Hox3 genes coordinate mechanisms of genetic suppression and activation in the generation of branchial and somatic motoneurons

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Summary

In the developing hindbrain, the functional loss of individual Hox genes has revealed some of their roles in specifying rhombomere (r) identity. However, it is unclear how Hox genes act in concert to confer the unique identity to multiple rhombomeres. Moreover, it remains to be elucidated how these genes interact with other transcriptional programs to specify distinct neuronal lineages within each rhombomere. We demonstrate that in r5, the combined mutation of *Hoxa3* and *Hoxb3* result in a loss of *Pax6*- and *Olig2*-expressing progenitors that give rise to somatic motoneurons of the abducens nucleus. In r6, the absence of any combination of the Hox3 paralogous genes results in ectopic expression of the r4-specific determinant *Hoxb1*. This ectopic expression in turn results in the differentiation of r4-like facial branchiomotoneurons within this rhombomere. These studies reveal that members of the Hox1 and Hox3 paralogous groups participate in a 'Hox code' that is necessary for coordinating both suppression and activation mechanisms that ensure distinction between the multiple rhombomeres in the developing hindbrain.

Key words: Motoneurons, Hox3, Mouse

Introduction

An evolutionarily conserved function of Hox genes is to assign positional information to body segments along the anteroposterior (AP) axis of developing organisms (Ferrier and Holland, 2001; Hafen et al., 1984; Lewis, 1978; McGinnis and Krumlauf, 1992; Rozowski and Akam, 2002; Weatherbee et al., 1998). In vertebrates, segments or rhombomeres (r) of the developing hindbrain have been used as a model to understand the role of Hox genes in segmental patterning (Barrow et al., 2000; Bell et al., 1999; Carpenter et al., 1993; Davenne et al., 1999; Gaufo et al., 2000; Gavalas et al., 1998; Goddard et al., 1996; Mark et al., 1993; McClintock et al., 2002; Pata et al., 1999; Studer et al., 1998; Studer et al., 1996). A plethora of genetic and embryological experiments have established that Hox genes are necessary and sufficient to confer a unique identity to rhombomeres in early stages of hindbrain segmentation, as well as cell identity in later stages of development. The general phenomenon of Hox gene induced homeosis – transformation of one body part to another – initially observed in *Drosophila*, appears to be equally applicable in complex structures such as the vertebrate hindbrain. In the mouse, for example, absence of *Hoxb1* function in r4 results in a lack of facial branchiomotoneurons (BMNs) owing to an apparent segmental transformation of r4- to an r2-like rhombomere identity (Goddard et al., 1996; Studer et al., 1996). Conversely, after segmentation has established an apparent normal r2 in chick embryos, gain of *Hoxb1* function results in the ectopic differentiation of r4-like facial BMNs in r2 (Bell et al., 1999). Thus, although studies of loss of Hox gene function has revealed an important role in early segmental patterning, the latter study also implicates a significant role for Hox genes in the differentiation of unique cell types later in hindbrain development.

There is also evidence that combinations of various Hox genes can elicit emergent phenotypes beyond the contributions of individual genes (Condie and Capecchi, 1994; Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). For example, the absence of either *Hoxa1* or *Hoxb1* function leads to some degree of rhombomere identity transformation and cell loss is observed (Carpenter et al., 1993; Gaufo et al., 2000; Goddard et al., 1996; Mark et al., 1993; Pattyn et al., 2003; Studer et al., 1996). However, the combined absence of both genes leads to severe abnormal programmed cell death resulting in the deletion of multiple rhombomeres and subsequent reorganization of the hindbrain (Gavalas et al., 1998; Rossel and Capecchi, 1999). Therefore, the interaction of these genes is equally important for the determination of segmental specification as is the contribution of each individual Hox gene to individual rhombomere identity. It remains to be determined how the various Hox genes expressed in the hindbrain, r2 to r7, interact with each other to determine distinction of each rhombomere as well as integration of function across rhombomeres.

Similarly, given that Hox genes are co-expressed with molecules involved with generic neural specification programs in later stages of development, it is likely that they can also interact with these programs to establish unique cell identities within each rhombomere (Davenne et al., 1999; Gaufo et al., 2000; Osumi et al., 1997; Pattyn et al., 2003; Takahashi and Osumi, 2002). For example, neural progenitors expressing the homeodomain proteins Nkx2.2 and Phox2b give rise to all BMNs present in various rhombomeres of the hindbrain.

The interaction between these molecules with different combinations of Hox proteins expressed at different rhombomeres may give rise to functionally distinct BMNs, such as facial BMNs unique to r4 where *Hoxb1* is expressed (Gaufo et al., 2000; Goddard et al., 1996; Pattyn et al., 2003; Studer et al., 1996). Likewise, somatic motoneurons (SMNs) derive from neural progenitors expressing the homeodomain proteins Pax6 and the bHLH Olig2 are also present at various rhombomeres. In this case, however, the Hox genes involved in their differentiation into functionally unique SMNs within each rhombomere have not been determined (Guidato et al., 2003).

In summary, given that Hox gene expression persists throughout hindbrain development, it is plausible that it plays at least two distinct roles in neuronal specification. First, Hox genes are required for assignment of rhombomere identity and in their absence, for example *Hoxa1* and *Hoxb*, such patterning is highly perturbed (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). Second, they may play a role in differentiating functionally unique motoneurons at a later stage of development (Bell et al., 1999; Guidato et al., 2003). The goal of this study was to identify what combination of Hox genes are necessary to distinguish rhombomeres r4 to r6, and how these genes effect the production of motoneuron subtype programs unique to these rhombomeres. To achieve this, we first focused on Hox3 paralogous genes (*Hoxa3*, *Hoxb3* and *Hoxd3*), expressed in caudal rhombomeres, and analyzed the affects of different combinations of Hox3 mutations on the segmental identities of r4, r5 and r6. Then, to test the role of these Hox genes in neural specification, we looked for the presence or absence of specific SMNs in r5 with relation to different combinations of null mutations of the Hox3 genes. We observed that loss of any of the Hox3 genes results in a r6- to r4-like change in cell fate; different double mutation showed a graded increase in cell fate change. We also observed a gene dosage dependence of SMN specification of *Hoxa3* and *Hoxb3* in r5. This specification appears to be mediated through the control of the *Pax6/Olig2* regulatory pathway for SMN formation, suggesting a direct influence of Hox3 genes on SMN fate. Together, these observations reveal two functions for Hox3 paralogous genes in the developing hindbrain in defining segmental identity across multiple rhombomeres and controlling cell fate within an individual rhombomere.

Materials and methods

Mouse lines

Mice harboring mutations for *Hoxb1*, *Hoxa3*, *Hoxb3*, *Hoxd3* and *Pax6* were previously reported (Ericson et al., 1997; Gaufo et al., 2000; Greer et al., 2000; Manley and Capecchi, 1998). The Hoxa3^{CFP} reporter mouse expresses an in-frame fusion between the C terminus of the Hoxa3 protein and the N terminus of ECFP. This allele was generated by the targeted insertion of the *ECFP* gene (Clontech) to the second exon of *Hoxa3*. This fusion has been shown not to affect *Hoxa3* function (K.R.T. and M.R.C, unpublished). Single- or compound-mutant embryos were generated from intercrossings of either single- or double-heterozygote mice bearing the mutant allele(s) of interest. Mice were mated overnight and the detection of plugs the following morning was considered 0.5 days post-coitum [embryonic day 0.5, (E0.5)]. Genotyping was performed as previously described (Gaufo et al., 2000; Greer et al., 2000; Manley and Capecchi, 1998). A minimum of two embryos were analyzed for each time point and experimental group.

In situ hybridization and immunohistochemistry

For whole-mount in situ hybridization, E11.5 neural tubes were dissected in cold PBS and fixed in cold 4% formaldehyde for 2-3 hours and processed for *Hoxa3*, *Hoxb3* and *Hoxd3* RNA in situ hybridization as previously described (Manley and Capecchi, 1998). Detection of Hoxb1^{GFP} was detected by confocal microscopy on E11.5, live-dissected flat-mount hindbrain preparations. E9.25-E11.5 embryos were harvested as described above and processed for cryostat sectioning. Frozen, 10 μm transverse and coronal sections were labeled with rabbit anti-Hoxb1 (1:250, Covance), rabbit anti-Phox2b (1:1000, a gift from C. Goridis), rabbit anti-Olig2 (1:5000; a gift from H. Takebayashi), mouse anti-TuJ1 (1:1000, Covance), rat anti-Hoxb4 (1:25; Developmental Studies Hybridoma Bank, DSHB), mouse anti-Nkx2.2 (1:25, DSHB), mouse anti-Isl1/2 (1:25; DHSB), rabbit anti-Chx10 (1:2500; a gift from S. Morton and T. Jessell), mouse anti-MPM2 (1:8000; Upstate Biotechnology), rabbit anti-activated Caspase-3 (1:50; NEB Cell Signaling), TUNEL (manufacturer's protocol, Roche), mouse anti-NeuN (1:250; Chemicon), rabbit anti-ChAT (1:1000; Chemicon) and αBTX (1:500; Molecular Probes). Immunolabeled sections were developed with Alexa-fluor (1:1000; Molecular Probes) and Cy5 (1:1000; Jackson Immunoresearch)-conjugated secondary antibodies. Images of fluorescent-labeled sections were captured with the BioRad MRC 1024 confocal microscope and processed with Adobe Photoshop and Microsoft Powerpoint software.

Results

Segmental expression of paralogous Hox3 genes coincide with domains of motoneuron subtypes in the hindbrain

Our analyses focused on the function of Hox genes in the central region of the hindbrain spanning r4 to r6, where two motoneuron subtypes are characterized by their specific distribution and the expression of distinct combinations of genes encoding Hox and non-Hox homeodomain proteins (Briscoe et al., 2000; Carpenter and Sutin, 1983; Gaufo et al., 2000; Pattyn et al., 2000; Pattyn et al., 1997; Saper, 2000) (Fig. 1A-E). The facial BMNs can be identified by their unique r4 origin and their posterolateral migration into r6 (Fig. 1B, green) (Gaufo et al., 2000). In the same panel, the relative location of the abducens somatic motoneurons (SMNs) is diagrammed to show their positions relative to the BMNs. In contrast to BMNs, SMNs are born in a ventromedial position in r5 and migrate laterally within the same rhombomere. In transverse sections of the ventral hindbrain of E11.5 control embryos labeled for Hoxb1^{GFP}, Phox2b and Isl1/2, the differentiation and migration of the BMNs can be further characterized by their lateral location and combined expression of Hoxb1 (green), Phox2b (blue) and Isl1/2 (red) in r4 to r6 (triple labeling seen as white) and the SMNs are labeled singly by Isl1/2 in r5 (Fig. 1C-E). In E11.5 control hindbrain flatmount, the relative high levels of Hoxa3, Hoxb3 and Hoxd3 expression are restricted to the r4/r5, r6/r7 and r5/r6 boundaries, respectively, suggesting that Hox3 genes may have a role in the specification of multiple rhombomere identities as well as the motoneuron subtypes that are derived from these rhombomeres (Fig. 1F-H).

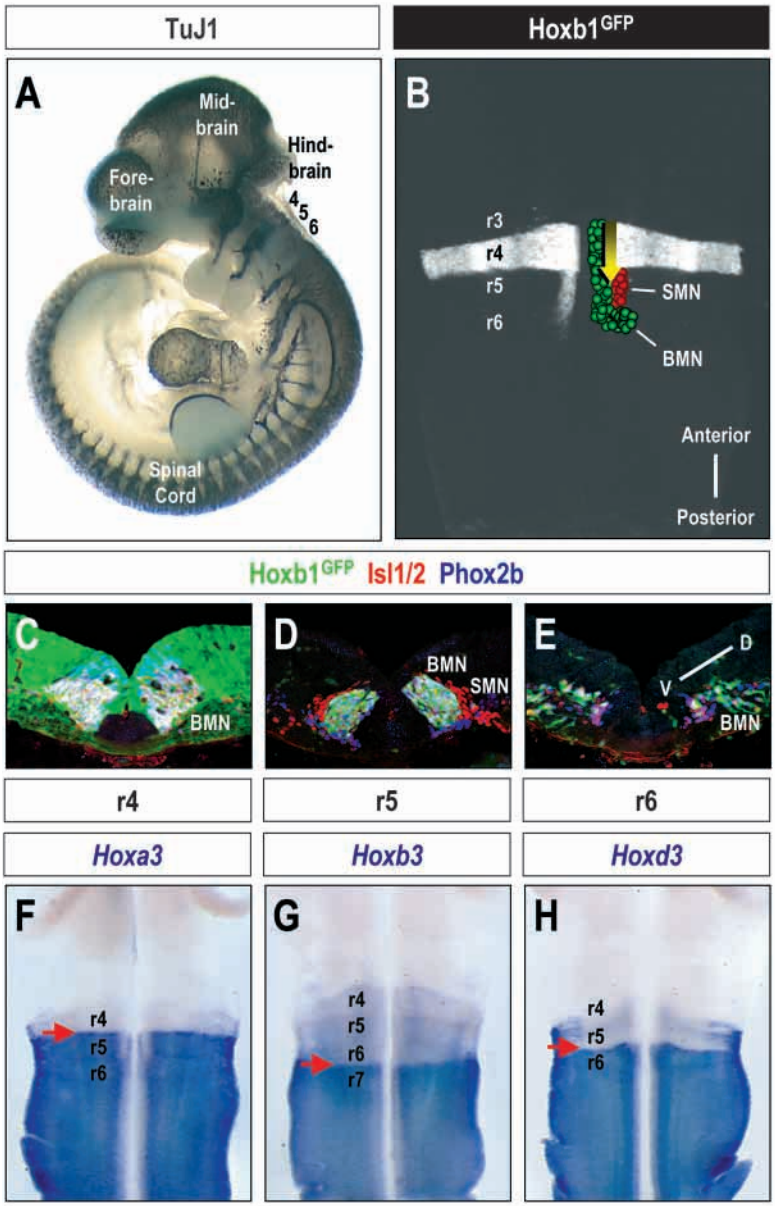
Hox3 genes mediate segment specific suppression of *Hoxb1*

Analysis of patterning defects along the AP axis among mice individually homozygous for *Hoxa3*, *Hoxb3* or *Hoxd3* revealed

a mild ectopic expression of Hoxb1 in single mutants (data not shown). However, the ectopic expression of Hoxb1 was exacerbated in E11 embryos harboring different combinations of double mutations in the Hox3 genes, with *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos showing the greatest effect (Fig. 2A-D; data not shown). The systematic elimination of *Hoxa3* and *Hoxd3* alleles reveal a gene dosage-dependent effect in the severity of ectopic Hoxb1 expression (Fig. 2A-D). Labeling of Hoxb4, which demarcates the boundary between r6 and r7 suggests that the ectopic expression of Hoxb1 is restricted to r6 (Fig. 2E,F) (Gould et al., 1998). Hoxb4 also labels neurons in the marginal layer of r6, presumably neurons that have migrated from r7, which are reduced in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ mutant embryos. The Hox3 genes may thus function to regulate the anterior migration of these neurons. With respect to this study, however, the appearance of ectopic Hoxb1-expressing cells in the inner ventricular and outer marginal neuroepithelial layers suggest that these cells are initially born and differentiate in r6.

Segmental formation occurs normally in *Hoxa3* and *Hoxd3* double mutant embryos

To determine if the loss of *Hoxa3* and *Hoxd3* result in early segmental defects, we analyzed the expression patterns of Hoxb1, Krox20 and Hoxb4 in coronal sections of E9.25-9.5 *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos. In both control and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, Hoxb1 expression was normal in r4 and in neural crest cells migrating from r4 (Fig. 2G,H). Krox20 expression in r3 and r5 and the expression of Hoxb4 at the border of r6 and r7 also appear normal in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos compared with controls (Fig. 2I,H). In r6, however, the expression of Hoxb1 persists in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos at a period when the expression of Hoxb1 is normally down regulated posterior to r4 (Gaufo et al., 2000) (Fig. 2H, bracket). Although segmentation and rhombomere periodicity appear normal in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, the persistence of Hoxb1 expression in r6 indicates an early defect in segmental identity.



Ectopic *Hoxb1* expression is associated with r4-like facial branchiomotoneuron differentiation and migration pattern in r6

We next examined the functional consequence of the ectopic expression of Hoxb1 in r6 of *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos. To address this issue, we focused on two well-defined functions of Hoxb1 in r4: (1) the expression requirement of Nkx2.2, Phox2b and Isl1/2 among BMNs; and (2) the posterior migration of Hoxb1-expressing BMNs from r4 to the ventrolateral region of r6 (Gaufo et al., 2000; Goddard et al., 1996; Pattyn et al., 2000; Studer et al., 1996) (Fig. 1C-E). In control embryos, a small population of these r4-derived Hoxb1-expressing BMNs can be seen adjacent to the Nkx2.2-expressing progenitor domain in ventral r6 (Fig. 3A). In *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, Hoxb1 is ectopically expressed in an expanded domain of Nkx2.2-expressing cells, indicative of BMN progenitors (Fig. 3B) (Gaufo et al., 2000; Pattyn et al., 2000). Co-localization of Isl1/2 with these same Hoxb1-expressing cells suggests that these progenitors are differentiating into motoneurons (Fig. 3D). Furthermore, the ectopic expression

Fig. 1. Expression of Hox and non-Hox genes among motoneuron subtypes in the central hindbrain. (A) Lateral view of an E11 embryo stained with TuJ1 showing the region of the central hindbrain labeled by 4, 5 and 6 to indicate the individual rhombomeres examined in this study. (B) Flat-mount preparation of an E11.5 embryo containing a targeted Hoxb1^{GFP} reporter (white). Hoxb1^{GFP} labels the progenitors and differentiating branchiomotoneurons (BMN) in r4 and their migratory course (arrow) towards the ventrolateral region of r6. Two classes of motoneurons, facial BMNs (green) and abducens somatic motoneurons (SMNs, red), are represented by spheres superimposed over the expression of Hoxb1^{GFP} to illustrate their spatial relationship. (C-E) Transverse sections of E11.5 embryo spanning r4, r5 and r6 labeled with Hoxb1^{GFP} (green), Phox2b (blue) and Isl1/2 (red). (F-H) E11.5 hindbrain flat-mount RNA in situ hybridization for *Hoxa3*, *Hoxb3* and *Hoxd3*. V, Ventral; D, Dorsal.

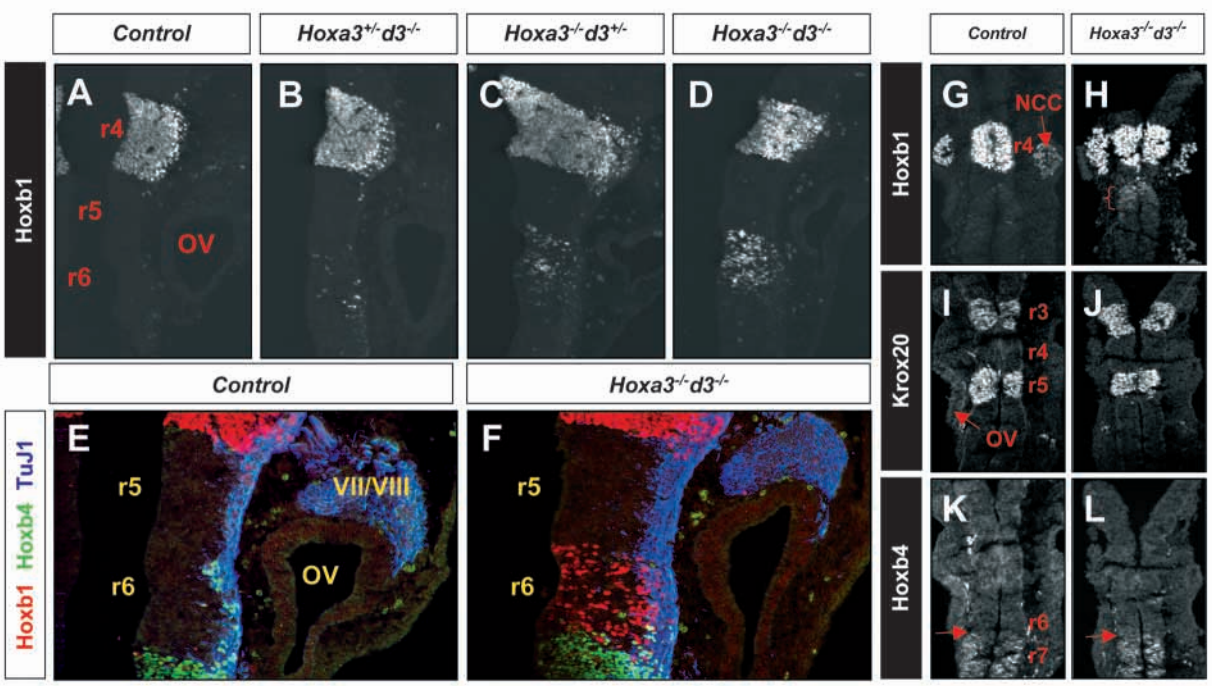


Fig. 2. Loss of *Hoxa3* and *Hoxd3* results in ectopic expression of Hoxb1 in r6 with normal rhombomere periodicity. (A-D) Coronal hindbrain sections, immunolabeled for Hoxb1 of E11 control and mutant embryos with incremental loss of *Hoxa3* and *Hoxd3* alleles. (E,F) Coronal hindbrain sections of E11 control and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos labeled for Hoxb1 (red), Hoxb4 (green) and TuJ1 (blue). Hoxb4 labels the boundary between r6 and r7. The VIIth and VIIIth cranial sensory ganglia (VII/VIII) are located ventrolateral to r4 and anterior to the otic vesicle (OV), which borders r5 and r6. (G-L) Coronal hindbrain sections of E9.25 control and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos labeled for Hoxb1 (G,H), Krox20 (I,J) and Hoxb4 (K,L). Hoxb1 is expressed in r4 and in migrating neural crest cells (NCC). The otic vesicle (OV) borders r5 and r6. The bracket in H highlights the ectopic expression of Hoxb1 in r6. The arrows in K and L represent the boundary between r6 and r7.

of Phox2b and Isl1/2 suggest that these neurons are differentiating specifically into BMNs (Fig. 3F).

These findings suggest that Hoxb1 is capable of inducing the ectopic expression of BMN-specific genes within r6. However, it does not provide evidence for the capacity of these motoneurons to adopt functional properties, such as posterior migration, unique to BMNs in r4. To examine the possibility that the ectopic Hoxb1-expressing BMNs in r6 have adopted an r4-like migratory capacity, we looked for their presence in r7 as a consequence of migration from r6. The expression of Hoxb4 was used to demarcate the posterior boundary of r6 and also to label cells specific to r7 (Gould et al., 1998). Transverse sections of E11.5 control embryos show the expression of Hoxb1 in r6 is restricted to a small population of BMNs that have migrated from r4; whereas in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, Hoxb1 is expressed ectopically at a high level throughout r6 (Fig. 3G,H). Analysis of r7 shows no Hoxb1-expressing neurons in control embryos (Fig. 3I). By contrast, Hoxb1-expressing motoneurons, as confirmed by co-expression with Isl1/2 (data not shown), are observed in r7 of *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos (Fig. 3J). Moreover, the ectopic Hoxb1-expressing cells in r7 do not express Hoxb4, suggesting that these cells have migrated from r6 and are not derived from r7.

To address the possibility that the observed Hoxb1-expressing cells in r7 of *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos were derived from r4 and migrated aberrantly through r6, we analyzed coronal sections of younger E10.5 embryos at a period when the Hoxb1-expressing BMNs have initiated their migration from r4 (Fig. 3K,L). In both control and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, the expression of Hoxb1 in r4 is normal. In r5, the initial migration of Hoxb1-expressing cells is also normal in both groups. In r6, the ectopic expression of Hoxb1 in *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos is evident compared with control embryos. In r7 of *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos, small clusters of Hoxb1-expressing neurons, negative for Hoxb4, are clearly visible (Fig. 3L). This observation thus precludes the possibility that the ectopic Hoxb1-expressing cells in r7 have migrated from the BMN population derived from r4. Together, these observations provide strong evidence that the ectopic expression of Hoxb1 in r6 is sufficient to activate a BMN differentiation and migration program normally unique to r4. These findings are consistent with a previous report demonstrating that the localized or global misexpression of Hoxb1 in r2 of the chick embryo is sufficient to transform cells in this rhombomere into r4-like BMNs (Bell et al., 1999). Moreover, the posterior migration of r4-like BMNs from r6 into r7 suggests that r7, like r5, produces local environmental cues required for the migration of even-numbered derived 'facial' BMNs (Garel et al., 2000; Studer, 2001).

Combined functions of *Hoxa3* and *Hoxb3* are necessary for the specification of r5-derived motoneurons

Among the Hox3 genes, only *Hoxa3* and *Hoxb3* are expressed

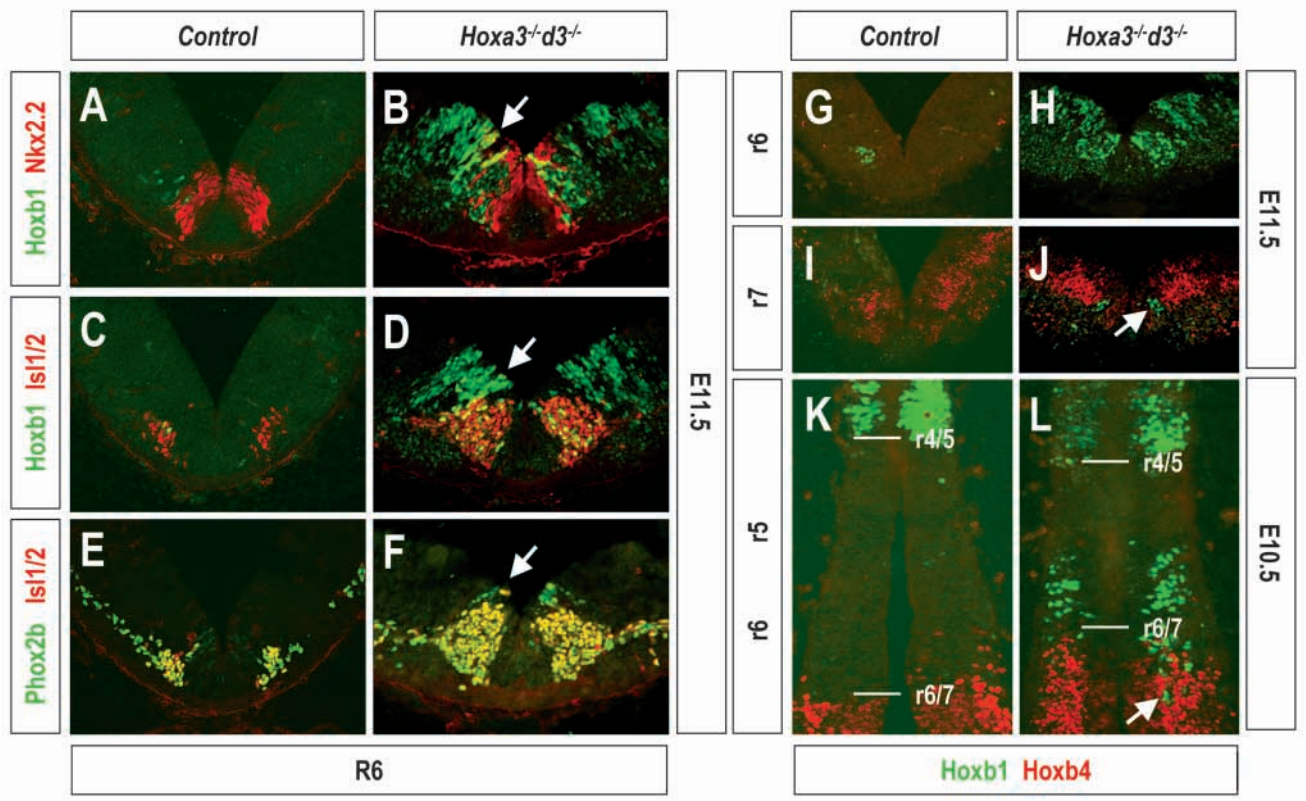


Fig. 3. Ectopic expression of Hoxb1 in r6 is associated with activation of r4-like facial branchiomotoneuron differentiation and migration. (A-F) Transverse sections of ventral r6 in E11.5 control (A,C,E) and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos (B,D,F) labeled for Hoxb1 (green), Nkx2.2 (red), Isl1/2 (red) and Phox2b (green). The arrows in B, D, F represent ectopic progenitors in the ventricular layer of the neuroepithelium in r6. (G,H) Transverse sections of ventral r6 and r7 in E11.5 control (G,I) and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos (H,J) labeled for Hoxb1 (green) and Hoxb4 (red). (K,L) Coronal sections of E10.5 control and *Hoxa3*$^{-/-}$*d3*$^{-/-}$ double mutant embryos labeled for Hoxb1 (green) and Hoxb4 (red). The arrows in J and L represent ectopic Hoxb1-expressing cells in r7 that have presumably migrated from r6.

in the ventral r5 region from which SMNs are derived (Fig. 1D,F-H). The loss of *Hoxa3* alone, results in a reduction in the number of SMNs (data not shown), whereas the elimination of both *Hoxa3* and *Hoxb3* in E11.25-E11.5 mutant embryos lead to the complete loss of SMNs, as identified by expression of the homeodomain HB9 (Fig. 4A,B) (Arber et al., 1999). By contrast, r4-derived Phox2b-expressing BMNs and r5-derived visceromotoneurons labeled by Isl1/2 and Phox2b are unaffected (data not shown). The specific loss of SMNs in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos closely resembles the phenotype of embryos bearing independent mutations for *Olig2* and *Pax6* (Fig. 4C) (Lu et al., 2002; Novitch et al., 2001; Takebayashi et al., 2002; Zhou and Anderson, 2002). However, in the case of the *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos, the loss of SMNs is restricted to r5.

To define the stage-specificity of the defects in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos, we first characterized the normal progenitors and neuronal subtypes in ventral r5 at the onset of SMN specification, prior to the migration of r4-derived BMNs into this region (Fig. 4D-I; data not shown). In E10.25 control embryos, three distinct progenitor domains and the neuronal subtypes that arise from them can be identified along the DV axis by their expression of specific homeodomain proteins (Fig. 4D-I) (Briscoe et al., 2000). In the ventral region of r5 just dorsal to the floor plate, the progenitors for V3 interneurons (pV3), SMN (pSMN) and V2 interneurons (pV2) can be identified by their expression of Nkx2.2, low levels of Pax6 and high Pax6, respectively (Fig. 4D). The pSMN can be further characterized by the expression of Olig2, the high expression of which appears to encompass the low Pax6-expressing region (Fig. 4E). These progenitors give rise to SMNs of the abducens nucleus, which are specifically labeled by HB9 (Fig. 4F). The more ventral V3 interneurons can be identified by their expression of Phox2b (Fig. 4G). These interneurons presumably give rise to serotonergic neurons (Hendricks et al., 2003; Pattyn et al., 2003). The SMNs and V3s in r5 are both labeled by the pan motoneuron marker Isl1/2 (Fig. 5J,K). The V2 interneurons, as in the spinal cord, are located immediately dorsal to SMNs and can be identified by the expression of Chx10 (Fig. 4H). Using a Hoxa3^{CFP} reporter, the expression of Hoxa3^{CFP} and HB9 were found to be co-localized in the outer marginal layer of the neuroepithelium (Fig. 4I). Although the relative high expression levels of Hoxa3^{CFP} are found amongst differentiated cells, its ubiquitous expression throughout the neuroepithelium, from progenitors to differentiated neurons, suggests a much broader regulatory role in neuronal patterning.

Hoxa3 and *Hoxb3* are early determinants of somatic motoneuron fate

As described earlier, the restricted loss of motoneurons in r5 of *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos was specific to SMNs

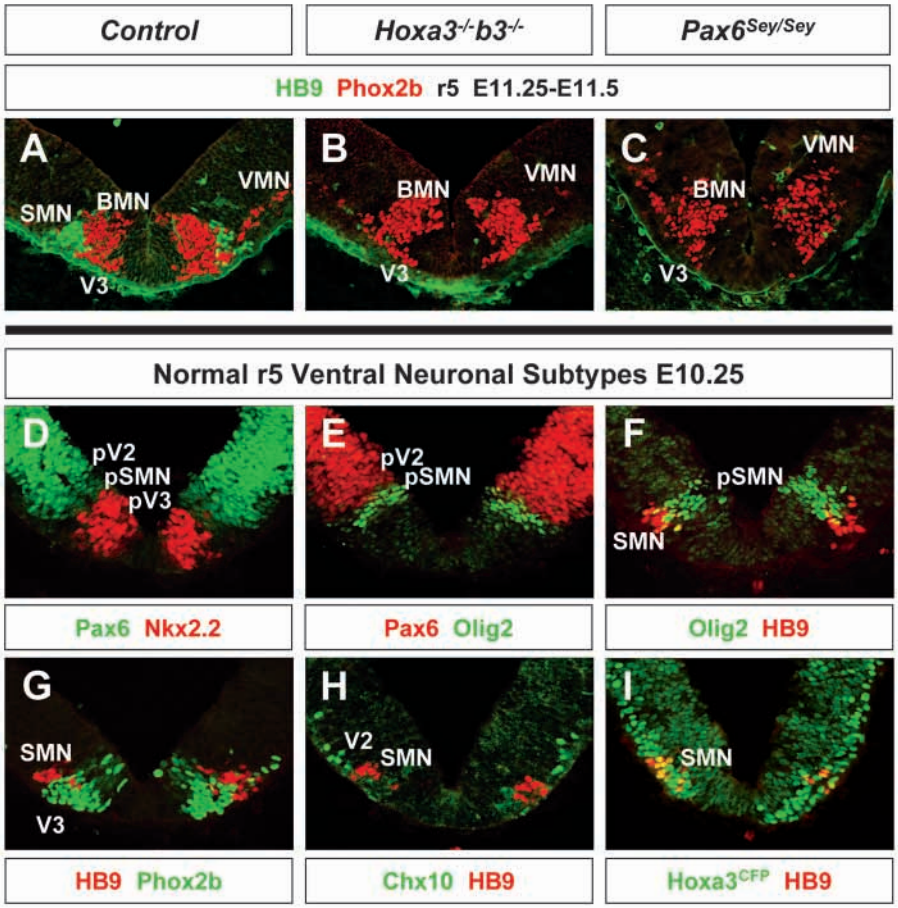


Fig. 4. Hox3 and *Pax6* are necessary for the development of somatic motoneurons in r5. (A-C) Transverse sections of ventral r5 in E11.25-E11.5 control, *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double and *Pax6*$^{Sey/Sey}$ mutant embryos labeled for HB9 (green) and Phox2b (red). The SMNs are missing in both *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double and *Pax6*$^{Sey/Sey}$ mutant embryos. The ventrolateral expression of Phox2b in both mutant embryos is spared, suggesting that the early differentiation of visceromotoneurons in r5 is unaffected by either mutations. (D-I) Characterization of ventral progenitors and neuronal subtypes in transverse sections of ventral r5 in control E10.25 embryos prior to the migration of r4-derived BMNs. The progenitors for V3 interneurons, SMNs and V2 interneurons are characterized by the expression of Nkx2.2 (red), low and high Pax6 (green) levels, respectively (D). Olig2 (green) labels the pSMN domain (red; green fluorescent intensity of Olig2 masks the low red fluorescence of Pax6 staining), immediately ventral to the high Pax6 pV2 domain (E). pSMN domain and SMNs are labeled by HB9 (red) and Olig2 (green), respectively (F). The SMNs and the V3 interneurons can be distinguished from each other by their non-overlapping expression of HB9 (red) and Phox2b (green), respectively (G). The V2 interneurons can be distinguished from the HB9-expressing SMNs (red) by their specific expression of Chx10 (green). A CFP reporter for Hoxa3 (green) labels all cells in r5 with relatively high expression levels among postmitotic cells, which include the HB9-expressing SMNs (red, I).

labeled by HB9, with no apparent loss of Phox2b expression amongst the more ventral V3 interneurons (Fig. 4A,B). To determine the onset of these defects, we analyzed E10.25 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos for the expression of Olig2, which specifically labels pMNs (Fig. 5A-D). The expression of Olig2 and HB9 show a gene dose-dependent response to the sequential loss of *Hoxa3* and *Hoxb3* alleles. The complete loss of Olig2 expression in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos, therefore suggests that *Hoxa3* and *Hoxb3* are upstream of the *Olig2*-dependent specification of SMN progenitors.

To determine the cause of the Olig2-specific loss in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos, we examined two possibilities: (1) programmed cell death; and (2) transformation in neuronal fate. To address the first possibility, we analyzed E10.25 embryos for the expression of TUNEL and activated caspase 3, both markers for programmed cell death (Fig. 5E-H). No differences were observed across experimental groups. Analysis of older *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos (E10.5-E11.25) also showed no differences in TUNEL and caspase 3 expression (data not shown). An assay for cell proliferation using the mitosis marker MPM2, showed normal cell division in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos compared with controls (Fig. 5E-H). The absence of aberrant cell death among the experimental groups left the possibility that the loss of SMNs in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos may be the result of a change in neuronal fate. To address this issue, we examined the expression pattern of Pax6, which identifies progenitors of SMNs and V2s. In E10.25 *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos, the normal low Pax6 expression in the pSMN domain is qualitatively similar to the more dorsal high Pax6 expression in the pV2 domain (Fig. 5I,M). The molecular change in the pSMN domain to that of the pV2 domain is substantiated by appearance of Chx10-expressing V2 interneurons immediately dorsal to V3 interneurons, a region normally occupied by SMNs (Fig. 5K,O). The ectopic appearance of Chx10-expressing V2 interneurons in the region normally occupied by HB9-expressing SMNs was also confirmed in E11.5 *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos compared with controls (Fig. 5L,P). In a gene dose-dependent manner, *Hoxa3* and *Hoxb3* are thus required for the specification of progenitors that will give rise to SMNs of the abducens nucleus.

The loss of SMN precursors of the abducens nucleus was also confirmed in late stage, E18 *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos. In the upper medulla, the r5-derived abducens nucleus can be easily identified by its stereotypic relationship with axons from the r4-derived facial nucleus. The axons of the facial nucleus, known as the genu, circumscribe the abducens nucleus in a medial to lateral pattern (Carpenter and Sutin, 1983). The

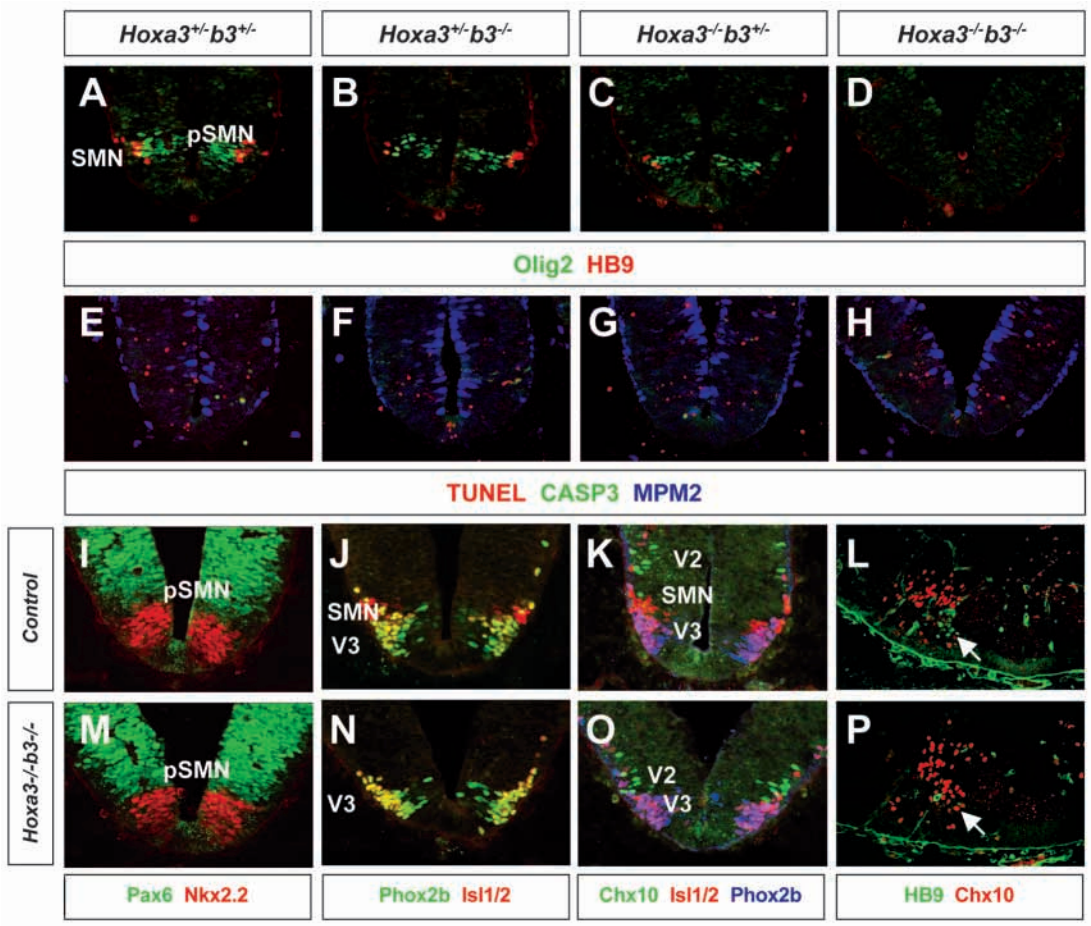


Fig. 5. Specification of somatic motoneurons in r5 is regulated by *Hoxa3* and *Hoxb3* in a gene dose-dependent manner. (A-D) Transverse sections of ventral r5 in E10.25 embryos harboring various mutant alleles in Hoxa3 and Hoxb3 labeled for Olig2 (green) and HB9 (red); (E-H) TUNEL (red), activated caspase 3 (green, CASP3) and the mitotic marker, MPM2 (blue). (I-P) Transverse sections of ventral r5 in E10.25 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos labeled for Pax6 (green) and Nkx2.2 (red) (I,M), Phox2b (green) and Isl1/2 (red) (J,N), Chx10 (green) and Isl1/2 (red), and Phox2b (blue) (K,O); and E11.5 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos labeled for HB9 (green) and Chx10 (red) (L,P). Cell counts for Chx10-expressing V2 interneurons were determined for representative control (*Hoxa3b3*-double heterozygote) and mutant (*Hoxa3b3*-double homozygote) embryos. Values for the control, 40.0±1.9 (mean/side±s.e.m.), versus double-mutant, 61.6±2.3, were statistically significant (P<0.005). The arrows in panels L and P represent clusters of HB9- and Chx10-expressing SMNs and V2 interneurons, respectively.

genu of the facial nerve is clearly visible in both E18 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos in the region devoid of NeuN expression, a nuclear marker for differentiated neurons (Fig. 6A,B, arrow). The early embryonic loss of r5-derived progenitors and precursors of the abducens nucleus is substantiated by the absence of choline acetyltransferase (ChAT) expression in *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos (Fig. 6C,D). These findings are supported by an associated reduction in the expression of TuJ1, a pan-neuronal marker, and acetylcholine receptors (AChR), as visualized by α-bungarotoxin (αBTX), in transverse sections of the lateral rectus muscle, innervated normally by the abducens nerve (Fig. 6E-J). The binding of αBTX in the lateral rectus of *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos is consistent with the finding that prepatterning of AChRs occurs in the target muscle independent of motor innervation (Yang et al., 2001). The remaining expression of TuJ1 in the lateral rectus of *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos may represent peripheral processes from sensory neurons (i.e. trigeminal ganglia). However, the close proximity of these processes with αBTX suggests a contribution from a motoneuron source, perhaps the aberrant innervation by axons from other cranial motoneurons.

Discussion

In this report, we provide genetic evidence that the paralogous Hox3 genes influence neuronal identity in two crucial ways. In r6, the Hox3 genes are required to genetically suppress the expression of the r4-specific determinant *Hoxb1* (Fig. 7A,B). In ventral r5, the combined functions of *Hoxa3* and *Hoxb3* are necessary for the specification of *Pax6*- and *Olig2*-dependent somatic motoneuron progenitors that give rise to the abducens nucleus (Fig. 7C,D). In their absence, the domain normally occupied by somatic motoneuron progenitors appears to be programmed for a V2 interneuron fate. Together, these data provide evidence that the coordinated activities of Hox3 genes along the AP axis are required to establish the unique identities of r5 and r6.

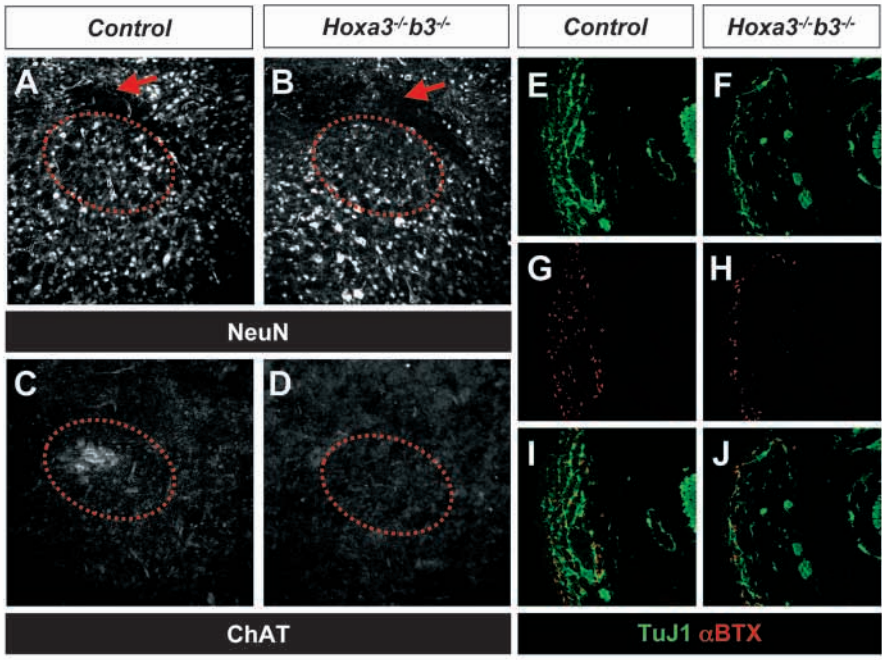


Fig. 6. *Hoxa3* and *Hoxb3* are necessary for the formation of the abducens nucleus and normal target innervation. (A-D) Transverse sections of the upper medulla in E18 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos labeled for the neuron-specific nuclear marker, NeuN (A,B) and choline acetyltransferase (ChAT) (C,D). In the control embryo, the region of the abducens nucleus (dotted outline) can be identified by its stereotypic relationship with the axons of the genu of the facial nucleus (arrow; devoid of NeuN expression) and the expression of ChAT (A,C). In the *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryo, the loss of ChAT expression in the region normally occupied by the abducens nucleus is not associated with the loss of NeuN-expressing neurons and the formation of the genu (B,D). (E-J) Transverse section through the proximal lateral rectus muscle in E18 control and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double mutant embryos labeled for TuJ1 (green) and α-bungarotoxin binding (αBTX, red). (I,J) Merged images of E,G and F,H, respectively.

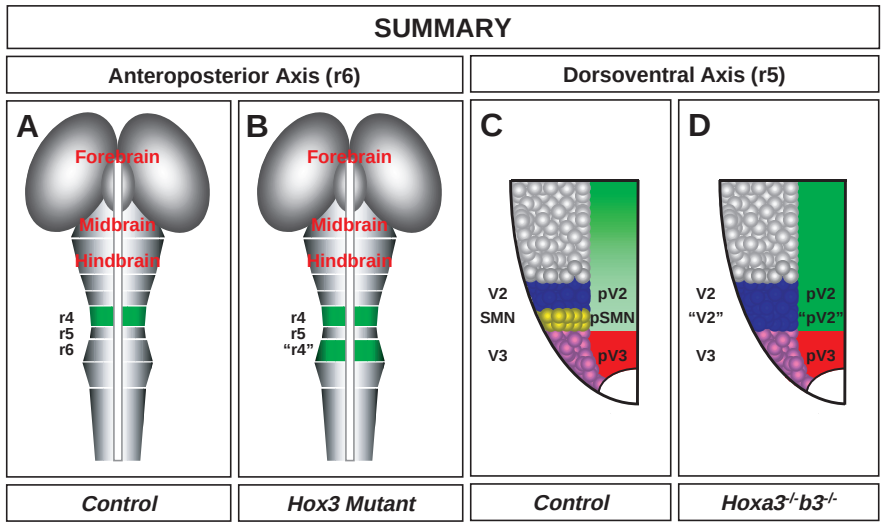


Fig. 7. The paralogous Hox3 genes play two crucial roles in neuronal fate specification. (A,B) Along the AP axis of the hindbrain, the paralogous Hox3 genes are required to suppress the expression of *Hoxb1* (green) in r6. The loss of Hox3 genes results in the ectopic expression of *Hoxb1* associated with the activation of r4-like facial BMN differentiation and migration program in r6 – characteristic of a homeotic transformation. Although the Hox3 genes do not influence r4 directly, the observation that it is required to genetically suppress the r4-program in r6 ensures that r4 maintains its unique identity. The Hox3 genes thus influence the identities of at least r4, r5 and r6 during hindbrain development. (C,D) Along the DV axis of r5, the combined functions of *Hoxa3* and *Hoxb3* are necessary for the specification of somatic motoneuron progenitors (pSMN) of the abducens nucleus. Mutations of *Hoxa3* and *Hoxb3* are associated with the ectopic expression of the more dorsal high expression of the Pax6 V2 interneuron progenitor (pV2) domain (dark green) into more ventral the pSMN domain (light green). Subsequently, V2 interneurons are ectopic in the domain normally occupied by SMNs.

Hox-regulated genetic suppression is an evolutionary conserved process required for segmental identity

Hox gene loss-of-function and transgenic regulatory analyses have so far revealed positive regulatory roles for Hox genes in neuronal subtype specification, segmental patterning and crossregulatory interactions between Hox genes during hindbrain patterning (Gavalas et al., 1998; Maconochie et al., 1997; Manzanares et al., 2001; Mark et al., 1993; McClintock et al., 2002; Popperl et al., 1995; Rossel and Capecchi, 1999; Studer et al., 1998; Studer et al., 1996). Single and compound mutations in Hox genes have demonstrated their requirement for the survival of AP-restricted populations of cells to patterning of entire segments of the hindbrain. These experiments provide ample evidence for the role of Hox genes in general cell survival and the cellular diversification within AP-restricted segments. However, they do not sufficiently explain how the Hox genes regulate identity amongst functionally related groups of neurons (i.e. motoneurons) along the AP axis of the neural tube.

Work in *Drosophila*, however, has provided insight into how the Hox genes may use the mechanism of genetic suppression to determine segmental identity (Hafen et al., 1984; Lewis, 1978; Struhl and White, 1985; Weatherbee et al., 1998). For example, *Ubx* normally suppresses multiple genes in the wing developmental pathway within the metameric segment that gives rise to the haltere such that in the *Ubx* mutant these genes are de-suppressed and the more posterior haltere is transformed

into the more anterior wing (Weatherbee et al., 1998). The suppression of the wing program by *Ubx* is analogous to our current observation of the r6-specific suppression of the facial BMN specification program by the Hox3 genes. This important crossregulatory phenomenon among members of the Hox3 and Hox1 paralogous members ensures the individual identity of rhombomeres. Interestingly, the observation that *Hoxb1* is suppressed specifically in r6 may reflect a developmental ground state common amongst even-numbered rhombomeres. Thus, as in other serially homologous structures, the periodicity of even- and odd-numbered rhombomeres exhibits common functions (Casares and Mann, 2001; Lumsden, 1990; Trainor and Krumlauf, 2000; Waskiewicz et al., 2002).

Hox3 genes are upstream of *Pax6*- and *Olig2*-dependent somatic motoneurons

The phenotypes of mice harboring independent mutations for the Hox3 paralogs *Pax6* and *Olig2* suggest that these genes are part of a common regulatory network necessary for determining the fate of SMNs (Ericson et al., 1997; Lu et al., 2002; Osumi et al., 1997; Takahashi and Osumi, 2002; Zhou and Anderson, 2002). Although these genes are necessary for the specification of SMNs, the regulatory process by which they attain this goal is qualitatively different. As described in this study and in a previous report, Hox3 genes and *Pax6* are upstream of *Olig2* expression (Novitch et al., 2001). In the loss of *Hoxa3* and *Hoxb3*, however, *Pax6* is expressed at ectopically high levels in the pSMN domain, suggesting that *Hoxa3* and *Hoxb3* genetically suppress *Pax6* expression levels in the pSMN domain. Our previous report also demonstrated the de-suppression of *Pax6* expression in the *Nkx2.2*-expressing BMN progenitor domain in r4 of *Hoxb1* mutant embryos (Gaufo et al., 2000). These observations are analogous to the role of the *Drosophila* Hox gene, *Antp*, in suppressing the activity of *eyeless*, the homologue of *Pax6* in vivo and in vitro (Plaza et al., 2001). However, a direct interaction between the mammalian Hox and *Pax* genes remains to be tested.

In contrast to the combined loss of *Hoxa3* and *Hoxb3*, the *Pax6* mutation results in the expansion or de-suppression of the ventral *Nkx2.2* pV3 domain into the more dorsal SMN progenitor domain in r5 (data not shown) (Ericson et al., 1997; Takahashi and Osumi, 2002). Consequently, the loss of Hox3 and *Pax6* genes leads to ectopic differentiation of V2 and V3 interneurons, respectively. The comparison of the Hox3 and *Pax6* genes demonstrates that they are necessary for the formation of SMNs, but they differ significantly at the level of specifying SMN progenitors. Genetically, Hox3 genes suppress the dorsal *Pax6* pV2 domain in the more ventral pSMN domain, whereas *Pax6* suppresses the ventral *Nkx2.2* pV3 domain in the more dorsal pSMN domain. In contrast to *Pax6*, the functions of *Olig2* appear similar to *Hoxa3* and *Hoxb3* in its capacity to specify pSMNs. In both *Olig2*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ mutant embryos, the loss of SMNs is associated with the de-suppression of *Pax6* and the subsequent ectopic differentiation of V2 interneurons (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). In both mutants, the more ventral *Nkx2.2*-expressing V3 progenitor domain appears unaffected, unlike that observed in the *Pax6* mutant. The functional similarities between the Hox3 and *Olig2* genes with respect to the specification of SMN progenitors in r5 suggest interactions between the pathways mediated by these genes.

The observation that the gene-dose dependent loss of *Hoxa3* and *Hoxb3* strongly correlates with the loss of Olig2/Hb9 and ectopic Chx10 expression suggests a direct role in SMN fate decisions. Furthermore, the expression of the Hox3 genes among progenitors and differentiating neurons in r5 (Fig. 1F-H, Fig. 4I) supports a possible role for Hox3 genes at later stages of motoneuron differentiation. This hypothesis has received additional support by the recent report that *Hoxa3* gain-of-function in chick r1-r4 is sufficient to generate SMNs in these rhombomeres (Guidato et al., 2003). Roles for the Hox3 genes at different stages of motoneuron specification would be consistent with the observation for the multi-level developmental regulation of the *Drosophila* wing by the Hox gene, *Ubx* (Weatherbee et al., 1998). However, the question remains whether the effect of Hox3 gene mutations is direct or results from a gradual titration of r5 to a more anterior rhombomere – a hallmark of a homeotic transformation. The present use of conditional mouse models should clarify more precisely the role of the Hox3 genes on distinct stages of motoneuron patterning.

A general function for paralogous Hox genes in the coordination of activation and suppression along the anteroposterior axis

The 13 mammalian Hox paralogs, each containing two to four genes, are expressed in a nested pattern along the AP axis of the neural tube, from the caudal-most tip of the spinal cord to the level of r2 of the hindbrain (Davenne et al., 1999; Economides et al., 2003). To date, the only published reports addressing the role of paralogous Hox genes on neuronal patterning have been the knockouts of the paralogous Hox1 and Hox2 genes (Davenne et al., 1999; Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). The characterization of the Hox1 paralogous mutants, *Hoxa1* and *Hoxb1*, highlights the basic requirements for these genes in normal hindbrain patterning (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). During early hindbrain patterning, prior to the closure of the roof plate, *Hoxa1* and *Hoxb1* are involved in establishing the AP-restricted identities of at least rhombomeres 3, 4 and 5. Equally important to this role is the requirement for *Hoxa1* and *Hoxb1* in cell proliferation and survival. In the absence of *Hoxa1* and *Hoxb1*, the hindbrain undergoes abnormal programmed cell death associated with deletions of multiple rhombomeres and subsequent reorganization (Rossel and Capecchi, 1999). The deletion of multiple hindbrain segments precludes analysis of later patterning events associated with the specification of a multitude of neuronal subtypes along the DV axis.

In contrast to the paralogous Hox1 mutants, the numbers and periodicity of rhombomeres appear to be normal in paralogous Hox2, *Hoxa2* and *Hoxb2* mutant embryos (Davenne et al., 1999). The normal features of the rhombomeres thus allow for the examination of later events in neuronal patterning. Indeed, the analysis of Hox2 mutant embryos led to the discovery that the program mediated by Hox genes along the AP axis appear to influence later patterning events along the DV axis. The present study, however, defines the possible mechanisms by which Hox genes may control distinct aspects of AP and DV patterning. For example, the functions of *Hoxa3* and *Hoxb3* appear to influence early neuronal fate decisions by regulating a developmental program common with a SMN-specific

determinant, *Olig2* (Lu et al., 2002; Novitch et al., 2001; Takebayashi et al., 2002; Zhou and Anderson, 2002). However, owing to the extended expression of the Hox3 genes during embryogenesis, their precise role in neuronal specification remains to be characterized. Nevertheless, the direct or indirect regulation of *Olig2* expression, a putative transcriptional repressor, by the early actions of *Hoxa3* and *Hoxb3* highlights the complex interplay between mechanisms of activation and suppression in the progressive specification of motoneurons.

The role of the paralogous Hox1, Hox2 and Hox3 groups in the positive regulation of segmental formation and DV patterning programs explain only in part the genetic mechanism by which segmental neuronal identity is achieved (Davenne et al., 1999; Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). Combined with the observations revealing the role of Hox genes in genetic suppression along the AP axis, a general mechanism emerges of how neuronal identity is acquired. Remarkably, the fundamental principles of Hox gene function involved in this developmental process are programmed within a single paralogous Hox group (where a distinct combination, a 'Hox code', may perform distinct roles). The functions mediated by the Hox3 genes in r5 and r6 may represent a phenomenon reiterated by other Hox paralogs along the entire AP axis of the neural tube. Ultimately, this developmental process could lead to the unique identities of neurons along the entire AP axis of the embryo.

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