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Networks within networks: The neuronal control of breathing

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Abstract

Breathing emerges through complex network interactions involving neurons distributed throughout the nervous system. The respiratory rhythm generating network is composed of micro networks functioning within larger networks to generate distinct rhythms and patterns that characterize breathing. The pre-Bötzinger complex, a rhythm generating network located within the ventrolateral medulla assumes a core function without which respiratory rhythm generation and breathing cease altogether. It contains subnetworks with distinct synaptic and intrinsic membrane properties that give rise to different types of respiratory rhythmic activities including eupneic, sigh, and gasping activities. While critical aspects of these rhythmic activities are preserved when isolated in *in vitro* preparations, the pre-Bötzinger complex functions in the behaving animal as part of a larger network that receives important inputs from areas such as the pons and parafacial nucleus. The respiratory network is also an integrator of modulatory and sensory inputs that imbue the network with the important ability to adapt to changes in the behavioral, metabolic, and developmental conditions of the organism. This review summarizes our current understanding of these interactions and relates the emerging concepts to insights gained in other rhythm generating networks.

Keywords

Breathing; Respiratory rhythm generation; Pre-Botzinger complex and interactions

Introduction

Behaviors are continuously adapted to changes in an organism's internal and external environment. Not surprisingly, a large degree of plasticity characterizes all levels of neuronal integration from the molecular, cellular to the network and ultimately behavioral level. Common principles of behavioral plasticity have been described in numerous invertebrates (Harris et al., 2010; Marder and Goaillard, 2006; Nadim et al., 2008; Nusbaum, 2002; Ramirez and Pearson, 1993) and mammalian model systems including humans (Lee et al., 2009; Macfarlane and Mitchell, 2009; Millhorn et al., 1980; Peng et al., 2003). In this review, we will focus on modulatory processes that are critical for the neuronal control of mammalian breathing, but we will also describe how insights gained in the respiratory system relate to other networks and behaviors. Breathing is well integrated with many other behaviors and needs to adapt continuously to changes in the metabolic and behavioral demands of an organism. Both the respiratory frequency and amplitude of breathing are adapted to behavioral conditions like posture, physical activity, sleep, or speech. In fact, breathing is so sensitive to an organism's internal state that the characteristics of breathing

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can reveal whether someone is calm, agitated, or scared. As in most animal behaviors, plasticity in the respiratory system seems to depend on a variety of amines, steroids, and peptides that exert their modulatory actions by acting on a large number of ion channels, receptors, and second messenger systems (Doi and Ramirez, 2008). Neuromodulatory processes play equally important roles during well-oxygenated and hypoxic conditions, and when disturbed neuromodulation has been associated with a number of pathophysiological conditions ranging from Rett syndrome (Viemari et al., 2005a) to Sudden Infant Death Syndrome (SIDS) (Paterson et al., 2009).

The mammalian respiratory network and the nuclei controlling neuromodulation are widely distributed along the neural axis. Important for breathing are the neuronal networks located in the ventral respiratory column (VRC) (Alheid et al., 2002; Feldman and Del Negro, 2006; Feldman et al., 2003; McCrimmon et al., 2004). These networks (Fig. 1) include, from rostral to caudal, the retrotrapezoid nucleus/parafacial respiratory group complex (RTN/pFRG), the Bötzinger complex, the pre-Bötzinger complex (pre-BötC), the rostral ventral respiratory group (rVRG), and the caudal VRG (cVRG) (Alheid et al., 2002; Feldman and Del Negro, 2006; Feldman et al., 2003; McCrimmon et al., 2004; Onimaru and Homma, 2003; Onimaru et al., 2006; Smith et al., 1991; Thoby-Brisson et al., 2009). The distinction between these networks is based on a histological characterization (Alheid et al., 2002; Gray et al., 1999; Guyenet et al., 2002), and their distinct functional properties (Gray et al., 1999, 2001; Janczewski and Feldman, 2006; Janczewski et al., 2002; McCrimmon et al., 2004; Onimaru and Homma, 2003; Onimaru et al., 2006; Thoby-Brisson et al., 2005, 2009). An important role in respiratory rhythm generation and modulation has also been described for the Kölliker-Fuse nucleus and the parabrachial complex; both are located in the dorsal pons (Alheid et al., 2004; Dick et al., 1994; Kobayashi et al., 2005; Milsom et al., 2004). The role of these neurons in the modulation and generation of eupnea (Chamberlin and Saper, 1994; Dutschmann and Herbert, 1996; St-John and Paton, 2004; Von Euler and Trippenbach, 1976) and in the transition phase between inspiration and expiration (Cohen, 1971; Morschel and Dutschmann, 2009; Von Euler and Trippenbach, 1976) has received considerable attention. Other areas involved in breathing include the cerebellum (Harper, 2000a; Harper et al., 2000b), the neocortex (Davenport et al., 2010; Von Leupoldt et al., 2010), as well as the periaqueductal gray, which is particularly important for the integration of speech and breathing (Subramanian and Holstege, 2010).

An area that is both essential and sufficient for generating the respiratory rhythm is the pre-BötC, a network located within the ventrolateral medulla (Smith et al., 1991). Disruption of rhythmic activity in the pre-BötC causes irreversible loss or major disruption of breathing *in vivo* (Gray et al., 2001; McKay et al., 2005; Ramirez et al., 1998c; Tan et al., 2008). Isolated in a brainstem slice preparation, the pre-BötC continues to generate respiratory activity (Fig. 2) (Ramirez et al., 1996; Smith et al., 1991). In these slices, respiratory rhythmic activity can be recorded directly from the surface of pre-BötC (Lieske et al., 2000) or from the hypoglossal motor nucleus, which receives respiratory rhythmic input via an interneuronal population located outside the hypoglossal nucleus (Koizumi et al., 2008).This *in vitro* approach has greatly facilitated our understanding of the cellular mechanisms of respiratory rhythm generation and neuromodulation. Although we still lack a deep understanding of how the different areas of the respiratory network interact and how they are altered by neuromodulation, the pre-BötC itself has provided an important avenue to study not only the fundamental elements of respiratory rhythm generation, but also how a single neuronal network can generate multiple rhythmic activity patterns as previously demonstrated in invertebrate neuronal networks (Harris-Warrick and Johnson, 2010; Marder and Goaillard, 2006; Meyrand et al., 1991; Ramirez, 1998a). This review will focus on our current understanding of respiratory rhythm generation in the pre-BötC and its alteration by

reconfiguration, neuromodulation, and plasticity (Fig. 1). We will also consider the possible roles of these mechanisms in physiological and pathophysiological conditions.

Network reconfiguration

The notion that the respiratory network can reconfigure to generate different forms of breathing such as eupnea, sighs, and gasps was introduced approximately a decade ago (Lieske et al., 2000). Since that time, much has been learned not only about the mechanisms of reconfiguration within the pre-BötC (Pena et al., 2004a), but also about the role of the pons and other areas in reconfiguring the network from the eupneic into the gasping state (Paton et al., 2006).

Under well-oxygenated conditions, the pre-BötC generates two distinct rhythms: a faster small amplitude rhythm ("fictive eupnea") and a much slower large amplitude rhythm (Fig. 2; "fictive sighs"). Several studies have shown that these activities originate from a multifunctional network located within the pre-BötC that can be partly preserved in the *in vitro* transverse medullary slice (Lieske and Ramirez, 2006a; Lieske et al., 2000; Ruangkittisakul et al., 2008; Tryba et al., 2008). Although the majority of neurons are activated during both eupneic and sigh rhythmic activities, pharmacological manipulations suggest that both activities emerge through distinct mechanisms. Fictive sigh, but not eupneic activity, is critically dependent on synaptic mechanisms involving the P/Q type calcium channels (Cav2.1). Interestingly, only a relatively small subpopulation of respiratory neurons receive glutamatergic inputs that depend on P/Q type calcium currents (Lieske and Ramirez, 2006a), suggesting that the pool of respiratory neurons contains a subset of neurons with specialized synapses that are critical for sigh rhythm generation. These synapses depend also on the activation of mGluR8 receptors (Lieske and Ramirez, 2006b), while NMDA-dependent mechanisms play an important role in the generation of eupneic activity (Lieske and Ramirez, 2006b; McCrimmon et al., 1997). Thus, in the respiratory network an overlapping pool of neurons generates different rhythmic activities using different behavior-specific types of synaptic mechanisms. This is a common principle that has also been described in the spinal cord (Berkowitz et al., 2010), the neocortex (Kramer et al., 2008; Wulff et al., 2009), and various invertebrate species (Haque et al., 2006; Jing and Gillette, 2003; Wood et al., 2000).

As the respiratory network responds to hypoxia, the breathing frequency *in vivo* transitions into an augmentation followed by depression (Fig. 2A; England et al., 1995; Haddad and Mellins, 1984; Neubauer et al., 1990), a sequence that is also seen in the isolated pre-BötC (Telgkamp and Ramirez, 1999). During the depression phase, the inspiratory burst changes from an augmenting, bell-shaped burst to a decrementing burst which is one of the characteristic features of gasping (Fig. 2A and B; Lieske et al., 2000; Pena et al., 2004a). The transition into the gasping activity pattern can be gradual, both *in vitro* and *in vivo*. During the transition, bursts with varying rise time and burst duration are typical, a characteristic that was referred to as pre-gasping *in vivo* (Wang et al., 1993). Hypoxia causes also a characteristic decrease in synaptic inhibition, which has been observed under both *in vivo* and *in vitro* conditions (England et al., 1995; Ramirez et al., 1998b; Richter et al., 1991; Thoby-Brisson and Ramirez, 2000; Völker et al., 1995). The depression of synaptic inhibition contributes to the reconfiguration of the respiratory network by altering the discharge pattern of a variety of neurons. Late-inspiratory neurons discharge earlier during inspiration (England et al., 1995). Many expiratory and inspiratory neurons in the ventrolateral medulla become inactive before cessation of phrenic and/or hypoglossal (XII) activity (Ballanyi et al., 1994; England et al., 1995; Ramirez, 1998a; Richter et al., 1991; Telgkamp and Ramirez, 1999; Thoby-Brisson and Ramirez, 2000). While XII neurons exhibit a massive potentiation of the rhythmic bursts both *in vivo* and *in vitro* (Ramirez et

al., 1997; Telgkamp and Ramirez, 1999), respiratory neurons in the ventrolateral medulla respond inconsistently and become either weakly de- or hyperpolarized (Ramirez et al., 1998b; Richter et al., 1991; Thoby-Brisson and Ramirez, 2000).

Within the pre-BötC, neurons can be differentiated into nonpacemaker and pacemaker neurons dependent on their ability to intrinsically generate bursting activity. Since nonpacemaker and pacemaker neurons are differentially affected by hypoxia and neuromodulators, they will be considered in more detail in this review. In isolation from fast synaptic transmission, nonpacemaker neurons either enter a tonic firing state or become quiescent while pacemaker neurons retain spontaneous bursting properties (Pena et al., 2004a). Pacemaker neurons can be further identified into cadmium sensitive (CS) and cadmium insensitive (CI) pacemaker neurons. Bursting in CS pacemakers seems to depend on a nonspecific cation current (I_{CAN}) while burst properties of CI pacemaker appear to be mediated via the persistent sodium current (I_{NaP}) (Pena et al., 2004a). Inhibition of these currents in the respective pacemaker subtypes eliminates their ability to spontaneously burst in synaptic isolation (Chevalier et al., 2008; Del Negro et al., 2002a, 2005; Pena et al., 2004a; Tryba and Ramirez, 2004). However, it is important to emphasize that neither I_{CAN} nor I_{NaP} are exclusive currents mediating pacemaker properties. These inward currents play also critical roles in amplifying synaptic inputs not only in pacemaker neurons, but also nonpacemaker neurons (Del Negro et al., 2002b, 2005; Ramirez et al., 1996; Rubin et al., 2009). Indeed, the role of bursting properties in amplifying synaptic inputs has been described in many neuronal systems, such as the locust flight system in which sensory synaptic inputs are amplified in a nonlinear manner by intrinsic bursting mechanisms (Ramirez and Pearson, 1991, 1993). As will be discussed later in this review, bursting properties can be induced and suppressed by neuromodulators, which imbues neuronal networks with the ability to amplify specific synaptic pathways in a state and behavioral dependent manner. In the locust flight system, bursting properties are induced at the onset of flight to nonlinearly amplify sensory inputs (Ramirez and Pearson, 1993). During locomotion intrinsic membrane properties boost synaptic input also in the spinal cord (Brownstone et al., 1992). These inward currents play important roles also in a variety of other mammalian systems, such as in networks involved in mastication (Kolta et al., 2007). In the neocortex, these currents depolarize neurons toward their firing threshold (Pennartz et al., 1997) and boost synaptic input (Schwindt and Crill, 1999; Stuart and Sakmann, 1995). Pharmacological blockade of I_{NaP} has been shown to modulate locomotion generated in the spinal cord (Darbon et al., 2004; Tazerart et al., 2007) and it can suppress slow oscillations generated in cortical networks (van Drongelen et al., 2006).

It is impossible to negate the fact that a substantial portion of neurons that are activated during respiration possess bursting properties. Moreover, the majority of neurons possess I_{NaP} or I_{CAN} or both, and the balance between these two major inward currents and a set of outward currents determines whether a neuron is a pacemaker or nonpacemaker (Koizumi et al., 2010). This conclusion has important implications for rhythm generation in general (Hudson and Prinz, 2010), which makes it difficult to differentiate between pacemakers and nonpacemakers as the balance between inward and outward currents is gradual. Nonpacemakers without any bursting properties and pacemakers with strong bursting properties are the extremes of a gradient of neurons possessing different degrees of bursting properties. Consequently, it is probably impossible to unambiguously dissect the relative contribution of pacemaker versus nonpacemakers. The ratio between pacemakers and nonpacemakers is not fixed, but dependent on the metabolic and modulatory state of the network, since neuromodulators, such as NE, SP, or 5-HT, can induce bursting in nonpacemakers (Pena and Ramirez, 2002, 2004b; Viemari and Ramirez, 2006). In an attempt to determine the relative contribution of these inward currents to respiratory rhythm generation, various laboratories have employed substances that are known to block these

currents. Blocking either I_{NaP} with Riluzole or the I_{CAN} by Flufenamic acid alone does not block fictive eupnea (Pena et al., 2004a), but both substances together lead to the cessation of respiratory rhythmic activity indicating that these conductances are critical for respiratory rhythm generation. A recent study extended these observations by demonstrating that applying Substance P or low doses of AMPA in the presence of Riluzole and Flufenamic acid could restimulate rhythmogenesis (Del Negro et al., 2005). Hence, the relative importance of pacemaker neurons in well-oxygenated conditions is the matter of an ongoing debate.

It is, however, clear that the relative contribution of these currents changes considerably during the transition from eupneic to gasping activity (Fig. 1). While eupneic activity involves the activation of neurons possessing I_{NaP} and I_{CAN}-dependent bursting mechanisms, pacemaker neurons that depend on I_{CAN} selectively hyperpo-larize during hypoxia rendering the network more dependent on I_{NaP} during gasping, a finding that has been confirmed *in vitro, in situ*, and *in vivo* (Paton et al., 2006; Pena and Aguileta, 2007; Pena et al., 2004a, 2008).

Pacemaker neurons may also differentially contribute to the generation of eupneic versus sigh activity, because the generation of sighs is more sensitive to the manipulation of the I_{NaP} current (Tryba et al., 2008). Thus, lessons learned from the respiratory network indicate that the different network states are characterized by differential contribution of different types of bursting mechanisms.

Neuromodulation and rhythm generation

In the respiratory network, the same neuromodulator differentially acts on a variety of receptor subtypes, thereby exerting specific and sometimes even diverging effects on different parameters of the network output. Perhaps, best understood in the neuronal control of breathing is the role of catecholaminergic neurons that are found in the brainstem and project onto neurons of the respiratory network (Doi and Ramirez, 2008; Hilaire, 2006; Hokfelt et al., 1984; VanderHorst and Ulfhake, 2006; Viemari and Hilaire, 2002; Viemari and Ramirez, 2006). These neurons are clustered in noradrenergic (Fig. 1; A1, A2, A5, A6, and A7) and adrenergic (C1 to C3) nuclei. In the pons, the activity of A6 (i.e., the locus coeruleus) neurons is modulated by hypoxia both *in vivo* (Guyenet et al., 1993) and *in vitro* (Nieber et al., 1995; Yang et al., 1997). Although some A6 neurons are excited while others inhibited during hypoxia, A6 exerts an overall stimulatory effect on breathing. Electrical stimulation of the locus coeruleus increases breathing frequency (Doi and Ramirez, 2010), whereas genetic alteration of A6 decreases respiratory activity (Viemari et al., 2004; Viemari et al., 2005a). Unlike the A6 nucleus, all neurons in A5 appear to be activated by hypoxia *in vivo* (Guyenet et al., 1993) and inhibit breathing. Thus, lesions of A5 increase the respiratory frequency *in vitro* (Viemari and Hilaire, 2002) and reduce the posthypoxic frequency depression (Coles and Dick, 1996). Thus, while both A5 and A6 contain noradrenergic neurons that modulate respiratory network activity, their effects are very diverse even though they are modulated under similar conditions. In mutants where A5 or A6 neurons are altered (Viemari et al., 2004, 2005a), the hypoxic response is blunted supporting a role of both groups in oxygen sensing. A5 and A6 are not the only noradrenergic nuclei modulating respiration: In the medulla, endogenous release of NE from A1/C1 neurons stimulates the respiratory rhythm *in vitro* (Zanella et al., 2006). Exogenously applied NE on medullary slices stimulates the respiratory rhythm by a direct effect on inspiratory neurons (Viemari and Ramirez, 2006). Because hypoxia increases TH expression (Peyronnet et al., 2003; Roux et al., 2000, 2003) and Fos-like immunoreactivity (Erickson and Millhorn, 1994; Teppema et al., 1997), it is likely that neurons in the A1/C1 and A2/C2 region are activated by low oxygen levels. This modulatory role may be disturbed in certain

neurological disorders. In Mecp2 mutant mice, a model for Rett syndrome, the number of tyrosine hydroxylase positive neurons in A1/C1 and A2/C2 and the level of norepinephrine in the medulla is decreased (Viemari et al., 2005b), yet these mice show an increased ventilatory response to hypoxia (Roux et al., 2008; Voituron et al., 2009).

Mechanistically, NE stimulates inspiratory nonpacemaker and pacemaker neurons contained within the pre-BötC acting presumably via α1, α2, and β-noradrenergic mechanisms (Viemari and Ramirez, 2006). NE induces I_{CAN}-dependent bursting properties in active nonpacemaker neurons, and it depolarizes CI pacemakers and increases their burst frequency. In CS pacemakers, NE increases only the amplitude of the depolarizing drive potential and the number of action potentials during the burst. However, in contrast to the situation in CI pacemakers NE does not affect the burst frequency in CS pacemakers. This differential effect is preserved at the network level, since only the modulation of the burst amplitude but not the frequency depends on the activation of I_{CAN} (Viemari and Ramirez, 2006). This leads to the important conclusion that different network parameters are differentially modulated by the same neuromodulator acting on different cellular targets.

NE is not the only bioamine acting on the respiratory network. Like other catecholaminergic neurons, serotonergic neurons are also found in the brainstem and project to neurons involved in breathing (Dahlstrom and Fuxe, 1964; Fuxe, 1965; Holtman et al., 1990; VanderHorst and Ulfhake, 2006). Serotonergic neurons are contained in nuclei numbered from B1 to B9, from the caudal to the rostral axis (Dahlstrom and Fuxe, 1964). The nuclei can also be referred to as raphe pallidus (B1), raphe obscurus (B2), and raphe magnus (B3). The action of these groups on breathing is diverse and often data are contradictory probably due to species differences, state of the animals (awake or sleep), or type and level of anesthesia (Besnard et al., 2009; Doi and Ramirez, 2010; Holtman et al., 1986; Lalley, 1986; Sessle et al., 1981). For instance, in rats under volatile anesthesia (Besnard et al., 2009) electric stimulation of raphe magnus and obscurus induced apnea, whereas stimulation of the raphe pallidus induced tachypnea. On the other hand, in mice anesthetized with urethane (i.p.) electric stimulation of the raphe magnus increases respiratory frequency (Doi and Ramirez, 2010). In cats, stimulation of raphe pallidus and obscurus (Holtman et al., 1986; Lalley, 1986) increases phrenic discharge amplitude and frequency, whereas they decrease during stimulation of the raphe magnus (Lalley, 1986; Sessle et al., 1981). Similarly, exogenous application of serotonergic agents *in vitro* has various actions on respiratory activity (Di Pasquale et al., 1992, 1994; Hilaire et al., 1997; Morin et al., 1990; Schwarzacher et al., 2002). In brainstem slices containing the pre-BötC, exogenous application of 5HT2A agonist or blockade of serotonergic reuptake increases inspiratory frequency (Pena and Ramirez, 2002). Consistent with these results, blockade of the activation of 5HT2A receptors by endogenous release of serotonin decreases respiratory frequency. This has been recently confirmed in rats using transverse brainstem slices and *in situ* preparations and extended to 5HT2C and 5HT4 receptors (Ptak et al., 2009). Indeed, neurons from the raphe obscurus show a tonic activity and emit projections to the pre-BötC, which innervates the raphe reciprocally.

Neuromodulation cannot be discussed without also emphasizing the importance of peptidergic modulation. Perhaps, the most studied peptide in the respiratory network is substance P (Gray et al., 2001; Hayes and Del Negro, 2007; Morgado-Valle and Feldman, 2004; Pena and Ramirez, 2004b). Neurons releasing substance P are localized in the nucleus of solitary tract (NTS), the nucleus ambiguous (NA), the raphe, the dorsal motor nucleus of the vagus (X), and the hypoglossal nucleus (Ribeiro-da-Silva and Hokfelt, 2000). Substance P is often coreleased with other neurotransmitter. For example, neurons in the raphe contain 5-HT and substance P (Kachidian et al., 1991; Ptak et al., 2009) and they project directly on the pre-BötC. NK-1 receptors are strongly expressed on pre-BötC neurons as well as

serotonergic neurons of the raphe (Alheid and McCrimmon, 2008; Gray et al., 2001; Stornetta et al., 2003; Wang et al., 2001). Substance P activates the inspiratory frequency at the network and behavioral level (Del Negro et al., 2005; Doi and Ramirez, 2010; Gray et al., 2001; Pena and Ramirez, 2004b; Thoby-Brisson et al., 2005). At the cellular level, substance P slowly depolarizes nonpacemaker neurons, leading to an increase of the firing rate of action potentials. Substance P also dramatically activates CS pacemakers and to a lesser extent CI pacemakers, causing an increase in burst amplitude, frequency, and duration (Pena and Ramirez, 2004b).

This discussion leads to the important conclusion that every parameter in rhythm generation is controlled by multiple modulators. It must be emphasized that for simplicity we focused in this review only on three neuromodulators, even though there are many additional modulators that play equally important roles in the neuronal control of breathing (Doi and Ramirez, 2008). Another important conclusion is that the same modulator can exert many different effects on rhythmic activity. Thus, the influence of any given neuromodulator occurs in concert with many other aminergic and peptidergic substances (Fig. 1). The complexity of neuromodulation as described here for the respiratory network is reminiscent to the complexity that is well documented in invertebrate neuronal networks (Nusbaum, 2002; Thirumalai and Marder, 2002) and has important implications for all neuronal networks. Different sets of modulators with diverse, convergent, and divergent actions will define different states of a rhythm generating network that may change dependent on the metabolic, developmental or behavioral conditions of an animal. These network states involve a complex orchestration of large sets of different ion-channels, multiple receptors, and numerous neuropeptides and biogenic amines in addition to those described here (Doi and Ramirez, 2008; Grashow et al., 2009; Thoby-Brisson and Simmers, 1998). A modulatory state will, therefore, not only determine how a rhythm is generated, but will also determine the responsiveness of neuronal networks to inputs. Many recent experimental and computational studies suggest that neuronal networks respond differently to inputs if their state is altered by modulators (Destexhe and Contreras, 2006; MacLean et al., 2005; Nadim et al., 2008; Prescott and De Koninck, 2003). In the respiratory network, numerous excitatory and inhibitory inputs that arise from multiple anatomical regions of the brain to modulate breathing in amplitude, frequency, and regularity have been described (Doi and Ramirez, 2008).

Unfortunately, only little is known how different competing inputs interact in the context of different neuromodulators. In the respiratory network, excitatory inputs mediated by substance P are only critical for respiratory rhythm generation when levels of serotonin or NE are low, that is, under conditions that resemble more closely the sleep state (Jones, 2005). By contrast, when 5-HT2a receptors and α-1 receptors are fully activated, that is in a state when serotonin and norepinephrine levels are high, inputs mediated by substance P are not critical for respiratory rhythm generation (Doi and Ramirez, 2010). Thus, the convergence of different neuromodulatory inputs (Fig. 1) provides a safety net in case a given modulator or receptor is disturbed. It may also explain the state-dependency of a variety of disorders. In SIDS, there is mounting evidence for a role for 5-HT (Broadbelt et al., 2009; Duncan et al., 2010; Kinney et al., 2009; Paterson et al., 2006; Rand et al., 2007). SIDS (and also sleep apnea) occurs during sleep, when aminergic and presumably also SP levels are reduced. Thus, it seems that the respiratory network is critically dependent on an individual neuromodulator only in a reduced “modulatory state.” By contrast, in the awake animal, it is unlikely that a disruption of a single neuromodulator will have much of a critical effect, since the level of other converging modulators is relatively high. Thus, understanding the convergence of different neuromodulators is not only an interesting basic scientific issue, but will also be important for gaining insights into the neuropathology of breathing disorders.

The data summarized above indicate that it is not possible to associate specific network states with the action of individual neuromodulators. Instead, a network state is defined by a complex set of different modulators with highly diverse, convergent, and divergent actions. A particular neuromodulator can differentially act on a variety of receptor subtypes, thereby exerting specific, yet sometimes diverging effects on different parameters of the respiratory network (e.g., frequency vs. amplitude). Conversely, different neuromodulators, such as serotonin, SP, or NE, can exert similar effects via different cellular mechanisms. The specificity and types of these modulatory effects are not necessarily preserved across different animal species even if compared between related rodent species and strains. These conclusions are reminiscent of findings in invertebrate systems. Like in the respiratory system, modulatory responses can vary between different species and even across individuals of the same species at different developmental stages (Newcomb and Katz, 2007, 2009; Rehm et al., 2008). Also similar to the situation in the respiratory network is the observation that different neuromodulatory inputs can exert distinct or comparable activity patterns from the same neuronal ensemble (Saideman et al., 2007). Like in these invertebrate networks, it is remarkable that despite the large number of concurrent and rather diverse modulatory processes the various parameters of rhythm generating networks are relatively tightly maintained even when exposed to extreme conditions. This can be illustrated for the control of the respiratory frequency in humans. The breathing frequency of children under normal conditions is 45±13 breaths/min. Acute exposure to 3109 m increases the frequency range on average by only 86 breaths/min to 51.9±15 breaths/min (Yaron et al., 2003). Under extreme altitudes, such as climbing to the Everest (8000 m), only one individual of the American Medical Research Expedition raised the breathing rate to up to 86 breaths/min (West, 2010). Thus, despite the presence of numerous modulatory systems affecting breathing frequency, the range over which this particular parameter is modulated is relatively narrow under normal as well as extreme conditions. For the invertebrate model system, it has been suggested that homeostatic mechanisms play critical roles in maintaining different parameters in a tightly controlled range (Rehm et al., 2008). Although little is known about how these mechanisms regulate the various parameters of the mammalian respiratory rhythm, they must play critical roles in the homeostasis of breathing (Fig. 1) as will be discussed below.

Homeostatic plasticity and other forms of long-term plasticity

Homeostatic plasticity is a fundamental mechanism that has been demonstrated in a variety of neural networks. It is crucial to maintain network stability and it affects nearly every aspect of circuit development and function (Fig. 1). One principle mechanism is the activity dependent scaling of neuronal receptors to maintain neurons in a certain firing range (Turrigiano et al., 1998). But, multiple forms of homeostatic plasticity have been described in a variety of brain areas (Aizenman et al., 2003; Desai et al., 1999; Ibata et al., 2008; Koch et al., 2010; Marder and Goaillard, 2006; Stellwagen and Malenka, 2006). Homeostatic plasticity has been shown to regulate pre- and postsynaptic scaling of excitation and inhibition. Such changes involve alterations in ion-channel composition which tune and anchor intrinsic neuronal excitability to a defined range of activity. The mechanisms that define and regulate these setpoints of cellular activity are not well understood. Alterations in internal calcium levels $[Ca^{2+}]_i$ have been discussed as a possible mechanism balancing synaptic homeostasis in neuronal networks (Turrigiano, 2008). In fact, since calcium fluctuations are directly related to the activity of the cells, it has been hypothesized that calcium plays important roles in regulating many forms of plasticity (Grubb and Burrone, 2010; Lisman et al., 2002; Malenka and Bear, 2004; Zhang and Linden, 2003). Consistent with this hypothesis, blockade of calcium channels can trigger upscaling of excitatory synaptic terminals (Ibata et al., 2008). Moreover, pharmacological blockade of calcium dependent kinases (i.e., the CaMK- family) prevents the effects of activity deprivation on

excitatory synaptic transmission (Thiagarajan et al., 2002). Besides intracellular calcium as an activity sensor, multiple other molecules have been implicated to be involved to mediate homeostatic synaptic plasticity (Aoto et al., 2008; Koch et al., 2010; Rutherford et al., 1998; Stellwagen and Malenka, 2006). Interestingly, some of these molecules are part of the inflammatory pathway and include prostaglandin-E2 (PGE2) and tumor necrosis factor-α (TNF-α) (Koch et al., 2010; Stellwagen and Malenka, 2006). Activation of glia cells and TNF-α signaling are necessary for synaptic upscaling in cortical neurons, since blocking the activation of TNF-α receptors prevents the upscaling (Stellwagen and Malenka, 2006). Recently PGE2, the major reaction product of the Cyclooxygenase-2 enzymes (COX-2) was reported to acutely inhibit network activity in the neocortex, but if chronically applied led to a predominantly presynaptic increase in excitatory synaptic transmission (Koch et al., 2010). The COX-2 pathway is directly activated by hypoxia through the hypoxia induced factor-1α (HIF-1α) and PGE2 (Fig. 1) has been reported to be an important regulator of the respiratory rhythm generator (Hofstetter et al., 2007). Thus, the link of activity-dependent scaling and inflammation could potentially be important for understanding pathophysiological changes that occur in the cardiorespiratory control following chronic intermittent hypoxia (IH).

In the context of cardio-respiratory homeostasis, several studies described long-term regulatory processes for the NTS, an area that is critical for sensory integration not only in the context of breathing but also other autonomic functions (Greenberg et al., 1999a, 1999b; Kline et al., 2007; Zhou et al., 1997). Activity-dependent long-term depression (LTD) can be elicited in a subset of NTS neurons (Zhou et al., 1997), and chronic exposure to IH causes an activity dependent synaptic downscaling of excitatory synaptic transmission (Kline et al., 2007).

Perhaps, the best studied form of long-term plasticity (Fig. 1) in the respiratory system is the response to intermittent hypoxia. Investigating the neuronal consequences of repetitive episodes of hypoxia (Acute Intermittent Hypoxia, AIH) is clinically very relevant, as this condition is associated with a variety of breathing disorders including Rett syndrome and obstructive sleep apnea (Lee et al., 2009; Weese-Mayer et al., 2006). AIH causes persistent increases in respiratory frequency and amplitude of integrated motor neuronal bursts *in vivo* (Baker and Mitchell, 2000; Hayashi et al., 1993; Millhorn et al., 1980; Turner and Mitchell, 1997). These changes persisting for ≤90 min are collectively referred to as long-term facilitation (LTF) (Fuller et al., 2000; Powell et al., 1998). The degree of influence varies with preparation (Bach and Mitchell, 1996; Turner and Mitchell, 1997), animal strain (Fuller et al., 2000), gender (Zabka et al., 2006), and experimental conditions (Baker and Mitchell, 2000). AIH causes changes at multiple levels of the respiratory system, including the carotid body (Dogas et al., 1995; Powell et al., 1998), and motor nuclei (Kinkead et al., 1998). The likely site for the long-term frequency modulation is the pre-BötC, since intermittent hypoxia causes a long-lasting frequency increase within the pre-BötC that persists for ≤90 min after repetitive hypoxic episodes (Blitz and Ramirez, 2002). AIH causes immediate changes in the modulatory milieu and long-term changes involving gene expression. For this to occur, the intermittent pattern and not duration of hypoxia is critical, as even prolonged hypoxic exposure does not evoke LTF (Baker and Mitchell, 2000). AIH causes the intermittent production of reactive oxygen species (MacFarlane and Mitchell, 2009; Pawar et al., 2009) and release of aminergic neuromodulators. Blockade of serotonin receptors abolishes both motor amplitude and frequency LTF *in vivo* (Bach and Mitchell, 1996; Kinkead et al., 1998). It has been hypothesized (Bach and Mitchell, 1996; Fuller et al., 2000), that LTF is induced by chemoreceptor activation of serotonergic raphe neurons (Erickson and Millhorn, 1994; McCrimmon et al., 1997; Pawar et al., 2009). However, not only serotonin, but also repeated exposure to norepinephrine elicits LTF *in vitro* (Bocchiaro and Feldman, 2004; Neverova et al., 2007). Both modulatory systems seem to interact

(Kinkead et al., 2001). There is accumulating evidence indicating that long-lasting changes in intracellular signaling molecules involving a reactive oxygen species activated PKC pathway cause new synthesis of BDNF acting on TrkB receptors (Fig. 1), which in turn will increase postsynaptic glutamate receptor density and thereby increases glutamatergic synaptic transmission (Baker-Herman et al., 2004). Thus, taken together these studies suggest that neuronal networks involved in respiratory control are regulated by multiple forms of long-term synaptic plasticity.

Conclusion

The convergence of reconfiguration, neuromodulation, state dependency, and homeostatic plasticity (Fig. 1) provides multiple mechanisms by which the pre-BötC is capable of generating multiple, dynamically responsive yet stable sets of respiratory rhythmic activity. As discussed, the pre-BötC retains its ability to generate stable rhythmicity throughout changes in the oxygen environment using both network reconfiguration and neuromodulation. The ability of the pre-BötC to retain stable rhythmicity and transmit this activity to a variety of motor outputs is not a trivial property, as many neuronal networks within the mammalian nervous system shut down during hypoxia in an attempt to conserve the energy during oxygen limiting conditions. This is readily seen in the hippocampus (Krnjevic and Leblond, 1987; Garcia et al., 2010), neocortex (Jiang and Haddad, 1992), and striatum (Calabresi et al., 1995). Hence, sustaining stable rhythmicity in the pre-BötC even during severe levels of hypoxia underscores the importance of this network, as loss of the respiratory rhythmic activity would ultimately lead to death. This robustness may be one reason why the pre-BötC is capable of generating behaviorally relevant rhythmicity even when isolated from the rest of the nervous system (Fig. 2), thus facilitating a rigorous cellular and network analysis. In the intact animal, the pre-BötC constitutes a core network which operates within a larger network of interconnected nuclei that contribute not only to the generation of the respiratory rhythm, but also to the plasticity and state-dependency that characterizes breathing (Fig. 1). The principles gained in the respiratory network apply to all neuronal network functions not only to the mammalian nervous system, but also to the networks of invertebrates. The emerging conclusions gained by studying the respiratory network are surprisingly complex, and we are clearly only touching the surface of our understanding. Yet, this degree of complexity is obviously necessary to guarantee that the respiratory network is very adaptive and at the same time sufficiently stable to maintain regular network activity even under adverse environmental and behavioral conditions.

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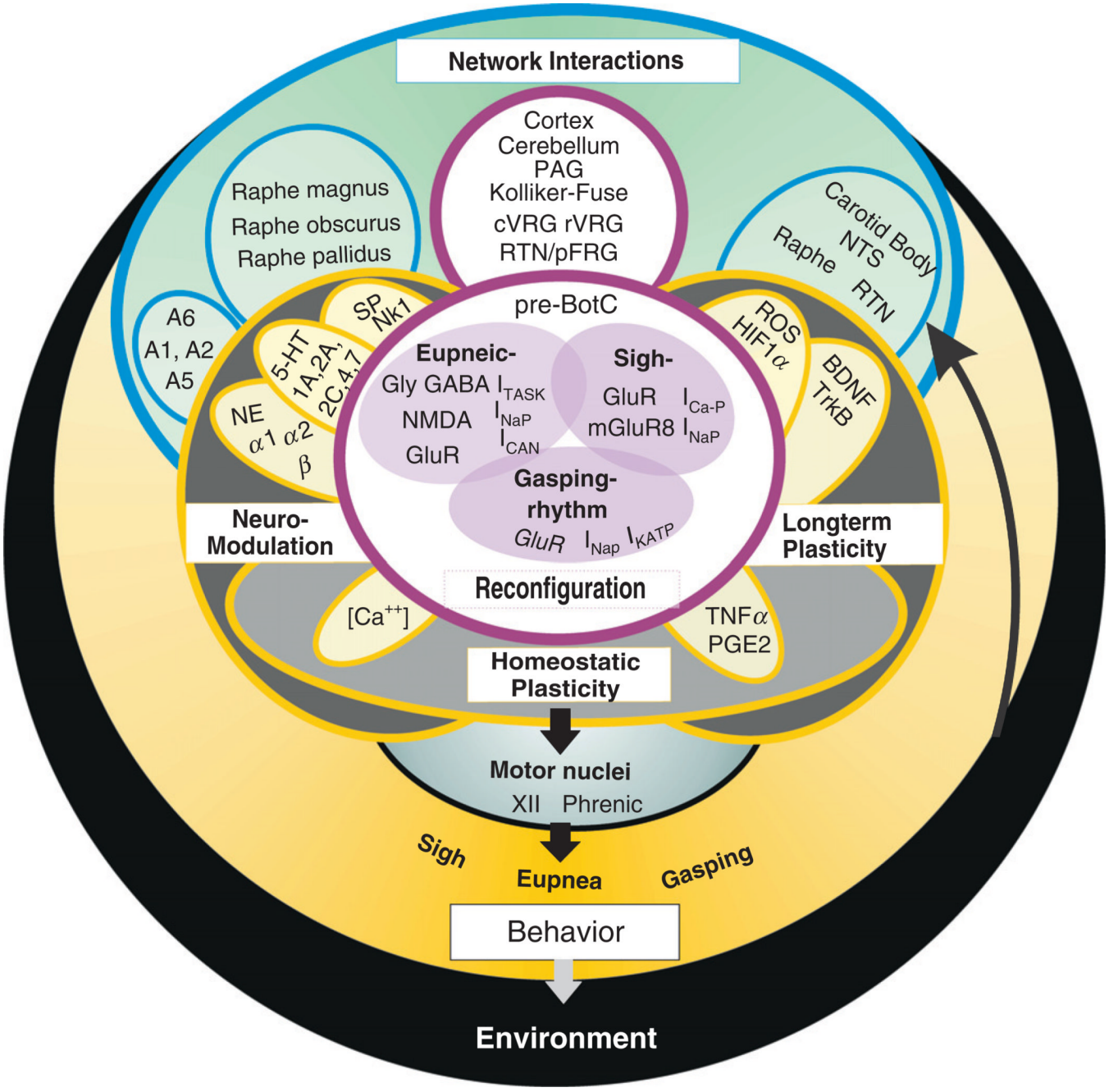

Fig. 1. Networks within networks

A schematic illustrating some of the interactions that are involved in the neuronal control of breathing. As complex as this schematic may appear, it still represents only a fraction of the known interactions. The interactions depicted in this figure are clustered according to their function in the neuronal control of breathing. *Network interactions* that are important for the generation of the respiratory rhythm include the interactions between the cortex, cerebellum, PAG (Periaqueductal Gray), Kölliker-Fuse, cVRG (caudal Ventral Respiratory Group), rVRG (rostral Ventral Respiratory Group), RTN (retrotrapezoid nucleus)/pFRG (parafacial respiratory group), and the pre-Bötzinger complex (pre-BötC). While the pre-BötC is capable of generating three distinct rhythmic activities via network reconfiguration

(eupneic-, sigh-, and gasping-rhythm) even in isolation (see also Fig. 2), in the intact animal other networks will also contribute to the reconfiguration and the shaping of the respiratory rhythms that are transmitted to the *motor nuclei*. The extent and significance of these contributions to the overall motor output and ultimately the behavior is a topic of intense investigations. Respiratory rhythm generation is also the target of three types of modulatory processes: *Neuromodulation* characterizes the modulatory processes occurring on a moment-to-moment basis and it is mediated via numerous aminergic and peptidergic substances acting on various receptor subtypes. For simplicity, the schematic illustrates only three neuromodulators: NE (norepinephrine) acting on α1, α2, and β noradrenergic receptors; 5-HT (serotonin) acting on 5-HT1A, 5-HT2A, 5-HT2C, 5-HT4, and 5-HT7 receptors; and SP (substance P) acting on the NK1 receptor. These neuromodulators are released by nuclei that include the Raphe magnus, obscurus, and pallidus and the noradrenergic regions: A1, A2, A5, and A6. For further explanations see text. *Long-term plasticity* characterizes the modulatory processes that lead to long-term changes in respiratory activity, which includes, for example, long-term facilitation of the respiratory frequency and amplitude which is evoked by intermittent hypoxia (see text for details). Long-term plasticity is mediated among other molecules by BDNF acting on the TrkB receptor and reactive oxygen species (ROS) as well as HIF1α. Critical areas involved in long-term plasticity are the carotid body and the pre-BötC as well as a variety of motor nuclei. Important chemosensory areas include the NTS (nucleus tractus solitarius), the RTN, and the Raphe nuclei besides the carotid body. These chemosensitive areas are important sensors for inputs from the environment, which is in part affected by the breathing behavior itself. *Homeostatic plasticity* characterizes regulatory processes that are critical for stabilizing network activity in the context of respiratory rhythm generation as well as neuromodulation and long-term plasticity. Unfortunately, little is known about the homeostatic mechanisms that are specifically relevant for the neuronal control of breathing, but much is already known in other networks in particular in the networks located in the neocortex and hippocampus.

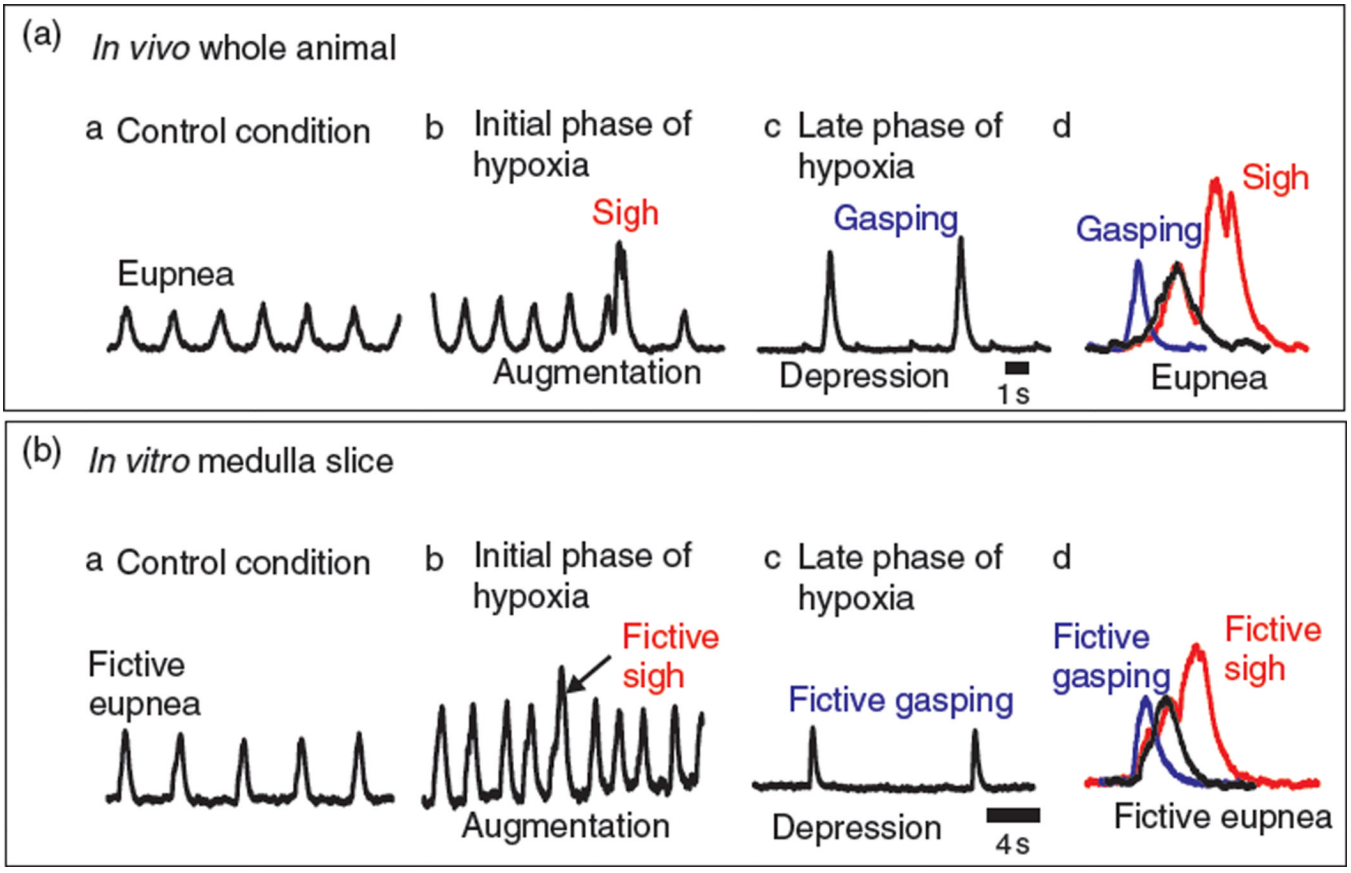

Fig. 2.

Isolating the pre-Bötzinger complex in a single medullary brainstem slice preserves multiple rhythms that reflect breathing rhythms found *in vivo* and illustrates reconfiguration of a multifunctional network. (A) Integrated traces from an *in vivo* electromyogram (∫EMG) recorded from respiratory muscles show multiple rhythmic behaviors for breathing. (a) In control conditions, eupnea, "the normal breathing rhythm" conditions, is characterized by augmented bell-shaped waveform. (b) During the transition to hypoxia (initial phase of hypoxia), the frequency of eupnea and sigh rhythms become faster (i.e., augmentation). The sigh rhythm consists of large amplitude complex waveforms. While slower in frequency, sigh rhythms are generated together with the eupnea rhythm (for detail see text). (c) During hypoxia, the frequency of the breathing rhythm is slow (late phase of hypoxia) (i.e., depression) and the waveforms can be clearly distinguished from the eupneic bursts based on their fast rise time. Collectively, these waveforms, during the depression, are described as the gasping rhythm. (d) Overlay of representative eupneic (black line), sigh (red line), and gasping (blue line) waveforms found in the respiratory rhythm *in vivo*. (B) Integrated activity (∫pre-BötC) of extracellular recordings obtained from the surface of the pre-Bötzinger complex within the medullary brainstem slice illustrates that this neuronal network generates several patterns of neuronal activity that are reminiscent to breathing behaviors found *in vivo*. (a) In control conditions, fictive eupnea is generated. The waveforms in fictive eupnea are similar to that of eupnea, having an augmented bell-shaped waveform. (b) Large amplitude, complex bursts, described as resembling sighs in both frequency and waveform. Similar to that *in vivo*, the fictive sigh rhythm is commonly increased during the augmentation phase during the transition to hypoxia (initial phase of hypoxia). (c) During hypoxia, the frequency of the fictive respiratory rhythm slows (late phase of hypoxia, i.e., depression) and the waveforms of the population rhythm changes

from the augmented bell-shaped waveform of fictive eupnea to a fictive gasping waveform. Similar to the gasping rhythm *in vivo*, fictive gasping possess waveforms that have fast rise times. (d) Overlay of representative fictive eupneic (black line), fictive sighs (red line), and fictive gasping (blue line) waveforms illustrates the ability of pre-Bötzinger complex to generate multiple rhythms that likely contribute to *in vivo* breathing rhythms. Moreover, these waveform patterns represent the summation of different, yet overlapping mechanisms involving network reconfiguration and neuromodulation of the pre-Bötzinger complex (see text for full discussion).