001; Wang et al., 2001; Guyenet et al., 2002; Stornetta et al., 2003; Llona and Eugenín, 2005; McKay et al., 2005; Tan et al., 2008; Cui et al., 2016). 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.
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., 2014) 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. 2). 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 (S1 Video) 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 4 and 8). The effects on $T_i$ and $f_R$ match the second prediction (Fig. 8). 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. 2A2). 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 (Moore et al., 2013; Deschênes et al., 2016) 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. 4A1,2). 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 preBöttC 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 preBöttC 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 preBöttC neurons is insufficient to suspend rhythmogenesis. According to our measurements and calculations, the light intensity at the preBöttC was adequate to evoke near maximum Arch-mediated current (see Materials and Methods). However, if Arch-EGFP expression were limited within the Dbx1 preBöttC 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 (Pierani et al., 2001; Bouvier et al., 2010; Gray et al., 2010). We activated CreER\(^{T2}\) 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\(^{T2}\) 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.,
as well as excitatory inputs from the pons and raphé (Viemari et al., 2003; Hilaire et al., 2004; Ptak et al., 2009) 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 ~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 ~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 (Green et al., 1981; Erhardt et al., 1984; Flecknell, 2015), 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 (Stornetta et al., 2002; Guyenet et al., 2013). 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 $Dbx1^{CreERT2;Ai35D}$ 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 (Wang et al., 2014; Revill et al., 2015). 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.
References


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Chapter 2

Introduction

In the first chapter, we demonstrated photoinhibition of Dbx1-derived preBötz neurons suppresses the respiratory rhythm. However, this study leaves caveats and questions to be answered. First, we were unable to rule out potential contributing factors of non-preBötz neurons whose axons and axon terminals project into the preBötz and express archaerhodopsin. This could disfacilitate the preBötz through the absence of excitatory synaptic inputs in vivo. Second, photoinhibition of Dbx1-derived preBötz neurons was unable to abolish ventilation in the awake mouse as seen by the lack of effect on $V_T$ and $MV$. It is conceivable that archaerhodopsin mediated hyperpolarization was unable to completely inhibit Dbx1-derived preBötz neurons and halt rhythmogenesis.

To address these concerns, we conducted an additional optogenetic study determining the effects of photoinhibiting the preBötz. In doing so, we can conclude that inhibition of non-preBötz axons and axon terminals expressing archaerhodopsin is not central for the phenotypes previously observed if we see respiratory behaviors identical to those seen when photoinhibiting Dbx1-derived preBötz neurons. Furthermore, we provide future directions on conducting this study in awake adult mice. If photoinhibition of the entire preBötz cannot cease rhythmogenesis of the awake mouse, then it is apparent that the levels of hyperpolarization attained by archaerhodopsin are indeed insufficient to achieve this. For the present optogenetic study, we expressed archaerhodopsin exclusively in the preBötz by transfecting neurons with a recombinant adeno-associated virus (rAAV) containing a synapsin driven promoter region and genes for archaerhodopsin and GFP.
AAV Technologies

rAAV delivery systems are highly advantageous for use in optogenetic studies. rAAV is unable to replicate and thus expression of its genes is localized to the region of viral injection. Additionally, administering AAV to the central nervous system appears to be nontoxic and does not elicit an inflammatory response [10]. Lastly, using a synapsin driven promoter region results in neuron specific infection of AAV [11].

Methods

Mice

The Institutional Animal Care and Use Committee at the College of William and Mary approved these protocols. We ordered male and female CD-1 mice from Charles River at 3-4 weeks old and weighing approximately 20 g. Male and female mice were kept separate and housed 2 mice per cage in a vivarium. Cages allowed free access to food and water. Mice were aged until 8-20 weeks old and weighed between 25 g and 45 g, at which point we performed surgical protocols for AAV injection and fiber optic implantation. After surgery, mice were housed in cages in a BSL-2 hood with 12-h light cycle.

AAV-Injection

We initially anesthetized mice via intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. To ensure mice were deeply anesthetized throughout the surgery, mice were administered an additional 50 mg/kg ketamine and 5 mg/kg xylazine every 45 minutes after the initial injection until the surgery was complete. All surgical tools were sterilized with 70% ethanol and hot bead sterilizer prior to surgery. After trimming and sterilizing the skin on the
head, we secured the mice into a stereotaxic apparatus and exposed the skull with a midline incision extending from the eyes to the rostral end of the neck. We then performed 0.5-mm-diameter bilateral craniotomies 1.2-1.4 mm lateral to the midline at the interface of the interparietal bone and posterior neck muscles. Additionally, we scoured the surface of the skull at this point to increase the surface area for a cyanoacrylate adhesive we later use while implanting the fiber optics. We loaded a 0.5 μL Neuros Syringe with 60-75 nL of AAV-hSyn-EGFP or AAV-hSyn-eArch3.0-EGFP (titers between 1-8 x 10^{12} vg/ml) and lowered the syringe 4.8-5.1 mm from the surface of the brain at the same coordinates used for the craniotomies. The syringe descended at a velocity of 10 μm/sec and at the end, the AAV virus was administered into the preBötC at an approximate rate of 60 nL/min. The syringe was left in place for 10 minutes after injection to reduce backflow. The syringe was retracted at a velocity of 10 μm/sec and cleaned with H_2O_2, dH_2O, and acetone before an identical injection was made on the opposite side.

**Implantation of Fiber Optics**

Immediately after viral injection, we proceeded to implant the fiber optics constructed from joining ceramic ferrules with multimode fibers. Using the same coordinates for craniotomies and viral injection, we lowered the first fiber optic to a depth 400 μm dorsal to the injection depth at a rate of 10 μm/sec. We secured the fiber optic in place by applying the cyanoacrylate adhesive around the fiber optic and onto the skull. The second fiber optic was then lowered and secured at the opposite side using the same methods as the first. The wound was closed using surgical staples and a tissue adhesive. The mice were placed in cages under a BSL-2 hood, and their recovery was monitored daily. Two weeks after surgery, the ferrule ends were connected to a 200-mW, 589-nm diode-pumped solid-state laser with a custom patch cord. We
screened for breathing phenotypes during 30-s bouts of light application and later performed nosecone recordings on those with a visible response to light application.

**Nosecone Recordings**

Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine. Once anesthetized, we connected their implanted ferrules to the 589-nm laser and fitted them with a nose cone. We ran breathing air through the nose cone and measured airflow using a 1-liter respiratory flow head and differential pressure transducer. We used PowerLab to store our data and manage experimental settings.

For our experimental paradigm, we illuminated the preBötC with either 0.2-s or 5-s bouts. For each bout, we collected data with light intensities equal to 100%, 75%, and 50% of maximum laser output. We continued experiments with the 5-s bout and each intensity until we obtained 5 airflow recordings during which there was no movement in a 10-s window before, during, or after illumination. For the 0.2-s bout, we continued experiments until we collected 100 recordings without movement before, during, or after illumination.

**Histology**

After completing experiments, we administered a lethal dose of pentobarbital (100 mg/kg) via intraperitoneal injection. We transcardially perfused the mice with a working solution of 1X phosphate-buffered saline. Once the liver cleared, we changed the perfusate to 4% paraformaldehyde in 1X phosphate-buffered saline. The brain was removed and placed in 4% paraformaldehyde overnight at 4°C. We then washed the brain in 1X phosphate-buffered saline 3 additional times for 10 minutes during each wash. Free floating transverse or sagittal sections containing the preBötC were taken using a vibratome. Sections were mounted on glass slides and
cover-slipped using Fluoroshield. Tissue sections were visualized using confocal microscopy. Image brightness and contrast were adjusted in Photoshop.

**Data Analysis**

We calculated tidal volume ($V_T$), inspiratory duration ($T_i$), and minute ventilation (MV) from the airflow signal using LabChart7. We used Microsoft Excel to organize airflow data and conduct phase shift analyses. Figures were created using Microsoft Excel, Adobe Illustrator, IGOR Pro 6, or Photoshop CS6.

**Results**

**Determination of AAV Injection Coordinates**

We considered successful viral injections as those that transfected neurons confined to the preBötC. To determine the volume and proper site of viral injection, we initially used AAV-hSyn-EGFP during our trials to identify the transfection region of our injections. In our first trials, we bilaterally injected 100 nL of AAV-hSyn-EGFP 1.5 mm from the midline at the interface of the interparietal bone and the neck muscles. The injections were 4850 μL from the surface of the brain. In figure 9, the section shows GFP expression along the left border, indicating the injection was too lateral from the midline. It is likely that GFP expression is absent right of the midline because the injection was similarly too lateral. However, the section still contains well developed loops from the inferior olive on the ventral border and a smooth fourth ventricle on the dorsal border, indicating the injection along the rostrocaudal axis is accurate.
**Figure 9.** GFP expression reveals transfected neurons from 100 nL AAV-hSyn-EGFP injections in this transverse section. Injection was made 1.5 mm lateral to the midline at the interface of the interparietal bone and neck muscles and was 4850 μL deep.

In subsequent trials, we retained the interparietal bone-neck muscle reference while shifting the injections medially. Figure 10 shows the GFP expression from 100 nL bilateral injections 1.2 mm from the midline and 4850 μL from the surface of the brain. Again, the inferior olive and smooth fourth ventricle are present on this transverse section and verify the accuracy along the rostrocaudal axis. The lateral positioning of injections also appears correct as GFP expression is present ventral to the humps of the dorsal border. However, expression in this section is not confined to the preBötC. The depth of the injection reaches the preBötC, but GFP expression extends dorsally. This suggests that the volume of virus administered was too great. Additionally, backflow of viral particles likely occurred due to inadequate time for diffusion before retracting the syringe.
Figure 10. AAV-hSyn-EGFP injections 1.2 mm lateral to the midline reveal better positioning. However, transfection is widespread from the ventral edge to dorsal edge.

Figure 10 demonstrated accurate positioning of AAV injections, but transfection was not localized to the preBötC. Moving forward, we worked to constrain transfection to the preBötC by reducing the viral volume injected to 60 nL and leaving the syringe in place post-injection for 10 minutes. Figure 11 shows a sagittal slice to analyze the extent of viral transfection. Also, note that we switched to AAV-hSyn-eArch3.0-EGFP to see its specific transfection pattern. Here, we see that these adjustments result in transfection constrained to the preBötC along the rostrocaudal axis. Furthermore, transfection does not extend dorsally as seen in figure 10. These findings demonstrate a reliable protocol of transfecting preBötC neurons with AAV in a localized manner.
Figure 11. Sagittal slice is used to determine spread of transfection along rostrocaudal axis. 60 nL AAV-hSyn-eArch3.0 injection is confined to the preBötC region.

**Long Pulses Suppress Respiratory Activity**

After surgical procedures to inject AAV-hSyn-ARCH-EGFP in the preBötC and implant fiber optics, we waited at least 2 weeks after surgery for mice to recover and AAV to transfect neurons. We measured breathing before, during, and after 5-s pulses of 589-nm light on the preBötC using a nose cone on sedated mice. Measurements taken before 5-s pulses were used as controls. Figure 12 shows air flow traces and other respiratory metrics during this time frame. Immediately after illuminating preBötC neurons with 100% laser output, we saw a marked reduction in the amplitude of airflow fluctuations. This was also seen with 75% and 50% laser output, however, the reduction in amplitude became more attenuated as laser output dropped. Analyses showed the mouse breathed with $V_T = 0.23\pm0.03$ mL, $MV = 36\pm6$ mL/min, and $T_i = 220\pm50$ ms before preBötC illumination with 100% laser output. During illumination, $V_T$
decreased to 0.11±0.03 mL, MV decreased to 17±6 mL/min, and $T_i$ increased to 260±140 ms. After illumination, $V_T$ increased to 0.24±0.03 mL, MV increased to 48±6 mL/min, and $T_i$ decreased to 190±20 ms. Tables 1-3 in the appendix organize these data along with the data for 75% and 50% laser output.
Figure 12. Top traces show airflow recordings and breathing metrics with 100% laser output for 5-s. Middle traces are with 75% laser output and bottom traces are with 50% laser output.
Figure 13. Overall tidal volume measurements before (ctl), during (stim), and after (recov) illumination of preBötC.

Figure 14. Overall minute ventilation measurements before, during, and after illumination of preBötC.

Figure 15. Overall inspiratory period measurements before, during, and after illumination of preBötC.
Short Pulse

On the same mouse, we illuminated the preBötC with 0.2-s pulses of light randomly distributed throughout the respiratory cycle and measured the shift in respiratory phase. To calculate the phase, we took the peaks of the two complete respiratory cycles preceding a photostimulation and extrapolated the expected respiratory cycle that would occur had there been no photostimulation. Then, we took the time between two peaks and divided it equally over 360° to transform time to phase. Phase shift was calculated by subtracting the phase of the induced peak by the phase of the expected peak.

Figure 16. Schematic of phase shift calculation. Expected peak airflows were extrapolated from the preceding respiratory cycle and phase shift was calculated by the observed peak airflow phase subtracted by the expected peak airflow phase.

Figure 17 plots the phase shift vs. photostimulations at random points during the respiratory cycle. Photostimulation from 0° to approximately 125° resulted in phase advance during which the expiratory effort was shortened and peak inspiratory airflow occurred earlier.
than expected. Photostimulation from 125° to approximately 300° delayed the peak inspiratory airflow by apparently lengthening the inspiratory phase. From 300° to 360° we see that some neurons are still phase delayed. However, a cluster of neurons in this range are also centered around a phase shift of 0°, meaning their peak inspiratory airflow occurred when expected.

\[\text{Figure 17. Plot of phase shift in response to 0.2-s pulses of light at random points during the respiratory cycle.}\]

**Discussion**

Previously, we suppressed Dbx1-derived preBötC neurons of intact mice and saw perturbed rhythmogenic function. Optogenetic activation of archaerhodopsin expressed in these neurons resulted in apnea, prolonged T\(_i\), and decreased f\(_R\). The experimental design, however, could not exclude contributions from inhibiting Dbx1 axons and axon terminals that project into the preBötC from outside and express archaerhodopsin. This could result in disfacilitation of non-Dbx1 populations within the preBötC through the absence of excitatory inputs. We
addressed this by transfecting most and only preBötC neurons with serotype 5 rAAV containing genes for archaerhodopsin and GFP.

Long pulse illumination of archaerhodopsin expressing preBötC neurons resulted in rhythmogenic perturbations similar to those when only activating archaerhodopsin in Dbx1-derived preBötC neurons. Apnea persisted for the duration of the long pulse illumination, T₁ was lengthened, and MV increased. Our series of histological experiments show that we can transfect neurons in a region confined to the preBötC. Thus, we interpret the respiratory data from this optogenetic experiment as proof that disfacilitation does not play an essential role in the breathing perturbations seen in the previous study.

A primary concern of this study, however, would be the possibility of AAV entering axons that project into the preBötC and undergoing retrograde transport to non-preBötC neurons. The serotype of AAV used in our study, AAV5, has the potential to be retrogradely transported in specific regions of the mouse brain such as the entorhinal cortex and the dentate gyrus [12]. However, it is unclear whether AAV5 can be retrogradely transported in the regions surrounding the preBötC. Regardless, this should not be a point of concern. Assuming that AAV5 can be retrogradely transported, the virus would first need to be transported to the nucleus of the neuron, and then translated archaerhodopsin proteins would have to be transported back to the axons and axon terminals before presenting a problem in this study. The union of these events appears unlikely. Analyses of sections in figure 11 provide no evidence of retrograde transport. We expect GFP expression in the somata of neurons to occur before expression in the axons. However, we do not see expression in cell bodies outside of the preBötC. This leads us to conclude that AAV5 is not retrogradely transported from axons in the region around the preBötC.
During the long pulse experiments, we noted that airflow metrics immediately after illumination differed from the measurements before illumination, especially when illumination was with 100% laser output. MV and VT during the recovery period were greater than during the control period. Further, $T_i$ was less during the recovery period compared to the control period. These effects are likely due to chemosensory inputs driving inspiratory efforts. While illuminating the preBötC, inspiratory efforts cease. Metabolism continues, however, producing CO$_2$, H$_2$O, and ATP. CO$_2$ builds up from the inability to exhale, affecting the blood bicarbonate buffer system shown in the equation below.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

Le Chatlier’s principle predicts an equilibrium shift in the forward direction as the CO$_2$ concentration increases. H$^+$ is produced which lowers pH and risks widespread damage from acidosis. In response to CO$_2$ and H$^+$, excitatory synaptic inputs to rhythm generating neurons of the preBötC increase and continue to accumulate while illuminating the preBötC [13]. They may be unable to overcome the hyperpolarizing current mediated by archaerhodopsin though. Once we stop illuminating the preBötC, archaerhodopsin inactivates. In the absence of hyperpolarizing current, preBötC becomes overexcited and increases inspiratory efforts until CO$_2$ is removed and pH returns to baseline levels.

In our short pulse experiments, we saw that stimulations soon after peak airflow resulted in phase advance and stimulations later induced phase delay. In the first scenario, the pulse likely occurs during the expiratory phase of the cycle and accelerates expiration, leading to the earlier onset of inspiration. In the second scenario, the pulse likely occurs during inspiration. The short hyperpolarizing current induced by briefly activating archaerhodopsin proteins stifles inspiratory
efforts momentarily, however inspiration continues after the proteins are inactivated. Now, the bifurcation of data from stimulations during phases between 300° and 360° depicts two possible outcomes. First, events of phase delay probably illustrate the same scenario where inspiratory efforts are paused and then lengthened. The cluster around a phase shift of 0° likely indicates that the inspiratory period had committed far enough to peak at the expected time. Activation of archaerhodopsin may be unable to induce hyperpolarization quickly enough to prolong the inspiratory period.

The results we currently have cannot fully explain why $T_v$ and $MV$ are unchanged when optogenetically inhibiting Dbx1-derived preBötC neurons in the awake mouse. Moving forward, we aim to perform the present optogenetic study on awake mice using plethysmography recordings too. This will allow us to directly compare airflow measurements between suppressing the preBötC and suppressing Dbx1-derived neurons of the preBötC. Additionally, we plan on conducting another optogenetic study using an AAV construct that would allow us to express channelrhodopsin in Dbx1 neurons of the injection site. Here, we will be able to see the effects of depolarizing specifically Dbx1 preBötC neurons. Lastly, some Dbx1-derived neurons can have nonrhythmogenic functions. We intend to investigate genetic subsets of Dbx1 preBötC neurons to try and find a genetic marker more specific to the rhythmogenic population.
References


Appendix

Tables 1, 2, and 3 summarize airflow metrics throughout long pulse experiments.

**Table 1. Overall Minute Ventilation (L/min)**

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<th>Mean</th>
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<td>100%</td>
<td>75%</td>
</tr>
<tr>
<td>Ctl</td>
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<td>0.0385</td>
</tr>
<tr>
<td>Stim</td>
<td>0.0170</td>
<td>0.0191</td>
</tr>
<tr>
<td>Recov</td>
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<td>0.0480</td>
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</table>

**Table 2. Overall Tidal Volume (L)**

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<th>Mean</th>
<th>STDEV</th>
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<tr>
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<td>0.00023137</td>
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<td>9.68782E-05</td>
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<tr>
<td>Recov</td>
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<td>0.000238007</td>
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**Table 3. Overall Inspiratory Period (ms)**

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<tr>
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<tr>
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<tr>
<td>Recov</td>
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