

Review

Developmental basis of the rostro-caudal organization of the brainstem respiratory rhythm generator

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The *Hox* genetic network plays a key role in the anteroposterior patterning of the rhombencephalon at pre- and early-segmental stages of development of the neural tube. In the mouse, it controls development of the entire brainstem respiratory neuronal network, including the pons, the parafacial respiratory group (pFRG) and the pre-Bötzinger complex (preBötC). Inactivation of *Krox20/Egr2* eliminates the pFRG activity, thereby causing life-threatening neonatal apnoeas alternating with respiration at low frequency. Another respiratory abnormality, the complete absence of breathing, is induced when neuronal synchronization fails to develop in the preBötC. The present paper summarizes data on a third type of respiratory deficits induced by altering *Hox* function at pontine levels. Inactivation of *Hoxa2*, the most rostrally expressed *Hox* gene in the hindbrain, disturbs embryonic development of the pons and alters neonatal inspiratory shaping without affecting respiratory frequency and apnoeas. The same result is obtained by the *Phox2a*^{+/-} mutation modifying the number of petrosal chemoafferent neurons, by eliminating acetylcholinesterase and by altering *Hox*-dependent development of the pons with retinoic acid administration at embryonic day 7.5. In addition, embryos treated with retinoic acid provide a mouse model for hyperpnoeic episodic breathing, widely reported in pre-term neonates, young girls with Rett's syndrome, patients with Joubert syndrome and adults with Cheyne–Stokes respiration. We conclude that specific respiratory deficits *in vivo* are assignable to anteroposterior segments of the brainstem, suggesting that the adult respiratory neuronal network is functionally organized according to the rhombomeric, *Hox*-dependent segmentation of the brainstem in embryos.

Keywords: *Hox*; *Krox20/Egr2*; retinoic acid; acetylcholinesterase; Joubert syndrome; Cheyne–Stokes

1. INTRODUCTION

Strong regulatory constraints couple the expression pattern in *Hox* gene clusters to the progression of embryogenesis in the brainstem (Lumsden & Krumlauf 1996). In vertebrates, the formation of rhombomeres (r) is accompanied by a sequential activation of these genes from 3' to 5' in the clusters. As a result, early structures are given an anterior identity with 3' *Hox* genes as key determinants, while progressively later structures start expressing more 5' *Hox* genes and acquire a more posterior identity. Thus, *Hox* genes play a key role in anteroposterior patterning of the rhombencephalon at pre- and early-segmental stages of development (embryonic days E 7.5–E 9.5 in mice, before the neurogenesis starts). *Hoxa1* and *Hoxb1* are expressed up to the rhombomeric r3/r4 boundary, and they are directly downregulated in r3 by the zinc-finger transcription factor *Krox20*

(Schneider-Maunoury *et al.* 1998), also named *Egr2*. This signalling pathway is required for the proper development of r3–r5 and boundaries between rhombomeres. *Hoxa2*, the most anterior *Hox*, is expressed up to the r1/r2 boundary, and it is upregulated by *Krox20/Egr2* in r3. Other *Hox* are prevalent caudal to r5 in the so-called 'post-otic' rhombomeres (i.e. located caudal to the otic vesicles). Thus, in all vertebrates, the branchiomotor nuclei conform to this rhombomeric pattern with a two-segment periodicity. Trigeminal motoneurons originate from r2 and r3 and send their axons to an exit point in r2. In mammals, the facial branchial nucleus originates in r4, forming with adjacent rhombomeres the 'parafacial' level of the hindbrain. The ventral and dorsal motor nuclei of glossopharyngeal–vagal nerves are post-otic in origin (Lumsden & Krumlauf 1996).

By investigating the respiratory behaviour of *Krox20*^{-/-} and *Hoxa1*^{-/-} mutants (Jacquin *et al.* 1996; Dominguez del Toro *et al.* 2001), we have established links between the transient rhombomere-nested expression of these genes and deletions, neoformations and reconfigurations of the respiratory rhythm

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generator (reviewed at post-natal stages by Bianchi *et al.* 1995; Feldman & Del Negro 2006). These mutations helped in the unveiling of a vital role of the rhombomeric parafacial domain for the specification of an adapted respiratory rhythm at birth (Borday *et al.* 2004, 2005; Chatonnet *et al.* 2006), which requires activity of the parafacial respiratory group (pFRG, Chatonnet *et al.* 2003b; Mellen *et al.* 2003; Onimaru & Homma 2003). Comparisons with mutations affecting adjacent embryonic territories now allow us to differentiate two additional regulatory levels controlling breathing, including: (i) caudally, at the vagal glossopharyngeal (post-otic) level, the inspiratory oscillator, named the pre-Bötzing complex (pre-BötC, Smith *et al.* 1991; Thoby-Brisson *et al.* 2005; Thoby-Brisson & Greer 2008) necessary for breathing (Gray *et al.* 2001; McKay *et al.* 2005; Wallen-Mackenzie *et al.* 2006; Tan *et al.* 2008) and (ii) the rostral pons (close to the midbrain/hindbrain boundary, McCrimmon *et al.* 2004; Potts *et al.* 2005), altering the shape of inspiratory efforts, without affecting respiratory frequency (Chatonnet *et al.* 2007; Guimarães *et al.* 2007). The present paper concentrates on this pontine respiratory control and suggests interactions with the peripheral reflex and central cholinergic controls of breathing.

2. PARAFACIAL CONTROL OF RESPIRATORY RHYTHM FREQUENCY: INDUCTION REQUIRES *KROX20/EGR2* AND *HOXA1* BUT NOT *KREISLER/MAFB*

The rhombomere-related formation of the pFRG was first postulated from the observation of *Hoxa1* (Dominguez del Toro *et al.* 2001) and *Krox20/Egr2* (Jacquin *et al.* 1996) homozygous mice mutants that revealed the anti-apnoeic and respiratory frequency promoting functions of the pFRG at birth (figure 1a). Furthermore, the finding of an embryonic parafacial oscillator now clearly shows that pFRG derives from, and requires, *Krox20*-expressing progenitors (Thoby-Brisson *et al.* in press). *In vivo/in vitro* analysis also shows that the more caudal medulla including the preBötC is not affected by the *Krox20*^{-/-} and *Hoxa1*^{-/-} mutations (Jacquin *et al.* 1996; Dominguez del Toro *et al.* 2001). In fact, mutations disrupting preBötC function, e.g. the glutamatergic synchronization between preBötC, eliminate breathing behaviour (Wallen-Mackenzie *et al.* 2006). Thus, the rhombomeric organization supports the hypothesis of a dual control for respiratory frequency by distinct neuronal oscillators, pFRG and preBötC during foetal and early post-natal life (Chatonnet *et al.* 2003b; Mellen *et al.* 2003; Onimaru & Homma 2003).

Rhombomeres are polyclonal developmental compartments that include a large variety of cell types and lineages that are all affected in *Hoxa1* and *Krox20/Egr2* (Lumsden & Krumlauf 1996). To further investigate the effect of rhombomere reduction during the development of the parafacial system, we used *kreisler* homozygous mutants with vestigial (almost absent) r5 (Manzanares *et al.* 1997; Sadl *et al.* 2003) and *Krox20*^{Cre/flox} hypomorphic mutants (Voiculescu

et al. 2000; Taillebourg *et al.* 2002) resulting in a reduced, though not absent, r3 territory. *Krox20*^{Cre/flox} mutants were obtained by combining two previously developed *Krox20* alleles, a Cre knock-in and a floxed allele. Compound heterozygous *Krox20*^{Cre/flox} mutants express *Krox20* only transiently, leading to a severe reduction in r3 (figure 1b). Analysis of the *kreisler* homozygous (Chatonnet *et al.* 2002) and *Krox20*^{Cre/flox} mutants (Chatonnet *et al.* 2007) revealed robustness of the dual rhythm generator in neonates, which is able to maintain a physiological respiratory frequency despite dramatic alterations of the brainstem anatomy. Both mutants exhibited a normal respiratory frequency (figure 1c) and normal durations of apnoeas during the first post-natal day. Therefore, a severe reduction of r3 or r5 is compatible with a normal control of the respiratory rhythm, suggesting that a subset of progenitors specifically generating the pFRG remains active in *kreisler* and *Krox20* hypomorphic mutants. This lineage is now being identified and followed during migration in collaboration with Jean-François Brunet and Christo Goridis (Ecole Normale Supérieure, Paris): these neurons derive from *Krox20*-expressing progenitors and co-migrate caudally with the facial branchiomotor nucleus to reach a final position near the ventral surface of the brainstem; these neurons are characterized by intrinsic membrane pacemaker properties and expression of the developmental gene *Phox2b* (Thoby-Brisson *et al.* in press).

3. PONTINE INSPIRATORY CONTROL: DISTINCT *HOXA2*- AND *KROX20/EGR2*-RELATED RESPIRATORY PHENOTYPES

We compared the phenotypes of mice carrying targeted inactivations of *Krox20/Egr2* (affecting the pFRG development, as discussed earlier) and *Hoxa2*, the only *Hox* gene expressed up to r2 and controlling the development of the rostral pons (Chatonnet *et al.* 2007). In addition to breathing, we investigated the impact of such mutations on the neural circuits controlling jaw opening, compatible with *Hoxa2*-dependent trigeminal defects (r2r3 derived) and direct upregulation of *Hoxa2* by *Krox20* in r3. We found that *Hoxa2* mutants displayed an impaired oro-buccal reflex similar to *Krox20* mutants (jaw opening in figure 2a), but that the respiratory phenotypes were different. Inactivation of *Hoxa2* did not affect the breathing frequency, but, instead, led to a larger-than-normal inspiratory amplitude (tidal volume in figure 2b). We analysed the distribution of EGFP⁺ cells in knock-in mice in which EGFP expression was selectively induced in r2-derived *Hoxa2*-expressing cells upon Cre-mediated recombination (in collaboration with F. Rijli, see Oury *et al.* 2006). This was done by mating the *Hoxa2*^{EGFP(lox-neo-lox)} knock-in allele with an r2-specific Cre transgenic line (Chatonnet *et al.* 2007). This mating scheme resulted in full *Hoxa2* targeted inactivation and, concomitantly, allowed selective tracing of the *Hoxa2*-expressing EGFP⁺ cells in the r2-derived progeny in controls (figure 2c) and homozygous mutants (figure 2d). Tracing of r2-specific progenies of *Hoxa2*-expressing

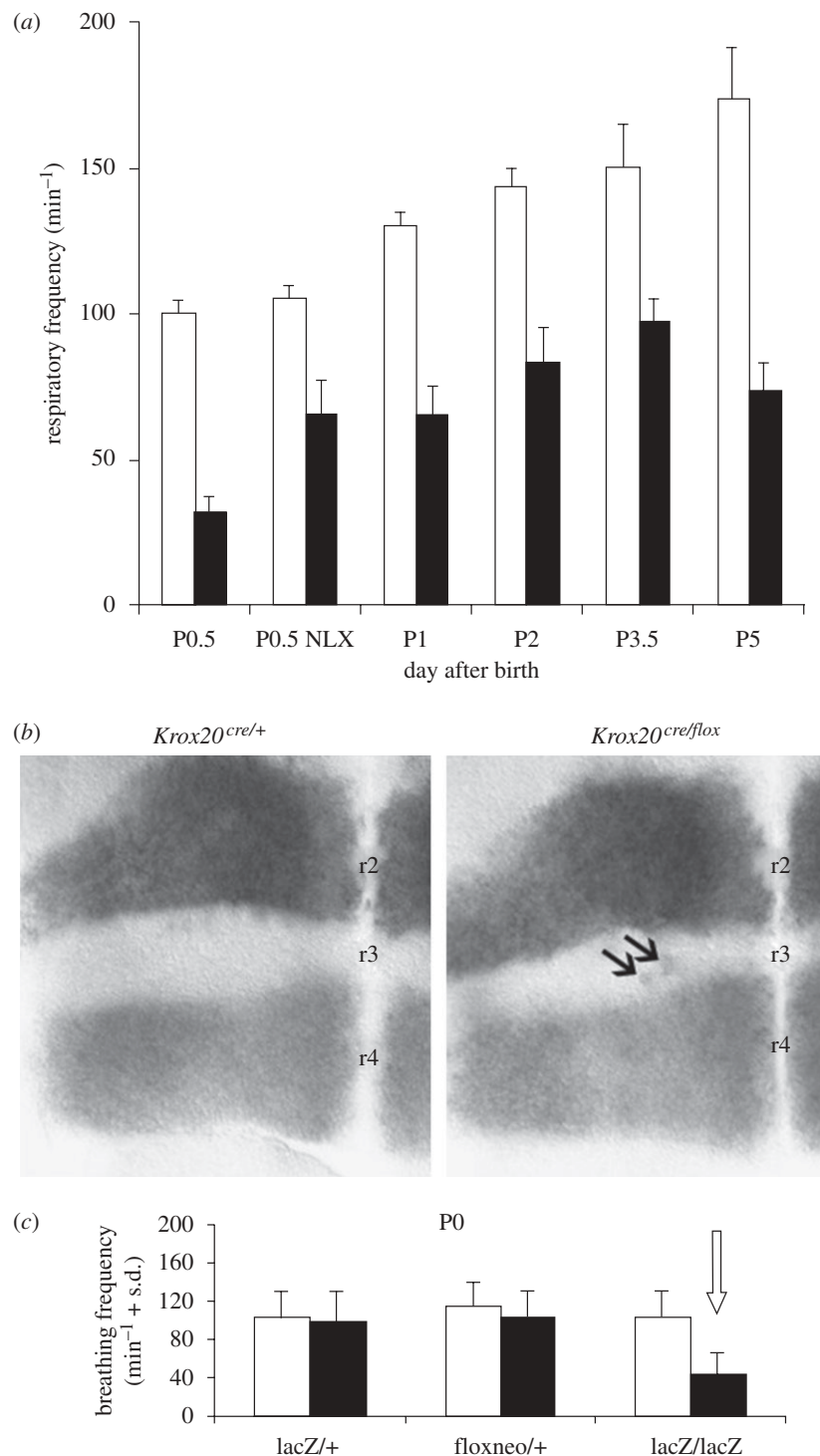


Figure 1. Parafacial control of respiratory rhythm frequency. (a) Average (\pm s.d.) respiratory frequency measured by plethysmographic recording of wild-type (white, $n = 17$) and homozygous mice in which *Krox20* (also called *Egr2*) is inactivated (black, $n = 10$; mutations by P. Charnay *et al.* Paris). *Krox20* controls *Hox* gene expression in rhombomeres r3 and r5 and the development of r3 and r5, including the parafacial neuronal oscillator (pFRG) located ventral to the facial branchiomotor nucleus. In contrast to the silencing of the preBötC (Wallen-Mackenzie *et al.* 2006), elimination of the pFRG does not completely abolish breathing: respiration persists although at a very low frequency. During the first post-natal day (P0.5), mutants exhibit long apnoeas, so that survival requires naloxone administration (NLX; adapted from Jacquin *et al.* 1996; Chatonnet *et al.* 2003a, 2007). (b) Size of rhombomere r3 estimated at a segmental embryonic stage (E9.5) on flat-mounted hindbrains by labelling adjacent rhombomeres as follows: r4 by *in situ* hybridization with an *Hoxb1* probe and r2 by detection of the alkaline phosphatase activity from an r2-specific transgene. The negative territory located in-between corresponds to the *Krox20*-expressing rhombomere r3: it is reduced by the hypomorphic mutation *Krox20^{Cre/flox}* (on the right) compared with heterozygous *Krox20^{Cre/+}* embryos (on the left). A few mis-specified (*Hoxb1*-positive) cells are also observed within r3 in embryos (arrows, modified from Chatonnet *et al.* 2007). (c) The low breathing frequency of homozygous *Krox20* mutants (lacZ/lacZ, right) is not seen in *Krox20* heterozygous (lacZ/+) and hypomorphic (floxneo/+) neonates. These observations indicate that a subpopulation of *Krox20*-expressing progenitor cells, left active in hypomorphic and heterozygous mutants, is required for the development of a parafacial, anti-apnoeic and rhythm promoting control of respiration (adapted from Jacquin *et al.* 1996; Chatonnet *et al.* 2003a, 2007). Open squares, WT; filled squares, mutant.

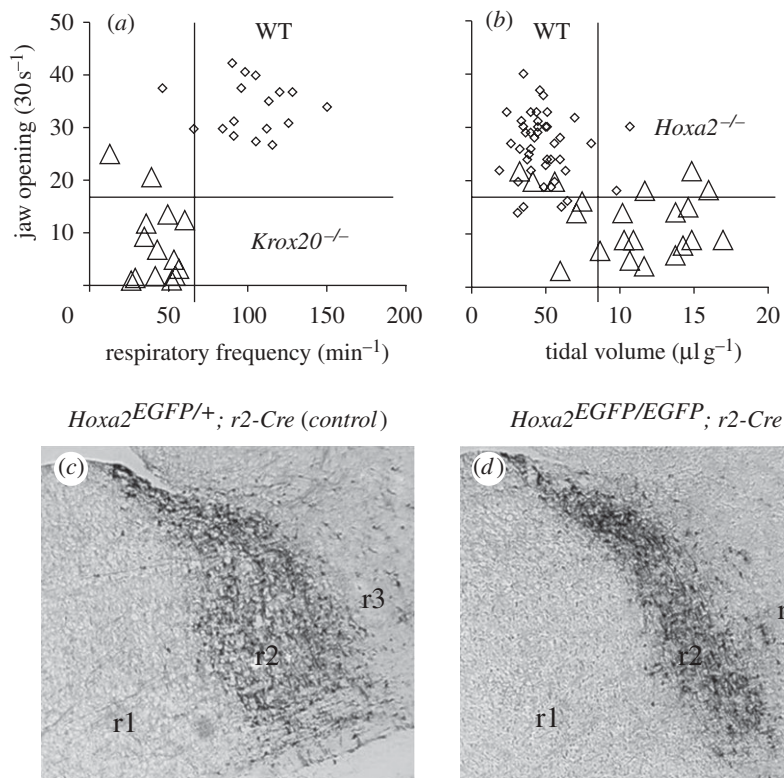


Figure 2. Pontine control of the tidal volume at birth (adapted from Jacquin *et al.* 1996; Chatonnet *et al.* 2003a, 2007). Top: impaired oro-buccal reflex and respiratory parameters in the *Hoxa2* homozygous mutant (a), compared with *Krox20* mutants (b); each symbol represents a neonate; triangles, mutant; diamonds, wild-type), during the first hours following birth. Ordinates show the number of jaw openings within 30 s, induced by a gentle mechanical stimulation of the mouth: the observed deficit in both mutants (less than 20 openings per 30 s in a series) is compatible with *Hoxa2*-dependent trigeminal defects (r2 and r3 derived) and direct upregulation of *Hoxa2* by *Krox20* in r3. Regarding breathing (abscissas: frequency in (a); tidal volume in (b)), *Hoxa2*^{-/-} mutants show large inspiratory amplitudes (b), but none of them show the low respiratory frequency that results from the elimination of the pFRG in *Krox20*^{-/-} (a). Bottom: increase in rhombomere r1 at the expense of r2 by homozygous inactivation of *Hoxa2* (d) compared with heterozygous (normal, c). Parasagittal sections (rostral is on the left and dorsal on the top) showing the r2-derived territory by immunodetection of EGFP in mice produced by mating *Hoxa2*^{EGFP(*lox-neo-lox*)^{-/-}} mutants with an *r2::Cre* transgenic line (mutations by P. Rijli, Strasbourg). The homozygous mutants exhibit irreversible abnormality of the pons linked to the hypertrophy of the r1-derived territory (on the left; position of the r2/r3 boundary, on the right, is unchanged). These observations identify *in vivo* the respiratory-related function of pontine circuits deriving from r1r2.

cells indicated that the control of inspiratory activity resided in rostral pontine areas (Chatonnet *et al.* 2007). Inspiratory deficit was ascribed to the expansion of structures deriving from r1 (the catecholaminergic Locus Coeruleus, the cholinergic pediculo-pontine tegmentum, the pontine respiratory groups parabrachial medial and Kölliker–Fuse nuclei), at the expense of r2 (see reduction of r2 in figure 2d).

Our data point to the importance of rhombomere-specific genetic control in the development of modular neural networks in the mammalian hindbrain: we show that inspiratory shaping and respiratory frequency are under the control of distinct *Hox*-dependent segmental cues and pontine (*Hoxa2*-dependent), parafacial (*Hoxa1*-dependent) and post-otic segments of the neural tube.

4. LOSS OF ACETYLCHOLINESTERASE OR MUSCARINIC RECEPTORS INCREASES INSPIRATORY DEPTH OF BREATHING

We have studied impairment of the cholinergic system within the brainstem by the (r1 derived)

pediculo-pontine tegmentum in adults after homozygous inactivation of the gene encoding the enzyme acetylcholinesterase (Chatonnet *et al.* 2003a; Boudinot *et al.* 2004a,b, 2005). In these mice, inspirations were deeper than normal, while rhythm frequency appeared normal and no apnoeas were seen. It has long been known that blockade of acetylcholinesterase by organo-phosphorus compounds produces rapid death by respiratory failure (reviewed in Brimblecombe 1981). We have shown *in vitro* that one of the mechanisms by which respiratory failure is prevented is the decreased sensitivity of preBötC neurons to nicotinic and muscarinic agonists in the preBötC (Chatonnet *et al.* 2003a). Indeed, muscarinic receptors are involved. We found that when muscarinic M1 receptors are eliminated, mice show unaffected respiratory frequency but elevated tidal volume at rest and unaffected ventilatory responses to hypoxia and hypercapnia *in vivo*. Mice knock-out for the M3 receptors had elevated tidal volume at rest and a slightly decreased tidal volume response slope to hypercapnia and responses to hypoxia (Boudinot *et al.* 2008). The results suggest that M1–M3 muscarinic receptors play significant

roles in the regulation of the depth of inspiration at rest and that M3 receptors are activated in the afferent pathway originating from peripheral chemoreceptors, thus linking pontine inspiratory shaping and sensory afferent synaptic integration (Boudinot *et al.* 2008). These results suggest that cholinergic neurons of the pediculo-pontine tegmentum contribute with the pontine respiratory group (parabrachial medial and Kölliker–Fuse nuclei) to alter inspiratory depth after the abnormal *Hox* control of the pontine development.

5. PRE-SEGMENTAL RETINOIC TREATMENT REDUCES INSPIRATIONS AND CAUSES EPISODIC BREATHING

The experiments on the *Krox20/Hox* signalling discussed earlier suggest that segmental signalling at end-segmental stages (E 9.5) is sufficient to induce the development of a parafacial rhythm generator. However, segmentation of the hindbrain starts earlier at E 7.5 in mice. Retinoic acid treatment at E 7.5 with low doses (0.5 mg kg^{-1} , one to two orders of magnitude lower than the teratogenic dose) was found to be convenient in mice to further investigate the importance of this early developmental stage for the breathing behaviour (Guimarães *et al.* 2007). Retinoic acid at E 7.5 is known to affect the spread of *Hox* and *Gbx2* expression domains into the anterior hindbrain, followed by a retraction, leaving behind a posteriorized expression pattern (Li & Joyner 2001). In keeping with these data on embryo, we found rostral pontine abnormalities that persisted until adulthood and were opposite to those observed in *Hoxa2* mutants: reduced tidal volume (with unchanged respiratory frequency) and reduced size of the pontine parabrachial respiratory area. The treatment also changed the shape of the pediculo-pontine tegmentum and induced a displacement of the superior cerebellar peduncle with respect to the midline. Strikingly, periods of quiet breathing with small tidal volume consistently alternated with sequences of hyperpnoeic episodic breathing (figure 3), a cyclic waxing and waning of inspirations (Guimarães *et al.* 2007). This is a clinical trait widely reported in pre-term neonates, in patients with Joubert or Rett syndromes and in adults (Cheyne–Stokes respiration) with congestive heart failure and brainstem infarction. Human and animal studies have provided evidence supporting the importance of hypoxia, altitude acclimatization and hypocapnia in the induction of hyperpnoeic episodic breathing, so that ventilatory instability may result from the unbalanced control of the rhythm generators by peripheral and central afferent controls (Han *et al.* 2002). In mice treated with retinoic acid, exaggeration of this Cheyne–Stokes pattern upon silencing chemosensory receptors by hyperoxia (figure 3) suggests that the rostral pontine deficit interacts with peripheral chemosensory pathways.

Interestingly, anatomical anomalies of the superior cerebellar peduncle in mice also resemble the molar tooth sign reported in the Joubert syndrome, in which mal-oriented superior cerebellar peduncles give the appearance of a ‘molar tooth’ on axial brain MRI through the junction of the midbrain and

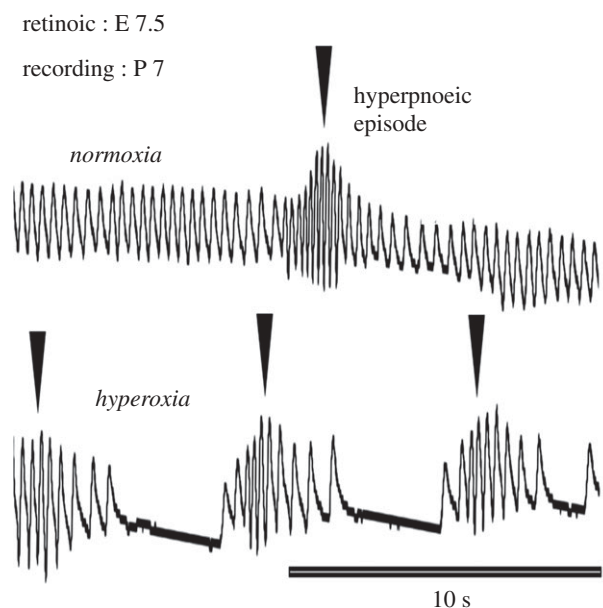


Figure 3. Deficit of the pontine control of inspiration may cause hyperpnoeic episodic breathing at post-natal stages (adapted from Guimarães *et al.* 2007). Plethysmographic records (one week after birth) of hyperpnoeic episodic breathing (triangles show single episodes) in normoxia (top trace) and hyperoxia (bottom trace). This pattern was consistently induced by treating embryos with retinoic acid at early pre-segmental stages (E 7.5, with doses as low as 0.5 mg kg^{-1}). This treatment induces anatomical abnormalities restricted to the rostral pons resembling those in the human Joubert syndrome (Guimarães *et al.* 2007). Outside sequences of episodic breathing, the control of inspiration was altered (with small tidal volumes), but the respiratory frequency was normal. Apnoeas and survival were normal at neonatal stages. The hyperoxic test (bottom) aggravates the hyperpnoeic episodic pattern (apnoeas between episodes are not seen in normoxia), showing that both the pontine control (eliminated by the treatment with retinoic acid) and the peripheral chemosensory control (eliminated by hyperoxia) stabilize breathing.

hindbrain (Gleeson *et al.* 2004). The Joubert syndrome is clinically heterogeneous with breathing abnormalities including episodic hyperpnoeas (in 60–80% of the patients) in the neonatal period, oculomotor apraxia (abnormal eye movements in 75% of the patients), ataxia and mental retardation (Joubert *et al.* 1969). This is also a genetically heterogeneous syndrome, with different known loci (Ferland *et al.* 2004; Lagier-Tourenne *et al.* 2004). Although candidate gene approaches have failed so far to detect mutations in *wnt1*, *en1*, *en2* and *fgf8* genes of the patients (Blair *et al.* 2002), observations in mice suggest a possible link with the large *Hox*-related genetic network affected by retinoic acid during early embryonic development.

6. CONTROL OF INSPIRATIONS BY PERIPHERAL CHEMORECEPTOR AFFERENT NEURONS

From the results mentioned earlier, we suspect an interaction between rostral pontine structures and sensory afferents in the nucleus tractus solitarii, in which

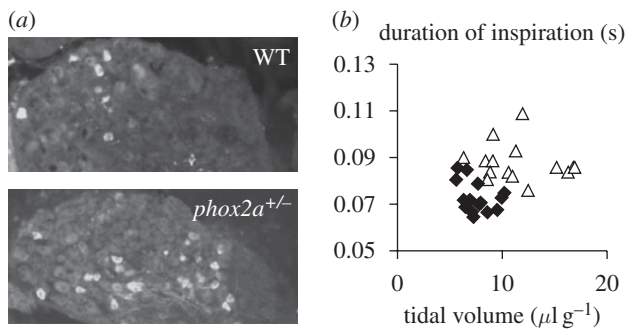


Figure 4. The *Phox2a* heterozygous mutation increases the number of peripheral chemoreceptor afferent neurons in the petrosal ganglia (a) and impairs inspiratory control (b; adapted from Wrobel *et al.* 2007). (a) Peripheral chemoreceptor afferent cell bodies identified by immunohistochemistry of tyrosine hydroxylase in the petrosal ganglion at post-natal day 10. The effect of the mutation was rather selective because the size of the ganglion and the total number of unstained neurons were not affected. (b) Respiratory deficits in mutant include long inspirations (in ordinates) and large amplitudes (tidal volume in abscissa). Large amplitudes (illustrated here at post-natal day 1) were seen until adulthood. Respiratory frequency, neonatal apnoeas and survival were normal. Interestingly, the reverse phenotype (a small tidal volume) has been reported after the decrease (instead of increase) in the peripheral chemoreceptor afferent population by the invalidation of the BDNF (Erickson *et al.* 1996). Diamonds, WT; triangles, *Phox2a*^{+/-}.

the inspiratory shape is regulated by lung stretch receptors of the Breuer–Hering reflex, under control by peripheral chemoreceptor afferent neurons originating from the carotid bodies. Development of the petrosal ganglion, containing chemoreceptor afferent neurons, depends on the brain-derived neurotrophic factor (BDNF) as well as on the expression of the *Phox2a* and *Phox2b* transcription factors that are important for cell survival and specification of the dopaminergic fate (Brunet & Pattyn 2002). Our study of the petrosal ganglion was also prompted by previous findings of a small respiratory amplitude when the peripheral chemoreceptor afferent population was reduced by apoptosis following inactivation of the gene encoding the BDNF (Erickson *et al.* 1996, see also Thoby-Brisson *et al.* 2003). Mutations of *Phox2b* induce brainstem anomalies associated with defects of breathing behaviour at birth (Dubreuil *et al.* 2009). The role of *Phox2a* proteins in the establishment of brainstem cardiorespiratory neuronal pathways was unknown, largely because of the lethality of homozygous mutants. We therefore examined the effects of a haploinsufficiency of the *Phox2a* gene. *Phox2a* heterozygous mice survive and exhibit significantly larger and longer inspirations (figure 4b) compared with wild-type animals during both normoxic breathing and in response to hypoxia. This phenotype, accompanied by an unaltered frequency, is evident at birth and persists afterwards (it was followed until post-natal day 10). Morphological analysis of *Phox2a*^{+/-} animals reveals no anomaly in the pons (parabrachial medial and Kölliker–Fuse nuclei, pediculo-pontine tegmentum, superior cerebellar peduncles) or in the locus coeruleus region, but

highlighted an increase in the number of cells expressing the catecholamine-synthesizing enzyme—tyrosine hydroxylase, a marker of peripheral chemoreceptor afferent neurons (Katz & Black 1986; Erickson *et al.* 1996), in the petrosal sensory ganglion (figure 4a). These data indicate that *Phox2a* plays a critical role in the ontogeny of the reflex control of inspiration and that abnormality may independently affect rhythm generators and pontine/sensory inspiratory controllers as well as their peripheral reflex control.

7. CONCLUSION

Although transient during early development, the rhombomeric patterning strongly and irreversibly influences the establishment of the neuronal circuitry of the mature brainstem. Reconfiguration of neurons and synapses during foetal and post-natal development is unable to restore viable neural functions when the basic scaffold is altered during early development. Thus, from studies of hindbrain development, we are gaining an understanding of how genes govern the early embryonic specification of neuronal networks, how they might specify patterns of motor activities operating normally throughout life and how early developmental dysfunction might cause hereditary syndromes including post-natal breathing abnormalities relevant to those seen in humans.

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