Topical Review

Preparing for the first breath: prenatal maturation of respiratory neural control

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By birth, the regulatory neural network responsible for respiratory control is capable of generating robust rhythm-driving ventilation that can adjust to homeostatic needs. The advent of *in vitro* models isolated from prenatal rodents has significantly advanced our understanding of these processes. In this topical review, we examine the development of medullary respiratory rhythm-generating centres and phrenic motoneurone–diaphragm properties during the prenatal period.

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Most mammalian brain structures do not become fully functional until they undergo significant postnatal development. While the neuronal and muscular components of the respiratory system mature postnatally, they must be developmentally advanced and functional by birth to generate a rhythm that allows for gas exchange in a highly compliant chest wall and to integrate swallowing and other behaviours with breathing. Neonatal intensive care units regularly deal with premature infants with inadequate prenatal maturation of the respiratory apparatus. Their maladies typically result from deficiencies of central respiratory rhythmogenesis or activation of respiratory musculature. Understanding the pathogenesis of breathing problems in the newborn and development of effective pharmacological treatment will greatly benefit from knowledge of *in utero* development of neural respiratory control.

The instrumentation of fetal sheep for physiological measurements allowed for the landmark studies demonstrating that mammals generate episodic fetal breathing movements (FBMs) that are modulated with changes in EEG and metabolic states (Dawes *et al.* 1970; Jansen & Chernick, 1991). Current understanding of neural circuits underlying FBMs and the development of these circuits to work at birth has resulted predominantly from the subsequent development of *in vitro* rodent models (Suzue, 1984; Smith *et al.* 1991). Specifically, rhythmically active brainstem–spinal cord or medullary slice preparations of rodents are amenable to intracellular recording, dynamic imaging and manipulation of specific neuronal groups by focal lesioning or pharmacological manipulation. In this review we will emphasize data from rodent models and discuss prenatal maturation of respiratory neural control.

Fetal respiratory activity in rodent preparations

Respiratory rhythmogenesis first emerges in the rat at embryonic day (E) 16.5–17 (Fig. 1). This observation was initially based on *in vitro* electrophysiological recordings of the isolated rat fetal brainstem–spinal cord (Greer *et al.* 1992; DiPasquale *et al.* 1992), and has been confirmed in ultrasound recordings of rat FBMs *in utero* from anaesthetized dams (Kobayashi *et al.* 2001) and direct electrophysiological recordings from the pre-Bötzinger complex (pre-BötC) in fetal medullary slices (Pagliardini *et al.* 2003). Fitting with the shorter gestation period of the murine model, the first indication of inspiratory discharge *in utero* and *in vitro* starts at E15 in the fetal mouse (Viemari *et al.* 2003; Thoby-Brisson *et al.* 2005).

Episodic respiratory activity, a characteristic feature of fetal breathing in mammals including rats (Jansen & Chernick, 1991; Kobayashi *et al.* 2001), is not seen *in vitro*. This presumably reflects the lack of intact supramedullary structures that impart the episodic nature of FBMs (Blanco, 1994), and their suppression during hypoxia (Johnston & Gluckman, 1989; Ackland *et al.* 1997). Regardless of pattern, however, two features common to fetal breathing *in vitro* and *in utero* are that respiratory network activity is depressed and that this depression decreases, or excitation increases, as the fetus develops. In rats, between E17 and E20, the rate of FBMs

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increases ~10-fold *in utero* (Kobayashi *et al.* 2001) and the frequency of inspiratory bursting increases ~5-fold *in vitro* (Greer *et al.* 1996; Onimaru & Homma, 2002; Pagliardini *et al.* 2003; Ballanyi, 2004). The amplitude and duration of respiratory bursts also increase with gestational age, while variability in both frequency and amplitude decreases (Greer *et al.* 1992; DiPasquale *et al.* 1996; Hilaire & Duron, 1999).

Ontogeny of the pre-Bötzinger complex

Sequential serial sectioning from the rostral and caudal aspects of the isolated brainstem–spinal cord neonatal rat preparation developed by Suzue (1984) revealed that a restricted area of the medulla containing the pre-BötC (Fig. 2*A*) was necessary and sufficient for generating respiratory rhythm *in vitro* (Smith *et al.* 1991; reviewed in Rekling & Feldman, 1998; Feldman *et al.* 2003). A central role of the pre-BötC for inspiratory rhythmogenesis in perinates and adults is widely accepted although there remains considerable debate about the cellular mechanisms (Richter & Spyer, 2001; Feldman *et al.* 2003; Ezure, 2004; Duffin, 2004; Ramirez *et al.* 2004; Del Negro *et al.* 2005). Whether the pre-BötC cooperates with a second, more rostral system in the region of the parafacial respiratory group (pFRG) is currently also under investigation (Janczewski *et al.* 2002; Onimaru & Homma, 2003; Mellen *et al.* 2003). Data from imaging, electrophysiological and pharmacological studies suggest that neurones within the pFRG are intrinsically rhythmogenic and control expiratory musculature (Onimaru & Homma, 2002, 2003, 2005; Janczewski *et al.* 2002).

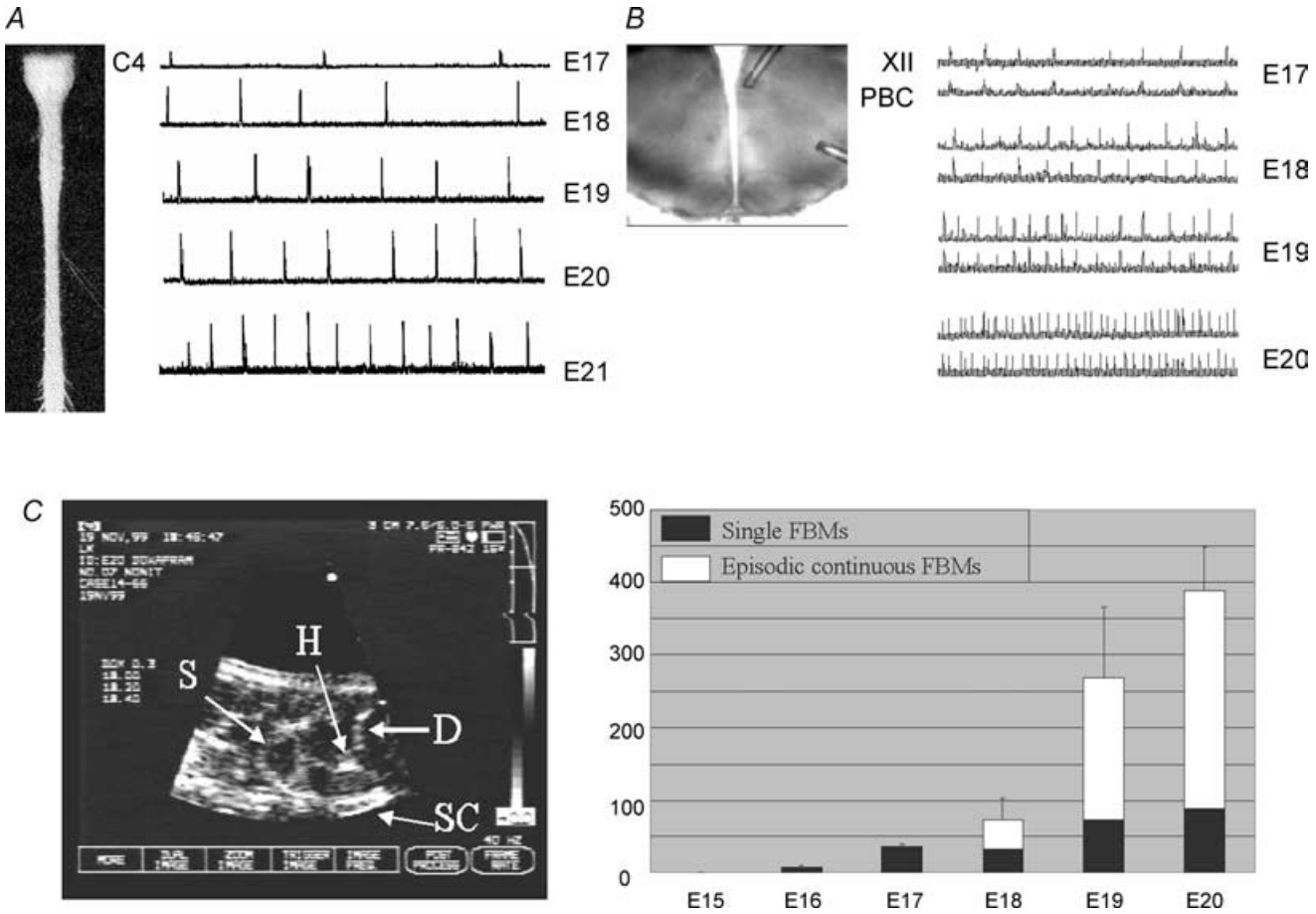

Figure 1. *In vitro* and *in vivo* recordings of fetal respiratory activity in rat
A, rectified and integrated suction electrode recordings of C4 ventral root activity from prenatal brainstem–spinal cord preparations. *B*, rectified and integrated suction electrode recordings made from the pre-BötC (PBC) and XII motoneurone pool of prenatal medullary slice preparations. Rhythmic respiratory discharge commenced at E17 and the frequency and amplitude of inspiratory bursting increased in an age-dependent manner in both types of *in vitro* preparations. *C*, ultrasound image of fetal rat used to measure the incidence of fetal breathing movements. Right panel shows graph depicting the age-dependent increase in FBMs from E16–E20. FBMs occurred as isolated single movements or as episodes of clustered movements lasting 40–180 s. Abbreviations: H, heart; D, diaphragm; S, stomach; SC, spinal cord.

Molecular markers can delineate the critical population of pre-BötC neurones as pioneered by Feldman and colleagues for immunolabelling for neurokinin-1 receptors (NK1R; Gray *et al.* 1999). Subsequent studies supported the idea that there is a population of small fusiform, glutamatergic neurones in the pre-BötC expressing NK1R that have characteristics consistent with their involvement in rhythmogenesis (Pilowsky &

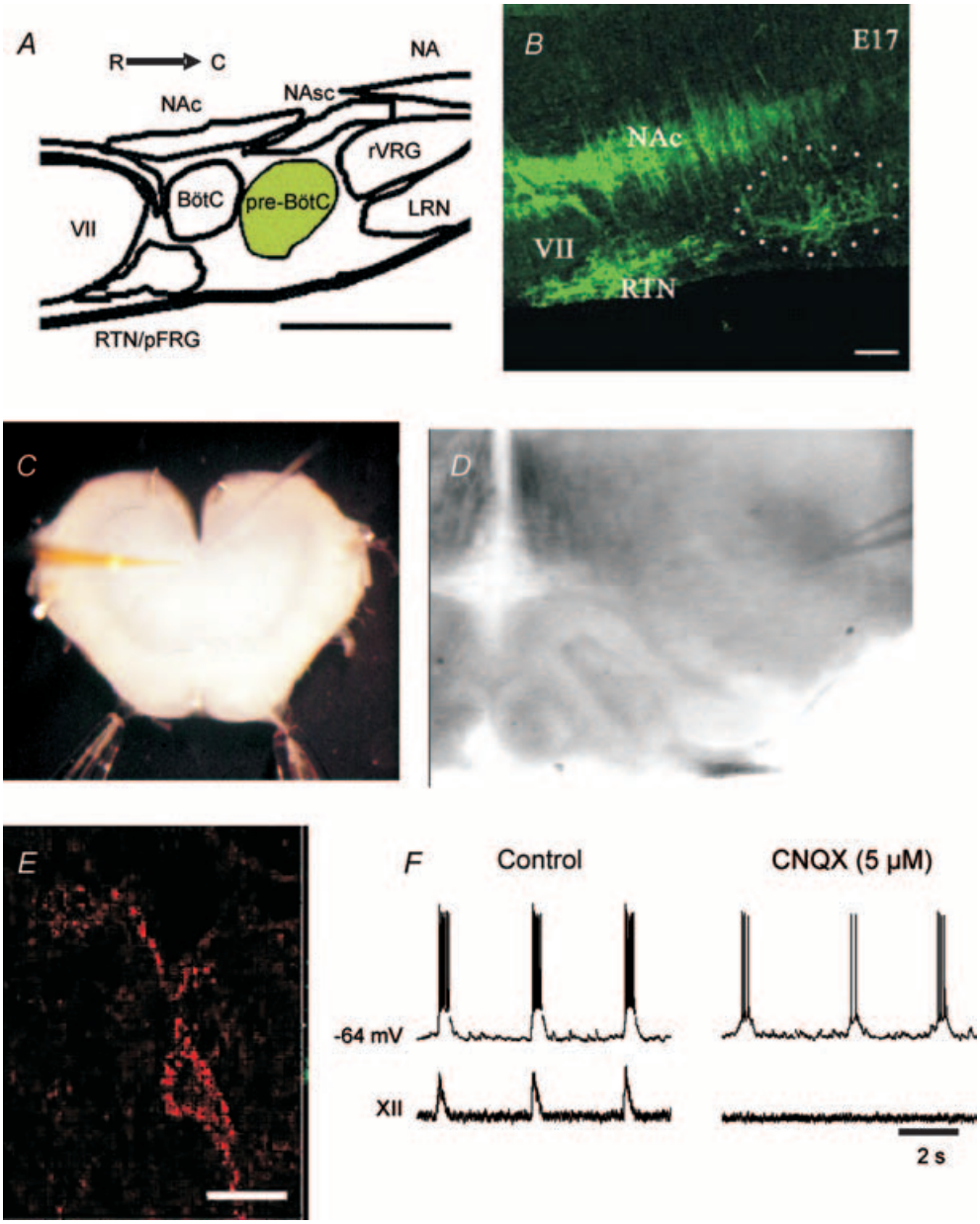

Figure 2. Identification and targeting of pre-BötC neurones within the ventrolateral medulla of perinatal rats

A, an illustration in sagittal view of the primary structures in the ventrolateral medulla along the rostral (R)–caudal (C) axis. *B*, immunolabelling for NK1R (green) in E17 sagittal section of the ventrolateral medulla. The dotted circle demarcates the approximate area of the pre-BötC (calibration bar = 100 μm). *C*, photomicrograph of medullary slice preparation used for whole-cell recordings. *D*, IR-DIC image of the ventrolateral medulla with recording electrode positioned in the vicinity of the pre-BötC. *E*, neurone within the pre-BötC that has been fluorescently tagged via the internalization of TMR-conjugated SubP. *F*, whole-cell recording of membrane potential from a Tetramethylrhodamine (TMR)-SubP-positive neurone and simultaneous recording of integrated XII nerve inspiratory activity before and during block of rhythmic activity by bath application of CNQX. The neurone continues to burst after application of CNQX, consistent with it having pacemaker properties. Abbreviations: VII, facial nucleus; NAc, nucleus ambiguus, pars compacta; NAsc, nucleus ambiguus, semicompact NA, nucleus ambiguus; RTN, retrotrapezoid nucleus; pFRG, parafacial respiratory group; BötC, Bötzinger complex; pre-BötC, pre-Bötzinger complex; rVRG, rostral ventral respiratory group; LRN, lateral reticular nucleus.

Feldman, 2001; Wang *et al.* 2001; Guyenet *et al.* 2002; Stornetta *et al.* 2003). Further evidence came from experiments in adult rats demonstrating that bilateral destruction of NK1R-expressing neurones within the pre-BötC by substance P (SubP) conjugated to saporin results in ataxic breathing during wakefulness (Gray *et al.* 2001).

The NK1R expression data provided the basis for a study of the ontogeny of the pre-BötC in the rat (Pagliardini *et al.* 2003). A combination of immunohistochemical labelling and neuronal birth-dating with bromodeoxyuridine (BrdU) indicated that the NK1R-positive, pre-BötC neurones are born within the ventricular zone on E12 and E13, approximately 2 days later than adjacent NK1R-positive neurones within the semicompact division of the nucleus ambiguus. The neurones reach the region of the pre-BötC between E16.5 and E18 (Fig. 2*B*), which coincides with the time that respiratory-related neural discharge is first detected electrophysiologically. Thoby-Brisson *et al.* (2005) used a combination of NK1R labelling, electrophysiological recordings and calcium imaging to demonstrate a similar developmental profile of pre-BötC anatomy and functional development in mice. Less is known about the prenatal development of the pFRG. However, imaging of respiratory network activity in rat with voltage-sensitive dyes suggests that rhythmic activity of the pFRG commences at E18, approximately 1 day later than in the rat pre-BötC (Onimaru & Homma, 2005).

While the anatomical aspects of pre-BötC prenatal development have been amenable to study, important questions regarding the ontogeny of neuronal properties and synaptic connectivity within the pre-BötC have been difficult to address. DiPasquale *et al.* (1996) demonstrated that the firing frequency and stability of medullary respiratory neuronal activity increases markedly from E18 to birth (reviewed in Hilaire & Duron, 1999). However, there have been no reports during the prenatal period of intracellular or whole-cell analyses of identified NK1R-expressing pre-BötC neurones. The fact that the pre-BötC contains a functionally heterogeneous pool of neurones has been an impediment to progress. However, a recent technical advancement that identifies NK1R-expressing neurones for whole-cell patch-clamp recording, based on their internalization of fluorescently conjugated SubP, should greatly facilitate the analysis of these key pre-BötC neurones (Fig. 2*B–F*; Pagliardini *et al.* 2005*a*). Further, both the activity and structure of populations of pre-BötC neurones can be assessed online with two-photon calcium imaging prior to whole-cell recording (Ruangkittisakul & Ballanyi, 2006). Thus, it should now be possible to more clearly examine the spatiotemporal pattern of expression of multiple voltage- and ligand-gated ion channels in the different populations of respiratory neurones.

Neurochemical control of fetal respiratory rhythm

A fundamental issue pertaining to the ontogeny of respiratory rhythmogenesis is whether fetal networks oscillate at a slower rate than neonatal networks because: (i) of age-dependent differences in the neurones and/or the network underlying rhythmogenesis; (ii) fetal network activity is suppressed by endogenous inhibitory modulators; or (iii) modulatory systems that provide excitatory drive to the respiratory networks are not well developed. Certainly the increase in respiratory frequency and stability that accompanies development *in utero* will, in part, reflect network maturation. However, it also appears to reflect the latter two options. Fetal rhythm-generating centres can oscillate at frequencies comparable to those of the neonate if agonists of excitatory modulators or antagonists of inhibitory modulators are administered. For example, *in utero*, the frequency of FBMs in rats is increased by administration of the respiratory stimulants doxapram and aminophylline (Kobayashi *et al.* 2001). Application of the excitatory neuromodulators serotonin (5-HT), thyrotropin-releasing hormone (TRH) and SubP *in vitro* also markedly increases the frequency of prenatal respiratory rhythm (Greer *et al.* 1996; Pagliardini *et al.* 2003; Ballanyi, 2004).

The primary excitatory phasic drive that maintains the oscillatory state in the pre-BötC of neonates arises from activation of non-NMDA glutamatergic receptors (Greer *et al.* 1991; Funk *et al.* 1993). This has also been demonstrated for prenatal *in vitro* preparations (Thoby-Brisson *et al.* 2005). Further conditioning is provided by a diverse group of neuromodulators, including those acting via cyclic nucleotides, including noradrenaline, opioids, prostaglandins and SubP (Moss & Inman, 1989; Richter *et al.* 1997; Ballanyi *et al.* 1997, 1999). The medullary raphe nuclei are an important source of neuromodulatory input regulating respiratory rhythmogenesis (Bonham, 1995). The three major neurotransmitters released from the raphe complex, 5-HT, TRH and SubP, all have excitatory actions on respiratory rhythmogenesis from early fetal stages when rhythmic respiratory activity first appears (DiPasquale *et al.* 1994; Al-Zubaidy *et al.* 1996; Pagliardini *et al.* 2003). The neurotransmitters γ-aminobutyric acid (GABA) and glycine are the principal mediators of fast chloride-mediated inhibitory transmission in the mammalian central nervous system. They modulate mammalian respiratory rhythmogenesis and the patterning of motor output (Johnson *et al.* 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Parkis *et al.* 1999; Ritter & Zhang, 2000). Changes in membrane potential in response to $GABA_A$ and glycine receptor ligands are determined by the chloride equilibrium potential which depends on a developmental decrease in the expression of the Na^+–K^+–$2Cl^-$ cotransporter (NKCC), which elevates

intracellular [Cl^-], and concomitant up-regulation of the K^+–Cl^- cotransporter (KCC2), which lowers intracellular [Cl^-] (Payne *et al.* 2003). Data from *in vitro* preparations indicate that the transition from a depolarizing to a hyperpolarizing action via chloride-mediated conductances within the pre-BötC occurs prenatally (Brockhaus & Ballanyi, 1998; Ren *et al.* 2001).

Prenatal maturation of respiratory motoneurones and muscle

The phrenic and hypoglossal motoneurone groups have been studied extensively in the newborn and adult period (reviewed in Berger *et al.* 1996; Rekling *et al.* 2000; Cameron & Nunez-Abades, 2000). However, exploration of prenatal development has primarily been limited to phrenic motoneurone (PMN) and diaphragm muscle properties. By E17, when PMNs are first recruited for the generation of FBMs, the motoneurones have migrated to their position in the ventral horn, extended intramuscular branches throughout the full extent of the developing diaphragm, received synaptic input from spinal afferents, undergone the major period of naturally occurring cell death and expressed the necessary ionic conductances for the generation of sodium-dependent action potentials (Harris & McCaig, 1984; Allan & Greer, 1997*a*,*b*; Martin-Caraballo & Greer, 1999). While PMNs do not receive inspiratory drive prior to E17, they are recruited prior to E17 as part of a robust, regular rhythmic motor pattern that is generated along the full extent of the developing spinal cord and medulla (Greer *et al.* 1992; Ren & Greer, 2003). Spontaneous embryonic rhythmogenesis, quite distinct from FBMs, is postulated to play a key role in regulating the events involved in the early development of neuronal circuits and establishing motoneuronal phenotype (reviewed in Ben-Ari, 2001).

While PMNs are functional at E17 and can induce diaphragmatic contractions, there are several key characteristics of the PMN–diaphragm unit that mature prior to birth. These include the following. (1) Morphologically, the distinct rostrocaudal bundling of PMN dendrites, which may facilitate the synchronized activation of PMNs by descending synaptic drive, occurs during the 48–72 h following the onset of descending inspiratory drive (Allan & Greer, 1997*b*). (2) At the inception of inspiratory drive, PMNs have relatively depolarized resting membrane potentials and very high input impedance (Martin-Caraballo & Greer, 1999). Both of these characteristics increase the propensity for reaching firing threshold despite the rather weak inspiratory drive currents at this point in development (DiPasquale *et al.* 1996). (3) PMN firing rates increase 2-fold during the last few days *in utero* with the expression of new ion channels, most notably Ca^{2+}-activated K^+ conductances (Martin-Caraballo & Greer, 2000, 2001). (4) The speed of twitch contraction and the range and absolute amount of force generated by diaphragm muscle fibres change in concert with PMN properties (Martin-Caraballo *et al.* 2000). Thus, prior to birth, PMN and diaphragm properties ensure gross movement of the ribcage despite relatively weak synaptic drive. By birth, the motoneurone and muscle properties allow for the generation of more substantial ribcage movements that can be graded to meet the varied demands of breathing *ex utero*. Our current working hypothesis states that the rapid maturation of PMN–diaphragm properties is in part due to phenotypic changes induced by synaptically mediated events associated with inspiratory drive transmission (e.g. Ca^{2+} influx, neurotrophic factor release).

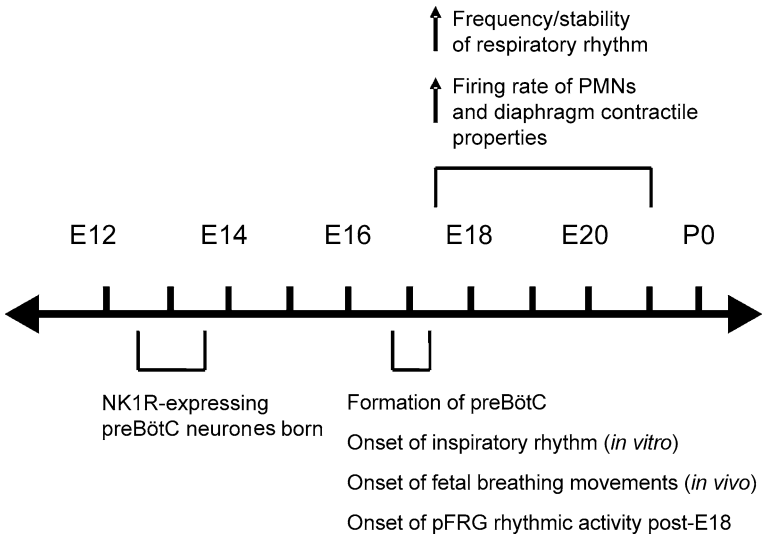

Figure 3
Time line illustrating key events in the development of respiratory neuronal activity in fetal rats.

Summary and future perspectives

Rodent *in vitro* models are providing significant advances in our understanding of respiratory neural control during the prenatal period. There is now solid foundational data regarding the anatomical development of respiratory neuronal populations, onset of rhythmic respiratory drive, the actions of neuromodulators that control respiratory frequency and functional development of the phrenic nerve and diaphragm musculature (Fig. 3). The advent of experimental approaches to examine putative rhythmogenic neurones within the pre-BötC should facilitate the understanding of their basic properties and developmental processes. Further, experimental approaches based on advances in developmental genetics are emerging. Large-scale screening of the spatiotemporal distribution of transcription factors in the developing brainstem should reveal additional pre-BötC neuronal markers (Gray *et al.* 2004). Genetic manipulations will provide the opportunity to inactivate, eliminate or alter specific neurones to dissect and understand central rhythm and pattern generating networks at the molecular, cellular, network and physiological levels (Kiehn & Kullander, 2004; Goulding & Pfaff, 2005). An increasing number of genetic mutations in mouse models are associated with hypoventilation, which has been linked to abnormalities of the central control of respiratory rhythmogenesis (reviewed in Blanchi & Sieweke, 2005). Data from these models will provide insight into the transcriptional control mechanisms underlying respiratory network development and breathing disorders in the perinate, including apnoeas of prematurity, congenital central hypoventilation syndrome and Prader Willi syndrome (Ren *et al.* 2003; Pagliardini *et al.* 2005*b*; Blanchi & Sieweke, 2005). Finally, there is increasing awareness that an inhospitable *in utero* environment has long-term consequences on physiological function in adulthood (reviewed in Barker, 2004). The respiratory control system, including normoxic ventilatory parameters and responses to hypoxia, is clearly influenced by perturbations during the newborn period (reviewed in Mitchell & Johnson, 2003; Gozal, 2004) and there is emerging evidence for long-term plasticity in response to intermittent prenatal hypoxia (Gozal *et al.* 2003) that warrants further investigation.

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