REVIEW
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Defining the Rhythmogenic Elements of Mammalian Breathing

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Breathing's remarkable ability to adapt to changes in metabolic, environmental, and behavioral demands stems from a complex integration of its rhythm-generating network within the wider nervous system. Yet, this integration complicates identification of its specific rhythmogenic elements. Based on principles learned from smaller rhythmic networks of invertebrates, we define criteria that identify rhythmogenic elements of the mammalian breathing network and discuss how they interact to produce robust, dynamic breathing.

Introduction

Breathing is vital. Its pivotal role in regulating blood gases positions this rhythmic behavior at the core of physiology and pathophysiology. Much has been learned about the neural networks that generate the breathing rhythm, and several comprehensive reviews discuss our current understanding (1, 7, 22, 32, 51, 103, 104, 113, 119). Reviewing the mechanisms underlying respiratory rhythmogenesis is timely, since many new, and often unexpected, insights have been gained due to the advent of transgenic and optogenetic approaches. But it has been a long journey from the early discovery that the isolated brain stem is sufficient to generate a basic rhythm (2) to our current understanding of how the underlying networks are integrated to generate breathing in the intact organism. Along this journey, there have been many controversies, confusions, and discussions about where and how the respiratory rhythm originates (31, 32, 93, 106, 115, 119, 131). Indeed, several key questions continue to be debated and seem to remain unresolved despite a wealth of new insights. For example, we continue to debate whether the respiratory rhythm is based on synaptic inhibition, recurrent excitation, or pacemaker properties (1, 54, 75), a topic already discussed in 1960 (125). Defining the role of the pons in respiratory rhythmogenesis is another issue that has been discussed since the early 1900s (26, 28, 71, 72). The role of inhibitory mechanisms as an inspiratory off-switch was first hypothesized by Euler (155) and is still considered as a possible mechanism for rhythmogenesis (27, 33, 100, 168). Many of these questions remain unresolved because the criteria used for rhythm generation are vaguely defined or misunderstood. Thus the purpose of this review is to systematically address and define the key processes that govern the generation of breathing in the context of our current understanding.

On the surface, breathing appears to be a relatively simple rhythmic behavior that, like locomotion, controls skeletal muscles. Although breathing can be voluntary, conscious awareness is not required for it to continuously adapt to changing metabolic, environmental, and behavioral demands. Breathing's remarkable ability to adjust in a cycle-to-cycle and well-coordinated manner may be best exemplified for human speech or singing (63) (FIGURE 1A). During the inspiratory phase before vocalization, the amount of inhaled air is accurately adjusted to the length of the subsequent sentence. Inspiration must also be precisely timed between sentences to produce seemingly uninterrupted speech or singing. Vocalization occurs following inspiration during a breathing phase referred to as postinspiration (28, 137), when expiratory airflow is precisely regulated by the diaphragm, upper airway muscles, and vocal cords to produce sounds. Speech becomes more difficult under conditions of high metabolic demand, such as exercise (120), when breathing must become more rapid (79), and thoracic and abdominal muscles are recruited to forcefully expel air from the lungs, a breathing phase called active expiration (FIGURE 1B). The three breathing phases, inspiration, postinspiration, and active expiration, can be reconfigured and recombined to produce a breathing rhythm that is surprisingly dynamic and adaptable (103). For example, during severe hypoxia, breathing switches to gasping, a one-phase inspiratory rhythm (156) (FIGURE 1C).

Breathing's complex control is often differentiated into two principle processes: rhythm vs. pattern generation (FIGURE 1, D AND E). In general, rhythm-generating mechanisms control breathing frequency and are involved in reconfiguring breathing into a one-, two-, or three-phase rhythm (103). In contrast, regulation of tidal volume and the coordinated and differential activation of the many upper airway and respiratory pump muscles is considered "pattern

generation" (33). The notion of distinct rhythm- and pattern-generating mechanisms is supported by a variety of neuronal and modulatory properties that differentiate rhythm vs. pattern (34, 141) (FIGURE 1D). These two principle processes are differentially controlled by rhythmogenic microcircuits, premotor, and motoneuron pools (117). However, rhythm and pattern generation are not always separate and distinct. Processes of rhythm and pattern generation can be interdependent such that manipulations that influence the breathing rhythm also alter its pattern or vice versa (9) (FIGURE 1E). Indeed, neuronal networks that generate the rhythm within the CNS are generally referred to as "central pattern generators," which is consistent with the notion that processes of rhythm and pattern generation are intermingled. This review will focus primarily on rhythm generation, since it aims to define the criteria required to be considered "rhythmogenic." As we discuss the neuronal origins of mammalian breathing, we consider fundamental principles learned in smaller, well-characterized rhythm-generating networks of invertebrates.

Defining a Rhythm-Generating Network, and the Concept of a Central Pattern Generator (CPG)

Graham Brown first proposed the concept that rhythmic behaviors arise from specialized networks in the CNS, referred to as central pattern generators (CPGs) (37, 135, 161). A CPG must fulfill one criterion: the putative CPG needs to generate a behaviorally relevant rhythmic activity even when physically isolated from all other central and peripheral inputs. However, after Graham Brown's proposal, it took several years before convincing evidence for a CPG was demonstrated. Adrian and Buytendijk discovered that the isolated, completely deafferented brain stem of the goldfish continued to generate a respiratory-related rhythm (2); but these experiments did not specifically identify the region/network within the brain stem responsible for rhythmogenesis. It took another 60 years before it was discovered that isolated slices from the medulla of neonatal rodents contained a small network, the so-called pre-Bötzinger complex (preBötC), that was sufficient to generate a breathing-related rhythm (134).

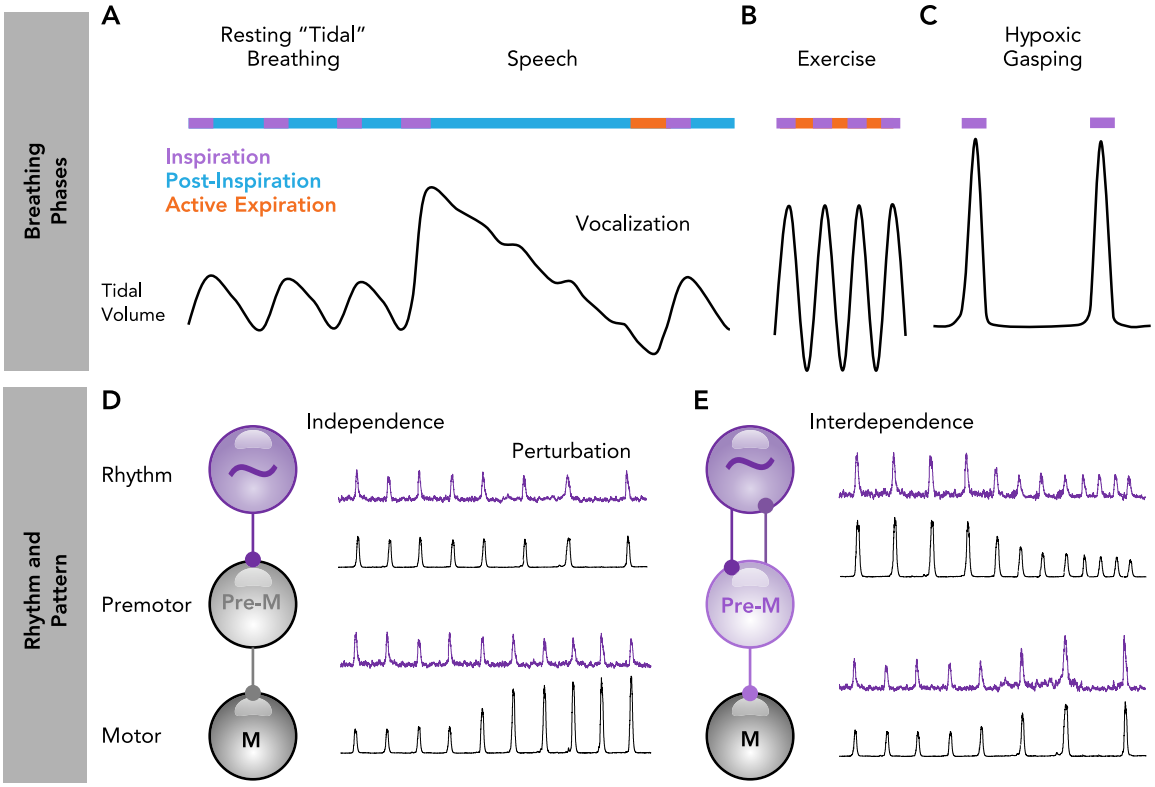

FIGURE 1. **Breathing phases arise from a combination of rhythm- and pattern-generating mechanisms**
A–C: schematic of breathing phase coordination and reconfiguration during rest, speech, exercise, and severe hypoxia. *A*: during rest, breathing alternates between inspiration (purple) and postinspiration (blue). Prior to speaking, the depth of inspiration (tidal volume) is adjusted to the anticipated length of the vocalization. Vocalization occurs during an extended postinspiratory phase. *B*: during heavy exercise, breathing generally alternates rapidly between inspiration and active expiration (red) to match increased metabolic demands. *C*: during severe hypoxia, breathing reconfigures to produce a slow one-phase inspiratory rhythm. *D–E*: processes of rhythm and pattern generation can be independent or interdependent. *D*: when independent, perturbations of rhythm (purple) do not alter pattern (black), and perturbations of pattern do not alter rhythm. *E*: when interdependent, perturbations of rhythm also alter pattern, and perturbations of pattern also alter rhythm.

Although the persistence of rhythmic activity in isolation may seem like a straight-forward criterion for a CPG, demonstrating its behavioral relevance can be more difficult. One complication is that the rhythmic output of an isolated CPG may depend on the specific experimental context. In the preBötC, not only one but three distinct types of rhythms can be generated. The rhythm that underlies the inspiratory component of normal eupneic activity and sigh activity are concurrently generated under control conditions, whereas gasping activity is generated during hypoxia (66). These in vitro rhythms are often cautiously referred to as "fictive" activities since there is no actual drive to the muscles, and they may only be a representation of the actual behavior in vivo. Furthermore, in the intact system, a CPG will be under the influence of many neuromodulatory and synaptic inputs that may significantly alter the characteristics of its rhythmic output. Thus demonstrating the behavioral relevance of a putative CPG often requires interrogating the functional consequences of manipulating it in the context of the whole animal. Indeed, the discovery of the preBötC led to numerous follow-up studies and 25 years of intense research aimed at better defining this "in vitro" rhythm and its role in vivo (39, 78, 112, 140, 160). As a result, it is now generally accepted that the preBötC is a microcircuit that is central to the generation of mammalian breathing.

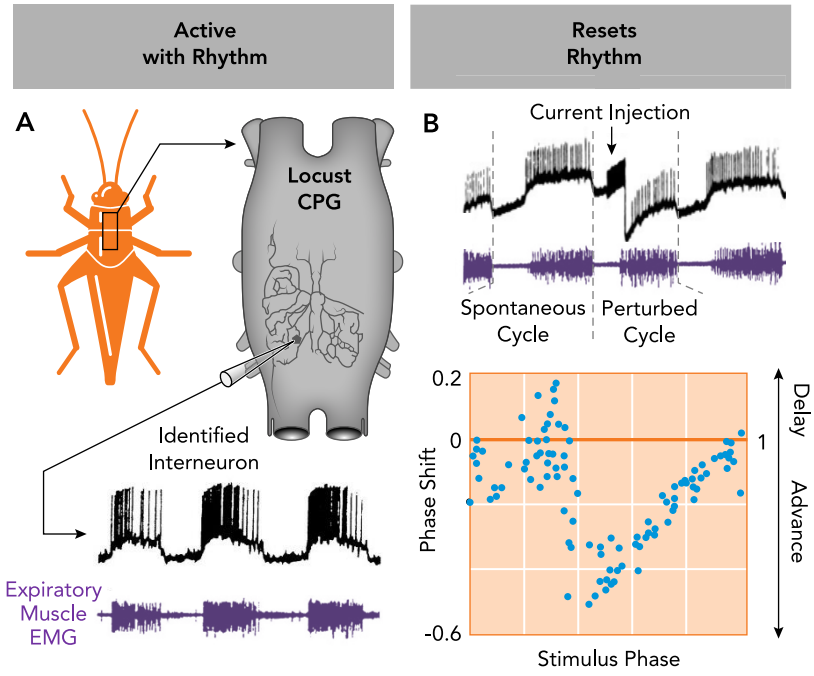

FIGURE 2. **Characteristics of an identified rhythmogenic neuron in the respiratory central pattern generator of the locust**
A: the neuron (subesophogeal ganglion interneuron 378) is active in phase with the rhythm (*criterion 1*), as indicated by intracellular recording of spiking activity (black) concurrent with expiratory muscle EMG activity (purple). *B*: stimulation of the neuron resets the respiratory rhythm (*criterion 2*). Direct current stimulation of subesophogeal ganglion interneuron 378 shortens the perturbed respiratory cycle relative to a spontaneous cycle. Resetting characteristics for the neuron are visualized in a phase-shift plot, demonstrating stimulus phase-dependent resetting of the rhythm. Data pooled from experiments in three different animals. *A* and *B* are adapted from Ref. 102 with permission from the *Journal of Neurophysiology*.

Defining the Neuronal Elements of a Rhythm-Generating Network

Today, there is overwhelming evidence that CPGs exist for most, if not all, rhythmic motor behaviors in invertebrates and vertebrates. Yet, identifying the CPG for a rhythmic behavior is only a first step toward understanding how a neural rhythm is generated. Next is to identify the specific element(s) within the CPG that make it rhythmic. As first established in invertebrate model systems, two criteria must be met to define a neuron as an important element of a rhythm-generating network: *1*) a neuron or group of neurons must be active in phase with the rhythm and *2*) the neuron(s) need to reset the rhythm in a characteristic phase-dependent manner in response to a brief stimulus. Neurons that fulfill these two characteristics typically also modulate the frequency of the rhythm during a sustained stimulus and entrain the rhythm when stimulated repetitively. These criteria are illustrated for an anatomically identified neuron in the respiratory network of the locust, which is rhythmically active in phase with expiration (FIGURE 2A, *criterion 1*) and resets the rhythm when stimulated with a current pulse (FIGURE 2B, *criterion 2*). The resetting characteristics of these stimulations are visualized in a phase-shift curve that reveals a neuron's ability to advance or delay the rhythm depending on the stimulus phase, providing critical insights into a neuron's role in rhythmogenesis (102, 108).

Whether a given neuron, neuron group, or microcircuit is obligatory for rhythmicity is often also used as a criterion to identify rhythmogenic mechanisms. However, this can be a source of considerable confusion. Common pitfalls associated with this strategy are illustrated using a small invertebrate network, in which all neuronal elements of the CPG are well defined (FIGURE 3A) (76). The network diagram shown in FIGURE 3B illustrates the connectivity of the network that generates the pyloric rhythm within the stomatogastric ganglion (STG) of crustacean. Within this rhythmogenic network, two types of electrically coupled neurons, the AB and PD neurons, form the pacemaker kernel of this CPG (30, 44, 48). These neurons possess intrinsic bursting properties (83, 123) and are rhythmically active in phase with the pyloric rhythm, and stimulating these neurons resets the rhythm (48). Yet, when lesioning AB, PD, or other core neurons within this network, additional rhythmogenic mechanisms allow the pyloric rhythm to continue (FIGURE 3B) (48, 129). Thus

these neurons are not "obligatory" for rhythmogenesis, even though they are interconnected with all other rhythmogenic neurons and are important elements of the normal operation of this rhythm-generating network. These experiments have important general implications: although lesioning a neuron or neuron group can provide insight into its functional role (29, 30, 129), the persistence of a rhythm, i.e., the finding that a neuron or neuron group is not obligatory for rhythmicity, does not negate its important role in rhythmogenesis.

Conversely, there are many reasons why a neuron or neuron group may be obligatory for rhythmicity, but not rhythmogenic. For example, removing descending drive to the STG stops rhythmic activity produced by the pyloric network (FIGURE 3C). However, rhythmicity can be reestablished by tonic, non-rhythmic electrical stimulation of the descending input. Therefore, it is a tonic neuromodulatory influence rather than a rhythmic drive that makes this descending input obligatory (129). Indeed, the synaptic and intrinsic properties of the pyloric neurons are tuned such that their rhythmogenic properties are dependent on neuromodulation (143). Although rhythmicity ceases upon acute removal of neuromodulatory input, after several days, rhythmicity returns spontaneously in a neuromodulator-independent mode as mechanisms of homeostatic plasticity reestablish a balance of ionic and synaptic conductances (65, 73, 143) (FIGURE 3D). Indeed, it seems that rhythmogenic networks are more plastic and adaptable if their membrane properties are tuned to be neuromodulator-dependent. These

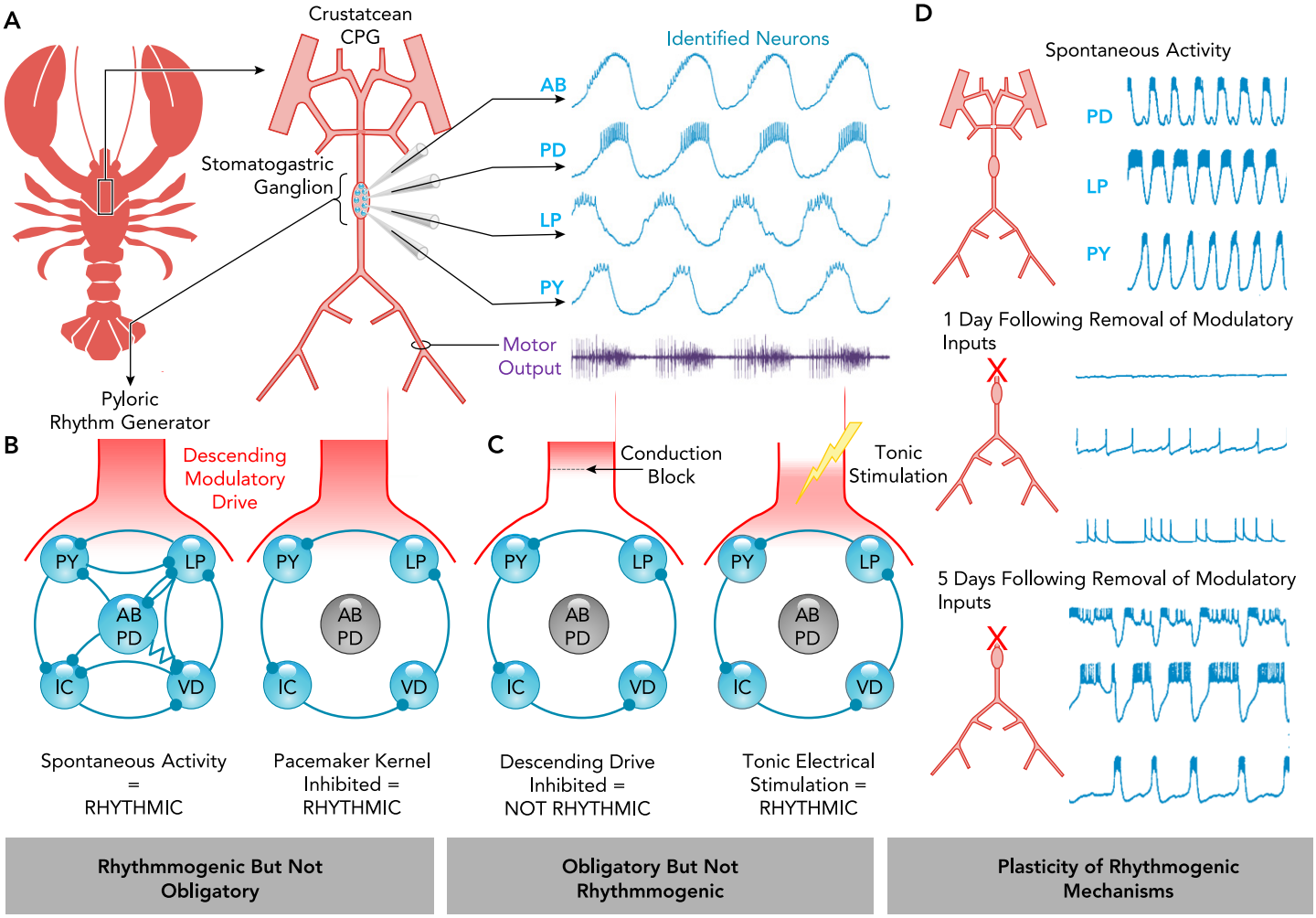

FIGURE 3. **The rhythmogenic role of an element may not be determined solely based on whether it is obligatory**
A: schematic of the crustacean stomatogastric nervous system and intracellular recordings of identified stomatogastric ganglion neurons of the pyloric rhythm generator (AB, anterior burster; PD, pyloric dilator; LP, lateral pyloric; PY, pyloric). The electrically coupled AB and PD neurons have autonomous bursting properties and comprise the "pacemaker kernel." These neurons are active in phase with rhythmic motor output (purple) and can also reset the rhythm. Adapted from Ref. 77, with permission from the *Annual Review of Physiology*. *B–C*: schematic of the pyloric rhythm-generating circuit. All synapses are inhibitory; resistor indicates electrical connections. *B*: in the presence of descending neuromodulatory drive, silencing the AB/PD pacemaker kernel does not abolish rhythmogenesis. *C*: subsequent removal of neuromodulatory drive eliminates the rhythm; however, it can be restored with artificial tonic stimulation (129). *D*: the extent to which a given mechanism is obligatory for rhythmogenesis may be altered by plasticity. Spontaneous rhythmicity of the pyloric rhythm generator is initially lost following removal of neuromodulatory inputs. However, rhythmicity returns as the rhythm-generating properties of the network adapt. Adapted from Ref. 143, with permission from the *Journal of Neurophysiology*.

considerations illustrate why being "obligatory" or "not obligatory" for rhythmogenesis cannot be unambiguously used to either identify or negate a neuron's role as a rhythmogenic element. Instead, for a neuron or neuron group to be considered rhythmogenic, it must be active in phase with the network rhythm and be able to reset the rhythm when stimulated.

Defining Rhythmogenic Elements in the Mammalian Respiratory Network

Similar to the crustacean model network discussed above, there are numerous neuromodulatory inputs to the rhythmogenic networks underlying mammalian breathing (25, 77). Based on the principles learned in invertebrates, removal of modulatory inputs should be expected to produce drastic changes in the breathing rhythm. Indeed, early lesion experiments that sectioned the brain stem at the mid-pontine level together with the transection of the vagus nerves caused "apneusis," prolonged inspiratory activity (71, 72, 98). These findings led to the concept of a "pneumotactic center" within the pons and contributed to the idea that the pons has an important role as an "inspiratory off-switch mechanism." However, such lesion experiments can only provide limited insights into the rhythmogenic role of the pons. Many early investigators were fully aware of these limitations. In von Euler's publication (155) on the potential existence of a pontine "off-switch" mechanism, he carefully stated, "it must be emphasized, however, that so far there is only correlational evidence for the proposed functions of any of these neurons." Yet, these concepts remain influential to this day, despite convincing studies demonstrating that the medulla can generate rhythmic breathing in the absence of the pons and sensory feedback (14, 46, 125). Although there is no doubt that the pons, including the Kölliker-Fuse region, plays an important role in the integration of sensory inputs and the modulation of the respiratory rhythm (26), it has yet to be convincingly demonstrated that the pons is a respiratory CPG, i.e., can be rhythmogenic when isolated. Moreover, evidence that selectively stimulating identified rhythmically active pontine neurons can reset the respiratory rhythm is, to the best of our knowledge, still missing. Thus we conclude that the pons provides important neuromodulatory inputs to the rhythm-generating neurons of the medulla, but whether the pons, or a region within the pons, plays a role in generating the breathing rhythm remains to be determined.

The mammalian breathing CPG, as identified within the medulla (FIGURE 4A), lacks the anatomical specificity of smaller invertebrate rhythm-generating networks that allow repeated identification of individual neurons with consistent functional roles (see FIGURES 2 AND 3). However, the criteria used to define an element of a rhythm-generating network may still be applied to specific subtypes of neurons in the mammalian medulla. Two landmark studies identified a specific class of excitatory, glutamatergic neurons within the preBötC (13, 38). These neurons are derived from precursors that express the transcription factor Dbx1. Many Dbx1-lineage neurons are rhythmically active in phase with inspiration (3, 45, 97) (FIGURE 4B), and optogenetic stimulation of these neurons resets the rhythm produced by the preBötC in isolated medullary slices in vitro, as well as the breathing rhythm produced by intact mice in vivo (9, 19, 149, 150) (FIGURE 4C). Thus these neurons fulfill the criteria indicative of elements of a rhythm-generating network. In addition to these principle criteria for rhythmogenesis, Dbx1 neurons also seem to be obligatory, because silencing these neurons in medullary slices (157, 158) or in adult mice (150) stops or significantly disrupts the breathing rhythm. Thus there is little doubt that Dbx1 preBötC neurons are core elements of the respiratory rhythm-generating network. It must be emphasized, however, that Dbx1 neurons are not a functionally homogenous population. Some Dbx1 neurons may play a more significant role in pattern vs. rhythm generation (or vice versa) (19, 117, 158), and some may have other non-respiratory functions such as modulation of arousal (163). Further identification and characterization of the specific subset of Dbx1 neurons that contribute to rhythmogenesis should be an important avenue of continued research.

The preBötC also contains many types of neurons that are not derived from Dbx1 precursors. Among preBötC neurons that are functionally active in phase with inspiration, an estimated 37% are inhibitory (9). These neurons, characterized by the vesicular GABA transporter (Vgat), are activated concurrently with Dbx1 neurons (FIGURE 4B), and optogenetic stimulations of these neurons elicit a stimulus phase-dependent reset of the breathing rhythm both in vitro and in vivo (FIGURE 4C) (9, 130). Thus inhibitory preBötC neurons, like Dbx1 neurons, fulfill the principle criteria that define a neuron as being an element of a rhythm-generating network.

Excitatory and inhibitory neurons are both important for controlling rhythmicity in the preBötC, and their interactions have been explored in modeling studies (43). However, these neuronal populations have different functional roles with distinct resetting characteristics (FIGURE 4C). Interestingly,

during the inspiratory phase, concurrent with preBötC population bursts, stimulation of excitatory Dbx1 neurons delays the onset of the subsequent breath. In contrast, the equivalent stimulation of inhibitory Vgat neurons advances the next breath (9). During the expiratory phase, between preBötC population bursts, stimulation of Dbx1 neurons can advance the subsequent breath. However, as will be discussed in detail below, this effect is limited by refractory properties of Dbx1 neurons. Conversely, stimulation of inhibitory neurons during the expiratory phase has distinct effects depending on the experimental preparation: in vivo, the next breath is delayed (9, 130), and during the much slower frequencies generated in vitro, post-inhibitory rebound advances the next breath (9, 17). These experiments serve as an important general reminder that, unless specific neurons are selectively stimulated during their active phase, it is difficult to determine whether and how they contribute to rhythmogenesis. Furthermore, since it has been repeatedly demonstrated that blockade of synaptic inhibition may alter, but does not stop, the preBötC rhythm (54, 166), these experiments further illustrate how a given element may play an important role in rhythmogenesis without being obligatory.

The Relationship Between Synchronization and the Duration of a Rhythmic Cycle

Mechanisms of Refractoriness

Like other rhythmic systems (64, 74, 126, 138, 162, 167), the preBötC has an inherent refractoriness

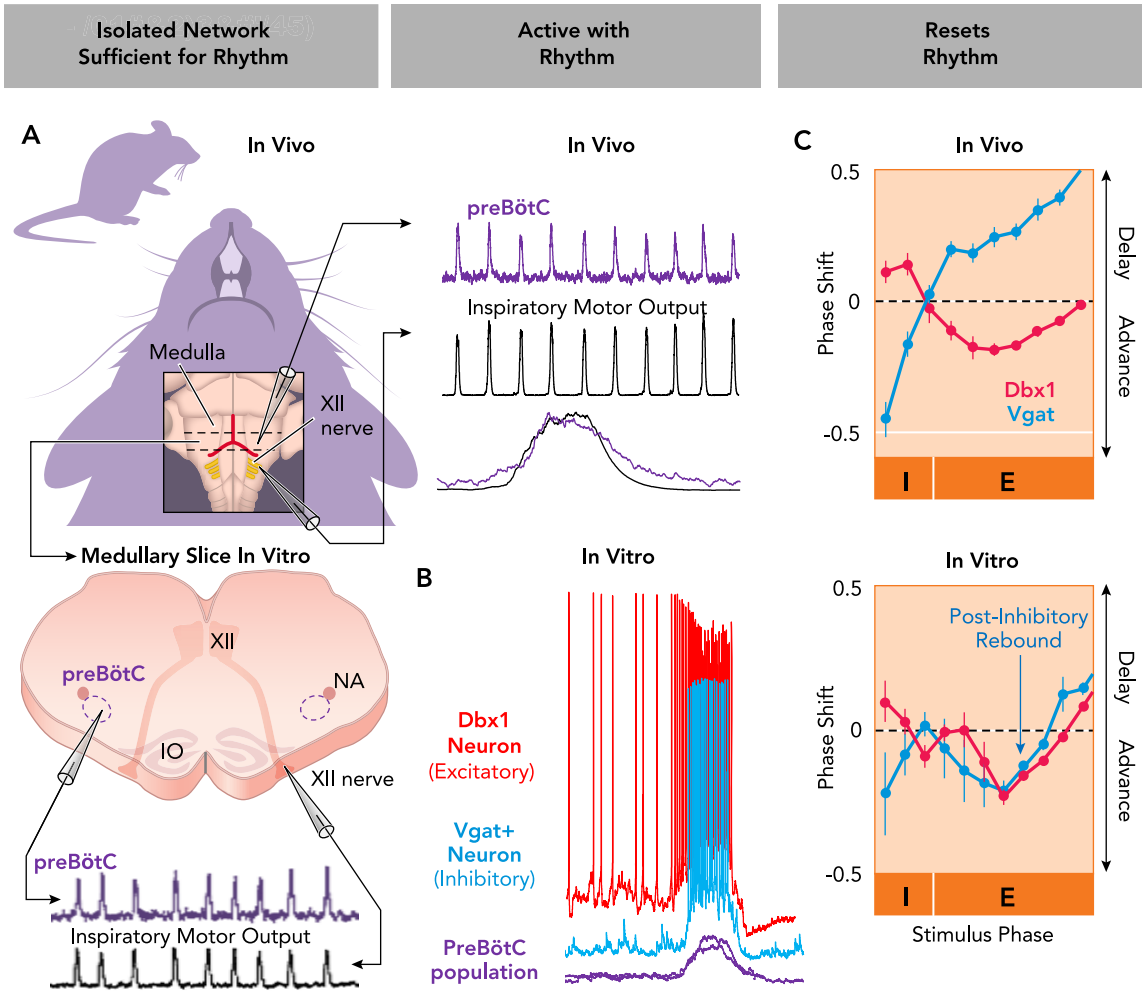

FIGURE 4. **Rhythmogenic roles of excitatory and inhibitory neurons in the mammalian breathing CPG**
A: the pre-Bötzinger Complex (preBötC) continues to produce a rhythm (purple) that is in phase with inspiratory hypoglossal (XII) motor output (black) when isolated in medullary slices from neonatal mice. The preBötC is also active in phase with inspiratory motor activity in intact adult mice. *B*: excitatory (red) and inhibitory (blue) preBötC neurons are concurrently active in phase with rhythmic inspiratory activity (purple). Intracellular current-clamp recordings of genetically identified Dbx1+ (excitatory) and Vgat+ (inhibitory) neurons active with integrated extracellular preBötC population bursts generated in vitro. *C*: optogenetic stimulation of excitatory (Dbx1+) or inhibitory (Vgat+) preBötC neurons resets the breathing rhythm in vivo and in vitro. Stimulations can advance or delay the next inspiratory cycle depending on stimulus phase (I, inspiratory phase; E, expiratory phase). Note that postinhibitory rebound advances the next cycle during relatively slow rhythms in vitro but not in vivo. Figure adapted from Ref. 9, with permission from *Nature Communications*.

(4). During rhythmogenesis, recurrently connected excitatory Dbx1 neurons (and non-Dbx1 neurons) periodically synchronize and desynchronize within the preBötC. Following a synchronized population "burst," during the early expiratory phase, stimulation of Dbx1 neurons typically fails to evoke another burst, whereas stimulation during late expiration almost always evokes a burst (FIGURE 5A) (9, 62). This refractory period following synchronized preBötC activity is determined by at least two mechanisms (FIGURE 5B). *1*) High firing rates during each inspiratory burst deplete presynaptic vesicles, limiting synaptic transmission between excitatory neurons until the vesicle pool is restored (40). This has been observed in vitro as a progressive reduction in evoked EPSP amplitude recoded in Dbx1 neurons during repeated electrical stimulation of excitatory presynaptic inputs (62). *2*) The depolarizing "drive potential" during each inspiratory burst activates intrinsic membrane properties that cause a transient hyperpolarization and reduced excitability of excitatory neurons until resting membrane potential is restored. In Dbx1 neurons following blockade of synaptic transmission, a depolarizing current step is followed by afterhyperpolarization and reduced spiking evoked during smaller current pulses (9). Identifying the specific ionic conductance(s) underlying this intrinsic membrane property should be an important next step in unravelling the mechanisms of refractoriness in the preBötC.

The extent to which refractory mechanisms influence rhythmogenesis is determined by the amount of synchronized activity during each preBötC population burst. This is illustrated for different patterns of preBötC activity in FIGURE 5C. During normal "eupneic" bursts (106, 147), recur-

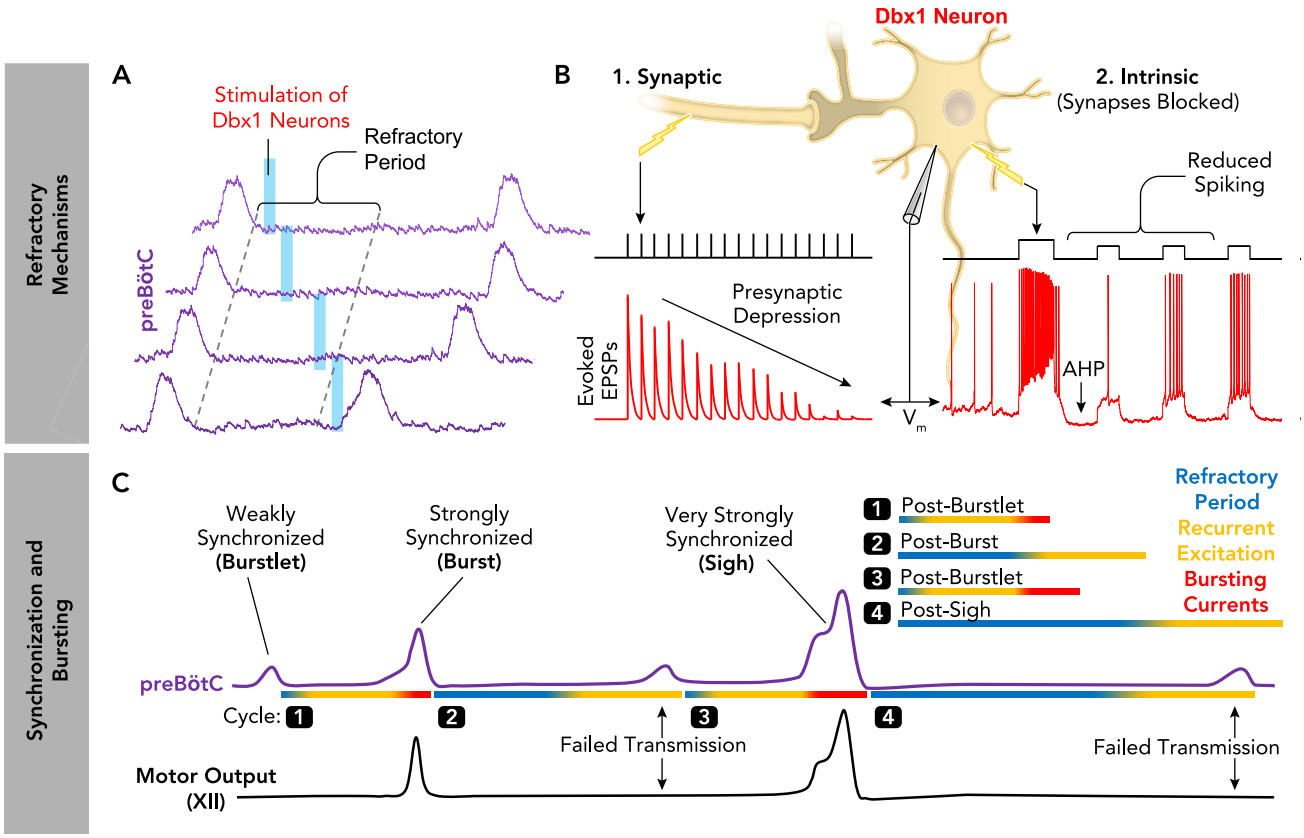

FIGURE 5. **The relationship between synchronization, bursting, and refractory mechanisms in the preBötC**
A: the preBötC has an inherent refractoriness. The probability of evoking preBötC population bursts by optogenetic stimulation of Dbx1 neurons is transiently reduced following spontaneous preBötC bursts, i.e., during the refractory period (9) *B*: synaptic and intrinsic properties of excitatory Dbx1+ neurons contribute to the refractory period (*1*). Schematic demonstrating presynaptic depression of excitatory synaptic transmission between preBötC neurons. The amplitude of evoked excitatory postsynaptic potentials (EPSPs; red) recorded in Dbx1 neurons is progressively reduced during repeated electrical stimulations (black) of presynaptic inputs (62). *2*: intrinsic properties transiently reduce the excitability of Dbx1 neurons following preBötC bursts. Intracellular current-clamp recording of a synaptically isolated Dbx1 neuron demonstrating membrane afterhyperpolarization (AHP) and transiently reduced spiking following an initial, larger depolarizing burst (9). *C*: schematic representing the different amounts of synchronization during distinct patterns of preBötC activity (purple) and associated hypoglossal (XII) motor output (black). During burstlets, synchronization is weak, bursting currents are only activated in some neurons, and the refractory period is minimal. During normal "eupneic" bursts, synchronization is strong and bursting currents are activated in many neurons, resulting in a period of relative refractoriness. During periodic sigh bursts, synchronization is very strong and bursting currents are strongly activated, leading to large depolarizing drive potentials in preBötC neurons and an exaggerated refractory period. Note that strong synchronization facilitated by activation of bursting currents promotes successful transmission of preBötC activity to XII motor output.

rent synaptic excitation and intrinsic bursting currents (discussed in detail below) strongly synchronize preBötC neurons, leading to a refractory period. As neurons recover from the refractory period, recurrent excitation can begin to initiate another cycle. During much weaker synchronizations, referred to as "burstlets" (56), low spiking rates and small drive potentials in excitatory neurons are expected to elicit a relatively short refractory period. The opposite is expected during very strongly synchronized bi-phasic sigh bursts generated by the preBötC (147), when long refractory periods likely result in a "post-sigh apnea" (9). These considerations illustrate how rhythm generation (i.e., cycle period) and pattern generation (i.e., burst amplitude) can be interdependent processes (see FIGURE 1E).

Intrinsic Bursting

An important mechanism that promotes synchronization is the activity of intrinsic bursting properties, sometimes also referred to as "pacemaker properties." First described in small invertebrate neuronal networks (see, e.g., Refs. 5, 122), intrinsic bursting is a fundamental feature of most, if not all, neuronal networks (8, 21, 69, 70, 114, 142, 146, 148). Although, these intrinsic bursting properties allow neurons to generate bursts of action potentials in the absence of synaptic input, this is rarely the case in a functional network where neurons are bombarded by excitatory and inhibitory synaptic inputs. In this context, bursting neurons play a critical role as nonlinear amplifiers of synaptic drive (107). In the preBötC, it is thought that intrinsic bursting properties mediate the transition of weakly synchronized burstlets into normal inspiratory bursts (111) (FIGURE 5C). The transition to the burst also depends on the interplay between concurrent excitation and inhibition, which will vary from cycle to cycle for each individual neuron. If inhibition is predominant or excitability is low, the bursting threshold may not be reached in many neurons, which can lead to a burstlet at the population level (56, 57, 110, 111). We refer to this balance between excitation, inhibition, and intrinsic bursting as the rhythmogenic triangle (FIGURE 6): the onset and synchronization of a rhythmic cycle, as well as the resulting refractory period, will depend on the close interdependence between these three principle rhythmogenic mechanisms (103).

Intrinsic bursting can be mediated by a variety of voltage- or calcium-dependent mechanisms. In the preBötC, the persistent sodium current (I_{NaP}) is an important voltage-dependent bursting mechanism, whereas the calcium-activated nonselective cation current (I_{CAN}) is largely voltage-independent, but dependent on the intracellular calcium concentration (10, 18, 45, 94). Neuromodulators (e.g., norepinephrine, serotonin, and substance P) can differentially modulate I_{NaP}- and I_{CAN}-dependent bursting properties, allowing the network to amplify synaptic inputs in a voltage- and calcium-dependent manner (94–96, 151–153). Whether these bursting properties can be homeostatically adjusted, similar to bursting in the pyloric rhythm generator of the crustacean (see FIGURE 3C), remains unknown. Nevertheless, the ability to differentially modulate bursting characteristics among subsets of preBötC neurons is likely one mechanism that contributes to the impressive flexibility of the breathing rhythm.

Integrated Local Inhibition

Synaptic inhibition plays a critical role in regulating synchronization (43). Bursting properties of preBötC neurons are suppressed by inhibition (148), and in the absence of synaptic inhibition, Dbx1 neurons become hyperactive and synchronize more efficiently. However, this results in long refractory periods and, in vivo, such slow breathing frequencies are not physiologically sustainable. Conversely, increasing the activity of inhibitory neurons during inspiration weakens synchronization, which dramatically accelerates breathing (9). Indeed, modulation of refractory mechanisms can explain the phase-dependent resetting characteristics of inhibitory neurons (see FIGURE 4C). Activation during inspiration weakens synchronization of the inspiratory network and reduces the refractory period, whereas activation of inhibitory neurons during expiration slows breathing, presumably by disrupting the propagation of excitatory activity through the network. Thus, by regulating the synchronization of excitatory neurons, the activation of bursting properties, and the subsequent refractory period, inhibitory neurons exert a powerful influence on breathing frequency. However, there must be a precise balance of excitation and inhibition in the preBötC, as indicated by computational models (43): too much inhibition integrated within the network desynchronizes and irregularizes the rhythm until it eventually falls apart. Future modeling studies should be useful to better understand how the balance between excitation and inhibition controls refractory mechanisms, cycle duration, and the stability of the preBötC rhythm.

Network Connectivity

As mentioned previously, the preBötC lacks the same level of anatomical specificity available in smaller invertebrate rhythm-generating networks (see FIGURES 2 AND 3). As a result, we are far from having established a detailed connectome of the rhythmogenic network that gives rise to mammalian breathing. Moreover, it is unknown to what

extent network connectivity is unique among individuals and whether connectivity may be altered throughout life by synaptic plasticity. However, a functionally important property of the preBötC seems to be sparsity. Combined modeling and experimental studies suggest that sparse connectivity contributes to the high degree of stochasticity and onset variability that is observed in the activity of individual preBötC neurons in medullary slices (15, 16), but also among neurons recorded in vivo (68, 87, 127). These computational models predict that networks with sparse connectivity can have weakly synchronized cycles (i.e., burstlets), and that close to 300 excitatory neurons need to synchronize to maintain network activity with regular burst frequency and amplitude (15, 16). These modeling studies also predict that connectivity within the preBötC is tuned near the limit of network stability, which may endow the network with a great deal of flexibility, but in some cases may make it vulnerable to pathology. For example, the preBötC rhythm becomes irregular, and weak synchronizations become more common in neonatal mice chronically exposed to intermittent episodes of hypoxia (35, 36). These weak burstlets generated in the preBötC fail to fully activate hypoglossal (XII) motoneurons, leading to breathing cycles without XII motor output (110) (FIGURE 5C). This finding has important clinical implications in the context of obstructive sleep apnea, where patients experience chronic intermittent hypoxia and have reduced inspiratory drive to muscles of the upper airway (innervated by the XII nerve), making it prone to collapse (105).

Integrated Rhythmogenesis

Overall, existing data suggest that respiratory rhythmogenesis is not the deterministic result of a single homogenous mechanism. However, history has shown that semantics can cause widespread confusion, and invertebrate networks can once again serve as an important reminder. In the STG, a "pacemaker kernel," consisting of the AB-PD bursting neurons, is important for rhythmogenesis. This may imply that bursting properties of these two cells are the primary determinants of rhythmogenesis. Yet, many characteristics of bursting

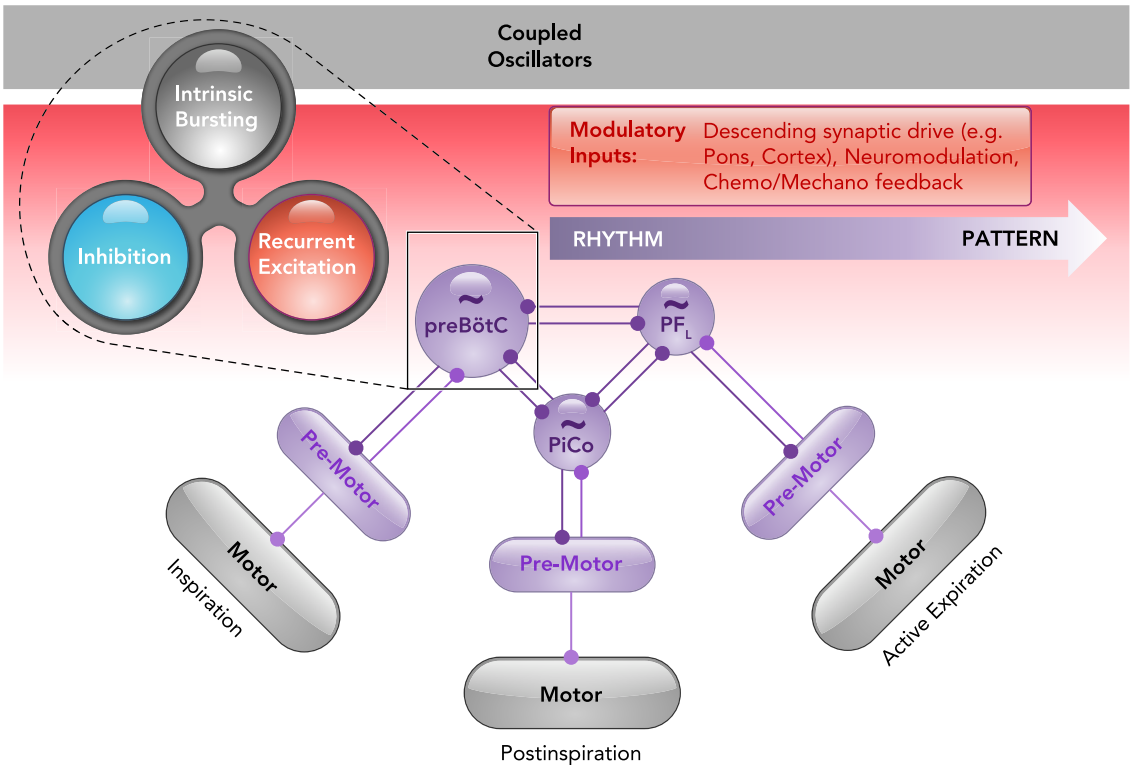

FIGURE 6. **A contemporary view on the origins of mammalian breathing**
The triple oscillator hypothesis (6) proposes that each breathing phase is generated by a distinct microcircuit in the medulla: the preBötzinger complex (preBötC) generates inspiration, the postinspiratory complex (PiCo) generates postinspiration, and the lateral parafacial region (pF$_L$) generates active expiration. Each oscillator is coupled by excitatory and inhibitory connections, although inhibition typically dominates to coordinate the timing of each breathing phase. As shown in the preBötC, rhythmicity within each microcircuit is controlled by a balance between recurrent synaptic excitation, inhibition, and intrinsic bursting properties, i.e., the rhythmogenic triangle (103). A gradient of rhythm (purple) and pattern (gray) generating properties can be independent or interdependent depending on the connectivity between specific rhythm, premotor, and motor elements. The activity of rhythm- and pattern-generating elements can be differentially tuned by various modulatory inputs (red) to endow breathing with exquisite metabolic-, state-, and behavior-dependent control.

neurons and their temporal activation depend on the dynamic integration of multiple neuromodulatory and synaptic mechanisms as well as the interaction with other rhythmogenic neurons of the STG network (see FIGURE 3A). Thus the desire to identify and attribute rhythmogenesis to one essential rhythmogenic mechanism may not reflect how a network actually operates. A popular hypothesis for respiratory rhythmogenesis, the so called "group pacemaker hypothesis," posits that periodic inspiratory bursts originate from intrinsic currents that are ordinarily latent until synaptically evoked in the context of network function (116). This hypothesis describes the integration between intrinsic and synaptic mechanisms, but by emphasizing the synaptic mechanisms that are necessary to activate the "ordinarily latent, intrinsic" properties, it infers that excitatory synaptic transmission is the primary rhythmogenic mechanism (23, 24, 121). However, we suggest that assigning a primary rhythmogenic mechanism can be misleading. Instead, the emphasis should be on the dynamic integration between synaptic excitation, inhibition, and intrinsic bursting, which assembles the respiratory rhythm in a cycle-to-cycle manner. Any given cycle may occur with or without bursting properties (103) and also with or without synaptic inhibition (e.g., in gasping). But it is the dynamic interplay between heterogeneous mechanisms that imbues rhythmogenesis with the ability to support breathing during the many physiological and pathophysiological conditions that may occur throughout life.

Coupled Oscillators and the Multiphase Breathing Rhythm

Thus far we have focused on mechanisms that govern the inspiratory rhythm generated by the preBötC. However, despite being a core rhythmogenic microcircuit, the preBötC is just one component in a wider network; and mammalian breathing does not only consist of inspiration but also postinspiration and active expiration (118, 119). Coordination of the three breathing phases requires a different role for synaptic inhibition. Instead of controlling synchronization as discussed above, synaptic inhibition needs to control the temporal sequence of each breathing phase (119). However, consistent with the compartmental model (124, 131–133), computational network models predict that inhibition cannot generate multiple phases within the preBötC alone (43). Specifically, to generate neurons that are inhibited during inspiration and active during expiration, the fraction of inhibitory neurons within the network needs to be increased. Yet, increasing inhibition desynchronizes network activity, and only a small number of neurons (maximally ~15%) can be forced to fire during the expiratory phase before rhythmicity falls apart (43). Indeed, experimental data indicate that only a small fraction of preBötC neurons are active out of phase with inspiration (15, 16, 131). Thus the computational model makes an important prediction: to produce a multiphase rhythm, multiple independently rhythmic networks must be coupled by synaptic inhibition. Such networks have been identified in the medulla as the postinspiratory complex (PiCo), active during postinspiration (6, 7), and the lateral parafacial nucleus (pF_L), a subdivision of the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) region that is recruited during active expiration (49, 50).

The PiCo, located rostral to the preBötC, continues to produce a rhythm when isolated in a medullary slice, contains neurons that are rhythmically active in phase with postinspiration, and stimulation of these neurons resets the rhythm in vitro and in vivo (6). Thus the PiCo fulfills all criteria that define a rhythmogenic network or CPG. PiCo neurons (or at least a subset of them) are characterized by a glutamatergic, cholinergic transmitter phenotype. Cholinergic signaling seems to have primarily modulatory functions, whereas, like the preBötC, glutamatergic transmission is required for synchronization of the network. GABAergic inhibition establishes the phase relationship between the PiCo and the preBötC; however, to what extent inhibition also regulates synchronization and refractory mechanisms within the PiCo is still unknown. Further characterization of the rhythmogenic elements within PiCo and their role in the generation of postinspiration and potentially other behaviors, such as vocalization, will be critical to gain further insights into the functional implications of this medullary microcircuit.

The RTN/pFRG is located rostral to the preBötC and ventral to the PiCo (49, 50, 52). This region has been subdivided into rhythmic lateral (pF_L) and non-rhythmic ventral (pF_V) areas (49). However, the degree to which these areas are distinct is a matter of debate. The pF_L is typically silent but becomes rhythmic when disinhibited (92). In adult rodents, rhythmicity in the pF_L depends on (presumably tonic) excitation from the preBötC (50). However, in younger rodents, the pFRG region can generate a rhythm without rhythmic preBötC activity (52, 139), even when physically isolated from the preBötC (91). In vivo, neurons in the pF_L fire during active expiration, and stimulation of the pF_L evokes expiratory activity and resets the breathing rhythm (92), presumably through inhibitory interactions with the preBötC (49). Neurons that give rise to rhythmicity within the pF_L may

(90) or may not (92) include neurons that express Phox2b. However, like Dbx1 neurons in the preBotC, neurons within the pF_L and parafacial region in general are heterogeneous, and unraveling exactly which neuronal subtypes are responsible for rhythmogenesis, chemosensation, and blood pressure regulation is an important research avenue (41, 42, 90, 91).

As illustrated in the invertebrate STG network (102), CPGs are often tuned to neuromodulation to allow precise control of rhythmic behavior. In this context, it is interesting that the three coupled oscillators for breathing are differentially tuned with regard to their sensitivity to neuromodulators. For example, the PiCo is strongly stimulated by norepinephrine and inhibited by somatostatin and the μ-opioid agonist DAMGO (6). In contrast, the pF_L is strongly activated by acetylcholine (12) but is insensitive to opioids (52, 80). The enhanced sensitivity of the PiCo and pF_L to neuromodulation may contribute to their higher threshold for rhythmicity, which is likely an important property allowing these networks to be rhythmic only during the appropriate metabolic, environmental, or behavioral context. The preBötC is also regulated by a host of neuromodulators (25). However, the preBötC has a relatively low threshold for rhythmicity and less dependence on neuromodulatory input, reflecting its role as the "master clock" for breathing-related behaviors (86) and the paramount importance of the inspiratory phase in mammalian breathing.

Conclusions and Open Questions

A contemporary view on the origins of mammalian breathing is conceptualized in FIGURE 6. The organization of the breathing network is consistent with the idea that rhythmic activity emerges from the interactions between three coupled oscillators, in which each phase, inspiration, postinspiration, and active expiration, is generated within the medulla by its own dedicated microcircuit, referred to as the "triple oscillator hypothesis" (7). The dynamic balance between recurrent synaptic excitation, inhibition, and intrinsic bursting properties, i.e., the "rhythmogenic triangle" (103), controls synchronization within each microcircuit, although this concept has yet to be tested in the PiCo and pF_L. Mutually inhibitory interactions between the microcircuits seem to control the temporal sequence of the rhythm generated by the network. However, interactions between the PiCo and pF_L have not been described. Rhythmogenic microcircuits project to premotor and motor pools, and the connectivity of a given element will determine its role in rhythm generation, pattern generation, or both. The complex integration of the network with the rest of the central and peripheral nervous system allows exquisite modulation of all rhythm- and pattern-generating elements. This complex organization may have evolved in mammals to match their increased metabolic and behavioral demands on breathing (104).

An important next step in understanding the breathing rhythm will be to examine in further detail how the three rhythmogenic microcircuits interact. Indeed, the triple oscillator hypothesis and the concept of the rhythmogenic triangle raise many unresolved questions: How is the so-called Bötzinger complex (124, 131, 132) integrated with the three rhythmogenic microcircuits? Are there separate sets of inhibitory neurons dedicated to controlling synchronization within the microcircuits and temporal control between them? How dynamic are these interactions? Do burstlets, bursts, and sighs generated by the preBötC differentially activate or inhibit the other rhythmogenic microcircuits? A sigh associated with arousal may be differentially connected to the expiratory networks than a sigh of relief. Do hypoxic and hypercapnic conditions differentially alter and reconfigure these microcircuits? How do the roles of rhythmogenic elements within and the interactions between each microcircuit change during the variety of non-ventilatory behaviors, such as coughing and swallowing, that are known to involve reconfiguration of the network (11, 67, 99, 128)? As discussed here for the preBötC, it will be important to unravel to what extent the neurons active during non-ventilatory behaviors can be identified as belonging to multiple CPGs. Specifically, do neurons that reset breathing also reset other rhythmic behaviors like swallowing and coughing? The discovery of more functionally specific molecular and genetic markers should allow exploration of these possibilities. Along the way, principles learned from invertebrate networks can continue to provide guidance in unraveling the reconfiguration and control of different, yet partially overlapping, rhythmic behaviors (102, 108, 109, 159).

Many additional open questions relate to the supramedullary control of breathing and the functional integration of the breathing rhythm with higher brain regions. It is well known that breathing is under cognitive and emotional control, likely involving descending control from regions such as the parabrachial nucleus, the hypothalamus, the periaqueductal gray, various limbic regions, and the prefrontal and anterior cingulate cortex (20, 26, 28, 47, 58, 84, 89, 136, 165). All of these regions are likely connected with the rhythmogenic microcircuits in the medulla to regulate breathing; and this detailed connectivity is beginning to be worked out (164). There are also important ascending influences of the breathing rhythm that may contribute

to the function of supramedullary regions. Activity in the prefrontal cortex, the locus coeruleus, hippocampus, and olfactory bulb can all be modulated by the breathing rhythm (55, 59, 61, 81, 85, 88). Indeed, breathing has been described as a global timing mechanism in the brain (144, 145). Thus, in addition to its critical role in regulating blood gasses, breathing likely also plays an important role in regulating higher brain functions and emotions. Yet, how the rhythmogenic microcircuits in the medulla are connected with supramedullary regions and the functional implications of this ascending integration are only beginning to be revealed (163). Perhaps one day we will understand the neuronal mechanisms of why we sigh when we are sad, in love, or relieved (101); why we sometimes feel "inspired"; and why panic attacks and fear can be controlled with breathing (60, 82, 154). Although it has taken almost a century to unravel the basic medullary circuitry underlying generation of the mammalian breathing rhythm, we are in an exciting time when powerful new experimental approaches promise rapid progress toward understanding these important unanswered questions. ■

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