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The Dynamic Basis of Respiratory Rhythm Generation: One Breath at a Time

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Abstract

Rhythmicity is a universal timing mechanism in the brain, and the rhythmogenic mechanisms are generally dynamic. This is illustrated for the neuronal control of breathing, a behavior that occurs as a one-, two-, or three-phase rhythm. Each breath is assembled stochastically, and increasing evidence suggests that each phase can be generated independently by a dedicated excitatory microcircuit. Within each microcircuit, rhythmicity emerges through three entangled mechanisms: (*a*) glutamatergic transmission, which is amplified by (*b*) intrinsic bursting and opposed by (*c*) concurrent inhibition. This rhythmogenic triangle is dynamically tuned by neuromodulators and other network interactions. The ability of coupled oscillators to reconfigure and recombine may allow breathing to remain robust yet plastic enough to conform to nonventilatory behaviors such as vocalization, swallowing, and coughing. Lessons learned from the respiratory network may translate to other highly dynamic and integrated rhythmic systems, if approached one breath at a time.

Keywords

breathing; microcircuits; coupled oscillators; rhythm generation; excitation/inhibition balance; synchronization

INTRODUCTION

Understanding how the brain generates behavior requires knowledge and integration of the underlying processes at the genetic, molecular, cellular, and network levels. Some behavioral models in invertebrates and vertebrates are particularly amenable to a rigorous analysis of these processes. For example, breathing in mammals can be considered a simple rhythmic behavior, whereby respiratory muscles move air into and out of the lungs. However, such focus on the stereotypic nature of breathing largely ignored that breathing is remarkably complex and dynamic. Breathing can occur in the form of a single, bi-, or triphasic rhythm, and it is multifunctional. Eupneic breathing, gasping, and sighing as well as vocalizations are functionally distinct breathing behaviors (Hernandez-Miranda et al. 2017, Ramirez 2014,

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Subramanian et al. 2016). Moreover, breathing serves as a timing mechanism for behaviors such as sniffing (Rojas-Libano et al. 2014, Tsanov et al. 2014) and whisking (Deschenes et al. 2016, Kleinfeld et al. 2014) and is highly integrated with other functions of the central nervous system (CNS). Respiratory-related oscillations are found in the olfactory bulb (Jessberger et al. 2016, Kay & Lazzara 2010), locus coeruleus (Ballantyne et al. 2004), hippocampus (Dubois et al. 2016, Lockmann et al. 2016, Nguyen Chi et al. 2016), insula (Radna & MacLean 1981), amygdala (Masaoka et al. 2014, Zelano et al. 2016), and neo-cortex (Zhong et al. 2017). The complexity and dynamic nature of breathing are also reflected in its underlying neuronal mechanisms (Feldman et al. 2013, Ramirez et al. 2012). As discussed here, breathing is assembled in a state-dependent and stochastic manner through interactions within and between different rhythmogenic microcircuits (Anderson & Ramirez 2017, Ramirez et al. 2016). These interactions depend on the evolutionary and developmental contexts of the organism, as well as the momentary metabolic, environmental, and behavioral demands.

Thus, although breathing was initially touted as a model for studying simple behaviors, there is no such thing as simple behavior (Hamood & Marder 2014). The cellular repertoire and connectivity that give rise to a single breath must be sufficiently complex to allow an opera singer to turn a breath into an emotionally packed operatic experience. In this review we focus on breathing as a model to unravel the dynamic complexity of brain rhythmicity, which constitutes a universal entity essential for synchronizing and coordinating neuronal activities and behaviors such as rhythmic motor behaviors (Katz 2016), sensory-motor processing (McCormick et al. 2015), learning, memory consolidation, and other higher brain functions (Buzsáki & Moser 2013,Buzsáki & Schomburg 2015, Cheron et al. 2016, Mendoza & Merchant 2014). Therefore, unraveling the mechanisms of breathing rhythmicity can reveal important generalizable insights into how the nervous system processes and integrates complex behavioral information.

BREATHING: THE DYNAMIC ASSEMBLY OF THREE DISTINCT PHASE MODULES

The process of breathing can be divided into three dynamically regulated phases (Richter & Smith 2014). In humans, but not all vertebrates (Jenkin & Milsom 2014), a breath begins by drawing air into the lungs during a phase called inspiration. To prolong gas exchange, inspiration is typically followed by postinspiration, an expiratory phase that slows the release of air through the activation of upper airway muscles (Dutschmann et al. 2014). Postinspiration is an active process, which is most evident during singing, when muscles can be rhythmically activated multiple times during the postinspiratory phase of a single respiratory cycle (Watson et al. 2012) (Figure 1*a*). This suggests that postinspiration can be independently rhythmic and not strictly dependent on a preceding inspiration. Under increased metabolic demand a third phase, active expiration, is recruited. During this phase, activation of internal intercostal and abdominal muscles forcefully expels air from the lungs. In some vertebrates the respiratory cycle can begin with active expiration (Jenkin et al. 2017), and neonatal rats can generate cycles of active expiration even when inspiration is

suppressed by opioids (Janczewski & Feldman 2006). Thus, evidence suggests that all breathing phases can function independently.

Breathing phases can also reconfigure and synchronize to generate a single bi- or triphasic rhythm (Figure 1*b*). Postinspiratory activity joins inspiration during gasping (Ramirez et al. 1998a, St-John 1998) and active expiration during coughing (Shannon et al. 2000). Moreover, there can be considerable stochasticity in breath-to-breath waveforms (Figure 1*c*). Thus, breathing is composed of three independent phase modules that are assembled and reconfigured in a cycle-to-cycle manner to orchestrate a behavior that can switch between one-, two-, and three-phase rhythms.

DEFINING THE SUBREGIONS OF THE VENTRAL RESPIRATORY COLUMN

The core circuits for respiratory rhythm generation are distributed along the medullary ventral respiratory column (VRC) (Figure 2) and often described in terms of functionally and chemically defined subregions (Alheid & McCrimmon 2008). These subregions include (from rostral to caudal) the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) (Guyenet et al. 2009, Huckstepp et al. 2015, Pearce et al. 1989), postinspiratory complex (PiCo) (Anderson et al. 2016), Bötzinger complex (BötC) (Schreihofer et al. 1999), preBötzinger complex (preBötC) (Schwarzacher et al. 2011, Smith et al. 1991), rostral ventral respiratory group, and caudal ventral respiratory group.

Some subdivisions of the VRC have also been included in the so-called compartmental model for respiratory rhythm generation (Smith et al. 2007, 2009, 2013). In this model, rhythmogenesis emerges through inhibitory interactions and the interdependence between three phases. For example, postinspiratory activity switches off inspiratory activity, and inhibitory rebound turns on postinspiratory activity. This presumed interdependence is not necessarily consistent with the behavior in which each respiratory phase can apparently function independently under certain conditions, or with recordings in vivo that show remarkable cycle-to-cycle variability specifically in postinspiratory neuronal activity (Orem & Trotter 1992). The compartmental model is also challenged by the repeated demonstration that neither rhythmogenesis, burst initiation, nor termination is abolished after blocking synaptic inhibition (Janczewski et al. 2013, Ramirez & Viemari 2005, Ramirez et al. 1996, Shao & Feldman 1997, Zanella et al. 2014). However, blocking synaptic inhibition is not the ideal approach for testing rhythmogenic inhibitory mechanisms (Marchenko et al. 2016), because a redundant network may simply switch from an inhibition-dependent to an inhibition-independent mode after inhibition is lost.

An alternative model, the triple oscillator hypothesis, proposes that each breathing phase is generated independently by excitatory microcircuits located in the preBötC, RTN/pFRG, and PiCo (Anderson & Ramirez 2017, Anderson et al. 2016, Huckstepp et al. 2016, Janczewski & Feldman 2006, Smith et al. 1991, Tan et al. 2008). Silencing the preBotC, PiCo, and lateral pFRG eliminates inspiration, postinspiration, and active expiration, respectively (Anderson et al. 2016, Silva et al. 2016, Tan et al. 2008). Each of these microcircuits can generate rhythmicity dependent on glutamatergic interactions (Anderson et al. 2016, Chen et al. 2013, Smith et al. 1991). In the preBötC, the critical glutamatergic neurons are

characterized by the homeobox gene *Dbx1* (Bouvier et al. 2010, Gray et al. 2010, Picardo et al. 2013) (Figure 2*a*), and the PiCo is characterized by glutamatergic-cholinergic neurons (Anderson et al. 2016) (Figure 2*b*). Neurons within RTN/pFRG regions are associated with the transcription factor Phox2b (Figure 2*c*), but it is uncertain which of these neurons are rhythmogenic, chemosensitive, or both (Onimaru et al. 2009, Pagliardini et al. 2011, Silva et al. 2016).

It would be an oversimplification to imply that these microcircuits have well-defined borders. The preBötC contains several overlapping subpopulations of glutamatergic neurons (Cui et al. 2016; Feldman & Kam 2015; Gray et al. 2001; Hayes et al. 2017; Tan et al. 2008, 2012; Yackle et al. 2017), and no one marker exclusively defines the preBötC (Feldman & Kam 2015). For example, Dbx1 neurons extend rostrally and caudally beyond the presumed preBötC (Gray et al.2010), and there is also a gradient from the more ventrally located rhythmogenic preBötC to the more dorsally located hypoglossal premotor Dbx1 neurons (Revill et al. 2015, Wang et al. 2014) (Figure 2*a*). Thus, neurons may gradually transition from more rhythmogenic to less rhythmogenic at its boundaries. Moreover, neurons located outside the presumed preBötC may become rhythmogenic under certain conditions, which is supported by several findings: Acute preBötC lesions abolish eupneic activity (Ramirez et al. 1998b, Tan et al. 2008, Wenninger et al. 2004) but not gasping (Ramirez et al. 1998b), eupneic breathing can recover if the preBötC is lesioned slowly (Forster et al. 2014, Krause et al. 2009), and the effect of lesioning the preBötC is dependent on the sleep state (McKay & Feldman 2008). Thus, the borders of the preBötC may be dynamically regulated in a behavior- and state-dependent fashion. These considerations suggest that the concept of discrete microcircuits is only a temporary framework that will evolve as we gain more detailed insights into their composition, boundaries, and dynamic regulation.

THE TRIANGLE OF RHYTHMOGENESIS

Although glutamatergic synaptic transmission is critical for rhythmogenesis, the respiratory network is heterogeneous (Figure 3*a*) and glutamatergic mechanisms never function alone. The degree of synchronization within the network depends on many factors, but at the core are three interwoven mechanisms: (*a*) glutamatergic transmission, which synchronizes respiratory neurons; (*b*) intrinsic bursting, which promotes synchronization; and (*c*) concurrent inhibition, which opposes synchronization and intrinsic bursting. Each component of this rhythmogenic triangle (Figure 3*b*) is dynamically regulated in a cycle-to-cycle manner to control the regularity, frequency, activity onset and termination, and stochasticity of respiratory neurons.

The respiratory rhythm shows remarkable cycle-to-cycle fluctuations in the degree of synchronization and neuronal activation patterns (Carroll & Ramirez 2013, Carroll et al. 2013, Eugenin et al. 2006, Harris et al. 2017, Lindsey et al. 1997, Mellen 2010, Mellen & Mishra 2010, Nieto-Posadas et al. 2014, Oke et al. 2015, Orem & Trotter 1992, Yackle et al. 2017). Instead of clustering into discrete categories (Smith et al. 2007, 2009), respiratory neurons can show activities spanning different phases, and some neurons can be rhythmically active in wakefulness but not REM (rapid-eye movement) sleep (Orem & Trotter 1992). Within the preBötC, respiratory neurons exhibit varied discharge patterns

along a continuum, from strongly inspiratory, to weakly inspira-tory, to tonic spiking, to expiratory (Carroll & Ramirez 2013) (Figure 4*a*). Even neurons active during the same respiratory phase exhibit a wide variety of activation patterns, burst durations, mean firing rates, onset and offset times, and roundness of burst shapes (Figure 4*b*). Moreover, individual neurons can miss a cycle and exhibit a large cycle-to-cycle jitter in activity onset (Carroll & Ramirez 2013, Kam et al. 2013a, Nieto-Posadas et al. 2014, Yackle et al. 2017) (Figure 4*c*). The stochastic onset variability of preBötC neurons can be modeled by a network configuration of sparsely connected excitatory neurons (Carroll & Ramirez 2013) (Figure 5*a*), which necessitates that several hundred excitatory neurons are connected to consistently generate synchronized bursts at the population level (Carroll & Ramirez 2013).

Within the preBötC, synchronization is delayed during each cycle by concurrent inhibition, resulting in a preinspiratory, weakly synchronized ramp that transitions into a fully synchronized population burst (Figure 5*b*) (Kam et al. 2013a, Koch et al. 2011, Ramirez & Richter 1996, Ramirez et al. 2004). The transition from the weakly synchronized preinspiratory ramp to a fully synchronized population burst can be abrupt. Yet consecutive cycles can reach different degrees of synchronization, resulting in amplitude fluctuations of population bursts. Thus, full synchronization should not imply maximal synchronization, during which all inspiratory neurons are synchronized. Indeed, it is unlikely that a maximal synchronization state is ever reached during eupneic breathing, because much larger inspiratory population bursts can be generated during a sigh (Ramirez 2014).

Fluctuations in synchronization are not rare, and cycles of apparently weak synchronization can be seen not only in the isolated network but also in vivo (Morris et al. 2001, Orem & Trotter 1992). Conditions such as long-term facilitation can increase synchrony (Morris et al. 2001), whereas the probability of weak synchronization can increase after exposure to intermittent hypoxia (Garcia et al. 2016, Zanella et al. 2014), increased postnatal age (Ramirez et al. 1996), low K^+ concentrations (Kam et al. 2013a,b), or decreased glutamatergic synaptic transmission (Harris et al. 2017). During some cycles, individual inspiratory neurons can fail to discharge or generate only a few action potentials (Figure 5*a,b*), as first demonstrated by Ramirez & Richter (1996) (see also Carroll & Ramirez 2013, Harris et al. 2017). Cycles in which inspiratory neurons remain weakly synchronized have also been called burstlets (Cui et al. 2016; Kam et al. 2013a,b). Such cycles are characterized by low-amplitude bursts in integrated preBötC population activity that fail to transmit to respiratory motor output (Bacak et al. 2016; Carroll & Ramirez 2013; Garcia et al. 2016, 2017; Harris et al. 2017; Zanella et al. 2014).

It has been proposed (Kam et al. 2013a) that preinspiratory ramps, or burstlets, and fully synchronized population bursts serve fundamentally different functions. Burstlets are considered rhythmogenic, but population bursts are viewed as pattern formation (Cui et al. 2016). This is an intriguing idea and the reader is referred to Kam et al. (2013b) for further explanation. While these two network states may indeed exert differential influences on rhythm and pattern formation, a strict separation is not possible. All rhythmogenic neurons that are activated during the preinspiratory ramp are also activated during the fully synchronized burst. Moreover, the same synaptic and intrinsic membrane properties that generate the ramp are also engaged during the burst, which inevitably influences the timing

of the next burst (see figure 2*a,e* in Kam et al. 2013a). Thus, we propose that the weakly and fully synchronized network states emerge through the same rhythmogenic process, in which concurrent inhibition delays a full synchronization, until a sufficient number of connected neurons trigger a snowball event that culminates in the fully synchronized state. An abrupt transition is promoted by the nonlinearity of intrinsic bursting properties (Figure 5*b*) (Ramirez & Richter 1996). At what point the preinspiratory ramp transitions into the population burst depends on the rhythmogenic triangle (i.e., the overall balance of synaptic inhibition, synaptic excitation, and excitability of intrinsic bursting) (Ramirez & Richter 1996, Ramirez et al. 2004).

Transitioning from the weakly to the fully synchronized population burst is of great functional importance because failure to transition results in failure to transmit inspiratory activity from the preBötC to the hypoglossal nucleus (XII), causing a XII apnea (Garcia et al. 2016, Kam et al. 2013a, Ramirez & Richter 1996, Ramirez et al. 1996). This is particularly relevant in the context of obstructive sleep apnea (OSA). In order to vocalize, the human pharynx is flexible and prone to collapse. Collapse is prevented through the coordinated activation of the diaphragm and muscles that control the upper airway, such as the genioglossus (driven by the XII). Failure to activate the XII during inspiration allows negative pressure generated during contraction of the diaphragm to collapse the pharynx and obstruct the airway, the key symptom of OSA (Ramirez et al. 2013). Important aspects of this disease, including airway obstructions (Peng et al. 2017), can be studied in rodents intermittently exposed to hypoxia for several days (chronic intermittent hypoxia, CIH). Exposure to CIH significantly increases the number of failed transitions into fully synchronized population bursts. As a result, the inspiratory burst does not transmit from the preBötC to the XII (Garcia et al. 2016, Ramirez & Richter 1996, Ramirez et al. 1996), causing the XII apnea, which is a major contributor to OSA (Ramirez et al. 2013).

INTRINSIC BURSTING: AN IMPORTANT DYNAMIC, NONLINEAR MEMBRANE PROPERTY

As in many neuronal networks (Koch et al. 2011, Schwindt & Crill 1999, Stuart & Sakmann 1995, van Drongelen et al. 2006), the synchronization of preBötC neurons is facilitated by intrinsic bursting properties. In the preBötC, a subset of neurons continue to burst in the absence of synaptic inputs; this bursting depends on two types of inward currents, the persistent sodium current (INaP) and the calcium-activated nonselective cation current (ICAN) (Figure 5*c*) (Peña et al. 2004, Thoby-Brisson & Ramirez 2001). Whereas ICAN seems to modulate primarily the burst shape and amplitude, INaP activation affects the frequency (Viemari & Ramirez 2006). But as discussed above, a strict functional separation of rhythm and pattern-generating mechanisms is probably an oversimplification.

Intrinsic bursting neurons are typically called pacemaker neurons, but this nomenclature has been the source of considerable confusion. Burstiness among inspiratory neurons ranges from weak, irregular bursting to strong, regular bursting (Carroll & Ramirez 2013). Although bursting properties can be sensitive to the blockade of either ICAN or INaP currents (Peña et al. 2004), there is considerable heterogeneity in peak inward and outward

current densities among inspiratory neurons. Bursting neurons tend to have higher current densities of INaP than nonbursting neurons do (Del Negro et al. 2002), but the current density of ICAN has not been systematically studied.

The large variability in the ratio of inward to outward currents is a fundamental neuronal property. Even in individually identified neurons of genetically identical animals, there is remarkable heterogeneity in the balance of different ionic currents and their conductances (Golowasch 2014; O'Leary et al. 2013, 2014; Rotstein et al. 2016; Swensen & Bean 2005; Temporal et al. 2012). Many solutions of cellular mechanisms with considerable animal-to-animal variability can generate apparently identical rhythmic outputs, suggesting that parameter nonuniqueness is a fundamental mechanism of system robustness and versatility (Prinz 2017). Moreover, acute lesioning of a given conductance can lead to compensatory changes (Etheredge et al. 2007, Grashow et al. 2010, Haedo & Golowasch 2006, Koch et al. 2013, O'Leary et al. 2013) that may operate even in a cycle-to-cycle manner (Olypher & Calabrese 2007, Rotstein et al. 2016). This finding calls into question the value of trying to identify one particular cellular mechanism that is essential for rhythmogenesis in a redundant and degenerative network (Mellen 2010).

A related yet different issue is whether pacemaker, or bursting, neurons actually pace the respiratory rhythm. Pacemaker neurons are generally more excitable. Thus, pacemaker neurons are more likely to lead a given cycle than nonbursting neurons are (Carroll & Ramirez 2013). Yet all neurons in the respiratory network are stochastically activated. Bursting or nonbursting neurons could lead a given cycle and contribute to the onset of events leading to a synchronized population burst (Carroll & Ramirez 2013). This is consistent with the observation that both bursting and nonbursting neurons are activated during the preinspiratory period (Cui et al. 2016, Feldman & Kam 2015, Kam et al. 2013a). Thus, the respiratory rhythm does not emerge through any one mechanism. Instead, each breath is assembled stochastically through the integration of various bursting properties as well as synchronizing and desynchronizing synaptic properties, as conceptualized in the triangle of rhythmogenesis (Figure 3*b*).

INTERDEPENDENCE BETWEEN INTRINSIC BURSTING AND NETWORK STATE: WHEN THE GOOD TURNS BAD

In the respiratory network, bursting neurons possess special membrane properties that allow them to burst even at physiological concentrations of potassium (Tryba & Ramirez 2004, Tryba et al. 2003), yet bursting properties are actively weakened by concurrent inhibition (Tryba et al. 2003) (Figure 6*c*). Thus, except for occasional ectopic bursts (Ramirez et al. 2004), pacemaker neurons typically require glutamatergic excitatory input, neuromodulatory input, or removal of inhibitory input in order to burst. Any of these factors can change. Bursting can be induced by substance P (Peña & Ramirez 2004) or norepinephrine (NE) (Viemari & Ramirez 2006), which promotes synchronization and regularizes the respiratory rhythm. By contrast, strengthening synaptic inhibition by exposing the network to acute intermittent hypoxia (AIH) biases the network toward weakly synchronized cycles. Exposing the network to AIH and NE enhances bursting and inhibition concurrently, which

increases the gain of the fluctuations between failed and synchronized states and results in an increased amplitude irregularity (Zanella et al. 2014). Weakening bursting properties with riluzole, which blocks INaP (Peña et al. 2004), decreases the gain of fluctuations caused by CIH, which decreases amplitude irregularity (Garcia et al. 2017). This result is the opposite of the application of riluzole in control conditions (Peña et al. 2004). Thus, whether any aspect of the rhythmogenic triangle or a given neuromodulator stabilizes or destabilizes the rhythm depends on the network state (Zanella et al. 2014). This concept may help explain the state dependency of various respiratory disorders. Specifically, a neuromodulator that normally stabilizes the respiratory network may cause instability in pathological conditions associated with intermittent hypoxia. In Rett syndrome, for example, breathing irregularities occur during wakefulness (Viemari et al. 2005; Weese-Mayer et al. 2006, 2008), whereas OSA occurs primarily during REM sleep (Ramirez et al. 2013). Both states are characterized by elevated levels of excitatory neuromodulators, including NE.

CONCURRENT INHIBITION AND EXCITATION AND THE CONTROL OF SYNCHRONIZATION

Concurrent inhibition and excitation is a network property frequently found in the CNS (Chagnac-Amitai & Connors 1989, English et al. 2014, Kolind et al. 2012, Petersen et al. 2014, Zheng & Raman 2011). In general terms, concurrent excitation and inhibition prevents excessive spiking and changes in membrane potential (Kolind et al. 2012, Shadlen & Newsome 1998, van Vreeswijk & Sompolinsky 1996), and it is a major contributor to stochasticity (Rudolph et al. 2005, Yarom & Hounsgaard 2011). This network mechanism is also critical for gain control (Brunel et al. 2001, Burkitt 2006, Chance et al. 2002, Destexhe et al. 2003), which is consistent with the role of inhibition in the respiratory network (Dogas et al. 1998). Concurrent excitation and inhibition arises from local connections within the preBötC (Figure 6*a,b*) (Harris et al. 2017, Morgado-Valle et al. 2010). In some respiratory neurons the strength of inhibition can outweigh the concurrent excitation, resulting in an expiratory discharge pattern (Figures 4*a*, 6*c*, 8a). But in most excitatory preBötC neurons this network configuration results in an augmenting inspiratory burst (Figure 6*c*). This augmenting shape, also called eupneic activity, is characteristic of the inspiratory pattern during normal breathing.

The network transitions from eupnea to gasping during severe hypoxia (Figure 7) (Lieske et al. 2000). This reconfiguration is associated with varied changes in the spiking activity of, and the interactions between, preBötC neurons (Figure 7*b,c*) (Nieto-Posadas et al. 2014), with a bias toward reduced synaptic inhibition (Figure 7*d*) both in vitro (Lieske et al. 2000) and in vivo (Richter et al. 1991). The loss of inhibition in hypoxia also results in a phase shift that turns expiratory neurons into inspiratory neurons (Figure 7*e*). Similar changes occur when blocking inhibition pharmacologically (Lieske et al. 2000). However, reconfiguration in hypoxia likely involves more than reduced inhibition (Peña et al. 2004), and reconfiguration within the preBötC constitutes only one aspect of a wider network reconfiguration that includes other microcircuits and afferent inputs to generate the emergent hypoxic response. These considerations are consistent with the broader concept that breathing is assembled from different, principally independent phase modules that can be

reconfigured and reassembled to generate different forms of breathing behaviors, such as eupnea and gasping (Figure 1*b*), but also behaviors closely associated or coordinated with breathing, such as coughing or swallowing (Pitts et al. 2012). Indeed, phase as an independent behavioral module also facilitates the ability of behaviors to reconfigure, another general principle (Earhart & Stein 2000, Frigon 2009, Jing et al. 2007, Li 2015, Liao & Fetcho 2008, Ramirez 1998, Soffe 1993, Weimann et al. 1991, White & Nusbaum 2011).

MULTIPLE, COUPLED MICROCIRCUITS ARE REQUIRED TO GENERATE A MULTIPHASE RHYTHM

Local inhibition exerts powerful control over synchronization and burst generation (Figure 8). Increasing the strength or proportion of inhibitory neurons desynchronizes the respiratory rhythm, leading to increased variability and eventually to cessation of rhythmogenesis. Thus, there is a small parameter space in which rhythmogenesis can be maintained in the presence of inhibition. Inhibitory neurons incorporated within the network also lead to the emergence of neurons with an expiratory discharge pattern (Figure 8*a*) (Harris et al. 2017). However, there is also a limited window in which inhibition can support a discharge pattern that is not in phase with the main population rhythm, because network rhythmicity is lost if the percentage of expiratory neurons reaches ~20% (Figure 8*b*). It is estimated that 25–50% of preBötC neurons are inhibitory (Morgado-Valle et al. 2010, Winter et al. 2009), and multi-array recordings from the preBötC indicate that approximately 9% of neurons are expiratory (Figure 4*a*) (Carroll & Ramirez 2013). Thus, as suggested by network models (Harris et al. 2017), the preBötC operates on the edge of synchrony, which perhaps contributes to its remarkable flexibility. Indeed, this may be a general network phenomenon. In the neocortex, rhythmic up and down states are also characterized by concurrent inhibition and excitation (Shu et al. 2003), and neurons that discharge out of phase (i.e., during the down state) are rare but do exist (van Drongelen et al. 2003).

These considerations lead to the conclusion that local concurrent inhibition and excitation is not sufficient to allow a single microcircuit to generate a robust multiphase rhythm (Harris et al. 2017). This has important ramifications for rhythm generation in general and the organization of the respiratory network in particular. To generate more than one phase requires a variety of network mechanisms (Tupal et al. 2014), including long-range inhibition, that can establish the phase relationships between different excitatory microcircuits. As indicated by computational modeling, the balance between the strength of local versus long-range inhibition can determine the regularity of rhythmogenesis and the generation of different phases (Figure 9*a*) (Harris et al. 2017). Although this model tested the interactions between only two microcircuits, it is conceivable that long-range inhibitory interactions between three microcircuits may also generate a three-phase rhythm, as proposed by the triple oscillator hypothesis (Figure 9*b*) (Anderson & Ramirez 2017).

The concept of coupled oscillators is consistent with the organization of other rhythmogenic networks, such as the locomotor rhythm and circadian rhythms (Bywalez et al. 2012, Cangiano et al. 2012, Grillner & El Manira 2015, Im & Taghert 2010, Lee et al. 2009). This concept also explains many behavioral features of breathing (e.g., how a given phase can be

drastically weakened or strengthened in a cycle-to-cycle manner), because in contrast to existing network models, the generation of a given phase is not strictly dependent on the presence of another phase. Moreover, local concurrent inhibition and excitation can locally generate inspiration with eupneic characteristics independent from the influence of other breathing phases (Ramirez & Lieske 2003). A higher threshold for the activation of the pFRG network may explain why active expiration is recruited during high metabolic demands, and why it depends on the preBötC in older animals (Huckstepp et al. 2016). Indeed, the differential modulatory sensitivity of these oscillators (Anderson et al. 2016, Doi & Ramirez 2008) may allow the CNS to differentially activate or inhibit a given phase. Further, weakened long-range inhibitory interactions between oscillators might explain why postinspiration and expiration can overlap (McCrimmon et al. 2000), and may provide a conceptual framework to understand how the respiratory network reconfigures during nonventilatory behaviors such as coughing or swallowing (Segers et al. 2012). From an evolutionary perspective, it is conceivable that the CNS recruited and combined different microcircuits as breathing evolved from gill breathing in fish, to transitional gill and lung breathing in amphibians, to various distinct forms of breathing in reptiles and birds, and to diaphragmatic breathing in mammals (Ramirez et al. 2016).

Further experimental and computational studies are necessary to integrate these relatively novel concepts and to reconcile some of the fundamental differences between the triple oscillator hypothesis and compartmental models. For example, rhythmogenesis in the compartmental model emerges through inhibitory interactions driven by excitation from the pons. Therefore, unravelling the role of the pons will be instrumental in finding common ground in respiratory rhythm generation (Dutschmann et al. 2014). Often thought to be essential for the termination (off-switch) of inspiration, the pons may have various important roles in regulating, stabilizing, and shaping ongoing respiratory activity. Indeed, structures such as the pons may be critical for exerting stability and limiting the variability within the medullary networks (see, e.g., Dhingra et al. 2016, 2017). Disruption in these pontomedullary interactions may also be primarily responsible for the breathing disturbances in Rett syndrome (Abdala et al. 2016). The pons also influences other aspects of the respiratory rhythm, including the frequency of inspiratory and expiratory activities (Zuperku et al. 2017) and coordination with nonventilatory behaviors such as swallowing (Bonis et al. 2011).

CONCLUDING REMARKS

The apparent stereotypy of breathing inferred that the underlying mechanisms are simple. The discovery of the preBötC (Smith et al. 1991) fit this mindset, as it suggested that breathing emerges from one rhythmogenic kernel that is both sufficient and necessary for rhythmogenesis. The criterion of necessity also guided the search for one essential rhythmogenic mechanism. The hypothesis that this rhythmogenic mechanism is realized by pacemaker properties was rejected early on and replaced by ideas such as the group pacemaker hypothesis, the burstlet hypothesis, and the hypothesis that the rhythm emerges through inhibitory interactions, all of which are likely important contributors to respiratory rhythmogenesis. But the premise has to be reconsidered: Neither a single rhythmogenic mechanism, nor a single rhythmogenic network, is likely. Indeed, there is no simple

behavior, not even in the smallest invertebrate. As for breathing, it may temporarily seem stereotypic. But in a state-dependent manner breathing must be able to reconfigure and switch between one-, two-, and three-phase rhythms, and during vocalization it must serve our highest intellectual or artistic control. To cover this wide behavioral spectrum, each cycle emerges from a dynamic interplay between rhythmogenic and rhythmoinhibitory mechanisms that are targeted by numerous modulators and synaptic interactions between microcircuits distributed over numerous CNS regions. Although this review focused on the dynamic complexity observed at the neuronal network level, the striking variability and stochasticity may not automatically translate into variability at the motor output or behavioral level, as each level of integration has its own regulatory processes, as elegantly illustrated in the characterization of amplitude variability of transdiaphragmatic pressure (Medina-Martínez et al. 2015). As modern optogenetic and molecular tools increasingly allow us to bridge different levels of integration and to characterize the dynamic complexity of breathing in freely behaving animals, the field has reached an important turning point. We are gaining insights into the intriguing relationship between breathing, emotion, and attention (Burke et al. 2014, Li et al. 2016, Ramirez et al. 2012, Yackle et al. 2017). But this is just the beginning, as we still have only a limited mechanistic understanding of the link between the hippocampus, amygdala, insula, and respiratory rhythm generating networks.

This review provides a snapshot of our current understanding of the network interactions that characterize breathing. We are fully aware that models such as the triple oscillator hypothesis or the rhythmogenic triangle are still rooted in a reductionistic mindset. However, most progress in the field emerged from simplified hypotheses that could be challenged, overthrown, or built upon. Thus, we expect that the concepts discussed here will continue to be revised as we gain a better understanding of a neuronal network that is so intimately linked to life and all its complexity.

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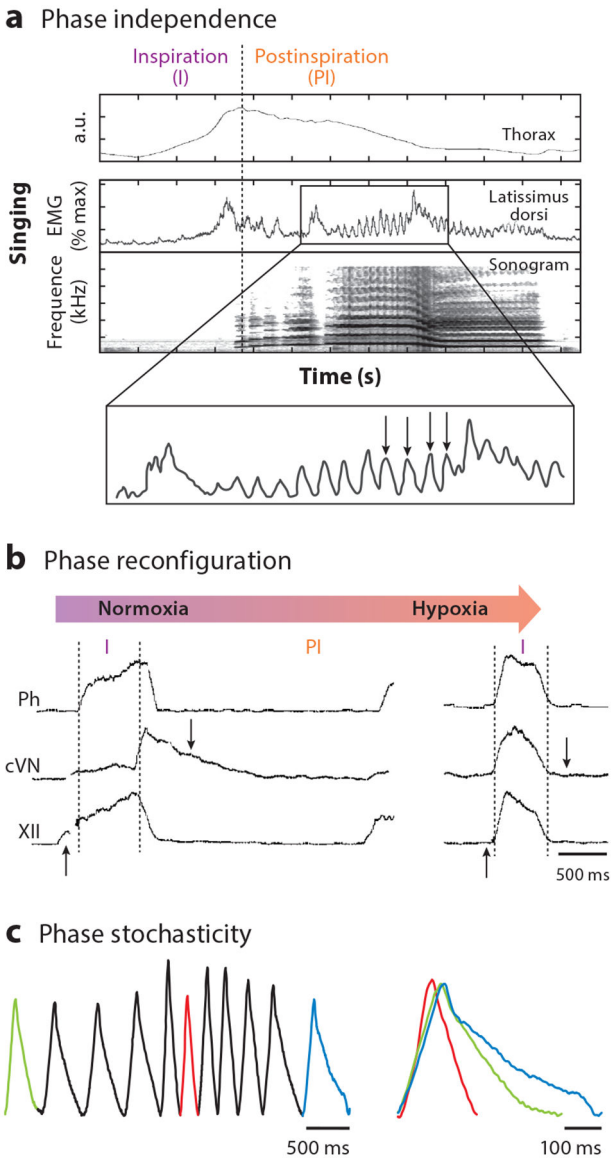

Figure 1.

Respiratory phases can act independently, reconfigure, and exhibit stochasticity. (*a*) Respiratory-related electromyographic activity of the latissimus dorsi (LD) muscle during a single breath in a trained classical singer performing an exercise from “Una voce poco fa” from *The Barber of Seville* by Gioachino Rossini. Note the repeated bouts of LD activity during postinspiration (*arrows* in expanded view) that correlate with vibrato visible in the corresponding sonogram (figure adapted with permission from Watson et al. 2012). (*b*) Experiment from an in situ arterially perfused rat preparation showing recordings of activity from the phrenic (Ph) nerve, innervating the diaphragm; cervical vagal nerve (cVN), containing a branch that innervates laryngeal muscles; and hypoglossal (XII) nerve, innervating the tongue, under normoxic and hypoxic conditions. During normoxia, postinspiratory and preinspiratory activity are prominent in cVN and XII recordings, respectively (*arrows*). But activity in the cVN and XII becomes primarily inspiratory during

hypoxia, demonstrating the ability of the respiratory network to reconfigure from a three-phase rhythm to a one-phase rhythm (figure adapted with permission from Paton et al. 2006). (*c*) Plethysmography recording from an awake behaving mouse reveals stochasticity in breath waveforms.

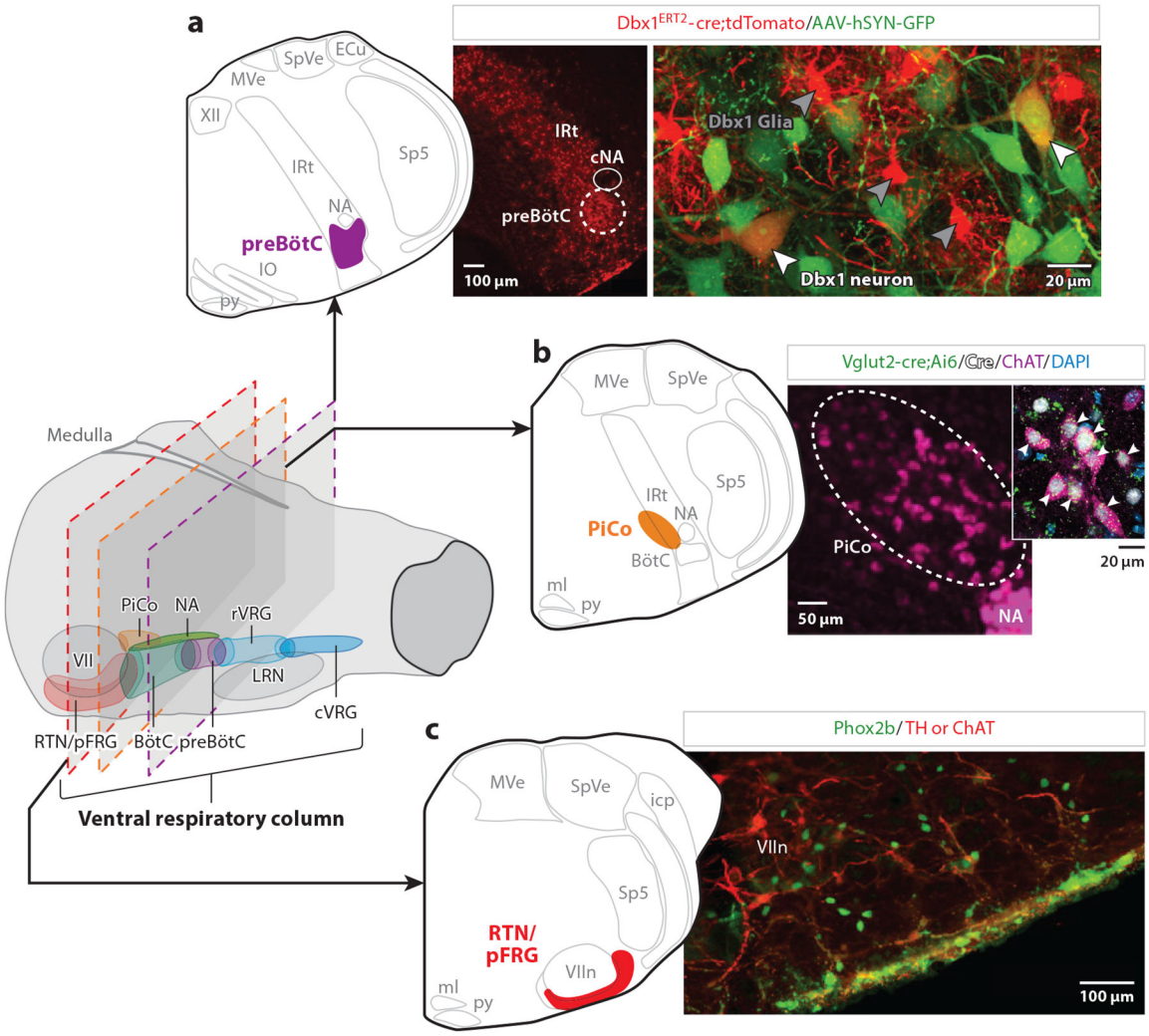

Figure 2.

The ventral respiratory column (VRC) in the medulla provides the basis for respiratory rhythm generation. The VRC contains (from rostral to caudal) the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), encompassing the ventral borders of the facial nucleus (VII); the postinspiratory complex (PiCo), caudal to VII and dorsomedial to the nucleus ambiguus (NA); the Bötzinger complex (BötC) and preBötzinger complex (preBötC), ventromedial to the NA; and the rostral and caudal ventral respiratory groups (rVRG and cVRG, respectively), dorsal to the lateral reticular nucleus (LRN). The borders of VRC compartments are indistinct. However, genetic and molecular markers that characterize the rhythmic regions of the VRC have been described. (*a*) In the preBötC, rhythmic excitatory neurons are derived from precursors that express the transcription factor Dbx1 during development (*white arrowheads* mark Dbx1 neurons colabeled with the neuron-specific AAV-hSYN-GFP). Neurons along the inter-reticular zone (IRt), including XII premotor neurons, and some glia (*gray arrowheads*), are also derived from Dbx1-expressing cells (figure adapted from Kottick et al. 2017 with permission). (*b*) PiCo neurons are defined by coexpression of vesicular glutamate transporter 2 (Vglut2) and choline

acetyltransferase (ChAT). White arrows specify neurons containing nuclei expressing Cre (under control of the Vglut2 promoter) and ChAT within the surrounding cytoplasm (figure adapted from Anderson et al. 2016). (*c*) The RTN/pFRG contains neurons that express Phox2b but not tyrosine hydroxylase (TH) or ChAT (figure adapted from Stornetta et al. 2006 with permission). Other abbreviations: AAV-hSYNGFP, adeno-associated virus-human synapsin 1-green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; ECu, external cuneate nucleus; icp, inferior cerebellar peduncle; IO, inferior olive; ml, medial lemniscus; MVe, medial vestibular nucleus; py, pyramidal tract; Sp5, spinal trigeminal tract; SpVe, spinal vestibular nucleus; XII, hypoglossal motor nucleus.

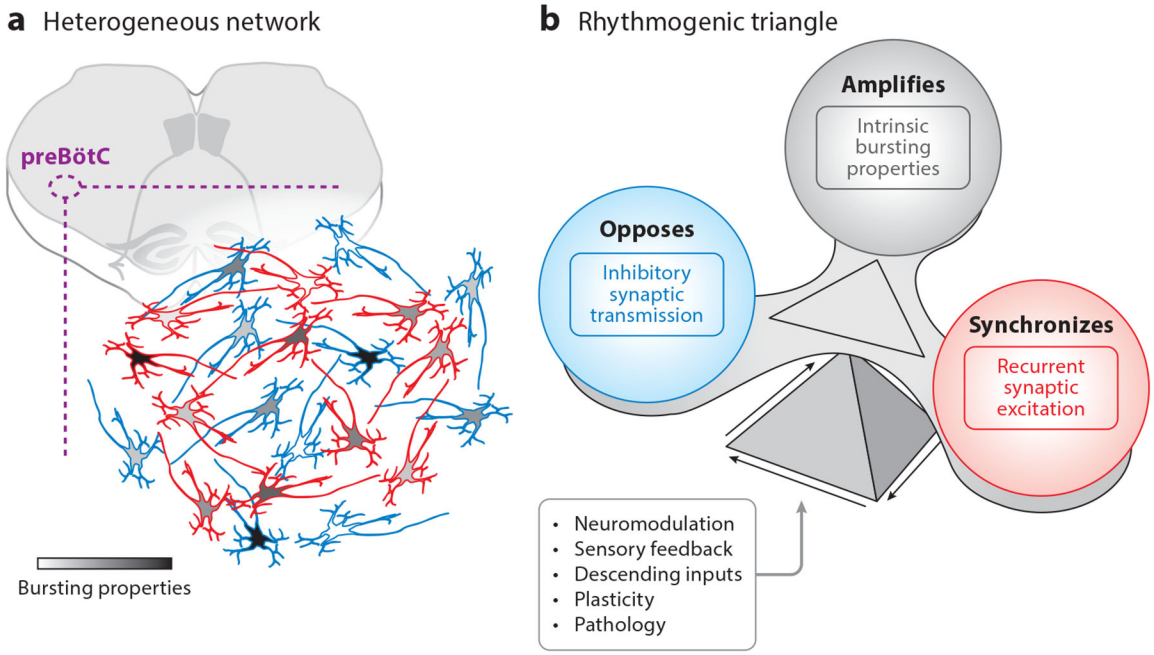

Figure 3.

Interactions in a heterogeneous network give rise to a rhythmogenic triangle. (*a*) The isolated preBöTc network contains excitatory and inhibitory neurons with varied degrees of burstiness. (*b*) Network synchrony is regulated by the rhythmogenic triangle: Glutamatergic synaptic transmission is essential for synchronization, intrinsic membrane bursting conductances enhance spiking and amplify synchronization, and concurrent inhibition opposes synchronization and increases variability. The balance of these components can be tuned to adapt breathing for specific behaviors and states by, for example, neuromodulation, sensory feedback, descending inputs, and plasticity; whereas pathology can disrupt this balance, leading to breathing disturbances that can be behavior- and state-dependent. Abbreviation: preBötC, preBötzinger complex.

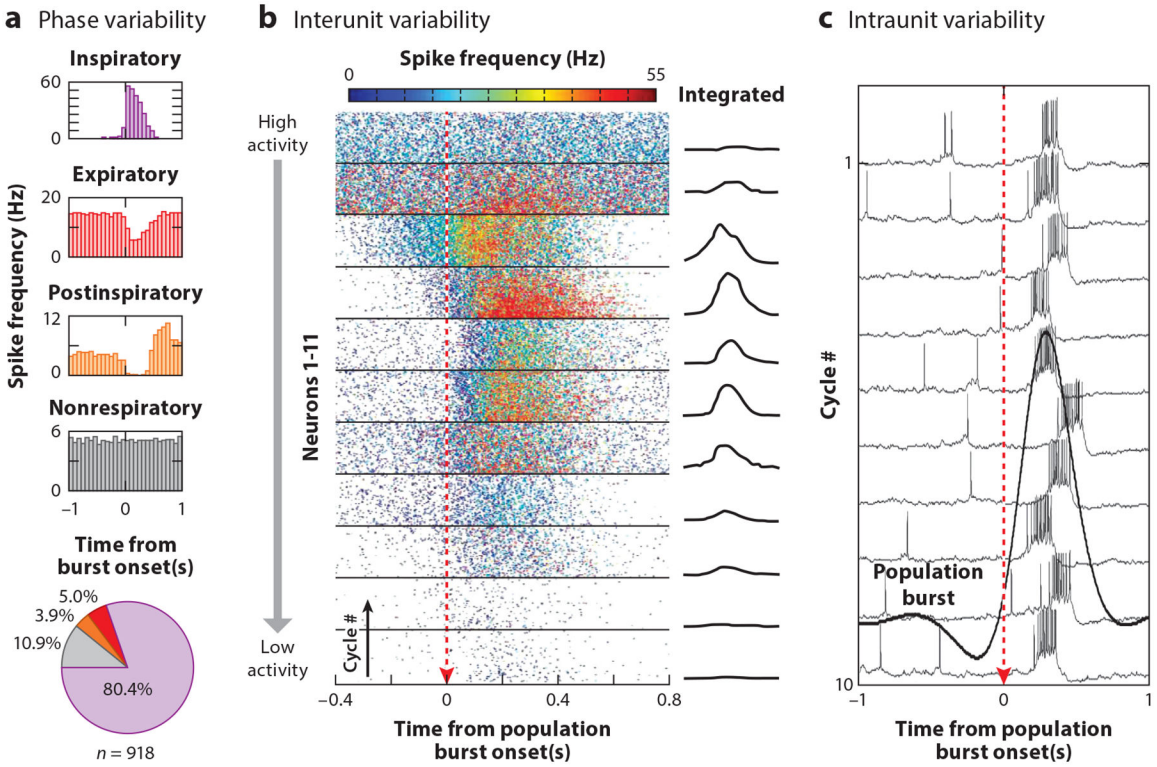

Figure 4.

Respiratory rhythm-generating networks contain neurons with variable firing patterns and stochastic activity. (*a*) Multielectrode array recordings from the preBötTc (77 experiments with 918 units) reveal that most neurons are active in phase with inspiration. (*b*) Spike rasters of 11 simultaneously recorded inspiratory neurons over 150 respiratory cycles demonstrating substantial variability in the amount of spiking activity, phase modulation, burst onset, and burst shape and duration between neurons. Red arrow indicates preBötC population burst onset. Integrated spiking activity of each neuron is shown on the right. Panels *a* and *b* adapted from Carroll et al. 2013. (*c*) Intracellular recording of a single inspiratory neuron over 10 respiratory cycles illustrating the stochasticity of burst onset relative to preBötC population activity (*red arrow*). Abbreviation: preBötC, preBötzinger complex.

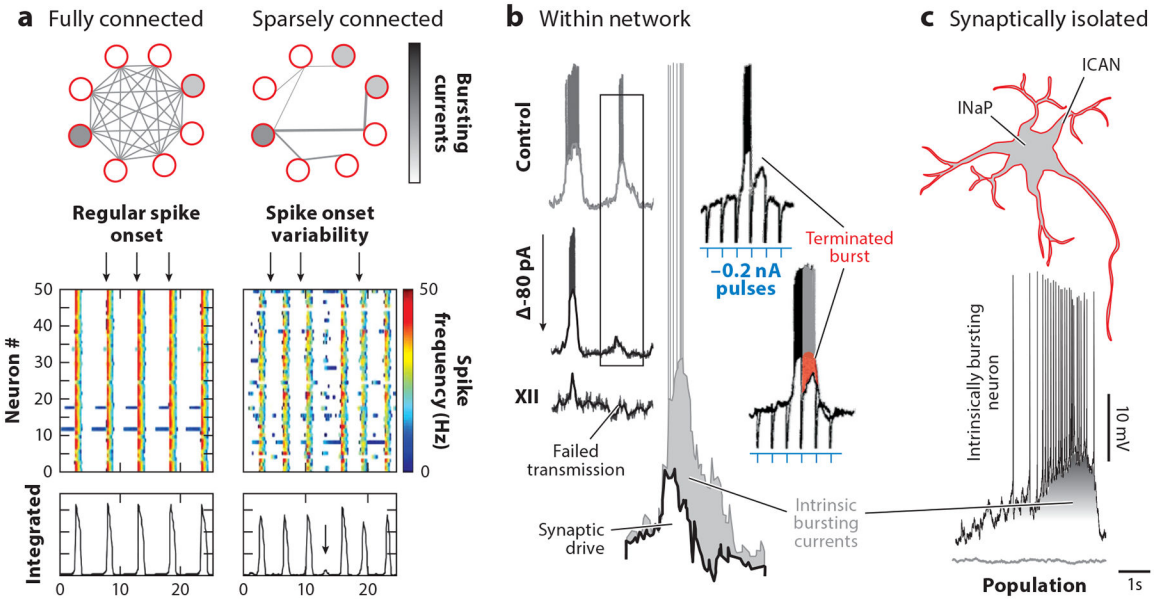

Figure 5.

Network connectivity and bursting properties influence stochasticity and the transition to a population burst. (*a*) Comparison between fully and sparsely connected preBötC models containing excitatory neurons and bursting currents. Sparse network connectivity reproduces the burst onset variability (*arrows*) characteristic of the preBöTc (see Figure 4b,c). Sparsity can also prohibit the network from fully synchronizing in some cycles, resulting in a weak population burst (*arrow* in integrated model activity). (*b*) Within the network, bursting currents amplify excitatory synaptic drive and facilitate transmission of preBötC population activity to motor output. The effect of bursting currents can be observed by comparing burst activity of a neuron under control conditions and in the presence of a slight hyperpolarizing current in which weak inspiratory activity is no longer able to translate synaptic drive into a burst (illustrated in the expanded overlay) and preBöTc activity is not successfully transmitted to XII motor output (*left*). The amplifying influence of bursting properties is also indicated by the ability to prematurely terminate an ongoing burst with a brief hyperpolarizing current pulse (*right*). Figures adapted with permission from Ramirez & Richter (1996). (*c*) The presence of persistent sodium current (INaP) and calcium-activated nonselective cation current (ICAN) gives rise to a gradient of burstiness among preBötC neurons. Some neurons, called intrinsic or endogenous bursting neurons, continue to burst in the absence of synaptic interactions. Abbreviations: preBötC, preBötzinger complex; XII, hypoglossal motor nucleus.

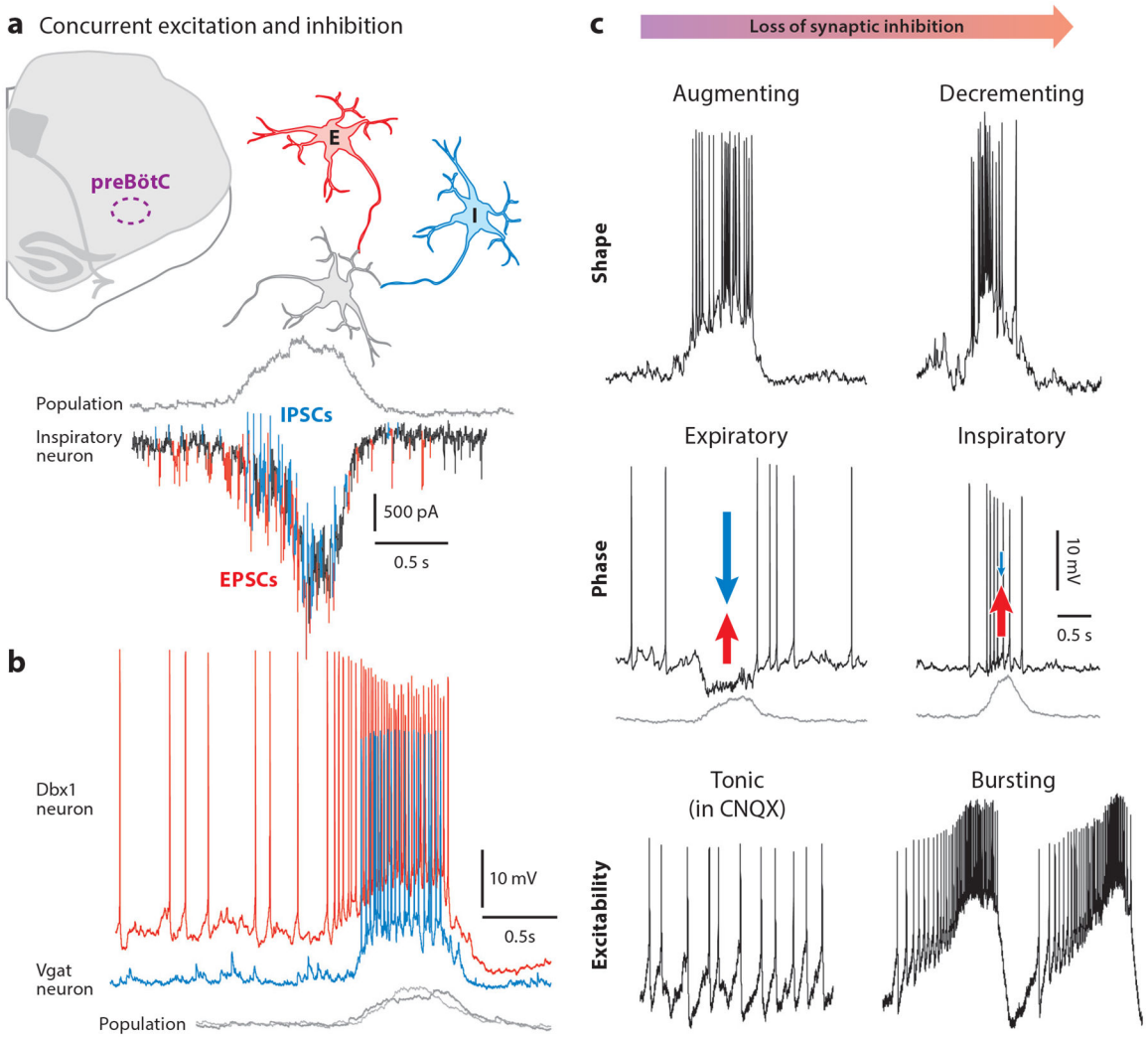

Figure 6.

Neurons receive concurrent excitation and inhibition from within the isolated preBötC, a critical feature regulating burst shape, phase, and excitability. (*a*) Voltage clamp recording from an inspiratory preBötTc neuron showing concurrent EPSCs (*red*) and IPSCs (*blue*) synchronized with integrated preBötC population activity (*gray*). (*b*) Activity of optogenetically identified excitatory Dbx1- and inhibitory Vgat-expressing neurons during preBötC population bursts. (*c*) Loss of synaptic inhibition transforms the augmenting burst shape of inspiratory preBötC neurons into a decrementing shape; expiratory neurons become inspiratory (*red* and *blue arrows* reflect the relative influence of excitatory and inhibitory synaptic inputs, respectively). With glutamatergic synaptic transmission blocked (CNQX), tonic activity in some inspiratory neurons is transformed into endogenous bursting (figure adapted from Tryba et al. 2003). Abbreviations: E, excitation; EPSCs, excitatory postsynaptic currents; I, inhibition; IPSCs, inhibitory postsynaptic currents; preBötC, preBotzinger complex.

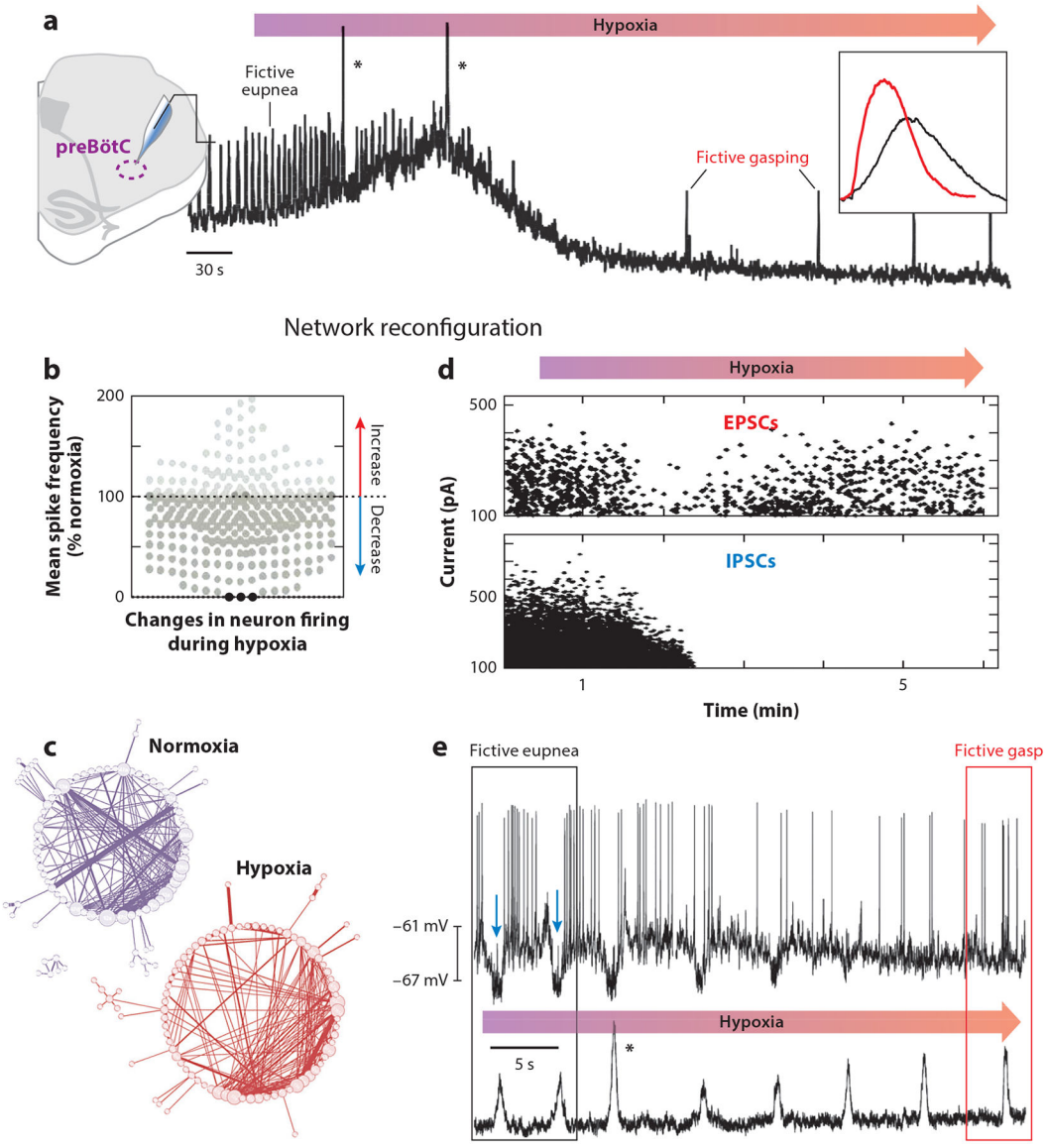

Figure 7.

The preBötC network reconfigures in hypoxia. (*a*) Population activity in the isolated preBötC at baseline and during the transition to hypoxia. Fictive eupneic bursts transition to fictive gasping. Averaged eupnea (*black*) and gasping (*red*) burst waveforms illustrating changes in burst rise time and duration are shown on the right. (*b*) Multi-array recordings reveal that, although the average activity of the preBötC is reduced in hypoxia, individual respiratory elements have varied responses to hypoxia. Some exhibit increased mean firing rate, whereas others are completely suppressed (*black dots*). (*c*) Graphic representation of preBötC network configurations in normoxia and hypoxia. Respiratory elements are represented as circles with a diameter proportional to the number of functional connections as determined by cross-correlation analysis. Significant correlations are represented as connecting lines, with the weight of the line reflecting the strength of each connection. Note that reconfiguration of the network involves changes in the number and strength of the

connections between individual elements, but that the overall number of elements and connections within the network remains relatively unchanged (panels *b* and *c* adapted with permission from Nieto-Posadas et al. 2014). (*d*) Quantification of EPSCs and IPSCs from inspiratory preBötC neurons during the transition to hypoxia demonstrates that changes in activity are not evenly distributed among excitatory and inhibitory neurons. (*e*) Activity of an expiratory preBötC neuron during network reconfiguration in hypoxia. Note the loss of inhibition during inspiration (*blue arrows*), resulting in a phase transition from expiratory activity during fictive eupnea to inspiratory activity during fictive gasping. Asterisk indicates fictive sigh bursts. Abbreviations: EPSCs, excitatory postsynaptic currents; IPSCs, inhibitory postsynaptic currents; preBötC, preBötzinger complex.

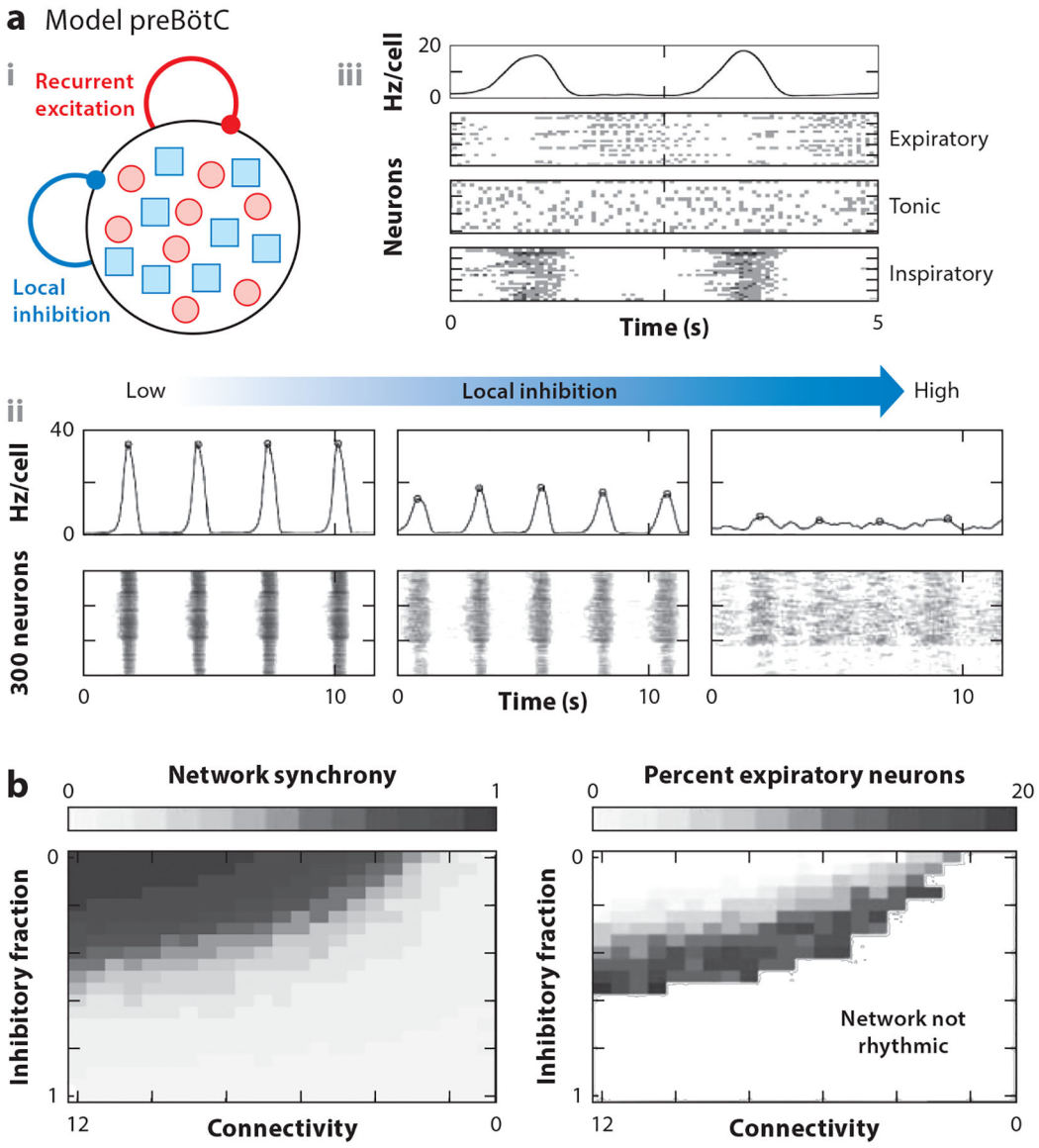

Figure 8.

The preBötC operates on the edge of synchrony. (*a*) Representation of increasing amounts of heterogeneity included in preBöTc network models. (*a, i*) Schematic of a model preBötC examining the role of inhibitory neurons in rhythm generation. (*a, ii*) Integrated rhythmic model preBötC activity and spike rasters with an increasing fraction of inhibitory neurons. (*a, iii*) Represents the same network parameters shown in the middle panels of *a,ii*, demonstrating the emergence of neurons with expiratory activity. (*b*) As the fraction of inhibitory neurons and the sparsity of network connectivity increase, network synchrony is reduced and the percentage of neurons with expiratory activity increases to 20%, at which point synchrony is lost and the network is no longer rhythmic (figures adapted with permission from Harris et al. 2017). Note that approximately 25–50% of the neurons in the preBötC are inhibitory (Morgado-Valle et al. 2010, Winter et al. 2009) and approximately

10% of neurons are expiratory (see Figure 3) (Carroll et al. 2013), suggesting the preBötC operates near the edge of synchrony. Abbreviation: preBötC, preBötzinger complex.

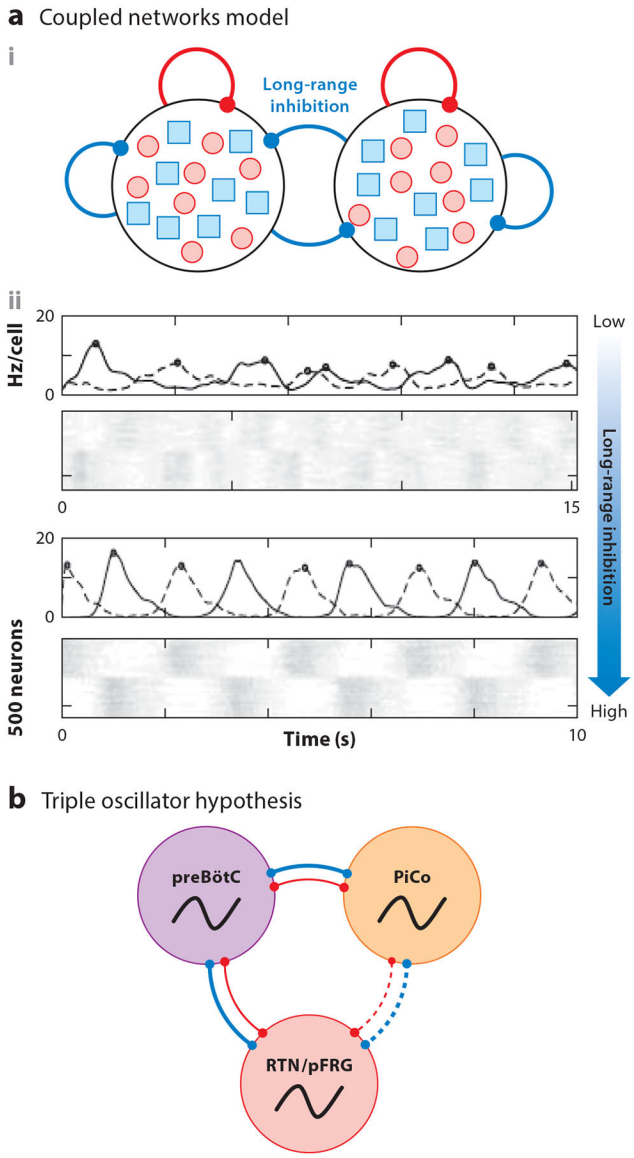

Figure 9.

Coupled rhythmogenic networks are required to generate a multiphase breathing rhythm.(*a, i*) Computational model of two rhythmic networks, containing recurrent excitation and local inhibition, coupled by long-range inhibition. (*a, ii*) Comparison of simulated rhythmic activity in networks with strong local inhibition and weak long-range inhibition (*top*), and in networks with weak local inhibition and strong long-range inhibition (*bottom*) (figure adapted with permission from Harris et al. 2017). (*b*) Schematic representation of the triple oscillator hypothesis, in which the preBötC, PiCo, and, under high metabolic demand, RTN/pFRG interact via mutually inhibitory and excitatory connections (connections indicated by *dashed lines* are speculative). Inhibition dominates under normal conditions, resulting in a multiphase rhythm; but like the rhythmogenic triangle, the balance of excitation and inhibition between oscillators can be shifted to facilitate phase reconfiguration such as

during hypoxia (see Figure 1b). Abbreviations: PiCo, postinspiratory complex; preBötC, preBötzinger complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group.