PREBÖTZINGER COMPLEX AND PACEMAKER NEURONS: Hypothesized Site and Kernel for Respiratory Rhythm Generation

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ABSTRACT

Identification of the sites and mechanisms underlying the generation of respiratory rhythm is of longstanding interest to physiologists and neurobiologists. Recently, with the development of novel experimental preparations, especially in vitro en bloc and slice preparations of rodent brainstem, progress has been made. In particular, a site in the ventrolateral medulla, the preBötzinger Complex, is hypothesized to contain neuronal circuits generating respiratory rhythm. Lesions or disruption of synaptic transmission within the preBötzinger Complex, either in vivo or in vitro, can abolish respiratory activity. Furthermore, the persistence of respiratory rhythm following interference with postsynaptic inhibition and the subsequent discovery of neurons with endogenous bursting properties within the preBötzinger Complex have led to the hypothesis that rhythmogenesis results from synchronized activity of pacemaker or group-pacemaker neurons.

INTRODUCTION

Breathing is a fundamental physiological process produced by movements generated and controlled by efferent signals from the nervous system. Of particular interest is understanding its underlying mechanisms in humans, and a substantial and insightful literature exists describing the phenomenology of the neural control of breathing in human subjects. Unfortunately, such experiments rarely

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illuminate the actions of the brain at molecular, synaptic, cellular, and network levels. Until activity at the level of single neurons is routinely and noninvasively measured in humans, animal models, which permit the application of a broad range of neurobiological techniques, must serve as surrogates. For studies of neural control of breathing, anesthetized or decerebrate cats were the model of choice from 1920 to about 1985; more recently, rodents have become increasingly favored. By 1986, the location of neuronal populations that contain the basic circuits generating respiratory rhythm and pattern in the brainstem and spinal cord were well understood from in vivo experiments (Figure 1), as were interconnections, projections, firing patterns, and basic pharmacology of respiratory neurons found in these regions (see 1–6). However, in vivo preparations are poorly suited for thorough investigations of the synaptic and cellular physiology of neurons, and preparations allowing more detailed studies were needed to advance the understanding of control of respiration at the cellular and network levels. Here we review recent progress identifying critical sites and possible cellular mechanisms involved in the generation of the respiratory rhythm that have relied on novel preparations developed over the past decade, i.e. the in vitro en bloc brainstem/spinal cord preparation (7, 8) and the medullary slice isolated from neonatal rodents (9). These novel preparations generate a respiratory-related rhythm in vitro, and their exploitation has led to useful observations of the cellular and synaptic physiology of brainstem and spinal cord respiratory neurons and has suggested two hypotheses that we discuss: (*a*) Site hypothesis—the preBötzinger Complex is the site for respiratory rhythm generation; (*b*) rhythmogenesis hypothesis—pacemaker or group-pacemaker neurons are the cellular kernel for respiratory rhythm.

RESPIRATORY RHYTHMOGENESIS: LESSONS FROM IN VIVO STUDIES

Neuronal mechanisms cannot be fully studied unless the relevant sites are known. In order to understand how the respiratory rhythm is generated, the site(s) responsible for rhythm generation must be identified with precision sufficient to allow targeted cellular studies. For two millennia, there has been reasonable evidence that respiratory rhythm is generated within the brainstem. Our contemporary view is based on the observations in mammals, including traumatically injured humans, that respiratory movements of the chest and upper airways persist following transection of the neuraxis rostral to the brainstem, but that only upper airway respiratory movements persist following spinomedullary transection (1).

The brainstem contains several anatomically distinct groups of neurons (Figure 1) involved in various aspects of the neural control of breathing, such
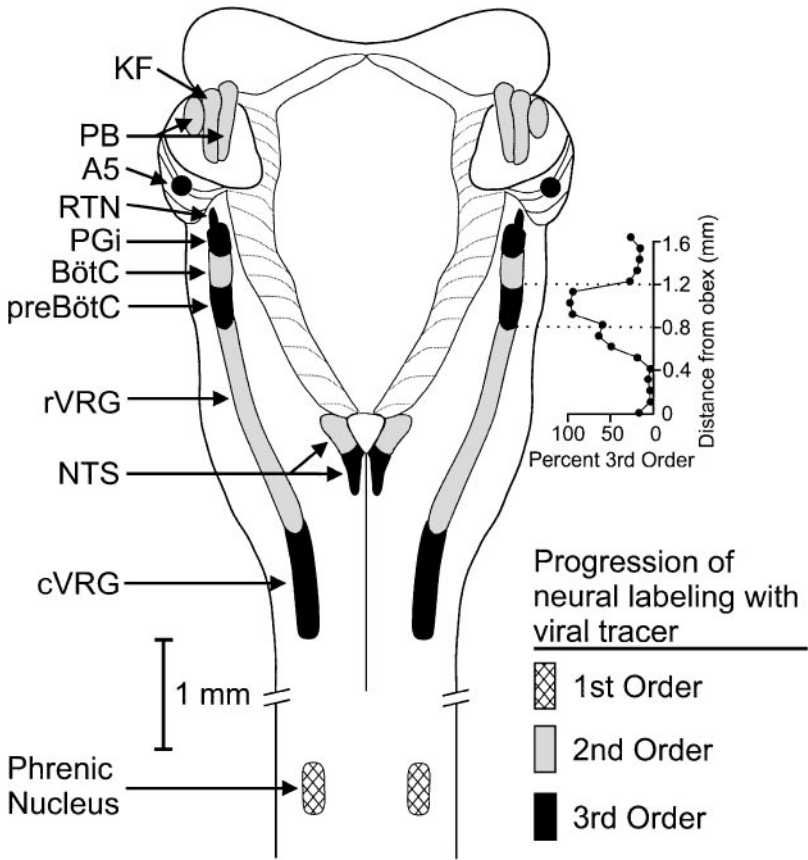
Figure 1   Dorsal view of brainstem and cervical spinal cord, indicating regions involved in control of breathing and progression of labeling with a viral tracer injected into the phrenic nerve. The percentage of labeled 3rd Order neurons (propiobulbar neurons) in the preBötzinger Complex and adjacent regions is plotted in the inset at right. Note that the preBötzinger Complex contains almost 100% 3rd Order neurons, whereas adjacent regions, rVRG, and Bötzinger Complex contain 0–20%. BötC, Bötzinger Complex; cVRG, caudal ventral respiratory group; KF, Kölliker-Fuse nucleus; NTS, nucleus tractus solitarius; PB, parabrachial nuclei; PGi, paragigantocellular reticular nucleus; preBötC, preBötzinger Complex; RTN, retrotrapezoid nucleus; rVRG, rostral ventral respiratory group (modified from 21).

as central chemoreception, afferent signal processing, rhythm generation, and motor pattern formation. Studies both in vivo and in vitro suggest a single site in the rostral medulla as critical in rhythmogenesis. The site, named the preBötzinger Complex (9, 10), is ventral to the compact division of the nucleus ambiguus, midway between the facial nucleus and the obex, caudal to the Bötzinger Complex (containing predominantly expiratory neurons) and rostral to the rostral ventral respiratory group (VRG), which contains predominantly inspiratory bulbospinal neurons (Figure 1).

Although the specific hypothesis concerning the preBötzinger Complex came from in vitro experiments and was widely disseminated in 1991 (9; see below), a retrospective analysis of earlier work in vivo points to a site in the rostral ventrolateral medulla with a central role in rhythmogenesis. Below are some observations from in vivo experiments (cat, rat, or rabbit) consistent with the preBötzinger Complex hypothesis.

1. Correlation analysis of interactions between inspiratory neurons in the ventrolateral medulla reveals that in most coupled pairs, there is an excitatory projection from a rostral neuron to a more caudal neuron (11); Segers et al (11) suggest a rostrocaudal polarization in the VRG column, with more rostral neurons driving more caudal neurons. The firing pattern of neurons located in the most rostral VRG, i.e. the preBötzinger Complex, differs from the firing pattern of neurons in the immediately rostral Bötzinger and immediately caudal VRG regions (12–14). The preBötzinger Complex contains pre-I neurons that fire before the onset of inspiratory activity, early inspiratory neurons, and postinspiratory neurons, all classes of cells proposed to be involved in respiratory phase transitions (1, 15–17). Prior to these studies, most recordings of ventrolateral respiratory neurons were much more caudal (e.g. 18, 19), skewing our view of the types, numbers, and distributions of respiratory neurons.

2. The preBötzinger Complex contains very few bulbospinal neurons and a high percentage of propriobulbar neurons (neurons with axonal arborizations in the medulla), as demonstrated by retrograde transport of fluorescent markers and trans-synaptic transport of pseudorabies virus (9, 20, 21). Thus a region 800 to 1200 μm rostral to the obex in rat, i.e. the preBötzinger Complex, contains almost 100% propriobulbar neurons, whereas the adjacent Bötzinger Complex contain $\approx$20% and the adjacent rostral ventral respiratory group (rVRG) close to 0% (21; Figure 1). These data suggest the ventrolateral respiratory cell column is heterogeneous and that most of the column, with the exception of the preBötzinger Complex, serves as a premotor nucleus controlling respiratory motoneurons.

3. Local cooling or injection of procaine (a reversible Na^+ channel blocker) into the medial area of the nucleus retrofacialis, located proximal to the preBötzinger Complex at its rostral boundary, abolishes respiration in rabbits (22, 23). Bilateral lesion of the retrofacial nucleus by radiofrequency lesions or injections of kainic acid result in cessation of all phasic phrenic discharges in cats (24).

4. Blocking excitatory synaptic transmission in vivo by microinjection of excitatory amino acid antagonists in the retrofacial area of cat (25) or rat (26) brainstem leads to perturbed rhythmogenesis and occasionally apnea. Injection of muscimol (a $GABA_A$ agonist) into the preBötzinger Complex of rats in vivo eliminates respiratory activity (27). Blockade of synaptic transmission by unilateral injection of ω-conotoxin GVIA into the adult cat preBötzinger Complex induces central apnea (28).

5. Respiratory activity persists in rostral cranial nerves after transections of the cat medulla near the obex or after destruction of neurons in the dorsal respiratory group (DRG) by kainic acid or electrocoagulation (29–31). This demonstrates that more caudal parts of the ventrolateral medulla (caudal ventral respiratory group; cVRG) and the dorsal respiratory group (DRG) are not principal sites for rhythmogenesis.

Thus the picture emerging from in vivo studies is that the rostral ventrolateral medulla may indeed have an obligatory role in rhythmogenesis, whereas more caudal and dorsal medullary structures may not.

NEURAL CONTROL OF RESPIRATION: IN VITRO MODELS

In 1984, Suzue reported that an in vitro en bloc preparation of the newborn rat brainstem and spinal cord maintained in vitro generates a spontaneous rhythmic motor output (7). Short rhythmic bursts of activity representing respiratory motor outflow are present in spinal ventral roots and cranial nerves and are in synchrony with upward movements of the (still attached) thorax (see also 8, 32). This in vitro approach was extended with the development of the transverse brainstem slice preparation, where the rhythm-generating circuits remain intact, and an endogenous respiratory-related motor output is present in rootlets of the hypoglossal (XII) nerve (9). These preparations have attracted the interest of increasing numbers of investigators who exploit them to address basic questions regarding the neural control of breathing. Six classes of problems are presently being addressed: (*a*) Where and how is respiratory rhythm generated? (*b*) What are the cellular mechanisms underlying modulation of respiratory rhythm?

(*c*) How is respiratory motoneuronal excitability controlled? (*d*) What are the sites and mechanisms of central chemoreception? (*e*) How are pulmonary afferent signals processed? and (*f*) What are the developmental sequelae underlying the fetal ontogenesis and postnatal maturation of respiratory control mechanisms?

Rhythmogenesis and the preBötzinger Complex

The idea that a compact region in the rostral ventrolateral medulla is essential for rhythmogenesis originated from in vitro studies. A series of transection studies in the newborn rat en bloc in vitro preparation (otherwise impossible in the intact animal) suggested a critical site in the rostral medulla (9). With the en bloc brainstem and spinal cord placed in a Vibratome™, respiratory rhythm persists following serial microsectioning of thin ($\approx$50–75 μm) transverse sections starting at the pontomedullary border and proceeding caudally until the caudal part of the retrofacial nucleus (rostral nucleus ambiguus; rNA) is reached. Further sectioning perturbs and then abolishes the rhythm. Similarly, caudal to rostral microsectioning, starting at the spinomedullary border, does not perturb the respiratory-related rhythm (recorded in the facial nerve) until at a level 200 μm caudal to the caudal part of the rNA. Horizontal sectioning of the medulla, dorsal to nucleus ambiguus, does not abolish the rhythm in the remaining ventral half (9, 33). These lesion experiments place a site critical for rhythm generation ventral to the rNA (RFN), midway between the facial nucleus and the obex, sandwiched between Bötzinger Complex and rVRG. In the newborn rat, this corresponds to a region between $\approx$400 and $\approx$600 μm rostral to the obex (Figure 2). A brainstem slice framed by these sections (350 μm) can generate a respiratory-related rhythmic motor output on

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Figure 2 Sagittal and transversal view of the location of the preBötzinger Complex. *A* Sagittal view of the ventral medulla showing the preBötzinger Complex and neighboring regions in the newborn rat. Note that the preBötzinger Complex is ventral to the nucleus ambiguus approximately midway between the facial nucleus and the obex. cNA, caudal nucleus ambiguus; LRN, lateral reticular nucleus; rNA, rostral nucleus ambiguus; rVRG, rostral ventral respiratory group; VII, facial nucleus (modified from 4). *B* Transverse section of rat brainstem at the level of the preBötzinger Complex (see *A*). Data from three types of experiments give overlapping localization of the preBötzinger Complex to the region ventral to NA. + symbols are locations of respiratory-modulated neurons in neonatal rat en bloc brainstem (modified from 32). 0 symbols are sites where injection of AMPA receptor antagonist slows then stops respiratory motor outflow in neonatal rat brainstem slice (modified from 34). X symbols represent 3rd order neurons in adult rats labeled after pseudorabies virus injections into diaphragm or phrenic nerve (modified from 21). These sites have been mapped by scaling the larger adult brainstem to the size of the neonatal brainstem and aligning with the NA as the reference point. *Inset* Triangles indicate sites where lesions affect gasping but not eupnea in adult rats. *Circles* indicate sites affecting eupnea but not gasping in adult rats. (Sites redrawn from 105.)
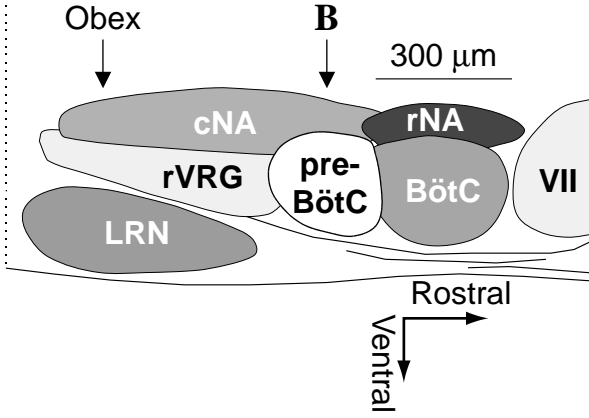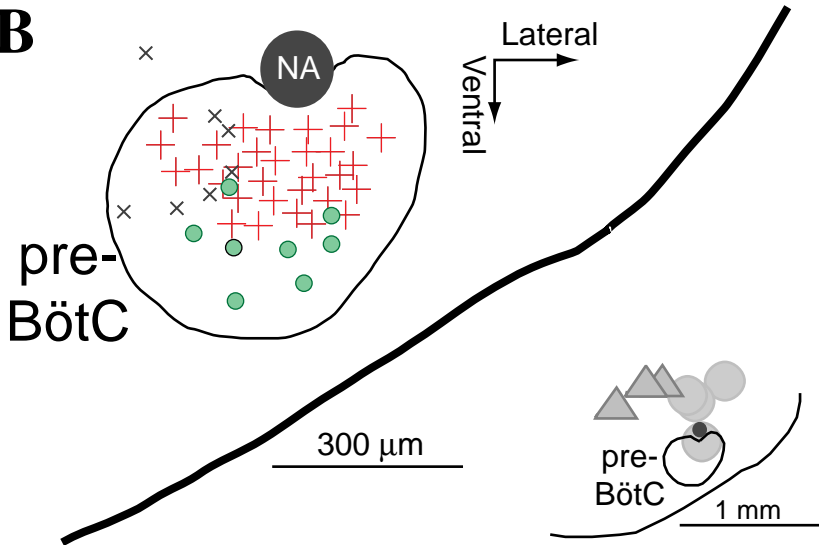
hypoglossal nerves (9). The critical site was named the preBötzinger Complex. [Given conventional neuroanatomical terminology, this region, caudal to the Bötzinger Complex, should be called the postBötzinger Complex. However, this region was identified and named by physiologists (10) who put primacy in this site as the (putative) kernel of respiratory rhythm, preceding all other sites in the timing of activity.] The importance of this region for rhythmogenesis is confirmed by injection of CNQX (a non-NMDA receptor antagonist) into the preBötzinger Complex in the slice, which completely abolishes the respiratory rhythm recorded from the hypoglossal roots (9, 34; Figure 2). These observations also establish that synaptic interactions between propriobulbar respiratory neurons in the preBötzinger Complex involve excitatory amino acids acting on AMPA channels, whose normal function is essential for rhythm generation. Conversely, synaptic inhibition mediated by the inhibitory amino acid neurotransmitters GABA or glycine is not essential for rhythm generation in the neonatal rodent, because GABA/glycine antagonists or changes in extracellular $[Cl^-]$ do not abolish respiratory rhythm (26, 35, 36).

To understand the cellular basis of rhythmogenesis, the electrophysiological, biochemical, and synaptic properties of neurons within the preBötzinger Complex need to be delineated; this work is still in its early stages. The firing pattern of neurons in the rostral ventrolateral medulla, including the VRG and the preBötzinger Complex, has been analyzed in relation to respiratory cycle phase. Several distinct respiratory-related neuron types are observed, including neurons with endogenous bursting properties (32, 33, 37–40). The intrinsic properties of inspiratory neurons found in the VRG and preBötzinger Complex reveals that different types of inspiratory neurons have A-like, I_h-like, high- and low-voltage activated Ca^{2+}, and persistent Na^+ currents (41–45). Finally, the synaptic connections between certain subtypes of respiratory neurons in the VRG within the brainstem in vitro are beginning to be elucidated (46–48). The functional phenotype (rhythm-generating, pattern-forming, premotor) of those neurons with these various properties is still unknown. Nonetheless, many models for respiratory rhythm generation have been proposed for which such properties are stipulated to be present in neurons with particular functional roles (e.g. 4, 15, 49).

Neuromodulators Affecting Rhythm

In in vitro preparations, a large number of neuromodulators, including 5-HT, histamine, acetylcholine, glutamate, substance P, thyrotropin-releasing hormone (TRH), noradrenaline, GABA, glycine, and [Met5] and [Leu5] enkephalin affect the frequency and/or amplitude of the respiratory rhythm (50–64). Several of these neuromodulators (GABA, catecholamines, TRH, substance P, μ-opioids) modulate respiratory frequency by acting on neurons within the preBötzinger Complex, activating $G_{i/o}$-protein-dependent mechanisms (59, 60,

64). For example, TRH increases respiratory frequency (50, 59, 64, 65), and putative group-pacemaker neurons (see below) are depolarized postsynaptically by this small neuropeptide. Thus postsynaptic depolarization of neurons intimately involved in rhythm generation may be a general cellular mechanism for increasing frequency (57, 59).

Synaptic Drive to Respiratory Motoneurons

Whole-cell patch recordings from respiratory-modulated spinal and cranial motoneurons have provided insights into the pharmacology and modulation of endogenous respiratory synaptic drive. The inspiratory synaptic drive to hypoglossal and phrenic motoneurons is mediated by an excitatory amino acid mainly acting on AMPA receptors. This excitatory synaptic drive is under pre- and postsynaptic modulatory control through activation of 5-HT, TRH, noradrenaline, adenosine, and metabotropic glutamate receptors (34, 66–75). Premotor neurons projecting to spinal and cranial respiratory motoneurons are located in the VRG and DRG (21, 76). The electrophysiological properties of the neurons located in the DRG have been investigated by combining retrograde labeling of premotor neurons in the ventral nucleus tractus solitarius (vNTS) (the anatomical location of the DRG) and intracellular recordings. These neurons are under modulatory control by $GABA_B$ receptors, which may control repetitive firing activity and participate in presynaptic inhibition (77–80).

Central Chemoreception and Pulmonary Reflexes

Central chemosensitivity remains functional, although blunted, in vitro, and a significant number of ventral medullary neurons in en bloc preparations are intrinsically chemosensitive (81–87). A decrease in pH (induced by increasing $[CO_2]$ in the superfusate) can depolarize or hyperpolarize respiratory neurons via postsynaptic actions (87). These responses are seen only in neurons with dendrites extending to the surface of the medulla, suggesting that the channels mediating chemosensitivity are located in distal dendrites.

When the lungs are left attached to the brainstem-spinal cord in vitro preparation, the classical Breuer-Hering expiratory prolongation (88, 89) and inspiratory shortening (N Mellen & JL Feldman, personal communication) reflexes in response to lung inflation can be elicited. The expiratory prolongation requires activation of $GABA_A$ receptors (88, 90).

Maturation of Respiratory Control Mechanisms

The use of in vitro preparations from animals of different pre- and postnatal ages reveals developmental changes in such properties as hypoxia tolerance, network connections, and neuromodulation (73, 91–99). Respiratory motor activity emerges early in the third trimester ($\approx$E17–E18 in rat) (91, 94), preceded by widespread rhythmic motor patterns that may represent activity in generalized primordial rhythm-generating networks (100). Rhythmic activity

in these perinatal animals under in vitro conditions can be maintained by anaer-
obic metabolism and depends on high levels of glucose in the bathing solution,
whereas aerobic metabolism dominates in the adult animals (92, 101).

The role of glycinergic inhibition in rhythmogenesis at different develop-
mental stages is controversial. Slices from mice up to the age of P21 display
rhythmogenesis (73, 99), and a study using a tilted-sagittal slice from rats of
various postnatal ages suggests that glycinergic synaptic inhibition is essential
for rhythmogenesis in animals older than 15 days (97). However, block of
glycinergic transmission does not abolish respiratory rhythm in mice between
P0 and P22 in the transverse slice in vitro (99). The major difference between
these two studies is the amount of respiratory-related structures contained in
the preparation; the blockage of rhythm in the tilted-sagittal slice in older ani-
mals may be due to an effect of glycinergic antagonist on neuronal structures
upstream to the preBötzinger Complex.

CRITIQUE OF IN VITRO MODELS

The respiratory-related motor rhythm in in vitro preparations differs from that
in intact animals. Under typical experimental conditions, the rhythm in vitro
is slower than the resting frequency in vivo in the newborn rat: 9 cycles/min
in vitro versus 46 cycles/min in vivo (32). This marked change in frequency is
partly explained by removal of afferents (vagotomy in particular, which removes
afferent signals from the lungs) and the low temperature under which the in
vitro preparations are kept ($\approx 28°C$; at body temperature, respiratory rhythm
is present at higher frequency but persists for less than one hour) (32). Thus
bilateral vagotomy in newborn rats in vivo decreases respiratory frequency
to a level comparable to the frequency in vitro at the same temperature, and
decreasing the temperature from 35 to 27°C in vitro decreases the frequency
by $\approx 40\%$ (32, 102).

The pattern of discharge on the phrenic and cranial nerves during inspiration
changes from an incrementing pattern in vivo to a decrementing pattern in vitro.
Since the discharge pattern of motor nerves during gasping is also decrementing
and at a low frequency, the rhythm in vitro could represent gasping (103).
However, a careful analysis suggests otherwise.

1. Lesion studies demonstrate that the preBötzinger Complex is essential for
   eupnea, not gasping. Respiratory-related activity in the slice disappears
   following complete bilateral removal of the preBötzinger Complex (104).
   Subsequent exposure of the slice to anoxic conditions elicits a different,
   more gasp-like pattern of motor output. These discharges differ from the
   normal rhythm in the slice by having a steeper onset and a larger amplitude.
   In rats, electrolytic lesions of the lateral tegmental field dorsomedial to the

nucleus ambiguus (gasping center; 103), quite distant from the preBötzinger Complex (which is ventral and ventromedial to nucleus ambiguus), abolish gasping but not eupnea (103, 105; Figure 2). Although we consider this evidence against the idea that rhythm in the slice is gasping, St John and colleagues reach a different conclusion (103); because the preBötzinger Complex is the source of rhythm in the slice and because they surmise that their gasping center is synonymous with the preBötzinger Complex, the rhythm must be gasping. We believe that their conclusions are not warranted by their data: St John and colleagues (103, 105) do not recognize the obvious distinction between the lateral tegmental field dorsomedial to the nucleus ambiguus (gasping center) and the preBötzinger Complex, which is ventral and ventromedial to the nucleus ambiguus (Figure 2). This is particularly curious because in their own experiments, lesions closer to the preBötzinger Complex abolish eupnea (see figure 4 in Reference 105). So even if there is a gasping center, it is not the preBötzinger Complex.

2. Removal of afferent inputs, such as vagotomy, have significant effects in vivo; thus a transformed pattern in vitro in the absence of all afferent input should be expected. The decrementing phrenic nerve motor discharge envelope in vitro may be induced by the loss of afferent input from the lungs, because vagotomy in vivo in young animals (<4 days) transforms respiratory motor discharge from an augmenting to a decrementing pattern (32; for example, see 106). In addition, arterially perfused in vitro preparations of adult mice or guinea pigs show either incrementing discharge envelopes or change from incrementing to decrementing envelopes throughout the recording period while the periodicity remains stable (107, 108). These observations suggest that the decrementing discharge envelope is a result of a transformed eupneic pattern and does not reflect a radical shift towards an independently generated gasp-like pattern.

3. Severe hypoxia or anoxia in the preBötzinger Complex could underlie gasping, which follows severe hypoxia in vivo, but this is not the case under standard in vitro conditions either in en bloc or in slice preparations. Careful measurements of O_2 partial pressure and pH in en bloc preparations show that the neurons located more superficial than 700 μm (including the preBötzinger Complex) are functioning under aerobic conditions (109). In slices, the diffusion distances for O_2 to deeper structures is greatly reduced due to the large rostral and caudal surfaces, and the rhythm in slices as thin as 350 μm is similar to that observed in en bloc preparations.

In conclusion, in vitro preparations have become valuable models for studies of respiratory rhythmogenesis and cellular physiology of respiratory neurons.

We believe that detailed data at the cellular and molecular level obtained in these in vitro preparations will set the stage for understanding respiration in more intact animals, provided that care is taken in extrapolating data obtained from such reduced systems.

## CELLULAR BASIS OF RESPIRATORY RHYTHM GENERATION: THE PACEMAKER HYPOTHESIS

Oscillations of neural activity ranging from 500/s to 1/day are a basic feature of brain function. In brain regions where neural oscillations are fairly well understood, a detailed description of intrinsic neuronal properties, synaptic physiology, and connections have been crucial in forming constrained models that reproduce experimental data. One example is the synchronized oscillations in the thalamus, which result from interactions between intrinsic conductances and network interactions among thalamocortical and perigeniculate neurons (110, 111). The same kind of detailed information about the respiratory neurons and their interconnections is lacking, and the mechanisms underlying respiratory rhythmicity are therefore unknown. However, each investigator has a favorite hypothesis and many models have been proposed (4, 15–17, 26, 44, 49, 59, 112–114). Universally these models are based on incomplete cellular data and assumptions about connectivity and remain highly speculative, but they have been useful in focusing efforts to design experiments relevant to their elaboration and testing.

Pacemaker Neurons

The notion that neurons with pacemaker properties (relatively slow oscillations of membrane potential by cyclic activation and inactivation of intrinsic conductances or intracellular messengers, e.g. I_{Ca}, cAMP) may be involved in respiratory rhythmogenesis, initially speculative (e.g. 115), became a serious hypothesis following the observation that attenuation of Cl^--mediated synaptic inhibition does not block the respiratory-related rhythm in the en bloc in vitro preparation (26, 35, 36). Thus when the extracellular $[Cl^-]$ is reduced (36) or set at nominally zero (26), the burst discharge pattern of respiratory neurons is augmented, but the underlying rhythm in respiratory motor nerve output persists. With separate or subsequent antagonism of inhibitory amino acid receptors, i.e. $GABA_A$, $GABA_B$, and/or glycine, the rhythm remains. Other types of postsynaptic inhibition or even presynaptic inhibition could be involved, but these observations suggest that conventional reciprocal postsynaptic inhibition between groups of respiratory neurons is not the cellular basis for rhythm, making neurons intrinsically capable of generating cyclic discharges, i.e. pacemaker neurons, reasonable candidates. With the subsequent identification of

the preBötzinger Complex as a putative site for rhythmogenesis, an obvious test was to determine if pacemaker neurons were present there, and they were (9). When respiratory-related rhythm in the slice is suppressed, a subclass of inspiratory-modulated neurons within the preBötzinger Complex displays oscillatory discharges in a membrane voltage window between -45 and -55 mV. The expression in these neurons of properties consistent with voltage-dependent channels with a region of negative slope in the current-voltage relationship is a characteristic signature of endogenously oscillating pacemakers (4, 5). At more hyperpolarized membrane potentials (-60 mV) these neurons show no spike activity, and when recorded from in rhythmic slices, exhibit trains of low amplitude EPSPs in phase with the inspiratory discharges on the XII nerve. The existence of neurons capable of endogenous bursting has been further substantiated by experiments showing that neurons in the preBötzinger Complex produce regular rhythmic bursts when synaptically isolated by a low-Ca^{2+}/high-Mg^{2+} solution (40). The conductances that give rise to the bursting and, more importantly, the synaptic connections among the pacemaker neurons and with the rest of the respiratory network are yet to be determined. Critical tests of the obligatory role of pacemaker neurons in respiratory rhythmogenesis will probably require such information.

The discovery of pacemaker neurons in the preBötzinger Complex led to the hypothesis that a hybrid network of pacemaker and more mundane neurons were responsible for rhythmogenesis (4, 26; Figure 3A). Thus pacemaker neurons receive tonic excitatory inputs necessary to bring the membrane potential into the voltage window where bursting occurs; the characteristic current-voltage profile (negative slope conductance) provides a means to control the bursting frequency by tonic depolarizing or hyperpolarizing inputs. Because permissive inputs may be necessary to maintain bursting, these neurons are classified as conditional bursting pacemakers. Synchronization of their oscillatory activity is proposed to be a consequence of recurrent excitatory synaptic coupling. Because of the variability of intrinsic and afferent properties, there may be a dispersion in the membrane potential and consequently the functional state (quiescent, oscillatory, beating) of different pacemaker neurons. As a wave of excitation at the onset of inspiration spreads through the network of coupled pacemaker neurons, the differences in state may lead to a spatial and temporal dispersion of onset times of spiking within the population, with some neurons bursting early and some later. In this scheme rhythmogenesis is a direct result of endogenous pacemaker properties of a certain class of neurons, but tonic excitatory input to and interconnections among pacemaker neurons is a prerequisite for synchronization of the rhythm, with inhibitory inputs regulating the shape, duration, and interval between bursts.
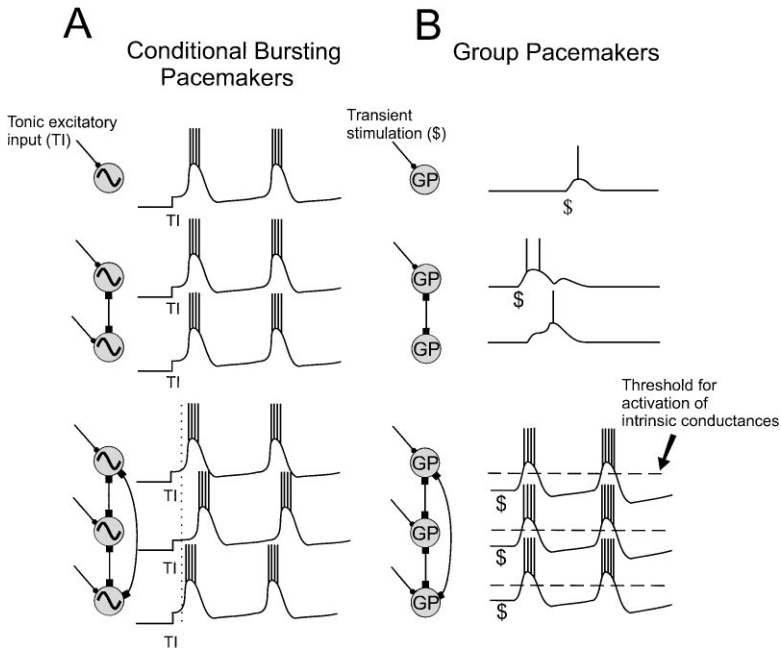
Figure 3   Two models for respiratory rhythmogenesis: *A* Pacemaker model. When synaptically isolated, single conditional bursting pacemaker neurons are capable of bursting by virtue of non-linear intrinsic properties. Tonic excitatory input may be necessary to depolarize the neurons to a level where bursting occurs, as indicated. The oscillation of two coupled pacemaker neurons would synchronize through mutual excitatory connections. The oscillation of many coupled pacemaker neurons would synchronize, although there could be a temporal and amplitude dispersion of the on- and offset of individual bursting because the strength of the tonic inputs might vary. *B* Group-pacemaker model. Single group-pacemaker neurons are incapable of intrinsic bursting, either when stimulated ($) by transient (or tonic) excitatory input or intracellular current. Two coupled group-pacemaker would excite each other through recurrent synaptic connections, but the associ-ated depolarization would be too small for activation of burst firing and subsequent long-lasting hyperpolarization. Transient (or tonic) stimulation of several coupled group-pacemaker neurons would lead to self-sustained burst firing, as phasic synaptic input would recruit depolarizing and hyperpolarizing intrinsic conductances.

Onimaru and coworkers have described a group of neurons (Pre-I neurons) with endogenous bursting properties that fire preceding and succeeding cervical root (C4) inspiratory activity but are silent during inspiration (38). These neurons are proposed to be the primary respiratory rhythm-generating neurons by virtue of their endogenous bursting properties (39, 114). However, this class of cells is generally found more rostral in the medulla than the preBötzinger Complex in the en bloc preparation and has yet to be observed (or reported) in

the oscillating slice preparation (40, 44), raising doubts about their obligatory role in rhythmogenesis.

Group-Pacemaker Neurons

A different class of neurons with special voltage-dependent conductances in the preBötzinger Complex may underlie respiratory rhythmogenesis (44, 59). Three different types of inspiratory neurons (types 1–3), located rostral to and within the preBötzinger Complex, can be identified based on their characteristic response to current injection and membrane potential trajectory throughout the respiratory cycle (44). Approximately 50% of the type-1 neurons have endogenous bursting properties and an A-like current. Type-2 neurons have an I_h-like current, and type-3 a low-voltage-activated Ca^{2+}-like current. All three types receive excitatory synaptic input before the onset of the XII nerve inspiratory burst, but with very different latencies (type-1: ≈400 ms; type-2: ≈170 ms; type-3: ≈100 ms). Of interest are the type-1 neurons, with the earliest onset prior to inspiration, because they exhibit a prolonged postinspiratory hyperpolarization that lasts throughout expiration. This long-lasting hyperpolarization does not reverse at hyperpolarized potentials (JC Rekling, unpublished data) and therefore appears to result from activation of an intrinsic conductance by the phasic depolarization produced by the inspiratory synaptic drive. Interestingly, long-lasting hyperpolarizations in type-1 neurons are not elicited by transient somatic current injection, i.e. with the same duration as an inspiratory burst, suggesting that the currents flowing or transmitters released during inspiration recruit a different set of intrinsic conductances than current injection at the soma. Thus the oscillation of membrane potential in these neurons is driven by depolarization at the onset of inspiration triggered by excitatory input, and hyperpolarization between inspirations due to an intrinsic conductance activated by the inspiratory depolarization (Figure 3B). Given these properties, such neurons could form a group-pacemaker driving respiratory rhythm (59). In the group-pacemaker hypothesis, type-1 neurons, mutually interconnected by excitatory connections, initiate inspiration by generating a population burst of activity from positive, i.e. recurrent, feedback. Endogenous membrane properties would then amplify the depolarizing action of recurrent synaptic input before and at the onset of inspiration. During inspiration, various conductances would be activated in these neurons (e.g. I_{Ca^{2+},K^+}, or electrogenic pumps) that terminate the inspiratory burst and produce an obligatory and prolonged hyperpolarization. During the initial part of the long-lasting hyperpolarization, type-1 neurons remain below threshold for firing; as these neurons slowly depolarize during expiration, the most excitable type-1 neurons start to spike, leading to another positive feedback cycle initiating the next inspiration. Respiratory frequency would be regulated by tonic excitatory and inhibitory inputs

to type-1 neurons by affecting the time it takes for their membrane potential to reach threshold for spikes and for activating conductances that terminate their bursts. The fact that TRH depolarizes type-1 neurons postsynaptically and increases the frequency of the rhythm (59) is consistent with this latter proposal. An important distinction of the group-pacemaker hypothesis is that individual neurons need not generate cyclic burst discharges when synaptically isolated or in response to tonic (or transient) input and that rhythm generation is a property that emerges from the recurrent interactions among these neurons. The intrinsic conductances serve to phase-lock mutually interconnected type-1 neurons during inspiration and expiration, and phasic recurrent synaptic input may be necessary for full activation of these properties.

CONCLUSION

In vivo and in vitro experiments point to a single site in the rostral ventrolateral medulla, the preBötzinger Complex, as critical for respiratory rhythmogenesis. The development of preparations that continue to generate a respiratory-related rhythm in vitro has been instrumental in forming and testing this hypothesis. The persistence of rhythmogenesis after removal of Cl$^-$-mediated inhibition and the presence of conditional bursting pacemakers and putative group-pacemakers in the preBötzinger Complex have led to the hypotheses that respiratory rhythm may be generated by interconnected pacemaker cells or group-pacemaker cells. In the long run, the impetus that these hypotheses have given investigators to exploit in vitro preparations and to tackle fundamental problems of neural control of breathing may be as important as whether these hypotheses are correct.

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