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Understanding the Rhythm of Breathing: So Near, Yet So Far

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Keywords

Abstract

Breathing is an essential behavior that presents a unique opportunity to
understand how the nervous system functions normally, how it balances
inherent robustness with a highly regulated lability, how it adapts to both
rapidly and slowly changing conditions, and how particular dysfunctions re-
sult in disease. We focus on recent advancements related to two essential sites
for respiratory rhythmogenesis: (*a*) the preBötzinger Complex (preBötC) as
the site for the generation of inspiratory rhythm and (*b*) the retrotrapezoid
nucleus/parafacial respiratory group (RTN/pFRG) as the site for the gen-
eration of active expiration.

INTRODUCTION

Understanding the mechanisms leading from DNA to molecules to neurons to networks to behavior is a major goal for neuroscience but is largely out of reach for many fundamental and interesting behaviors. The neural control of breathing may be a rare exception, presenting a unique opportunity to understand how the nervous system functions normally, how it balances inherent robustness with a highly regulated lability, how it adapts to both rapidly and slowly changing conditions, and how particular dysfunctions result in disease. Why can we assert this? First and foremost, the functions of breathing are clearly definable, starting with its regulatory role of maintaining blood (and brain) O_2, CO_2, and pH; failure is not an option. Breathing is also an essential component of many vocal and emotive behaviors, e.g., crying, laughing, singing, and sniffing, and must be coordinated with vital behaviors such as suckling and swallowing, even at birth. Second, the regulated variables—O_2, CO_2, and pH (and temperature in nonprimate mammals)—are continuous and are readily and precisely quantifiable, as are ventilation itself along with the underlying rhythmic motor activity, i.e., respiratory muscle EMGs. Third, we breathe all the time, except for short breaks such as those during breath holding (which can be especially long in diving or hibernating mammals) or sleep apnea. Mammals (including humans) breathe in all behavioral states, e.g., in sleep-wake, in rest-exercise, in panic/fear-calm, during anesthesia, and even following loss of cortical function or decerebration. Moreover, essential aspects of the neural mechanisms driving breathing, including rhythmicity, are present at levels of reduction down to a medullary slice. Fourth, the relevant circuits exhibit a remarkable combination of extraordinary reliability, starting ex utero with the first air breath (intermittent breathing movements actually start in utero during the third trimester) and continuing for as many as $\sim 10^9$ breaths, as well as considerable lability, responding rapidly (in less than one second) and with considerable precision over an order of magnitude in metabolic demand for O_2 (~ 0.25 to ~ 5 liters of O_2 per minute). Breathing does indeed persist! Finally, breathing is genetically determined to work at birth, with a well-defined developmental program underlying a neuroanatomical organization with apparent segregation of function; i.e., rhythmogenesis is separate from motor pattern (burst shapes and coordination) generation. Importantly, single-human-gene mutations can affect breathing, and several neurodegenerative disorders compromise breathing by direct effects on brain stem respiratory circuits.

Figure 1 presents a broad overview of the central pattern generator for breathing. We distinguish rhythmogenesis from the more complex process of pattern generation, which is the production of precisely coordinated and timed motor nerve burst patterns across a broad array of muscles pumping the lung or controlling airflow resistance. A substantial portion of the nervous system that controls breathing movements is not involved in rhythmogenesis but instead transforms rhythmic signals into the appropriate pattern of muscle contraction. These critical but nonrhythmogenic structures include motor and premotor neurons. In addition, there are sensory neurons, including mechanoreceptors and chemoreceptors. Here we focus on recent advancements related to two essential sites for rhythmogenesis: (*a*) the preBötzinger Complex (preBötC) in the generation of inspiratory rhythm and (*b*) the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) in the generation of active expiration. We first discuss their function, anatomy, and physiology, providing a foundation for us to then discuss their developmental genetics.

Caveat lector: Making sense of the literature on respiratory rhythmogenesis is challenging. The ultimate goal is to understand normal and pathological breathing in humans. However, very little experimentation relevant to rhythmogenesis can be done in humans, in whom the location and relatively small size of the relevant neural structures in the brain stem and the prevalence of
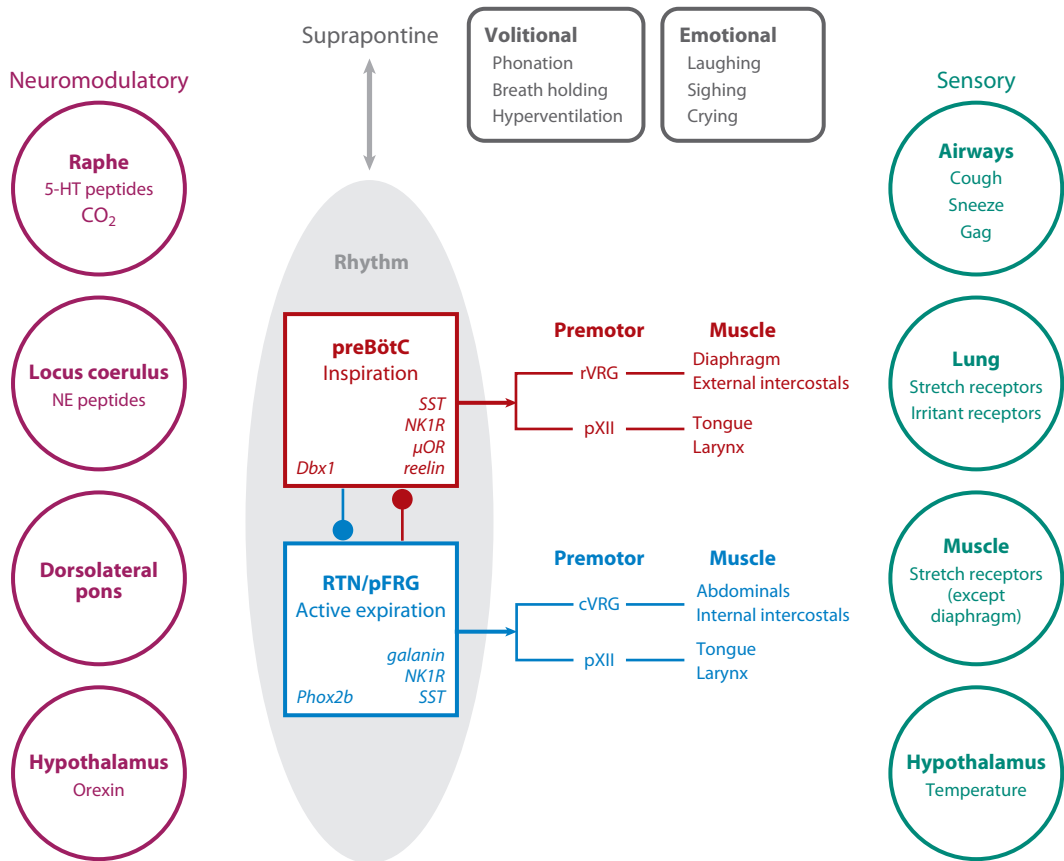
Figure 1

Overview of the central pattern generator for breathing. Core rhythm-generating circuits appear to have two distinct brain stem oscillators: the endogenously active preBötzinger Complex (preBötC) (*red box*) and the conditionally active retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) (*blue box*). The preBötC drives inspiratory activity by projections to various premotor populations [rostral ventral respiratory group (rVRG), parahypoglossal (pXII)] that in turn project both to inspiratory muscles that pump air, e.g., the diaphragm and external intercostals, and to inspiratory muscles that modulate airflow resistance, e.g., laryngeal and tongue muscles. The RTN/pFRG has a similar functional path to expiratory muscles. preBötC and RTN/pFRG progenitors and neurons express distinct transcription factors (*Dbx1* or *Phox2b*) and other genes (*right; italics*). Numerous neuromodulatory (*left*), suprapontine (*top*), and sensory (*right*) influences are shown. (*Left*) Neuromodulatory influences. Respiratory pattern is highly labile. When you go from quiet sitting to slow walking, your O_2 consumption increases approximately threefold, and if your ventilation does not increase rapidly, you will probably pass out within 100 m. Peptides, serotonin, norepinephrine, and other endogenous neuromodulators—originating in projections from, for example, the raphe, locus coerulus, and hypothalamus—can affect rhythmogenesis. These actions are essential for normal regulation and may go awry in diseases affecting breathing. The dorsolateral pons, aka pontine respiratory group, including the Kölliker-Fuse and parabrachial nuclei, is also an important modulatory source. (*Top*) Suprapontine inputs are related to volition and emotion. (*Right*) Sensory inputs are essential for the proper regulation of blood gases and for mechanical adjustments related to posture, body mechanics, and likely metabolic efficiency.

heartbeat- and breathing-induced movements prevent invasive techniques and render noninvasive neuroimaging techniques difficult (1). All nonhuman in vivo experimental models are studied while breathing or studied in vitro or in situ while generating breathing-related neural activity, with breathing rhythm and pattern highly dependent on the details of the experimental preparation. In more or less intact mammals, breathing is markedly affected by species (and even by strain),

body temperature, blood gas level, anesthetic (with different anesthetics having different actions on breathing), paralysis, decerebration, integrity of peripheral nerves such as the carotid sinus and vagus nerves (which can be intact or cut), and sleep-wake state. In in situ preparations, the blood-brain barrier is compromised. In rhythmic slice preparations, slight variations in the rostral or caudal borders can markedly affect the pattern of motor output, as does the composition of the bathing fluid, which can vary considerably in K^+ (3–9 mM) and Ca^{2+} (0.8–2.5 mM) between different laboratories (2–7). Unfortunately, we tend to pick and choose among results that align with our biases, often ignoring confounds that temper or invalidate the comparisons, and to dismiss those results that do not agree.

The terminology for breathing patterns, once well defined and essential for straightforward comparison of experimental results (8), has devolved. During eupnea, commonly understood to mean quiet breathing or resting respiration in fully intact mammals, neural output to respiratory muscles is regular: Rhythmic bursts of motor activity to the diaphragm and external intercostal muscles drive inspiratory airflow, and expiratory airflow results from passive recoil of the lung and rib cage. The term eupnea has now been stretched to include the respiratory motor nerve patterns in hypothermic, aortic perfused, vagotomized, blood-brain barrier–compromised rodent preparations that generate respiratory output patterns that are quite different from those in intact rodents at rest (9). Further confusing the issue is the addition of the term fictive, such as fictive eupnea, to describe the pattern of XII nerve activity in rhythmically active slice preparations (10). As another example, gasping in its long-standing definition described the very stereotyped last breaths in anoxic mammals just before they would asphyxiate, absent a rescue of some type. However, gasping now refers to various respiratory motor nerve patterns in highly reduced preparations (10–12), often to dismiss such activity as irrelevant to understanding mechanisms underlying eupnea, however defined; we strongly disagree with the shift in the use of the term gasping. Experimental limitations and discontinuity of definitions and terminology notwithstanding, the advances in the past six years provide considerable new insights into the sites and mechanisms underlying breathing.

PREBÖTZINGER COMPLEX AND INSPIRATION

There is a broad consensus (with plenty of quibbling over details) for the answer to the age-old question, "Where does breathing originate?" The paradigms underlying investigation of the neural control of breathing underwent a shift with the establishment of in vitro experimental models relevant to breathing (5, 13). Such models led to the identification of the preBötC and the hypothesis that it was the kernel for respiratory rhythmogenesis (5, 14). In previous reviews (15–17), we discussed key experiments that tested this hypothesis in vivo. To better understand recent work, we note three findings. (*a*) The preBötC is both necessary and sufficient for the generation of inspiratory motor, i.e., hypoglossal nerve (XIIn), output in in vitro rodent slice preparations (5, 14). (*b*) Juvenile rats in vivo with brain stem transection just rostral to the preBötC continue to generate rhythmic inspiratory-dominated breathing patterns (18), as do in situ heart-brain stem preparations (19). (*c*) Neurotoxic lesioning of a peptide receptor–defined subset of preBötC neurons results in ataxic breathing in otherwise intact rats. Approximately 300 glutamatergic preBötC neurons per side express the neurokinin-1 receptor (NK1R), for which substance P (SP) is the ligand. A toxin, saporin, conjugated to SP (SP-SAP) selectively kills, over a period of days, neurons that express NK1R. Injection of SP-SAP into the preBötC in intact adult rats induces, after several days, a disturbed breathing pattern during sleep; with more complete lesions, ataxic breathing occurs during wakefulness (20), and apneas (the complete cessation of breathing) occur during sleep (21).
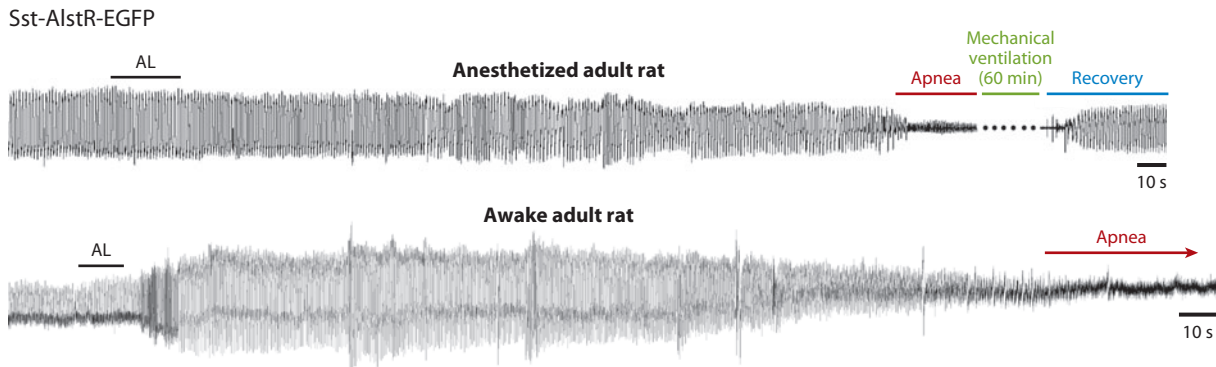
Figure 2

The preBötC is essential for breathing in adult rats. Rapid silencing of allatostatin receptor (AlstR)-expressing preBötC somatostatin (Sst) neurons induces persistent apnea (lasting >45 min with mechanical ventilation) in anesthetized (*top*) or awake (*bottom*) adult rats. Neurons were transfected by local, bilateral injection into the preBötC of an AAV2 virus expressing AlstR under a Sst promoter. The traces are plethysmographic recordings. The allatostatin ligand (AL), when administered intracerebrocisternally, induces a gradual decline of frequency and tidal volume until apnea develops after several minutes. After ~60 min of mechanical ventilation, the rats resume spontaneous breathing. EGFP denotes enhanced green fluorescent protein. From Reference 24.

Further (Smoking-Gun) Evidence for the preBötC as the Kernel for Inspiration

Saporin-lesioned rats survive, albeit with a markedly pathological ataxic breathing pattern (21), for up to 12 days, leaving the unlikely possibility that another site drives breathing under normal conditions. Severe hypoxia and hypercapnea caused by increasingly long and frequent apneas (20) can cause neuronal death. Moreover, slow neurodegeneration with saporin makes it difficult to separate the specific and immediate effects of the loss of preBötC NK1R$^+$ neurons from secondary effects, including plasticity and bystander death. To eliminate these confounding effects of slow degeneration, a largely overlapping subpopulation of glutamatergic preBötC neurons that express the neuropeptide somatostatin (Sst) (~300 per side) (22, 23) can be transiently suppressed by transfection with the *Drosophila* allatostatin receptor (AlstR) and subsequent exposure to the allatostatin ligand (AL). AL does not appear to activate any mammalian receptors. Rat neurons made to express the AlstR hyperpolarize when exposed to AL. When AL is introduced into the brain stem of control adult rats, there is no effect on breathing. However, in anesthetized or awake adult rats whose preBötC Sst neurons express AlstR, AL rapidly induces a remarkably persistent and profound apnea that requires mechanical ventilation to prevent asphyxiation (**Figure 2**) (24). Implicit in this observation is that these rats generate no breathing movements; there are no volitional or emotive movements, sighs, or gasps. These compelling data substantiate the essential role for preBötC neurons in normal breathing, i.e., eupnea.

Respiratory Rhythm Generation in the preBötC

If understanding the neural control of breathing requires solving a series of problems, foremost is delineating the mechanism for rhythmogenesis. More generally, rhythms, oscillations, and periodic bursting of various sorts are central to almost all aspects of brain function, ranging from rhythmically patterned movements (25), to cortical and cerebellar rhythms associated with signal processing and state, to slower processes with daily or seasonal rhythms. Breathing presents a unique window into neural rhythmogenesis because of a convergence of its essential properties: robustness, lability, a localized and identified rhythm generator (preBötC) [a key piece of the

puzzle lacking for locomotion or chewing (26)], and various levels of reduction in vitro that provide exceptional access to neurons and networks for imaging and electrophysiology while maintaining spontaneous breathing-related motor output.

We postulate that rhythm is generated at the core of the respiratory central pattern generator and that this core is not concerned primarily with details of the output pattern. Our view is that timing signals normally originate in the preBötC and are broadcast to the rest of the network, perhaps by a subset of glutamatergic neurons with widespread projections (27) that transform the timing signals into appropriate patterns of muscle contraction (**Figure 1**). Adopting this point of view allows one to focus on the oscillation per se and on its neural origins. As mentioned above, the highly reduced slice preparation is experimentally optimal to elucidate such mechanisms. Our explicit assumption is that mechanisms gleaned in slices represent the foundation for rhythmogenic mechanisms in vivo. This point of view is opposite to assertions that rhythm generated in the slice is irrelevant to eupneic breathing in intact mammals (see below and References 11 and 19). In spite of all the inherent experimental advantages and the deceptive simplicity of rhythmogenesis, understanding rhythmogenesis in the preBötC in vitro is a tough problem.

Before the preBötC hypothesis (5, 14), proposals for rhythmogenesis focused on building ball-and-stick models consisting of populations of respiratory-modulated point/spherical neurons, i.e., neurons without dendrites, classified according to firing pattern with simple cellular properties, e.g., no voltage dependency, no ion selectivity, no metabotropic actions, and straightforward excitatory or inhibitory connections (8, 28–30). Once the intrinsic membrane properties of preBötC neurons were measured in vitro, these schematic models were elaborated. Currently, there are two major viewpoints for the mechanism of preBötC rhythm generation on the basis of the widely accepted finding that the preBötC rhythm is generated by glutamatergic interneurons (31, 32). Their distinction lies in whether the inspiratory burst initiates due to a small population of specialized pacemaker neurons (discussed below) or results from excitatory interactions among preBötC neurons that express synaptically triggered burst-generating conductances.

Role of Glutamatergic Pacemaker Neurons in Respiratory Rhythm Generation

We evaluated the evidence for and against possible roles of pacemaker neurons more than a decade ago in a previous *Annual Review of Physiology* review (16). These critiques, however, remain relevant to more recent work that pertains to the preBötC, as well as to pacemaker-driven models of locomotor and oral-motor rhythm generation (26, 33–35). The heuristic power of the relatively straightforward pacemaker hypothesis may, in part, explain its persistence in the face of significant conflicting data. Over the past decade, some investigators have moderated their view, interpreting newer data as demonstrating that pacemakers and their underlying intrinsic conductances can contribute to rhythmogenesis but conceding that they are not essential (19, 36–38). Yet many authors assert that the preBötC rhythm is pacemaker driven and to interpret their data or build their models in that framework (12, 39–44).

preBötC rhythms in vitro continue without significant changes in frequency after blockade of chloride-mediated inhibition (45–48). This finding rules out mechanisms predicated on phase transitions that require conventional postsynaptic inhibition. The original observation catalyzed a search for and subsequent identification of preBötC neurons with bursting pacemaker properties that may best be defined by the ability to generate (*a*) rhythmic membrane polarization absent rhythmic input and/or (*b*) ectopic bursts of rhythmic activity when depolarized (5, 49–51).

Bursting in preBötC neurons has two underlying ionic mechanisms. The first is voltage sensitive and depends on subthreshold activation of persistent Na^+ current (I_{NaP}) in neurons with sufficiently low leakage-like K^+ current (38, 51–53). I_{NaP}-dependent bursting is present in a subset

of preBötC neurons at least from birth as well as throughout the medulla postnatally; however, it is not a specialized property of preBötC neurons (52, q.v. 54, 55). The second bursting mechanism depends on a Ca^{2+}-activated nonspecific cationic current (I_{CAN}) (50), whose activation mechanism in the absence of synaptic input depends on voltage-gated Ca^{2+} channels. Bursting of this type is less voltage sensitive (see figure 4*c* in Reference 39; see also Reference 49) and emerges in a subset of preBötC neurons after postnatal day 4 (P4) (42, 49) with its relative prevalence influenced by neuromodulation (12, 39, 41, 56, 57). Under most experimental conditions in slices from neonatal rodents, ~5–25% of all preBötC inspiratory neurons (glutamatergic or glycinergic) exhibit bursting pacemaker properties (38, 42, 49, 58, 59). However, the logic in the literature gets murky as one proceeds from the observation that pacemakers are present to conclusions that these pacemaker neurons generate the rhythm.

Arguments for the pacemaker hypothesis generally hinge on two observations. First, pacemaker neurons oscillate with a period and burst duration that match the duty cycle of the respiratory rhythm in vitro. Second, conditions that regulate the period and burst duration of synaptically isolated pacemaker neurons, e.g., high K^+, neuromodulators, and ion channel agonists/antagonists, have similar effects on respiratory rhythm in vitro. These arguments assume that pacemaker neurons are glutamatergic/excitatory, which, alas, is not the case. In transgenic mice that express enhanced green fluorescent protein (EGFP) in glycinergic neurons, in which ~50% of preBötC neurons fluoresce (60), ~23% of inspiratory glycinergic preBötC neurons have voltage-dependent pacemaker properties (59).

Tests of the pacemaker hypothesis are not straightforward in execution or in interpretation. Antagonists of I_{NaP} or I_{CAN} such as riluzole or flufenamic acid invariably preclude intrinsic bursting in isolated pacemaker neurons but do not always perturb or stop the rhythm when bath applied or locally infused into the preBötC of slices or en bloc preparations or in situ (38, 42, 49, 61–63). Nevertheless, under the best of circumstances (which is likely never the case), rhythm cessation after drug exposure is a necessary but not sufficient condition if pacemakers are essential for rhythmogenesis. That is because I_{NaP} and I_{CAN} antagonists markedly affect other membrane properties and depress excitatory transmission in all neurons. Their net effect is a widespread decrease in neuronal excitability that is not limited to particular respiratory or even nonrespiratory nuclei (61, 64). Additionally, local infusion of an I_{NaP} antagonist into the midline nucleus raphe obscurus also stops the rhythm (62, 65). Therefore, rhythm cessation by drug application cannot be attributed to effects solely on preBötC neurons, and the results of pharmacological tests are at best equivocally in support of the pacemaker hypothesis. The conclusions that I_{NaP} pacemaker neurons drive gasping-like behavior in reduced in vitro and in situ preparations (11, 42) and that I_{NaP}-dependent pacemaker activity drives spinal locomotor patterns (34, 66) are similarly unconvincing.

Can Respiratory Rhythm Be Generated Without Pacemaker Neurons and Without Synaptic Inhibition?

Previously (16, 17) we described a schematic group-pacemaker mechanism (7) that subsequently gave rise to explicit models for respiratory rhythm generation (67, 68). Before explaining how a group pacemaker works, we should note that there are few definitive data in support of the group-pacemaker model. It is at present a working hypothesis and framework for further testing. In the model, a fraction of glutamatergic preBötC neurons fire tonically at a low rate (<1 Hz) between inspiratory bursts. Percolating activity in this interconnected subset of respiratory neurons (69) increases via positive feedback. The part of the cycle in which positive feedback dominates other network constituents is properly considered the preinspiratory phase because it precedes the inspiratory burst by 300–400 ms. According to the group-pacemaker hypothesis, recurrent

excitation builds up during the preinspiratory phase and leads to a network-wide synchronous inspiratory burst phase. Consistent with this model, a ramp-like increase in the baseline membrane potential accompanied by low-rate spontaneous spiking during the preinspiratory phase is observed in so-called type 1 neurons, which are thought to be rhythmogenic on the basis of NK1 receptor expression (see above) and physiologically verified excitatory synaptic interconnections in the preBötC (**Figure 3d**) (69).

The termination of the inspiratory burst is still unresolved. Synaptic depression is a possible mechanism that would halt recurrent excitation (67). Burst termination may also involve activity-dependent outward currents evoked by intense spiking, including Na^+/K^+ ATPase electrogenic pump current, Na^+-dependent K^+ current, and ATP-dependent K^+ current (6, 70–73).

I_{CAN}, Burst Generation, and Dendritic Excitability

Active dendritic membrane properties, in concert with intracellular signaling, can couple synaptic input to burst generation. Because inspiratory bursts are much larger in magnitude (and intraburst spiking is more intense: 20–100 Hz) compared with temporal summation of excitatory postsynaptic potentials (EPSPs) and low-rate (2–5-Hz) spiking during preinspiratory activity, the onset of the inspiratory burst reflects the recruitment of postsynaptic conductances that amplify synaptic drive but are unrelated to I_{NaP} (62). Morphology may play a role in synaptic amplification. Rall's analyses of dendritic properties (e.g., Reference 74), the advent of dendritic patch-clamp recordings, and new laser-imaging technologies demonstrate a wide array of excitable properties in dendrites that significantly influence synaptic integration, specifically the spatiotemporal summation of EPSPs (75). In preBötC neurons, the activation of AMPA receptors as well as of group I metabotropic glutamate receptors (mGluRs) evokes postsynaptic Ca^{2+} transients that in turn evoke I_{CAN}, either locally at the dendritic input site (76, 77) or via propagating Ca^{2+} waves (**Figure 3a,b**) (68). The net result is that, during the inspiratory burst phase, excitatory synaptic inputs evoke inward currents that promote robust inspiratory burst generation often recognized by depolarization block of spiking during the inspiratory phase (**Figure 3a,b,d**). Somatic Ca^{2+} transients, in contrast, do not appear to play a critical role (78). The underlying ion channels appear to be members of the transient receptor potential (TRP) family, most likely TRPM4 and TRPM5 (68, 73, 79) or possibly TRPC3 and TRPC7 (40). Inspiratory bursts with depolarization block are shown in a representative excitatory (glutamatergic) rhythmogenic preBötC neuron (rhythm-generating neurons are derived from precursors that express Dbx1; see below) in **Figure 3c,d**. The dendrites of Dbx1$^+$ neurons are planar and radiate dorsoventrally 300–500 μm, often beyond the borders of the preBötC (**Figure 3c,e**). Dendritic excitability is critically important in understanding neuron computation in general (80). In a rhythmic central pattern generator, the ability to summate and synchronize excitatory inputs in support of periodic burst generation may be a function particularly well suited for dendrites, which we have just begun to exploit in order to elucidate key aspects of respiratory rhythmogenesis (81).

Role of Inhibition in Rhythmogenesis and Pattern Formation

Inhibition is an essential element that underlies rhythmic movements in invertebrates, in which most central-pattern-generator neurons are inhibitory (82). For locomotion in mammals, reciprocal inhibition was first proposed as the mechanism for the alternating rhythm around each joint (83, 84). Although this mechanism remains hypothetical, inhibition clearly underlies left-right limb alternations (85, 86). For breathing, postsynaptic inhibition is postulated, sometimes stipulated, as an essential element for rhythmogenesis (87–89). Inhibitory currents and potentials
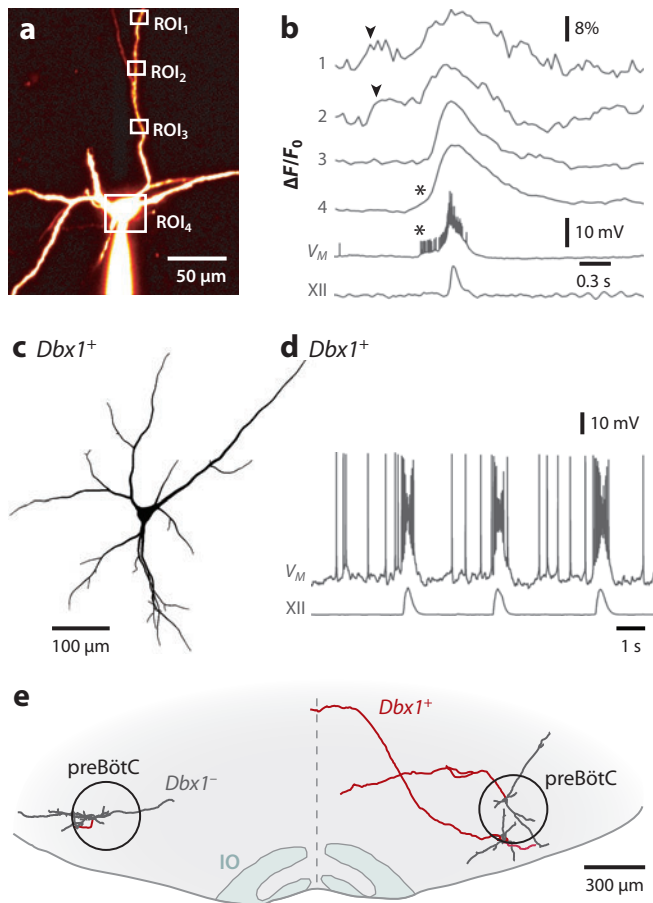
Figure 3

Inspiratory burst generation: the role of dendrites and the properties of $Dbx1^+$ preBötC neurons.
(*a*, *b*) Dendritic two-photon Ca^{2+} imaging and somatic patch clamp. (*a*) preBötC neuron filled with
fluorescent dye from a somatic whole-cell recording. Numbered regions of interest (ROIs) correspond to the
numbers in panel *b*. (*b*) Dendritic Ca^{2+} transients (*arrowheads*) precede somatic bursts and XII motor output.
Asterisks indicate somatic spike-driven Ca^{2+} transients. From Reference 81. (*c*,*d*) Morphology (*c*) and
physiology (*d*) of a $Dbx1^+$ preBötC neuron. Drive potentials of ∼25-mV amplitude and depolarization block
of spiking are indicative of I_{CAN} (Ca^{2+}-activated nonspecific cationic current) activation during the
inspiratory burst. (*e*) Transverse view of a mouse slice (ventral) showing two Dbx1$^+$ neurons (*right*) and a
$Dbx1^-$ neuron (*left*) recorded and biocytin reconstructed. All three are inspiratory neurons. $Dbx1^+$ neurons
are commissural (axons in *red*). Abbreviations: IO, inferior olive; V_M, membrane voltage; XII, hypoglossal
motor root.

are readily observed in respiratory-modulated brain stem neurons in intact mammals and are quite
prominent when recorded under barbiturate anesthesia (30). The core mechanism for rhythm
generation in one current, widely disseminated model is an inhibitory ring of three distinct
neuronal populations of preBötC and BötC (just rostral to the preBötC and containing a high
concentration of inhibitory respiratory-modulated neurons) neurons that sequentially inhibit
each other and transform a presumptively tonically active subpopulation of excitatory preBötC
neurons into an inspiratory-modulated one (19, 90). Block of synaptic inhibition throughout the
entire neuraxis of a reduced in situ–perfused rat preparation produces apnea with tonic firing

of preBötC and BötC neurons (19). Under certain pathological conditions, such as when the hindbrain is transected just rostral to preBötC in this in situ preparation, the inhibitory ring is presumably broken. According to this model, preBötC pacemaker neurons are released from tonic excitation and endogenously burst, driving a respiratory rhythm by a completely different mechanism. This presumptively pacemaker-driven rhythm is strikingly different from that presumably produced by the inhibitory ring in the following ways: (*a*) Inspiratory and expiratory outputs discharge synchronously, (*b*) inspiratory bursts change shape from incrementing to decrementing, and (*c*) respiratory frequency drops. According to the model, removing the inhibitory ring should produce apnea (11, 19, 90). However, apnea apparently does not occur. In anesthetized, adult, spontaneously breathing, vagus-intact rats, effectively complete antagonism of $GABA_A$ and glycine receptors in the preBötC and BötC slows down the rhythm to that of a vagotomized rat; i.e., no apnea is induced (90a). This result suggests that postsynaptic inhibition within the preBötC (and the BötC) is not essential for the generation of respiratory rhythm, consistent with the group-pacemaker model. The primary role of inhibition appears to be in shaping the pattern of respiratory motor output and in assuring its stability, but not in the generation of rhythm per se.

Opiates and the preBötC

Opiates are a class of compounds that can depress breathing such that, at too high a concentration, asphyxiation occurs, as can happen when opiates are administered as an analgesic or are taken recreationally. μ-Opioid receptors are present on preBötC neurons and when activated reduce their excitability (91). These preBötC μ-opioid receptors appear to be responsible for the respiratory depressive effects of opiates (92). A functional antagonist of the μ-opioid receptor–signaling pathway in preBötC neurons is the $5HT_4$ receptor, which does not appear to be present in pain pathways (93). In the adult rat, systemic administration of a $5HT_4$ agonist reverses the respiratory depressive effects of systemic administration of fentanyl (a potent μ-opioid agonist) without a significant reduction in analgesia. More to the point, depression of breathing by systemic administration of fentanyl is fully reversed by focal injection into the preBötC of naloxone, a μ-opioid antagonist (92). These observations provide further proof of the importance of the preBötC in breathing and suggest the potential of protocols to reduce the dangers of opiate analgesics.

REDISCOVERY OF EXPIRATION

Until recently, the paradigm for rhythmogenesis was that, regardless of the underlying mechanism, all phases of respiratory motor outflow originate from the same source(s). Thus, the preBötC was envisioned to sequentially parcel out signals to produce the alternating rhythm of inspiratory and expiratory movements. This does not appear to be the case. The diaphragm, an extraordinarily powerful muscle for inspiratory movements, is a defining characteristic of mammals. Breathing is the only mammalian behavior requiring the continuous movement of skeletal muscles; breathing consumes ~7% of metabolic output at rest, and that number is much higher in pulmonary disease (94). In mammals, the most efficient pattern for rhythmic breathing at rest is active inspiration, which is generated by forces resulting from diaphragmatic and external intercostal muscle contraction acting to expand the lung, alternating with passive expiration due to the forces generated by the elastic recoil of the inspiratory muscles, lung, and rib cage. Modest increases in ventilation near resting values, such as during slow walking in humans, are typically accomplished by increasing inspiratory (tidal) volume, combined with decreasing expiratory duration, with expiration remaining (predominantly) passive (95). During exercise, e.g., when one chases prey or

flees predators, there is a transition from passive to active expiration (96) due to the onset of contraction of the abdominal and internal intercostal muscles. In the past several years, there has been a significant paradigm shift, with the current view that rhythm originates from two distinct but coupled oscillatory populations with segregated function, i.e., one population for inspiration and one for expiration (**Figure 1**).

This shift began with the observation that both in vitro and in vivo, low-dose opioids can produce an occasional dropout of the inspiratory phase, i.e., quantal slowing (97), which requires structures rostral to the preBötC (18). This observation led to the hypothesis of an opiate-insensitive second oscillator that drives active expiration (18, 97). The location of this second oscillator was suggested by observations in in vitro en bloc preparations, in which a population of neurons rostral to the preBötC and mostly ventral to the facial nucleus (VIIn), dubbed the parafacial respiratory group (pFRG), was active prior to the onset of inspiratory activity (98). The pFRG overlaps with (and may even be identical to) the retrotrapezoid nucleus (RTN) (see below), which is postulated to be a site for central chemoreception for CO_2 (99–101). Pending resolution as to their unique identity, we refer to this region as the RTN/pFRG (15, 102). The nonchemosensitive function of the RTN/pFRG was initially hypothesized to be the primary source of inspiratory rhythm (98). However, in juvenile rats generating active expiration, transection of the brain stem just caudal to the RTN/pFRG abolishes active expiration without much effect on inspiratory motor activity (18). The RTN/pFRG is postulated as a conditional oscillator for active expiration that is quiescent under certain conditions (18). Reduced preparations containing the RTN/pFRG but lacking the preBötC generate rhythmic output from VII motor roots under opioid inhibition (103), consistent with the picture of the RTN/pFRG as an independent oscillator. The absence of significant expiratory neuronal activity in the RTN/pFRG of normocapnic, normoxic, adult, anesthetized, mechanically ventilated rats (104, 105) reflects the absence of active expiration (17). Thus, stimulating the lateral RTN/pFRG via local disinhibition or photoactivation with channelrhodopsin transforms a resting breathing pattern with no active expiration into one with active expiration associated with the activation of late-expiratory RTN/pFRG neurons (**Figure 4**) (106). These results are consistent with the presence in the RTN/pFRG of an expiratory oscillator that when turned on promotes active expiration. Although elevated CO_2 stimulates active expiration, elevated CO_2 is not a prevalent stimulus in the normal life of most mammals. Rather, we suggest that descending signals related to exercise (107) or to a powerful emotional response, e.g., fear, are likely to turn on the RTN/pFRG oscillator to produce active expiration. Moreover, active expiration sometimes occurs in anesthetized mammals (108–110) or in highly reduced preparations (111–113), suggesting that multiple mechanisms may gate its activity.
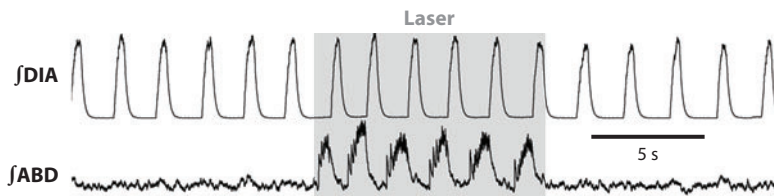

Figure 4

Passive expiration transformed into active expiration. The anesthetized adult rat has active inspiration, reflected in integrated diaphragm EMG (DIA), but passive expiration, reflected in tonic integrated abdominal EMG (ABD). Photoactivation of lateral RTN/pFRG neurons transfected with ChR2 (*gray band*) induces active expiration.

Expiration, Like Gaul, Is Divided into Three Parts

Regardless of its origin, expiration has subphases. In particular, the transitions at the beginning and end of the expiratory phase are referred to as the postinspiratory (early-expiratory) and preinspiratory (late-expiratory) periods. In the postinspiratory period, following a sharp decline in phrenic nerve activity, inspiratory airflow stops and expiratory airflow begins. Just after this point, there is often a small, postinspiratory burst of phrenic nerve activity. This burst causes a lengthening contraction of the diaphragm that slows expiratory airflow. During the preinspiratory phase, (*a*) in vitro, type 1, putatively rhythmogenic preBötC neurons exhibit a ramp-like slow depolarization (**Figure 3d**) (7), and (*b*) in vivo, stimuli that when delivered earlier in expiration affect expiratory duration (T_E) are ineffective, e.g., Reference 114. Whether postinspiratory output constitutes a distinct phase of breathing rhythmogenesis (a three-phase model) (19, 90) or a motor subcomponent of the expiratory phase (a two-phase model) (18) is controversial. The timing of abdominal activity depends, in part, on the experimental condition; some experiments report purely preinspiratory activity (108, 110) or a mixture of pre- and postinspiratory activity (18, 111). Similar flexibility in motor patterns is present in reduced neonatal preparations (4, 113).

RESPIRATORY CONNECTOME

preBötC Intrinsic Connections

Regardless of the critical cellular properties, the connectivity among preBötC neurons must play an essential role in the stability and lability of the rhythm and perhaps in rhythmogenesis itself. This information has been challenging to obtain. Type 1 inspiratory preBötC neurons, on the basis of a small sample in rhythmically active mouse slices, synaptically project to approximately one out of six neurons and form electrical synapses with approximately one out of six neurons that are also type 1 but are different from the first set of neurons (69, 115). Each rhythm-generating preBötC neuron also projects an axon toward the midline, presumably to the contralateral preBötC (**Figure 3e**). Putative rhythm-generating neurons in organotypic cultures of the preBötC form clusters of approximately seven neurons, each projecting to four neighbors. Therefore, connectivity estimates range from 15% to 67%. The topological pattern of connectivity in neural networks (as in all networks) influences whether and how individual elements, e.g., neurons, can synchronize their activity (116). Therefore, the specific topological pattern of connectivity is at least as important as a simple tally or an estimate of connection probability in understanding network function. For example, the robustness of network function under attack, i.e., destruction of its constituent nodes, depends on topology (117). Cumulative single-neuron laser ablation of ~18% of inspiratory preBötC neurons causes irreversible cessation of rhythm in slices (118), but to what extent the underlying details of the wiring diagram determine the robustness (fragility) of the rhythmogenic network remains unknown. Robustness is an important characteristic essential both for understanding the neural basis for periodic behaviors like breathing and for interpreting and treating neurodegenerative disorders (119).

The preBötC and the RTN/pFRG Interconnect

Glutamatergic, Sst-expressing, inspiratory-modulated preBötC neurons project to the RTN/pFRG (27). Glutamatergic, galanin-positive RTN/pFRG neurons project to the preBötC (120). Whether these neurons are involved in the rhythmogenic aspects of the RTN/pFRG remains to be established. The lack of identification of inhibitory connections between these two structures is puzzling; the preBötC and the RTN/pFRG oscillate in antiphase postnatally. A

polysynaptic inhibitory pathway is possible and may involve a relay in the BötC. Significant resources are being devoted to determining the connectome for cortical columns; the challenges are heightened by the paucity of information concerning the sophisticated signal processing that is occurring. We suggest that the preBötC presents a much more straightforward challenge of considerably less complexity, as the signal processing and output are well understood, and may be useful as an intermediate step in the development of brain connectomics.

GENETICS OF THE RESPIRATORY NETWORK

Our understanding of the developmental genetics of brain stem circuits underlying the neural control of breathing is rapidly advancing, with considerable potential to inform our understanding of the preBötC and RTN/pFRG. Brain stem organization is remarkably consistent among vertebrates (121, 122), a consequence of conserved developmental programs that control stereotyped patterns of gene expression, including critical transcription factors (TFs) (123, 124). At first approximation, every brain stem neuron results from a specific sequence and combination of TF expression on the basis of its physical location during neurogenesis (125). Rostrocaudally, the brain stem is organized into 7 to 8 domains (rhombomeres) associated with conserved and discrete patterns of TF expression (126). Medullary and caudal pontine respiratory populations are distributed across rhombomeres 5–7. Dorsoventrally, the brain stem neural tube can be divided into at least 13 distinct dorsoventral domains on the basis of TF expression (**Figure 5a**) (127). Each of these domains produces distinct classes of neurons with their own neurotransmitter identity (**Figure 5a**), morphology, and migratory pattern (**Figure 5b**). Neurons of the ventral respiratory column (119) and the adjacent RTN/pFRG are derived predominantly from four distinct progenitor domains (**Figure 5a–c**).

Genetics of the preBötC

We make the case that the preBötC is the rhythmogenic source of inspiratory drive. Yet it is a small portion of the rostral ventral respiratory column (119), which mostly contains bulbospinal premotoneurons receiving inspiratory drive from the preBötC. What is the developmental mechanism underlying this ventral respiratory column organization, and in particular, what makes the preBötC special? Are its constituent neurons genetically distinct from adjacent respiratory premotoneurons? The expression in preBötC neurons of the receptor NK1R (91, 128–130), the peptide Sst (22, 24), and the glycoprotein reelin (131) distinguishes this structure from surrounding regions (**Figure 5d**); yet none of these markers appear to endow the preBötC with rhythmogenic properties. In mice, these genes are expressed on only a partially overlapping minority of preBötC excitatory neurons (~1,000 per side) (132). The role that different genetically defined subclasses of preBötC neurons play in rhythmogenesis is unknown. Genetic ablation of Sst or NK1R genes, but not of the neurons that would otherwise express these genes, does not appear to affect the formation of the preBötC and produces only mildly dysfunctional respiratory phenotypes (133–136). No intrinsic membrane properties, e.g., I_{NaP} or I_{CAN}, appear uniquely expressed in subsets of preBötC neurons.
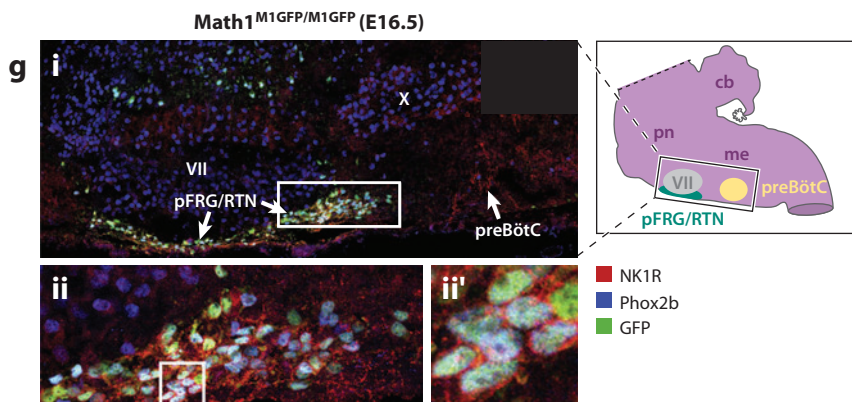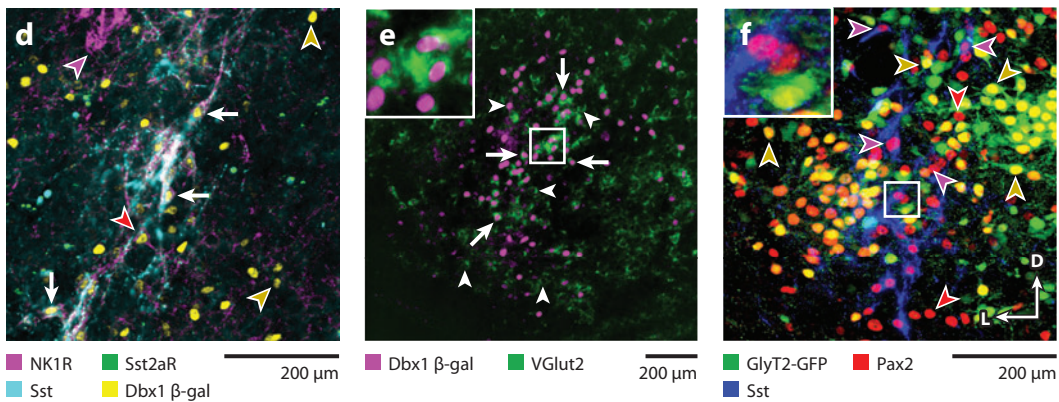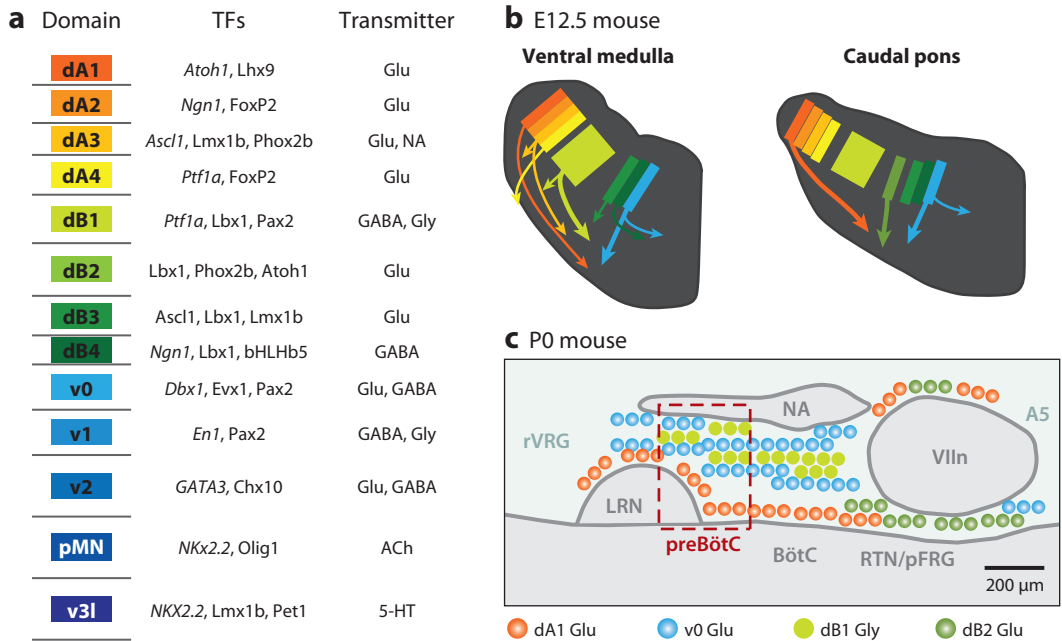
The ventrolateral medulla (VLM) contains both excitatory (glutamatergic) and inhibitory, mostly glycinergic, respiratory neurons (**Figure 5e,f**) (59, 60, 137, 138). Glutamate is the essential excitatory neurotransmitter for preBötC rhythmogenesis (31, 139). Genetic ablation of the vesicular glutamate transporter 2 (VGlut2, Slc17a6) completely eliminates respiratory-related motor activity both in vitro and in vivo (32). Respiratory dysfunction leading to death at or near birth is a consequence of the genetic ablation of a number of TFs (123, 140). In some cases (see

below), these TFs play a role in the specification of neurons that modulate preBötC function, but not in the specification of preBötC neurons. These TFs include those important for the specification of ventral medullary and pontine glycinergic neurons (Lbx1, Ptf1a) or glutamatergic neurons (Atoh1, Krox20/Egr2, Lbx1, Lmx1b, MafB/Kreisler, Phox2b, Tlx3, Tshz3) (100, 127, 140–151). None of these mutations, however, affect the specification of preBötC NK1R/Sst neurons. For example, Lmx1b and Tlx3 are expressed in and essential for the formation of ventral medullary catecholaminergic neurons that partially intermingle with preBötC neurons (152). Tlx3 mutation leads in vitro to respiratory instability that is alleviated by disinhibition. Similarly, subsets of Atoh1-derived neurons are present near the ventral medullary surface (153). Atoh1 mutant mice do not breathe in vivo at birth, yet a functional preBötC and rhythmic activity persist in vitro (141).

The TF Dbx1 is essential for preBötC development and respiratory function. Dbx1 is expressed throughout the brain stem and spinal cord in V0 neural progenitor cells (**Figure 5a**). The V0 domain consists of at least two subdivisions, dorsal and ventral (V0d and V0v) (154, 155). V0d neurons are predominantly GABAergic and are located near the dorsal midline. V0v postmitotic neurons express the TFs Evx1 and Evx2, and a subset also express VGlut2. In the spinal cord, V0v neurons are a small population that migrates toward the ventral midline. Within the medulla, however, the V0v glutamatergic subpopulation greatly expands and migrates laterally to the ventral medullary

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Figure 5

Genetic organization of brain stem respiratory regions. (*a*) (*Left*) Schematized description of brain stem progenitor domains for eight dorsal (dA1–dB4) and five ventral (v0–v3l) progenitor populations based on their relative locations within the brain stem progenitor region. (*Middle*) A partial list of transcription factors (TFs) expressed at some point within progenitors (*in italic font*) or postmitotic neurons (*in roman font*) within each domain. (*Right*) The neurotransmitter identity of neurons derived from each domain. Adapted from Reference 123. (*b*) The partial migratory path of ventral medulla (*left*) and caudal pons (*right*) neurons in the embryonic mouse brain stem. Colors correspond to those of the domains in panel *a*. Thick arrows correspond to populations important for breathing. The dB2 population (*light green*) is present only in the caudal pons. (*c*) The developmental origin and approximate anatomical locations of respiratory-related populations in the sagittal plane within the ventral medulla and caudal pons. Colors correspond to those of the domains in panel *a*. The legend describes transmitters released by these neurons. Within the ventral respiratory column, nearly all respiratory-related glutamatergic neurons are Dbx1 derived. The dotted red box shows the location of preBötC Sst-expressing neurons. The RTN/pFRG, in contrast, contains Dbx1, Atoh1, and Phox2b glutamatergic populations. The ventral medulla also contains many dB1-derived glycinergic neurons. (*d*) preBötC neurons are derived from Dbx1-expressing progenitors. The four-color confocal image shows coexpression of NK1R (*magenta*), Sst2aR (*green*), Sst (*cyan*), and β-gal (*yellow*) in the P0 Dbx1 β-gal mouse (adapted from Reference 132). Arrows indicate coexpression of all four genes. The red arrowhead indicates a Dbx1-derived, NK1R/Sst2aR-expressing neuron that lacks Sst. The yellow arrowheads indicate Dbx1-derived neurons lacking coexpression. The magenta arrowhead indicates a NK1R-expressing nucleus ambiguus neuron. (*e*) preBötC Dbx1 neurons are glutamatergic. The image shows β-gal (*magenta*) expression within the majority of VGlut2 (*green*)-expressing preBötC neurons (adapted from Reference 132). The inset is enlarged from the square in the main image. Arrows indicates coexpression. Arrowheads indicate absence of coexpression. (*f*) preBötC contains glycinergic neurons. The three-color confocal image shows Pax2 (*red*) and Sst (*blue*) immunoreactivity with intrinsic GFP from a P0 GlyT2-GFP transgenic mouse (59). Pax2 (*red arrowheads*) colocalizes with Sst (*magenta arrowheads*) or GFP (*yellow arrowheads*), but there is no GFP expression in Sst neurons. The inset is enlarged from the square in the main image. Scale bars in panels *c*, *d*, *e*, and *f*: 200 μm. D denotes dorsal, and L denotes lateral. (*g*) RTN/pFRG Phox2b neurons express Phox2b and Atoh1. (*i*) Magnification of the ventral respiratory column of the E16.5 $Math1^{M1GFP/M1GFP}$ hindbrain from the black rectangle on the model hindbrain (the *teal ventral region* is the pFRG/RTN, whereas the *yellow circle* indicates the preBötC), showing NK1R (*red*), Phox2b (*blue*), and Math1-EGFP (*green*) expression. NK1R is expressed in both pFRG/RTN and preBötC neurons. Magnified pFRG/RTN neurons from the caudal pole of VII (*white rectangle* in panel *i*) show colocalization of Math1-EGFP with Phox2b and NK1R. (*ii*) The three markers are merged. Further magnification from the white box in panel *ii* is shown in panel *ii′*. Image from Reference 141. Abbreviations: β-gal, β-galactosidase, A5, A5 catecholaminergic group; ACh, acetylcholine; BötC, Bötzinger Complex; cb, cerebellum; GABA, gamma aminobutyric acid; GFP, green fluorescent protein; Glu, glutamate; Gly, glycine; LRN, lateral reticular nucleus; me, medulla; NA, nucleus ambiguus; NK1R, neurokinin 1 receptor; pn, pons; preBötC, preBötzinger Complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; rVRG, rostral ventral respiratory group; Sst, somatostatin; Sst2aR, Sst2a receptor; VIIn, facial motonucleus; VGlut2, vesicular glutamate transporter 2.

a Domain   TFs   Transmitter

Domain	TFs	Transmitter
dA1	*Atoh1*, Lhx9	Glu
dA2	*Ngn1*, FoxP2	Glu
dA3	*Ascl1*, Lmx1b, Phox2b	Glu, NA
dA4	*Ptf1a*, FoxP2	Glu
dB1	*Ptf1a*, Lbx1, Pax2	GABA, Gly
dB2	Lbx1, Phox2b, Atoh1	Glu
dB3	Ascl1, Lbx1, Lmx1b	Glu
dB4	*Ngn1*, Lbx1, bHLHb5	GABA
v0	*Dbx1*, Evx1, Pax2	Glu, GABA
v1	*En1*, Pax2	GABA, Gly
v2	*GATA3*, Chx10	Glu, GABA
pMN	*NKx2.2*, Olig1	ACh
v3l	*NKX2.2*, Lmx1b, Pet1	5-HT

b E12.5 mouse

Ventral medulla   **Caudal pons**

c P0 mouse

rVRG   NA   A5   VIIn   LRN   preBötC   BötC   RTN/pFRG

200 μm

dA1 Glu   v0 Glu   dB1 Gly   dB2 Glu

d

NK1R   Sst2aR
Sst   Dbx1 β-gal   200 μm

e

Dbx1 β-gal   VGlut2   200 μm

f

GlyT2-GFP   Pax2
Sst   200 μm

D   L

Math1^{M1GFP/M1GFP} (E16.5)

g **i**

X   VII   pFRG/RTN   preBötC

cb   pn   me   preBötC   VII   pFRG/RTN

ii   **ii'**

NK1R
Phox2b
GFP

surface, producing a column extending the full length of the ventral respiratory column and including the RTN/pFRG, BötC, preBötC, and rostral ventral respiratory group (**Figure 5***b–e*).

Neurons with high levels of Sst in the neonatal mouse ventral medulla are limited to the (approximate boundaries of the) preBötC and are derived from Dbx1-expressing progenitors (**Figure 5***d*) (132, 156). These cells are ∼20% of preBötC Dbx1-derived neurons. The function and developmental relationship between preBötC Sst and other neurons within and adjacent to the preBötC are unknown. NK1Rs and somatostatin 2a receptors (Sst2aRs) are coexpressed on the majority of preBötC Sst neurons but are more broadly expressed within and adjacent to the preBötC (**Figure 5***d*). In addition, Dbx1 defines not only preBötC NK1R/Sst-expressing neurons but essentially all glutamatergic neurons of the ventral respiratory column (**Figure 5***e*). This finding suggests that, for breathing, rhythm-generating neurons and (a significant proportion of the) downstream premotor neurons are derived from a single developmental progenitor domain. This organization is in contrast to that proposed for models of locomotion in which rhythms are generated by the interactions of multiple developmental populations (86, 157). The genetic mechanisms specifying preBötC neurons from the larger Dbx1-derived population are unknown, but they may induce signaling cascades similar to those responsible for segmental patterning of homeobox genes in the brain stem and spinal cord (158).

The elimination of the commissural projections from only Dbx1-derived neurons by deletion of the axon guidance receptor Robo desynchronizes respiratory outflow between the left and right sides (**Figure 3***e*) (156). Genetic deletion of Dbx1 eliminates all preBötC glutamatergic respiratory neurons, including those expressing NK1R and Sst, with consequent complete elimination of inspiratory activity, both in vitro and in vivo (132, 156). Additionally, preBötC Dbx1-derived neurons are predominantly inspiratory modulated, at least in vitro (**Figure 3***d*) (132, 156). Together, these data indicate that both the generation and the coordination of inspiratory output depend on commissurally projecting Dbx1-derived neurons that are presumably within the preBötC (**Figure 3***e*).

The Mouse in the Room

In vivo experiments targeting respiratory neurons in goats suggest that under certain conditions, breathing does not require preBötC neurons (159–162). Here, a nonspecific excitotoxin, ibotenic acid (IA), is injected in slowly increasing quantities over 5 weeks (to avoid bilateral application of high dosages that can cause acute cardiac and respiratory failure). IA affects breathing during injection, but breathing returns to normal by the next day. This protocol results in a substantial, if not complete, loss of neurons within the presumptive preBötC, at least to the degree that markers and neuroanatomy in goats are homologous to those in rodents. In contrast, goats injected with SP-saporin into the same region followed 10–14 days later with a single large dose of IA cease breathing for at least 6 h without recovery, either leading to death or necessitating euthanasia. One interpretation is that normal breathing can be generated in the absence of preBötC neurons, given modest damage per event and sufficient time for respiratory network reorganization between events that possibly include neurogenesis. There are four alternative explanations. (*a*) With sufficient time, regions outside of the preBötC with a different developmental lineage can generate normal respiratory output. One possible source is neurons in the RTN/pFRG (see below). (*b*) The postlesion rhythm may be generated by neurons that are genetically related to preBötC neurons but that, at least in rats, do not normally generate inspiratory rhythm (24), i.e., some subset of the Dbx1-derived glutamatergic neurons that extend the length of the ventral respiratory column (**Figure 5***b*). This last explanation is consistent with the observation that saporin ablations that include but extend beyond the preBötC lead more rapidly to death than do smaller ablations (20, 163). (*c*) In goats, the boundaries of the preBötC, which have been well characterized only in

the rat and the mouse, extend beyond the IA lesion, and a prolonged recovery is required for the unlesioned remaining neurons to coordinate their activity for effective breathing movements. NK1R expression is not limited to the preBötC in mice, and in goats NK1R expression appears to be ubiquitous, so the use of NK1R expression to identify preBötC anatomical boundaries is questionable (162). (*d*) The rhythm is generated by a population at some distance from the preBötC, perhaps in the rostral pons, although there are currently no data to support this.

GENETICS OF THE RTN/pFRG: ACTIVE EXPIRATION, CENTRAL CHEMORECEPTION, AND CONGENITAL CENTRAL HYPOVENTILATION SYNDROME

There are two critical functions associated with the RTN/pFRG: the generation of active expiration and central chemoreception. The lateral RTN/pFRG is the most sensitive site for the induction of active expiration without a concomitant increase in inspiration (106); this region contains few, if any, Phox2b neurons (**Figure 5*a,c***). One of the most essential functions of breathing in mammals is maintenance of the partial pressure of blood/tissue CO_2 (at ~40 mm Hg in arterial blood in humans). Several brain stem populations of neurons, in particular the medial RTN/pFRG and the raphe, likely play a role in this central chemosensitivity (15). Blunted CO_2 chemosensitivity in humans is a defining characteristic of congenital central hypoventilation syndrome (CCHS) (164). CCHS is a rare disorder caused by an alanine expansion in the gene encoding Phox2b, a highly conserved and essential TF involved in the specification of brain stem visceral sensory and motoneurons (148). The size of the Phox2b expansion directly correlates with the severity of CCHS. In rodents, a small population of ~2,100 RTN/pFRG glutamatergic neurons are responsive to small changes in pH and express Phox2b (99, 100). This identification of a specific population of neurons as putative CO_2 sensors is a landmark finding of the first developmental marker for neurons that are important for breathing and whose dysfunction leads to a survivable breathing phenotype (147).

The case that RTN/pFRG neurons play a role in mediating chemosensitivity is compelling. Briefly, the elimination of ~70% of RTN/pFRG Phox2b neurons, by the use of the aforementioned saporin lesion technique, leads to an ~50% attenuation of the ventilatory response to increases in blood pH (165). Activation of medial RTN/pFRG Phox2b neurons directly increases inspiratory output (166) and can produce active expiration (167). In contrast, stimulation of all neurons in the non-Phox2b-expressing lateral RTN/pFRG reliably evokes active expiration but has little effect on inspiratory activity (106). In the adult rat, medial RTN/pFRG Phox2b neurons are not rhythmically active, even under conditions of strong chemosensitive drive. This is consistent with a role of the medial RTN/pFRG in chemosensitivity but not in rhythmogenesis or the production of active expiration (168, 169).

Like nearly all TFs, Phox2b is expressed in distinct subpopulations, only some of which maintain postnatal expression (170, 171). RTN/pFRG Phox2b neurons are generated from a caudal pontine progenitor population that coexpresses the TF Lbx1 (127, 143). These neurons migrate to their final location in the VLM (dB2 in **Figure 5*b,f***). The RTN/pFRG is absent in Lbx1 mutant mice as well as in Krox20-null mice that lack rhombomeres 3 and 5 (127, 143, 172). Phox2b gene function is necessary for RTN/pFRG formation, as its conditional elimination by using either Lbx1 (limited to the dB2 domain; see **Figure 5*a***) or Krox20 (limited to rhombomeres 3 and 5) cre recombinase mice prevents RTN formation and produces neonatal lethality and/or blunted chemosensitivity (147, 173).

Mouse mutants that recapitulate the most common CCHS mutation, an alanine expansion (Phox2b[27Ala]) in the germline, have a blunted inspiratory response to elevated CO_2 in in vitro

embryonic preparations. Although such mice are capable of at least some inspiratory activity, most die at or shortly after birth (147, 173). In this mutation, RTN/pFRG Phox2B neurons form dorsally but do not migrate to the ventral surface. This migration normally coincides with the expression of the TF Atoh1 within putative RTN/pFRG neurons, but Atoh1 expression is lost in these mice (141, 153, 173, 174). Loss of Atoh1 also prevents RTN/pFRG migration (141, 153, 173, 174). Surprisingly, the conditional expression of the Phox2b[27Ala] mutation within rhombomeres 3 and 5 is not neonatal lethal but results in the loss of Atoh1 expression and of RTN/pFRG neuron migration, as well as in blunted chemosensitivity in neonates. By 4 months of age, however, mice recover 60% of their CO_2 sensitivity (compared with wild-type controls) and have normal blood CO_2 levels at rest (173).

FETAL DEVELOPMENT OF THE RTN/PFRG

In utero, rhythmic respiratory motor output in mice begins at approximately embryonic day 15.5 (E15.5) coincident with the onset of respiratory activity in the embryonic preBötC (175). However, rhythmic brain stem activity is present by E14.5, i.e., ~1 day prior to the onset of preBötC activity, within the Phox2b-expressing region near the facial nucleus. This region is termed the embryonic parafacial oscillator (ePF) and is presumably the embryonic equivalent of the RTN/pFRG. In the later fetal period, the initially slow preBötC rhythm is coupled to a faster ePF rhythm (145). Unlike the preBötC rhythm, ePF rhythms do not require glutamatergic neurotransmission, as they persist following pharmacological blockade of glutamate receptors or genetic ablation of the glutamate transporter VGlut2 (32, 145). Rhythmically active ePF neurons express Phox2b and NK1R, but not Sst2a or μ-opioid receptors (132). In neonatal en bloc preparations, the majority of pFRG pre-I and respiratory-modulated tonic neurons express Phox2b (176, 177). Mutations that eliminate RTN/pFRG Phox2b neurons, such as those in Krox20 mutants, eliminate ePF activity, resulting in a slowed fetal preBötC rhythm (145). Together these data suggest that ePF and RTN/pFRG contain the same population of Phox2b-expressing neurons that have a vital perinatal role. As the ePF is an intrinsically rhythmic population coupled to breathing movements, its neurons represent excellent candidates for an evolutionarily conserved, independent expiratory oscillator (178). Significantly, however, fetal RTN/pFRG rhythms persist in Dbx1 mutant mice. These mice do not generate any rhythmic activity from VII or other respiratory motoneurons, suggesting that RTN/pFRG neurons alone are insufficient to assure the generation of active expiratory (or inspiratory) movements (156).

SEROTONIN, CENTRAL CHEMORECEPTION, AND SUDDEN INFANT DEATH SYNDROME

Raphe serotonergic neurons are also proposed to play a role in mediating CO_2 chemosensitivity (179). Raphe neurons have a discrete pattern of TF expression, including the combinatorial expression of the TFs Pet1 and Lmx1b (v3l in **Figure 5a**). Pet1 is expressed only in serotonin neurons, and its genetic ablation decreases their number by 70% (180). Lmx1b is expressed in several brain stem populations, but its conditional elimination from Pet1-expressing neurons completely eliminates all brain stem serotonergic neurons. In adult mice, such elimination produces an ~50% reduction in the ventilatory response to high levels of CO_2 but has no effect on baseline respiration (181). This is the same pattern of chemosensitivity seen in adult mice with Phox2b[27Ala/+] RTN/pFRG mutations. Partial serotonin neuron loss produces a similar but smaller effect (182). In neonates, the complete loss of serotonin increases and prolongs neonatal apnea and reduces ventilation at rest; ventilation nevertheless recovers to wild-type levels by adulthood (183). Selective silencing of

raphe neurons in adults depresses breathing and blunts chemosensitivity, indicating a continuing role for these neurons in respiratory modulation (184). In aggregate, these data strongly suggest that both RTN/pFRG and raphe neurons contribute to normal chemosensitivity and that the explicit role of either one may depend upon the age and state (e.g., sleep-wake, rest-exercise) of the animal (185). Medullary serotonergic neurons are present in vitro and can affect rhythmogenesis and motor output (28, 186, 187), representing mechanisms by which serotonin can regulate CO_2.

Serotonin dysfunction is hypothesized to underlie many cases of sudden infant death syndrome (SIDS) (188). In vitro, serotonin induces a depolarizing, nonselective cation current in preBötC neurons that enhances burst generation (65). The same or a very similar nonselective cation current is also a target of SP and is likely coreleased (under unknown conditions) by raphe neurons (40, 44, 115). These serotonergic inputs also enhance bursting pacemaker neurons by amplifying I_{NaP} and closing leakage K^+ currents (65, 189). Although the molecular identity of the nonselective cation current(s) described in these various studies is unknown, the current may be related to the leakage Na^+ current (190) or to a canonical transient receptor potential channel (TRPC) such as TRPC3 or TRPC7 (40).

ASTROCYTES AND CHEMOSENSATION

The mechanism(s) underlying central chemosensitivity in vivo is incompletely understood. In highly reduced preparations, both raphe and RTN/pFRG neurons are chemosensitive, but no protein or pathway within either of these populations has been unequivocally identified as the transducer of pH/CO_2 sensitivity. Hence whether these neurons are intrinsic chemosensors or part of a relay pathway is unclear, although the two mechanisms are not mutually exclusive and appear to act in concert through multiple transduction mechanisms (185). An alternate hypothesis is that pH and CO_2 are intrinsically sensed by a distinct population of astrocytes, which, by releasing ATP near the ventral surface, modulate the activity of nearby chemosensitive neurons and thus provide a CO_2-related stimulus that modulates breathing (191). The ventral medullary surface has several sites where local acidification increases ventilation (192, 193). At some of these sites, including adjacent to the RTN/pFRG, astrocytes respond to modest acidification (∼0.2 pH units) by releasing ATP. Also, there is an increase in ATP release as blood CO_2 increases in vivo, and local application of ATP receptor agonists increases ventilation (194). Optogenetic stimulation of astrocytes in the RTN/pFRG increases ventilation as well (191). A subset of brain stem astrocytes, including those adjacent to the RTN/pFRG, express connexin 26 (Cx26). Cx26 hemichannels are directly CO_2 sensitive and open to release ATP upon acidification (195, 196), and blocking gap junctions reduces CO_2 sensitivity akin to the action of purinergic receptor antagonists (197). Astrocytic gliotransmission thus provides a hypothetical mechanism linking CO_2 levels to changes in breathing.

RESPIRATORY NETWORKS AND HUMAN DISEASE

Increasingly, researchers working on the neural control of breathing are trying to understand the causes of, and to find rational therapies for, diseases that affect breathing. Central respiratory dysfunction is symptomatic of many diseases that can occur at almost any time during life, and unfortunately, we poorly understand the underlying causes (198, 199). This is true even for diseases in which causative single-gene mutations are known and relevant transgenic mice are available; examples include CCHS (see above), Rett syndrome, and Joubert syndrome (200–202). In Rett syndrome, due to a mutation in the MeCP2 gene, girls and, much more rarely, boys show prolonged periods of respiratory dysfunction during wakefulness and milder effects during sleep

(203, 204). MeCP2 mutant mice show similar respiratory deficits, and dysfunction in several different populations of glutamatergic and noradrenergic neurons is suggested as causative, although the respiratory phenotype may not manifest by effects in the brain stem or glia (203, 205–208). Similarly, breathing pathologies, especially prolonged and frequent apneas during sleep, are present in a number of neurodegenerative diseases such as Parkinson's, amyotrophic lateral sclerosis, and multiple-systems atrophy (MSA). Losses of putative preBötC NK1R or Sst neurons have been found in both Parkinson's- and MSA-affected brains (119, 209–212).

More problematic are diseases that likely have a strong, but currently unknown, genetic component(s), such as ROHHAD (rapid-onset obesity, hypoventilation, and hypothalamic dysfunction), and diseases in which the role of genetics is unknown, such as apnea of prematurity, Perry syndrome, SIDS, and sleep apnea (198, 213–215, 216, 217). These last two are the most prevalent diseases with a breathing phenotype: For live births, SIDS kills ∼6 in 1,000 infants <1 year of age (**http://www.sidscenter.org/Statistics/table1.html**), and sleep apnea affects 4–6% of the adult male population at all ages and an equivalent percentage of females postmenopause (**http://www.rightdiagnosis.com/s/sleep_disorders/prevalence-types.htm**). Sleep apnea is associated with significant increases in morbidity and mortality. As mentioned above, a leading hypothesis posits that SIDS is a dysfunction of serotonin in the infant brain stem (216). The relatively early stage of our knowledge of the respiratory neural control system as it relates to human disease has led to confusion about the underlying causes, which may consequently overemphasize possible future treatments to the detriment of both scientific progress and patient hopes.

CHALLENGES REMAINING

Although great progress has been made since the discovery of the preBötC, especially as related to key and specific brain stem sites generating and modulating respiratory rhythm and pattern, our understanding of the underlying mechanisms is still at an early stage. Key high-priority problems include identifying and understanding neurons important for the generation of expiratory activity and understanding the mechanisms of rhythm generation within the preBötC. Of all vital behaviors in mammals, the neural control of breathing may be the first to be solved.

DISCLOSURE STATEMENT

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