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Generation of a novel functional neuronal circuit in *Hoxa1* mutant mice

Abbreviated title: **Neuronal circuits function in Hoxa1 $^{-/-}$ mice**

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Keywords: Homeobox genes, *Hoxa1* knockout; Respiration, Suction, Rhythm generation, Rhombomeres, Neural progenitors, Migratory pathways, Neuronal networks, Reticular formation, Pons, Hindbrain, Brainstem, Newborn mice.

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ABSTRACT

The early organisation of the vertebrate brainstem is characterised by a cellular segmentation into compartments, the rhombomeres, which follow a metameric pattern of neuronal development. Expression of the homeobox genes of the *Hox* family precedes rhombomere formation and analysis of mouse *Hox* mutations revealed an important role in the establishment of rhombomere-specific neuronal patterns. However, segmentation is a transient feature and a dramatic reconfiguration of neurons and synapses takes place during fetal and postnatal stages. Thus, it is not clear whether the early rhombomeric pattern of *Hox* expression has any influence on the establishment of the neuronal circuitry of the mature brainstem. The *Hoxa1* gene is the earliest *Hox* gene expressed in the developing hindbrain. Moreover, it is rapidly downregulated. Previous analysis of mouse *Hoxa1-/-* mutants has focused on early alterations of hindbrain segmentation and patterning. Here, we show that ectopic neuronal groups in the hindbrain of *Hoxa1-/-* mice establish a supernumerary neuronal circuit, that escapes apoptosis and becomes functional postnatally. This system develops from mutant rhombomere 3 (r3)-r4 levels, includes an ectopic group of progenitors with r2 identity, and integrates the rhythm-generating network controlling respiration at birth. This is the first demonstration that changes in *Hox* expression patterns allow the selection of novel neuronal circuits regulating vital adaptive behaviors. The implications for the evolution of brainstem neural networks are discussed.

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In the hindbrain of the vertebrate embryo, Hox genes are segmentally expressed and loss- and gain-of-function mutations revealed their involvement in neuronal patterning (e.g. Mark et al., 1993; Carpenter et al., 1993; Studer et al., 1996; Goddard et al., 1996; Lumsden and Krumlauf, 1996; Gavalas et al., 1997, 1998; Rijli et al., 1998; Helmbacher et al., 1998; Davenne et al., 1999; Rossel and Capecchi, 1999; Jungbluth et al., 1999; Bell et al., 1999). Expression of Hoxa1 provides one of the earliest signs of regionalisation within the developing hindbrain. As early as 7.5 day-post-coitum (dpc), the Hoxa1 expression domain extends from the posterior end of the mouse embryo up to the presumptive r3/r4 border and is downregulated before rhombomere boundary formation (Murphy and Hill, 1991). This transient expression has a profound impact on hindbrain patterning, as Hoxa1 targeted inactivation results in severe reduction of r4 and r5 and their derived structures (e.g. the motor nucleus of the facial nerve) and in lethality shortly after birth (Mark et al., 1993; Carpenter et al., 1993). However, it is unclear how transient Hox expression before segment formation may influence the generation of functional neuronal networks in the postsegmental hindbrain (Fortin et al., 1999), and affect vital behaviors during postnatal life (Fortin et al., 2000). By examining hindbrain neural networks in Hoxa1$^{-/-}$ mice, we now identify ectopic groups of misspecified neurons which escape apoptosis (Rossel and Capecchi, 1999) during development and control the respiratory rhythm-generating neural network (Champagnat and Fortin, 1996) after birth.

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MATERIALS AND METHODS

Mouse lines and genotyping.

Hoxa1 mutant mice (Mark et al., 1993), embryos, and newborns were PCR genotyped as described (Gavalas et al., 1998). The r2-lacZ transgenic line was obtained by injection of a construct carrying a 2.5 kb BamHI *Hoxa2* genomic fragment (Frasch et al., 1995) cloned in a non native orientation into the BGZ40 plasmid (Studer et al., 1996), containing the human β-globin promoter driving *lacZ* expression. Transgenic *r2-lacZ* mice were bred with *Hoxa1*$^{+/-}$ mice to produce *Hoxa1*$^{+/-}$, *r2-lacZ* animals. The latter were bred with *Hoxa1*$^{+/-}$ animals to produce embryos with the desired genotype. Detection of the transgene was performed by PCR.

Whole-mount in situ RNA hybridisation, immunohistochemistry, and X-gal staining.

Whole-mount in situ RNA hybridisation was performed as described (Davenne et al., 1999) using the *Phox2b* (Pattyn et al., 1997) and *Hoxb1* (Studer et al., 1996) probes. Whole-mount immunohistochemistry using the anti-ISL1 monoclonal antibody (4D5) (Developmental Studies Hybridoma Bank) and X-gal staining were performed as described (Davenne et al., 1999). Hindbrains were dissected out and flat-mounted before being photographed. Postnatal neuronal groups (Jacquin et al., 1996) were identified on coronal, horizontal and parasagittal 40 µm thick sections processed alternatively using cresyl violet and polyclonal antibodies to choline acetyltransferase (Chemicon International Incorporated, 1:1000 in PBS, pH 7.4) and to tyrosine hydroxylase (Boehringer, 1:1000 in PBS), in the presence of Triton X-100 and

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subsequently revealed using the Vectastain ABC kit (Vector) as described (Jacquin et al., 1996). To study axonal pathways, the trigeminal motor root or the bulbar reticular area ventral to the ambiguus nucleus was pressure injected with DiI (5mg/ml in DMSO) after brain fixation. Incubation times (at 37°C) were 3 days after trigeminal injections and 4 days after bulbar injections.

Plethysmograph recordings and naloxone treatment in vivo.

We have used 231 mice from 34 Hoxa-1 litters. Sixty mice were wild-type, 124 heterozygous and 47 homozygous mutants, a proportion close to the mendelian expectation. Respiratory activity was measured every six hours using a modified barometric method previously employed in neonates (Jacquin et al., 1996). The whole body plethysmograph chamber (20 ml) equiped with a temperature sensor (LN 35 Z) was connected to a reference chamber of the same volume. The pressure difference between the two chambers was measured with a differential pressure transducer (Validyne DP 103-12) connected to a sine wave carrier demodulator (Validyne, CD15). Neonates were removed individually from the litter and placed in the plethysmograph chamber kept hermetically closed and maintained at 31°C during the recording session (2 min). During quiet breathing, a computer-assisted method was used to measure the duration of inspirations and expirations from which the respiratory frequency is derived. Naloxone was administered subcutaneously (3.33 mg/kg in 50 µl saline) using an Hamilton syringe at the end of the first plethysmographic recording, 1-2 hours after birth and the stimulatory effect on respiration was controlled 0.5-1 hour later.

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Network analysis in vitro.

The brainstem was removed as previously (Jacquin et al., 1996, 1999) and cut horizontally (Fig. 3f) under visual control with a vibratome (TPI, series 1000). The 1200 µm thick slice was transferred, dorsal side up, into a recording chamber and perfused with an artificial cerebrospinal fluid (pH 7.4) containing (in mM) 130 NaCl, 5.4 KCl, 0.8 KH_2PO_4, 26 $NaHCO_3$, 30 glucose, 1 $MgCl_2$ and 0.8 $CaCl_2$, saturated with carbogen (90% 0_2, 10% $C0_2$). Motor activities were recorded from the motor trigeminal roots using suction electrodes. Previous experiments (Jacquin et al., 1999) have demonstrated that the respiratory activity in vitro propagates to this nerve. The selected root was contralateral to the studied Rpc-α/SNS (Fig. 3G), to avoid stimulating directly the recorded motoneurons. Other electrodes were located on the dorsal surface under the visual guidance of a microscope (ACM, Zeiss) and locations were identified histologically. Neurons were recorded in the whole-cell configuration with patch-clamp electrodes as in Fortin et al. (1999). Electrodes containing 0.1-0.5 mM AMPA in artificial cerebrospinal fluid were used for pressure application (0.1 bar, 20 ms). Experiments were performed according to autorisation by the ministry of Research-Technology and Agriculture that provides autorisation to have the animal facilities.

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RESULTS

A supernumerary neuronal structure in the dorsal Pons of Hoxa1 $^{-/-}$ mice

Morphological analysis of the Pons at birth indicates a rather extensive cellular reorganisation in *Hoxa1*$^{-/-}$ mutants, affecting different cell types. First, in keeping with the heterogeneous antero-posterior (A-P) pattern of the ventricular zone, the anterior fourth ventricle exhibits a characteristic morphological abnormality in newborn mutants (compare Fig. 1A,B,C,D). Moreover, the size of the reticular formation is affected both dorsally and ventrally. Ventrally, a 40% reduction of the length of the ventral Pons (vP, rectangle in Fig. 1E-F) results from the elimination of r4- and r5-derived structures. In contrast, dorsally, a 6% increase of the postnatal A-P length of the Pons (dP, arrow in Fig. 1E-F) was observed, so that the ratio dP/vP in *Hoxa1*$^{-/-}$, although variable (average ± SEM: 1.45 ± 0.08, n=18), is much larger than in wild-type animals (0.77 ± 0.02, n=18).

We have localised in the dorso-lateral Pons the anatomical modifications underlying this dP increase. In wild-type mice, caudal to the trigeminal motor nucleus, the Parvocellular Reticular Formation (Rpc-α, “pc” in Fig. 2A-B) normally contains trigeminal pre-motor interneurons involved in feeding behaviors (Lund et al. 1998). The Rpc-α is likely derived from r3, since it is eliminated in *Krox-20*$^{-/-}$ mutants (Jacquin et al., 1996), in which pontine defects lead to an abnormal suction behavior after birth. In all *Hoxa1*$^{-/-}$ mice (n=10), the anatomy of the Rpc-α is reorganised (Fig. 2) and extended along the A-P axis, in keeping with the abnormalities of the r3-r4 region at early developmental stages (described below, see also Mark et al., 1993; Carpenter et al.,

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1993; Rijli et al., 1998; Gavalas et al., 1998; Helmbacher et al., 1998; Rossel and Capecchi, 1999). In particular, radial stripes of reticular formation and ectopic motoneurons alternate, forming a compound reticular and motor supernumerary neuronal structure (SNS). Most extensive labelling of ectopic SNS motoneurons included three distinct subnuclei (outlined in Fig. 2A-B) identified by analysis with anti-cholineacetyltransferase antibodies. In addition, injecting the fluorescent marker DiI into the trigeminal motor root (Fig. 2C-F) revealed that these ectopic subnuclei form a distinct dorsoventral trigeminal motor fasciculus running laterally in the SNS (Fig. 2D and F, stars) caudal to the normal root (Fig. 2E, star). Therefore, dP increase in *Hoxa1*$^{-/-}$ mice results from the generation of three additional trigeminal subnuclei alternating with stripes of reticular formation at the same location as the wild-type Rpcα.

Function of ectopic reticular neurons in the dorsolateral Pons of Hoxa1$^{-/-}$ *mice*

To further characterise the reticular cells of the SNS, we investigated their functional connectivity (summarised in Fig. 3G). The hindbrain was isolated in vitro during the first postnatal days (P0-P1) and the dorsal Pons was exposed in a thick horizontal slice (Fig. 3F), and made accessible to dorsal approach under microscopic control. This slice preparation also included the bilateral ventral respiratory group (VRG, stars in Fig. 3G) that generates a persisting rhythmic activity propagating to cranial (e.g. trigeminal) motor neurons from which it can be recorded (Jacquin et al., 1996, 1999). Neuronal populations immediately caudal to the trigeminal nucleus (which in wild-type include the Rpc-α premotor neurons; rectangle in Fig. 3G) were stimulated by pressure application of the glutamatergic agonist α-amino-3-hydroxy-5-methyl-4-

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isoxazoleproprionic acid (AMPA). The contralateral Vn was recorded to avoid direct stimulation of motoneurons.

AMPA-induced non-rhythmic trigeminal activities recorded from the contralateral trigeminal motor rootlet (the upward noisy deflection of the traces in Figs. 3A,C) indicate that normal premotor Rpc-α inputs to the trigeminal motoneurons (Lund et al., 1998) persist in *Hoxa1*$^{-/-}$ mutants. The Rpc-α normally lacks respiratory-related functions. AMPA application had no effect on rhythm frequency in the wild-type preparations (Fig. 3B). In the mutants, a robust increase in rhythm frequency is followed in all cases by a transient inhibition of the rhythm (Fig. 3C, D). This effect strongly suggests the presence of supernumerary functional efferent connections of the SNS to the rhythm generator, resembling the wild-type ventral pontine respiratory connections, located rostrally to the SNS and originating in r2 and r3 (Jacquin et al., 1996; Borday et al., 1997). Moreover, rhythmic activity recorded from single neurons in the SNS area (Fig. 3E) also indicated afferent connections from the rhythm generator. Furthermore, abnormal axonal pathways were found in the lateral Pons by injecting the fluorescent marker DiI into the VRG area (Fig. 3H-J). In the mutants, labelling from the VRG revealed a robust axonal pathway (Fig. 3I), not present in the wild-type (Fig. 3H), and running laterally in the Pons. Thus, in *Hoxa1*$^{-/-}$ mice the SNS exhibits a novel relationship with the respiratory rhythm generator, while preserving premotor connections with the trigeminal system.

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Embryological origin of the supernumerary neuronal system

The appearance of this ectopic neuronal system prompts the question of its embryological origin. We investigated the expression of rhombomere-specific molecular markers in *Hoxa1*$^{-/-}$ mutant hindbrains (Fig. 4). Rhombomere-restricted gene expression persists in the ventricular zone after the segmentation period (Wingate and Lumsden, 1996). In 11.5 day post coitum (dpc) mutants, expression of the r4 marker *Hoxb1* is drastically reduced and patchy along the dorso-ventral axis (compare Fig. 4A-B). To assay for r2 features, we generated a transgenic line containing the *lacZ* reporter under the control of an *Hoxa2* r2-specific enhancer (Frasch et al., 1995) (Fig. 4C). In *Hoxa1*$^{-/-}$ mutants, ectopic patches of cells expressing the r2 marker are present at the r4 axial level (compare Fig. 4C-D), remarkably similar to what is observed in *Hoxb1*$^{-/-}$ mice (Studer et al., 1996). In addition, patches of r2-like cells are also present at the level of r3, as previously described (Helmbacher et al., 1998). Thus, in the absence of *Hoxa1*, some neural precursors at the presumptive r3/r4 levels fail to activate or properly maintain their appropriate molecular programs and acquire an r2 identity.

To investigate the developmental fate of these ectopic r2-like precursors we examined motoneuron development in the hindbrain of *Hoxa1*$^{-/-}$ mice. In wild-type 11.5 dpc embryos, the *Phox2b* gene is expressed in migrating motoneurons (Pattyn et al., 1997). *Phox2b* expression in ventral r4 identifies facial motoneurons migrating caudally through r5 into r6 (bent arrow) to form the facial (VIIth) motor nucleus, whereas strings of *Phox2b*-positive cells in r2 are indicative of dorsal migration of trigeminal motoneurons (straight arrows, Fig. 5A). In the *Hoxa1*$^{-/-}$ mutant r4 region (Fig. 5B), a

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much reduced, though not abolished, *Phox2b* expression identifies a small number of facial motoneurons migrating caudally (bent and dashed arrow). In addition, an abnormal trigeminal-like lateral migration of cells can be detected (straight arrows, Fig. 5B), which is completed at about 12.5 dpc (Fig. 5D) and results in a characteristic dorso-lateral accumulation of ectopic *Phox2b*-positive cells (rectangle, compare Fig. 5C, D). This population includes ectopic motoneurons as assessed by anti-Islet1 immunohistochemistry (arrows; compare Fig. 5E, F). Remarkably, lack of caudal migration of facial motoneurons and lateral trigeminal-like migration are also observed in *Hoxb1*$^{-/-}$ mice (Studer et al., 1996). Thus, together with the above molecular analysis (Fig. 4), these data suggest facial-to-trigeminal changes in motoneuron subtype identity in *Hoxa1* mutants which could be induced by lack of *Hoxb1* activation in pre-r4 cells.

Persistence and functional role of the supernumerary neuronal system after birth

To investigate the functional role of the SNS in controlling respiratory and feeding rhythms *in vivo*, we have compared mutant and wild type behaviors in relation to the anatomical modification of the Pons. Although irregular after birth, the wild-type minute ventilation increases progressively and stabilises at the end of the first day (Fig. 6A, left). In contrast, mutants exhibited a variable neonatal respiratory frequency (NRF), 2-4 hours after birth, and eventually apneic breathing and death (Fig. 6A, right). A correlation was found in mutants between the NRF and the hindbrain anatomical index dP/vP (Fig. 6B, correlation coefficient: c=0.83 *vs.* c=0.32 in wild-type mice), indicating that there are pontine abnormalities accelerating spontaneous breathing at

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birth. In contrast, the suction behavior, estimated by the frequency of jaw openings induced by a buccal stimulus (Jacquin et al., 1996), was normal in the mutants and unrelated to dP/vP (c= 0.25). Furthermore, *Hoxa1*$^{-/-}$ newborns with a low NRF (<35/min, n=7) died within 2.5 ± 0.8 hours (Fig. 6C, lower left triangles) while those exhibiting a higher NRF (n=15) progressively increased their respiratory rate to normal values (Fig. 6A, C) and survived for 18 ± 7 hours. Thus, one possibility is that the appearance of the SNS may result in enhanced survival rates by significantly increasing NRF values, so that the rhythm promoting action of the SNS seems to compensate lethal apneic breathing resulting from vP hypoplasia. To further investigate this hypothesis, animals with highest NRF were submitted to naloxone administration, a treatment known to be effective on life-threatening pathologies resulting from the vP hypoplasia as, for instance, in *Krox-20*$^{-/-}$ mutants (Jacquin et al., 1996). A striking effect of naloxone administration was obtained in 2 out of the 5 treated *Hoxa1*$^{-/-}$ newborns (Fig. 6C, dots): one of them survived 4 days, whereas the other was sacrificed 12 days after birth. Interestingly, in this animal, histological analysis revealed the same pattern of SNS motoneurons (Fig. 6D) as observed at birth (Fig. 2). The survival of these motoneurons is noteworthy, considering the wave of apoptosis which normally removes abnormal motoneurons in the foetal hindbrain before birth (deLapeyrière and Henderson, 1997). Altogether, the present *in vitro* and *in vivo* observations demonstrate that the *Hoxa1* mutation results in the incorporation of a SNS, which originates from the mutant r3/r4 region, into the hindbrain neural network. As a consequence, the animal acquires a novel respiratory-related function enhancing survival, while not affecting suction, which is under the control of neuronal populations from the same region.

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DISCUSSION

These results allow a hypothesis compatible with the involvement of developmental control genes in the assembly of functional neuronal circuits (Tanabe and Jessel, 1996; Brunet and Ghysen, 1999). In fact, this work provides the first formal evidence that the selective modification of the expression pattern of a *Hox* gene whose expression is transient in the presumptive hindbrain, namely *Hoxa1*, is sufficient to incorporate a novel functional neural circuit in the mature hindbrain. This striking finding prompts the question of the cascade of regulatory events triggered by *Hoxa1* loss-of-function, leading to such long-term modification of hindbrain neural networks. Previous work demonstrated a role for *Hoxa1* in the activation of *Hoxb1* expression in the presumptive r4 (Studer et al., 1998). Thus, some of the long-term effects of the *Hoxa1* mutation could be due to the lack of *Hoxb1* activation in a subset of presumptive r4 cells, leading to r2-like specification. However, *Hoxa1*, unlike *Hoxb1,* appears to control both r4 and, indirectly, r3 development (Helmbacher et al., 1998; this study). Thus, it is tempting to speculate that regulatory changes in two adjacent rhombomeres may be required for the generation of a SNS. Interestingly, we have recently shown that assembling of a rhythm-promoting respiratory network also requires a two-segment functional unit in the chick (Fortin et al., 1999). In this respect, it will be interesting to compare the physiology of neuronal networks in *Hoxb1*$^{-/-}$ mutants to that of *Hoxa1*$^{-/-}$ mutants.

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Because hindbrain neurons control adaptive behaviors, these findings have considerable significance both on developmental and evolutionary grounds. The evolution of neural networks of multisegmental origin may be facilitated by the partitioning of the early hindbrain in a number of metameric units initially developing as independent modules (Lumsden, 1990; Clarke and Lumsden, 1993; Champagnat and Fortin, 1996). As a result, subsets of neurons may be developmentally isolated from each other and allowed to evolve independently. Our present data suggest that *Hox* genes may provide a genetic basis for segment-specific modulation of neuronal development and connectivity. Changes in *Hox* cis-regulatory modules and downstream targets have been suggested to underlie morphological changes of segmented structures in animal evolution (Gellon and McGinnis, 1998). Similarly, local changes in the regulation of *Hox* genes within the segmented hindbrain of vertebrates may open novel opportunities for the evolution of distinct subsets of neurons, without affecting the function of others, eventually resulting in novel functional features (see Brunet and Ghysen, 1999). In this respect, studies of conditional segment-specific *Hox* mutations, which may not result in lethality of the animal, will be important to further investigate adaptive mechanisms in development of hindbrain neuronal networks.

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FIGURE LEGENDS

Fig. 1. Distinct dorsal and ventral anatomical phenotypes in the *Hoxa1*$^{-/-}$ brainstem at birth. **A-D**: adjacent horizontal sections (A and C are dorsal to B and D, respectively; scale bar: 250µm) showing the ependymal epithelium at the anterior end of the fourth ventricle (see also E, F caudal to DTg and Fig. 2B for location): it forms, in WT mice (A, B, E), a single invagination closing in the dorsal Pons medially to the trigeminal motor nucleus and multiple invaginations (2 to 5) in *Hoxa1*$^{-/-}$ mice (C, D, F). **E, F**: Parasagittal sections of the hindbrain at P0 in WT (E) and *Hoxa1*$^{-/-}$ (F) littermates; A-P length of the Pons is differently affected dorsally (arrow, from the rostral limit of the hypoglossal nucleus, 12, to the caudal limit of the dorsal tegmental area, DTg) and ventrally (rectangle, from the rostral pole of the inferior olive, IO, to the caudal pole of the pontine nuclei, Pn); IP: interpeduncular nucleus, Sol: solitary nucleus.

Fig. 2. The dorsal anatomical phenotype in *Hoxa1*$^{-/-}$ mice at birth: identification of motoneurons showing location of the supernumerary neuronal structure (SNS). **A**: sagittal sections of the brainstem, cut parallel to Fig. 1, E and F. The drawings on the left (including the analysis of 5 *Hoxa1*$^{-/-}$ mice) include both lateral and medial structures (scale bar, 1 mm). Medial sections (in gray, showing the ventricular surface) are illustrated in Fig. 1, E and F: note that supernumerary motor (lateral) and ventricular (medial) structures are at the same antero-posterior level of the dorsal Pons. Lateral sections (on the right) show cholineacetyltransferase immunoreactive WT (+/+) ventral facial structures eliminated by the mutation: the branchial motor nucleus (VII), the

preganglionic nucleus (pg) and accessory nuclei (between VII and pg, extending close to the descending facial root, VIIn). In *Hoxa1*$^{-/-}$ (-/-), caudal to the trigeminal nucleus (V), the SNS includes three dorsal motor subnuclei (outlined and numbered) alternating with 2 unstained stripes of reticular formation. Abbreviations IP, Pn, IO: as in Fig. 1; X, XII: dorsal vagal and hypoglossal motor nuclei; SO: superior olive; pc: parvocellular reticular formation. **B**: horizontal sections cut parallel to the arrow in Fig. 1, E and F. Drawings on the left (including the analysis of 5 *Hoxa1*$^{-/-}$ mice) show the left part of the Pons (scale bar: 1 mm) and the relative positions of the V and VII nuclei and trigeminal nerve root (Vn). Note, close to the midline (dotted line), appearance of a supernumerary ventricular structure (illustrated in Fig. 1D) and elimination of the abducens motor nucleus (VI). The right part superimposes cholineacetyltransferase immunoreactive pontine neurons in WT (black) and *Hoxa1*$^{-/-}$ (red) littermates, from 4 horizontal sections sampling, in each littermate, the entire V nucleus and adjacent areas. Supernumerary motor nuclei n°1, 2 and 3 are at the same place as the WT Rpc-α (pc), VIIn and pg, respectively. **C-E:** horizontal sections showing retrograde DiI labelling of trigeminal and SNS motoneurons in a WT (C, arrow: 200µm) and an *Hoxa1*$^{-/-}$ (D, E) mouse. Labelling of the SNS shows the three ectopic trigeminal subnuclei (compare D to C) and a more ventral view (E) shows a supernumerary dorso-ventral fasciculus located laterally in subnucleus n°2 (star in D and E) and distinct from the WT-like Vn. **F**: medial half of a subnucleus n°2 at higher magnification (arrow: 67 µm, oriented as in C; the border of the V is in the upper left corner; subnucleus n°1 is lacking). The supernumerary motoneuron (triangle) shows an axon (stars) running in the direction of the lateral fasciculus.

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Fig. 3. Functional connectivity of reticular neurons in the *Hoxa1*$^{-/-}$ supernumerary neuronal structure at birth. **A-D:** modification of the contralateral trigeminal nerve activity (Vn) induced by excitating SNS neuronal cell bodies using brief (25 ms) pressure applications of AMPA in WT (A, B) and *Hoxa1*$^{-/-}$ (C, D) hindbrain slices *in vitro*. A, C: 4 samples of integrated Vn activity (2 min long) starting (from top to bottom) -2, 0, 3 and 5 min after AMPA application (time indicated on the left). In both WT and *Hoxa1*$^{-/-}$, the rhythm generator produces bursts of activity (fast upward deviations) and AMPA generates background non-rhythmic activity starting at time 0 min. B, D: temporal evolution (scale bar: 2 min) of average (± standard error) burst frequency from 5 experiments. Significant increase followed by inhibition ($p<.001$) indicates a functional connection *to* the rhythm generator in *Hoxa1*$^{-/-}$ but not in WT mice. **E:** Vn: integrated nerve activity; Em: membrane potential of a single (*Hoxa1*$^{-/-}$) neuron located in the SNS area (scale bars: 20mV, 1s): a connection *from* the rhythm generator results in a simultaneous Vn burst and neuronal depolarization inducing firing of action potentials. **F, G:** schematic presentation of the slice preparation in sagittal (F, arrowhead indicates the upper side) and horizontal (G) sections. Rectangle in G: approximate extent of the area affected by AMPA applications (arrowhead, more medial applications were ineffective); thin arrows: WT projections, preserved in mutants: these are either rhythmic, from the bilateral rhythm generator (stars) to the contralateral trigeminal nucleus (VMo) and Vn (recorded) or non-rhythmic premotor from Rpc-α/SNS to VMo; thick arrows: supernumerary connections in mutants including those from SNS to the rhythm generator and the trigeminal axons of SNS motoneurons. **H-J:** Sagittal sections (location in J; rostral to the left) of the most lateral 300µm of the Pons

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showing in mutant (I), but not in WT (H) animals, an axonal fasciculus stained after DiI injection in the area of the rhythm generator (lower right corner); scale bar: 200µm.

Fig. 4. Molecular and morphological patterning defects in *Hoxa1* mutant hindbrain. Dorsal view of 11.5 d.p.c. wild-type (WT, A, C) and *Hoxa1*$^{-/-}$ (B, D) mutant hindbrains hybridized with the r4-specific *Hoxb1* (A, B) or carrying a lacZ reporter under the control of an r2-specific enhancer (C, D). Vertical arrows indicate location of the motoneuron progenitor columns.

Fig. 5. The *Hoxa1*$^{-/-}$ supernumerary motoneurons: migration and final postnatal location. Dorsal view of 11.5 (A, B) and 12.5 (C-F) d.p.c. WT and *Hoxa1*$^{-/-}$ mutant hindbrains, respectively, flat-mounted and hybridized with *Phox2b* (A-D) or *Isl1* (E, F) probes; bent white arrows in A, B: caudal migration of facial (VII) motoneurons; straight arrows in A, B: dorsal migration of trigeminal (V) motoneurons and, in *Hoxa1*$^{-/-}$ (B), of supernumerary motoneurons from r4; rectangle in C, D and arrows in E, F: ectopic, dorso-lateral, accumulation of *Phox2b* and *Isl1* positive cells, not present in WT.

Fig. 6. The *Hoxa1*$^{-/-}$ breathing pattern after birth. **A:** samples of plethysmographic recording (inspiration upwards) 2, 6, 12, 18 and 24 hours after birth (p.n.) showing normal maturation in a WT and transient increase of frequency in a mutant (scale bars 20 µl, 1s). The mutant typically exhibits irregular breathing at birth (top trace), and eventually (bottom trace) apneic breathing and death. **B:** Individual *Hoxa1*$^{-/-}$ (empty triangles) and WT (black squares) mice identified by their respiratory rate at birth (ordinates) and the dP/vP index quantifying abnormality of the pontine A-P distances

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(see arrow (dP) and rectangle (vP) in Fig. 1E,F): a correlation exists in *Hoxa1*$^{-/-}$, not in WT mice. **C:** Temporal evolution of average respiratory frequency (± SEM) in *Hoxa1*$^{-/-}$ animals breathing faster or slower than 35/min at birth (empty triangles): slowest animals lack rhythm stimulation shown in A (6-18); fastest animals survive longer; death has been delayed by > 3 d.p.n. in 2 animals (dots) treated with subcutaneous naloxone (NLX). **D:** Supernumerary motoneurons in a NLX-treated animal sacrificed 12 days after birth: sagittal section (rostral to the left) showing cholineacetyltransferase immunoreactive motoneurons (arrowheads), caudal to the trigeminal motor nucleus (located in the upper left corner, scale bar: 100µm).