

# Brainstem respiratory networks: building blocks and microcircuits

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**Breathing movements in mammals are driven by rhythmic neural activity generated within spatially and functionally organized brainstem neural circuits comprising the respiratory central pattern generator (CPG). This rhythmic activity provides homeostatic regulation of gases in blood and tissues and integrates breathing with other motor acts. We review new insights into the spatial–functional organization of key neural microcircuits of this CPG from recent multidisciplinary experimental and computational studies. The emerging view is that the microcircuit organization within the CPG allows the generation of multiple rhythmic breathing patterns and adaptive switching between them, depending on physiological or pathophysiological conditions. These insights open the possibility for site- and mechanism-specific interventions to treat various disorders of the neural control of breathing.**

## Introduction

Breathing in mammals is the primal homeostatic process regulating levels of oxygen and carbon dioxide in the body that is critical for life. Respiratory movements occur automatically and continuously throughout life and are driven by the rhythmic motor activity generated within neural circuits in the brainstem and spinal cord. The underlying neural machinery is robust yet exquisitely flexible for physiological and behavioral integration. The respiratory neural control system not only performs a vital physiological function but is also engaged in volitional (e.g., speech and singing) and emotional (e.g., laughing and crying) motor behaviors. Understanding this neural circuitry may have far-reaching implications for other rhythmic motor behaviors and oscillatory circuits [1–3].

Respiratory movements, like other innate rhythmic motor behaviors such as locomotion, are produced by semi-autonomous neural networks referred to as central pattern generators (CPGs). These networks are the basic neural substrates for rhythmic motor pattern generation and sensorimotor integration [4]. They consist of core circuits of excitatory and inhibitory interneurons that

interact to generate rhythmic patterns of activity for coordinated motor output [5]. A major goal in the motor systems field is to unravel the architecture of these circuits and decipher how cellular-, circuit-, and systems-level properties are integrated functionally [1]. This is critical for revealing mechanisms of operation in both normal and disease states.

New insights into the architecture of respiratory CPG circuits have recently been obtained from the rapid convergence of electrophysiological, imaging, anatomical, genetic, developmental, and computational modeling approaches. Here, we review key developments, with a major focus on advances in understanding, including current hypotheses of circuit organization and operation. The important advantage of this system is that it can be studied not only in conscious and anesthetized animals *in vivo* but also in various reduced experimental preparations retaining circuit interactions *in situ* and *in vitro*. This has allowed high-fidelity measurements at cellular, synaptic, and circuit levels in the context of behaviorally meaningful network activity, which are essential for dissecting the logic of CPG circuits [1] and ultimately for designing novel therapeutic interventions.

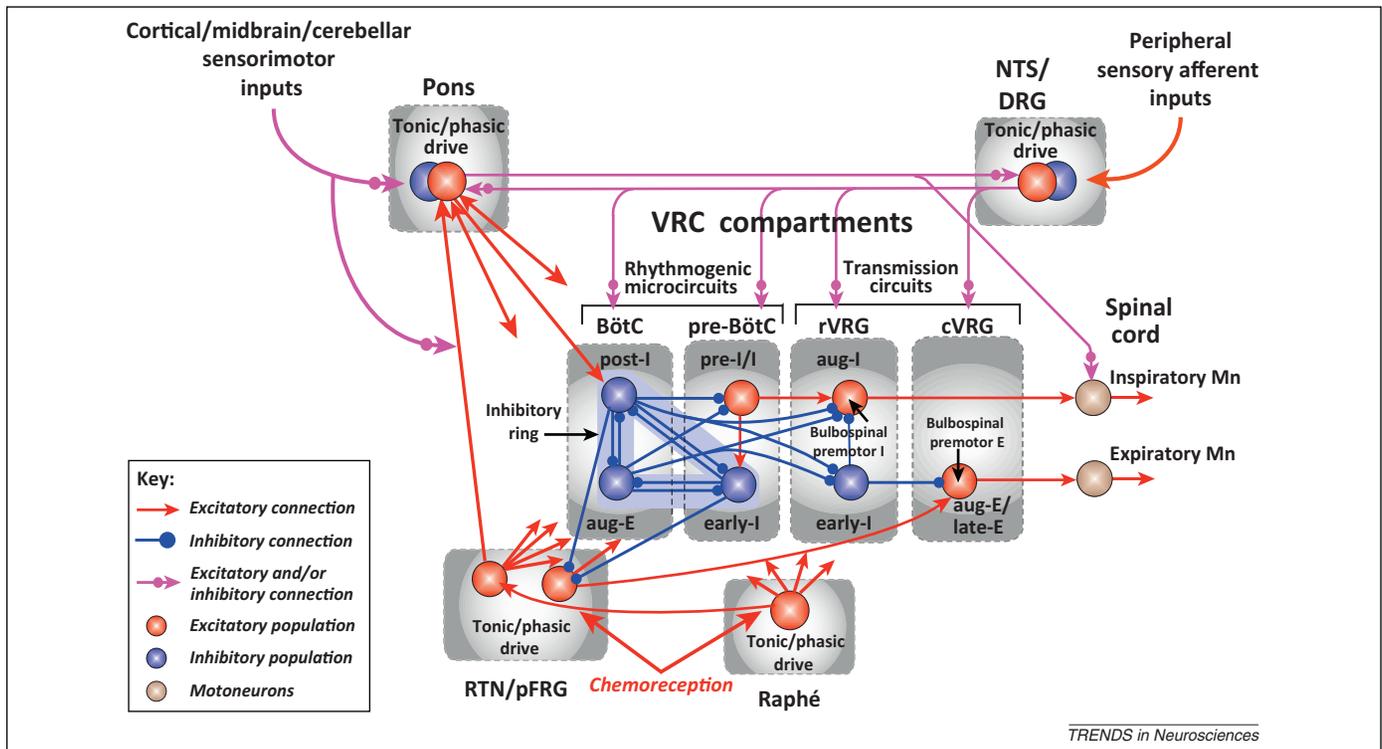
## The brainstem respiratory network is arrayed within structural–functional compartments

The brainstem circuits generating and controlling respiratory motor activity during normal eupneic breathing *in vivo* are distributed bilaterally in the pons and medulla oblongata. The current view is that each side of the medulla has a ventral respiratory column (VRC) of respiratory neurons, interacting within the VRC and interconnected with several pontine nuclei [6–9]. The VRC contains key interacting excitatory and inhibitory interneuron populations (Figure 1) representing the respiratory CPG. Numerous afferent systems, some of which funnel through the caudal nuclei of the solitary tract (NTS) [10], control these microcircuits including via interconnections with pontine circuits. They are also subject to behavioral control from supra-brainstem structures [11,12], including motor and sensory cortices, basal ganglion, cerebellum, and hypothalamus. The output of VRC circuits is transmitted through premotor networks to cranial and spinal motoneurons. The former control muscles of the upper airways, whereas the latter include phrenic, intercostal, and lumbar motoneurons,

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Keywords: breathing; brainstem; respiratory central pattern generator; breathing disorders



**Figure 1.** Schematic representation of compartmentalized microcircuits in the brainstem hypothesized to be involved in respiratory rhythm and pattern generation. Each side of the brainstem contains these compartments with extensive bilateral circuit interconnections (not shown) as well as reciprocal connections, a few of which are indicated. It is hypothesized that the pre-Bötzinger complex (pre-BötC) and Bötzinger complex (BötC) are the major components of the ventral respiratory column (VRC) with rhythmogenic microcircuits generating multiple respiratory patterns as described in the text (Figure 2). These circuits, shown in highly simplified form, incorporate the excitatory glutamatergic (red) pre-inspiratory-inspiratory (pre-I/I) population in pre-BötC and three inhibitory GABAergic or glycinergic (blue) populations within the pre-BötC–BötC that are postulated [7,13] to mutually inhibit each other [inhibitory ring of early-I, post-I, and augmenting expiratory (aug-E) neurons; see Box 2 for respiratory neuron nomenclature]. BötC expiratory neurons are a major source of synaptic inhibition of inspiratory neurons during expiration. The pre-I/I excitatory neurons of the pre-BötC, which is the excitatory kernel of the respiratory network, project via premotor circuits to cranial motoneurons (e.g., hypoglossal and vagus, not shown; Box 2) and to rostral ventral respiratory group (rVRG) excitatory [augmenting inspiratory (aug-I)] bulbospinal premotor neurons with projections to spinal cord inspiratory (phrenic) motoneurons (Mn, brown). The excitatory bulbospinal expiratory neurons of the caudal VRG (cVRG) project to thoracic and abdominal spinal expiratory motoneurons. Neurons within these compartments are considered to receive tonic, phasic, or rhythmic excitatory drive from the pontine, dorsal respiratory group (DRG), retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), and raphé compartments. Drives from the latter two compartments are regulated in part by blood or brain CO<sub>2</sub> levels (chemoreception), which may involve modulatory connections from the raphé to RTN/pFRG [78] as depicted. Some pontine and BötC neurons (not indicated in the schematic) have bulbospinal projections. The spinal cord also contains segmental interneuronal microcircuits (not represented) controlling motoneuronal activity. See Box 1 and Figure 2 for additional descriptions of the roles of the different populations of excitatory and inhibitory respiratory neurons. See Box 4 for issues related to supra-brainstem sensorimotor inputs (upper left) and regulation of breathing.

which innervate the diaphragm, thoracic, and abdominal respiratory pump muscles, respectively.

A current hypothesis about the structural–functional organization of this pontine–medullary respiratory network is that it contains hierarchically organized [13] functional compartments [6,7], spatially arranged bilaterally in the rostro–caudal direction along the neuraxis from the rostral pons to the caudal medulla (Box 1, Figure 1). Box 1 provides a neuroanatomical overview and gives a synopsis of regional properties that are essential for understanding the global operation of the network [6]. This compartmentalization has been inferred from recordings of neuron population activity that identified the predominant types of functionally related respiratory neurons in each compartment (Box 2) [6,13,14]. Further insight came from developmental [15–18], anatomical [6,10,19,20], ablation/lesion [13,21,22], pharmacogenetic [23,24], and optogenetic [25,26] studies that provide evidence of regional circuit specialization. It is hypothesized that these spatially and functionally distinct compartments represent neural building blocks of the respiratory CPG (Figure 1 and see below). They are reminiscent of the rhombomeric structure of the developing hindbrain in which the rostral–caudal

organization of the entire brainstem respiratory network, including in the pons, the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), the Bötzinger (BötC) and pre-Bötzinger (pre-BötC) complexes, appears to be pre-determined genetically [15]. Several ontogenetically based and other breathing disorders associated with some of these structures are described in Box 3; Box 4 provides ontogenetic perspectives.

#### Different breathing patterns are produced by reconfiguration of neural building block circuits

The normal breathing cycle consists of three main phases of neural activity [27]: inspiration (I), post-inspiration, and the later stage 2 of expiration (Box 2). This three-phase pattern is evident in the activity of simultaneously recorded motor outputs [8,13] and is also reflected in the activity profiles of interneuron populations within the VRC compartments, as determined by simultaneous neuron recordings at multiple VRC sites [14]. Populations of inspiratory interneurons are concentrated in the pre-BötC and rostral ventral respiratory group (rVRG), whereas expiratory neurons reside primarily in the BötC [post-I, augmenting expiratory (aug-E) neurons] and caudal VRG

### Box 1. Spatially arrayed compartments of the rodent brainstem respiratory network

Respiratory-related brainstem structures are shown in Figure 1 and described below.

**Pontine nuclei**, including Kölliker–Fuse (K-F) and parabrachial (PB) nuclei, comprise the pontine respiratory group (PRG), which regulates the inspiratory–expiratory phase transition [79]. The K-F also contains laryngeal premotor neurons controlling upper airway resistance [79] and spinal projecting neurons controlling phrenic motoneuron activity (see Figure 1 in main text).

The **retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG)** contains glutamatergic neurons expressing the transcription factor paired-like homeobox 2b (Phox2b) [16,25,71,80], many of which are rhythmically active intrinsically in perinatal rodents [70,71], and is called the pFRG. In adults, these neurons (called RTN) are tonically active [25,81] or respiratory-modulated [66], which may reflect developmental transformation [77]. The activity of these neurons is regulated by CO<sub>2</sub> or pH [81] and inputs from peripheral chemoreceptors [82]. The RTN performs a central chemosensory function [83,84]. In adult rats, a subset of parafacial neurons becomes rhythmically active in late expiration during hypercapnia [77] or when disinhibited [26,75] and may be responsible for abdominal muscle contraction during active expiration [38].

The **Bötzinger complex (BötC)** contains predominantly expiratory neurons [6,13,85]. BötC glycinergic or GABAergic neurons [86] inhibit inspiratory neurons and thus provide the inspiratory–expiratory phase alternation during normal breathing.

The **pre-Bötzinger complex (pre-BötC)** consists of bilaterally connected circuits [18,41] essential for normal inspiratory activity *in vivo* [21,38]. Pre-BötC glutamatergic neurons have widespread projections [20] and are the primary source of rhythmic inspiratory excitatory drive. The cellular composition is heterogeneous and includes glutamatergic populations expressing neurokinin-1 receptors (NK1R), somatostatin

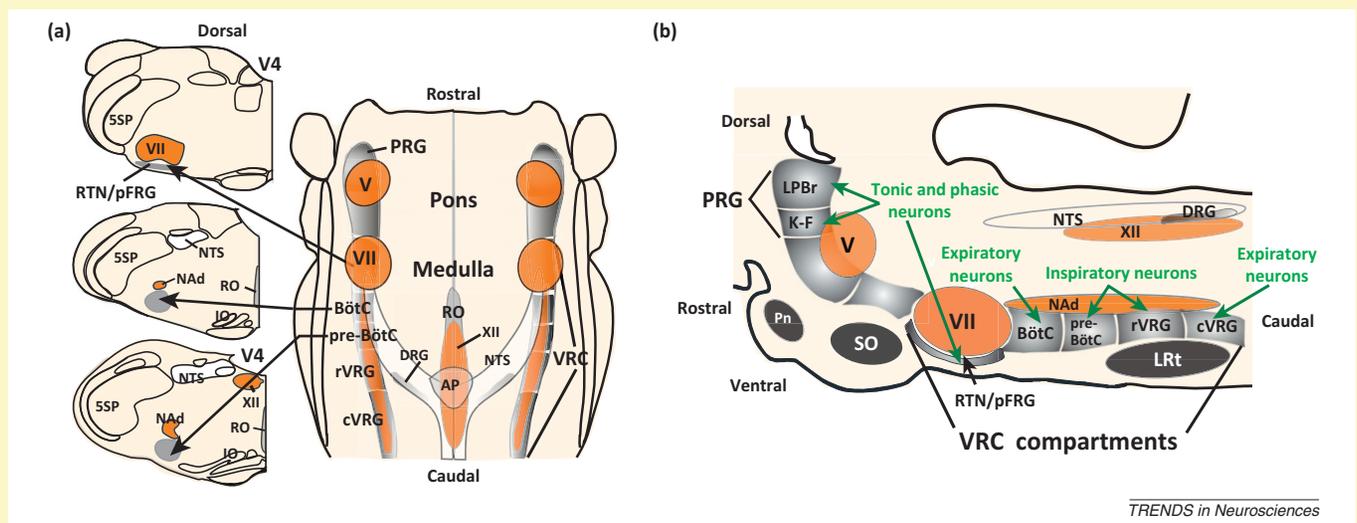
(SST), and the transcription factor developing brain homeobox 1 protein (Dbx1) [17,18], as well as subpopulations of inspiratory glycinergic [58,59] and GABAergic [60] neurons. The latter two types may provide inhibition of expiratory neurons during inspiration. The human pre-BötC has been delineated [87].

The **rostral ventral respiratory group (rVRG)** contains the main cluster of bulbospinal premotor inspiratory neurons [6] relaying inspiratory drive to spinal phrenic motoneurons innervating the diaphragm. rVRG neurons are driven by excitatory pre-BötC neurons and inhibited by expiratory BötC neurons (Figure 1); these inputs shape the characteristic ramping pattern of inspiratory rVRG activity.

The **caudal ventral respiratory group (cVRG)** comprises excitatory bulbospinal expiratory neurons that receive convergent inputs including those from the RTN/pFRG and BötC, which shape patterns of expiratory drives to spinal thoracic and lumbar expiratory motoneurons.

The **nucleus tractus solitarius (NTS)** in the dorsomedial medulla is the entry point of pulmonary mechanoreceptor, peripheral chemoreceptor, and other visceral sensory afferent inputs. Caudal NTS regions (cNTS) and the associated dorsal respiratory group (DRG) mediate afferent control of breathing via projections to pontine and VRC compartments [10] (Figure 1).

**Brainstem raphe nuclei** containing serotonergic neurons are involved in somatic and autonomic motor control and project to the VRC as well as respiratory motoneurons. These excitatory neurons [66] release serotonin and co-localized peptides, especially substance P and thyrotropin-releasing hormone (TRH) [88]. Some of these neurons have chemosensory properties [88,89]. They participate in chemosensory regulation of breathing [89] and stabilize breathing [67,90,91]. Raphe obscurus (RO) neurons are associated with sudden infant death syndrome (SIDS) [92,93].



**Figure 1.** Overview of bilaterally distributed brainstem respiratory compartments of the rat arranged from the rostral pons to the caudal medulla represented neuroanatomically by (a) horizontal (right) and several coronal (left) brainstem sections and (b) a parasagittal section through one side of the brainstem. Predominant locations of inspiratory, expiratory, tonic, and respiratory-modulated (phasic) interneurons are indicated in (b). Other abbreviations: AP, area postrema; LRt, lateral reticular nucleus; NAd, nucleus ambiguus, dorsal division; Pn, ventral pontine nucleus; SO, IO, superior and inferior olivary complexes; SPS, spinal trigeminal nucleus; V, motor nucleus of the trigeminal nerve; V4, fourth ventricle; XII, hypoglossal motor nucleus. Neuroanatomical representations are based on [6,7,10].

(cVRG; predominantly aug-E neurons) (Box 2), although the localization of these neuron types is not exclusive, with some spatial overlap [6,28]. Many models of the operation of VRC circuits attempt to explain how respiratory patterns emerge from spatiotemporal interactions of these inspiratory and expiratory neurons [29].

The normal (eupneic) three-phase respiratory pattern can be transformed to other rhythmic patterns with fewer active phases (Figure 2). Such transformations were recently demonstrated following brainstem transections

made at various levels in an *in situ* perfused rat brainstem–spinal cord preparation [13,30], which is an effective strategy for uncovering spatiotemporal features of network organization. The three-phase pattern is transformed to a two-phase inspiratory–expiratory pattern (lacking the post-I phase) generated by intact BötC and pre-BötC circuits after more rostral compartments are physically removed. This activity is subsequently transformed to one-phase rhythmic inspiratory patterns originating within pre-BötC circuits after the BötC compartment is removed.

Removal of the pre-BötC eliminates rhythmic inspiratory activity, as originally demonstrated *in vitro* [31] and consistent with experiments suppressing pre-BötC excitatory neuron activity *in vivo* [23]. Thus, these different patterns occur in register with specific compartments containing functionally distinct microcircuits (below). Importantly, similar transformations can also occur when the intact network goes through various physiological and/or metabolic disturbances [7]. For example, apneusis, a two-phase inspiratory–expiratory pattern [32], and gasping, one-phase inspiratory oscillations originating in the pre-BötC [33], are evoked during hypocapnia and severe hypoxia, respectively. These findings are important because researchers have been attempting to explain the neural substrates and mechanisms for these established motor patterns for decades.

These transformations suggest that there are multiple rhythmic pattern generation mechanisms inherent in the brainstem respiratory network. The latter can be broken down into a set of microcircuits that can at least theoretically explain operation of the network in intact, physically reduced, and pathophysiological states.

### A model of microcircuits generating different rhythmic patterns

The concept of functional compartmentalization incorporates the hypothesis that excitatory and inhibitory circuits within the pre-BötC and BötC are substrates for the generation of the different rhythmic motor patterns described above. Computational modeling studies suggest that a minimal circuit structure should include inhibitory expiratory (post-I and aug-E) neurons of the BötC and inhibitory

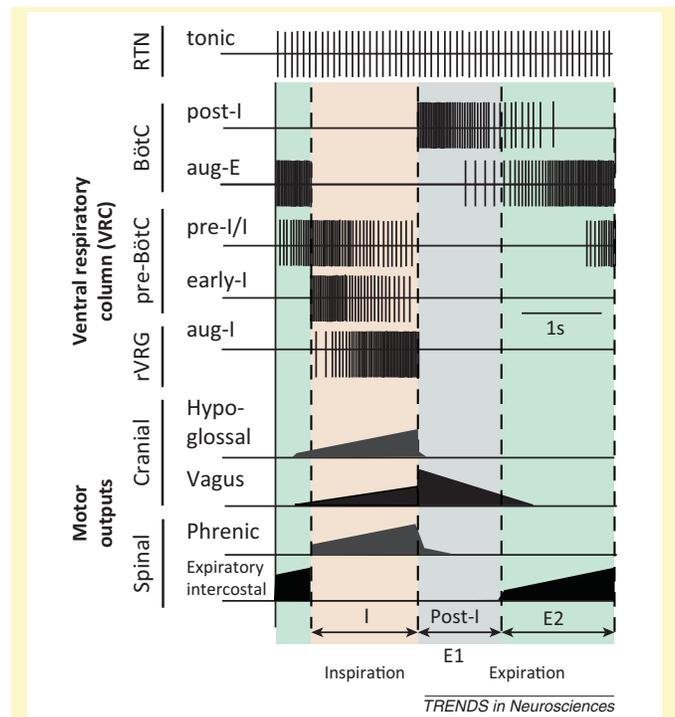
#### Box 2. The three-phase respiratory pattern and motor behavior

The spiking activities of respiratory CPG neurons in various compartments as well as cranial and spinal motor output patterns (Figure 1) during eupneic breathing *in vivo* in anesthetized mammals consist of three behaviorally distinct phases: inspiration (I), post-inspiration (post-I or stage 1 of expiration, E1), and stage 2 of expiration (E2). These phases translate into coordinated motor activity of the diaphragm, thoracic muscles, musculature of the upper airways (hypoglossal, glossopharyngeal, laryngeal) controlling airflow resistance, and expiratory muscles involved in breathing.

**Inspiration.** Bulbosplinal (aug-I) neurons in the rVRG drive the activity of phrenic motoneurons that control contraction of the diaphragm to inflate the lungs. The pre-inspiratory–inspiratory phase (pre-I/I) spiking activity of distinct populations of medullary neurons, which in the pre-BötC are considered critical for rhythm generation and initiation of inspiration (Figure 3), activates the tongue protrusor muscles via hypoglossal nerves. Hypoglossal tone is maintained throughout inspiration to prevent obstruction of the oropharyngeal airway. Branches of the vagus nerve innervate laryngeal muscles, controlling the glottis and vocal cords, which are abducted by vagal inspiratory activity to maintain upper airway patency.

**Post-inspiration.** This phase is critical for termination of inspiration. During this phase the larynx is initially adducted (to stall airflow, thereby prolonging the time for gas exchange) and then slowly abducted to allow exhalation.

**Stage 2 expiration.** Expiratory bulbospinal neurons in the BötC and cVRG are active during this phase and produce low levels of internal intercostal and abdominal muscle activity.



**Figure 1.** Three-phase organization of neuronal spiking and motor output patterns during the respiratory cycle. Neuronal spiking patterns in different brainstem compartments (top) and motor outputs (bottom), illustrated schematically, are typical of those recorded in anesthetized mammalian preparations (rat and cat). The motor output patterns illustrated depict the activity of cranial hypoglossal, central vagus, and spinal phrenic and expiratory intercostal nerves. Respiratory neurons active during the three phases are typically classified based on profiles of their firing patterns (e.g., decrementing or augmenting spiking frequency) and their predominant activity phase. As shown, pre-I/I neurons in the pre-BötC start firing before and continue into inspiration; early-I neurons have peak spiking in early inspiration followed by a decrementing pattern; rVRG aug-I neurons have an augmenting or ramping firing pattern; post-I neurons have a decrementing firing pattern; and aug-E neurons have a ramp firing pattern during E2. Note that the activity phases are not strictly sequential, but exhibit considerable temporal overlap.

inspiratory neurons in the pre-BötC, coupled in a ring-like network with mutual inhibitory interactions (Figure 2) [13,30,34]. It has been proposed that this inhibitory network interacts with the key excitatory pre-BötC inspiratory neurons to coordinate the generation of inspiratory and expiratory activity phases [13]. This is a versatile dynamic structure with multiple oscillatory loops that can account hypothetically for rhythmic pattern generation in intact and reduced states of the network. It is hypothesized that various excitatory drives originating from, for example, the pontine, RTN/pFRG, and raphé compartments (Figure 1 and see below) control the operation of the coupled pre-BötC–BötC microcircuits. The pre-BötC contains the excitatory kernel of glutamatergic interneurons that are critical for rhythm generation (Box 1).

#### Intrinsic rhythmogenic properties of the pre-BötC

Since the discovery of the pre-BötC [31] there has been intense interest in this structure, particularly since experimental studies in rodents have established that there is a critical interconnected bilateral network of excitatory neurons coupled by ionotropic glutamatergic synaptic mechanisms [17,18,35] and a subset of these neurons exhibit intrinsic bursting or pacemaker-like properties *in vitro*

### Box 3. Clinical disorders of brainstem respiratory control

Disorders of the neural control of breathing, only a few of which are indicated below, have a major impact on health and can be life-threatening. Some disorders result from mutations of single genes, including transcription factors affecting critical groups of neurons (such as in the RTN). A prominent disorder is sleep apnea and its sequelae of hypertension and heart failure. Finally, respiratory depression accompanying analgesia is also a significant clinical problem.

**Congenital central hypoventilation syndrome (CCHS)** causes respiratory arrest during sleep and is fatal if untreated [94]. Mutation of the human *PHOX2B* gene has been identified in patients suffering from CCHS [16,94,95]. An animal model suggests that CCHS is due to a *Phox2b* gene mutation that deletes neurons expressing this transcription factor in the RTN/pFRG [71]. This reduces phrenic nerve activity, alters respiratory frequency, and attenuates system responses to CO<sub>2</sub>.

**Obstructive sleep apnea (OSA)**, the most common type of sleep-related breathing disorder, is caused by obstruction of the upper airway due to withdrawal of excitatory drives and respiratory activity of hypoglossal motoneurons [96] and is characterized by repetitive pauses in breathing during sleep. OSA is associated with reduced blood oxygen saturation, and elevated heart rate and blood pressure,

which increase the risk of cardiovascular disease. Experimental studies show that just 10 days of exposure to chronic intermittent hypoxia, to mimic disturbances in OSA, produces hypertension and increased abdominal expiratory activity at rest in rats [97]. It is hypothesized that this hypertension results from alterations in the respiratory modulation of sympathetic nerve activity [97].

**Rett syndrome (Rett)** is a neurodevelopmental disorder in females caused by mutation of the methyl CpG binding protein 2 (*MECP2*) gene encoding the transcription factor MECP2 [98]. Patients exhibit episodes of breath-holding and life-threatening breathing arrhythmia. Sudden death can occur [98]. Studies using a mouse model of Rett (*mecp2*<sup>-y</sup> knockout) confirmed that the respiratory abnormalities originate in the brainstem and result from excessive expiratory, particularly post-inspiratory, activity [99,100].

**Analgesia and respiratory depression.** Many pain-relieving drugs include opioids that can suppress breathing via pharmacological actions at brainstem (e.g., in the pre-BötC [101]) and possibly supra-brainstem [11] sites. In animal models, studies to identify pharmacological tools protecting against this depression indicate a therapeutic potential of serotonin receptor agonists [90,101,102] and drugs that alter glutamate receptor deactivation [103] in respiratory networks.

and *in situ* [36,37]. Pre-BötC circuits can generate inspiratory oscillations when isolated in slices *in vitro* [31,38] or uncoupled from more rostral compartments *in situ* [13]. Importantly, this *in vitro* rhythmic activity persists after pharmacological disruption of inhibitory synaptic interactions [38].

The underlying rhythm-generating cellular and excitatory network mechanisms have been debated for nearly two decades [38], centering on the role of neuronal pacemaker properties and excitatory interactions. The

generation of rhythmic bursts of activity in excitatory networks involves mechanisms for regenerative initiation, termination, and recovery of population-level bursts [39]. Early computational models [40] suggested a persistent (slowly inactivating) Na<sup>+</sup> current (*I*<sub>NaP</sub>) as the essential membrane current in pre-BötC neurons mediating these mechanisms for inspiratory burst generation. These models, which can account for both intrinsic oscillatory bursting behaviors at the cellular level and network level observed experimentally [36,41], propose that bursting is

### Box 4. Outstanding questions

- **How are the microcircuits in different brainstem respiratory compartments genetically specified and developmentally assembled?**

To assure survival, respiratory circuits must be functionally assembled by genetically encoded processes before birth. This includes segmentalization and positioning of neurons based on functional phenotype. Although several genes that regulate respiratory neuron positional fate [e.g., *Phox2b*, *Dbx1*, *Lbx1* (ladybird homeobox 1), *Math1* (mouse atonal homologue 1)] [16,18,104,105] have been identified, the ontogenetic programs that spatially organize the different populations of excitatory and inhibitory circuit interneurons need to be deciphered. This knowledge may provide a site-specific genetic basis for targeted therapeutic intervention.

- **What is the connectome of brainstem respiratory circuits?**

Although elaborate wiring diagrams of brainstem circuits have been proposed [13,106], many of the circuit connections have not been established definitively. New methods for neuron population-specific connectivity mapping [107,108] and optogenetic manipulation [25,26,66,109] should advance our understanding of network architecture.

- **What intrinsic neuronal biophysical properties and synaptic or circuit mechanisms are critically involved in respiratory rhythm generation?**

Despite the identification of the pre-BötC as a critical structure for rhythmogenesis, there is currently no consensus on how the basic inspiratory rhythm emerges from the dynamical interplay of cellular biophysical and synaptic interactions in any state (*in vitro*, *in situ*, or *in vivo*).

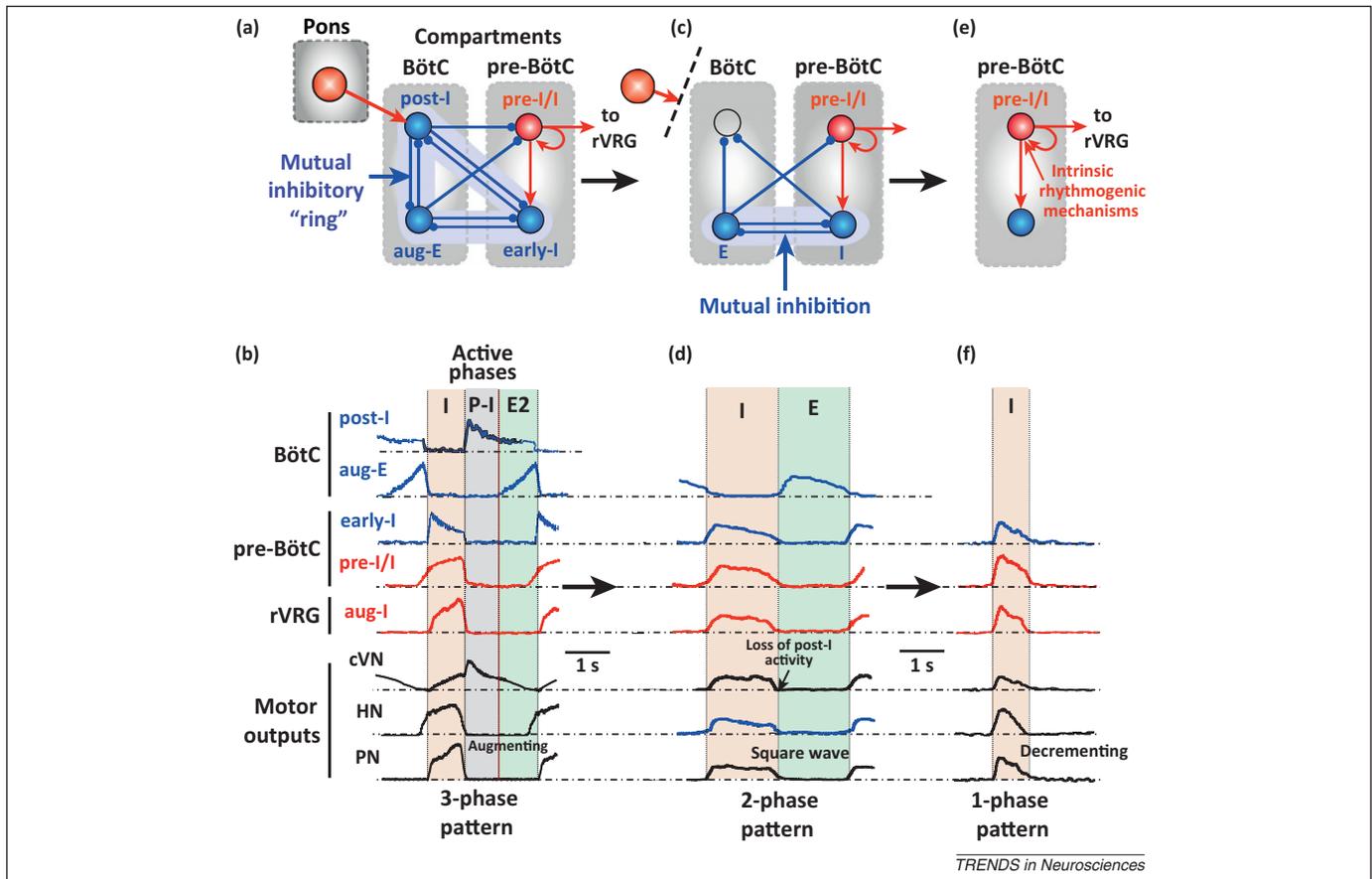
- **What is the integrated cellular- and circuit-level logic of the neuromodulatory control of breathing in health and disease?**

Respiratory CPG circuits are functionally plastic and dynamically tuned to support different states according to metabolic or

behavioral demands, including sleep-wake or arousal and disease states. These adaptive modifications are controlled in part by multiple endogenous neuromodulators, such as amines and neuropeptides released from medullary raphe serotonergic neurons, brainstem catecholamine systems (e.g., locus coeruleus, medullary C1 neurons) [110,111], cholinergic neurons [112], and hypothalamic (e.g., orexin- or hypocretin-releasing) neurons [113], which may act in concert, but the mechanisms remain poorly understood. Abnormal neuromodulatory control is implicated in many breathing disorders including CCHS, SIDS, Rett, sleep-disordered breathing, and multiple system atrophy (MSA). Understanding the logic of neuromodulation will lead to rational design of therapeutic pharmacological interventions [64,90].

- **What is the structural-functional neuroanatomy of cortical and other supra-brainstem structures controlling breathing?**

In humans, breathing is controlled involuntarily as well as voluntarily and in coordination with other behaviors (e.g., speaking, singing, laughing, coughing, and eating). Functional magnetic resonance imaging (fMRI) in awake humans suggests that the respiratory rhythm automatically generated in brainstem circuits is modulated by signals from cortical (primary motor and sensory cortices, limbic system) and subcortical (thalamus, basal ganglia, cerebellum, hypothalamus [12,114]) networks. How cortico-bulbar and spinal pathways directly controlling cranial and spinal respiratory motoneurons are integrated with their control via brainstem CPG circuits is not yet understood. Imaging-based mapping of cortical and subcortical structures engaged for respiratory control is a current frontier in understanding the neural substrates for breathing in humans.



**Figure 2.** Hypothesized minimal architectures and components of pre-Bötzinger complex (pre-BötC)–Bötzinger complex (BötC) microcircuits with associated patterns of respiratory activity in different states of rhythmic pattern generation. (a) It is postulated that the normal three-phase respiratory pattern is generated by interconnected inhibitory (blue) populations forming a mutual inhibitory ring-like structure of early inspiratory (early-I), post-I, and augmenting expiratory (aug-E) neural populations that interacts with the excitatory (red) pre-I/I population within the pre-BötC. The latter consists of synaptically coupled glutamatergic neurons with local and bilateral interconnections. It is hypothesized that the normal operation of these circuits requires excitatory inputs or drives from the more rostral pontine circuits, and from the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) and raphe nuclei (not shown). (b) The three-phase pattern is depicted by a composite of integrated neuron population activities in BötC, pre-BötC, and rostral ventral respiratory group (rVRG) compartments (the latter is not shown in the schematic) and motor output patterns of phrenic (PN), hypoglossal (HN), and central vagus (cVN) nerves. Recordings depicted were obtained from arterially perfused *in situ* brainstem–spinal cord preparations from 4-week-old rats that generate a respiratory pattern similar to that in anesthetized juvenile or adult rats *in vivo* [13]. (c,d) The three-phase pattern is transformed to a two-phase inspiratory–expiratory pattern lacking the post-I phase (d) after removing the pons via ponto–medullary transection (angled dashed line in c). This eliminates pontine excitatory drive required for generation of post-I activity (Box 1). It has been proposed that this two-phase pattern involves mutual inhibitory interactions between active pre-BötC inspiratory and BötC expiratory neurons that also interact with pre-BötC excitatory pre-I/I neurons, as illustrated in c. (e,f) A medullary transection at the rostral pre-BötC boundary transforms the two-phase pattern to one-phase inspiratory oscillations driving all motor outputs. These one-phase oscillations arise from intrinsic rhythmic mechanisms operating in the mutual excitatory network within the pre-BötC compartment (pre-I/I population; schematic in e), which is sufficient to drive inspiratory activity in the rVRG. This inspiratory activity, as well as the capability of more caudal structures to generate rhythmic motor output, is eliminated by a transection at the pre-BötC–rVRG boundary. Interestingly, the two-phase motor nerve discharges have a square wave-like burst profile (shown in d), whereas the one-phase pattern is strongly decrementing as indicated in f, both of which differ from the augmenting or ramping activity profiles in the normal three-phase pattern (b, bottom two traces). The one-phase oscillatory pattern generated by the pre-BötC *in situ* is remarkably similar to the pattern generated by the pre-BötC isolated in neonatal rodent slices *in vitro*. Adapted, with permission, from [13].

initiated by a subthreshold voltage-dependent activation of  $I_{NaP}$ . Its slow voltage-dependent inactivation and dynamic interactions with a  $K^+$ -dominated outward leak current cause burst termination. The latter mechanisms control the interburst period and regeneratively lead to the next burst. Electrophysiological studies *in vitro* have now documented that a tetrodotoxin (TTX)-sensitive  $I_{NaP}$  and  $K^+$ -dominated leak currents are co-expressed ubiquitously in pre-BötC inspiratory neurons [36,42]. Modeling studies indicate that a heterogeneous excitatory network of neurons incorporating these membrane currents can produce rhythmic population bursting over a broad range of frequencies [43].

The necessity of  $I_{NaP}$  for pre-BötC rhythm generation, at least *in vitro*, has been questioned on the basis of observations that  $I_{NaP}$  blockers do not perturb inspiratory rhythm

generation in slices from mouse medulla [44,45]. However, inspiratory rhythm is completely disrupted by pharmacological suppression of  $I_{NaP}$  in isolated neonatal rat pre-BötC *in vitro* [36] and in the juvenile rat *in situ* [13]. This contradiction remains unresolved. Inspiratory bursting during gasping that is thought to originate in the pre-BötC and induced by hypoxia also involves  $I_{NaP}$ -dependent bursting mechanisms both in neonatal mice slices *in vitro* [46] and in the juvenile rat *in situ* and *in vivo* [47], suggesting state-dependent expression of this mechanism [13].

Other rhythmic mechanisms based critically on neuronal  $Ca^{2+}$  dynamics have been proposed. A subset of pre-BötC neurons with  $Ca^{2+}$ -dependent intrinsic bursting properties, hypothesized to involve a  $Ca^{2+}$ -activated non-selective cationic current ( $I_{CAN}$ ) [48–50], has been found in

neonatal mouse slices *in vitro*.  $I_{CAN}$ -like channels, members of the transient receptor potential (TRP) channel family, have been tentatively identified in pre-BötC neurons of mice *in vitro* [51] although their molecular identity is debated [52]. Two models of  $I_{CAN}$ -dependent rhythm generation have been proposed: the dual pacemaker neuron model [53] and the group-pacemaker model [45]. In the former, two populations of synchronized and differentially controlled pacemaker neurons [50,54], one with  $I_{NaP}$ -dependent and the other with  $I_{CAN}$ -dependent intrinsic bursting properties, interact within the pre-BötC and collectively produce inspiratory rhythm in the excitatory network. In the latter group-pacemaker model, as the name implies,  $I_{CAN}$  and excitatory network interactions produce bursting that emerges from circuit properties, not involving mechanisms generating neuronal intrinsic bursting *per se*. It is hypothesized that this rhythmogenic mechanism depends on synaptic activation of ionotropic and metabotropic glutamatergic receptors (mGluR1/5) [45,51] and IP3 receptor signaling [55], which initiates release of intracellular  $Ca^{2+}$ , including within dendrites [51,53,56], to activate  $I_{CAN}$  for inspiratory burst initiation [53]. It is also postulated that strong depolarization via  $I_{CAN}$  transiently causes voltage-dependent spike inactivation (depolarization block of spike-generating  $Na^+$  channels), which contributes to burst termination [57]. An essential role for an mGluR activation-based network mechanism in rhythm generation *in vitro* has been questioned on the basis of recent pharmacological experiments [52].

Thus, although the isolated pre-BötC clearly exhibits intrinsic oscillatory properties and various cellular and synaptic properties of pre-BötC neurons have been identified or postulated, the underlying rhythmogenic mechanisms are not definitely established (Box 4). Furthermore, a major challenge in the field has been to understand how the pre-BötC operates when integrated in the more intact system [7], where interactions with other microcircuits such as in BötC, RTN/pFRG, and pons are likely engaged in generating the normal breathing pattern.

### Reciprocal synaptic inhibition is essential for inspiratory–expiratory pattern generation

In the intact respiratory system, inhibitory circuit interactions operating in conjunction with pre-BötC excitatory circuit mechanisms contribute to the coordination and shaping of respiratory phases [27]. As discussed above, we have hypothesized that this involves key inhibitory microcircuits in the pre-BötC and BötC (Figure 2). Inhibitory glycinergic or GABAergic inspiratory (early-I) and expiratory (post-I, E-2) neurons have been observed in the pre-BötC and BötC, respectively [58–61]. There is also evidence of mutual inhibition between BötC expiratory (post-I and aug-E) neurons [62]. Our recent computational models with different levels of cellular biophysical and network complexity illustrated in principle how a ring-like architecture (Figure 2) of reciprocal inhibitory interactions in the pre-BötC and BötC may contribute to generation of the three-phase rhythmic pattern [30,34,63,64]. In this modeled intact system, under some conditions the intrinsic neuronal or circuit mechanisms of the pre-BötC discussed above are not sufficient for inspiratory burst termination

[13]. Inhibition developing in late inspiration and post-inspiration therefore plays a major role in terminating the inspiratory phase, whereas the escape from inhibition and firing of pre-BötC pre-I/I excitatory interneurons during the later part of expiration drives the onset of inspiration. Dynamic events during a three-phase respiratory cycle, as explained hypothetically by these models, are outlined in Figure 3.

In summary, once the pre-BötC excitatory network is embedded in the intact system, inhibitory synaptic interactions operating in concert with intrinsic cellular properties and extrinsic excitatory inputs (described below) likely underlie the basic three-phase respiratory pattern.

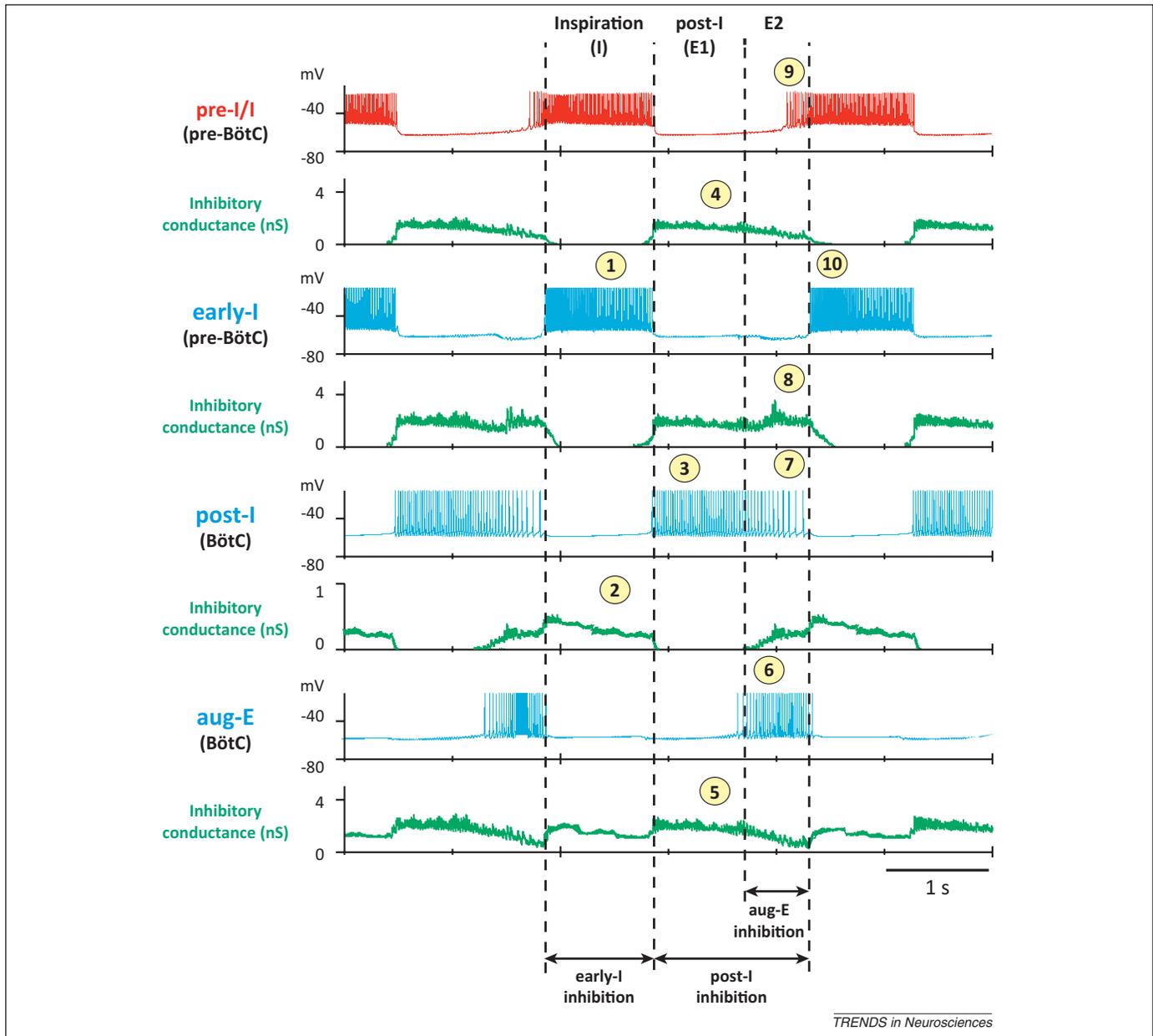
### Excitatory drives control respiratory rhythm and pattern

All concepts of respiratory rhythm and pattern generation incorporate the idea that excitatory drives to VRC circuits regulate network activity. The pontine, RTN/pFRG, and raphé nuclei are considered major sources of excitatory drives (Figure 1), likely acting in concert. These structures contain spontaneously active neurons with tonic and respiratory phasic spiking patterns. Anatomical evidence from anterograde and retrograde labeling has revealed extensive projections from these regions to the VRC [25,65] including to the BötC and pre-BötC. Electrophysiological studies showing respiratory modulated activity in these regions also indicate reciprocal interconnections [14,66,67].

The drive inputs from pontine circuits are involved in various aspects of behavioral control of respiratory pattern [32]. Some of the inputs are sensitive to the levels of blood or brain  $CO_2$  or pH (e.g., the RTN/pFRG, raphé; Box 1) and  $O_2$  (e.g., inputs originating from peripheral chemoreceptors via NTS). Input from peripheral chemoreceptors is one of the most potent drives for breathing [68]. Optogenetic-based selective photostimulation of the population of RTN  $CO_2$ -sensitive glutamatergic neurons that express the Phox2b transcription factor (Box 1) augments inspiratory discharge frequency and amplitude [25,69]. Selective pharmacogenetic-based inhibition of these neurons reduces inspiratory and post-inspiratory activity and can even convert the three-phase to a two-phase rhythmic pattern [24]. Activation of raphé obscurus serotonergic neurons [67], including selective photostimulation *in vivo* [65], augments inspiratory discharge frequency and amplitude.

### Coupling oscillators to generate new patterns of rhythmic expiratory activity

Other oscillatory mechanisms involving RTN/pFRG in perinatal [70] and mature rodents [22] have been proposed. A subpopulation of the Phox2b-expressing neurons has intrinsic oscillatory bursting properties in embryonic [71] and neonatal [72] rodents *in vitro*, which appear to be dependent on  $I_{NaP}$ , at least in embryonic mice [71]. These rhythmic cells also exhibit chemosensory properties [72,73]. Several different concepts of the physiological role of RTN/pFRG oscillations have been proposed. These include the hypothesis that in neonatal rodents the pFRG represents a primary inspiratory oscillator and entrains pre-BötC oscillations for stable breathing in neonatal rats [70]. In mature animals the RTN/pFRG is considered to



**Figure 3.** Synaptic inhibition is essential for orchestrating phase transitions to generate the three-phase respiratory pattern. This figure shows the membrane potential trajectories including spiking patterns and the temporal patterns of postsynaptic inhibitory conductances in single representative neurons from the four main neuron populations in the pre-Bötzinger complex (pre-BötC) and Bötzing complex (BötC) circuitry from a computational model [13,30], which is used here as an aid to explain the possible dynamic operation of these microcircuits. Numbers indicate sequential events, beginning with the inspiratory (I) period. (1) During the I phase, the activity of the pre-BötC early-I inhibitory population declines, in part because of intrinsic spike frequency adaptation. This provides a slow disinhibition of the BötC post-I neurons, which is evident in the trajectory of the post-I neuron inhibitory conductance (2). Once the post-I inhibitory neurons start firing (3), inspiratory activity is inhibited by the abrupt rise in inhibitory conductance in pre-I/I (4) and early-I neurons, which terminates inspiration. Augmenting expiratory (aug-E) neurons are also inhibited (5) and prevented from spiking by the abrupt rise in inhibitory postsynaptic conductance. With the progressive reduction in post-I neuron spiking frequency (adaptation) and inhibition, the activity of the aug-E neuron population (6) develops later in expiration, forming the second expiratory (E2) phase. Aug-E neuron activity produces a rise in inhibitory conductance in post-I neurons, further reducing their spike frequency (7), and in early-I neurons (8), transiently preventing their reactivation. During the E2 phase, the pre-I/I neurons of the pre-BötC, which are probably only weakly inhibited by aug-E neurons and receive high excitatory drive, escape from inhibition and start firing (9). This provides strong excitation of early-I inhibitory neurons. The rapid onset of early-I spiking (10) initiates a wave of inhibition of post-I and aug-E neurons that completes the E-to-I transition. The process is then repeated. All neurons in this model receive tonic excitation, which facilitates the phase transitions by promoting escape from inhibition. These model-based descriptions of the generation of three-phase respiratory activity are generally in accord with the classical descriptions of Richter and colleagues [27], updated here with regard to the involvement of specific compartmental microcircuits.

contain a separate expiratory rhythm generator that is coupled with the pre-BötC inspiratory rhythm generator (dual oscillator hypothesis [22,38]). Computational models have treated the RTN/pFRG as a conditional oscillator that can synchronize its activity with VRC circuits when the RTN/pFRG oscillator becomes active [63,74–76], which in adults only occurs at high levels of respiratory drive.

Experimental studies in mature rats [74,75] have demonstrated that a subpopulation of RTN/pFRG cells are inactive under normal conditions and become rhythmically active during hypercapnia (elevated  $\text{CO}_2$ ) [24,74] or local disinhibition [26,75]. It is hypothesized that this excitatory late-expiratory (late-E, or pre-inspiratory) activity emerging in RTN/pFRG drives bulbospinal expiratory neurons in

the cVRG, which receives excitatory projections from RTN [25], and drives activity of abdominal nerves (AbN) controlling expiratory pump muscles. The late-E AbN bursts observed experimentally immediately precede inspiratory bursts in the phrenic nerve and have an activity pattern and temporal relationships with the inspiratory activity that are distinct from the typical pattern of aug-E activity of BötC [74] or cVRG under normocapnic conditions. This emergent late-E AbN activity represents forced expiration that occurs naturally during hypercapnia, hypoxia, or exercise to effectively increase lung ventilation. This activity does not occur after removal [22] of the RTN/pFRG region or pharmacological inhibition [74], including selective pharmacogenetic inactivation of Phox2b-expressing RTN neurons [24]. Conversely, optogenetic-based photostimulation of these latter neurons [26,69] induces rhythmic late-E AbN activity.

Thus, convergent lines of evidence suggest that the RTN/pFRG contains rhythmogenic neurons, some of which function as a conditional expiratory oscillator in adults. The role of this oscillatory mechanism may be to orchestrate coordinated activity of spinal and cranial premotor circuits to produce a rhythmic pattern of active expiration as dictated physiologically [74]. The interactions of RTN/pFRG and VRC circuits that generate this rhythmic motor output remain to be delineated. Whether the oscillatory neurons driving forced expiration or the adult Phox2b-expressing chemosensory neurons in RTN/pFRG represent mature versions of the embryonic or neonatal oscillatory cells that transform developmentally also requires experimental clarification [77].

### Summary

Neurophysiologists have investigated mechanisms underlying breathing for more than a century. Here, we considered how the system can be broken down into structural-functional elements and synthesized according to currently available neurobiological approaches. The emerging concept of spatially organized brainstem compartments and their interacting microcircuits provides a framework for understanding the operation of the respiratory CPG and should assist in the identification of loci and mechanisms that fail in disease states.

By nature, respiratory circuits are oscillatory and functionally plastic. Multiple rhythmogenic mechanisms exist, allowing a variety of breathing behaviors, which is a basic design principle. Many outstanding issues remain (Box 4) and the challenge for the future will be to define more specifically the conditions for engaging these mechanisms physiologically and how they are perturbed under various pathophysiological conditions. This will require more detailed information on the integration of cellular biophysical properties, synaptic interactions, neuromodulatory control, and circuit dynamics under different behavioral conditions, especially in conscious animals. As we have alluded to, site-specific targeting of different excitatory and inhibitory neuronal populations via pharmacogenetic- and optogenetic-based manipulations to define functional roles now becomes essential. Computational approaches, also reviewed, will become increasingly important for understanding the integration and functional role of various

cellular and circuit properties. Only then will we be in a position to translate our understanding of microcircuit organization, including its genetic basis, into putative therapeutic strategies.

### Acknowledgments

This work was supported in part by the Intramural Research Program of the NIH, NINDS, and R01 NS057815 and R01 NS069220 to I.A.R. J.F.R.P. was supported by a Royal Society Wolfson Research Merit Award. A.B. was supported by a Feodor Lynen Research Fellowship from the Alexander von Humboldt Foundation.

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