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 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.


2. PARAFACIAL CONTROL OF RESPIRATORY BREATHING.

peripheral reflex and central cholinergic controls of breathing. (Chatonnet, 2004; Potts et al., 2007; Ta\"n
1996; Dominguez del Toro 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; Guimara\'es 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 HOX1 BUT NOT KREISLER/MARB

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 preB\ötC is not affected by the Krox20\textsuperscript{-/-} and Hoxa1\textsuperscript{-/-} mutations (Jacquin et al. 1996; Dominguez del Toro et al. 2001). In fact, mutations disrupting preB\ötC function, e.g. the glutamatergic synchronization between preB\ötC, 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 preB\ötC during foetal and early post-natal life (Chatonnet et al. 2003b; Mellen et al. 2003; Onimaru & Homma 2003).

Rhombomeres are polycyonal 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 k\textit{kreisler} homozygous mutants with vestigial (almost absent) r5 (Manzanares et al. 1997; Sadl et al. 2003) and Krox20\textsuperscript{Cre/flox} hypomorphic mutants (Voiculescu et al. 2000; Taillebourg et al. 2002) resulting in a reduced, though not absent, r3 territory. Krox20\textsuperscript{Cre/flox} mutants were obtained by combining two previously developed Krox20 alleles, a Cre knock-in and a floxed allele. Compound heterozygous Krox20\textsuperscript{Cre/flox} mutants express Krox20 only transiently, leading to a severe reduction in r3 (figure 1b). Analysis of the k\textit{kreisler} homozygous (Chatonnet et al. 2002) and Krox20\textsuperscript{Cre/+} 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 k\textit{kreisler} and Krox20 hypomorphic mutants. This lineage is now being identified and followed during migration in collaboration with Jean-Fran\c{c}ois Brunet and Christo Goridis (Ecole Normale Sup\textc{e}rieure, 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 Hox2- 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 Hox2a, 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 Hox2a-dependent trigeminal defects (r2r3 derived) and direct upregulation of Hoxa2 by Krox20 in r3. We found that Hox2a 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 Hox2a 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\textsuperscript{+} cells in knock-in mice in which EGFP expression was selectively induced in r2-derived Hox2a-expressing cells upon Cre-mediated recombination (in collaboration with F. Rijli, see Oury et al. 2006). This was done by mating the Hoxa2\textsuperscript{EGFP(lox-neo-lox)}\textsuperscript{-/+} 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\textsuperscript{+} cells in the r2-derived progeny in controls (figure 2c) and homozygous mutants (figure 2d). Tracing of r2-specific progenies of Hoxa2-expressing

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Figure 1. Parafacial control of respiratory rhythm frequency. (a) Average (+ 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 preBoëtC (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 Krox20Cre/flox (on the right) compared with heterozygous Krox20Cre/+ 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 Krox20Cre/+ 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.

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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 homoyzgous inactivation of the gene encoding the enzyme acetylcholinesterase (Chatonnet et al. 2003). 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 preBo¨tC neurons to nicotinic and muscarinic agonists in the preBo¨tC (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 hindbrain (Gleeson et al. 2004). The Joubert syndrome is clinically heterogeneous with breathing abnormalities including episodic hypopneas (in 60–80% of the patients) in the neonatal period, oculo-motor 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. 2002; 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
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 +/-.

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 +/-.

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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