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34

Abstract

Sighs prevent the collapse of alveoli in the lungs, initiate arousal under hypoxic conditions, and even express sadness and relief. Sighs are periodically superimposed on normal breaths, known as eupnea. Implicated in the generation of these rhythmic behaviors is the preBötzinger complex (preBötC)¹. Yet how this small microcircuit can produce two rhythms with strikingly different periodicities remains unresolved. Our computational simulations predict that sighs are generated by the coincidence of two temporally distinct calcium oscillations and are in agreement with experimental evidence suggesting that astrocytes drive sigh behavior through slower, extrinsically driven calcium oscillations that link the eupnea and sigh rhythms. We found that purinergic signaling is necessary to generate spontaneous and hypoxia- induced sighs, and photo-activation of preBötC astrocytes is sufficient to elicit sigh activity. We conclude that sighs are an emergent property of the preBötC network generated by neuroglial interactions, where the distinct modulatory responses of neurons and glia allow for both rhythms to be independently regulated.

Main

A question that is fundamental to the understanding of respiratory physiology is how the activity of a single population of neurons can be reconfigured to produce multiple behaviors, as is observed with eupnea, sighs, and gasps². The sigh is a critical component of breathing, serving to periodically reinflate collapsed alveoli in the lungs. The precise mechanisms by which sighs are generated remain unclear. However, it is understood that sighs and eupnea can be modulated separately³⁻⁷ and are characterized by distinct intrinsic⁸ and synaptic mechanisms within the preBötC⁹.

Contrary to this idea, it has been suggested that the control of sighing is driven by peptidergic projections from the Retrotrapezoid Nucleus (RTN) to the preBötC⁷. However, lesioning the RTN does not alter sigh frequency¹⁰, and sighs are preserved in wedges that physically isolate the preBötC⁵. These findings led us to combine computational and experimental approaches to revisit the question of how sighs can be generated locally.

Characterization of sighs: sighs are not generated through inhibitory mechanisms

Sighs vary in shape and not every large amplitude burst is a sigh. Thus, we analyzed characteristics that distinguish sighs from eupneic bursts generated by the preBötC in brainstem slices from neonatal mice. Under control conditions, sigh amplitude, burst duration, and post-burst interval were significantly greater than eupneic bursts, and occurred on a much slower time scale (Fig. 1a, b). Variation in sigh shape is a result of different degrees of connection to the eupneic rhythm; some sighs appeared as connected double bursts, with a large amplitude burst superimposed on a eupneic burst ('biphasic'), whereas others occurred as a single large amplitude burst ('monophasic') (Extended Data Fig. 1a). Biphasic and monophasic sighs did not differ in burst amplitude, burst duration, or post burst interval. Under control conditions *in vitro*, we found that $78.5 \pm 5.8\%$ of total sighs were biphasic and $21.4 \pm 5.8\%$ were monophasic. The prevalence of each sigh shape was variable between slices, and faster eupneic rhythms tended to have more monophasic sighs (Extended Data Fig. 1b). Previous hypotheses suggested that sighs are generated through inhibitory mechanisms¹¹. We show that pharmacological blockade of GABAergic and glycinergic synaptic inhibition with strychnine and gabazine, referred to as "inhibition block", did not eliminate sighs, change their frequency, or dissociate them from eupneic bursts (Fig.

1c)¹². Sigh amplitude, post-burst interval, duration, and shape were also unaffected by inhibition block. However, the amplitude of eupneic bursts increased relative to sighs¹³, thereby decreasing the ratio of sigh to eupnea amplitude (Extended Data Fig. 1c). Moreover, sigh frequency could still be increased pharmacologically following inhibition block with the β -adrenergic agonist isoproterenol (Fig. 1c), a potent sigh modulator⁶.

An excitatory preBötC model combines Ca^{2+} oscillations to generate sighs

Based on these results, we designed an *in silico* model of the preBötC network comprised of 300 excitatory model neurons. To test the feasibility of calcium oscillations to generate sigh-like network activity, the *in silico* excitatory preBötC network modifies an existing model¹⁴ to include a current gated by intracellular calcium ($\text{Ca}^{2+}_{\text{in}}$; Extended data Fig. 1d). $\text{Ca}^{2+}_{\text{in}}$ evolves on both fast and slow timescales (Fig. 1d-f, Table 1, 2). The fast calcium signal ($\text{Ca}^{2+}_{\text{fast}}$) is linked to inactivation of persistent sodium channels (Eq. 17) and activates during periods of burst firing (i.e. eupnea)¹⁵. The slow calcium ($\text{Ca}^{2+}_{\text{slow}}$) follows a slow oscillatory pattern^{15,16} that is extrinsically driven and synchronized across all neurons. This slow Ca^{2+} oscillation occurs independently of neuronal activity in the network and follows previously described dynamics¹⁶. Thus, the total $\text{Ca}^{2+}_{\text{in}}$ of each neuron of the respiratory network is a superposition of calcium dynamics on these two timescales (Eq.17).

Model neurons were defined as intrinsically bursting, tonic, or quiescent (B, T, Q) based on each neuron's firing pattern in synaptic isolation (Fig. 1d, Extended Data Fig. 3). Simulations including Ca^{2+} gated channels did not produce sigh bursts in the absence of Ca^{2+} oscillations. Neither the addition of a fast, eupnea-linked Ca^{2+} oscillation, nor a slow, extrinsically driven Ca^{2+} oscillation was sufficient to produce sigh bursts on their own. However, model networks including both fast and slow oscillations

reliably produced periodic sigh-like bursts similar to those observed *in vitro*⁵ (Fig. 1d, e). Indeed, compared to eupneic bursts, sigh bursts in the computational model were larger in amplitude and exhibited prolonged burst durations and post-burst intervals (Extended Data Fig. 1e). Modelled sighs were also temporally linked to the eupneic rhythm often occurring as an augmented population burst superimposed on a normal eupneic burst. Consistent with experimental findings *in vitro*, inclusion of both slow and fast Ca^{2+} components produced sigh-like bursts in “bursting” neurons even when synaptically isolated from the network (Fig. 1d)⁵. Furthermore, the model produced a small subpopulation of “sigh-only” neurons (18/300, 6%) that were active during sighs but not eupnea (Fig. 1e, f)⁵. These neurons were not defined to be “sigh-only” *a priori*.

To further test the role of Ca^{2+} oscillations in producing the sigh rhythm, Ca^{2+} dynamics were manipulated in the model network. We altered the amplitude of the slow Ca^{2+} oscillation by modifying the parameter k_1 , the amplitude of the fast Ca^{2+} oscillation by modifying k_2 , and the frequency of the slow Ca^{2+} oscillation by modifying the intracellular calcium release parameter $[\text{IP3}]$ ¹⁶. Increasing the amplitude of $\text{Ca}^{2+}_{\text{slow}}$ increased the probability of sighs (Fig. 2a, b). When the amplitude of $\text{Ca}^{2+}_{\text{slow}}$ was small, total Ca^{2+} exceeded the Ca^{2+} gated channels’ threshold and only evoked a sigh when the $\text{Ca}^{2+}_{\text{slow}}$ burst coincided with the eupnea linked $\text{Ca}^{2+}_{\text{fast}}$ oscillation, thereby linking the timing of sigh bursts to the burst phase of the eupnea rhythm (Fig. 2c). Indeed, sighs rarely occurred when the $\text{Ca}^{2+}_{\text{slow}}$ peak occurred early in eupneic phase (see Methods), that is, when the $\text{Ca}^{2+}_{\text{slow}}$ transient immediately followed a eupneic burst (Fig. 2c, Extended Data Fig. 2a)^{16,17}. Increasing $\text{Ca}^{2+}_{\text{slow}}$ amplitude increased the probability of a given $\text{Ca}^{2+}_{\text{slow}}$ oscillation evoking a sigh by lengthening the time-window within the eupneic cycle during which the total $\text{Ca}^{2+}_{\text{in}}$ could exceed the threshold for Ca^{2+} gated channel activity (Extended Data Fig. 2a). The morphology of modeled sighs also

depended on the amplitude and eupneic phase of the $\text{Ca}^{2+}_{\text{slow}}$ oscillation (Extended Data Fig. 2b, d). Biphasic sighs occurred more often at low $\text{Ca}^{2+}_{\text{slow}}$ amplitudes and when the $\text{Ca}^{2+}_{\text{slow}}$ oscillation coincided with or slightly preceded the onset of a eupneic burst. Conversely, monophasic sighs occurred more frequently when $\text{Ca}^{2+}_{\text{slow}}$ amplitude was high, and when the $\text{Ca}^{2+}_{\text{slow}}$ oscillation peaked between eupneic bursts. While holding the dynamics of the $\text{Ca}^{2+}_{\text{slow}}$ oscillation fixed, sighs also emerged from the model preBötC network when the amplitude of $\text{Ca}^{2+}_{\text{fast}}$ was increased; however, sighs were only generated within a small range of $\text{Ca}^{2+}_{\text{fast}}$ amplitudes (Extended Data Fig. 3). Increasing the frequency of $\text{Ca}^{2+}_{\text{slow}}$ while holding the $\text{Ca}^{2+}_{\text{slow}}$ amplitude constant led to a corresponding increased sigh frequency from low to moderate $\text{Ca}^{2+}_{\text{slow}}$ frequencies (Extended Data Fig. 3). At higher $\text{Ca}^{2+}_{\text{slow}}$ frequencies sighs were eliminated, and eupnea was disrupted (Fig. 2d).

Ca^{2+} dynamics drive sigh behavior *in silico* and *in vitro*

Our *in silico* preBötC model network made three predictions for the role of an extrinsically driven calcium oscillation in generating sigh bursts. Increases in $[\text{Ca}^{2+}]_{\text{in}}$ should: 1) increase sigh occurrence, 2) decrease the prevalence of biphasic sigh bursts, and 3) abolish sighs above a certain critical concentration. To test these predictions experimentally, we used norepinephrine (NE), a neuromodulator known to increase sigh frequency^{6,18} and up-regulate $\text{Ca}^{2+}_{\text{in}}$ concentration¹⁹ through second messenger pathways via activation of adrenergic receptors¹⁷. NE increased the frequency of sighs in a manner consistent with the simulated manipulation of $\text{Ca}^{2+}_{\text{slow}}$ frequency (Fig. 2d, e). Furthermore, increasing [NE] *in vitro* and increasing slow Ca^{2+} oscillation amplitude *in silico* had similar effects on sigh shape, shifting sighs from mostly biphasic to mostly monophasic (Fig. 2e, f). Consistent with simulated population activity observed at high

Ca²⁺_{slow} frequencies (Fig. 2d), inter-sigh interval decreased with increasing [NE] until the concentration was too high and resulted in cessation of sighs, leaving only eupneic activity (Fig. 2e, g). Collectively, these experimental and computational manipulations of Ca²⁺ suggest sigh generation is driven by the presence of a slow, rhythmic signaling mechanism that causes synchronized increases in [Ca²⁺]_{in} among preBötC neurons.

Astrocytes modulate sighing through purinergic signaling

We hypothesized that glia could be the source of the Ca²⁺_{slow} transients that are synchronized across the respiratory rhythm generating network. Astrocytes respond to metabolic changes in their extracellular environment by increasing intracellular Ca²⁺²⁰ through G-protein coupled receptor cascades. In the respiratory networks^{21,22}, and throughout the central nervous system^{23,24}, astrocyte responses spread through gap junctions²⁵ and involve the release of ATP which modulates neural activity via purinergic mechanisms²⁶⁻²⁸. Therefore, we tested the contribution of purinergic signaling to sigh generation *in vitro* by pharmacological manipulation of ADP and ATP receptors P2Y1 and P2X, respectively (Fig. 3a, b)²⁹. In rhythmic brainstem slices containing the preBötC, activation of purinergic P2Y1 receptors (P2Y1_R) with MRS2365 significantly increased sigh frequency (Fig. 3a), and subsequent blockade of P2Y1 receptors (MRS2279), but not P2X receptors (TNP-ATP), abolished sighs. The P2Y1_R antagonist also blocked sighs induced with the β-adrenergic agonist isoproterenol (Fig. 3b). These findings suggest that P2Y1- but not P2X-dependent purinergic signaling is important for the generation of spontaneous sighs.

To test if purinergic P2Y1 inhibition would also block sighs generated during the central hypoxic response *in vitro* (Fig. 3d), eupnea and sigh activity was recorded before and during an initial bout of severe hypoxia (SH). After a recovery period, MRS2279

was applied prior to a second hypoxic episode. Inhibition of P2Y₁ signaling eliminated or reduced sighs in 8 of 9 slices. We performed immunohistochemistry to confirm P2Y_{1R} expression in the brainstem^{29,30}. We found that P2Y_{1R} was widely expressed in the preBötC and appeared to colocalize with both neurons and astrocytes (Fig. 3c). Furthermore, inhibition of P2Y_{1R} significantly reduced the augmentation of eupneic frequency characteristic of the initial phase of the hypoxic response²⁶. In control experiments where no drugs were applied, similar effects were not observed during the second exposure to severe hypoxia (Fig. 3d).

Astrocytes are sufficient for sigh generation within the preBötC network

To further test the potential role of astrocytes in sigh generation, we tested whether selective manipulation of astrocytes^{31,32} would be sufficient to produce sighs. In brainstem preBötC slices from *Aldh1l1*^{Cre};*Rosa26*^{ChR2:EYFP} mice, we found that brief (200ms) light pulses evoked both eupnea and sigh bursts recorded from the contralateral preBötC (Fig. 4a,b). Our photostimulation parameters were able to reliably evoke bursts in 12/25 slice preparations. In the remaining slices, longer duration or higher intensity light pulses were effective in evoking eupnea and sigh bursts. Sighs evoked by photoactivation of *Aldh1l1*-expressing cells were indistinct from spontaneous sighs in burst amplitude and post burst interval (Fig. 4a). However, the duration of evoked sigh bursts was shorter relative to spontaneous sighs, consistent with a small increase in the proportion evoked monophasic sighs. These experiments suggest that *Aldh1l1* astrocytes are sufficient to initiate both sigh and eupneic bursts that are largely indistinguishable from spontaneously occurring activities.

Blocking P/Q-type calcium channels inhibits sighs⁹, and P/Q type (Cav2.1) knockout mice do not generate sighs *in vitro* or *in vivo*, and ultimately die of atelectasis⁸.

The effect of P/Q-type channel can also be mimicked by the general Ca^{2+} channel blocker Cd^{2+} which selectively inhibits sighs at low concentrations^{5,33}. Therefore, 4 μM Cd^{2+} was applied during optogenetic stimulation to test if astrocyte stimulation would continue to evoke sighs (Fig. 4b, Extended Data Fig. 4a). In Cd^{2+} , photostimulation failed to evoke sighs. However, we observed evoked double bursts and bursts that appeared to have slightly larger amplitudes than eupneic bursts. We refer to these bursts as 'sigh attempts'. Sigh attempts were less likely to occur than sighs evoked under baseline conditions; 4.7% of stimulations evoked a sigh attempt in Cd^{2+} , compared to 24.1% in baseline. The amplitude of sigh attempts in Cd^{2+} was significantly reduced from baseline sighs but remained slightly larger than evoked eupnea. The duration of sigh attempts in Cd^{2+} was statistically indistinct from eupnea duration. Moreover, sigh attempts in Cd^{2+} lacked an extended post burst interval ("post-sigh apnea") that is characteristic of normal sighs.

We also tested if the P2Y1_R antagonist MRS2279 would affect the ability of astrocytes to drive sighing (Fig. 4b, Extended Data Fig. 4a). Following P2Y1_R inhibition, sighs evoked during photostimulation of *Aldh1l1* astrocytes were completely blocked in all but one slice ($n = 7$). Consistent with evoked sigh attempts observed in Cd^{2+} , sigh attempts in MRS2279 were smaller in amplitude than baseline sighs and had burst durations similar to evoked eupnea. However, post-burst intervals after sigh attempts in MRS2279 remained longer than those after eupnea (Fig. 4b). Collectively, these data indicate that P2Y1_R - and Ca^{2+} -dependent signaling are important mechanisms that allow astrocytes to drive sighing activity in the preBötC.

Next, we asked if light activation of astrocytes *in vivo* would have the same effect on sigh modulation ($n = 9$). Indeed, we found that there was a higher probability of evoking sighs *in vivo* than *in vitro* (Fig. 4c, Extended Data Fig. 4c), and in general, sigh

characteristics similarly matched *in vitro* data. Evoked sighs *in vivo* could be unambiguously distinguished from eupnea by characterizing the diaphragm amplitude (Fig. 4d). Notably, evoked eupnea in simultaneous recordings of the hypoglossal nerve (XII) tended to be larger in amplitude than spontaneous eupnea, which made distinguishing sighs from eupnea more difficult based solely on XII recordings (Extended Data Fig. 4b).

Interestingly, MRS2279 bilaterally injected into the preBötC *in vivo* was less effective in completely blocking spontaneous or evoked sighs ($n = 3/6$, Extended Data Fig. 4c, d); contrary to the very reliable inhibitory effect observed *in vitro* (Fig. 4b). This may not be surprising given that numerous descending inputs that are present *in vivo* and endogenously modulate sighs³⁴ including bombesin-like peptides⁷ and noradrenergic inputs⁶ will likely reduce the ability to block sighs by purinergic mechanisms *in vivo*. Corresponding to this variability, we observed a higher probability of evoked burst failures altogether in the presence of MRS2279 (Extended Data Fig. 4c). This weaker stimulation corroborates the control of sighs from outside areas⁷, which are eliminated from *in vitro* slice experiments. Our data indicate that sighs are an emergent property involving local neuroglial interactions mediated by purinergic signaling and modulatory inputs intrinsic and extrinsic to the preBötC.

Materials and Methods

Computational Model

The computational model for the present study incorporates a shared external source of Ca^{2+} oscillations to elicit global synchronization across the respiratory network. Equations were adopted from an original model of the respiratory network¹⁴. The membrane potential of each compartment and gating variables are described in

equations 5 - 16. A Ca^{2+} dependent current which has bell shaped Ca^{2+} activation/inactivation was incorporated into each neuron. Based on the previous experimental observations indicating that the sigh frequency is independent from eupneic frequency² and that sighs are inhibited by Ca^{2+} channel blockers and absent in P/Q type Ca^{2+} channel knock-out mice⁸, we modeled the dynamics of Ca^{2+} oscillations as a superposition of two independent components: 1) slow oscillations with dynamics governed by IP_3 channels in endoplasmic reticulum (ER)^{16,35} and 2) fast Ca^{2+} that are associated with eupneic activity and are regulated by the dynamics of the persistent sodium current^{4,35}. The slow component is defined by:

$$\frac{dC}{dt} = f_i \cdot (J_{ERin} - J_{ERout}) \quad \text{Eq.1}$$

$$J_{ERin} = \left(L + P \left(\frac{I \cdot C \cdot l}{(I + k_I)(C + k_a)} \right)^3 \right) \left(\frac{C_t - C}{\sigma} - C \right) \quad \text{Eq.2}$$

$$J_{ERout} = \left(\frac{V_e C^2}{k_e^2 + C^2} \right) \quad \text{Eq.3}$$

$$l' = A \cdot (k_d - l \cdot (C + k_d)) \quad \text{Eq.4}$$

where C is the shared external calcium signal, J_{ERin} and J_{ERout} are the fluxes of Ca^{2+} into and out of the ER, respectively, and l is the IP_3 channel gating variable. The parameter f_i is the ratio of bound to free $[\text{Ca}^{2+}]$, L is the leak ER permeability, P is the maximum permeability of the IP_3 channels, I is the concentration of IP_3 channels, k_I is the dissociation constant of IP_3 receptor by IP_3 , k_a is the dissociation constant of the IP_3 receptor for Ca^{2+} , C_t is the maximal $[\text{Ca}^{2+}]$ in the external source, σ is the ratio of the volume of the ER to the volume of the cytosol, V_e is the maximal SERCA pump rate, k_e is the SERCA pump coefficient, A is a scaling factor, and k_d is the constant of IP_3 receptor inactivation by Ca^{2+} .

Each neuron is a single compartment neuron modeled by the following equations:

$$\frac{dV}{dt} = \frac{-1}{C_m} (I_{Na} + I_K + I_{NaP} + I_{leak} + I_{syn} + I_{Cas}) \quad \text{Eq.5}$$

279 The sodium, potassium, persistent sodium, leak, synaptic, and Calcium sensitive
280 currents are, respectively:

$$I_{Na} = g_{Na} \cdot m_{\infty}(V)^3 \cdot (1 - n(V)) \cdot (V - E_{Na}) \quad \text{Eq.6}$$

$$I_K = g_K \cdot n(V)^4 \cdot (V - E_K) \quad \text{Eq.7}$$

$$I_{NaP} = g_{NaP} \cdot m_{p\infty}(V) \cdot h(V) \cdot (V - E_{Na}) \quad \text{Eq.8}$$

$$I_{leak} = g_{leak} \cdot (V - E_{leak}) \quad \text{Eq.9}$$

$$I_{syn} = \sum_i^k g_{syn_i} \cdot (V - E_{syn}) \quad \text{Eq.10}$$

$$I_{Cas} = g_{Cas} \cdot m_{Cas}(Ca)^2 \cdot h_{Cas}(Ca) \cdot (V - E_{Cas}) \quad \text{Eq.11}$$

281 where g_x represents the maximal conductance of each current, E_x represents the
282 reversal potential of the respective channel.

283 The dynamic activation (n, m_{Cas}) and inactivation (h, h_{Cas}) of the currents are governed
284 by:

$$\frac{dx}{dt} = \frac{x_{\infty} - x}{\tau_x} \quad \text{Eq.12}$$

285
286 for $x = \{n, h, m_{Cas}, h_{Cas}\}$. Time constants τ_x are

$$\tau_x = \frac{\overline{\tau_x}}{\cosh\left(\frac{V - V_x}{2\sigma_x}\right)} \text{ for: } x = \{n, h\} \quad \text{Eq.13}$$

287

288 and

$$\tau_x = \tau_{0_x} + \frac{\overline{\tau_x}}{1 + \exp(\xi_x([Ca]_{in} - [Ca]_x))} \text{ for: } x = \{m_{Cas}, h_{Cas}\} \quad \text{Eq.14}$$

289 Steady state voltage dependent activation or inactivation, x_{∞} is defined as:

$$x_{\infty} = \frac{1}{1 + \exp\left(\frac{V - V_x}{\sigma_x}\right)} \text{ for: } x = \{m, m_p, h, n\} \quad \text{Eq.15}$$

290 and

$$x_{\infty} = \frac{1}{1 + \exp(\theta_x([Ca]_{in} - [Ca]_x))} \text{ for: } x = \{m_{Cas}, h_{Cas}\} \quad \text{Eq.16}$$

291

292 The values of $[Ca]_{m_{Cas}}$ and $[Ca]_{h_{Cas}}$ define the opening and closing (respectively) of the
 293 Ca^{2+} “activation window” (the range of $[Ca]_{in}$ values that open the Ca^{2+} sensitive
 294 channel), the values of $\xi_{m_{Cas}}, \xi_{h_{Cas}}$ define the kinetics of the Ca^{2+} sensitive channel
 295 activation and inactivation (respectively), and the values of $\theta_{m_{Cas}}, \theta_{h_{Cas}}$ define the
 296 steepness of the sigmoidal activation/inactivation of the Ca^{2+} sensitive channel.

297 Intracellular calcium concentration dynamics are described as:

$$\frac{d[Ca]_{in}}{dt} = \frac{1}{\tau_{Ca}} (k_1 \cdot C + (k_2 \cdot m_{p\infty} \cdot h) - [Ca]_{in}) \quad \text{Eq.17}$$

298 Where the first term describes the slow external calcium dynamics and the second term
 299 describes the fast, bursting driven internal dynamics. The variables l, C are those from
 300 the slow, external calcium oscillation. k_1, k_2 govern the relative influence of the slow and
 301 fast calcium sources, respectively, on the total intracellular calcium levels: $[Ca]_{in}$.
 302 Synapses from a source neuron (i) to a target neuron (j) were governed by the
 303 equations³⁶:

$$\frac{ds_i}{dt} = \frac{(1 - s_i)m_{\infty_{syn}}(V_i) - s_i}{\tau_{syn}} \quad \text{Eq.18}$$

$$m_{\infty_{syn}} = \frac{1}{1 + \exp\left(\frac{V_i - Q_s}{\sigma_{syn}}\right)} \quad \text{Eq.19}$$

$$g_{syn_i} = g_{syn_{max}} \cdot s_i \quad \text{Eq.20}$$

304

305 Networks were constructed of 300 neurons with randomly generated excitatory synaptic
 306 connections such that the average neuron had a total of 6 synaptic connections
 307 (incoming and outgoing inclusive). Eight unique network configurations (connectivity
 308 patterns) were generated to allow for multiple replicates in some experiments. Neurons

were defined such that their activity without synaptic inputs could be clearly defined as bursting, tonic, or quiescent by varying their leak conductance g_{leak} (Fig. 2a). Parameters that are shared across all neurons are given in Table 1. Network and cell specific parameters are given in Table 2. Simulations were constructed using the Brian2 python package³⁷ using the Runge-Kutta 4th order method with a fixed timestep of 0.25ms. Networks were simulated for 1000s, unless otherwise stated. After 1000s, synaptic conductance (g_{syn}) was set to 0nS to observe isolated neuron behavior. Spike times were computed as the time at which the cell voltage crossed a -20mV threshold. Population rate was computed by first taking the sum of the number of spikes in each timestep, then smoothing with a boxcar filter with width of 10-100ms. Eupneic phase was defined as the time difference between the peak of the Ca^{2+}_{slow} signal and the peak of the previous eupneic burst, divided by the mean eupneic interval.

Animals

All experiments and animal procedures were approved by the Seattle Children's Research Institute's Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines. Male and female mice were selected randomly. Experiments were performed in brainstem slices obtained from CD1 or transgenic mice age postnatal day 4-12 and bred at Seattle Children's Research Institute. Aldh1l1^{cre} mice were obtained from Jackson Laboratories (FVB-Tg (Aldh1l1-cre) JD1884Htz/J, Jax stock No. 023748). Cre mice were crossed with homozygous mice containing a floxed STOP channelrhodopsin2 fused to an EYFP (Ai32) reporter sequence. All mice were group housed with access to food and water *ad libitum* in a temperature controlled ($22 \pm 1^{\circ}\text{C}$) facility with a 12hr light/dark cycle.

In vitro transverse brainstem slice preparation and population recordings

The following techniques used are described in detail in a series of publications by the Ramirez laboratory^{38,4,9,9}. PreBötC slice thickness varied between 590-610µM. Animals were quickly decapitated, and brainstems were rapidly dissected in cold artificial cerebrospinal fluid (ACSF). Serial transverse slices were made from the appearance facial nerve until reaching the level of the preBötC, where a 580-610µM slice was made. Slices were transferred into the recording chamber with circulating ACSF with osmolarity of 305-320 osm/L, pH 7.4 and containing (in mM) NaCl (118), KCl (3), CaCl₂ (1.5), MgCl₂ (1), NaHCO₃ (25), NaH₂PO₄ (1), and D-glucose (30). Temperature was maintained at 29-31°C and ACSF was bubbled with carbogen (95% O₂ / 5% CO₂). During extracellular population recordings the superfused ACSF was circulating and recycled at a flow rate of 10-13ml/min. Activity was recorded from the caudal side of the transverse slice. Some experiments were performed with two slices at the same time using two extracellular electrodes. Extracellular raw traces were filtered and integrated. The data obtained were analyzed using GraphPad Prism 7 and PCLAMP 10 software which is available online at <http://www.moleculardevices.com/systems/conventional-patch-clamp/pclamp-10-software>. For unknown reasons, some preBötC slices do not have periodic sighs within the baseline recording period, or they occur at intervals >20 mins. These slices were not used for experiments. PreBötC slices were not used across multiple drug experiments. When selecting bursts for doublet analysis, bursts occurring in quick succession were only characterized as doublets if the first burst did not reach baseline before onset of the second burst.

In Vivo Anesthetized Preparation

In vivo anesthetized experiments were performed as previously published³⁹. Briefly, adult mice were anesthetized with urethane (1.5 mg/kg, i.p.) and placed supine on a custom surgical table. Adequate depth of anesthesia was determined via heart rate, breathing frequency, and response to toe pinch. Bipolar stainless steel electrodes were implanted in the diaphragm muscles to perform electromyographic recordings of inspiratory motor activities⁴⁰. The trachea was exposed through a midline incision and cannulated caudal to the larynx with a curved (180 degree) tracheal tube (24 G). The trachea and esophagus were removed rostral to the tracheotomy, and underlying muscles were removed to expose the basal surface of the occipital bone. Mice were supplied with 100% O₂ throughout the remainder of the surgery and experimental protocol. The portion of the occipital bone and dura overlying the ventral medullary surface were removed and the brainstem surface was perfused with aCSF (36 °C) equilibrated with carbogen (95% O₂, 5% CO₂). The hypoglossal nerve (XII) was then isolated unilaterally and recorded from using a fire-polished pulled glass pipette filled with aCSF. XII electrical activity was amplified (10,000×), filtered (low pass 300 Hz, high pass 5 kHz), rectified, integrated, and digitized (Digidata 1550 A, Axon Instruments). In animals in which we observed baseline sighs, MRS2279 (500μM, 41.3nL) and Evan's Blue dye or red fluorospheres were co-injected bilaterally in 3 positions around the preBötC using a NanoInject, as to avoid lesioning the preBötC core and altering the rhythm. In most instances, injections close to preBötC stimulated sighs immediately following placement of the cannula. Saline injected control animals did not have any alterations in baseline breathing, and we observed the same increase in sighs in most injections (n = 4/5). Similar to *in vitro* experiments, many animals *in vivo* did not have baseline sighs and therefore MRS2279 was not injected.

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385 A glass fiber optic (200µm core, 0.24NA) connected to a blue (447nm) high-
386 powered LED was positioned above the preBötC contralateral to the extracellular
387 electrode or XII nerve and positioned for maximal response. Power was set to 0.5
388 mW/mm². Fifty continuous 20s sweeps were recorded for each stimulation experiment
389 *in vitro*, and 20 sweeps for *in vivo* experiments. Laser power settings and pulse width
390 were selected based on previous experiments^{13,41}. In approximately 1/3 of
391 Aldh111^{cre}/Ai32 preBötC slices, we had difficulty evoking any responses unless
392 increasing the wavelength to a higher laser power (>0.5 mW/mm²- 1 mW/mm²). Despite
393 having robust respiratory rhythms, we did not continue to perform stimulations in these
394 slices. Control stimulations were performed in Aldh111^{cre -/-}/Ai32 littermates for *in vitro* (n
395 = 2) and adults for *in vivo* (n = 3) experiments. There were no observed non-specific
396 effects of light stimulation, even at maximum laser power.

397 Norepinephrine (1-40 µM, DL-Norepinephrine hydrochloride, cat. A7256),
398 Isoproterenol (Isoprenaline hydrochloride, cat. I5627), Strychnine (1 µM, cat. S0532),
399 and cadmium (4 µM cadmium chloride, cat. 20208) were purchased from Sigma.
400 Gabazine (1 µM, SR 99531 hydrobromide, cat. 1262), MRS2279 (20µM, P2Y1_R
401 antagonist, cat. 2158,), MRS2365 (20 µM, P2Y1_R agonist, cat. 2157) and TNP-ATP
402 triethylammonium salt (20 µM, P2X receptor antagonist, cat. 2464) were obtained from
403 Tocris. All drugs were diluted in water.

404

405 *Histology*

406 After experiments, animals were fixed by transcardial perfusion with cold ACSF
407 followed by 4% PFA in phosphate buffer solution (PBS). Medullary and cortical tissue

was carefully extracted and equilibrated in graded sucrose solutions to a final concentration of 30%, embedded in OCT solution, frozen at -80°C , and cryosectioned at $25\text{ }\mu\text{m}$ for imaging of immunofluorescence. Slides containing brainstem tissue slices were washed $3\times 10\text{ min}$ in PBS. Tissue slices were then permeabilized and blocked with 1% normal donkey serum in 0.5% Triton X-100/PBS for 1 h. Following blocking, tissue slices were incubated with the primary antibody for either NeuN (Millipore MAB377), P2Y1 (Abcam ab140859), or EGFP (Abcam ab6673) (1:500 in 1% normal donkey serum in 0.5% Triton X-100/PBS) overnight. After primary antibody incubation, tissue was washed $3\times 10\text{ min}$ in phosphate buffer solution (PBS), followed by secondary antibody incubation for 30 min. (Alexa Fluor 488, 594, or 647). Lastly, tissue was washed $3\times 10\text{ min}$ in PBS and hard set mounted (VECTASHIELD hardset mounting media with DAPI). Immunofluorescence images were acquired using an Olympus VS120 Virtual Slide Microscope Scanner. The excitation/emission filter sets used for imaging were DAPI: 380-405/410-480, FITC: 460-490/500-550, Cy3/TRITC: 540-570/580-640, and Cy5: 625-645/655-705. Laser scanning confocal imaging was performed using a Zeiss 710 34-channel Quasar LSC Microscope.

Sigh classification

Custom unsupervised methods were developed to distinguish between eupneic bursts and “sigh attempts” evoked by channelrhodopsin stimulation in *in vitro* recordings. First, bursts in the integrated pre-Bötzinger recording were detected such that the prominence of each peak was greater than 2.5 times the standard deviation of the integrated activity. The onset of the burst was defined as the time at which the trace reached 20% of the subsequent peak height. Bursts that were aberrantly large amplitude ($\sim >75$ times the mean amplitude) or very fast rising (reached peak amplitude

in less than 150 ms) were discarded as noise. The burst shape for all bursts was extracted from the onset to 1.5 s after onset of the burst. Burst shapes were then decomposed into principal components. The first n components (where n is the number of components needed to explain 95% of the variance) of the spontaneous bursts (i.e., not evoked by optogenetic stimulation) were used as features to fit a robust covariance estimator (sci-kit learn MinCovDet). Since the spontaneous bursts were observed never to be putative sighs, this covariance represents the prior distribution of eupnea, that is, the expected eupneic shape. We calculate the Mahalanobis distance (D) of each burst to the mean eupneic shape. D quantifies how similar each burst is to the “average” eupnea. We then determine a threshold as:

$$D_{thresh} = \widetilde{D_{spontaneous}} + 4 * IQR(D_{spontaneous}) \quad \text{Eq.21}$$

All evoked bursts where $D_{evoked} > D_{thresh}$ were considered outliers. This outlier detection included many false positive bursts in which the burst shape was different from the average eupnea, but upon inspection was not typical of a sigh (often long slow bursts or large amplitude noise). We boost this outlier detection with an identical method using instead the intuitive features of burst amplitude, full width at half max (FWHM) and time to next burst (post-burst apnea) as features to the covariance estimator. A burst was considered a sigh attempt when it was determined to be an outlier in both the classifier fit on burst shape and on the intuitive features. We imposed a set of final criteria where a sigh attempt must be larger in amplitude than 90% of the spontaneous bursts. To classify sighs in *in vivo* recordings, diaphragm EMG was recorded, rectified, and integrated as described above. Bursts in the integrated diaphragm EMG were detected, and each burst shape was extracted. The area under the curve was computed for each burst. Bursts were labeled as “sighs” if both the area under the curve and the

amplitude for a given sigh was >6x the median absolute deviation (MAD) of the 20 bursts preceding and following the given burst.

Statistical analysis

Analyses were performed using GraphPad Prism5 software. Groups were compared using the appropriate t-tests, one-way or two-way ANOVAs followed by Bonferonni's post hoc tests, if necessary. Statistical significance was set at $\alpha = 0.05$. Masking was not used during data collection or analysis.

Code availability

Source code is available at (<https://github.com/nbush257/sighnet>).

Data availability

The data presented in this manuscript is available upon request.

Caveats and limitations

The use of transverse preBötC slices as a model for respiratory rhythm generation is associated with significant caveats of which oxygenation⁴², incomplete network, low temperature, elevated [K⁺] saline are the most obvious concerns, and have been discussed in most of our publications^{2,4,42-44}. Although transverse slices generate rhythmicity in 3 mM K⁺ ⁴⁵⁻⁴⁷ rhythmicity is more reliable and consistently generated at 8 mM K⁺. Thus, in the present study we consistently use the extracellular concentration of 8 mM K⁺. To distinguish the fast-respiratory rhythm from the slow sigh rhythm, we refer to the fast-respiratory rhythm as “eupneic” activity throughout this study. However, we do not imply that this *in vitro* activity represents “eupnea” in the

482 behavioral sense. Rather this activity characterizes primarily the inspiratory component
483 of the respiratory activity generated within the preBötC^{2,48,49}. Furthermore, sighs were
484 characterized based on their appearance in the in vivo preparation and were carefully
485 distinguished using a novel pipeline aimed at reducing bias. However, in vivo recordings
486 should involve increased diaphragm activity along with the characteristics observed of
487 sighs, and thus in vitro work is limited in this sense. In our analysis, we did not consider
488 doublets to be sighs. Despite these caveats and limitations, the comparisons between
489 the experiments performed *in vitro* revealed many similarities to the testable predictions
490 set forth by the computational model.

491 **References**

- 492 1 Smith, J. C., Ellenberger, H. H., Ballanyi, K., Richter, D., W. & Feldman, J. L.
493 Pre-Bötzinger Complex: A Brainstem Region That May Generate Respiratory
494 Rhythm in Mammals. *Science* **254**, 726-729 (1991).
- 495 2 Lieske, S., Thoby-Brisson, M., Telgkamp, P. & Ramirez, J. Reconfiguration of the
496 neural network controlling multiple breathing patterns: eupnea, sighs and gasps.
497 *Nat Neurosci* **3**, 600-607 (2000).
- 498 3 Peña, F. & Ramirez, J.-M. Endogenous activation of serotonin-2A receptors is
499 required for respiratory rhythm generation in vitro. *The Journal of Neuroscience*
500 **22**, 11055-11064 (2002).
- 501 4 Peña, F., Parkis, M. A., Tryba, A. K. & Ramirez, J. M. Differential contribution of
502 pacemaker properties to the generation of respiratory rhythms during normoxia
503 and hypoxia. *Neuron* **43**, 105-117 (2004).
- 504 5 Tryba, A. K. *et al.* Differential modulation of neural network and pacemaker
505 activity underlying eupnea and sigh-breathing activities. *J Neurophysiol* **99**, 2114-
506 2125 (2008).
- 507 6 Viemari, J.-C., Garcia III, A. J., Doi, A., Elsen, G. & Ramirez, J.-M. β -
508 noradrenergic receptor activation specifically modulates the generation of sighs
509 in vivo and in vitro. *Frontiers in Neural Circuits* **7** (2013).
- 510 7 Li, P. *et al.* The peptidergic control circuit for sighing. *Nature* **530**, 293-297,
511 doi:10.1038/nature16964 (2016).
- 512 8 Koch, H. *et al.* Stable respiratory activity requires both P/Q-type and N-type
513 voltage-gated calcium channels. *J Neurosci* **33**, 3633-3645 (2013).
- 514 9 Lieske, S. & Ramirez, J.-M. Pattern-specific synaptic mechanisms in a
515 multifunctional network. II. Intrinsic modulation by metabotropic glutamate
516 receptors. *J Neurophysiol* **95**, 1334-1344 (2006).
- 517 10 Souza, G. *et al.* Breathing regulation and blood gas homeostasis after near
518 complete lesions of the retrotrapezoid nucleus in adult rats. *J Physiol* **596**, 2521-
519 2545, doi:10.1113/JP275866 (2018).
- 520 11 Chapuis, C., Autran, S., Fortin, G., Simmers, J. & Thoby-Brisson, M. Emergence
521 of sigh rhythmogenesis in the embryonic mouse. *J Physiol* **592**, 2169-2181,
522 doi:10.1113/jphysiol.2013.268730 (2014).
- 523 12 Borrus, D. S., Grover, C. J., Conradi Smith, G. D. & Del Negro, C. A. Role of
524 Synaptic Inhibition in the Coupling of the Respiratory Rhythms that Underlie
525 Eupnea and Sigh Behaviors. *eNeuro* **7**, doi:10.1523/ENEURO.0302-19.2020
526 (2020).

- 527 13 Baertsch, N. A., Severs, L. J., Anderson, T. M. & Ramirez, J. M. A spatially
528 dynamic network underlies the generation of inspiratory behaviors. *Proc Natl*
529 *Acad Sci U S A* **116**, 7493-7502, doi:10.1073/pnas.1900523116 (2019).
- 530 14 Butera, R. J., Jr., Rinzel, J. & Smith, J. C. Models of respiratory rhythm
531 generation in the pre-Botzinger complex. I. Bursting pacemaker neurons. *J*
532 *Neurophysiol* **82**, 382-397 (1999).
- 533 15 Toporikova, N., Chevalier, M. & Thoby-Brisson, M. Sigh and Eupnea
534 Rhythmogenesis Involve Distinct Interconnected Subpopulations: A Combined
535 Computational and Experimental Study(1,2,3). *eNeuro* **2**,
536 doi:10.1523/ENEURO.0074-14.2015 (2015).
- 537 16 Li, Y. X. & Rinzel, J. Equations for InsP3 receptor-mediated $[Ca^{2+}]_i$ oscillations
538 derived from a detailed kinetic model: a Hodgkin-Huxley like formalism. *J Theor*
539 *Biol* **166**, 461-473, doi:10.1006/jtbi.1994.1041 (1994).
- 540 17 Galaz-Montoya, M., Wright, S. J., Rodriguez, G. J., Lichtarge, O. & Wensel, T. G.
541 beta2-Adrenergic receptor activation mobilizes intracellular calcium via a non-
542 canonical cAMP-independent signaling pathway. *The Journal of biological*
543 *chemistry* **292**, 9967-9974, doi:10.1074/jbc.M117.787119 (2017).
- 544 18 Viemari, J.-C. & Ramirez, J.-M. Norepinephrine differentially modulates different
545 types of respiratory pacemaker and nonpacemaker neurons. *J Neurophysiol* **95**,
546 2070-2082 (2006).
- 547 19 Saez, J. C., Moreno, A. P. & Spray, D. C. Norepinephrine induces Ca^{2+} release
548 from intracellular stores in rat pinealocytes. *J Pineal Res* **16**, 57-64,
549 doi:10.1111/j.1600-079x.1994.tb00083.x (1994).
- 550 20 Angelova, P. R. *et al.* Functional Oxygen Sensitivity of Astrocytes. *J Neurosci* **35**,
551 10460-10473, doi:10.1523/JNEUROSCI.0045-15.2015 (2015).
- 552 21 Funk, G. D. Neuromodulation: purinergic signaling in respiratory control.
553 *Comprehensive Physiology* **3**, 331-363, doi:10.1002/cphy.c120004 (2013).
- 554 22 Ramirez, J. M., Severs, L., Ramirez, S. C. & Agosto-Marlin, I. M. Advances in
555 cellular and integrative control of oxygen homeostasis within the central nervous
556 system. *J Physiol*, doi:10.1113/JP275890 (2018).
- 557 23 Rodrigues, R. J., Marques, J. M. & Cunha, R. A. Purinergic signalling and brain
558 development. *Semin Cell Dev Biol* **95**, 34-41, doi:10.1016/j.semcdb.2018.12.001
559 (2019).
- 560 24 Del Puerto, A., Wandosell, F. & Garrido, J. J. Neuronal and glial purinergic
561 receptors functions in neuron development and brain disease. *Front Cell*
562 *Neurosci* **7**, 197, doi:10.3389/fncel.2013.00197 (2013).

- 563 25 Cotrina, M. L. *et al.* Connexins regulate calcium signaling by controlling ATP
564 release. *Proc Natl Acad Sci U S A* **95**, 15735-15740,
565 doi:10.1073/pnas.95.26.15735 (1998).
- 566 26 Rajani, V. *et al.* Release of ATP by preBotzinger complex astrocytes contributes
567 to the hypoxic ventilatory response via a Ca²⁺ -dependent P2Y1 receptor
568 mechanism. *J Physiol*, doi:10.1113/JP274727 (2017).
- 569 27 Chen, L. *et al.* K(ATP) channels of parafacial respiratory group (pFRG) neurons
570 are involved in H₂S-mediated central inhibition of respiratory rhythm in medullary
571 slices of neonatal rats. *Brain Res* **1527**, 141-148,
572 doi:10.1016/j.brainres.2013.07.009 (2013).
- 573 28 Rajani, V., Zhang, Y., Revill, A. & Funk, G. D. The role of P2Y1 receptor
574 signaling in central respiratory control. *Respiratory Physiology and Neurobiology*
575 **226**, 3-10 (2016).
- 576 29 Lurier, A. R. *et al.* P2Y1 Receptor Modulation of the Pre-Bötzinger Complex
577 Inspiratory Rhythm Generating Network In Vitro. *The Journal of Neuroscience*
578 **27**, 993-1005 (2007).
- 579 30 Huxtable, A. G. *et al.* Glia contribute to the purinergic modulation of inspiratory
580 rhythm-generating networks. *J Neurosci* **30**, 3947-3958,
581 doi:10.1523/JNEUROSCI.6027-09.2010 (2010).
- 582 31 Tien, A. C. *et al.* Regulated temporal-spatial astrocyte precursor cell proliferation
583 involves BRAF signalling in mammalian spinal cord. *Development* **139**, 2477-
584 2487, doi:10.1242/dev.077214 (2012).
- 585 32 Srinivasan, R. *et al.* New Transgenic Mouse Lines for Selectively Targeting
586 Astrocytes and Studying Calcium Signals in Astrocyte Processes In Situ and In
587 Vivo. *Neuron* **92**, 1181-1195, doi:10.1016/j.neuron.2016.11.030 (2016).
- 588 33 Krause, K. L. *et al.* Normal breathing pattern and arterial blood gases in awake
589 and sleeping goats after near total destruction of the presumed pre-Botzinger
590 complex and the surrounding region. *J Appl Physiol* (1985) **106**, 605-619,
591 doi:10.1152/japplphysiol.90966.2008 (2009).
- 592 34 Doi, A. & Ramirez, J. M. Neuromodulation and the orchestration of the
593 respiratory rhythm. *Respiratory physiology & neurobiology* **164**, 96-104,
594 doi:10.1016/j.resp.2008.06.007
595 S1569-9048(08)00166-3 [pii] (2008).
- 596 35 Thoby-Brisson, M. & Ramirez, J. M. Identification of two types of inspiratory
597 pacemaker neurons in the isolated respiratory neural network of mice. *J*
598 *Neurophysiol* **86**, 104-112 (2001).
- 599 36 Destexhe, A. Oscillations, complex spatiotemporal behavior, and information
600 transport in networks of excitatory and inhibitory neurons. *Phys Rev E Stat Phys*

601 *Plasmas Fluids Relat Interdiscip Topics* **50**, 1594-1606,
602 doi:10.1103/physreve.50.1594 (1994).

603 37 Stimberg, M., Brette, R. & Goodman, D. F. Brian 2, an intuitive and efficient
604 neural simulator. *Elife* **8**, doi:10.7554/eLife.47314 (2019).

605 38 Ramirez, J. M. & Richter, D. W. The neuronal mechanisms of respiratory rhythm
606 generation. *Current opinion in neurobiology* **6**, 817-825 (1996).

607 39 Baertsch, N. A., Baertsch, H. C. & Ramirez, J. M. The interdependence of
608 excitation and inhibition for the control of dynamic breathing rhythms. *Nat*
609 *Commun* **9**, 843, doi:10.1038/s41467-018-03223-x (2018).

610 40 Lemes, E. V., Colombari, E. & Zoccal, D. B. Generation of active expiration by
611 serotonergic mechanisms of the ventral medulla of rats. *J Appl Physiol* (1985)
612 **121**, 1135-1144, doi:10.1152/jappphysiol.00470.2016 (2016).

613 41 Baertsch, N. A. & Ramirez, J. M. Insights into the dynamic control of breathing
614 revealed through cell-type-specific responses to substance P. *Elife* **8**,
615 doi:10.7554/eLife.51350 (2019).

616 42 Hill, A. A., Garcia, A. J. r., Zenella, S. & Upadhyaya, R. Graded Reductions in
617 Oxygenation Evoke Graded Reconfiguration of the Isolated Respiratory Network.
618 *J Neurophysiol* **105**, 625-639 (2011).

619 43 Garcia, A. J., 3rd, Khan, S. A., Kumar, G. K., Prabhakar, N. R. & Ramirez, J. M.
620 Hydrogen peroxide differentially affects activity in the pre-Botzinger complex and
621 hippocampus. *J Neurophysiol* **106**, 3045-3055 (2011).

622 44 Zanella, S. *et al.* When norepinephrine becomes a driver of breathing
623 irregularities: how intermittent hypoxia fundamentally alters the modulatory
624 response of the respiratory network. *J Neurosci* **34**, 36-50,
625 doi:10.1523/JNEUROSCI.3644-12.2014
626 34/1/36 [pii] (2014).

627 45 Ruangkittisakul, A. *et al.* Generation of eupnea and sighs by a spatiochemically
628 organized inspiratory network. *J Neurosci* **28**, 2447-2458,
629 doi:10.1523/JNEUROSCI.1926-07.2008 (2008).

630 46 Ruangkittisakul, A., Kottick, A., Picardo, M. C., Ballanyi, K. & Del Negro, C. A.
631 Identification of the pre-Botzinger complex inspiratory center in calibrated
632 "sandwich" slices from newborn mice with fluorescent Dbx1 interneurons. *Physiol*
633 *Rep* **2**, doi:10.14814/phy2.12111 (2014).

634 47 Tryba, A. K., Pena, F. & Ramirez, J. M. Stabilization of bursting in respiratory
635 pacemaker neurons. *J Neurosci* **23**, 3538-3546, doi:10.1523/JNEUROSCI.23/8/3538 [pii] (2003).

636 48 Carroll, M. S. & Ramirez, J. M. Cycle-by-cycle assembly of respiratory network
637 activity is dynamic and stochastic. *J Neurophysiol* **109**, 296-305,
638 doi:10.1152/jn.00830.2011

639 jn.00830.2011 [pii] (2013).

640 49 Harris, K. D. *et al.* Different roles for inhibition in the rhythm-generating
641 respiratory network. *J Neurophysiol*, jn 00174 02017, doi:10.1152/jn.00174.2017
642 (2017).
643

Figure 1. Sigh generation in the preBötC can be mimicked in silico by introducing fast and slow Ca^{2+} oscillations into an excitatory network model. (a) integrated population activity of eupnea and sighs recorded from the transverse slice preparation. Inset shows overlaid 'fictive' eupnea (gray) and sighs (green) for comparison. (b) Amplitude, burst duration, post burst interval, and inter-event interval of eupnea and sighs differ significantly (paired t-test). (c) Inhibition block (strychnine and gabazine) does not alter sigh frequency at baseline or with β -adrenergic agonist isoproterenol (ordinary one-way ANOVA). (d) In silico generation of 'fictive' sighs is altered by adjusting modeled fast (eupnea associated) and slow (extrinsically driven) Ca^{2+} transients. Inclusion of both Ca^{2+} sources resulted in sigh-like bursts. Integrated population rates ($\downarrow$ P), and quiescent, bursting, and tonic neuron voltages (Q,B,T) are shown isolated and in network for different Ca^{2+} inclusions. (e) Integrated population activity and spiking raster for 300 simulated neurons. Purple shading highlights 18 "sigh-only" neurons (f) Median number of spikes per eupnea is plotted against median number of spikes per sigh burst; each dot represents one neuron. Sigh only neurons are shown in purple. Proportion of sigh-only neurons is 6% (18/300).

Figure 2. Sigh frequency and shape is similarly affected by manipulations of Ca^{2+} in vitro and in silico. (a) In silico population activity from simulations with increasing amplitude of $\text{Ca}^{2+}_{\text{slow}}$ signals (K1). (b) Sigh probability during an extrinsic Ca^{2+} burst as a function of slow Ca^{2+} amplitude (K1) represented by independent simulations (gray circles) and average of 8 simulations (black line). (c) Population traces (black), extrinsic Ca^{2+} (blue), and peak extrinsic Ca^{2+} (red) for sigh failure and success. (d) Population firing rates of simulated networks with increasing frequency of extrinsic slow Ca^{2+} transients as governed by increases in [IP3]. (e) Representative traces of *in vitro* preBötC population activity; increasing [NE] increases number of sigh bursts. (f) Sighs are more likely to be monophasic as slow Ca^{2+} amplitude (blue) or [NE] (green) increases. (g) Average inter-sigh interval decreases for *in silico* simulations (blue) as function of extrinsic Ca^{2+} oscillation frequency ([IP3]) with fixed amplitude and *in vitro* recordings as a function of [NE] (green).

Figure 3. P2Y_{1R} dependent signaling is necessary for spontaneous and hypoxia induced sigh generation in the preBötC. (a) Integrated preBötC activity depicting time course for P2Y_{1R} manipulation. MRS2365 significantly increased the number of sighs from baseline while P2Y_{1R} antagonist MRS2279 abolished all sighs. P2X_R antagonist (TNP-ATP) did not significantly affect sigh frequency; no significant effect on eupneic frequency was observed with any drug additions. Analysis of sigh and eupnea burst number is reported as number of bursts/20 min period (one-way ANOVA). (b) P2Y_{1R} antagonist MRS2279 eliminated the β -adrenergic agonist induced increase in sighs (one-way ANOVA). (c) PreBötC section immunostained with NeuN and EYFP (for Aldh1l1^{cre}Rosa26^{eYFP}). Inset is 40x confocal image of PreBötC slice with additional immunostaining for the P2Y_{1R}. (d) P2Y_{1R} inhibition blunts the augmented portion of the hypoxic response. Representative traces from transverse preBötC slices during initial bout of severe hypoxia (SH, 6mins), followed by a recovery period and second hypoxic challenge with MRS2279. Lower panel depicts control with two consecutive bouts of severe hypoxic challenge. Gaps in recordings were made to align control and experimental traces. Inset preBötC integrated traces have time scales of 10s. MRS2279 in addition of severe hypoxia significantly impaired the hypoxic response by eliminating sighs (left) and reducing the augmenting phase of the eupneic rhythm (right, both paired t-test).

Extended Data Figure 1. (a) Sighs observed in the transverse preBötC slice preparation are commonly ‘biphasic’ or ‘monophasic’ but retain the same distinguishing sigh characteristics (paired t-tests). Inset shows overlaid eupnea (gray) and sighs (green) for comparison. (b) Higher eupneic frequencies were positively correlated with a larger number of monophasic sighs. (c) Inhibition block does not alter sigh characteristics or frequency. No change in the amplitude of sighs was observed with inhibition block, however a significant increase in eupneic amplitude led to a decrease in the sigh:eupnea ratio. No change was observed in the sigh burst duration, sigh post burst interval, or sigh shape (ordinary one-way ANOVA). (d) Ca²⁺-sensitive cation channel steady state activation curves. Steady state activation $m_{Cas\infty}$, inactivation ($h_{Cas\infty}$), and total activation ($m^2_{Cas\infty} \cdot h_{Cas\infty}$) of the Ca²⁺-sensitive channels are shown as a function of intracellular Ca²⁺ concentration. Activation starts at zero for low [Ca]_{in}; inactivation starts at 1 for low [Ca²⁺]_{in}. These channels open only when the Ca²⁺_{in} levels fall in the center “activation window”. The curves follow the expressions in equation 16; the onset and offset of the “activation window” are governed by the parameters [Ca]_{mcas} and [Ca]_{hcas}, respectively. (e) *in silico* sigh burst amplitude, post-burst interval, and burst durations are larger than eupneic bursts, consistent with *in vitro* results (see Fig. 1a). Each dot represents one burst.

Extended Data Figure 2. (a-d) Varying rate and amplitude of $\text{Ca}^{2+}_{\text{slow}}$ in silico modulates sigh frequency and shape. Heatmaps show sigh probability (a) and sigh shape (b) as a joint function of $\text{Ca}^{2+}_{\text{slow}}$ amplitude and eupneic phase. Eupneic phase is shown for 2 cycles to visualize effects occurring near eupneic onset (phase = 0, 1, 2). Black regions denote bins where no sighs were evoked. Heatmaps show sigh frequency (c) and shape (d) as joint function of $\text{Ca}^{2+}_{\text{slow}}$ frequency ([IP3]) and amplitude (k1). Each heatmap bin represents average of 8 independent simulations. Burst shapes are weakly influenced by $\text{Ca}^{2+}_{\text{slow}}$ amplitude, not frequency. (e) Sigh burst amplitude, width, and post burst interval as a function of extrinsic $\text{Ca}^{2+}_{\text{slow}}$ amplitude. Each gray dot is the mean shape characteristic across a single 1000s simulation, and each connected black dot is the average of those simulation averages ($n = 8$). (g) Same as (f), but extrinsic $\text{Ca}^{2+}_{\text{slow}}$ frequency is varied.

Extended Data Figure 3. Modulating P/Q-type Ca^{2+} strength modulates network and sigh bursting behaviors. Example single neuron and population traces from networks while varying the strength of the P/Q type Ca^{2+} current (k2). Each row shows voltage (gray) and $[\text{Ca}]_i$ (magenta) traces for 3 example neurons (quiescent, bursting, and tonic, top to bottom); the integrated population spiking activity (black); and the slow extrinsically generated Ca^{2+} signal (blue). Far left columns show the synaptically isolated neurons, middle column shows the synaptically connected network, and far right column shows the integrated population activity for the connected network over an expanded time frame.

Table 1: Neuron and Calcium Parameters

Conductances		Gating and kinetics	
C_m	21pF	$\overline{\tau}_n$	10ms
g_{Na}	28nS	$\overline{\tau}_h$	10s
g_K	11.2nS	$\overline{\tau}_{m_{Cas}}$	134ms
g_{NaP}	3.3nS	$\overline{\tau}_{h_{Cas}}$	5.25s
$g_{syn_{max}}$	70nS	$\tau_{0_{m_{Cas}}}$	2ms
g_{Cas}	0.5nS	$\tau_{0_{h_{Cas}}}$	50ms
Reversal Potentials		$[Ca]_{m_{Cas}}$	500nM
E_{Na}	50mV	$[Ca]_{h_{Cas}}$	510nM
E_K	-85mV	$\xi_{m_{Cas}}$	-400 μM^{-1}
E_{leak}	-59mV	$\xi_{h_{Cas}}$	-250 μM^{-1}
E_{Cas}	0mV	$\theta_{m_{Cas}}$	-360 μM^{-1}
E_{syn}	0mV	$\theta_{h_{Cas}}$	420 μM^{-1}
Calcium Dynamics		V_m	-34mV
f_i	0.001pL $^{-1}$	V_n	-29mV
L	0.37pL/s	V_{m_p}	-40mV
P	31000pL/s	V_h	-48mV
k_I	1 μM	σ_m	-5mV
k_a	0.4 μM	σ_n	-4mV
C_t	1.25 μM	σ_{m_p}	-6mV
σ	0.185	σ_h	5mV
V_e	400aMol/s	τ_{syn}	15ms
k_e	0.2 μM	Q_s	15mV
A	0.5 $\mu M s^{-1}$	σ_{syn}	-3mV
k_d	0.4 μM		
τ_{Ca}	1s		

Table 2: Network Parameters

N = 300, $K_{avg} = 6$

Neuron Type	g_{leak}	% of Pop.
Tonic	$U(0.5, 1.5) \text{ nS}$	10%
Bursting	$U(2.5, 3.5) \text{ nS}$	60%
Quiescent	$U(4.0, 5.0) \text{ nS}$	30%

Figures

Figure 1

(see Manuscript file for figure legend)

Figure 2

(see Manuscript file for figure legend)

Figure 3

(see Manuscript file for figure legend)

in vitro

Figure 4

(see Manuscript file for figure legend)