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TOPICAL REVIEW

Facing the challenge of mammalian neural microcircuits: taking a few breaths may help

Jack L. Feldman and Kaiwen Kam

Systems Neurobiology Laboratory, Department of Neurobiology, David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, CA, USA

Abstract Breathing in mammals is a seemingly straightforward behaviour controlled by the brain. A brainstem nucleus called the preBötzinger Complex sits at the core of the neural circuit generating respiratory rhythm. Despite the discovery of this microcircuit almost 25 years ago, the mechanisms controlling breathing remain elusive. Given the apparent simplicity and well-defined nature of regulatory breathing behaviour, the identification of much of the circuitry, and the ability to study breathing *in vitro* as well as *in vivo*, many neuroscientists and physiologists are surprised that respiratory rhythm generation is still not well understood. Our view is that conventional rhythmogenic mechanisms involving pacemakers, inhibition or bursting are problematic and that simplifying assumptions commonly made for many vertebrate neural circuits ignore consequential detail. We propose that novel emergent mechanisms govern the generation of respiratory rhythm. That a mammalian function as basic as rhythm generation arises from complex and dynamic molecular, synaptic and neuronal interactions within a diverse neural microcircuit highlights the challenges in understanding neural control of mammalian behaviours, many (considerably) more elaborate than breathing. We suggest that the neural circuit controlling breathing is inimitably tractable and may inspire general strategies for elucidating other neural microcircuits.

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Corresponding author J. L. Feldman: Systems Neurobiology Laboratory, Department of Neurobiology, David Geffen School of Medicine at the University of California Los Angeles, Box 951763, Los Angeles, CA 90095-1763, USA. Email: feldman@ucla.edu

Abbreviations AP, action potential; BötC, Bötzinger complex; CPG, central pattern generator; Dbx1, developing brain homeobox 1; EB, endogenous bursting/burster; IBI, interburst interval; I-burst, inspiratory burst; I_{CAN}, Ca^{2+}-activated non-specific cation current; I_{NaP}, persistent Na^{+} current; NK1R, neurokinin 1 receptor; preBötC, preBötzinger Complex; SST, somatostatin; VRC, ventral respiratory column; XII, hypoglossal; XIIn, hypoglossal nerve.

Introduction

'A few of the earlier natural philosophers have dealt with respiration; some of them have offered no explanation why this phenomenon occurs in living creatures; others have discussed it without much insight, and with insufficient experience of the facts.'

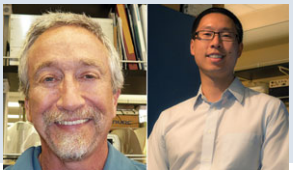
Aristotle, *On Respiration*, ~350 BC

'Hopefully, not us.'

The authors, AD 2014

The complexity of the mammalian brain spans the entire scale of biological function from molecules to behaviour (Koch, 2012). Least understood are the mechanisms transforming the activity of networks of

Jack L. Feldman is Distinguished Professor of Neurobiology at the David Geffen School of Medicine, University of California Los Angeles. His research interests are in the neural control of breathing and the physiology of mammalian microcircuits. **Kaiwen Kam** is an Assistant Project Scientist in Dr Feldman's laboratory and will soon be Assistant Professor of Cell Biology and Anatomy at the Chicago Medical School at Rosalind Franklin University. His research focuses on understanding how cellular and synaptic mechanisms give rise to emergent properties in neural circuits controlling rhythmic movements in mammals.



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neurons into the vast array of distinct behaviours, each typically a set of movements in response to environmental stimuli or internal state (BRAIN Working Group, 2013). We consider the network of all neurons that participate in a given behaviour to be its *neural circuit*. In mammals, neural circuits are composed of large, heterogeneous populations of hundreds to thousands of neurons dynamically interacting with each other over time scales of milliseconds to years within and across brain regions, in topologically complex (and, at present, computationally refractory) networks (Fig. 1; Koch, 2012). The necessity of generating and synchronizing activity and coordinating signal processing within and across populations presents novel and perhaps unique challenges for understanding mammalian neural circuits (Ainsworth *et al.* 2012; Kumar *et al.* 2013).

One approach to managing this complexity is to break the neural circuit into computational modules (Carandini, 2012; Koch, 2012), now often referred to as *neural microcircuits* (Grillner *et al.* 2005). This modular approach maps a signal-processing function to a smaller population of neurons within a defined region of brain that is, *de facto*, more amenable to analysis and perturbation (Koch, 2012). Commonly, since even small brain regions have thousands of neurons, microcircuits are further abstracted by lumping the properties of presumptively homogeneous categorical neuronal populations into representative 'neurons' (Fig. 1*A*; Grillner *et al.* 2005; Kumar *et al.* 2013). These 'neurons' are connected together and depicted in wiring diagrams that convey in a conceptual model how categorical neuronal elements might interact (Grillner *et al.* 2005). Such abstractions facilitate an intuitive and easy to grasp understanding of the role of microcircuits in behaviour.

Despite the advantages of such abstractions, compromises in detail can mask consequential aspects of microcircuit physiology, disconnecting microcircuits from underlying molecular and cellular mechanisms that implement the computation and breaking the link that microcircuits form between genes, single neurons and behaviour. Determining which details are consequential and which can be safely discarded is a fundamental issue (Abbott, 2008). To what extent can the microcircuit and the computation it performs be delimited as a module (Carandini, 2012; Koch, 2012) if there are lateral and recurrent, excitatory and inhibitory connections that cross modular boundaries (Horton & Adams, 2005)? Does the microcircuit perform different neural computation(s) across behaviours and states (Briggman & Kristan, 2008; Carr & Frank, 2012)? Is there consequential diversity in neuronal properties, e.g. protein expression, physiology, morphology, or connectivity, within apparently homogeneous populations, and are these identities stable over time (Fig. 1; Markram *et al.* 2004; Grillner *et al.* 2005)? How are results from *in vivo* and reduced preparations, different developmental time points, or different strains or species compared and reconciled across experimental conditions? Can microcircuit dynamics, i.e. the flow of activity that underlies signal generation and processing, be deduced from (static) wiring diagrams with lumped categorical elements (Carandini, 2012; Morgan & Lichtman, 2013; Kopell *et al.* 2014)? How sensitive is the microcircuit to

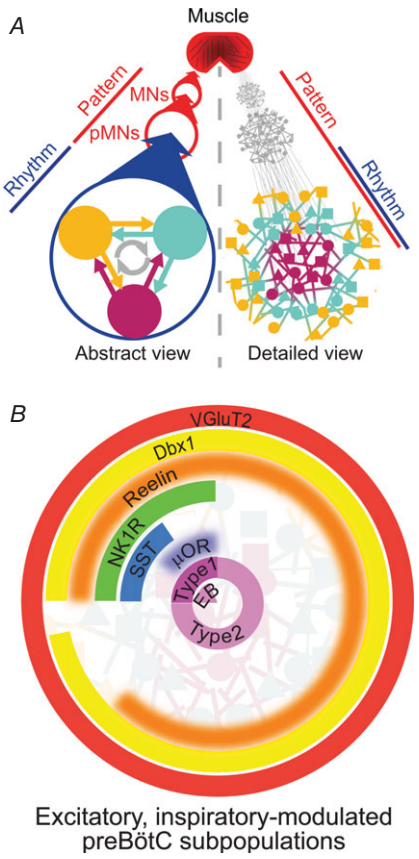

Figure 1. Abstract representations of the respiratory CPG
A, left, a common wiring diagram for the minimal respiratory neural circuit from rhythmogenesis to muscle involving several interconnected subpopulations, e.g. SST^+, $NK1R^+$, Type 1, within the rhythmogenic preBötC, projecting to premotoneuronal (pMN) and motoneuronal (MN) populations innervating respiratory muscles, e.g. tongue and diaphragm. Rhythm- and pattern-generating functions common to all CPGs are assumed to be segregated. Right, these representations obscure the microcircuit properties required for understanding mechanisms of neural dynamics and may overlook microcircuit connectivity, neuronal heterogeneity (represented by different shapes) and multifunctionality, such as the overlap of rhythm- and pattern-generating functions within the preBötC. *B*, proportional representations of molecularly and functionally defined excitatory, inspiratory-modulated preBötC populations. Radially overlapping concentric arcs represent populations with multiple properties. Blurred arcs, e.g. Reelin, are estimated or assumed. Data are extrapolated from rat and mouse. Functional categories, e.g. Types 1 and 2, and EB (endogenous bursters), are described in the text.

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the spatiotemporal pattern of inputs (Briggman & Kristan, 2008; Marder *et al.* 2014)? How is microcircuit output distributed during behaviour (Kumar *et al.* 2013)?

Recent studies of the neural circuits controlling breathing and, particularly, the microcircuit generating respiratory rhythm, are unmasking the limitations of common abstractions and assumptions applied to microcircuits. We describe the multidimensional problem of defining the rhythmogenic microcircuit in breathing and its constituent elements and functions; how hypotheses and models based on conceptually straightforward mechanisms are unlikely to explain respiratory rhythmogenesis; and the downstream transformations of rhythmic activity that can obscure the role of the microcircuit in behaviour. These issues invalidate the simplistic view of respiratory rhythm generation as the product of axiomatic mechanisms, such as pacemakers, inhibition and bursting, in 'neurons' that stand in for well-defined, clearly categorized populations. Instead, the breathing rhythm is an emergent property of the microcircuit (Suki *et al.* 2011), dependent on neuronal heterogeneity, recurrent and lateral connections, and other consequential details. The minimal neural circuit controlling breathing therefore permits close examination of the physiology of mammalian microcircuits in a simpler (but not simple) system that nonetheless preserves emergent properties that may be unique to mammals.

Why breathing?

Behaviour provides a critical, but broad constraint for adjudicating the relevance of neural activity, identifying regions constituting the neural circuit, and parsing out the component neural computations (Carandini, 2012). Basic rhythmic movements, such as breathing, walking and chewing, are among the simplest behaviours, with the capacity to operate independently of sensory input and with limited degrees of freedom in a stereotyped, well-defined, and measurable motor output (Feldman & Grillner, 1983; Grillner, 2006; Grillner & Jessell, 2009). The neural computations performed by circuits controlling rhythmic movements, known as central pattern generators (CPGs), are essentially limited to: (1) rhythm generation – the transformation of tonic drive (without the need for rhythmic afferent input) into the production of repetitive signals that determine the period of the cycle; and (2) pattern generation – the distribution and modulation of this signal to activate participating muscles with appropriate force and timing. Rhythmic motor patterns can range from the basic, bilaterally symmetric, finely tuned (for energy efficiency) breathing pattern with an active inspiratory phase and passive expiratory phase at rest, to more complex patterns, such as quadrapedal locomotion involving gait-dependent left–right, forelimb–hindlimb and flexor–extensor alternation or synchrony as well as coordination of proximal and distal muscles of each limb around multiple joints, i.e. hip, knee and ankle (Grillner, 2006; Andersson *et al.* 2012; Bachmann *et al.* 2013; Talpalar *et al.* 2013).

Separable microcircuits are hypothesized to mediate rhythm generation and patterning in mammalian CPGs (Feldman, 1986; McCrea & Rybak, 2008). While great progress has been made in illuminating patterning microcircuits (Grillner & Jessell, 2009; Garcia-Campmany *et al.* 2010; Kiehn, 2011), for most mammalian rhythmic behaviours, including locomotion and mastication, rhythmogenic microcircuits have resisted discovery (Grillner & Jessell, 2009). The coarse neuroanatomy of the CPGs for these behaviours is incomplete, and where, beyond being in spinal cord or brainstem, the rhythmic kernel is localized, if it is localized at all, has not yet been definitively determined (Grillner & Jessell, 2009). Rhythmogenic microcircuits in mammals should meet the following four criteria.

(1) Inhibition, silencing, or destruction of key elements of the microcircuit significantly perturbs or even stops rhythm.
(2) The microcircuit projects oligosynaptically to appropriate motoneuronal populations.
(3) Modulatory afferents affect frequency.
(4) The isolated microcircuit (when sufficiently driven) generates rhythmic activity.

Unique among mammalian CPGs, the respiratory CPG has a *localized* (<1 mm^3/side in rodents; ~ 1.5 mm^3/side in humans) rhythmogenic microcircuit, the preBötzinger Complex (preBötC; Feldman *et al.* 1990; Smith *et al.* 1991; Schwarzacher *et al.* 2011). Acute silencing of a targeted subpopulation preBötC neurons abolishes the breathing rhythm in awake, behaving rodents (criterion 1; Tan *et al.* 2008). A well-established *in vitro* slice preparation containing the preBötC, when sufficiently driven (usually with elevated extracellular K^+), recapitulates key aspects of rhythmic inspiratory behaviour, including motor nerve output, and rhythmic activity persists in the preBötC when it is further isolated by microdissection from surrounding tissue (criterion 4; Smith *et al.* 1991; Johnson *et al.* 2001). preBötC neurons generate rhythmic bursts of action potentials (APs) transmitted via premotoneurons to motoneurons to produce muscle contraction leading to inspiratory airflow and modulation of airflow resistance (criterion 2; Smith *et al.* 1991; Dobbins & Feldman, 1994, 1995; Koizumi *et al.* 2008). Neuromodulatory, suprapontine, cerebellar, and sensory inputs can modify the frequency and pattern of breathing to regulate blood gases and pH and to coordinate breathing with other

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movements and behaviours (criterion 3; Heywood *et al.* 1996; Huckstepp & Dale, 2011; Feldman *et al.* 2013; Moore *et al.* 2013, 2014).

As basic breathing behaviour is conserved across mammals (Milsom, 2010), translating findings from healthy and respiratory-related disease model organisms to humans should be straightforward. Conditions in which neural circuits controlling breathing are disturbed (recently reviewed in Feldman *et al.* 2013), e.g. in single gene disorders (Amir *et al.* 1999; Amiel *et al.* 2003; Weese-Mayer *et al.* 2003; Gallego, 2012), with sleep apnoea (Javaheri & Dempsey, 2013), following opiate intoxication (Stuth *et al.* 2012), or in sudden infant death syndrome (Lavezzi & Matturri, 2008; Weese-Mayer *et al.* 2008) have severe consequences for human health. Despite the development of rodent models for these conditions (Dubreuil *et al.* 2008; Calfa *et al.* 2011; Davis & O'Donnell, 2013), our current understanding of such disturbances of respiratory behaviour is insufficient for developing effective therapies or treatments and will depend on a better grasp of basic mechanisms underlying the neural control of breathing, including respiratory rhythmogenesis.

How the preBötC generates rhythm is, perhaps surprisingly, still unknown (Feldman *et al.* 2013). Localization and isolation of the kernel from the extensive regulatory, sensory and state-dependent feedback associated with respiratory physiology *in vivo* has not led to a straightforward mechanism analogous to any found in invertebrate CPGs or other biological oscillators (Monfredi *et al.* 2010; Selverston, 2010; Feldman *et al.* 2013). Nonetheless, the accessibility and tractability of *in vitro* experiments in slice (Smith *et al.* 1991), brainstem–spinal cord ('en bloc'; Smith & Feldman, 1987; Thoby-Brisson *et al.* 2009; Bouvier *et al.* 2010), and *in situ* (Paton, 1996) perinatal rodent preparations constitutes the best opportunity with present technology for revealing cellular and microcircuit mechanisms that can be tested/verified *in vivo* in intact awake, sleeping, anaesthetized or decerebrate mammals (often rodents).

An important caveat. The literature on respiratory rhythmogenesis is full of disparate, even contradictory, data and associated interpretations. An obvious but non-trivial explanation is that divergent results are due to differences in experimental preparation or condition. Details of experimental protocol, both conspicuous, e.g. species differences, *in vitro vs. in vivo*, neonate *vs.* adult, and less conspicuous, e.g. type of anaesthetic, strain of mouse, presence/absence of peripheral nerves, indeed count, and we describe some examples below. Our advice is to read the label before consuming the literature and be careful when you mix and match!

Defining the preBötC

The preBötC evaded identification until 1990 (Feldman *et al.* 1990), eluding even Ramón y Cajal (Ramón y Cajal, 1904). A functionally and molecularly heterogeneous bilateral population (~1000–3000 neurons in rodents) within the ventral respiratory column (VRC), a rostrocaudal column of respiratory-related neurons in the ventrolateral medulla, comprises the rodent preBötC (Feldman *et al.* 2013). The preBötC is ventrolateral to nucleus ambiguus with enriched inspiratory-modulated propriobulbar neurons (Smith *et al.* 1991; Monnier *et al.* 2003), caudal to the Bötzinger complex (BötC), which is populated primarily with expiratory-modulated glycinergic neurons, and rostral to the rostral ventral respiratory group (rVRG), which contains glutamatergic bulbospinal premotor neurons (Smith *et al.* 1991; Stornetta *et al.* 2003; Tan *et al.* 2012). A homologous structure is present in humans (Schwarzacher *et al.* 2011). In mice, the preBötC becomes rhythmically active late in embryonic development (embryonic day 15.5; Thoby-Brisson *et al.* 2009), coincident with the onset of fetal breathing movements that are necessary for proper development of lung, respiratory muscles, and the respiratory CPG itself (Kobayashi *et al.* 2001; Feldman *et al.* 2009); in humans this happens in the third trimester (Greer, 2012).

While these landmarks localize the general region of the preBötC, the lack of a cytoarchitectonically distinct nucleus and the neuronal heterogeneity in this area makes determination of constituent neurons and borders challenging (perhaps unsurprising for an area bounded dorsally by the nucleus ambiguus). Indeed, within this region are neurons that are not respiratory-modulated (Johnson *et al.* 1994; Ramirez *et al.* 1997; Shao & Feldman, 1997), and neurons from adjacent areas, including C1 (Guyenet *et al.* 2013), BötC (Schwarzacher *et al.* 1995), and rVRG (Tan *et al.* 2012) are probably intercalated among inspiratory-modulated preBötC neurons at their overlapping borders.

The preBötC is, nonetheless, a distinct area, functionally separable from surrounding neuronal populations. Perturbations within the preBötC produce markedly different responses compared with when they are targeted to adjacent regions (McCrimmon *et al.* 1986; Monnier *et al.* 2003), and rostrocaudal boundaries where rhythm is maintained in slices from perinatal rodents are now well-defined (Ruangkittisakul *et al.* 2006, 2011, 2014). We can therefore describe respiratory-related neurons in this region by their anatomy, physiology, and/or molecular identity, fully acknowledging that we are referencing the core of the preBötC and not necessarily its full real-estate.

Several functionally defined neuronal subtypes comprise the preBötC. Limited electrophysiological samples of brainstem neurons yield several classifications

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of neurons with firing patterns phase-locked to respiratory motor outflow. Early experiments categorized preBötC neurons based on peak activity during the respiratory cycle, i.e. inspiratory, expiratory, pre-inspiratory, post-inspiratory and phase-spanning (Smith *et al.* 1990; Schwarzacher *et al.* 1991; Johnson *et al.* 1994; Bianchi *et al.* 1995; Sun *et al.* 1998). Further subtypes are distinguished based on incrementing or decrementing firing patterns (Richter, 1996; Lindsey *et al.* 2012; Richter & Smith, 2014). Inspiratory preBötC neurons are also classified into Type 1 and Type 2 neurons, which differ in the duration of pre-inspiratory firing, membrane properties, including the expression of the hyperpolarization-activated cation current (I_h) and the A-type K^+ current (I_A), and voltage trajectory following the inspiratory burst *in vitro* (Fig. 1*B*; Rekling *et al.* 1996). Inspiratory-modulated preBötC neurons may also be divided into those with or without endogenous bursting (EB; pacemaker) properties (Fig. 1*B*; Smith *et al.* 1991; Johnson *et al.* 1994; Del Negro *et al.* 2001; Thoby-Brisson & Ramirez, 2001). These EB properties primarily depend on either of two well-studied currents, the Ca^{2+}-activated non-specific cation current (I_{CAN}) or the persistent Na^+ current (I_{NaP}), that are not exclusively expressed by EB neurons and are, in fact, present in many, if not all, preBötC inspiratory neurons *in vitro* (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.* 2002*a*, 2005; Rybak *et al.* 2003; Pace *et al.* 2007*a*).

A unique molecular marker or transcriptional lineage defining the preBötC has not yet been discovered, but expression patterns of some proteins differentiate the preBötC from surrounding regions. Molecular markers, first the neurokinin 1 receptor (NK1R; Gray *et al.* 1999, 2001), and subsequently the peptide somatostatin (SST; Stornetta *et al.* 2003; Wei *et al.* 2012), and the glycoprotein Reelin (Tan *et al.* 2012) identify subpopulations of preBötC glutamatergic neurons, which express vesicular glutamate transporter 2 (VGluT2; Fig. 1*B*; Wallen-Mackenzie *et al.* 2006). Neurons expressing μ opioid receptors (μORs), which overlap significantly with NK1R-expressing neurons and processes (Fig. 1*B*; Gray *et al.* 1999), the somatostatin 2a receptor (SST2aR; Gray *et al.* 2010), and tyrosine kinase B (Thoby-Brisson *et al.* 2003; Bouvier *et al.* 2008) are also found in the preBötC. These neurons are derived from precursors expressing the transcription factor developing brain homeobox 1 (Dbx1; Bouvier *et al.* 2010; Gray *et al.* 2010). Dbx1-derived ($Dbx1^+$) neurons populate the entire nervous system, but are prominent in the VRC (Gray, 2013). Glycine transporter 2 (GlyT2) identifies preBötC glycinergic neurons, which are about half of all preBötC neurons (Winter *et al.* 2009), some with inspiratory-modulated activity *in vitro* (Winter *et al.* 2009; Morgado-Valle *et al.* 2010; Koizumi *et al.* 2013). A small subset of preBötC neurons also express glutamic acid decarboxylase 67 (GAD67), which specifies γ-aminobutyric acid (GABA)ergic neurons, and some of these are also weakly inspiratory and overlap with glycinergic neurons (Kuwana *et al.* 2006; Koizumi *et al.* 2013; Rahman *et al.* 2013).

In most cases, the molecular identities or firing pattern of preBötC neurons do not uniquely map to functionally definable subpopulations. *In vitro*, both glutamatergic and glycinergic preBötC neurons can have inspiratory-modulated activity, which can be variable from cycle to cycle, and a small percentage of both can be EBs (Morgado-Valle *et al.* 2010; Carroll & Ramirez, 2013; Carroll *et al.* 2013; Kam *et al.* 2013*a*; Picardo *et al.* 2013). $Dbx1^+$ glutamatergic neurons are more functionally and morphologically homogeneous, most have a pre-inspiratory firing pattern and commissural connections and lack dendritic spines (Picardo *et al.* 2013). However, even $Dbx1^+$ neurons are divided into Type 1 (some of which are EBs) or Type 2 (Rekling *et al.* 1996; Picardo *et al.* 2013), which may correlate with some $Dbx1^+$ neurons serving a rhythmogenic role and others a premotor role (Wang *et al.* 2014). Additionally, neurons with inspiratory- or expiratory-modulated firing patterns, throughout the VRC from the rostral pons to the spinomedullary junction, can have very different roles in the production of respiratory pattern (Segers *et al.* 2008; Mellen & Mishra, 2010). In particular, bulbospinal and other premotoneurons proximal to the preBötC have inspiratory-modulated firing patterns but are not rhythmogenic. A recent attempt to find discrete classes of inspiratory-modulated preBötC neurons from multicellular recordings of firing patterns was unsuccessful, suggesting that inspiratory-modulated neurons fall along a continuum of firing behaviour (Carroll *et al.* 2013, but see section 'preBötC connectivity – a critical microcircuit parameter' below, for caveats).

Whether molecular markers are sufficient to determine functional classes of preBötC neurons remains to be established. Neurons with differing levels of expression of the same channels can have distinct electrophysiological properties (Golowasch *et al.* 2002; Schulz *et al.* 2006) and different combinations of channels can produce similar electrophysiological properties, e.g. I_{CAN}- and I_{NaP}-dependent EBs (Del Negro *et al.* 2002*b*, 2005). Second order parameters, e.g. variability in expression level of a protein across a population, may be critical for unravelling the relationship between molecular and functional categories (Rybak *et al.* 2004; Marder & Taylor, 2011). Receptor markers such as NK1R or SST2aR confer unique input specificity to a subpopulation of preBötC neurons, but, in the absence of such input, the function of these neurons may be indistinguishable from neurons lacking such receptors (Hayes & Del Negro, 2007). Indeed, preBötC neurons probably participate in multiple signal processing functions, and distinct roles for many subpopulations may only be elicited under specific conditions

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or for behaviours other than normal breathing at rest (Lieske *et al.* 2000; Briggman & Kristan, 2008).

While the neuronal heterogeneity and ambiguity of cytoarchitectonic boundaries of the preBötC may be considerable, such issues are not unique to the preBötC (Markram *et al.* 2004; Horton & Adams, 2005; DeFelipe *et al.* 2013; Huang, 2014; Seung & Sumbul, 2014), and are greatly mitigated by the context provided by breathing behaviour. By bounding the circuit and utilizing a presumptively straightforward, spatiotemporally constant tonic excitatory drive as input with behaviourally relevant respiratory rhythm as output, we can focus specifically on how this rhythm emerges from the confederation of molecularly and functionally heterogeneous preBötC neurons. Until recently, most research has focused on a few likely mechanisms, and without success. In what follows, we discuss how several of these mechanisms, first pacemakers, then inhibition, and finally even bursts, are, in fact, unlikely to be critical for rhythmogenesis.

Pacemaker properties are not necessary for respiratory rhythmogenesis

Shortly after the preBötC was discovered (Feldman *et al.* 1990), excitatory preBötC neurons with conditional EB properties were hypothesized to serve as pacemakers driving rhythm (Smith *et al.* 1991), analogous to the cardiac sinoatrial node (Monfredi *et al.* 2010) and some invertebrate CPGs (Selverston, 2010). While the pacemaker hypothesis is compelling, easy to grasp, and, for a long time, widely considered the favoured mechanism for rhythmogenesis, neither EBs nor their associated conductances are obligatory for rhythmogenesis (recently reviewed in Feldman *et al.* 2013). Briefly, the loss of rhythmic activity observed during widespread application of pharmacological blockers of EB conductances *in vitro* or *in vivo* can be attributed to depression of tonic excitatory drive into the preBötC and to non-specific effects on excitability (Del Negro *et al.* 2005; Pace *et al.* 2007*b*; Montandon & Horner, 2013). When blockade of EB conductances is restricted to preBötC or excitability is increased, rhythmic activity persists (Pace *et al.* 2007*b*; Montandon & Horner, 2013).

What then are EBs and the associated 'pacemaker' currents doing? While EB neurons are a limited subset of all preBötC neurons, I_{NaP} and I_{CAN} are ubiquitous (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.* 2002*a*, 2005; Rybak *et al.* 2003; Pace *et al.* 2007*a*). Their widespread expression suggests that these conductances serve as one of many mechanisms for controlling excitability, possibly to ensure either the stability (Purvis *et al.* 2007) or the robustness of preBötC rhythmogenesis, or to amplify activity to ensure transmission of preBötC signals to other neuronal populations (Mellen, 2008, 2010). The specific role of these and other conductances and these neurons may depend on the state of the network, e.g. during sleep, rest, or exercise, or the developmental stage (Del Negro *et al.* 2005; Montandon & Horner, 2013). How EBs might function under *in vivo* conditions and in adult mammals is unknown; at present EB preBötC neurons have not been identified or characterized *in vivo*, at any age. Even if present, how such neurons would operate within a heterogeneous network is unlikely to be straightforward (Boyett *et al.* 2000). With pacemaker neurons unlikely to drive rhythmogenesis, we next consider another likely suspect, inhibition.

Fast inhibitory synaptic transmission is not necessary for rhythmogenesis

The earliest proposals for the neural basis of rhythmic mammalian movements were based on mutually inhibitory populations underlying locomotion (Brown, 1914). This 'half-centre' model was later modified to a threshold hypothesis for breathing, where inspiratory activity grows until it reaches a threshold to trigger massive inhibition that resets it to zero with a refractory interval before it can grow again (Rubio, 1972; Bradley *et al.* 1975; Feldman & Cowan, 1975). A contemporary extension of this idea postulates that normal breathing rhythm relies on an 'inhibitory ring' of three populations of inhibitory neurons in the preBötC and neighbouring BötC (Smith *et al.* 2007; Richter & Smith, 2014).

Brainstem respiratory neurons receive fast inhibitory synaptic input mediated by GABA and glycine (Richter, 1982; Ballantyne & Richter, 1984; Paton & Richter, 1995; Shao & Feldman, 1997). In an *in situ* perfused neonatal rodent preparation, blocking inhibition throughout the entire neuraxis with $GABA_A$ and glycine receptor antagonists or low extracellular Cl^- abolishes respiratory-related activity (Hayashi & Lipski, 1992; Smith *et al.* 2007). This observation cannot exclusively be interpreted as demonstration of the necessity of inhibition for rhythmogenesis, since systemic blockade of inhibition will disinhibit preBötC neurons (Shao & Feldman, 1997), with the potential to shift the excitability of almost any type of rhythm generator into a tonic, even inactive, regime (Del Negro *et al.* 2001).

In contrast, in rhythmic medullary slices, a normal rhythm persists following blockade of fast synaptic inhibition (Feldman & Smith, 1989; Shao & Feldman, 1997). Similarly, a largely normal respiratory rhythm persists following well-controlled pharmacological blockade of $GABA_A$ and glycine receptors limited to preBötC and BötC in spontaneously breathing adult rats with vagus and carotid sinus nerves intact, preserving normal lung reflexes and chemo-/baroreception (Fig. 2; Janczewski *et al.* 2013). The changes induced by block of

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inhibition in these rats, increased inspiratory amplitude and decreased respiratory frequency, are similar to the effects of cutting the vagus nerve, which contains pulmonary afferents. Moreover, this blockade is profound as it abolishes the potent pulmonary afferent-mediated lung inflation reflex that under control conditions can produce apnoea (Fig. 2, compare top and bottom traces). Following subsequent vagotomy, block of inhibition does not change rhythm. Therefore, postsynaptic inhibition within the preBötC and/or BötC is NOT required for a normal breathing rhythm in intact rodents. The 'inhibitory ring' model (see section 'Can models show us the way?' below; Smith *et al.* 2007), as well as any other model requiring postsynaptic inhibition within the preBötC (e.g. Feldman, 1976) fails this critical test of necessity in the intact rat. The relevance of the 'inhibitory ring' model may therefore be limited to the reduced *in situ* preparations that provide the experimental basis for this concept (Smith *et al.* 2007) and to highly reduced *in vivo* preparations. This model cannot, however, be representative of basic mechanisms for generation of respiratory rhythm in intact mammals.

What then is inhibition doing in the respiratory CPG? Fast synaptic inhibition is generally accepted to play a key role in shaping motor nerve burst pattern and coordinating activity between motoneuron pools (Feldman & Smith, 1989; Grillner, 2006), and certainly this is the case for breathing, as fine tuning the motor output is a key element in the incredible mechanical efficiency of breathing (at rest ∼7% of total body metabolism, close to the minimal estimated energy for optimal breathing movements; Otis *et al.* 1950). In addition to being essential for the lung inflation reflex (Ezure & Tanaka, 2004; Janczewski *et al.* 2013), inhibition in the preBötC appears important for modulating the breathing frequency (Paton & Richter, 1995), for ensuring that spurious signals do not inappropriately affect ongoing respiratory activity (Busselberg *et al.* 2001), and in producing apnoeas (Lawson *et al.* 1991) required by such behaviours as swallowing and breath holding (Saito *et al.* 2002; Janczewski *et al.* 2013; Bautista & Dutschmann, 2014; Richter & Smith, 2014). Rhythmogenesis therefore does NOT depend on inhibition or pacemaker neurons. Next, we consider whether bursting is necessary.

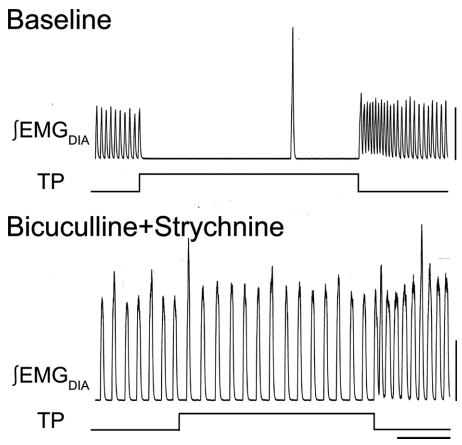

Figure 2. Inhibition is not essential for normal rhythmogenesis in adult rat
Block of postsynaptic inhibition within preBötC and BötC in vagus intact adult rat slows breathing frequency to that of vagotomized rat and completely blocks the Breuer–Hering inflation reflex. Top, lung inflation resulting from increased tracheal pressure (TP; 10 cmH_2O) causes apnoea as reflected in loss of activity in diaphragmatic EMG (∫EMG$_{DIA}$). Bottom, after microinjection of bicuculline and strychnine, $GABA_A$ and glycine antagonists, rhythm persists but lung inflation no longer has an effect on rhythm. Scale bars, 100 arbitrary units, 10 s. Adapted from Janczewski *et al.* (2013).

Burstlets, not bursts, are rhythmogenic

In invertebrates, coordinated bursts of APs, either in phase with motor output in pacemaker-driven CPGs or out-of-phase with motor output in inhibitory network-driven CPGs, are essential for rhythmogenesis (Getting, 1989; Marder & Calabrese, 1996; Selverston, 2010). Elucidating the mechanisms initiating and terminating preBötC neuronal bursts that result in inspiratory motor nerve bursts *in vivo*, *in situ* and *in vitro* has been a major focus in the search for how preBötC generates rhythm (Feldman *et al.* 2013). While revealing cellular mechanisms underlying burst amplitude, duration and firing pattern (Pace *et al.* 2007*a*; Del Negro *et al.* 2009; Richter & Smith, 2014), this approach has so far been less successful in illuminating rhythmogenic mechanisms (Feldman *et al.* 2013).

The presumption that preBötC burst-generating mechanisms are rhythmogenic, while generally accepted, is not warranted. In many intact mammals and *in vitro*, burst shape parameters, such as burst amplitude and duration that reflect AP frequency and/or number of active neurons, may stay the same despite large changes in breathing frequency; conversely, changes in integrated burst shape parameters in motor output can occur without significantly affecting frequency (Clark & von Euler, 1972; Del Negro *et al.* 2009). *In vitro*, preBötC burst shape parameters, including the amplitude and duration of the inspiratory drive potential recorded in individual preBötC neurons, can be substantially altered without concomitant changes in frequency, e.g. blockers of metabotropic glutamate receptors and inositol 1,4,5-trisphosphate (IP_3) receptors that significantly change preBötC burst shape do not affect frequency *in vitro* (Pace *et al.* 2007*a*). The separate modulation of burst shape and frequency suggests that determination of the time to the onset of the next burst is (at least partially) independent of the pattern-generating

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mechanisms that underlie bursting *per se* (Del Negro *et al.* 2009).

We recently discovered that bursts of preBötC population activity are not unitary events, but consist of two separable components: large amplitude inspiratory bursts (I-bursts) that are ultimately transmitted to hypoglossal (XII) motoneurons *in vitro* and all inspiratory motoneurons *in vivo*, and burstlets, of much smaller amplitude, that appear as pre-inspiratory activity and are rhythmogenic (Fig. 3). Burstlets are revealed by systematically altering respiratory CPG excitability *in vitro* and *in vivo* (Kam *et al.* 2013*a*). At conventional levels of extracellular K^+ and Ca^{2+} *in vitro*, there is a 1:1 correspondence between preBötC bursts and XII nerve (XIIn) motor bursts (Fig. 3*A*; Ramirez *et al.* 1996; Kam *et al.* 2013*a*). The XIIn interburst interval (IBI), unimodal under baseline conditions *in vitro*, becomes longer and, somewhat surprisingly, multimodal when excitability is lowered through decreases in extracellular K^+. These modes are multiples of the shortest IBI, i.e. 'quantized' (Fig. 3*B*; Kam *et al.* 2013*a*), so that, at times when a XIIn burst is expected but absent, burstlets occur in preBötC, and the number of preBötC burstlets during a XIIn IBI correlates linearly to the duration of the IBI (Kam *et al.* 2013*a*). Importantly, burstlets are neither an *in vitro* nor developmental epiphenomenon and can be elicited under specific conditions *in vivo* (Kam *et al.* 2013*a*). When preBötC bursts occur, burstlets appear as the low-level pre-inspiratory activity immediately preceding I-bursts (Fig. 3*C*; Kam *et al.* 2013*a*). Furthermore, a preBötC rhythm comprising only burstlets is possible when Cd^{2+} is bath-applied (Fig. 3*D*; Kam *et al.* 2013*a*). That rhythmic burstlet activity persists following the disappearance of bursts is incompatible with bursts, an essential element of many, if not most, models of rhythmogenesis (e.g. Butera *et al.* 1999; Rybak *et al.* 2004; Purvis *et al.* 2007), being necessary for generation of rhythmic activity (Fig. 4).

The difference in burstlet and burst amplitude (which are population measures) is primarily determined by significantly increased AP frequency in neurons during I-bursts compared to burstlets and much less so by recruitment of more neurons (Kam *et al.* 2013*a*). Ninety per cent of inspiratory preBötC neurons are active during both, with recruitment of the remaining ~10% during I-bursts (Kam *et al.* 2013*a*). preBötC neuron I-burst APs are preceded by a period of low frequency pre-inspiratory APs, comparable in duration and frequency to their activity during burstlets, suggesting that burstlets are the pre-inspiratory activity that triggers bursts (Kam *et al.* 2013*a*).

The generation of I-bursts from burstlets is a threshold process, akin to AP generation from EPSPs in single neurons (Fitzhugh, 1955). We speculate that when a burstlet exceeds an as yet undefined threshold, distinct mechanisms, probably involving both the activation of persistent inward conductances, e.g. I_{NaP} and I_{CAN}, and the recruitment of additional neurons, generates an I-burst (Feldman *et al.* 2013; Kam *et al.* 2013*a*). Following each burst, the activity of the network is reset, and a refractory period is observed (Del Negro *et al.* 2009; Kam *et al.* 2013*a*). However, this refractory period, which may set a minimum interval between bursts, is only a fraction of

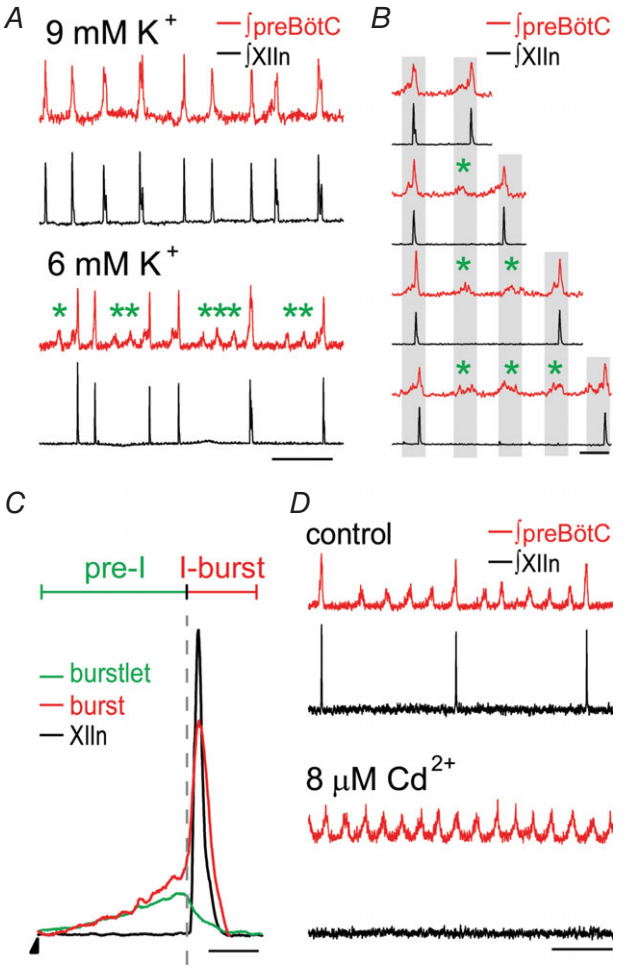

Figure 3. Bursts are not essential for rhythmogenesis in transverse medullary slices *in vitro*
A, rhythmic burstlets are distinct from bursts in medullary slices *in vitro*. In 9 mM K^+/1.5 mM Ca^{2+}, XIIn and preBötC bursting are synchronous and regular. In 6 mM K^+/1.5 mM Ca^{2+}, XIIn bursting is highly variable, while preBötC rhythm with both bursts and burstlets (*), which do not produce XIIn bursts, is more regular. Integrated preBötC and XII activity is shown, which represents suprathreshold, i.e. action potential, activity. Scale bar, 10 s. *B*, traces showing interburst intervals (IBIs) with different numbers of burstlets. Scale bar, 2 s. *C*, superimposed average waveforms of XIIn bursts (black) and preBötC burstlets (green) and bursts (red) aligned by the start of burstlet/pre-inspiratory preBötC activity (arrowhead). Vertical dashed line represents start of XIIn burst. preBötC bursts can be divided into a pre-inspiratory (pre-I) phase and an inspiratory burst (I-burst) phase. Scale bar, 0.5 s. *D*, Cd^{2+} selectively eliminates preBötC and XII bursts, but burstlets persist (bottom). Scale bar, 10 s. Adapted from Kam *et al.* (2013*a*).

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the IBI *in vitro* and does not determine burstlet or burst timing (Del Negro *et al.* 2009; Kam *et al.* 2013*b*). We liken rhythmogenic burstlet activity to a preBötC 'metronome' maintaining an underlying beat, with preBötC I-bursts and downstream premotoneuronal (that could include preBötC neurons) and motoneuronal networks transforming this timekeeping signal into a physiologically appropriate pattern for breathing and other orofacial behaviours (Fig. 4; Moore *et al.* 2013, 2014).

Importantly, the burstlet hypothesis suggests a unitary rhythmogenic signal underlying inspiration over a wide dynamic range of frequencies and considerably constrains the space of possible rhythmogenic mechanisms to those not requiring the high levels of neuronal activity seen during bursting. The lower firing frequency of individual neurons during burstlets compared to bursts probably precludes the involvement of conductances with high voltage or high Ca^{2+} thresholds (Richter & Smith, 2014). Indeed, the relative paucity of activity that is nonetheless distributed throughout the preBötC microcircuit suggests an emergent mechanism for rhythmogenesis.

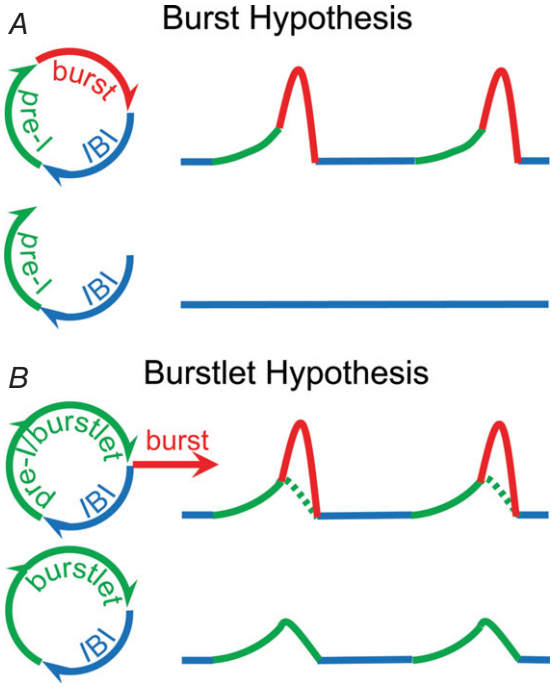

Figure 4. The burstlet hypothesis for rhythmogenesis fundamentally breaks with the burst hypothesis
A, top, in the burst hypothesis, the interburst interval (IBI; blue) is a quiescent phase that leads to pre-inspiratory (pre-I) activity (green) and then the necessary generation of a high amplitude burst (red). Bottom, blockade of bursts in this model would lead to interruption of the rhythmogenic cycle and cessation of rhythmic activity. *B*, top, in the burstlet hypothesis, the IBI (blue) leads to burstlets (green), which are sufficient for rhythmogenesis. Generation of high amplitude bursts (red) is a distinct step and not essential for generation of rhythmic activity. When bursts occur, burstlets appear as pre-I activity (dotted green line) preceding bursts. Bottom, by lowering excitability or adding Cd^{2+}, bursts disappear but rhythmic preBötC burstlets remain, contradicting the burst hypothesis.

What constitutes an emergent mechanism in the preBötC? Single EPSPs in preBötC neurons (~3 mV) are too small to produce an AP, since the typical threshold depolarization is ~10 mV (Rekling *et al.* 2000). Temporal summation of EPSPs arising from a single input neuron may also be insufficient to generate an AP, being limited by the typical maximum firing frequency of preBötC neurons (~30–40 Hz *in vitro*; Rekling *et al.* 1996; Kam *et al.* 2013*b*), since the resultant interspike intervals (~30 ms) are too long. Instead, the activity of a few convergent neurons could depolarize a target neuron sufficiently to produce APs – a classic 'emergent' property (Ratte *et al.* 2013). As network activity grows, APs of individual neurons may become more synchronized, increasing temporal convergence of synaptic inputs leading to greater postsynaptic depolarization that (more readily) crosses the AP threshold. Increased synchrony could accelerate the collective growth of activity amongst already active neurons and facilitate the triggering of bursts by burstlets. The novelty of this mechanism is that it would not require either increased firing in active neurons or recruitment of quiescent neurons for burstlet growth or burst triggering. Whatever the mechanism, how this emergent growth process plays out at different breathing frequencies (10-fold range in humans from rest to extreme exercise, and 100-fold range across species: 3–5 Hz in mice to 0.05 Hz in whales; Mortola & Limoges, 2006; Forster *et al.* 2012; Berndt *et al.* 2014) or behaviours, e.g. eupnoea, sighs, gasps, cough, speech (Bartlett & Leiter, 2012), particularly when firing frequencies of individual neurons are low (Kam *et al.* 2013*a*), critically depends on microcircuit connectivity parameters (Feldt *et al.* 2011).

preBötC connectivity – a critical microcircuit parameter

Basic parameters of connectivity, such as the number of inputs and outputs for each neuronal subtype and how neuron subtypes are interconnected, can be described anatomically, i.e. based on structural contacts between two neurons, functionally, i.e. based on a statistical association between the firing of neurons, or effectively, i.e. based on physiological, electrical or chemical synaptic connection between neurons (Feldt *et al.* 2011). While the influence of neurons is not restricted to proximate synaptic connections, e.g. paracrine release of neuromodulators or peptides (Zoli *et al.* 1999), anatomical and functional connectivity are necessary parameters for constraining models, and effective connectivity parameters, such as the strength of connectivity and synaptic properties, e.g. probability of release, number of synapses, receptor kinetics, are essential data for fully understanding how

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dynamics arise from network connectivity (Song *et al.* 2005; Feldt *et al.* 2011). The network structure as determined by the connectivity distribution, e.g. random, small world, local, or all-to-all, has profound impact on how activity propagates through the network (Sporns *et al.* 2004); once determined it allows the application of graph theoretical approaches for network analysis (Feldt *et al.* 2011, but see Mitra, 2014).

Excitatory preBötC neurons are recurrently connected (Rekling *et al.* 2000; Hayes & Del Negro, 2007) with fast excitatory synaptic transmission necessary for rhythmogenesis (Greer *et al.* 1991). In a small sample of dual whole cell patch recordings in preBötC *in vitro*, a subset of pre-inspiratory neurons are effectively connected to 13% of other pre-inspiratory neurons by one-way excitatory chemical synaptic connections and to a distinct 13% of other pre-inspiratory neurons by weak gap-junction connections (Rekling *et al.* 2000). However, in a larger sample of spike time correlations obtained from multielectrode extracellular recordings in preBötC *in vitro*, a much lower functional connectivity of 1% is estimated (Carroll & Ramirez, 2013). Yet another different, more complex network structure is observed in medullary slice cultures, where neurons show small world anatomical connectivity with neurons grouped into local, highly connected ($\sim$4 connections per neuron) clusters (Hartelt *et al.* 2008).

What might account for these divergent observations? The laboriousness of dual patch recordings, which provides direct physiological evidence of effective connectivity, limits the estimates of effective connectivity to small samples that may not be representative of the overall network. The multielectrode extracellular approach, in contrast, is indirect (Schwindel *et al.* 2014) and relies on proper assignment of APs to each stipulated neuron (Lewicki, 1998). High temporal synchrony and changes in spike shape (Lewicki, 1998; Carroll *et al.* 2013; Kam *et al.* 2013*a*; Picardo *et al.* 2013) can confound clustering and dimensionality reduction techniques such as independent components analysis (Valmianski *et al.* 2010), and complicate the parcelling of APs among different stipulated neurons (Lewicki, 1998). We suggest that the conclusion of 1% connectivity is too low, perhaps by an order of magnitude; we cannot readily reconcile such low connectivity with the successful electrophysiological detection of synaptically connected pairs from random sampling (Rekling *et al.* 2000). Finally, connectivity in slice cultures is unlikely to reflect network connectivity in acute *in vitro* preparations and *in vivo* due to immense sprouting and regrowth in cultured slices and the significant flattening of the three-dimensional structure (Zimmer & Gähwiler, 1984; Frotscher & Gähwiler, 1988). While the notion of clustering is intriguing, serious consideration should require validation in more intact networks.

Recent efforts to establish anatomical connectivity in mammalian networks at the electron microscopic (Kleinfeld *et al.* 2011) or light microscopic (Oh *et al.* 2014) levels will provide necessary information for understanding neural microcircuits. However, microcircuit structure is not sufficient for deducing network dynamics that emerge from the interplay of the electrophysiological and synaptic properties of neurons and the connectivity (Kopell *et al.* 2014). As the non-linearities of emergent behaviour typically defy intuition, we consider next the role of simulations in providing insight into how cellular and network properties interact to generate rhythmic population activity.

Can models show us the way?

Computational simulations of biologically plausible models can be useful for exploring rhythmogenic mechanisms (Abbott, 2008). Models of the respiratory CPG range from abstract models that test the feasibility of hypothetical mechanisms to attempts at biologically realistic models based on large datasets. The broader utility of these models, however, ultimately depends on their ability to motivate novel, non-trivial experimental tests of proposed mechanisms.

Some models for breathing, including the aforementioned 'inhibitory ring' model (Smith *et al.* 2007), incorporate large amounts of data related to cellular parameters, and crude (because that is the best we have) and largely incomplete measures of connectivity between respiratory-related brainstem areas. However, the considerable amount of data necessary for a biologically realistic model (Markram, 2006; Marder & Taylor, 2011) is not yet available for the respiratory CPG. Many critical parameters have yet to be experimentally determined, so these parameters are typically fitted to a specific dataset, i.e. the values are chosen so that the simulation resembles some limited range of experimental phenomenology. Unfortunately, these fits are done without any analysis of whether the chosen parameters represent unique solutions, or whether the model is stable with physiologically reasonable changes in the parameter set (Marder & Taylor, 2011), as may be associated with experimental perturbations, development, or increased ventilatory demand such as during exercise or changes in sleep–wake state.

In the absence of values derived from experimental data, parameterization of models is necessary to explore their dynamic range and sensitivity to given parameter sets (Nowotny *et al.* 2007; Marder & Taylor, 2011). Such parameterization should be accompanied by detailed comparison and analysis of model output across parameter sets and with experiments (Nowotny *et al.* 2007). Many regions of multidimensional parameter

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space inherent in a complex model can produce similar behaviours, i.e. solutions are highly degenerate (Prinz *et al.* 2004; Nowotny *et al.* 2007; Mellen, 2010), and there is no *a priori* guarantee that any given solution is stable to small perturbations in parameters. Not all rhythmic outputs are equivalent (Nowotny *et al.* 2007), and model behaviour that resembles experimental results with apparently subtle differences may be satisfactorily close or may, instead, reflect significant flaws in the model. Rigorous protocols for evaluating models, such as the 'verification, validation, and uncertainty quantification' (VVUQ) paradigm (Pathmanathan & Gray, 2014), are needed to determine the success of these large, multineuronal simulations in representing biological reality. We should recognize the limits of models that reproduce some phenomenology, but stipulate parameters or mechanisms that have yet to be or cannot realistically be determined or validated experimentally (Mitra, 2014).

Smaller models focused on the preBötC have been developed. As with the more comprehensive models, their plausibility is based on whether they reproduce some set of experimental data. For example, several models can produce burstlet/burst patterns, or 'irregular bursting,' when the variance of the distribution of I_{NaP} conductances or synaptic strengths are adjusted (Butera *et al.* 1999; Rybak *et al.* 2004; Purvis *et al.* 2007). A major caveat for these particular models is their reliance on pacemaker conductances, specifically the slow deactivation kinetics of I_{NaP}, to generate rhythmic bursting, which is not consistent with the pharmacological data demonstrating that such conductances are not necessary (Pace *et al.* 2007*b*; Feldman *et al.* 2013). A recent model, incorporating both I_{NaP} and I_{CAN}, explores the effects of these and other various cellular parameters on network dynamics and can reproduce rhythmic bursting independent of these conductances (Jasinski *et al.* 2013). However, the network connectivity in this model is all-to-all (Jasinski *et al.* 2013), which does not reflect the limited experimental data on preBötC connectivity (see previous section 'preBötC connectivity – a critical microcircuit parameter'), and its stipulation can significantly affect computed network dynamics (Butera *et al.* 1999; Shao *et al.* 2006).

In contrast to biologically realistic models, abstract, or 'toy' models of the preBötC can explore how a specific parameter or mechanism might generate or affect rhythmic bursting, permitting formal dynamical systems analysis of network behaviour (Weiss *et al.* 2003; Sherman, 2011). In one example, simplified model neurons placed in a network with small world connectivity can produce network-wide bursting under some conditions without invoking conductances with slow kinetics (Shao *et al.* 2006). In another model, networks of simplistic threshold bursting neurons with a slow adaptation process that terminates firing demonstrate that complex dynamics can arise solely from particular connectivity patterns (Schwab *et al.* 2010). Here, a network with all-to-all connectivity fails to reproduce the complex quasiperiodic dynamics observed in higher excitability regimes *in vitro* (Del Negro *et al.* 2002*c*). However, in a network with heterogeneous connections, these dynamics are observed, with the transition from a stable oscillatory state to a more complex dynamical state governed by a discontinuous function of network size and excitability, a novel, non-trivial and testable prediction.

Rhythmogenesis may also emerge from interactions between preBötC neurons if synaptic drive dynamically modifies neuronal conductances to generate bursts (Rekling & Feldman, 1998) or burstlets. This group pacemaker hypothesis (Rekling & Feldman, 1998) is most relevant when considering the interaction between synaptic input and the membrane properties of individual neurons (Rubin *et al.* 2009). How this mechanism might apply in a preBötC neuronal microcircuit was explored later in a network simulation where the size of the population and the degree of connectivity were varied (Wang *et al.* 2014). In this model, a rhythmic bursting regime that overlapped with the experimentally determined size of the rhythmogenic Dbx1$^+$ population and the 13% effective connectivity identified by paired recordings was found, demonstrating the plausibility of the group pacemaker mechanism (Wang *et al.* 2014). Importantly, when model results were compared with experimental data, discrepancies motivated further experiments that identified a novel premotor subpopulation of Dbx1$^+$ neurons (Wang *et al.* 2014).

Critical data for testing this model was obtained from dynamic, targeted perturbations that imposed critical constraints on neural circuit dynamics (Wang *et al.* 2014). New techniques that permit more complex interrogation of network properties with single cell resolution and high spatiotemporal specificity show great promise in addressing the functional aspects of microcircuits. We describe such experiments aimed at understanding the preBötC.

Targeted photoablation and patterned photostimulation can uniquely reveal microcircuit properties

With sparse information on neuronal heterogeneity and connectivity, perturbations with high spatiotemporal resolution can illuminate the underlying circuitry and constrain models (Wang *et al.* 2014). In rodents, bulk manipulations of preBötC neurons are limited in their ability to reveal critical microcircuit properties since functionally diverse, anatomically intercalated, and molecularly heterogeneous preBötC neurons are lumped together. A precise understanding of microcircuit mechanisms requires perturbations targeted to limited

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subsets of neurons, defined molecularly, functionally and/or spatially (Huang & Zeng, 2013).

One novel approach for preBötC microcircuit dissection is sequential targeted removal of neurons by laser photoablation (Hayes *et al.* 2012). Sequential ablation of ~120 inspiratory-modulated neurons *in vitro* results in the loss of rhythmic motor output (Hayes *et al.* 2012; Wang *et al.* 2013). When lesioning is restricted to Dbx1$^+$ (glutamatergic) preBötC neurons, rhythm stops after ablating ~85 neurons, suggesting that rhythmogenic circuits are more sensitive to the specific loss of Dbx1$^+$ neurons than to generic inspiratory-modulated neurons (Wang *et al.* 2013, 2014), half of which are inhibitory (Winter *et al.* 2009). A more detailed analysis of how preBötC activity changes during photoablation may shed light on how the dynamics degrade (Gray *et al.* 2001; Wenninger *et al.* 2004; McKay *et al.* 2005; Tan *et al.* 2008) to provide additional constraints for preBötC models (e.g. Schwab *et al.* 2010; Wang *et al.* 2014). Moreover, further controls addressing damage to neighbouring neurons due to ATP or free radical release following photoablation (Shah & Jay, 1993) would eliminate the possibility that the effects are due to spread of the perturbation beyond the targeted neuron. Nonetheless, the preBötC is resistant to moderate lesioning, whether by cumulative single cell or targeted subpopulations (Gray *et al.* 2001; Wenninger *et al.* 2004; McKay *et al.* 2005; Tan *et al.* 2008), robustly maintaining proper network function in the face of limited neuronal loss, as may occur throughout life.

A potentially significant advance for investigation of rhythmogenic mechanisms is the ability to rapidly activate/inhibit functionally and/or molecularly defined neurons, perturbing the underlying microcircuits with single neuron and millisecond resolution. Optogenetic techniques partially achieve such control (Yizhar *et al.* 2011); however, axonal trafficking, an advantage for inter-regional circuit mapping (Petreanu *et al.* 2007), limits its utility in the brainstem where fibres of passage and somatodendritic compartments intermingle. An advanced optical technique, holographic photostimulation (Lutz *et al.* 2008; Zahid *et al.* 2010), permits simultaneous, spatiotemporally precise individual activation of small numbers of preBötC neurons (Kam *et al.* 2013*b*) that may mimic one way the network can be physiologically activated to trigger an inspiratory burst. Here, a laser beam is reflected off a spatial light modulator displaying an algorithmically generated hologram that produces the desired light pattern in the target plane, i.e. small (10 μm diameter) laser spots over designated (~1–10) neuronal somas, that excite the target neuron by the highly localized release of caged glutamate without significant effects on surrounding neurons (Lutz *et al.* 2008; Zahid *et al.* 2010).

We utilized holographic photostimulation to address how preBötC network activity evolves from relative silence during the IBI to generate bursts by determining the threshold behaviour (de la Prida *et al.* 2006), i.e. the response of the network when the minimal number of neurons required to produce an inspiratory burst are activated. We found that simultaneous excitation of just 4–9 targeted preBötC inspiratory neurons during the IBI, ~1% of the population, reliably initiates ectopic, network-wide bursts resulting in XIIn activity (Fig. 5*A* and *B*) that resemble endogenous bursts in amplitude, duration and shape (Fig. 5*C*; Kam *et al.* 2013*b*). Evoked XIIn bursts are substantially delayed relative to excitation onset, ~255 ms (up to 500 ms; Fig. 5*D*), much longer than expected solely from oligosynaptic spread of activity (Kam *et al.* 2013*b*). This latency is dependent on the number of neurons excited (Fig. 5*D*). The relatively low threshold balances stability, buffering the network against spurious activation due to the non-synchronous activity of single neurons, with lability, permitting rapid changes in frequency or acute burst generation in response to environmental or metabolic changes, or cortical or other suprapontine input. As inspiratory-modulated activity was the only criterion for target neuron selection and with such a low threshold number for burst initiation, we

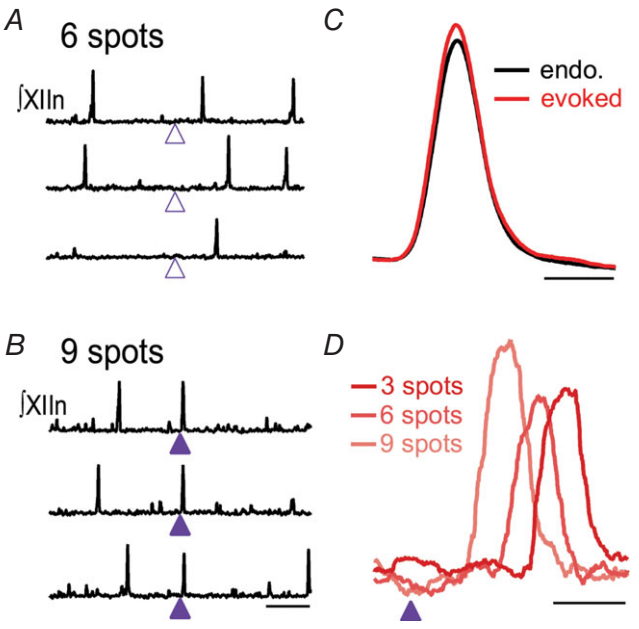

Figure 5. Activation of <10 inspiratory preBötC neurons during their silent phase can induce an inspiratory burst
XIIn activity in 3 patterned photostimulation trials targeting 6 (*A*) or 9 (*B*) neurons. Laser stimulation produced (filled arrowheads) or failed to produce (open arrowheads) a burst within 1 s of the pulse. Scale bar, 5 s. *C*, evoked bursts (averaged, red) do not differ from endogenous (endo.) bursts (black). Scale bar, 0.2 s. *D*, evoked XIIn bursts as a function of number of spots (*N*) in a different experiment where threshold is 3 neurons reveal a substantial *N*-dependent delay between laser stimulation (filled arrowhead) and burst generation. Scale bar, 0.2 s. Adapted from Kam *et al.* (2013*b*).

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consider it unlikely that the capacity for burst initiation is restricted to a specific subpopulation of preBötC neurons. Instead, we suggest that almost any excitatory, inspiratory-modulated preBötC neuron can participate in triggering bursts, which in network terms provides functional redundancy and robustness, particularly to modest neuron loss (Gray *et al.* 2001; McKay *et al.* 2005).

We speculate that the similarities in latency to bursting onset (~255 ms), and burstlet and pre-inspiratory (100–400 ms) duration, indicate a common mechanism (Kam *et al.* 2013*a*,*b*). These data suggest a model whereby the rhythmogenic microcircuit, initially quiescent, yields to spontaneous activity (due to an as yet undetermined post-burstlet process) occurring in a small number of neurons that have convergent inputs to other preBötC neurons and fire APs in a cluster tight enough to evoke APs in their targets (see section 'Burstlets, not bursts, are rhythmogenic' above), leading to a slowly growing phenomenon that appears as a burstlet or pre-inspiratory activity. A simple model for many natural processes that describes phase transitions, such as the transition from quiescence to bursting, in networks is percolation (Suki *et al.* 2011). The initial spread of activity may therefore correspond to a percolation-like phenomenon, constrained by the long latency to bursting observed with threshold stimulation, which may illuminate preBötC dynamics through analogy with other physical systems (Breskin *et al.* 2006). Microcircuit properties, such as connectivity, that govern the hypothesized dynamic percolation of activity through preBötC neurons that generates burstlets remain to be determined.

After rhythm: generation of pattern by the respiratory CPG

While rhythmogenesis, which we postulate is expressed as burstlets, is the exclusive purview of the preBötC, preBötC bursts serve as only the first stage in a process that spans several regions in shaping the temporal pattern, strength and distribution of activity to other microcircuits and ultimately to motoneuron pools (Fig. 1*A*; Dobbins & Feldman, 1994, 1995; Koizumi *et al.* 2008; Kam *et al.* 2013*a*). The progression of this activity through even the few regions separating the preBötC from motor output involves the non-linear transformation of activity through neuronal populations (Dobbins & Feldman, 1994, 1995; Koizumi *et al.* 2008; Kam *et al.* 2013*a*). Little is known about whether there are specialized projection neurons in the preBötC, how these neurons converge or diverge onto premotoneurons, and how premotoneurons then transmit activity to motoneurons. Instead, we illustrate the complexity that can arise when activity is passed across microcircuits with three potential pitfalls: (1) rhythmogenic microcircuit activity may not be transmitted faithfully to the motor output; (2) motor output may be generated independently from the activity of the rhythmogenic microcircuit(s); and (3) microcircuit activity *in vitro* may not squarely map to behaviour *in vivo*.

In addition to a threshold for burst generation within the preBötC, a threshold for transmission of rhythmic activity probably mediated at multiple microcircuit levels, i.e. preBötC, premotoneuronal and motoneuronal, regulates the activity that reaches motor output (Dobbins & Feldman, 1994, 1995; Koizumi *et al.* 2008; Kam *et al.* 2013*a*). During normal breathing, every beat produces motor output; however, under lower excitability conditions, a failure in transmission of this timekeeping signal can occur when a preBötC burst is not triggered (Fig. 4). Raising excitability can lower this transmission threshold, permitting even smaller burstlet activity to appear in XIIn output *in vitro* or diaphragm activity and airflow *in vivo* (Kam *et al.* 2013*a*). Activity recorded in preBötC may therefore not result in motor output (Ramirez *et al.* 1996; Kam *et al.* 2013*a*). Conversely, motor output may not faithfully reflect activity occurring even two synapses upstream (Kam *et al.* 2013*a*).

Burst failure produces longer IBIs at integer multiples of the basic burstlet frequency, observed in the respiratory CPG as 'quantal slowing' *in vitro* (Mellen *et al.* 2003) and *in vivo* (Janczewski & Feldman, 2006) and, we speculate, in other CPGs as 'deletions' (Stein, 2005; Zhong *et al.* 2012). Occurrences of these skipped breaths may underlie the occasional prolonged absence of inspiration that defines intermittent apnoea, such as is characteristic of central sleep apnoea (Davis & O'Donnell, 2013). While 'deletions' are commonly interpreted as indicative of separate circuits or layers governing rhythm and pattern generation (Feldman, 1986; McCrea & Rybak, 2008), for breathing, distinct preBötC burstlet and I-burst-generating mechanisms (Fig. 3*C*) indicate that separable rhythm- and pattern-generating mechanisms may co-exist within a rhythmogenic microcircuit (Figs 1 and 4). In a more intact preparation, interactions with a second respiratory oscillator for 'active expiration' (Mellen *et al.* 2003; Janczewski & Feldman, 2006) can be another mechanism for skipped bursts.

The significance of 'deletions' deduced from discrepant activity between motor outputs implicitly assumes that all motor activity arises from the same underlying CPG (Mellen *et al.* 2003; Janczewski & Feldman, 2006; McCrea & Rybak, 2008). In embryonic or early neonatal *in vitro* preparations lacking more rostral brain areas or in transgenic mice with mutations that may affect other neural circuits (Gray *et al.* 2010; Tupal *et al.* 2014*a*,*b*), nerve activity may be highly variable, and its provenance may be unclear. Activity in respiratory motor nerves may be driven by reflexes *in vivo*, e.g. during emesis or Valsalva manoeuvres, or by spinal locomotor circuits in wild-type rodents *in vitro*, and not by brainstem respiratory circuits

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(Viala *et al.* 1979; Schomburg *et al.* 2003; Bartlett & Leiter, 2012). Moreover, motoneurons themselves may burst spontaneously without premotor input, especially when neuromodulators are added or excitability is increased (Cifra *et al.* 2009; Manuel *et al.* 2012). Simultaneous recording or silencing of the preBötC or other oscillators can ensure that nerve activity is respiratory-related and preBötC in origin.

preBötC activity is not limited to producing normal breathing, and the preBötC appears to generate multiple burst types that underlie the distinct inspiratory airflow (and associated motor activity) of sighing, gasping, gagging, coughing, sneezing and sniffing (Bartlett & Leiter, 2012). When studied *in vitro*, preBötC burst types are sometimes mapped to *in vivo* behaviour based primarily on similarities in shape (Lieske *et al.* 2000). Assignment of a particular burst shape in even the most slightly altered or reduced preparation to a given function or behaviour *in vivo* can be problematic (Kam *et al.* 2013*a*). Sensory or neuromodulatory afferents and efferent patterning microcircuits can dramatically alter how breath types and preBötC burst types appear when inputs and outputs are removed in reduced preparations (Janczewski *et al.* 2013; Kam *et al.* 2013*a*).

As burst shape is variable and malleable and shape-based criteria for burst types are poorly constrained, differentiating between burst types across experiments, or even across conditions within the same experiment, is hardly unambiguous. Treating distinct burst types interchangeably (e.g. Rybak *et al.* 2004; Ruangkittisakul *et al.* 2008; Rose *et al.* 2009; Jasinski *et al.* 2013; Tupal *et al.* 2014*a*) may confound mechanistically distinct processes. Concomitant recordings of motor output, lacking in many studies (Lieske *et al.* 2000; Ruangkittisakul *et al.* 2008; Chapuis *et al.* 2014), would help determine the relationship between preBötC activity *in vitro* and motor output and airflow *in vivo*. For example, simultaneous recording of both preBötC and XIIn activity clearly delineates burstlets from bursts, and a distinct burst type, which we call doublets, closely resembles the double breaths that represent sighs in vagotomized animals (Kam *et al.* 2013*a*).

Beyond breathing: lessons for other neural microcircuits

Approaches for studying microcircuits were pioneered in invertebrates (see Getting, 1989; Marder & Calabrese, 1996; Selverston, 2010). The tremendous progress in elucidating specific network mechanisms in invertebrate CPGs has not, however, translated to a deep understanding of vertebrate, particularly mammalian, microcircuits or CPGs (Selverston, 2010). A major difference limiting the application of invertebrate mechanisms to mammalian microcircuits is the evolutionary increase in the number of neurons from invertebrate to mammalian CPGs (Katz *et al.* 2013). This expansion ultimately results in the dispersion of function and control from one or a few invertebrate neurons to tens or hundreds of mammalian neurons (Ainsworth *et al.* 2012), allowing for differential activity amongst these neurons and redundancy across neurons. With such networks, the vulnerability of the network to damage or disease is reduced, perhaps obviating the need for degenerate mechanisms to maintain robustness (Edelman & Gally, 2001; Marder & Taylor, 2011), while network lability is preserved. Where the invertebrate work continues to inform mammalian microcircuits, including respiratory microcircuits generating rhythm, is in relating biophysical detail to behaviour (Schulz *et al.* 2006), utilizing and testing models (Prinz *et al.* 2004; Nowotny *et al.* 2007), evaluating the role of neuromodulation (Marder *et al.* 2014), and confronting issues associated with biological complexity (Selverston, 2010; Marder & Taylor, 2011) that presage the challenges facing our efforts to understand the larger microcircuits found in mammals.

The CPG controlling breathing uniquely offers many of the advantages of invertebrate CPGs, i.e. smaller size, complete regional identification, well-defined function, *in vitro* accessibility, and behavioural simplicity, while retaining properties specific to mammalian brain. We hope we have made clear that understanding the neural control of breathing is still a tough problem. Nonetheless, the respiratory CPG remains a rich and inimitably tractable model system for establishing general approaches for understanding complex, emergent mechanisms in mammalian neural microcircuits.

One need not look far to appreciate the importance of respiratory rhythmogenesis for understanding other microcircuits and behaviours. As a continuous behaviour, breathing is constantly modulated, interacting with and sometimes controlling other behaviours, including respiratory-related behaviours, e.g. sniffing or coughing, orofacial movements, e.g. chewing, whisking or suckling, emotional behaviours, e.g. crying or sighing, and volitional movements, e.g. vocalization (Heywood *et al.* 1996; Bartlett & Leiter, 2012; Feldman *et al.* 2013; Moore *et al.* 2013, 2014). How these and other microcircuits interact depends critically on their interconnectivity and other details and properties described above, elaborating upon the basic emergent mechanisms suggested here for the preBötC.

The effort to elucidate rhythmogenic mechanisms in the preBötC may also inform the study of other rhythmic microcircuits, including locomotor CPGs (Grillner & Jessell, 2009) and cortical, thalamic and hippocampal microcircuits generating physiological and pathophysiological rhythmic activity (Buzsaki, 2006). The different mechanisms proposed for these various rhythmic microcircuits demonstrate that the same

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neural computation, namely rhythm generation, can be implemented in a number of ways (Buzsaki, 2006). However, issues common to rhythmic mammalian microcircuits can, nonetheless, be identified. How is activity synchronized across a population? How does the degree of connectivity affect rhythmogenesis? How do changes in drive affect frequency? Does rhythmic activity remain intrinsic to the microcircuit, serving as background activity to support microcircuit function or is the rhythmic activity transmitted to other microcircuits?

Finally, for these and other mammalian microcircuits, *caveat experimentor*. As with the respiratory CPG, axiomatic mechanisms derived from apparently parsimonious, heuristic, or phenomenological understandings of microcircuits may not withstand rigorous experimental testing and commonly abstracted biological details may in fact be consequential for a mechanistic understanding of the microcircuit (Kumar *et al.* 2013; Mitra, 2014). The lesson here is that defining microcircuit boundaries and functionality (Horton & Adams, 2005; Briggman & Kristan, 2008), classifying neuronal populations (Sharpee, 2014), comparing results across experimental conditions and preparations, deducing dynamics from connectivity (Kopell *et al.* 2014), and extracting how the microcircuit transforms the spatiotemporal activity patterns of physiological input into output appropriate for downstream microcircuits during behaviour are far from clear cut or straightforward.

Conclusion

Understanding neural circuits constitutes the next great challenge in basic neuroscience (BRAIN Working Group, 2013), and many novel concepts and principles related to mammalian microcircuit function remain to be discovered. For most behaviours, the lack of understanding at the microcircuit level is a roadblock for determining mechanisms by which genes, molecules, synapses and individual neurons initiate, produce, modulate and integrate behaviour. Microcircuits underlying respiratory rhythm generation offer an opportunity to bridge the gap between the dynamics of a mammalian behaviour and biological mechanisms that span the scale from protein expression to synapses, intrinsic neuronal properties to connectivity in populations, and microcircuits to behaviour.

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Additional information

Competing interests

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