5-2017

The neural basis of breathing rhythm: TRPM4 and TRPC3 ion channels contribute to inspiratory burst generation in Dbx1-derived interneurons of the preBötzingwer Complex in mice

Kaitlyn E. Dorst
College of William and Mary

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Part of the Molecular and Cellular Neuroscience Commons, and the Systems Neuroscience Commons

Recommended Citation
Dorst, Kaitlyn E., "The neural basis of breathing rhythm: TRPM4 and TRPC3 ion channels contribute to inspiratory burst generation in Dbx1-derived interneurons of the preBötzingwer Complex in mice" (2017). Undergraduate Honors Theses. Paper 1040.
https://scholarworks.wm.edu/honorstheses/1040

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
The neural basis of breathing rhythm: TRPM4 and TRPC3 ion channels contribute to inspiratory burst generation in Dbx1-derived interneurons of the preBötzing Complex in mice

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

by

Kaitlyn Elizabeth Dorst

Accepted for ________________________________
(Honors, High Honors, Highest Honors)

______________________________
Christopher Del Negro, Director, Ph.D.

______________________________
Margaret Saha, Ph.D.

______________________________
Eric Bradley, Ph.D.

______________________________
Diane Shakes, Ph.D.

Williamsburg, VA
May 3rd, 2017
The neural basis of breathing rhythm: TRPM4 and TRPC3 ion channels contribute to inspiratory burst generation in Dbx1-derived interneurons of the preBötzinger Complex in mice

Kaitlyn Dorst,
The College of William and Mary

Abstract

Breathing is a primal behavior that emanates from neural rhythms in a region of the ventral-lateral medulla named the preBötzinger Complex (preBötC). Dbx1-derived preBötC neurons comprise the underlying core oscillator. Although we understand the network (preBötC) and cellular (Dbx1) origins of breathing, its molecular (ion channel-level) mechanisms remain unknown. We hypothesized that transient receptor potential (TRP) ion channels are responsible for inspiratory burst generation in Dbx1-derived preBötC neurons (i.e. Dbx1 neurons). In this study, we evaluate the contributions of TRPM4 and TRPC3 channels in inspiratory burst generation using electrophysiological techniques. Pharmacological inhibition of these ion channels in vitro attenuates the drive potentials that underlie inspiratory bursts in Dbx1- preBötC neurons. This suggests that these specific TRP ion channels are important for inspiratory burst generation in Dbx1 preBötC neurons and thus mammalian breathing behavior.
Introduction

Breathing is an obviously vital behavior for all mammalian life. Its essence is a rhythm, specifically driving periodic tidal ventilation of the lungs. The essential active phase of breathing is inspiration; expiration is ordinarily passive. Although breathing relies on a physical plant that consists of lungs, thorax, diaphragm and other axial muscles, the rhythm and motor pattern emanates from neural circuits in the medulla. These respiratory circuits generate the behavior normally, and their dysfunction in the context of disease results in respiratory failure. Humans are susceptible to these diseases at all stages of life. Some examples include apnea of prematurity, SIDS in babies, Rett Syndrome in childhood, obstructive sleep apnea during adulthood, and neurodegenerative diseases in geriatric patients. The main goal of respiratory neurobiologists is to understand the neural underpinnings of breathing with explanations at the network, cellular and molecular (ion channel) levels.

The preBötzinger Complex (preBötC) is a region of the ventral medulla that contains the core inspiratory oscillator (Feldman and Del Negro, 2006; Feldman et al., 2013). This region is heterogeneous; it is composed of many different neuron types. Excitatory preBötC inspiratory neurons, which are essential for inspiratory behavior, arise from progenitors that express the embryonic transcription factor Dbx1 (Bouvier et al., 2010; Gray et al., 2010). Dbx1-knockout mice die at birth of asphyxia and do not form any recognizable
preBötC, thus indicating that the neurons that develop from Dbx1-expressing precursors are obligatory for respiratory rhythmogenesis.

Thus, we know the network (i.e., preBötC) and cellular origins (Dbx1 interneurons) of breathing. However, the molecular origins, i.e., and ion channel-level of inspiratory burst generation, remains unknown.

In order to identify the ion channel-level origins of breathing behavior, we need experimental access to individual preBötC neurons for intracellular recordings. Thin transverse slices containing the preBötC are our experimental models for breathing behavior (Funk and Greer, 2013; Smith et al., 1991). We utilized intersectional mouse genetics to cross genetically modified strains of mice in order to selectively identify Dbx1-derived neurons in our experimental slice preparations. The progeny produced by transgenic breeding results in expression of red tdTomato fluorescent protein in cells that arose from embryonically Dbx1-expressing progenitors (Figure 1A). We can then identify and record the output from Dbx1-derived inspiratory preBötC neurons using the whole-cell patch-clamp technique. Individual bursts generated from these neurons comprise of instances of transient depolarization called drive potentials. When these drive potentials reach spike threshold ($\theta = -45$ mV), action potential spiking is generated and is observed atop the drive potentials. Rhythmic activity of Dbx1 preBötC neurons is synchronous with the respiratory-related activity of the hypoglossal (XII) nerve. This nerve normally innervates the genioglossus muscle that modulates airway patency via lingual (i.e., tongue) movements during the inspiratory breathing
phase. The XII output in thin transverse slices serves as a guide for us to identify inspiratory neurons based on systemic behavior. This experimental preparation can allow us the means to study the possible molecular components of breathing behavior.

$\text{Ca}^{2+}$-activated non-specific cation current ($I_{\text{CAN}}$) contributes to more than 70% of the underlying drive potentials in Dbx1-derived preBötC neurons. Several members of the transient receptor potential (TRP) superfamily of ion channels are activated by $\text{Ca}^{2+}$ and underlie a variety of brain functions (Zheng, 2013). The melanostatin subfamily channel TRPM4, is activated by $\text{Ca}^{2+}$ and allows for the nonselective influx of monovalent cations. This causes membrane depolarization in a variety of rhythmic cells in cardiac and neural systems (Guinamard et al., 2011; Launay et al., 2002; Ullrich et al., 2005). We hypothesized that this is the main candidate ion channel responsible for generating drive potentials in Dbx1-derived inspiratory preBötC neurons (Crowder et al., 2007; Mironov, 2008; Mironov and Skorova, 2011). Nevertheless, in sequencing the transcriptome of Dbx1 preBötC neurons, we detected high RNA levels for the ‘canonical’ TRP, TRPC3 (Supplemental Fig. 1). Additionally, physiological evidence shows that TRPC3 may modulate preBötC rhythmicity (Ben-Mabrouk and Tryba, 2010). Thus, our study focuses on both TRPM4 and TRPC3 and their potential contributions to respiratory rhythm and breathing behavior.
Methods

Animal Ethics Statement

The Institutional Animal Care and Use Committee (IACUC) at the College of William and Mary approved all experimental procedures using animals.

Intersectional Mouse Genetic Breeding

We utilized intersectional mouse genetics in order to visualize Dbx1-derived neurons. We used tamoxifen-inducible Cre recombinase in order to induce conditional expression of tdTomato fluorescent protein in Cre-dependent reporters, obtained from The Jackson Laboratory (Bar Harbor, ME). Dbx1<sup>CreERT2</sup> females (Gray et al., 2010; Hirata et al., 2009; Ruangkittisakul et al., 2014) were bred with Rosa26<sup>tdTomato</sup> reporter males (Madisen et al., 2010, 2012). Tamoxifen was administered in pregnant females at embryonic day 9.5 in order to induce optimal expression of tdTomato fluorescent protein. The active Cre recombinase cleaves LoxP sites to remove a stop codon, thus initiating expression of the td-Tomato fluorescent-protein gene (Fig. 1A). The resulting progeny express td-Tomato in Dbx1-derived cells (neurons and glia) (Fig. 1B and 1C).
Animal Dissection and Slice Preparation

Neonatal mice, aged postnatal day 0-5, were anesthetized and dissected in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose. ACSF was bubbled with carbogen (95% O₂ and 5% CO₂) to equilibrate the pH (7.4). The brainstem and spinal cord were extracted from the mouse and then mounted for micro-sectioning on a cutting surface in a vibratome dish. We prepared 500-µm-thick transverse slices of tissue containing the preBötC for electrophysiological recording studies.

Electrophysiological Recordings

Slices obtained from micro-sectioning were placed in a recording chamber under perfusion of ACSF with elevated K⁺ concentration (i.e., 9 mM K⁺) at a flow rate of 5 ml/min. We increased the concentration of K⁺ in order to sustain activity from the slice (Funk and Greer, 2013; Ruangkittisakul et al., 2011; Smith et al., 1991). We recorded motor output from the hypoglossal (XII) nerve rootlet using a glass suction pipette. Output from the XII is synchronous to the rhythmic behavior exhibited by inspiratory neurons. We first identified Dbx1-neurons using epifluorescence (Fig. 1B). We then obtained recordings from inspiratory Dbx1-neurons using the whole-cell patch clamp technique (Fig. 1C). Glass patch pipettes were fashioned using a P-97 Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) with tip resistances between 3-5 MΩ. Recordings were
obtained using a patch clamp amplifier (HEKA Elektronic, USA Vendor: Instrutech Corp., Port Washington, NY). A baseline for cellular recordings was established at -60 mV.

Pharmacology

Various patch electrode internal solutions were employed. Normal K-gluconate patch solution was used for control recordings and contained the following (in mM): 140 potassium gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, and 2 Mg-ATP, dissolved in 20 ml dimethyl sulfoxide (DMSO).

BAPTA patch solution was made as follows (in mM): 125 potassium gluconate, 30 BAPTA (Sigma Aldrich, St. Louis, MO, Stock # 14510), 5 NaCl, 20 HEPES, 2 Mg-ATP, 0.3 Na$_3$GTP, dissolved in 20 ml in DMSO.

A 10 mM 9-phenanthrol stock solution was made by dissolving 0.388 g of 9-phenanthrol (Sigma 211281) in 20 ml DMSO. 2 ml of the 9-phenanthrol stock solution was added to 0.2 ml of normal K-gluconate patch solution in order to create 100 µM 9-phenanthrol patch solution.

We also made a 10 mM stock solution of flufenamic acid (FFA) by dissolving 0.115 g of FFA (Sigma F9005) in 20 ml DMSO. We then added 2 ml of the FFA stock solution to 0.2 ml of normal K-gluconate patch solution to make 100 µM FFA patch solution.

We made a 10 mM stock solution of Pyrazole 3 (Pyr3) by dissolving 10 mg of Pyr3 (Sigma 648490) in 483 µl of DMSO. We added 13 µl of the stock solution
to 60 ml of 9 mM K+ ACSF through the bath perfusion system to apply 10 µM Pyr3 to the slice. This is because Pyr3 directly binds to the extracellular site of TRPC3 (Kiyonaka et al., 2009; Zhou and Roper, 2014).

Analysis of Electrophysiological Recordings

We measured peak amplitude and area of inspiratory burst envelopes (i.e., the ‘drive potentials’) from Dbx1-preBötC neurons. We did similar analyses with the integrated XII motor nucleus output using LabChart software. We compiled all measurements in Microsoft Excel and computed burst period and frequency and statistics for group data. We had to exclude sigh bursts from analysis of eupneic (normal) bursts (and vice versa for the strictly sigh analyses). We were able to analyze the peak amplitude and area of the drive potentials and compare control and steady-state conditions of dialysis. A two-tailed t-test was used to determine statistical significance. Our quantitative data was incorporated into graphical format using IgorPro (v.6) software (Wavemetrics, Oswego, OR) and Adobe Illustrator (Adobe Systems Inc., San Jose, CA).
Results

Effect of TRP channels on normal bursts (eupnea):

We optimized our use of brainstem slice tissue to obtain experimental access to the preBötC. Our use of intersectional mouse genetics induced td-Tomato fluorescent protein expression in Dbx1-derived cells (Fig. 1A). Our target neurons could thus be identified using epifluorescence. We additionally used the nucleus ambiguus (NA) and the inferior olive (IO) as neuroanatomical markers because they collocate with the preBötC at the rostral surface of the slice preparation (Fig. 1B, Ruangkittisakul et al. 2014). Dbx1 preBötC neurons that were successfully patched were dialyzed with Alexa 488 dye through the patch pipette (Fig. 1C). As previously mentioned, inspiratory neurons within the preBötC are characterized by their rhythmic behavior that is synchronous with inspiratory-related XII motor output (Fig. 2).

We tested whether TRP channels, specifically TRPM4 and TRPC3, were implicated in inspiratory burst generation. In order to test this hypothesis, we dialyzed inspiratory neurons with various TRP antagonists through patch pipettes, or bath-applied TRP antagonists for those acting on the outside of the membranes. The control condition was reflected by the first set of ~n bursts after breaking the seal and achieving a whole-cell patch recording (prior to dialysis by the drug in the patch solution). Neurons were recorded for at least 20 minutes in order to observe steady-state conditions after achieving the whole-cell patch configuration and intracellular dialysis by drugs. For the present analysis, we only analyzed bursts
that were not sigh bursts (highlighted by arrows in Fig. 2). The following section describes the analyses of just the non-sigh bursts (i.e., eupnea).

We subjected Dbx1-derived inspiratory preBötC neurons to one of five experimental conditions. First, control neurons were dialyzed with normal K-gluconate patch solution (i.e., no drug, Fig. 3A). We observed no significant change in the amplitude (p = 0.06) or area (p = 0.22) of the drive potentials from control (0-5 minutes of whole cell recording) to steady state-conditions (5+ minutes of recording) (Fig. 2A).

The second condition employed BAPTA patch solution. BAPTA is a quaternary Ca\(^{2+}\) chelator. This experiment thus prevented intracellular Ca\(^{2+}\) from binding to its target, in this case ion channels that produce \(I_{\text{CAN}}\). Therefore, there was no monovalent cation influx that generated \(I_{\text{CAN}}\) in affected neurons. When targeted neurons were dialyzed with BAPTA, we observed a significant 48% and 33% decrease in drive potential amplitude (p = 0.01) and area (p = 0.02), respectively (Fig. 2B).

The third condition utilized flufenamic acid (FFA), which modulates a suite of TRP ion channels. This compound additionally has a broad range of targets other than TRP ion channels, thus results must be interpreted cautiously (Guinamard et al., 2013). When we dialyzed Dbx1-derived inspiratory preBötC neurons with FFA, we measured significant attenuation of drive potential amplitude (p = 0.002) and area (p = 0.05) by 40% and 36% respectively (Fig. 2C).
The fourth condition involved neurons dialyzed with 9-phenanthrol, which specifically inhibits TRPM4 (Grand et al., 2008; Guinamard et al., 2014). We observed significant attenuation of drive potential amplitude by 27% (p = 0.0008), but no significant attenuation in drive potential area (p = 0.268) (Fig. 2D).

The fifth experimental condition utilized Pyrazole 3 (Pyr3) to specifically inhibit TRPC3 in targeted neurons (Kiyonaka et al., 2009). Bath-applied Pyr3 of targeted neurons resulted in significant attenuation of both drive potential amplitude (p = 0.02) and area (0.02) by 30% and 39%, respectively (Fig. 2E).

Together, these data indicate that there is a substantial contribution of both TRPM4 and TRPC3 in normal inspiratory burst generation.

**Effect of TRP channels on sigh bursts:**

Sighing is another respiratory behavior that is exhibited by Dbx1-derived preBötC neurons. Sigh bursts are 2-5 times bigger than normal bursts and are additionally characterized by bimodal inspiration (‘doublet’) with a longer refractory period between bursts (Fig. 2, Li and Yackle, 2017; Li et al., 2016). We used these characteristics to identify and isolate sigh bursts from eupnea while performing our analyses. We also quantified the drive potential amplitude and area of sigh bursts in all experimental conditions in the same manner as our eupneic analyses. In control conditions there was no significant attenuation of drive potential amplitude (p = 0.09) and area (0.06) (Fig. 3A). In BAPTA conditions, there was significant attenuation of both the amplitude (p = 0.03) and area (p =
0.04) of the drive potential by 34% and 42%, respectively (Fig. 3B). In FFA, there was significant attenuation of the drive potential amplitude (p = 0.02) and area (p = 0.03) by 32% and 46%, respectively (Fig. 3C). In 9-phenanthrol, there was significant attenuation of the drive potential amplitude (p = 0.03) by 14%, but there was no significant decrease in the area (p = 0.93). In Pyr3, there was significant attenuation of both drive potential amplitude (p = 0.009) and area (0.005) by 23% and 26%, respectively.

Together, these data indicate that there is also substantial contribution of both TRPM4 and TRPC3 in sigh burst generation.
Discussion

Breathing behavior can be explained at the network, cellular, and molecular levels. The preBötzinger Complex is the region of the medulla that contains the underlying core oscillator. Neurons that comprise the oscillator arise form Dbx1-expressing progenitors. What remained a mystery was a molecular-level (i.e., ion channel) explanation for breathing-related rhythms. Elucidating the molecular point of origin of respiration is crucial for comprehensive understanding.

We hypothesized that TRPM4 and TRPC3 are key ion channels for inspiratory bursts that drive breathing movements. These hypotheses come from our previous electrophysiological evidence (Ben-Mabrouk and Tryba, 2010; Crowder et al., 2007; Mironov, 2008; Mironov and Skorova, 2011; Pace and Del Negro, 2008; Pace et al., 2007) as well as our RNA-Seq data (Supplemental Fig. 1). In order to confirm this hypothesis, we pharmacologically inhibited the function of these ion channels in vitro using the whole-cell patch-clamp technique in a brain slice preparation. Our data indicate significant contribution of both TRPM4 and TRPC3 in inspiratory burst generation.

It is noteworthy to acknowledge possible sources of error when we designed our experimental protocols. We understand that the preBötzinger Complex is heterogeneous and we only want to target Dbx1-expiratory neurons within the preBötzinger Complex. In order to eliminate side effects at the network level, we used the whole-cell patch-clamp technique to target individual neurons for experimental
Our exception to this was the series of Pyr3 experiments. Pyr3 is known to have extracellular affinity for TRPC3 (Kiyonaka et al., 2009). Intracellular application of Pyr3 has no affect on TRPC3 channels. Therefore, we had to apply the drug through the bath perfusion in order to elicit a response to the drug in the recorded neurons.

We also recognize that some of our compounds have broad targets. BAPTA sequesters intracellular Ca^{2+} to prevent $I_{\text{CAN}}$ activation and FFA inhibits multiple TRP and non-TRP channels. Both are considered general $I_{\text{CAN}}$ antagonists. The use of specific antagonists for TRPM4 (9-phenanthrol) and TRPC3 (Pyr3) allowed us to directly inhibit our candidate ion channels. Our control experiments did not show attenuation of the drive potential during dialysis. This demonstrates the efficacy of our experimental antagonists.

Inhibition of either TRPM4 or TRPC3 caused significant attenuation of the drive potentials generated by Dbx1-derived inspiratory preBötC neurons. However, rhythmic activity of the experimental neurons persisted during drug dialysis because the rest of the slice was unaffected. There are a few ideas that may explain this phenomenon. It is possible that multiple ion channels contribute to $I_{\text{CAN}}$ current—not just TRPM4 and TRPC3. Synaptic input to the recorded neuron can also give rise to $I_{\text{CAN}}$. Another possibility is that $I_{\text{CAN}}$ is a safety factor for the underlying glutamatergic currents. This would imply that $I_{\text{CAN}}$ current augments the drive potentials and is not vital for inspiratory burst generation, but this has yet to be test *in vivo.*
This study identified possible molecular origins of breathing behavior. The preBötC is the network origin, Dbx1 inspiratory neurons are the cellular origins, and TRPM4 and TRPC3 ion channels are molecular origins of breathing. In comparison, other motor behaviors such as chewing and locomotion can only be explained at the network level. The cellular and ion channel (molecular) origins of these other behaviors remain unknown. This implies that breathing may be the first and only motor behavior that can be defined at all levels of complexity—the network, cellular, and ion channel levels.
References


Pace, R.W., Mackay, D.D., Feldman, J.L., and Del Negro, C.A. (2007). Inspiratory bursts in the preBötzinger complex depend on a calcium-activated non-
specific cation current linked to glutamate receptors in neonatal mice. J. Physiol. 582, 113–125.


Figure 1: Identification of Dbx1-derived preBötzinger Complex neurons. (A) Transgenic breeding of $Dbx1^{CreERT2}$ females with $Rosa26^{tdTomato}$ males. (B) 10x magnified images of the experimental slice using transillumination and epifluorescence to identify Dbx1 cells. (C) 63x magnified Dodt imaging,
epifluorescence, alexa 488 (GFP) and merged channels of target neuron. Scale bar is 20 μm.
**Figure 2:** Pharmacological inhibition of TRP channels using the whole-cell patch clamp technique. (A) Control (n = 7) (B) BAPTA dialysis (n = 5) (C) FFA dialysis (n = 6) (D) 9-phenanthrol dialysis (n = 13) (E) Bath-applied Pyr3 (n = 10). (Center) Arrows indicate sigh bursts. (Right) Computed averages of experimental results are in red. * indicates statistical significance.
Figure 3: Quantification of sigh data from pharmacological experiments. (A) Control (n = 5) (B) BAPTA (n = 5) (C) FFA (n = 4) (D) 9-phenanthrol (n = 9) (E)
Pyr3 (n = 7). Computed averages of experiments are in red. * indicates statistical significance at $p < 0.05$. 
**Supplemental Figure 1:** RNA-seq of TRP channels determined the quantitative RPKM values of RNA in single samples (15 neurons each sample) and mean using a heat map. TRPC3 expression in both Dbx1 and non-Dbx1 populations of the preBötC is relatively high in comparison to other TRP channels.
Supplemental Figure 2: Immunolabelling of neurons within the preBötzinger complex (preBötC) demonstrates the presence of both TRPM4 (dark areas indicate expression) and TRPC3 (punctate, light areas indicate expression) proteins in Dbx1 cells. Arrows point to select Dbx1 cells that demonstrate localized antibody staining, indicating the presence of the respective TRP channel.