Neuronal defects in the hindbrain of *Hoxa1*, *Hoxb1* and *Hoxb2* mutants reflect regulatory interactions among these Hox genes

Anthony Gavalas[1],[*],[†], Christiana Ruhrberg[1],[*],[‡], Jean Livet[2], Christopher E. Henderson[2] and Robb Krumlauf[1],[3],[§]

[1]Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[2]INSERM U.382, Developmental Biology Institute of Marseille (CNRS-INSERM-Univ. Mediterranee) Marseille, France
[3]Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110, USA
[*]These authors contributed equally to this work
[†]Present address: MRC Center for Developmental Neurobiology, 4th Floor New Hunt's House, King's College London, London SE1 1UL, UK
[‡]Present address: Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, UK
[§]Author for correspondence (e-mail: rek@stowers-institute.org)

Summary

Hox genes are instrumental in assigning segmental identity in the developing hindbrain. Auto-, cross- and para-regulatory interactions help establish and maintain their expression. To understand to what extent such regulatory interactions shape neuronal patterning in the hindbrain, we analysed neurogenesis, neuronal differentiation and motoneuron migration in *Hoxa1*, *Hoxb1* and *Hoxb2* mutant mice. This comparison revealed that neurogenesis and differentiation of specific neuronal subpopulations in r4 was impaired in a similar fashion in all three mutants, but with different degrees of severity. In the *Hoxb1* mutants, neurons derived from the presumptive r4 territory were re-specified towards an r2-like identity. Motoneurons derived from that territory resembled trigeminal motoneurons in both their migration patterns and the expression of molecular markers. Both migrating motoneurons and the resident territory underwent changes consistent with a switch from an r4 to r2 identity. Abnormally migrating motoneurons initially formed ectopic nuclei that were subsequently cleared. Their survival could be prolonged through the introduction of a block in the apoptotic pathway. The *Hoxa1* mutant phenotype is consistent with a partial misspecification of the presumptive r4 territory that results from partial *Hoxb1* activation. The *Hoxb2* mutant phenotype is a hypomorph of the *Hoxb1* mutant phenotype, consistent with the overlapping roles of these genes in facial motoneuron specification. Therefore, we have delineated the functional requirements in hindbrain neuronal patterning that follow the establishment of the genetic regulatory hierarchy between *Hoxa1*, *Hoxb1* and *Hoxb2*.

Key words: Hox genes, Hindbrain, Neurogenesis, Neuronal patterning, Neuronal migration, Gene regulation, Neural development, Segmentation, Mouse

Introduction

The vertebrate nervous system contains a vast number of diverse neuronal cell types that serve distinct functions and interconnect in a precise and stereotyped manner. The foundations of this diversity and specificity are laid out during embryonic development, when the neural tube is specified along its anteroposterior (AP) and dorsoventral (DV) axes (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1997). Dorsal cell specification is controlled by secreted signals belonging to the TGFβ (Lee and Jessell, 1999) and Wnt (Megason and McMahon, 2002; Muroyama et al., 2002) families, and ventral cell specification depends upon the secreted molecule Shh (Jessell, 2000). These signals elicit the regionally restricted expression of a battery of transcription factors, such as members of the Pax family (Ericson et al., 1997; Osumi et al., 1997; Takahashi and Osumi, 2002), to further elaborate neuronal patterning. Hox genes play a general role in the processes that control the AP identity of the neural tube. Their expression patterns as well as gain- and loss-of-function experiments imply that they also have specific roles in patterning neurogenesis and neuronal differentiation (Davenne et al., 1999; Graham et al., 1991; Jungbluth et al., 1999; Studer et al., 1996). In addition to the nested patterns of Hox expression along the AP axis, their expression patterns also become restricted along the DV axis concomitant with the birth of major classes of neurons (Graham et al., 1991). While grafting and neural tube rotation studies suggest that the positional identity of cells in the neuroepithelium can be controlled independently along the AP and DV axes (Ensini et al., 1998; Simon et al., 1995), the patterning processes along the two axes ultimately have to be integrated to generate the different types of neurons in their appropriate position. How Hox genes function in coordinating AP and DV patterning in the neural tube is an important issue poorly understood.

The segmental structure of the hindbrain is particularly suited for studies aimed at elucidating the link between AP and

DV specification in the CNS. The hindbrain is transiently divided into a series of lineage restricted and morphologically distinct repeats, the rhombomeres (r1 to r8), each of which generates a similar set of neurons (Fraser et al., 1990; Lumsden and Keynes, 1989). However, the number, distribution and specialisation of these neurons are rhombomere-specific (Lumsden and Krumlauf, 1996). The specific identity of each rhombomere is imposed by the differential expression of Hox genes (Trainor and Krumlauf, 2000). During neurogenesis and neuronal differentiation, homeobox containing genes (i.e. *Phox2b*, *Isl1*), mammalian homologs of the *Drosophila* proneural genes, and members of the Notch signalling pathway are expressed in rhombomere specific longitudinal stripes (Davenne et al., 1999; Kusumi et al., 2001; Osumi et al., 1997; Pattyn et al., 1997). By analogy to the patterns seen in the spinal cord, these longitudinal domains are believed to prefigure sites of generation and differentiation of defined neuronal subtypes (Davenne et al., 1999; Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1997). Together these AP and DV expression patterns in the hindbrain appear to form a grid of distinct coordinates, onto which is superimposed the temporally and spatially ordered generation of specific neuronal subtypes.

Hindbrain motoneuron progenitors are born next to the floorplate and differentiate in a rhombomere-specific pattern (Lumsden and Keynes, 1989) into three functional classes: the somatomotor (sm) neurons, which innervate muscles of the body; the branchiomotor (bm) neurons, which innervate the muscles derived from the pharyngeal arches; and the visceromotor (vm) neurons, which innervate the sympathetic and parasympathetic ganglia. Each rhombomere will generate specific subtype(s) of motoneurons according to its AP level. Trigeminal (V) motoneurons are derived from r2/r3 and exclusively differentiate into the bm class. The abducens (VI) motoneurons, which are derived from r5, are composed exclusively from sm neurons. By contrast, facial (VII) motoneurons, derived from r4/r5, belong to both the bm and vm classes. According to their functional subclass, cranial motoneurons migrate to form nuclei in specific DV positions. In the mouse, motoneurons from r2 migrate dorsally to contribute to the Vth motor nucleus on the pial surface of the hindbrain. The abducens motoneurons assemble into nuclei close to their birthplace on the ventricular side of r5. The bm neurons derived from r4 undergo a complex migration moving caudally and laterally through r5 and r6 to form the VIIth motor nucleus on the pial side of in the posterior hindbrain (Auclair et al., 1996; Covell and Noden, 1989; Garel et al., 2000; McKay et al., 1997; Studer et al., 1996).

Mutational analyses of Hox genes involved in the early segmental patterning of the hindbrain such as kreisler and *Krox20* show that these genes have a profound impact on subsequent neuronal development (Cordes and Barsh, 1994; Manzanares et al., 1999; Schneider-Maunoury et al., 1997). Mutation of Hox genes also leads to later defects in neuronal patterning. Targeted inactivation of *Hoxa1* results in partial loss of r4 and r5, defects in the development of the facial motoneurons and in malformations of several cranial nerves (Carpenter et al., 1993; Gavalas et al., 1998; Mark et al., 1993). Loss of *Hoxb1* leads to early changes in the identity of r4 and impairs the development of facial motoneurons (Gaufo et al., 2000; Goddard et al., 1996; Studer et al., 1996). *Hoxa1* and

Hoxb1 also synergise in early patterning of the r4 territory (Gavalas et al., 1998; Studer et al., 1998) as well as the generation of the r4-derived neural crest (Di Rocco et al., 2001b). *Hoxa2* controls the identity to the r4-derived neural crest cells, r2 and r3 patterning and axonal pathfinding of a subset of the trigeminal motoneurons (Gavalas et al., 1997; Gendron-Maguire et al., 1993; Rijli et al., 1993). *Hoxa2* and *Hoxb2* have unique and overlapping roles in controlling neurogenesis and neuronal differentiation in multiple segments (Davenne et al., 1999). Furthermore, in chick, ectopic expression of *Hoxb1* and *Hoxa2* can confer specific motoneuron identities in the rostral hindbrain (Bell et al., 1999; Jungbluth et al., 1999). Although the phenotypes described above demonstrate that Hox genes play a role in neuronal development, it has not been shown whether they can directly instruct developing neurons to modify their behaviour, or if their influence is simply an indirect consequence of their ability to pattern the environment (Gavalas et al., 1998; Helmbacher et al., 1998; Manzanares et al., 1999; Studer et al., 1996).

Auto-, cross- and para-regulatory interactions among Hox genes contribute to the establishment and maintenance of their segmental expression patterns (Gould et al., 1997; Maconochie et al., 1997; Manzanares et al., 2001). For example, regulatory analyses have defined a hierarchy of direct interactions among the *Hoxa1*, *Hoxb1* and *Hoxb2* genes that control their segmental expression. Initially *Hoxa1* and *Hoxb1* are activated in neural tissue by retinoic acid (Dupé et al., 1997; Marshall et al., 1994; Studer et al., 1998). The subsequent maintenance of *Hoxb1* expression in r4 is dependent upon a highly conserved auto- and cross- regulatory element (r4 ARE) (Pöpperl et al., 1995). First Hoxa1 transactivates *Hoxb1* expression by binding to the *Hoxb1* r4 ARE in cooperation with co-factors (Pbx, Sox, Oct) (Di Rocco et al., 2001a; Di Rocco et al., 1997) and then Hoxb1 continues to maintain its own expression (Pöpperl et al., 1995; Studer et al., 1998; Studer et al., 1996). Similarly, the subsequent upregulation of *Hoxb2* expression in r4 is directly mediated by Hoxb1, through binding to an r4–specific enhancer at the 5′ of the *Hoxb2* locus (Ferretti et al., 2000; Maconochie et al., 1997). This regulatory hierarchy provides a mechanism for the synergy between *Hoxa1* and *Hoxb1* in patterning the r4 territory and formation of r4-derived crest (Di Rocco et al., 2001b; Gavalas et al., 1998; Studer et al., 1998; Studer et al., 1996).

To understand if this coordinated series of direct regulatory interactions also plays a role in neuronal patterning, we compared neurogenesis, neuronal differentiation and motoneuron migration in hindbrains lacking *Hoxa1*, *Hoxb1* or *Hoxb2*. Our comparative analysis revealed many similarities in the defects arising during neurogenesis and differentiation in r4 and differences in the relative degrees of severity correlate with their relative position in the hierarchy. Together these observations support the idea that these genes function in common pathways not only during the early phase of segmental patterning, but also in subsequent neuronal differentiation.

Materials and methods

Mouse strains

Embryos were obtained from overnight and 2 hour morning matings (8-10 am). Noon of the day that the vaginal plug was detected was
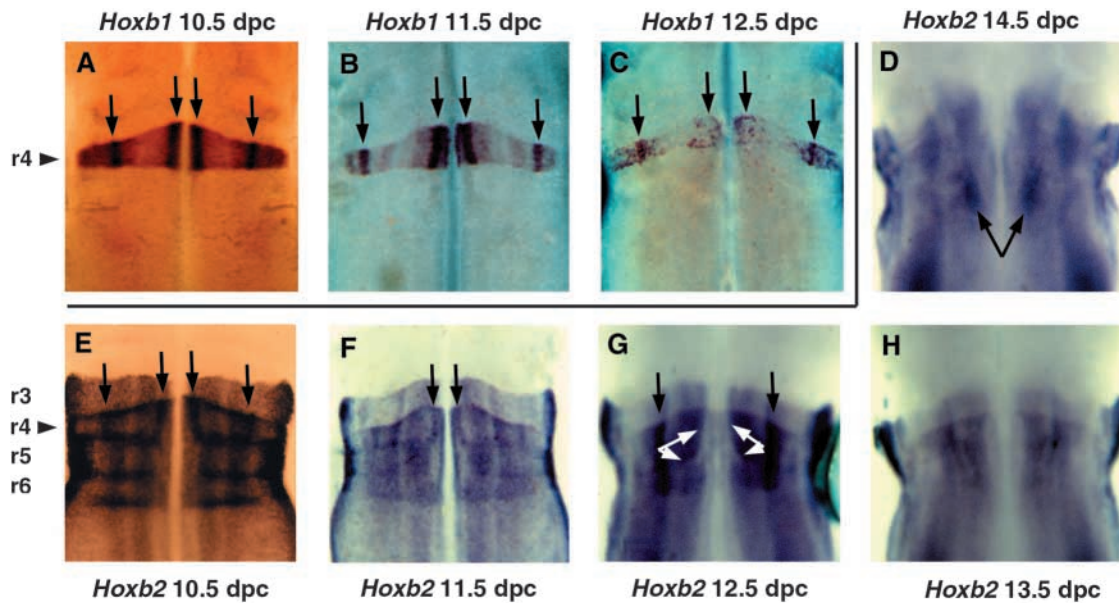
Fig. 1. Expression of *Hoxb1* and *Hoxb2* in the developing mouse hindbrain. (A-C) Ventricular views of flat-mounted wild-type mouse hindbrains stained with a *Hoxb1* riboprobe at 10.5 dpc (A), 11.5 dpc (B) and 12.5 dpc (C). (D-H) Ventricular views of flat-mounted wild-type mouse hindbrains stained with a *Hoxb2* riboprobe at 10.5 dpc (E), 11.5 dpc (F), 12.5 dpc (G), 13.5 dpc (H) and 14.5 dpc (D). (A-C) Between 10.5 dpc and 12.5 dpc, expression of *Hoxb1* was restricted to rhombomere 4. It was upregulated in ventral and dorsal columns (black arrows). At 11.5 dpc (B), the ventral expression domain was further subdivided into two adjacent stripes. At 12.5 dpc (C), *Hoxb1* expression was generally downregulated, but remained stronger in the ventral and dorsal columns. (D-H) Between 10.5 and 14.5 dpc, expression of *Hoxb2* resolved into a series of longitudinal stripes that persisted longer than that of *Hoxb1*. At 10.5 dpc, *Hoxb2* expression was stronger in the r4, r5 and r6 territories, where it was upregulated in specific ventral and dorsal columns (black arrows, E) that persisted to 11.5 dpc (black arrows, F). At 11.5 dpc (F), expression in the ventral domain resolved into two adjacent stripes similar to those of *Hoxb1*. At 12.5 dpc (G), *Hoxb2* expression was absent in a ventral territory corresponding to the path of facial branchial motoneuron (fbm) migration (white arrows). From 13.5 dpc, expression was downregulated throughout the hindbrain (H), whereas at 14.5 dpc (D), expression was upregulated in a region close to the final position of the facial motor nucleus (black arrows).

considered as day 0.5 dpc of development for embryos of the overnight matings. The genotype of the animals and the embryos was determined using PCR. The PCR conditions and primers for the *Hoxa1*, *Hoxb1* and *Hoxb2* mutations were as described before (Davenne et al., 1999; Gavalas et al., 1998). Genotyping of the *Bax* mutation (Deckwerth et al., 1998) was carried out using the same conditions and the primers were: 5′GTTGACCAGAGTGGCGTAGG3′ (common to both alleles), 5′GAGCTGATCAGAACCATCATG3′ (wild-type allele) and ACCCGCTTCCATTGCTCAGCGGTGC (mutant allele), which give rise to a 304 bp product for the wild-type allele and 507 bp product for the mutant allele. The HL5 *lacZ* transgenic line contains a β-galactosidase gene inserted in frame into an *Eco*RV fragment derived form the *Hoxb1* locus (Marshall et al., 1994).

Whole-mount in situ hybridisation

The following mouse cDNA templates were used: Hoxb1, Hoxb2, Math3, Phox2b, Gata2, Isl1, Lhx4, Er81, Met and Cad8. Antisense digoxigenin-labelled riboprobes were synthesised from linearised templates by the incorporation of digoxigenin-labelled UTP (Boehringer) using T3, T7 or SP6 polymerase. Processing of the embryos and hybridisation with 500 ng ml^{-1} of the probe was as previously described (Gavalas et al., 1998).

TUNEL assay

Embryos fixed in 4% PFA were embedded in 20% (w/v) gelatin and sectioned using a Leica vibratome at 50 μm. The TUNEL reaction was carried out on the sections as described (Maden et al., 1997) and peroxidase staining as described (Di Rocco et al., 2001b).

Results

Hoxa1, *Hoxb1* and *Hoxb2* are members of the same regulatory hierarchy that has been implicated in the segmental patterning of the vertebrate hindbrain. To understand how these genes and their genetic interaction impacts on neurogenesis in the developing hindbrain, we first examined how their expression patterns spatially and temporally overlapped with neuronal production and differentiation, and then analysed neuronal patterning in targeted mutants of these genes.

Expression patterns of *Hoxb1* and *Hoxb2* between 10.5 and 14.5 dpc

Analysis of *Hoxa1*, *Hoxb1* and *Hoxb2* expression patterns have previously been performed between 7.5 and 10.5 dpc, spanning the stages when the hindbrain is being segmentally patterned (Frohman et al., 1990; Murphy et al., 1989; Murphy and Hill, 1991; Wilkinson et al., 1989). To understand the potential roles that these genes may play during neurogenesis and neuronal differentiation, we extended this analysis to include later developmental stages. We focused our attention on *Hoxb1* and *Hoxb2,* as *Hoxa1* is no longer expressed in the hindbrain by 8.5 dpc (Murphy and Hill, 1991).

The early expression of *Hoxb1* was uniform throughout r4, but underwent dynamic changes between 9.5-12.5 dpc. Then expression became upregulated in paired ventral and dorsal longitudinal stripes (Fig. 1A,B). By 11.5 dpc, the ventral r4
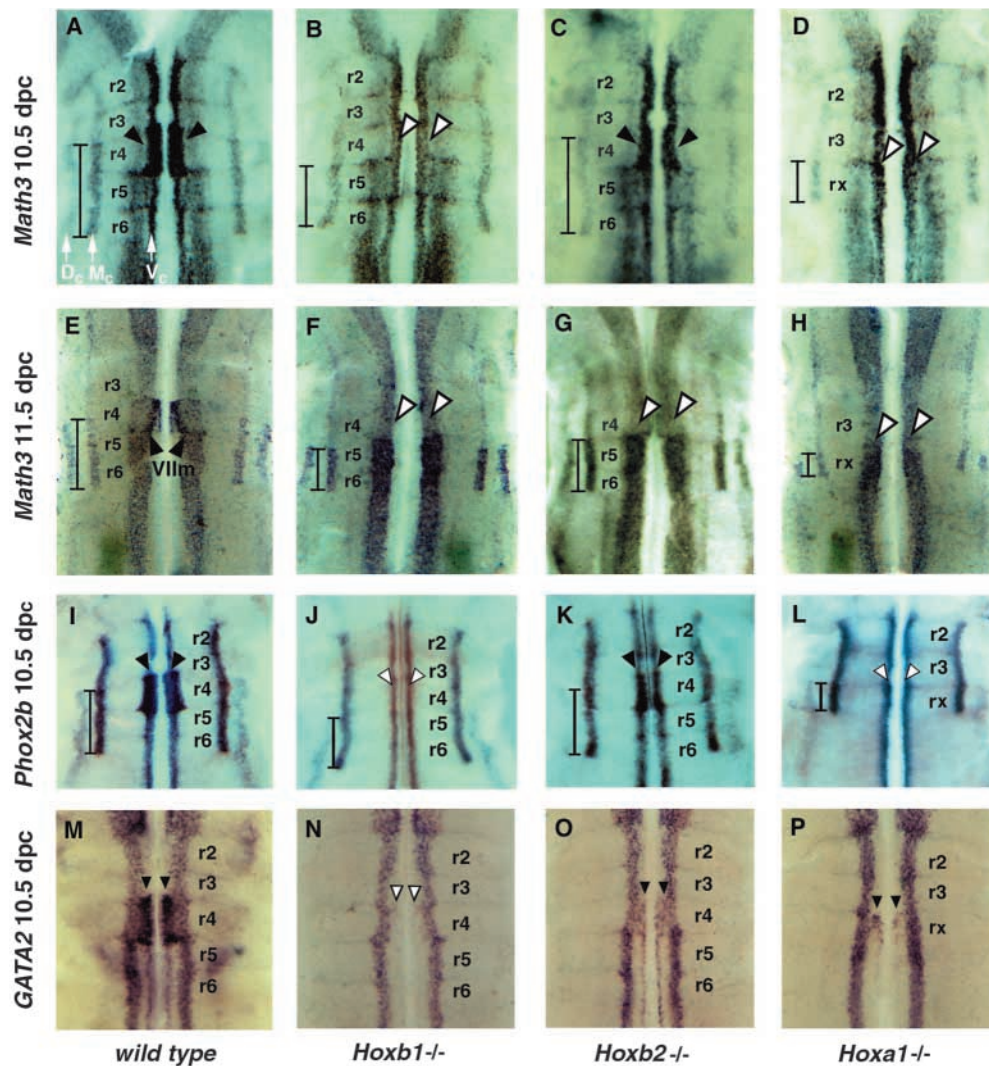
Fig. 2. Altered neurogenesis and neuronal differentiation patterns in the *Hoxa1*, *Hoxb1* and *Hoxb2* mutant hindbrains. Ventricular views of flat-mounted wild-type (A,E,I,J), *Hoxb1* (B,F,J,N), *Hoxb2* (C,G,K,O) and *Hoxa1* (D,H,L,P) mutant hindbrains labelled with a *Math3* (A-H), *Phox2b* (I-L) or *GATA2* (M-P) riboprobes. (A-H) At 10.5 and 11.5 dpc, *Math3* expression resolved into longitudinal stripes and was particularly strong in a ventral r4 column (Vc) (black arrowheads, A) and in a medial column (Mc) extending from r4 through to r6 (bar, A). There was also some upregulation at a more dorsal column (Dc). At 11.5 dpc, the ventral r4 column segregates into two adjacent stripes (black arrowheads, E). There is no r4-specific upregulation of the ventral column in either *Hoxb1* (white arrowheads, B,F) or *Hoxa1* (white arrowheads, D,H) mutants. Some upregulation was evident in *Hoxb2* mutants at 10.5 (black arrowheads, C), but not at 11.5 dpc (white arrowheads, G). Expression within the dorsal column extended throughout only two rhombomeres in the *Hoxb1* mutants (bar, B,F) and one rhombomere in *Hoxa1* mutants (bar, D,H), but appeared normal in the *Hoxb2* mutants (bar, C,G). (I-L) *Phox2b* expression at 10.5 dpc. In the wild-type hindbrain, expression was strongest in a ventral r4 column (black arrowheads), but it was also upregulated in a medial column extending from r2 to r6, and a dorsal column extending from r4 posteriorly (I). The medial and dorsal columns overlap in three rhombomeres: r4, r5 and r6 (black bar, I). In *Hoxb1* (J) and *Hoxa1* (L) mutants, there was no r4-specific upregulation (white arrowheads). The medial and dorsal columns overlap by only two rhombomeres in *Hoxb1* mutants (J, bar) and one rhombomere in *Hoxa1* mutants (L, bar). In *Hoxb2* mutants (K), expression levels in ventral r4 were lower than in wild type, but remained elevated when compared with neighbouring rhombomeres (black arrowheads). (M-P) *Gata2* expression at 10.25 dpc. In wild-type hindbrain, *Gata2* expression is strongly upregulated in ventral r4 (M, black arrowheads). *Gata2* expression is absent in this domain in *Hoxb1* mutants (N, white arrowheads) and slightly reduced in the *Hoxb2* mutants (O, black arrowheads). Both the width and the strength of this domain were greatly reduced in the *Hoxa1* mutants (P, black arrowheads).

domain had further segregated into two separate columns, a narrow one next to the floorplate and a broader column that curved away from the midline (Fig. 1B). By 12.5 dpc, *Hoxb1* expression in the hindbrain began to be downregulated and was not detectable at subsequent stages (Fig. 1C and data not shown).

In contrast to the earlier *Krox20*-dependent induction of *Hoxb2* in r3 and r5 (Vesque et al., 1996), expression between 10.5 and 12.5 dpc was upregulated in the r4, r5 and r6 territories (Fig. 1E-G). At 10.5 dpc the *Hoxb2* pattern had resolved into a series of longitudinal stripes, with a more complex and dynamic pattern that persisted longer than that of *Hoxb1* (Fig. 1D-H). Within r4, the expression pattern at 10.5 dpc was very similar to that of *Hoxb1* (Fig. 1A,E), consistent with their regulatory relationship (Maconochie et al., 1997). At

12.5 dpc *Hoxb2* expression was absent in the territory corresponding to the path of facial branchial motoneuron (fbm) migration (Fig. 1G). From 12.5 dpc onwards, expression in the other longitudinal stripes became diffuse and ill-defined (Fig. 1D,G,H). Together, the temporally dynamic and longitudinal organisation of these Hox expression patterns during neurogenesis provides a framework for examining neuronal patterning in mutants of these genes.

Altered patterns of *Math3* and *Phox2b* expression suggest a defect in the generation of r4 neurons

To analyse *Hoxa1*, *Hoxb1* and *Hoxb2* mutants for neurogenesis defects, we examined the expression of two genes that are expressed in early neuronal precursors in the hindbrain. The atonal-related gene *Math3* is upregulated in early post-mitotic
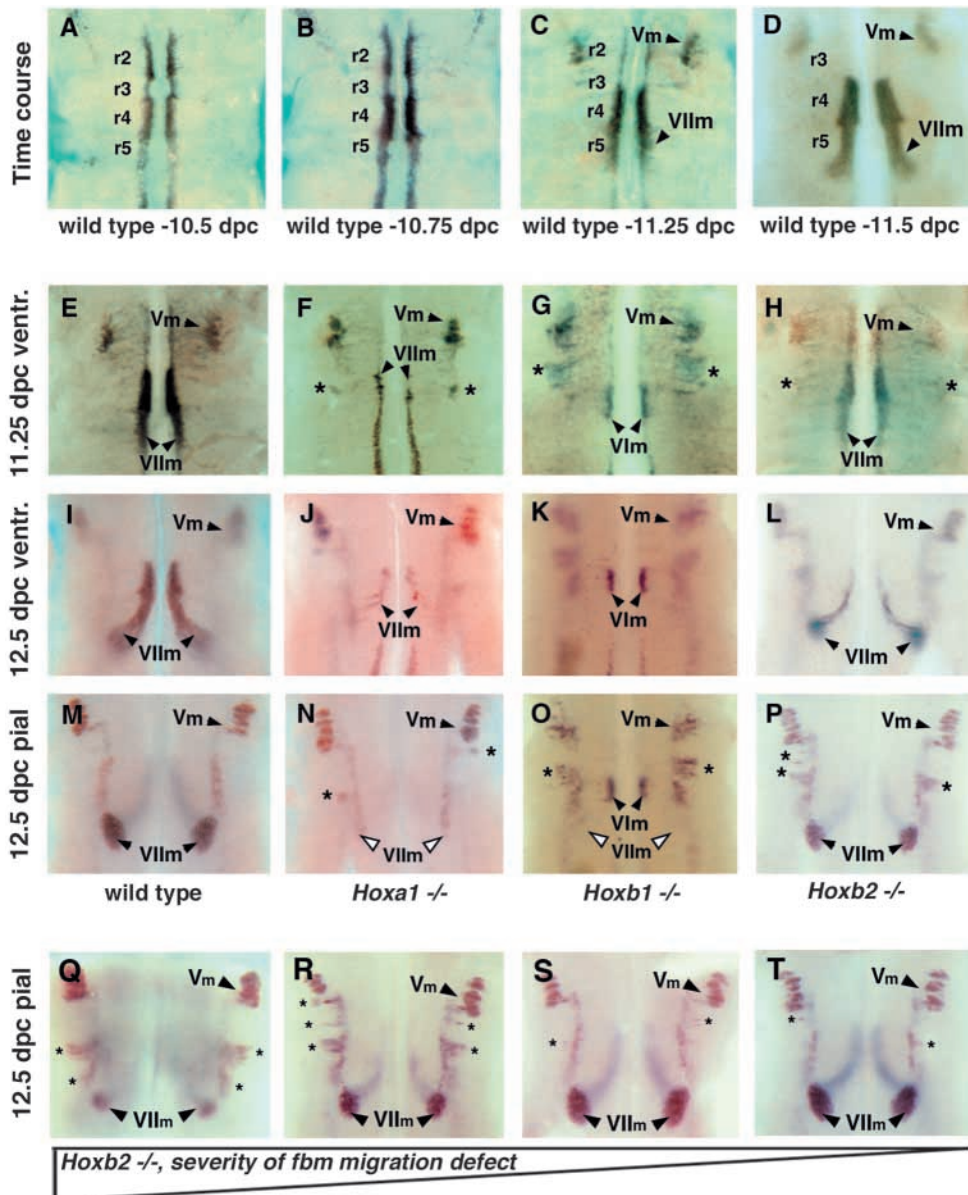
Fig. 3. Abnormal migration of r4-derived motoneurons in *Hoxa1*, *Hoxb1* and *Hoxb2* mutant hindbrains. (A-D) Time course of *Isl1* expression in wild-type flat-mounted hindbrains between 10.5 and 11.5 dpc (ventricular view). Expression was detected in the ventral domain of all rhombomeres, except rhombomere 1, and was strongest in r2 and r4, corresponding to the precursors of the trigeminal (Vm) and facial (VIIm) motoneurons, respectively. At 11.25 dpc, dorsal migration of the Vm and caudal migration of the VIIm began (C, black arrowheads) and continued until 11.5 dpc (D, black arrowheads) when Vm reached their final dorsal position. (E-H) Motoneuron distribution at 11.25 dpc in wild-type (E), *Hoxa1* (F), *Hoxb1* (G) and *Hoxb2* (H) mutant flat-mounted hindbrains (ventricular view). The caudally migrating VIIm population was strongly reduced in *Hoxa1* mutants (F, black arrowheads), absent in *Hoxb1* mutants and depleted in *Hoxb2* mutants (H, black arrowheads). The loss of the caudally migrating VIIm in the *Hoxb1* mutants unmasks the abducens motoneurons (VIm) (G, black arrowheads). Note the presence of ectopic motor nuclei in r4 in all three mutants (F-H asterisks). (I-P) Ventricular (I-L) and pial (M-P) views of motoneuron distribution at 12.5 dpc in wild-type (I, M), *Hoxa1* (J, N), *Hoxb1* (K, O) and *Hoxb2* (L, P) mutant flat-mounted hindbrains. At this stage, VIIm normally started migrating caudally and dorsally reaching the pial side of the hindbrain (I, M, black arrowheads). In the *Hoxa1* mutants, caudal migration was sparse and no VIIm had reached the pial side at this stage (N, white arrowheads). Some migrating motoneurons turned dorsally prematurely (J, black arrowheads). In the *Hoxb1* mutants, VIIm did not follow the normal migratory pathway (K,O, white arrowheads). This population was reduced in the *Hoxb2* mutants. At this stage, all three mutants formed ectopic motor nuclei in r4 (J-K; asterisks in N-P). (Q-T) Ventricular views of the *Isl1* expression pattern in flat-mounted *Hoxb2* mutant hindbrains at 12.5 dpc. The VIIm population following the normal migratory pathway was variably reduced (black arrowheads). The ectopic nuclei were also variable (asterisks) in both their density and AP position.

precursors (Takebayashi et al., 1997), and the paired homeobox gene *Phox2b* is expressed in both proliferating precursors and post-mitotic neurons (Dubreuil et al., 2000; Pattyn et al., 2000; Pattyn et al., 1997). In the wild-type hindbrain, *Math3* and *Phox2b* are each expressed in three longitudinal columns with defined AP positions (Fig. 2A,E) (see also Davenne et al., 1999)). Although it has not been established whether these columns overlap, we have termed them the ventral (Vc), medial (Mc) and dorsal (Dc) columns to refer to their respective DV positions (Fig. 2A, white arrows).

At 10.5 dpc, low *Math3* expression was present in a broad ventral domain throughout the hindbrain, but was higher in a narrow stripe adjacent to the floorplate. This expression was most prominent in r4, where it marked the territory that gives rise to the facial motoneurons (Fig. 2A, black arrowheads). At 11.5 dpc, ventral expression near the floorplate persisted only in r4 and resolved into two subdomains, highly reminiscent of the *Hoxb1* and *Hoxb2* patterns at this stage (compare Fig. 2E with Fig. 1B,E, black arrowheads).

In addition to this ventral pattern, expression was also prominent in paired medial (Mc) and dorsal (Dc) columns for both *Math3* and *Phox2b*. While the Mc population for each gene included r2 to r6, the dorsal column extended from r4 caudally, and therefore both columns overlapped in r4, r5 and r6 (Fig. 2A,E,I, black bar). Although *Math3* expression in the dorsal column was more clearly visualised at 11.5 dpc than at
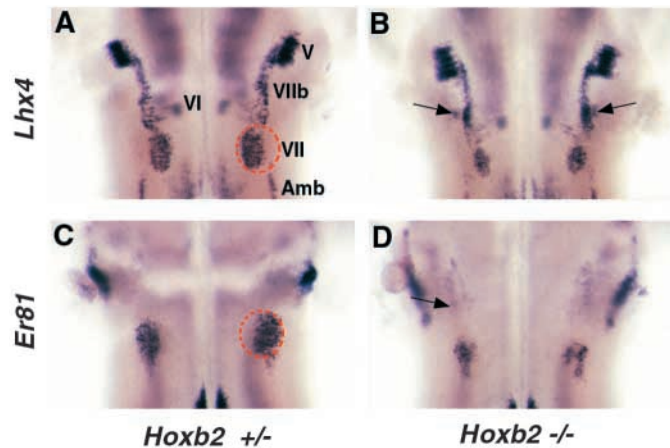
Fig. 4. Comparably low *Lhx4* and *Er81* expression in the facial motor nucleus of *Hoxb2* mutants. Ventricular view of *Lhx4* (A,B) and *Er81* (C,D) expression in the hindbrain of *Hoxb2* heterozygous (A,C) and mutant (B,D) embryos at 14.5 dpc. *Lhx4* is normally expressed in the trigeminal (V), abducens (VI), facial (VII), nucleus ambiguus (Amb) and dorsal r4/r5-derived [(VIIb) which are likely to include the superior salivatory nucleus] motoneurons (A). *Er81* is expressed only in the facial motor nucleus and the trigeminal nerve exit point. (C) Both genes define specific subpopulations within the facial nucleus (red circles in A,C). In *Hoxb2* mutants, expression of both markers is diminished in the facial nucleus, but only *Lhx4* is expressed ectopically in a dorsal population (compare arrows in B,D).

10.5 dpc, the *Phox2b* positive column was prominent at both 10.5 and 11.5 dpc (compare Fig. 2A with 2E,I; and data not shown).

In *Hoxb1* mutants, *Math3* and *Phox2b* expression was downregulated at both 10.5 and 11.5 dpc in the ventral, medial and dorsal columns of r4 compared with wild-type littermates, and, therefore, the r4 expression pattern now closely resembled that of r2 and r3 (compare Fig. 2A with 2B, 2E with 2F, and 2I with 2J). These altered expression patterns support the notion that the earlier homeotic transformation of r4 into r2 persists into the period of neurogenesis and neuronal differentiation (Studer et al., 1996).

Neurogenesis appeared less severely affected in *Hoxb2* compared with *Hoxb1* mutants. Although *Math3* and *Phox2b* expression was reduced in the ventral r4 domain at 10.5 dpc when compared with wild-type r4, it nevertheless remained stronger than in the neighbouring r3 and r5 domains (Fig. 2C) (see Davenne et al., 1999). By 11.5 dpc, the reduction of *Math3* expression in r4 had become more evident (Fig. 2G and data not shown). While *Math3* expression was reduced throughout the ventral r4 domain in 2/7 mutants (Fig. 2G), in 3/7 mutants upregulation was restricted to the posterior half of r4, and no *Math3* up-regulation at all could be detected in ventral r4 in 2/7 *Hoxb2* mutants (data not shown). The dorsal and medial columns had also receded caudally, as seen in *Hoxb1* mutants at that stage. The observation that neurogenesis defects in *Hoxb2* mutants were generally milder than in *Hoxb1* mutants and more prominent at later stages raised the possibility that only later-born neurons were affected (see below).

In *Hoxa1* mutants, the r4 domain is compressed to a very narrow stripe of cells (del Toro et al., 2001) and r5 is reduced and incorporated in r6 (Dollé et al., 1993; Mark et al., 1993)

to form a territory termed here rx (Fig. 2D,H,L). Accordingly, the r4-specific upregulation of *Math3* and *Phox2b* expression is lost in *Hoxa1* mutants (Fig. 2D,H,L; white arrowheads), and the medial and dorsal columns were shortened along the AP axis, so that they now overlapped by only one rhombomere rather than three rhombomeres width, as normally seen (compare Fig. 2A with 2D and 2E with 2H). Thus, the initial segmental patterning defects of *Hoxa1* mutants are paralleled by corresponding defects in subsequent neurogenesis patterns.

In summary, loss of *Hoxb1*, *Hoxb2* or *Hoxa1* impaired hindbrain neurogenesis in areas where their regulatory interactions have previously been shown to regulate segmental patterning. The region most severely affected was ventral r4, where motoneurons are formed, consistent with the observation that facial nerve development is disrupted in these mutants.

Patterns of *GATA2* expression suggest defects in the formation of r4 efferent neurons

Specification of r4 efferent neurons depends upon the presence of the zinc-finger transcription factor *Gata3*. In turn, *Gata3* expression in r4 is controlled by *Gata2*, a transcription factor of the same family that is expressed in ventral r4 between 8.5 and 10.5 dpc. The ventral-most expression domain of *Gata2* in r4 corresponds to the efferent motoneurons in this territory (Pata et al., 1999; Varela-Echavarria et al., 1996). Expression of *Gata2* in ventral r4 depends upon *Hoxb1* (Nardelli et al., 1999; Pata et al., 1999) and accordingly, was completely lost in *Hoxb1* mutants (Fig. 2N) (Pata et al., 1999) and reduced in *Hoxb2* mutants (Fig. 2O). The ventral-most domain of *Gata2* expression was strongly reduced, but not abolished in *Hoxa1* mutants (Fig. 2P). By contrast, the dorsal domain of *Gata2* expression, which corresponds to V2 interneurons (Ericson et al., 1997; Zhou et al., 2000), was not affected in any of the mutants. (Fig. 2N-P). These phenotypes are consistent with the idea that in *Hoxb1* mutants there is a more complete transformation of an r4 to r2 identity than in *Hoxb2* mutants, and that in *Hoxa1* mutants the r4 territory is reduced, but not lost.

Defects in hindbrain motoneuron migration in Hoxa1, Hoxb1 and Hoxb2 mutants between 10.5 and 12.5 dpc

To begin to understand how the observed changes in neurogenesis patterns impacted on subsequent development of hindbrain neurons, we decided to follow the migration patterns of postmitotic cranial motoneurons in our Hox mutants. All postmitotic motoneurons in the hindbrain and the spinal cord are characterised by the early expression of the LIM homeodomain protein Isl1 (Ericson et al., 1992; Karlsson et al., 1990; Tsuchida et al., 1994). In wild-type hindbrains motoneuron differentiation was initiated at 10.5 dpc in all rhombomeres, with elevated levels in r2 and r4 (Fig. 3A). Interestingly, at this stage the difference in *Isl1* expression levels between r4 and r2 appeared smaller than the difference observed in *Phox2b* and *Math3* expression levels between the two rhombomeres (compare Fig. 2A,I with Fig. 3A). This suggests that at this stage not all committed Math3$^+$ r4 motoneurons have fully differentiated into postmitotic Isl1$^+$ motoneurons. At 10.75 dpc (Fig. 3B), prospective trigeminal motoneurons began to migrate dorsally and half a day later they
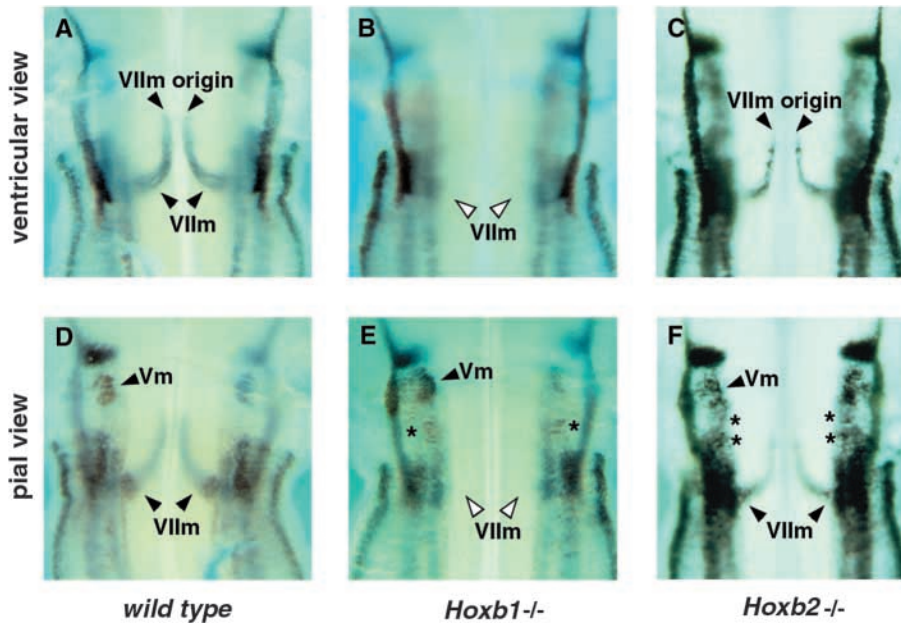
Fig. 5. Ectopic nuclei retained expression of *Phox2b* in wild-type, *Hoxb1* and *Hoxb2* mutant hindbrains at 12.5 dpc. Ventricular (A-C) and pial (D-F) views of flat-mounted wild-type (A,D), *Hoxb1* (B,E) and *Hoxb2* (C,F) mutant hindbrains at 12.5 dpc. At this stage, *Phox2b* was expressed in both Vm and VIIm in the wild type and the mutants (D-F, black arrowheads). Ectopic nuclei retained expression of *Phox2b* in both *Hoxb1* (E, asterisks) and *Hoxb2* (F, asterisks) mutants. Note the lack of *Phox2b* expression in the migratory path of the facial motoneurons.

started coalescing to form the trigeminal motor nucleus (Fig. 3C) next to the exit point of the trigeminal nerve. Migration of the trigeminal motoneurons appeared to be complete by 11.5 dpc (Fig. 3D). In contrast to the trigeminal motoneurons, facial motoneurons began their caudal migration at 11.25 dpc (Fig. 3C). Subsequently, they followed a curved path around the prospective abducens motor nucleus into a medial position of the pial side of the r6 territory where they form the facial motor nucleus (Fig. 3D,E,I,M) (Auclair et al., 1996; Studer et al., 1996). We initiated the analysis of motoneuron migration in our Hox mutants at 11.25 dpc, a time when most trigeminal motoneurons have completed their dorsal migration, but r4 motoneurons are migrating caudally.

In *Hoxa1* mutants, we detected two small populations derived from the level of r4. One group migrated dorsally, in a pattern similar to that of the r2 motoneurons, and a second population remained close to the floor plate and appeared to migrate caudally (Fig. 3F). As the r5 territory is largely eliminated in these mutants, the latter population corresponded to r4- rather than r5-derived motoneurons (Chisaka et al., 1992; Mark et al., 1993). In *Hoxb1* mutants the r4 motoneurons migrated dorsally (Fig. 3G) assuming a trigeminal-like migratory behaviour. Interestingly, in the *Hoxb2* mutants, a proportion of r4 motoneurons seemed to follow the normal path of migration, while a second population followed a trigeminal-like pattern of migration (Fig. 3H), consistent with a partial r4 to r2 transformation.

The dorsally migrating motoneurons detected in all three mutants appear to form an ectopic motor nucleus, first identified in the *Hoxb1* mutants at a position anterior to the normal facial motor nucleus (Goddard et al., 1996; Studer et al., 1996). Accordingly, we extended the analysis of *Isl1* expression to later stages of hindbrain development. At 12.5 dpc, r4 motoneurons migrated from the ventricular aspect of ventral r4 and r5 to a medial position at the pial side of r6 (Fig. 3I,M) to form the facial motor nucleus (Altman and Bayer, 1982; Auclair et al., 1996). During their migration, they assumed a caudal course with a dorsolateral deviation

travelling in a loop around the sixth motor nucleus (Altman and Bayer, 1982; Auclair et al., 1996; Goddard et al., 1996; Studer et al., 1996). Accordingly, at this stage the abducens motor nucleus is not distinguishable in whole mounts as the facial motoneurons migrate over the same area.

At 12.5 dpc only a few caudally migrating r4 motoneurons, which had not reached the pial side of the hindbrain, were seen in *Hoxa1* mutants (compare Fig. 3J,N). Their course of migration was shortened due to the overall reduction of the caudal hindbrain length in these mutants (Mark et al., 1993). Small ectopic nuclei were also detected (Fig. 3N), suggesting that the laterally migrating motoneurons detected earlier had indeed assumed a trigeminal-like behaviour. They were positioned between the trigeminal nuclei and the area where facial motor nuclei normally resided (compare Fig. 3N with 3M). Their AP position was variable between different embryos and even between the left and right side of the same embryo (Fig. 3N). Despite the low number of the r4-derived motoneurons the abducens motor nucleus could not be detected (see also below), and this is in accordance with the observation that the r5 territory is nearly eliminated in these mutants (Dollé et al., 1993; Mark et al., 1993). No r4-derived motoneurons were observed in the ventricular area of *Hoxb1* mutants at 12.5 dpc, while r5-derived abducens motoneurons were now clearly visible (Fig. 3K,O). In these mutants, no facial motor nuclei were formed in the appropriate site (compare Fig. 3O with 3M). Instead, ectopic nuclei were observed in more anterior positions (Fig. 3O) (see Studer et al., 1996)). In *Hoxb1* mutants, ectopic nuclei invariably resided in an area about half way between the normal positions of the trigeminal and facial motor nuclei. They were larger than the *Hoxa1* ectopic nuclei but remained smaller than the trigeminal nuclei. In all but one of six cases examined, *Hoxb2* mutants formed correctly positioned facial motor nuclei (Fig. 3L,P and data not shown). However, the number of caudally migrating pool of facial motoneurons was variable and reduced in most cases (compare Fig. 3I,O with 3L,P). *Hoxb2* mutants showed variability with respect to the number and position of ectopic nuclei, which
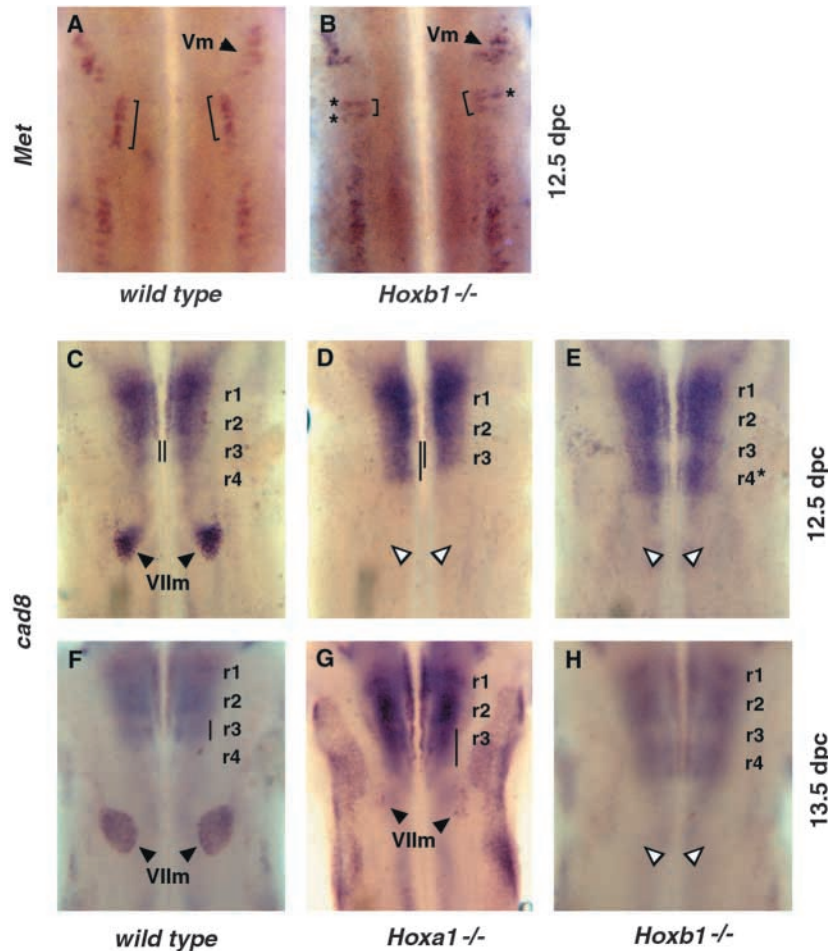
Fig. 6. Persistent identity changes detected by altered expression of *Met* and *Cad8* in wild-type and mutant hindbrains. (A,B) Ventricular views of *Met* expression in wild-type (A) and *Hoxb1* (B) mutant flat-mounted hindbrains at 12.5 dpc. In the wild type, *Met* was expressed in the Vm trigeminal (A, arrowhead) and the superior salivary nucleus (A, bracket) only. In the *Hoxb1* mutants, there were ectopic nuclei expressing *Met* (B, asterisks). Note also the reduction of the superior salivary nucleus (B, brackets). (C-H) Pial views of Cad8 expression in wild type (C,F), *Hoxa1* (D,G) and *Hoxb1* (E,H) flat-mounted hindbrains at 12.5 dpc (C-E) and 13.5 dpc (F-H). In wild-type hindbrains, *Cad8* was expressed in the ventral half or r1, r2 and r3 (C,F) and in the VIIm (C,F, black arrowheads) at 12.5 and 13.5 dpc. At 12.5 dpc, there was a longitudinal stripe of *Cad8* expression adjacent to the floor plate of r1 and r2, which extended into r3 at 13.5 dpc (C,F vertical bars). In *Hoxa1* mutants, r3 expanded caudally in both 12.5 (D) and 13.5 (G) dpc (compare the vertical bar in D with C, and in G with F), and VIIm were barely detectable only at 13.5 dpc. In *Hoxb1* mutants, Cad8 was ectopically expressed in r4 in a pattern reminiscent of r2 (E, r4*) at both 12.5 (E) and 13.5 (H) dpc, but no VIIm could be detected in either stage (E,H, white arrowheads).

always resided between the trigeminal and facial nuclei. Importantly, there was an inverse correlation between the size of the normally positioned facial nuclei and the number/size of the ectopic nuclei (compare Fig. 3Q-T).

These results revealed a striking commonality of phenotypes among the three mutants examined. Motoneurons derived from the r4 of *Hoxb1* mutants appear to be misspecified and follow an r2 migratory pathway. By contrast, in *Hoxa1* and *Hoxb2* mutants r4 derived motoneurons display both trigeminal and facial migratory behaviours, suggesting there are mixed identities or a partial transformation. In all mutants, ectopic motoneurons migrated dorsally to the r4/r5-derived vm neurons, suggesting that they retained a bm neuron identity.

Hoxb1 requires Hoxb2 for complete specification of facial motoneuron identity

Facial motoneurons depend upon *Hoxb1* for correct specification. The presence of two migratory populations in *Hoxb2* mutants could be explained by two alternative possibilities. Either cumulative levels of *Hoxb1* and *Hoxb2* activity may be necessary in all cells for robust specification of fbm fate or, alternatively, *Hoxb2* may be necessary to specify a subset of facial motoneurons. In the first case, molecular markers of fbms would be similarly affected, whereas in the second case they would be differentially affected. To address this issue we used riboprobes for *Lhx4* and *Er81*, which are expressed during the late phase of facial motor nucleus

development. *Lhx4*, a LIM homeobox-containing transcription factor, has been implicated in assigning motoneuron subtype identities in the spinal cord (Sharma et al., 1998), whereas *Er81*, an Ets transcription factor, has been implicated in the formation of functional circuits between proprioceptive afferent neurons and motoneurons of the spinal cord (Arber et al., 2000).

At 14.5 dpc *Lhx4* was expressed in the trigeminal as well as the superior salivary motor nucleus and the forming facial motor nucleus (Fig. 4A). By contrast, *Er81* was expressed in the facial motor nucleus (Fig. 4C). We examined the expression of these transcription factors in *Hoxb2* mutant embryos in order to determine whether any of the populations they mark was specifically affected. Expression of both markers was reduced in the forming facial motor nucleus, suggesting that the reduction of the nucleus was not due to preferential loss of a specific subpopulation (Fig. 4B,D). *Lhx4* (Fig. 4B), but not *Er81* (Fig. 4D), was expressed in the ectopic motoneurons. The position of the Lhx4+ ectopic cells is similar to that of the Isl1+ ectopic cells, and this is consistent with the idea that these motoneurons have acquired a trigeminal identity. Therefore, *Hoxb2* does not specify a subset of fbms, but rather the total dose of *Hoxb1* and *Hoxb2* activity is important for full fbm specification.

Persistent identity changes in *Hoxa1* and *Hoxb1* mutants

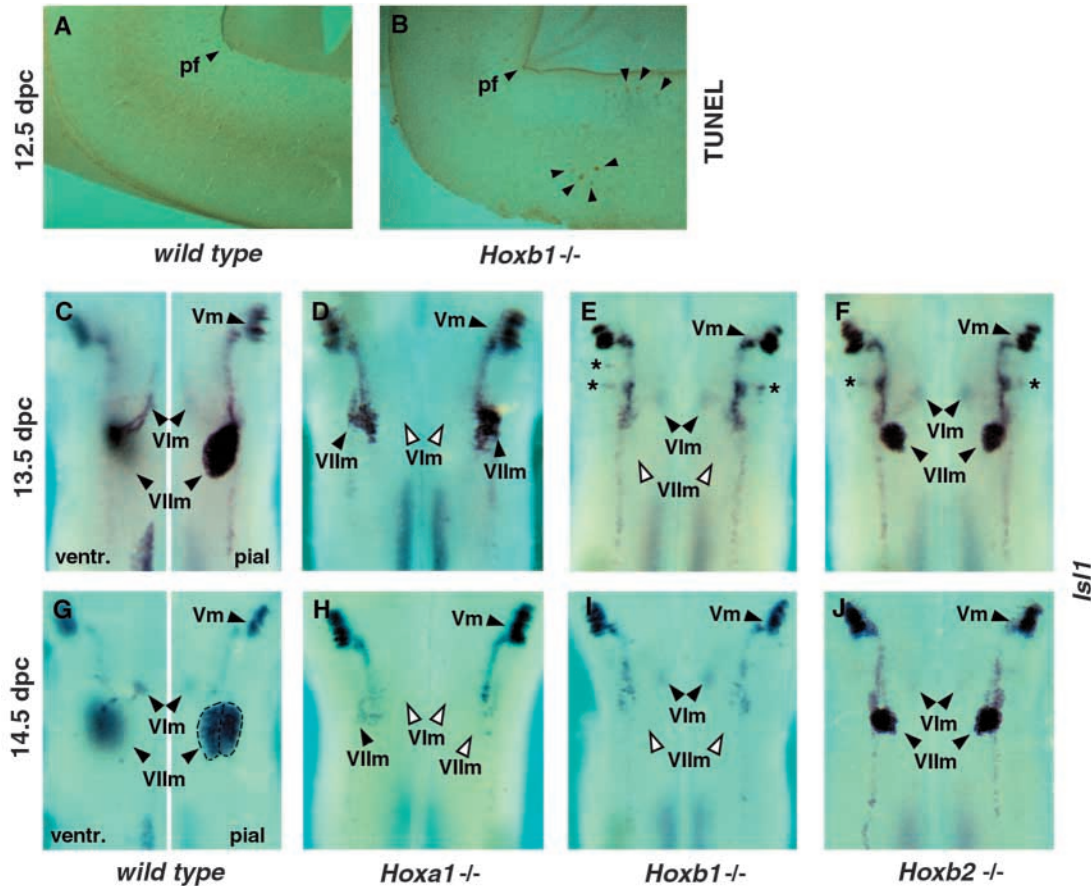The molecular analysis of the early hindbrain patterning in

Fig. 7. Ectopic cell death correlates with the loss of ectopic nuclei by 14.5 dpc. (A,B) TUNEL staining in parasagittal sections of wild-type and *Hoxb1* mutant hindbrains. There was ectopic cell death in the mutant (B, black arrowheads) caudally to the pontine flexure (pf). (C-F) Pial (ventricular where noted) views of *Isl1* expression in wild-type (C), *Hoxa1* (D), *Hoxb1* (E) and *Hoxb2* (F) mutant flat-mounted hindbrains at 13.5 dpc. By this stage, VIIm had normally completed their migration (C, black arrowheads). In *Hoxa1* mutants, a small VIIm of variable size could be detected (D, black arrowheads), whereas the VIm was absent (D, white arrowheads). In *Hoxb1* mutants, ectopic nuclei could still be detected (E, asterisks). The VIIm (E, white arrowheads), but not the VIm (E, black arrowheads) were absent. In *Hoxb2* mutants, ectopic nuclei could also be detected (F, asterisks), whereas the VIm and a small VIIm were present (F, black arrowheads). (G-J) Pial (ventricular where noted) views of *Isl1* expression in wild type (G), *Hoxa1* (H), *Hoxb1* (I) and *Hoxb2* (J) mutant flat-mounted hindbrains at 14.5 dpc. In the wild type, *Isl1* expression persisted in the Vm, VIm and VIIm (G, black arrowheads). In *Hoxa1* mutants, the VIIm were either missing or rudimentary (H, white and black arrowheads), whereas the VIm was absent (H, white arrowheads). In *Hoxb1* and *Hoxb2* mutants, no ectopic nuclei could be detected. The VIIm was absent in *Hoxb1* mutants (I, white arrowheads) and reduced in *Hoxb2* mutants (J, black arrowheads), while the VIm was present in both mutants (I,J, black arrowheads).

the Hox mutants and their aberrant migratory phenotypes suggested an r4 to r2 identity switch (Studer et al., 1996). The aberrant migratory phenotype could be due to either cell-autonomous changes in the fbm identity, changes in the resident territory or both. Furthermore, it was unclear whether molecular changes detected in early hindbrain development persisted during neuronal patterning as well. We addressed these issues by molecular analysis at late stages in hindbrain development.

All bm and vm neurons in the hindbrain express *Phox2b* at 10.5 dpc (Pattyn et al., 1997b) (Fig. 3A). At 12.5 dpc *Phox2b* expression persists only in trigeminal and facial motoneurons (Fig. 5A,D). To determine whether ectopic nuclei retained features of either of these neurons, we examined the expression of *Phox2b* in *Hoxb1* (Fig. 5B,E) and *Hoxb2* (Fig. 5C,F) mutants. In both mutants *Phox2b* expression was retained in the ectopic motoneurons, suggesting that they had either a

trigeminal or facial-like identity. The presence of laterally located neurons expressing *Phox2b* at that stage precluded an unambiguous analysis in the *Hoxa1* mutants in which the length of the hindbrain, and thus that of the Phox2b+ neuron-free area, is reduced.

The retention of *Phox2b* expression in the ectopic nuclei could either indicate that they had retained partial facial identity or that they had adopted a new, trigeminal-like identity. In order to distinguish between these two possibilities, we analysed the expression of the hepatocyte growth factor (HGF) tyrosine kinase receptor Met (Maina and Klein, 1999). *Met* is expressed in trigeminal motoneurons and in the vm neurons of the salivatory nucleus (Fig. 6A) (Caton et al., 2000), but not in the r4 derived facial motor nucleus.

We analysed *Met* expression in *Hoxb1* mutants, because large ectopic nuclei are readily detected. We could detect ectopic *Met* expression in the area of migration of the ectopic
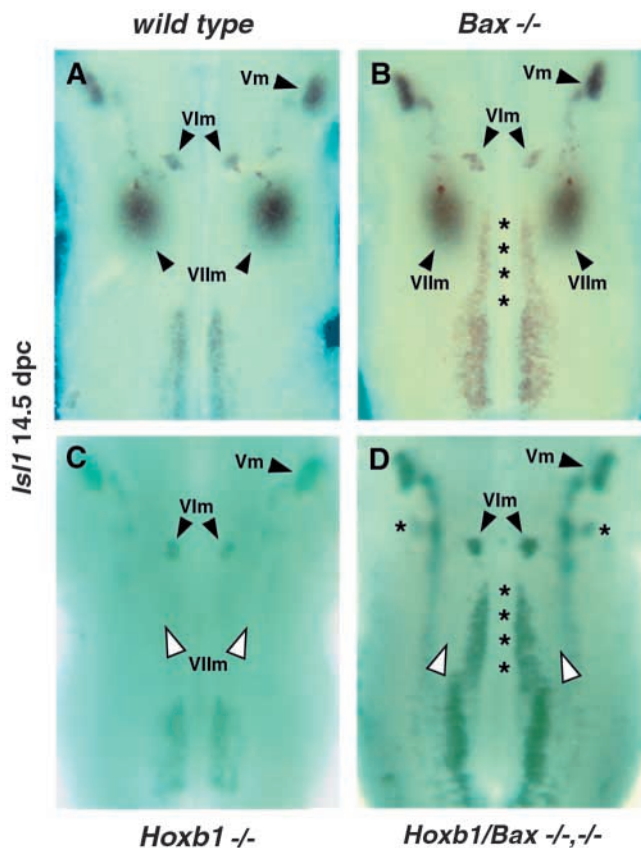
Fig. 8. *Bax* inactivation could prolong the survival of ectopic nuclei. (A-D) Ventricular views of *Isl1* expression in wild-type (A), *Bax* (B), *Hoxb1* (C) and *Hoxb1/Bax* (D) flat-mounted mutant hindbrains at 14.5 dpc. In both wild-type and Bax mutants, the Vm, VIm and VIIm could be detected (A,B, black arrows). In *Hoxb1* mutants only the Vm and VIm could be detected (C, black arrowheads), whereas the VIIm was absent (C, white arrowheads). In *Hoxb1/Bax* mutants, the Vm and the VIm could be detected (D, black arrowheads), but not the VIIm (D, white arrowheads). In *Bax* and *Hoxb1/Bax* mutants there was an additional ventral column of ectopic Isl1-positive cells in the caudal hindbrain (B,D, asterisks).

motoneurons (compare Fig. 4O with Fig. 6B). Furthermore, the salivatory nucleus was reduced in these mutants (Fig. 6B). This is in accordance with earlier patterning defects observed in the r5 territory of these mutants (Studer et al., 1996).

To further examine the identity of the ectopic nuclei, we analysed expression of cadherin 8 (Cad8) (Korematsu and Redies, 1997), a member of the Ca^{2+} dependent surface adhesion molecules (Takeichi et al., 1997). The mouse *Cad8* is expressed in the facial, but not the trigeminal motor nucleus from 12.5 dpc into postnatal life (Korematsu and Redies, 1997). In wild-type hindbrains, *Cad8* is expressed in the r1, r2 and r3 territories in a broad ventral stripe and in the facial motor nucleus (Fig. 6C,F). At 12.5 dpc there is also a narrow, longitudinal stripe of expression next to the floorplate in r1 and r2 but not r3 (Fig. 6C). At 13.5 dpc, this stripe extended into the r3 territory (Fig. 6F).

Only a rudimentary facial motor nucleus could be detected in some *Hoxa1* mutants at 13.5 dpc and none could be detected in *Hoxb1* mutants at either 12.5 or 13.5 dpc. In contrast to the

Isl1 expression analysis, the *Cad8* probe did not label the r4-derived ectopic nuclei in either *Hoxa1* or *Hoxb1* mutants (Fig. 6D,G,E,H), supporting the idea that they lacked facial identity. The early caudal expansion of the r3 territory in *Hoxa1* mutants was also detected at these stages by means of *Cad8* expression (compare Fig. 6D with 6C, and 6G with 6F). The extent of the expansion was variable and not always the same on the left and right sides of the same embryo (compare Fig. 6D with 6C, and 6G with 6F), in accordance with similar observations at earlier stages. In *Hoxb1* mutants, *Cad8* expression was normal in r1-r3, but upregulated in r4 at 12.5 dpc, resembling the r1/r2 expression pattern (compare Fig. 6E with 6C).

Taken together, these observations suggest that patterning defects in the r3-r5 territories persist well into the phase of neurogenesis and neuronal differentiation. As a direct consequence, r4 and r4-derived motoneurons, which migrate ectopically, have adopted an r2 and trigeminal-like identity, respectively.

Ectopic motor nuclei are lost by 14.5 dpc through cell death

Given that in these mutants there is no corresponding transformation of their innervation targets, namely the second arch (Goddard et al., 1996; Studer et al., 1996), we wondered whether this mismatch would eventually lead to apoptotic cell death of these neurons because of lack of proper trophic support. To analyse whether the ectopic motoneurons were cleared by cell death, we performed TUNEL assays at 12.5 dpc on hindbrain sections of wild-type and *Hoxb1* mutant embryos, which displayed the most prominent r4-derived ectopic nuclei. Ectopic cell death was detected in *Hoxb1* mutants at positions corresponding to the AP level of ectopic motoneurons (compare Fig. 7A with 7B). We then analysed the fate of motoneurons at later stages in wild-type and mutant embryos by *Isl1* expression analysis.

In the wild-type the migration of facial motoneurons from the ventricular to the pial side was completed between 13.5 and 14.5 dpc (Fig. 7C,G). At 14.5 dpc, the facial motor nucleus has started to segregate into two lobes that become more prominent at later stages (Fig. 7G and data not shown).

In *Hoxa1* mutants no ectopic nuclei were observed at these stages; however, a rudimentary facial motor nucleus of variable size was retained in most, but not all cases (Fig. 7D,H). This observation could explain disagreements among earlier reports on the presence or lack of the facial nucleus in these mutants (Carpenter et al., 1993; Mark et al., 1993). In both *Hoxb1* and *Hoxb2* mutants, the ectopic nuclei were still detectable at 13.5 dpc, but not at 14.5 dpc (compare Fig. 7E,F with 7I,J, respectively). In agreement with the *Isl1* expression at earlier stages, the facial motor nucleus was absent in *Hoxb1* mutants, but present, albeit variably reduced, in all *Hoxb2* mutants examined. The abducens motor nucleus (VIm) was present in *Hoxb1* and *Hoxb2* mutants (compare Fig. 7E,I and 7F,J with the wild type in 7C,G). This nucleus could not be detected in *Hoxa1* mutants, and was consistent with the loss of r5 in these mutants (7D,H).

Taken together, these results suggest that Isl1^{+} ectopic nuclei are not retained to any significant extent beyond 13.5 dpc in any of the mutants examined. This is in agreement with the idea that due to their switch in identity, they are unable to respond to trophic support from their innervation targets in the

second arch and die by apoptosis. We therefore decided to investigate whether a block in the apoptotic pathway could extend the presence of these nuclei in the hindbrain.

The presence of the ectopic nuclei can be extended by a block in the apoptotic pathway

Bax, a member of the Bcl2 family, is necessary for the programmed cell death of many neurons. Facial motoneurons in *Bax* knockout mice show increased numbers at birth and sympathetic neurons from these mice can survive for long periods in culture without trophic support. Thus, this mutation blocks the apoptotic pathway induced from lack of trophic support (Deckwerth et al., 1998). To test whether it was possible to extend the survival of the ectopic motoneurons we crossed this mutation (Deckwerth et al., 1998) into the *Hoxb1* mutant genetic background.

Loss of Bax function extended the survival of ectopic motor nuclei in the hindbrains of *Hoxb1* mutants to 14.5 dpc (compare Fig. 8C with 8D). Interestingly, we also found excess numbers of motoneurons in the ventral domain of the caudal hindbrain of *Bax*-null mutants between 12.5 and 14.5 dpc (compare Fig. 8A with 8C and data not shown). Their ventral position indicated that they were specified as sm neurons and therefore did not contribute to the IX and X/XI motor nerves, which are mixed branchiomotor and visceromotor nerves.

These findings underline the importance of programmed cell death in the developing hindbrain. This process is normally used to eliminate unnecessary somatomotoneurons in the caudal hindbrain but can be recruited to eliminate incorrectly specified branchio-motoneurons in Hox mutants.

Discussion

Regulatory analyses in transgenic mice have revealed that direct auto- and cross-regulatory relationships between the *Hoxa1*, *Hoxb1* and *Hoxb2* genes defined a transcriptional hierarchy important for patterning early segmental processes in the developing hindbrain. In this cascade, *Hoxa1* triggers early expression of *Hoxb1* in r4 and *Hoxb1* maintains its own expression, which in turn, is required for upregulation of *Hoxb2* in r4. These events are required to establish and maintain the regional character of r4 (Di Rocco et al., 1997; Maconochie et al., 1997; Studer et al., 1998). This pathway may also be important in regulating later events in neural patterning. In this study, we examined neurogenesis and motoneuron migration defects in mouse mutants of these three genes and found that there are strong similarities in the phenotypes that are correlated with their regulatory relationship. Common defects were largely concentrated to the r4 territory and included abnormalities in the patterns of neurogenesis and neuronal differentiation. The r4-derived motoneurons underwent a transformation towards an r2 identity. This altered identity was maintained until late stages of hindbrain development, at which point misspecified motoneurons from the transformed segment migrated to an ectopic position and were cleared by cell death. Using *Bax* mutants (Deckwerth et al., 1998), we found that survival of the misspecified motoneurons could be prolonged by a genetic block in the apoptotic pathway. These results suggest that the regulatory hierarchy between *Hoxa1*, *Hoxb1* and *Hoxb2* is not only required to control early hindbrain patterning, but also

shapes patterns of neurogenesis and the behaviour of differentiating neurons.

Common roles for *Hoxb1* and *Hoxb2*

Rhombomeric and neuronal patterning defects were milder in the *Hoxb2* mutants, compared with *Hoxb1*$^{-/-}$ embryos. Furthermore, there were no r4-derived phenotypes in *Hoxb2* mutants that were not detected in *Hoxb1* mutants. This is consistent with *Hoxb2* being a direct transcriptional target of *Hoxb1* (Maconochie et al., 1997) and raises a number of possibilities concerning the precise regulatory and functional relationships between *Hoxb1* and *Hoxb2*. *Hoxb2* may act synergistically with *Hoxb1* by regulating either distinct target genes or a set of common target genes in r4, so that their combined activities are required for the normal differentiation of r4-derived motoneurons. An alternative mechanism whereby *Hoxb2* may synergise with *Hoxb1* would be through a role for *Hoxb2* in maintaining *Hoxb1* expression.

To begin to distinguish between these possibilities, we monitored the r4 status in the *Hoxb2* mutants by assaying *Hoxb1* expression and the expression of a the r4-specific transgene (*HL5/lacz*), which is known to be a direct target of *Hoxb1* (Marshall et al., 1994; Popperl et al., 1995). Endogenous *Hoxb1* expression (Fig. 9A-F) and staining for the *HL5/lacZ* transgene (Fig. 9G-O) are initiated in the r4 of *Hoxb2* mutants (Fig. 3B) but are not maintained at appropriate levels in later stages. This demonstrates a direct or indirect requirement for Hoxb2 in maintaining Hoxb1 expression in r4 (Fig. 9P). The observation that *Hoxb1* expression is initiated normally in *Hoxb2* mutants, but is not maintained properly could explain the mixed behaviour of facial motoneurons. Those r4 motoneuron progenitors that retain sufficient *Hoxb1* activity adopt a normal fbm identity, while the rest adopt trigeminal motoneuron characteristics. We favour the idea that the effect of *Hoxb2* on *Hoxb1* expression is most probably indirect, through regulation of general aspects of r4 identity. Hoxb2 cannot bind the *Hoxb1* r4 regulatory element in vitro, although it is possible that Hoxb2 may bind to an as yet unidentified *Hoxb1* r4 regulatory element. In vivo, ectopic expression of *Hoxb2* does not ectopically activate *Hoxb1*, whereas *Hoxb1* does transactivate *Hoxb2* (Maconochie et al., 1997).

Neuronal differentiation defects and persistent identity changes in the hindbrain of Hox mutants

Retrograde tracing of axonal projections in the *Hoxa1*, *Hoxb1* and *Hoxb2* mutants suggested defects in fbm migration. *Hoxb1* mutants generate fbms that extend axons from r4 to the appropriate exit point but fail to generate both caudally migrating fbms (Studer et al., 1996). In *Hoxb2*, there is a reduction in the numbers of caudally migrating r4 motoneurons (Davenne et al., 1999) and consistent with reduction of r4 in the *Hoxa1* mutants, there is a greatly diminished r4 motoneuron population that migrates caudally. There were two populations in the expanded r3 territory of these mutants. One population projected axons in an r3-like pattern to the first arch and a second variable population projected axons in an even rhombomere-like pattern to the second arch (Gavalas et al., 1998). These populations were often, but not always, intermixed. The detection of an ectopic nucleus in *Hoxb1* (Studer et al., 1996) and *Hoxa1* (Mark et al., 1993) mutants at
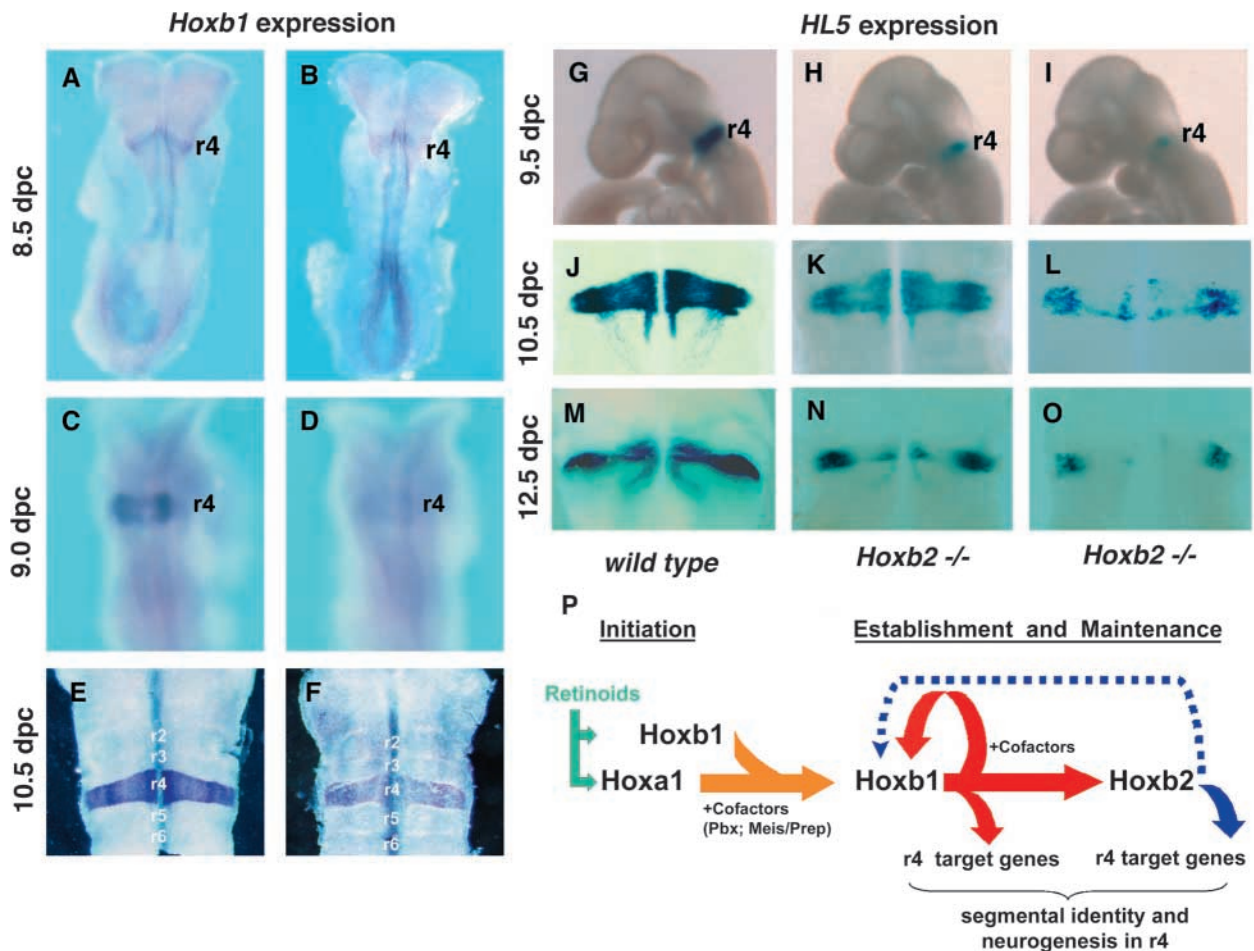
Fig. 9. Regulatory interactions among the *Hoxa1*, *Hoxb1* and *Hoxb2* genes. (A-F) Dorsal views of *Hoxb1* expression in wild-type (A,C,E) and *Hoxb2* mutants (B,D,F) at 8.5 (A,B), 9.5 (C,D) and 10.5 (E,F) dpc. Expression of *Hoxb1* is initiated (A,B) but not properly maintained (C-F) in the *Hoxb2* mutants. (G-O) Expression of a *Hoxb1 lacZ* transgene (HL5) in wild type (G,J,M), and *Hoxb2* mutants (H,I,K,L,N,O) at 9.5 (G-I), 10.5 (J-L) and 12.5 (M-O) dpc in side views of whole mount embryos (G-I) and dorsal views of flat mounted hindbrains (J-O). Expression of the HL5 transgene is variable in the *Hoxb2* mutants (H,I,K,L,N,O). Note also that *lacZ* expression is maintained in migrating fbms in the wild type (J,M) and in a proportion of migrating fbms in some (K,N), but not all (L,O), *Hoxb2* embryos. (P) The regulatory interactions among the three genes. *Hoxb1* and *Hoxa1* expression is initiated through the action of retinoids (green line). In turn, *Hoxa1*, *Hoxb1* and co-factors Pbx/Meis establish the expression of the latter in the r4 territory (orange line), which is subsequently maintained through *Hoxb1* autoregulation (red arrow). Hoxb2 directly or indirectly (broken blue arrow) feedback upon Hoxb1 to maintain its expression in r4. An indirect contribution could be achieved through a requirement for Hoxb2 in the maintenance of the r4 territory. Hoxb1 and Hoxb2 synergise to regulate target genes pertaining to segmental identity and neurogenesis in r4.

later stages suggested an abnormal migratory behaviour of presumptive facial motoneurons rather than a simple temporal delay of their migration.

To understand the fate of these motoneurons, we followed their migratory behaviour by monitoring *Isl1* expression (Ericson et al., 1997; Osumi et al., 1997) at different stages in all three mutants examined. The migratory pattern and final location of this population resembled that of r2-derived motoneurons. The size of the ectopic nucleus and the loss or reduction of the correctly migrating fbm population varied among the mutants in a manner that resembled an allelic series. The *Hoxb1* and *Hoxb2* phenotypes could be explained by a strong or weak transformation, respectively, of r4 into r2. The *Hoxa1* phenotype was consistent with a partial transformation of r4 into r2 and concurrent loss of cell sorting at the r4/r3 interface. Presumptive r4 cells that failed to activate *Hoxb1*

were specified as r2-like cells and intermingled with r3 cells (Helmbacher et al., 1998). This is consistent with the presence of cells with an r2 identity in this region (del Toro et al., 2001; Helmbacher et al., 1998). Therefore, the trigeminal-like ectopic nuclei observed in the *Hoxa1* mutants (Mark et al., 1993) (this study) appeared to be the progeny of misspecified presumptive r4 cells. Intermixing at the r3/r4 interface may lead to patterning defects in a subset of r3. This could explain the presence of ectopic motoneurons projecting via the trigeminal motor root in the brainstem of *Hoxa1*−/− pups (del Toro et al., 2001).

Cells of presumptive r4 able to activate *Hoxb1* give rise to the correctly migrating fbms in *Hoxa1* mutants. Consistent with this, we found a severely reduced facial motor nucleus in some but not all *Hoxa1* mutant embryos. Therefore, the *Hoxa1* r4 motoneuron phenotype is fundamentally similar to that of
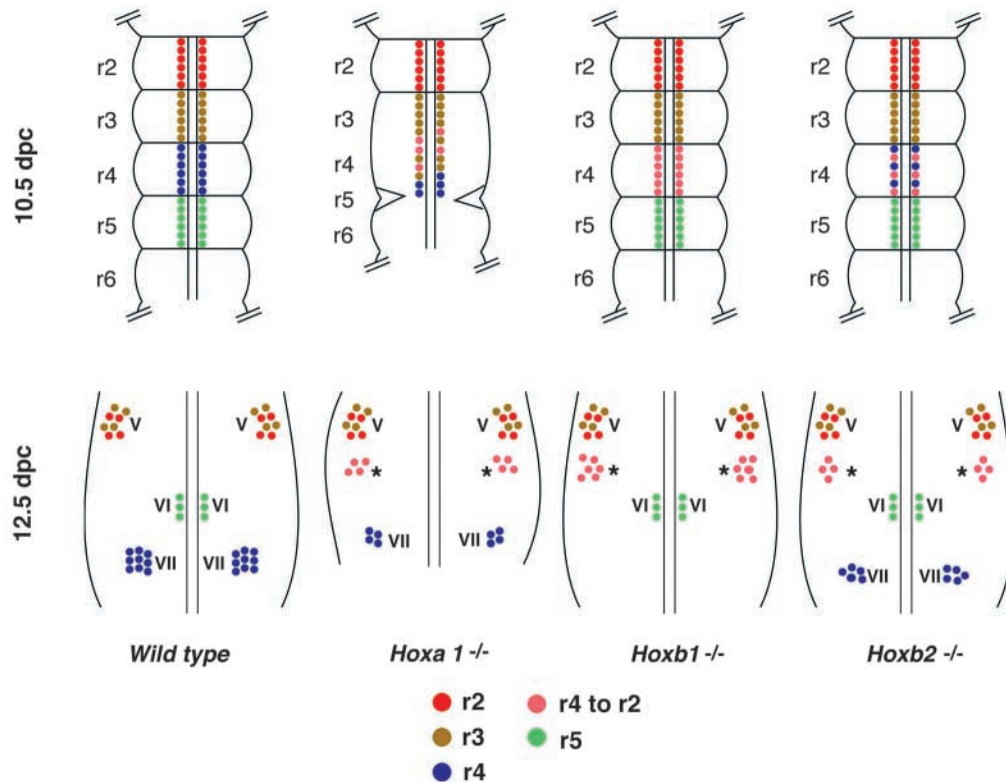
Fig. 10. Fate of hindbrain motoneuron precursors in *Hoxa1*, *Hoxb1* and *Hoxb2* mutants. A schematised view of early (10.5 dpc, top row) axial identity and subsequent (12.5, bottom row) fate of hindbrain motoneuron precursors in wild type, *Hoxa1*, *Hoxb1* and *Hoxb2* embryos. At 10.5 dpc, r2-r5 have normally acquired specific identities and the corresponding motoneuron precursors do not mix. The segregated precursor populations will give rise to the trigeminal (V, derived from r2 and r3), abducens (VI, derived from r5) and facial (VII derived from r4) motor nuclei. In *Hoxa1* mutants, the r3/r4 boundary does not form and most of the r5 territory is lost, resulting in a single rhombomere often referred to as rx. Some of the presumptive r4 precursors have acquired a partial r2 identity and mix with r3 precursors. The latter form an ectopic transient motor nucleus (asterisk) and sometimes a small facial (VII) motor nucleus. As a result of r5 strong reduction, the abducens motor nucleus (VIm) is lost. In *Hoxb1* mutants, presumptive r4 precursors are misspecified to an r2-like identity. As a result, they all migrate in a trigeminal like manner and form an ectopic, but transient, motor nucleus (asterisk), which is cleared by cell death. In *Hoxb2* mutants, some r4 precursors have acquired an r2-like identity, and as a result a transient ectopic motor nucleus (asterisk) is formed in addition to the facial motor nucleus.

Hoxb1 mutants, with the exception of a subset of r4 cells that are properly specified because of later activation of *Hoxb1* expression. These observations are consistent with a strong, but not absolute, requirement of *Hoxa1* for the activation of *Hoxb1* (Studer et al., 1996).

Transformation and changes in neuronal differentiation

The migratory patterns of the presumptive fbms in all three mutants suggested a transformation of their territory of origin (r4) towards an r2 identity. To further investigate the extent of this transformation we analysed patterns of neurogenesis and neural differentiation in these mutants and the results are summarized in Fig. 10. The r4 territory in the *Hoxb1* mutants assumed aspects of an r2 identity from an early stage in development (Studer et al., 1996). This altered specification persisted in later stages, as expression of both *Phox2b* and *Math3* followed an r2-like pattern along the full DV extent of the rhombomere. Furthermore, using *Gata2* (Nardelli et al., 1999; Pata et al., 1999) as a marker for the whole complement of r4 efferent motoneurons, we found that expression was reduced in ventral r4 and resembled that in the r2 territory. The

presumptive r4 territory in the *Hoxb1* mutants may have retained some aspects of r4 identity since expression of *Epha2*, an early marker of this territory, is not lost (Studer et al., 1998) and expression of *Hoxb2* is downregulated but not abolished (Maconochie et al., 1997). Therefore, some neuronal progenitors may still be born following an r4-specific program, but loss of *Hoxb1* may result in their early removal by cell death as observed (Gaufo et al., 2000). The reduction of the superior salivary nucleus, that is primarily derived from r5 (Jacob and Guthrie, 2000; McKay et al., 1997), in *Hoxb1* mutants is consistent with early patterning defects found in the r5 territory of these mutants. Signals derived from r4 may be necessary for correct patterning of this and other rhombomeres (Maves et al., 2002; Studer et al., 1996; Walshe et al., 2002).

Defects in *Math3* and *Phox2b* expression in *Hoxb2* mutants were evident at 10.5 dpc, but more pronounced at 11.5 dpc, and *Gata2* expression is only partially affected in these mutants. Overall, neurogenesis defects were similar to those of *Hoxb1* mutants, but milder and more apparent at a later stage. The segmentation defects in *Hoxa1* mutants (Mark et al., 1993) result in a drastically reduced r4 territory, and therefore it was difficult to determine whether r4-specific patterns of

neurogenesis and differentiation were implemented. Nevertheless, consistent with the hypothesis that the r4 program is executed although in a reduced territory of *Hoxa1* mutants, we were able to document r4-specific patterns of *Gata2* expression.

Molecular analysis confirmed that the initial changes in segmental identity persisted at later stages in hindbrain development. Ectopic motor nuclei in both *Hoxb1* and *Hoxb2* mutants retained expression of *Phox2b*, suggesting that they were specified as branchiomotoneurons. Similarly, the loss of *Cad8* expression, which is characteristic of fbms at their final position (Garel et al., 2000; Korematsu and Redies, 1997), and ectopic expression of the tyrosine kinase receptor *Met* (Caton et al., 2000) were consistent with an r4 to r2 transformation. Ectopic expression of *Cad8* in the ventral r4 territory of the *Hoxb1* mutants in an r2-specific pattern suggested that both cell-autonomous (see above) as well as non cell-autonomous defects may contribute in the misspecification of the r4-derived motoneurons. The caudal extension of *Cad8* expression was smaller in *Hoxa1* mutants, in agreement with a partial transformation of r4 into r2.

The *Hoxb2* motoneuron phenotype represented a milder version of the *Hoxb1* phenotype, as only a subpopulation of the presumptive fbms assumed migration and gene expression patterns corresponding to a trigeminal-like identity. Expression of *Lhx4* and *Er81* support the idea that the ectopic motoneurons have undergone an r4 to r2 change in identity. Their expression was similarly affected in the correctly specified fbms, suggesting a general, late *Hoxb2* requirement for fbm specification. This implies that loss of *Hoxb2* might reduce the total level of *Hoxb* activity to threshold levels resulting in a variable phenotype due to the stochastic variation of gene expression. This situation would not affect specific fbm subpopulation(s).

The migratory behaviour of r4 motoneurons and molecular analysis strongly suggested an r4 to r2 transformation of this territory in the mutants examined. How can we account for the anterior change in r4 identity in these Hox mutants? *Hoxa2* is the most likely candidate for mediating this transformation. Its ectopic expression can induce the generation of trigeminal motoneurons (Bell et al., 1999) and it is still expressed in presumptive r4 in the absence of *Hoxb1* expression (Di Rocco et al., 2001b). Therefore, in the absence of *Hoxb1* and the subsequent lack of *Hoxb2* upregulation, *Hoxa2* is the only gene expressed at a high level in presumptive r4, leading to transformation.

Loss of ectopic misspecified motoneurons by programmed cell death

The ectopic motoneuron population in the *Hoxb1* mutants can be readily detected by the *Isl1* riboprobe as a robust population until 12.5 dpc. Its size was rapidly reduced thereafter and became undetectable by 14.5 dpc. The detection of ectopic cell death in the same region suggested that this population was eliminated by cell death. Accordingly, by genetically blocking the apoptosis pathway through crossing *Hoxb1* mutants into a *Bax*$^{-/-}$ background (Deckwerth et al., 1998) we extended the survival of this ectopic population. Programmed cell death is used extensively to sculpt the central nervous system (deLapeyriere and Henderson, 1997; Pettmann and Henderson, 1998). Its role in the hindbrain is underscored by the

persistence of ectopic motoneurons in the r6 to r7/r8 region in the *Bax* mutant background. The higher than normal levels of cell death detected during early r4 development in the *Hoxb1* mutants (Gaufo et al., 2000) could reflect normal patterns of cell death consistent with the transformation of that segment into an r2 identity.

The re-specified motoneurons in r4 of the *Hoxb1* mutants still projected their axons exclusively to the second arch (A.G. and R.K., unpublished). Therefore, their respecification towards an r2 identity is not sufficient to change the pathfinding of their axonal projections. This is consistent with the finding that heterotopically transplanted trigeminal bms in the chick were unable to pathfind correctly to the first branchial arch and projected inappropriately to the second arch (Jacob and Guthrie, 2000). Consequently, as the regional identity of the second pharyngeal arch has not changed in *Hoxb1* mutants, the motoneurons generated in r4, which possess an r2 identity, innervate what they perceive as inappropriate muscle targets (Goddard et al., 1996; Studer et al., 1996). It will be interesting to determine whether these motoneurons would be rescued in a *Hoxa2* mutant genetic background where the second arch has assumed a first arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993).

As target-derived neurotrophic factors play an essential role in regulating motor and sensory neuron survival (deLapeyriere and Henderson, 1997; Oppenheim, 1996), it is very likely that the demise of the ectopic motoneurons in the *Hoxb1* mutants was due to the lack of neurotropic support. Mice lacking cyclin-dependent kinase 5 (*Cdk5*) form fbms that do not migrate out of r4 or even laterally within r4 (Ohshima et al., 2002). In these *Cdk5* mutant mice, the motoneurons have the correct r4 identity, and they remain in r4 like the motoneurons in *Hoxb1* mutants (Ohshima et al., 2002). In both *Cdk5* and *Hoxb1* mutants, axons project into the second pharyngeal arch, but unlike *Hoxb1* mutants the fbms in the *Cdk5* mutants survive to birth when the mice die. This illustrates that staying in the r4 territory is not a problem for motoneuron survival and implies that the reason for the programmed cell death of the ectopic motoneurons in *Hoxb1* mutants is a mismatch between motoneuron and target tissue identity.

Effectors of Hox genes in neurogenesis and neuronal specification

The results presented here demonstrated a variable r4 to r2 transformation, in the mutants examined, which persists at late stages of hindbrain neurogenesis and neuronal differentiation. Hox expression patterns and therefore the AP identity of neuronal progenitors have a direct bearing on the expression patterns of transcription factors such as *Pax6*, *Nkx2.2* and *Phox2b* that are involved in the specification of neuronal subtypes (Davenne et al., 1999; Gaufo et al., 2000) (this study). The hindbrain and the spinal cord are patterned along their DV axis by the action of signalling centres that reside at the roof plate, the notochord and the floor plate (Jessell, 2000; Lee and Jessell, 1999; Liem et al., 1997). Retinoic acid has been also implicated in the specification of a late-born subset of motoneurons (Pierani et al., 1999). However, there is no evidence for variable activity of these centers that could explain AP specific patterns of neurogenesis and neuronal differentiation. Therefore, the Hox-based system of AP specification is a plausible candidate

for imparting AP specific patterns of neurogenesis and neuronal differentiation.

Hox genes could influence the decisions made by neural progenitors cells in several ways. The late columnar expression of Hox genes (Davenne et al., 1999; Gaufo et al., 2000; Graham et al., 1991) (this study) implies a direct role for subsets of Hox genes in the specification of particular subsets of neuronal progenitors. Hox proteins could be modulating the response of neuronal precursors to neural tube patterning signals by regulating the expression of all or some of the components of the corresponding signal transduction machinery. They may also be directly modulating the transcription rate of genes involved in neurogenesis and neuronal specification. *Phox2b* could be such a target as it is necessary for the generation of all bm and vm neurons in the hindbrain (Pattyn et al., 2000) and it is clearly downregulated in the r4 of both *Hoxb1* and *Hoxb2* mutants. It has also been shown that *Phox2b* positively regulates exit from the cell cycle (Dubreuil et al., 2000) and therefore this may provide an indirect venue for Hox genes to influence the exit from the cell cycle. In both *Hoxb1* and *Hoxb2* mutants, the generation of neuronal precursors has been reduced, as suggested by the *Math3* downregulation, and this could also be due to effects on cell cycle regulation.

Motoneuron specification defects are manifested in the mutants examined here, by incorrect migratory routes and inappropriate expression of molecular markers. The onset of caudal migration of the fbms is affected in these mutants, possibly through the incorrect expression of cell-surface receptors that may guide the migration of these cells (Bloch-Gallego et al., 1999; Brose and Tessier-Lavigne, 2000; Jessen et al., 2002). Presumably, the subsequent crosstalk between caudally migrating facial bm neurons and their environment could correctly determine further steps along their differentiation pathway (Garel et al., 2000; Studer, 2001) without the direct involvement of either *Hoxb1* or *Hoxb2*. However, these migratory properties are not enough to determine facial identity as opposed to trigeminal identity as caudal migration in the chick does not occur to the same extent (Bell et al., 1999; Jacob and Guthrie, 2000; Jacob et al., 2000).

In summary, this study has revealed the important roles that the regulatory cascade involving *Hoxa1, Hoxb1* and *Hoxb2* plays in governing processes such as neurogenesis, migratory behaviour and specification in r4 of the developing hindbrain. This work highlights both unique and common functions of these genes. It is unlikely that they exert their activity in r4 by controlling a single downstream effector; therefore, they most likely have common regulatory input into multiple aspects of the decisions that pattern neuronal cells. Delineating these inputs and their effectors will be an important challenge for the future.

References

Altman, J. and Bayer, S. (1982). *Development of the Cranial Nerve Ganglia and Related Nuclei in the Rat*. Berlin: Springer-Verlag.

Arber, S., Ladle, D. R., Lin, J. H., Frank, E. and Jessell, T. M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* **101**, 485-498.

Auclair, F., Valdes, N. and Marchand, R. (1996). Rhombomere-specific origin of branchial and visceral motoneurons of the facial nerve in the rat embryo. *J. Comp. Neurol.* **369**, 451-461.

Bell, E., Wingate, R. and Lumsden, A. (1999). Homeotic transformation of rhombomere identity after localized *Hoxb1* misexpression. *Science* **284**, 2168-2171.

Bloch-Gallego, E., Ezan, F., Tessier-Lavigne, M. and Sotelo, C. (1999). Floor plate and netrin-1 are involved in the migration and survival of inferior olivary neurons. *J. Neurosci.* **19**, 4407-4420.

Brose, K. and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. *Curr. Opin. Neurobiol.* **10**, 95-102.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of *Hoxa-1 (Hox-1.6)* function results in the reorganization of the murine hindbrain. *Development* **118**, 1063-1075.

Caton, A., Hacker, A., Naeem, A., Livet, J., Maina, F., Bladt, F., Klein, R., Birchmeier, C. and Guthrie, S. (2000). The branchial arches and HGF are growth-promoting and chemoattractant for cranial motor axons. *Development* **127**, 1751-1766.

Chisaka, O., Musci, T. and Capecchi, M. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*. *Nature* **355**, 516-520.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Covell, D. A., Jr and Noden, D. M. (1989). Embryonic development of the chick primary trigeminal sensory-motor complex. *J. Comp. Neurol.* **286**, 488-503.

Davenne, M., Maconochie, M., Neun, R., Brunet, J.-F., Chambon, P., Krumlauf, R. and Rijli, F. (1999). *Hoxa2* and *Hoxb2* control dorsoventral patterns of neuronal development in the rostral hindbrain. *Neuron* **22**, 677-691.

Deckwerth, T. L., Easton, R. M., Knudson, C. M., Korsmeyer, S. J. and Johnson, E. M., Jr (1998). Placement of the BCL2 family member BAX in the death pathway of sympathetic neurons activated by trophic factor deprivation. *Exp. Neurol.* **152**, 150-162.

del Toro, E. D., Borday, V., Davenne, M., Neun, R., Rijli, F. M. and Champagnat, J. (2001). Generation of a novel functional neuronal circuit in Hoxa1 mutant mice. *J. Neurosci.* **21**, 5637-5642.

deLapeyriere, O. and Henderson, C. E. (1997). Motoneuron differentiation, survival and synaptogenesis. *Curr. Opin. Genet. Dev.* **7**, 642-650.

Di Rocco, G., Mavilio, F. and Zappavigna, V. (1997). Functional dissection of a transcriptionally active, target-specific Hox-Pbx complex. *EMBO J.* **16**, 3644-3654.

Di Rocco, G., Gavalas, A., Popperl, H., Krumlauf, R., Mavilio, F. and Zappavigna, V. (2001a). The recruitment of SOX/OCT complexes and the differential activity of HOXA1 and HOXB1 modulate the Hoxb1 auto-regulatory enhancer function. *J. Biol. Chem.* **276**, 20506-20515.

Di Rocco, G., Gavalas, A., Popperl, H., Krumlauf, R., Mavilio, F. and Zappavigna, V. (2001b). The recruitment of SOX/OCT complexes and the differential activity of HOXA1 and HOXB1 modulate the Hoxb1 auto-regulatory enhancer function. *J. Biol. Chem.* **1**, 1.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of *Krox-20* and *Hox* gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null *Hoxa-1 (Hox-1.6)* mutant embryos. *Proc. Natl. Acad. Sci. USA* **90**, 7666-7670.

Dubreuil, V., Hirsch, M., Pattyn, A., Brunet, J. and Goridis, C. (2000). The Phox2b transcription factor coordinately regulates neuronal cell cycle exit and identity. *Development* **127**, 5191-5201.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. (1997). *In vivo* functional analysis of the *Hoxa1* 3′ retinoid response element (3′ RARE). *Development* **124**, 399-410.

Ensini, M., Tsuchida, T., Belting, H.-G. and Jessell, T. (1998). The control of rostrocaudal pattern in the developing spinal cord: Specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm. *Development* **125**, 969-982.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early

stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256**, 1555-1560.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Ferretti, E., Marshall, H., Pöpperl, H., Maconochie, M., Krumlauf, R. and Blasi, F. (2000). Segmental expression of *Hoxb2* in r4 requires two distinct sites that facilitate co-operative interactions and ternary complex formation between Perp, Pbx and Hox proteins. *Development* **127**, 155-166.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse *Hox-2.9* gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110**, 589-607.

Garel, S., Garcia-Dominguez, M. and Charnay, P. (2000). Control of the migratory pathway of facial branchiomotor neurones. *Development* **127**, 5297-5307.

Gaufo, G. O., Flodby, P. and Capecchi, M. R. (2000). Hoxb1 controls effectors of sonic hedgehog and Mash1 signaling pathways. *Development* **127**, 5343-5354.

Gavalas, A., Davenne, M., Lumsden, A., Chambon, P. and Rijli, F. (1997). Role of *Hoxa-2* in axon pathfinding and rostral hindbrain patterning. *Development* **124**, 3693-3702.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F., Krumlauf, R. and Chambon, P. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Goddard, J., Rossel, M., Manley, N. and Capecchi, M. (1996). Mice with targeted disruption of *Hoxb1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.

Gould, A., Morrison, A., Sproat, G., White, R. and Krumlauf, R. (1997). Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev.* **11**, 900-913.

Graham, A., Maden, M. and Krumlauf, R. (1991). The murine Hox-2 genes display dynamic dorsoventral patterns of expression during central nervous system development. *Development* **112**, 255-264.

Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F., Chambon, P. and Charnay, P. (1998). *Hoxa1* and *Krox20* synergize to control the development of rhombomere 3. *Development* **125**, 4739-4748.

Jacob, J. and Guthrie, S. (2000). Facial visceral motor neurons display specific rhombomere origin and axon pathfinding behavior in the chick. *J. Neurosci.* **20**, 7664-7671.

Jacob, J., Tiveron, M. C., Brunet, J. F. and Guthrie, S. (2000). Role of the target in the pathfinding of facial visceral motor axons. *Mol. Cell. Neurosci.* **16**, 14-26.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A. and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat. Cell Biol.* **4**, 610-615.

Jungbluth, S., Bell, E. and Lumsden, A. (1999). Specification of distinct motor neuron identities by the singular activities of individual Hox genes. *Development* **126**, 2751-2758.

Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990). Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* **344**, 879-882.

Korematsu, K. and Redies, C. (1997). Restricted expression of cadherin-8 in segmental and functional subdivisions of the embryonic mouse brain. *Dev. Dyn.* **208**, 178-189.

Kusumi, K., Dunwoodie, S. L. and Krumlauf, R. (2001). Dynamic expression patterns of the pudgy/spondylocostal dysostosis gene Dll3 in the developing nervous system. *Mech. Dev.* **100**, 141-144.

Lee, K. and Jessell, T. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.

Liem, K. F., Jr, Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Maconochie, M., Nonchev, S., Studer, M., Chan, S.-K., Pöpperl, H., Sham, M.-H., Mann, R. and Krumlauf, R. (1997). Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. *Genes Dev.* **11**, 1885-1896.

Maden, M., Graham, A., Gale, E., Rollinson, C. and Zile, M. (1997). Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. *Development* **124**, 2799-2805.

Maina, F. and Klein, R. (1999). Hepatocyte growth factor, a versatile signal for developing neurons. *Nat. Neurosci.* **2**, 213-217.

Manzanares, M., Trainor, P., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C.-T., Sham, M.-H. et al. (1999). The role of *kreisler* in segmentation during hindbrain development. *Dev. Biol.* **211**, 220-237.

Manzanares, M., Bel-Vialer, S., Ariza-McNaughton, L., Ferretti, E., Marshall, H., Maconochie, M. K., Blasi, F. and Krumlauf, R. (2001). Independent regulation of initiation and maintenance phases of *Hoxa3* expression in the vertebrate hindbrain involves auto and cross-regulatory mechanisms. *Development* **128**, 3595-3607.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119**, 319-338.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370**, 567-571.

Maves, L., Jackman, W. and Kimmel, C. B. (2002). FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain. *Development* **129**, 3825-3837.

McKay, I., Lewis, J. and Lumsden, A. (1997). Organization and development of facial motor neurons in the *kreisler* mutant mouse. *Eur. J. Neurosci.* **9**, 1499-1506.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H. and Takada, S. (2002). Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev.* **16**, 548-553.

Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* **111**, 61-74.

Murphy, P., Davidson, D. R. and Hill, R. E. (1989). Segment-specific expression of a homeobox-containing gene in the mouse hindbrain. *Nature* **341**, 156-159.

Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F. Y. and Orkin, S. H. (1999). Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Dev. Biol.* **210**, 305-321.

Ohshima, T., Ogawa, M., Takeuchi, K., Takahashi, S., Kulkarni, A. B. and Mikoshiba, K. (2002). Cyclin-dependent kinase 5/p35 contributes synergistically with Reelin/Dab1 to the positioning of facial branchiomotor and inferior olive neurons in the developing mouse hindbrain. *J. Neurosci.* **22**, 4036-4044.

Oppenheim, R. W. (1996). Neurotrophic survival molecules for motoneurons: an embarrassment of riches. *Neuron* **17**, 195-197.

Osumi, N., Hirota, A., Ohuchi, H., Nakafuku, M., Iimura, T., Kuratani, S., Fujiwara, M., Noji, S. and Eto, K. (1997). Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development* **124**, 2961-2972.

Pata, I., Studer, M., van Doorninck, J. H., Briscoe, J., Kuuse, S., Engel, J. D., Grosveld, F. and Karis, A. (1999). The transcription factor GATA3 is a downstream effector of Hoxb1 specification in rhombomere 4. *Development* **126**, 5523-5531.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. *Development* **124**, 4065-4075.

Pattyn, A., Hirsch, M., Goridis, C. and Brunet, J. F. (2000). Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. *Development* **127**, 1349-1358.

Pettmann, B. and Henderson, C. E. (1998). Neuronal cell death. *Neuron* **20**, 633-647.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. (1999). A sonic hedgehog-independent, retinoid activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., Brenner, S., Mann, R. and Krumlauf, R. (1995). Segmental expression of *Hoxb1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/Pbx*. *Cell* **81**, 1031-1042.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-1349.

Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A. (1997). Segmental and neuronal architecture of the hindbrain of *Krox-20* mouse mutants. *Development* **124**, 1215-1226.

Sharma, K., Sheng, H. Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H. and Pfaff, S. L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell* **95**, 817-828.

Simon, H., Hornbruch, A. and Lumsden, A. (1995). Independent assignment of antero-posterior and dorso-ventral positional values in the developing chick hindbrain. *Curr. Biol.* **5**, 205-214.

Studer, M. (2001). Initiation of facial motoneurone migration is dependent on rhombomeres 5 and 6. *Development* **128**, 3707-3716.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384**, 630-635.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F., Chambon, P. and Krumlauf, R. (1998). Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Takahashi, M. and Osumi, N. (2002). Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development* **129**, 1327-1338.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M. and Kageyama, R. (1997). Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to Drosophila proneural gene atonal. *EMBO J.* **16**, 384-395.

Takeichi, M., Uemura, T., Iwai, Y., Uchida, N., Inoue, T., Tanaka, T. and Suzuki, S. C. (1997). Cadherins in brain patterning and neural network formation. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 505-510.

Tanabe, Y. and Jessell, T. (1997). Diversity and patterning inthe developing spinal cord. *Science* **274**, 1115-1123.

Trainor, P. and Krumlauf, R. (2000). Patterning the cranial neural crest: Hindbrain segmentation and *Hox* gene plasticity. *Nat. Rev. Neurosci.* **1**, 116-124.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of *LIM* homeobox genes. *Cell* **79**, 957-970.

Varela-Echavarria, A., Pfaff, S. L. and Guthrie, S. (1996). Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. *Mol. Cell. Neurosci.* **8**, 242-257.

Vesque, C., Maconochie, M., Nonchev, S., Ariza-McNaughton, L., Kuroiwa, A., Charnay, P. and Krumlauf, R. (1996). *Hoxb-2* transcriptional activation by *Krox-20* in vertebrate hindbrain requires an evolutionary conserved *cis*-acting element in addition to the Krox-20 site. *EMBO J.* **15**, 5383-5896.

Walshe, J., Maroon, H., McGonnell, I. M., Dickson, C. and Mason, I. (2002). Establishment of hindbrain segmental identity requires signaling by FGF3 and FGF8. *Curr. Biol.* **12**, 1117-1123.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.

Zhou, Y., Yamamoto, M. and Engel, J. D. (2000). GATA2 is required for the generation of V2 interneurons. *Development* **127**, 3829-3838.