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Microcircuits in respiratory rhythm generation: commonalities with other rhythm generating networks and evolutionary perspectives

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Abstract

Rhythmicity is critical for the generation of rhythmic behaviors and higher brain functions. This review discusses common mechanisms of rhythm generation, including the role of synaptic inhibition and excitation, with a focus on the mammalian respiratory network. This network generates three phases of breathing and is highly integrated with brain regions associated with numerous non-ventilatory behaviors. We hypothesize that during evolution multiple rhythmogenic microcircuits were recruited to accommodate the generation of each breathing phase. While these microcircuits relied primarily on excitatory mechanisms, synaptic inhibition became increasingly important to coordinate the different microcircuits and to integrate breathing into a rich behavioral repertoire that links breathing to sensory processing, arousal, and emotions as well as learning and memory.

Introduction

Rhythmicity is involved in almost all behaviors and brain functions [1[•],2]. This includes the generation of rhythmic behaviors, communication, encoding, attention, learning and memory [3,4]. Thus, understanding rhythmogenesis is a core issue in neuroscience. Rhythmogenesis can be studied in microcircuits isolated from invertebrates [5,6], mammalian [7[•],8[•],9,10,11[•],12], and non-mammalian vertebrates [13,14^{••},15[•],16] as well as fully integrated behavioral systems [17^{••},18–20,21[•],22,23]. Yet, the quest to unravel the mechanisms underlying rhythmogenesis has been a difficult journey. Concepts developed using intact systems have often conflicted with those obtained from isolated networks. In particular in mammalian studies, some discrepancies have been attributed to developmental differences, since many *in vivo* studies were conducted in adults, while *in vitro* experiments have often been limited to neonates [24[•]], a difficulty that can be overcome by studying non-mammalian model systems [25[•]].

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Conflicts of interest statement

The authors do not have any conflict of interest.

Mechanisms commonly found in rhythm generating networks include reciprocal inhibition [26*,27,28], rhythmic pacemaker properties [11*,29–33] and recurrent excitatory network mechanisms [10,34*,35,36*]. However, their roles within a given network vary and are often different than originally hypothesized. We learned that the relative contribution of neuronal mechanisms is not fixed, but dynamically regulated, resulting in state-dependent re-configuration of neuronal networks [37–40]. Mechanisms that are essential in one condition can become non-essential contributors in another state even within the same network [41].

This review will discuss the mechanisms underlying rhythm generation in a variety of brain networks with a focus on the respiratory rhythm-generating network. This network has well-defined physiological roles and it is amenable to a rigorous cellular analysis [17**,42–44]. Moreover, the respiratory rhythm is integrated with the activity of numerous networks distributed throughout the brainstem and forebrain [45*,46]. A comparative approach among vertebrates may provide important insights into how multiple rhythmic circuits become functionally intertwined to produce and coordinate ventilatory and non-ventilatory behaviors. By unraveling the complexities of the breathing rhythm, we may also gain insights into rhythmicity involved in other CNS functions ranging from locomotion to memory and emotion.

Rhythm generating networks and the role of synaptic inhibition

Most rhythm generating networks are heterogeneous, consisting of silent, tonic and rhythmically bursting neurons (Figure 1a) [2,47]. Neurons endowed with these discharge patterns form the building blocks of neuronal networks and are often incorporated into computational models. One of those models, the so-called half-center oscillator (HCO) was one of the first models to mechanistically explain rhythmogenesis [48–50] and has been particularly influential. In this model, two non-rhythmic cells or groups of cells are coupled by mutual inhibitory connections that give rise to antiphasic rhythmicity in the presence of an excitatory drive [51].

Mutually inhibitory connections are ubiquitous in rhythm generating networks [44,52,53], and variations of the HCO are found in some models of respiration [54] and locomotion [55]. However, to what extent reciprocal inhibition operates as a rhythmogenic mechanism is still an open question. In lamprey swimming, reciprocal inhibition regulates left and right coordination, yet each hemisegment can generate rhythmicity even without inhibition [56]. Similarly, in the respiratory network rhythmicity persists after blockade of synaptic inhibition in lamprey and frogs [25*,57], in isolated mammalian respiratory microcircuits [44] and in intact mammals [19,58*].

Yet, inhibitory mechanisms have important functions within the respiratory network [24*,58*]. Concrete insights were gained from studying the preBötzinger complex (preBötC; Figure 1), the first rhythmogenic microcircuit identified within the mammalian respiratory network [12,59]. This network, located in the ventrolateral medulla, is both necessary and sufficient for generating respiratory rhythm [9,12,60]. A large proportion of preBötC neurons are inhibitory (Figure 1b) [61], and optogenetic manipulations have revealed that these neurons provide important temporal cues by specifically mediating afferent input in

the intact network [19]. Another medullary region, the Bötzing complex (BötC; Figures 1 and 3c), contains primarily inhibitory neurons that are thought to play a critical role in forming the different phases of breathing [62]. However, BötC neurons are not well defined and optogenetic approaches probing the functional roles of these neurons are still missing.

Inhibitory mechanisms have also been studied in non-mammalian vertebrates. For the buccal rhythm generating network in bullfrogs, a sensitivity to changes in chloride-dependent conductances has been demonstrated [14**], but the exact role of inhibition remains unclear. In lamprey, respiratory frequency is controlled by inhibition, but like mammalian respiratory microcircuits, inhibition is not necessary for rhythmogenesis [16].

The role of excitatory mechanisms in rhythm generation

The critical role of synaptic excitation within the respiratory network has never been questioned. Two principle rhythmogenic mechanisms have been proposed for excitatory networks in general: (1) Interconnected endogenous bursting, pacemaker neurons: In its extreme, the temporal characteristics of amplitude and period are defined by the intrinsic membrane properties of pacemakers [63,64]. (2) Excitatory interactions between non-pacemakers: In this configuration temporal and amplitude characteristics are largely defined by synaptic dynamics [27]. Yet, neither of these ‘extreme’ network configurations is likely realized in actual networks, since synaptic and intrinsic mechanisms are intimately interwoven (Figure 2) [44].

Glutamatergic, non-NMDA-dependent, synaptic transmission is the essential rhythmogenic mechanism within the isolated preBötC, which is similar to other respiratory microcircuits in mammals [65**,66] and non-mammalian vertebrates [25*]. Synchronization is established by recurrent excitation [67], which leads to pre-synaptic facilitation [27] and the activation of burst generating conductances (Figure 2) [68,69]. Two conductances that promote the non-linear amplification of excitatory synaptic interactions are the persistent sodium (INaP) and calcium activated non-specific cation (ICAN) currents (Figure 1a) [70]. Conductances promoting bursting have also been implicated in respiratory rhythm generation in turtles [32].

Multiarray recordings reveal that the majority of preBötC neurons are weakly spiking or tonically spiking, while silent and intrinsic bursting neurons constitute a minority [71,72]. This distribution of spiking profile is likely the result of a gradient distribution of ionic conductances [30]. Indeed, a fundamental question is whether different ionic conductance ratios are randomly distributed among respiratory neurons, or whether this distribution follows specific rules. One possibility is that conductance ratio is regulated to shift the network towards intrinsically spiking modes based on energy consumption principles. For example, bursting activity patterns require a dynamic overlap between inward and outward currents [63,73], which is not energy efficient [74]. This could explain why bursting neurons are relatively rare within the preBötC. By contrast, a small overlap between Na⁺ and K⁺ currents gives the most efficient energy consumption; most likely such dynamics promote tonic spiking activity [74], which is also the activity most frequently observed in the pre-BötC [71,72].

However, preBötC neurons cannot simply be classified according to their discharge pattern in isolation since the strength of discharge can also vary with respect to a given respiratory phase [71]. Neurons within the preBötC exhibit a high degree of cycle-to-cycle variability with regard to which neurons lead each successive population burst, as well as the timing jitter of spike patterns of individual rhythmic neurons [72]. Based on this finding it can be concluded that neurons are stochastically activated during synchronized population bursts [72,75]. This suggests that there is not a particular cell type that plays a distinct functional role in kindling or terminating the rhythm, although neurons with increased excitability (which includes bursting neurons) tend to discharge earlier. The stochastic phase distribution of preBötC neurons seems to be a result of sparse connectivity within the network, which is consistent with cross-correlation analysis of 10,778 cells recorded in multicellular experiments, indicating a connectivity probability of only 1% [72].

The critical rhythmogenic preBötC neurons are derived from progenitors expressing the transcription factor Dbx1 during development (henceforth referred to as ‘Dbx1 neurons’). Identification of these and other respiratory neurons has greatly facilitated our understanding of synaptic excitation in rhythmogenesis [76^{**},77]. The synaptic interactions between glutamatergic Dbx1-derived neurons dynamically regulate both burst frequency and burst termination [76^{**}]. The volley of action potentials generated during synchronization of Dbx1 neurons depletes the ready-releasable pool of synaptic vesicles (i.e., pre-synaptic depression) resulting in a ‘refractory period’ for activating the subsequent Dbx1 burst (Figure 2) [34^{*}]. Thus, the degree of network synchronization likely influences the magnitude of pre-synaptic depression. Specifically, one might expect that a high degree of network synchrony may lead to a respiratory burst with larger amplitude, resulting in greater pre-synaptic depression and a longer refractory period. Interestingly, this refractory period lasts on average approximately 2 seconds *in vitro*, which is incompatible with breathing frequencies typically observed *in vivo*. This raises an important question: To what extent do synaptic dynamics support the broad range of breathing frequencies observed *in vivo*? The answer is likely complex, since aside from local excitatory and inhibitory mechanisms, the rhythmogenic properties of Dbx1 neurons are influenced by the activity of other microcircuits and a rich cellular milieu of neuromodulatory and glial interactions.

The role of glia in the generation of rhythmic activity

Neuron–glia interactions are increasingly being considered important for generating physiological and pathophysiological rhythmicity in the brain [78]. Although glia do not synchronize with each other through mechanisms of glutamatergic synaptic transmission, they are capable of synchronous activity via other mechanisms such as gap junctions [79]. The resulting Ca^{+2} oscillations or ‘waves’ between interconnected glia have important network functions [80^{*}], and models of astrocyte–neuron interactions suggest that cytosolic calcium dynamics within astrocytes can induce or modulate network synchronization by up- or down-regulating synaptic transmission [81,82].

In the mammalian respiratory network, there is ample evidence that aside from their well-known role in maintaining network homeostasis [83], glia are critical for enhancing the system’s responsiveness to pH, PCO_2 and PO_2 [84^{**},85^{*},86]. There is increasing awareness

that central neuronal networks are hypoxia sensitive [43], which may critically depend on glial cells [85*,86]. Interestingly, the processes involved in the hypoxic response share common pathways with the inflammatory response [87,88]. In this context, not only astrocytes, but also microglia may play important roles, as demonstrated for the release of pro-inflammatory cytokines within the preBötC [87,89]. An important, yet unresolved question is to what extent glial oscillations influence neuronal synchrony during the ongoing respiratory rhythm. Also unresolved is to what extent the role of glial cells is conserved throughout evolution.

Coupled oscillators in respiratory rhythmogenesis and their evolution

The preBötC likely originated from similar rhythm generating structures present in early vertebrates [15*]. In lamprey, the respiratory rhythm generator is located in the pons, in the so-called para-trigeminal respiratory group (pTRG) (Figure 3a). This differs from the respiratory rhythm-generating network of dogfish, carp and tench that seems to span the length of the brainstem [14**] including medullary regions. Evidence for a localized microcircuit comes from bullfrogs: the gill/buccal rhythmogenic network is located in rhombomere 7/8, similar to the preBötC [14**]. However, a fundamental problem in assessing homology is that some markers that define the preBötC, such as somatostatin, are not conserved among different vertebrate species [90]. The use of transcription factors expressed during development may provide more detailed insights into the evolution of neuronal networks as exemplified by comparative studies exploring the evolution of neurons expressing the transcription factor Phox2b [91*] or Atoh1 [77]. But to the best of our knowledge, this comparative information is currently missing for Dbx1 neurons.

In mammals, the preBötC has been considered the *noeud vital*, because of its important role in breathing [92]. Yet, this network is primarily responsible for inspiration, and multi-array recordings indicate that roughly 80% of preBötC neurons are active during the inspiratory phase (Figure 1b) [71,72]. There is increasing evidence that anatomically distinct microcircuits are responsible for generating the other phases of breathing.

A microcircuit in the parafacial respiratory group (pFRG) of the medulla, termed the pFL, generates active expiration (Figures 1c and 3c) [21*]. This respiratory phase is conditionally recruited during high metabolic demand (e.g., exercise). Like the preBötC, the pFL is an autonomous rhythm generator that depends on excitatory mechanisms, and is modulated by inhibition [93]. The pFL is temporally coupled with the preBötC [66], perhaps by a specific neuronal population that expresses the transcription factor Atoh1 [77]. A theoretical model incorporating these inspiratory and expiratory microcircuits hypothesizes a ‘hand-shake’ mechanism where the inspiratory CPG inhibits the expiratory CPG, which in turn excites the inspiratory network via post-inhibitory rebound [62]. However, to mimic the experimental behavior, the frequency of the expiratory CPG must be faster than the inspiratory network, and this has not yet been confirmed experimentally [62]. The pFL is located in rhombomere 4/5 and it has been hypothesized that the pFL is a homologue of the lung burst generator in frogs (Figure 3b) [13], but further research is necessary to better define this potential homology.

An additional mammalian microcircuit, the postinspiratory complex (PiCo), was recently discovered rostral to the preBötC. Optogenetic manipulations suggest that PiCo is both necessary and sufficient for generating postinspiration (Figure 1d) [65**], the dominant expiratory phase of breathing under control conditions. PiCo is an autonomous excitatory rhythm generator that is temporally coordinated with the preBötC through GABAergic inhibition [65**]. The discovery of this additional oscillator suggests that each of the three phases of breathing present in mammals is generated by a distinct excitatory CPG — the so-called ‘triple oscillator hypothesis’ (Figure 3c) [65**]. Although the presence of three rhythmogenic networks has also been predicted in frogs (Figure 3b) [13], it is too early to know whether PiCo is homologous to one of the networks identified in non-mammalian vertebrates.

From an evolutionary perspective, it has been hypothesized that, as breathing evolved from buccal/brachial ventilation to aspiration-driven ventilation, additional oscillatory networks located more caudally and closer to the cranial nerves became involved in rhythm generation (Figure 3) [14**,25*]. In mammals, we hypothesize that the three phases of breathing evolved through the recruitment of separate excitatory rhythmogenic micro-circuits in the medulla. Mechanisms of inhibition have likely evolved in parallel to integrate and coordinate these microcircuits. Indeed, modeling, recording and lesioning experiments implicate extensive inhibitory interactions between distinct compartments of the wider mammalian respiratory network [24*]. Yet, since each microcircuit evolved with its own rhythmogenic mechanisms, rhythmicity can still persist even if stripped from inhibitory control.

In addition to coordinating the numerous pump and upper airway muscles involved in the three phases of breathing, respiratory microcircuits had to become integrated with circuits underlying a multitude of non-ventilatory behaviors (Figure 1e). In mammals, each phase of breathing is associated with distinct behaviors and conditions. Inspiration is expressed during eupnea, gasping and sighing, and is associated with olfaction, whisking [94], arousal [95], and specific emotional conditions [96], while postinspiration is associated with swallowing, vocalization, breath-holding and coughing [97*]. Thus, it is likely that each respiratory microcircuit is specifically connected to the brain regions that control these behaviors. For example, sighs can be generated by distinct connectivity within [96,98], and outside the preBötC [17**]. Similarly, sensory behaviors such as whisking and sniffing [94,99] have distinct connectivity with the preBötC. Continuing to unravel how respiratory rhythm generating microcircuits interact with networks associated with other behaviors will be an important issue for future studies.

The emerging concept is that complex behaviors can be generated through interactions between multiple rhythmogenic microcircuits; each with their own functional properties that allow them to be differentially reconfigured under various modulatory conditions in the intact network. As organisms evolved and the behavioral repertoire increased, these microcircuits required increased coordination. This seems to be established by inhibitory interactions. By comparing rhythmogenic mechanisms and their integration across vertebrates, we may gain critical insights not only into the generation of breathing, but also into the evolution of rhythmicity that is so critical for higher order brain functions.

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Figure 1.

Microcircuits in mammalian respiratory rhythm generation. (a) Isolated preBötC neurons have various activity patterns including bursting ‘pacemaker’, tonic firing, and silent that are largely determined by conductance characteristics including persistent sodium (INaP) and non-specific cation (ICAN) currents (Adapted with permission from [96]). (b) The microcircuit constituting the preBötC consists of glia and both excitatory and inhibitory neurons that primarily fire (>80%) in phase with the inspiratory phase of breathing (Adapted with permission from [71]). (c and d) *In vivo* optogenetic manipulations of respiratory microcircuits coupled to the preBötC. (c) The parafacial lateral region (pFL; blue) is a conditional oscillator that generates active expiration visualized in abdominal (Abd) activity; excitation of this microcircuit elicits an Abd_{EMG} burst and inhibits diaphragm activity (Dia_{EMG}) (Adapted with permission from [100]). (d) The post-inspiratory complex (PiCo; purple) generates postinspiration visualized in the vagus nerve (X N); stimulation of this microcircuit elicits a vagal nerve burst and delays the onset of inspiratory activity observed in hypoglossal motor output (XII N). BötC, Bötzing complex; VRG, ventral respiratory group. (e) The contribution of each microcircuit to the generation of the respiratory rhythm is dynamically regulated and integrated with brain regions controlling distinct respiratory-related and non-respiratory behaviors.

Figure 2.

The anatomy of an inspiratory population burst. Recurrent synaptic excitation among sparsely connected neurons begins to increase action potential rates and build network excitability. As presynaptic Ca^{+2} summates, vesicle release probability increases, strengthening synaptic transmission. Burst generating Ca^{+2} and voltage gated conductances become increasingly active as neurons in the network depolarize, leading to non-linear amplification of action potential rates and network synchrony. The high rate of action potentials generated during the population burst depletes the ready-releasable pool of synaptic vesicles, which reduces synaptic transmission, leading to the loss of synchronization, termination of the burst, and a refractory period for inspiratory activity.

Figure 3.

Vertebrate rhythmogenic respiratory microcircuits. **(a)** Dorsal representation of respiratory rhythm generation in lamprey, which seems to be localized to a single microcircuit located in the pons — the para trigeminal respiratory group (pTRG). The pTRG utilizes excitatory mechanisms for rhythm generation and may be homologous to the mammalian preBötC. Respiratory activity in this primitive vertebrate generates gill movements, which can be observed in vagus nerve (X N) motor output (bottom trace) (Adapted with permission from [25]). **(b)** Dorsal representation of respiratory microcircuits located near cranial nerve nuclei in bullfrogs. It is thought that three distinct respiratory oscillators generate the buccal (orange), lung priming (purple), and lung powerstroke (blue) rhythms. These respiratory activities can be differentially observed in cranial nerve activity, as shown here in facial (VII N), vagus (X N) and hypoglossal (XII N) nerve motor output (Adapted with permission from [14]). **(c)** Sagittal representation of the three identified excitatory rhythmogenic respiratory microcircuits in the mouse. The ‘triple-oscillator’ hypothesis: three anatomically distinct coupled excitatory microcircuits generate the three phases of the mammalian breathing rhythm — the preBötC, PiCo, and pFL, generate inspiration (I), postinspiration (PI), and active expiration (AE), respectively. These breathing phases are observed in motor output from respiratory-related nerves (Abd, abdominal; Ph, phrenic; cVN, cervical vagus nerve), which is precisely coordinated to produce a breath (Adapted with permission from [101]). BötC, Bötzinger complex, NA, nucleus ambiguus; LRt, lateral reticular nucleus.