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Hoxb1 controls effectors of sonic hedgehog and Mash1 signaling pathways

Gary O. Gaufo, Per Flodby* and Mario R. Capecchi‡

Howard Hughes Medical Institute, Department of Human Genetics, University of Utah, Salt Lake City, UT 84112, USA
*Present address: Karolinska Institute, Department of Medical Nutrition, Novum, SE-141 86 Huddinge, Sweden
‡Author for correspondence (e-mail: mario.capecchi@genetics.utah.edu)

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SUMMARY

The diverse neuronal subtypes in the adult central nervous system arise from progenitor cells specified by the combined actions of anteroposterior (AP) and dorsoventral (DV) signaling molecules in the neural tube. Analyses of the expression and targeted disruption of the homeobox gene *Hoxb1* demonstrate that it is essential for patterning progenitor cells along the entire DV axis of rhombomere 4 (r4). Hoxb1 accomplishes this function by acting very early during hindbrain neurogenesis to specify effectors of the sonic hedgehog and Mash1 signaling pathways. In the absence of Hoxb1 function, multiple neurons normally specified within r4 are instead programmed for early cell death. The findings reported here provide evidence for a genetic cascade in which an AP-specified transcription factor, Hoxb1, controls the commitment and specification of neurons derived from both alar and basal plates of r4.

Key words: Hox genes, Hindbrain neurogenesis, Motoneurons, Mouse, *Hoxb1*

INTRODUCTION

The vertebrate hindbrain coordinates multiple complex functions, including somatic and visceral motor activities and the processing of sensory information. Formation of the neural circuits underlying these functions depends on the generation of distinct populations of neuronal subtypes that constitute these circuits (Altman and Bayer, 1980; Carpenter and Sutin, 1983; Paxinos, 1995; Ramón y Cajal, 1995). The molecular and cellular mechanisms that spatially and temporally specify these neurons are beginning to be identified. This advance has largely been made possible by assigning the plethora of neurally expressed transcription factors and receptor-ligand signaling systems to coherent molecular pathways that control the formation of the neuronal subtypes (Lumsden, 1990; Tanabe and Jessell, 1996; Kageyama and Nakanishi, 1997; Flanagan and Vanderhaeghen, 1998; Sasai, 1998; Edlund and Jessel, 1999).

Genes belonging to the Hox complex constitute one component of this molecular network. Gain- and loss-of-function analyses have identified Hox genes that are important to the regional specification of the hindbrain into compartmental units called rhombomeres (r) (Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996; Capecchi, 1997). Hox genes have been implicated both in the process of rhombomere formation as well as in the subsequent specification of cell identities within rhombomeres. For example, while loss-of-function mutations in *Hoxa1* result in the failure to form specific rhombomeres, disruption of *Hoxb1* leads to a failure to specify distinct motoneurons within r4 (Carpenter et al., 1993; Mark et al., 1993; Goddard et al., 1996; Studer et al., 1996; Pata et al., 2000). Gain-of-function experiments have also been informative. For example, ectopic expression of either *Hoxb1* or *Hoxa2* in r1 results in the apparent mis-specification of neurons within this non-Hox-expressing rhombomere to acquire characteristics normally associated with r4 or r2 branchiomotor neurons, respectively (Bell et al., 1999; Jungbluth et al., 1999). Although the data linking Hox genes to hindbrain neurogenesis is compelling, the ways in which these genes interact with the other molecular pathways that define neuronal subtypes has not been delineated.

We have examined the interaction of the *Hoxb1* mutation with two principal molecular pathways mediating neuronal subtype specification, the sonic hedgehog (Shh) and the Mash1 (Ascl1 – Mouse Genome Informatics)/Ngn signaling pathways. The former is required for motor and interneuron specification in the ventral neural tube (Ericson et al., 1996, 1997; Pierani et al., 1999), whereas the latter transcription factors are involved in the specification of very early neural progenitors, principally at the ventricular surface of the neural tube (Gradwohl et al., 1996; Lee, 1997; Ma et al., 1997). We have also established an epistatic relationship between *Hoxb1* and *Phox2b*/*Phox2a* (*Arix*/*Pmx2b* – Mouse Genome Informatics) in r4, the latter of which are required for specification of the branchial and visceral motoneurons in the brainstem (Pattyn et al., 2000).

Mash1 and Ngns are homologues of the basic helix-loop-helix (bHLH) *Drosophila acheate-scute* complex and *atonal*-like proneural genes (Jan and Jan, 1994; Ma et al., 1996, 1997; Anderson and Jan, 1997). In the mouse peripheral nervous system, *Mash1* and Ngns foster developmental programs that specify distinct neuronal subtypes. Thus *Mash1*-deficient mice lack peripheral neurons of the noradrenergic lineage

(Guillemot et al., 1993; Hirsch et al., 1998), whereas *Ngn1* (*Neurod3* – Mouse Genome Informatics) and *Ngn2* (*Atoh4* – Mouse Genome Informatics) mutants lack sensory ganglia (Fode et al., 1998; Ma et al., 1998, 1999). Within the central nervous system (CNS), these mammalian proneural genes are expressed in complementary as well as overlapping neural progenitor domains, suggesting that in the CNS they function in a more complex, combinatorial fashion with each other, compared to their distinct roles in peripheral neurogenesis.

The Shh-signaling pathway has been elegantly demonstrated to play a pivotal role in dorsoventral (DV) patterning of neurons within the spinal cord (Tanabe and Jessell, 1996; Davenne et al., 1999). In the ventral neural tube, the activity of Shh is graded ventral^{high} to dorsal^{low}, and is absolutely required for the induction of floor plate cells, motoneurons and interneurons (Chiang, 1996; Ericson et al., 1996, 1997; Briscoe et al., 1999; Pierani et al., 1999). In response to Shh, cells of the ventral neural tube differentiate into progenitors expressing the homeodomain transcription factors Nkx2.2 (Nkx2-2 – Mouse Genome Informatics) or Pax6. The progeny of these ventral progenitors express specific Lim-homeodomain transcription factors during their progressive differentiation into motor neurons or ventral interneurons, thus acquiring distinct Lim-homeodomain codes (Tsuchida et al., 1995; Pfaff, 1996; Tanabe and Jessell, 1996; Briscoe et al., 2000). The spatiotemporal expression pattern of Mash1 and Ngns suggests that they may coordinate with Nkx2.2 or Pax6 to specify distinct neural progenitors along the DV axis of the neural tube.

The molecules involved in neural determination and Shh signaling pathways are expressed along the full extent of the anteroposterior (AP) axis. This makes unlikely the involvement of these pathways in conferring distinct identities on the neural progenitors at specific AP levels. However, the AP-restricted expression patterns of Hox genes (Dollé et al., 1989; Graham et al., 1989), their ability to specify distinct neural subtypes and their correct spatiotemporal expression patterns during hindbrain neurogenesis make them ideal candidates for interacting with these pathways to confer AP identity on the neuronal progenitor cells (Davenne et al., 1999; this study). In this study we provide evidence that within r4 there is a requirement for Hoxb1 by both the Mash1/Ngn and Shh signaling pathways. Hoxb1 is needed to correctly specify early progenitor cells along the DV axis of r4 and subsequently for normal neuronal differentiation along the ventriculopial (VP) axis, the third coordinate of the developing neural tube.

MATERIALS AND METHODS

Targeting vector and generation of *Hoxb1^{GFP}* homozygous mice

In order to allow continuous monitoring of *Hoxb1* expression in live embryos and embryonic tissues, we generated an allele of *Hoxb1*, in which the reporter gene for green fluorescent protein (GFP), was targeted inframe into the *Hoxb1*-coding sequence (Fig. 1a and b). The genomic DNA used for vector construction was isolated from a 129Sv mouse library in lambda FIX II (Stratagene) (Goddard et al., 1996). A 12.3 kb segment of genomic sequence containing the *Hoxb1* locus was included in the targeting vector. A 2.25 kb *Sal*I fragment, derived from the vector pEGFPKT1LOXNEO (Godwin et al., 1998), including the gene coding for GFP sequence (*EGFP*, Clontech), followed by a *loxP*-flanked *neomycin*-resistance gene, was cloned into the unique *Eag*I site in exon I by blunt-end ligation, placing *EGFP* in frame with the *Hoxb1* gene (Fig. 1a). The targeting vector was linearized with *Xho*I and electroporated into R1 ES cells (Nagy et al., 1993). After double selection with G418 and FIAU (Mansour et al., 1988), surviving clones were analyzed by Southern blot. To identify homologous recombinants, a 0.6 kb 5′ flanking probe was used with *Bam*HI-digested DNA (Fig. 1b). To show that no random integration of the targeting vector had occurred and that only one copy of the *neo* gene was present, as a result of homologous recombination, a *neo* probe (a 0.75 kb *Pst*I fragment) was used to hybridize digested genomic DNA (data not shown). Positive clones were subsequently injected into C57BL/6J (BL6) blastocysts and the resulting chimeric males bred with BL6 females. Offspring harboring the targeted allele was identified by Southern blot using the same probe as described above (data not shown). A *neo*-resistance cassette, flanked by *loxP* sites, was located 3′ to the fusion gene.

Previously, we have encountered several examples where removal of the *neo* gene has been critical for appropriate expression of the reporter gene and/or to obviate problems associated with interference of the *neo* gene with the expression of neighboring genes (Barrow and Capecchi, 1996; Olson et al., 1996). For this reason, the *loxP*-flanked *neo* selection marker in the *Hoxb1^{GFPneo}* allele was removed by *Cre/loxP*-mediated recombination in vivo. This was accomplished by crossing females heterozygous for the *Hoxb1^{GFPneo}* allele with male mice transgenic for the *Cre*-deleter gene (Schwenk et al., 1995). The offspring from these intercrosses were screened for excision of the *neo* gene by PCR. The following neo primers, resulting in a 355 bp product, were used: 5′GTGCTCGACGTTGTCACTGAAG3′ (forward primer) and 5′CCATGATATTCGGCAAGCAGGC3′ (reverse primer). The PCR conditions were one cycle at 94ºC for 1 minute, then 94ºC, 30 seconds; 60ºC, 20 seconds; 72ºC, 1 minute for 28 cycles and finally 72ºC for 7 minutes. The *neo*-less *Hoxb1^{GFP}* mice were then backcrossed to BL6 mice for two generations. Heterozygous *Hoxb1^{GFP}* mice were subsequently intercrossed, which produced wild-type, heterozygous, and homozygous littermates in the expected Mendelian ratio. The mice were genotyped by PCR using a set of three primers. These were as follows: *Hoxb1* sense primer, 5′AGCGCCTACAGCGCCCCAACCTCTTTT3′ (nucleotides 153-179, upstream of the *Eag*I cloning site); *Hoxb1* antisense primer, 5′CTTGACCTTCATCCAGTCGAAGGTCCG3′ (nucleotides 615-64, downstream of the *Eag*I cloning site) (Frohman et al., 1990) and GFP antisense primer, 5′ATGGTGCGCTCCTGGACGTAGCCTT3′. The PCR conditions were one cycle at 94°C for 1 minute and then 94ºC, 30 seconds; 60ºC, 30 seconds; 68ºC, 2 minutes for 30 cycles. The wild type allele product was 489 bp and the Hoxb1GFP allele product was 353 bp (Fig. 1c). The phenotype of mice homozygous for the *Hoxb1^{GFP}* mutation was indistinguishable from mice homozygous for our previously described mutant alleles of *Hoxb1* (Barrow and Capecchi, 1996; Goddard et al., 1996; Rossel and Capecchi, 1999).

Fluorescence imaging

Embryos used for detection of GFP fluorescence were the progeny of *Hoxb1^{GFP}*-heterozygous intercrosses or crosses between *Hoxb1^{GFP}* homozygous males and C57BL/6J females. Embryos were harvested between embryonic days 8.5 and 14.5 (E8.5-E14.5), and maintained at room temperature in Leibovitz's L-15 medium during imaging with a Bio-Rad MRC 1024 Laser Scanning Confocal Imaging System connected to a Leitz Aristoplan microscope. *Hoxb1^{GFP}* homozygotes do not produce GFP protein in the hindbrain after E8.0 because maintenance of *Hoxb1* expression in r4 requires Hoxb1 autoregulation (Pöpperl et al., 1995).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in PBS and processed for single- or double-immunolabeling as transverse sectioned tissues. After immunolabeling, sections were then analyzed by confocal

microscopy. Antibodies were used at the following dilutions: rabbit polyclonal anti-Hoxb1 (Covance, Berkeley, CA), 1:200; mouse monoclonal anti-Shh, anti-HNF3β, anti-Pax6, anti-Nkx2.2 and anti-Isl1 (Developmental Hybridoma), 1:20; mouse monoclonal anti-Mash1, 1:20 (D. J. Anderson); and rabbit polyclonal anti-Phh3 (Upstate Biotechnology, Waltham, MA). Fluoroscein-, Texas Red-, or Cy5-conjugated secondary antibodies were obtained from Jackson Immunoresearch (Westgrove, PA).

Analysis of apoptosis

The TUNEL assay was used to detect apoptotic cell death in fixed, frozen transverse sections of E9.0-E12.0 embryos following the manufacturer's protocol (Roche).

In situ hybridization

Whole-mount RNA in situ hybridization was performed as previously described (Manley and Capecchi, 1995; Goddard et al., 1996).

RESULTS

Visualization of Hoxb1-expressing neuronal columns during hindbrain development

The dynamic changes in *Hoxb1* expression were monitored in live mouse embryos using a targeted allele of *Hoxb1* in which the vital reporter gene for GFP, was fused, in frame, with the first protein encoding exon of this gene (Fig. 1; for details see Materials and Methods). The sensitivity of the Hoxb1^{GFP} reporter allowed detection of cell body migratory processes and axon-specific labeling of r4-derived neurons in live embryonic tissues. In the present study we used the Hoxb1^{GFP} allele to monitor the spatiotemporal appearance of *Hoxb1*-positive neuronal columns during hindbrain development.

At E8.5 (six somites), Hoxb1^{GFP} was highly expressed along the entire AP neural axis up to the level of the presumptive r3-r4 boundary. Dorsal views show that Hoxb1^{GFP} was expressed uniformly in r4 (Fig. 2a, arrowheads). At this stage, reconstruction of the hindbrain from 5 μm sections showed that all discernible nuclei in r4, approximately 1000, express Hoxb1 (data not shown). Hoxb1^{GFP} could also be seen in a more anterior domain (r3) than has been previously reported (Fig. 2a, arrow) (Murphy et al., 1989; Frohman et al., 1990; Murphy and Hill, 1991). At the posterior boundary of r4, a transverse band of cells not expressing Hoxb1^{GFP} could be seen (Fig. 2a, lower arrowhead). This reflects the onset of the r4-restricted expression of *Hoxb1* seen in later stages.

By E9.5, the hindbrain neural tube has fully closed. In order to visualize *Hoxb1* expression in the ventricular neuroepithelium, a dorsal midline dissection along the AP axis of the hindbrain was made, and the neural tube was splayed open (flat mount) exposing the ventricular surface. At E9.5 (21 somites), two distinct Hoxb1^{GFP}-expressing columns, corresponding to the basal (ventral) and the alar (dorsal) plates of r4, were visible on each side of the neural tube (Fig. 2b). In slightly older embryos (E9.5, 27 somites), the formation of a Hoxb1^{GFP}-expressing intermediate column could be seen (Fig. 2c). To confirm that the Hoxb1^{GFP} signal accurately reflects the expression of endogenous Hoxb1 protein, immunolabeling of E10.5 and E11.5 wild-type mice was performed using a Hoxb1-specific polyclonal antibody (Goddard et al., 1996). At E10.5, the ventral, intermediate and dorsal columns, containing early differentiating neurons, were condensed, as compared

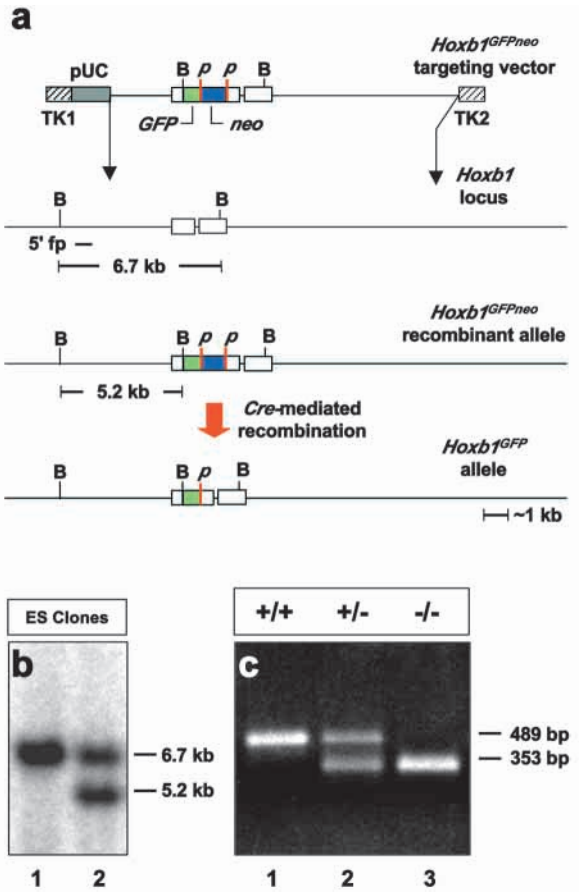

Fig. 1. Targeted disruption of the mouse *Hoxb1* gene . (a) Schematic representation of the *Hoxb1*^{GFPneo} targeting vector (top), *Hoxb1* locus (upper middle), *Hoxb1*^{GFPneo} recombinant allele (lower middle) and removal of *neo* by *Cre*-mediated recombination to produce *Hoxb1*^{GFP} allele (bottom). (b) Southern transfer analysis of *Bam*HI-digested genomic DNA from parental ES clone (lane 1) and an ES clone that has undergone homologous recombination (lane 2). Using a 5′ flanking probe (5′ fp), the wild-type gene is identified by a 6.7 kb *Bam*HI fragment, whereas the *Hoxb1*^{GFPneo} allele has a 5.2 kb fragment. (c) Genotype analysis of tail DNA by PCR from *Hoxb1*^{GFP} heterozygous crossing showing wild-type (lane 1, 489 bp band), heterozygous (lane 2, 489 and 353 bp bands) and homozygous (lane 3, 353 bp band) littermates. B, *Bam*HI; *p*, *loxP* site; *GFP*, green fluorescent protein gene; *neo*, neomycin resistance gene; TK, herpes simplex virus thymidine kinase gene.

with younger embryos (Fig. 2d and data not shown). Intense Hoxb1 labeling could also be seen in the anterior and posterior boundaries of r4. By E11.5, numerous Hoxb1-expressing columns containing later-born neurons had formed along the DV axis of r4 (Fig. 2e). Hoxb1^{GFP} was similarly expressed in live E10.5 and E11.5 *Hoxb1*^{GFP} heterozygous mice (data not shown).

In summary, continuous analysis of *Hoxb1* expression along the neural tube DV axis has shown that early in development, E8.0-E8.5, all of the cells in r4 express *Hoxb1*. Subsequent to this stage, *Hoxb1* expression first becomes concentrated to two zones within r4, the alar and basal plates, and then to a third, the intermediate zone. As neural development continues, more *Hoxb1*-expressing columns, of increasing refinement, become

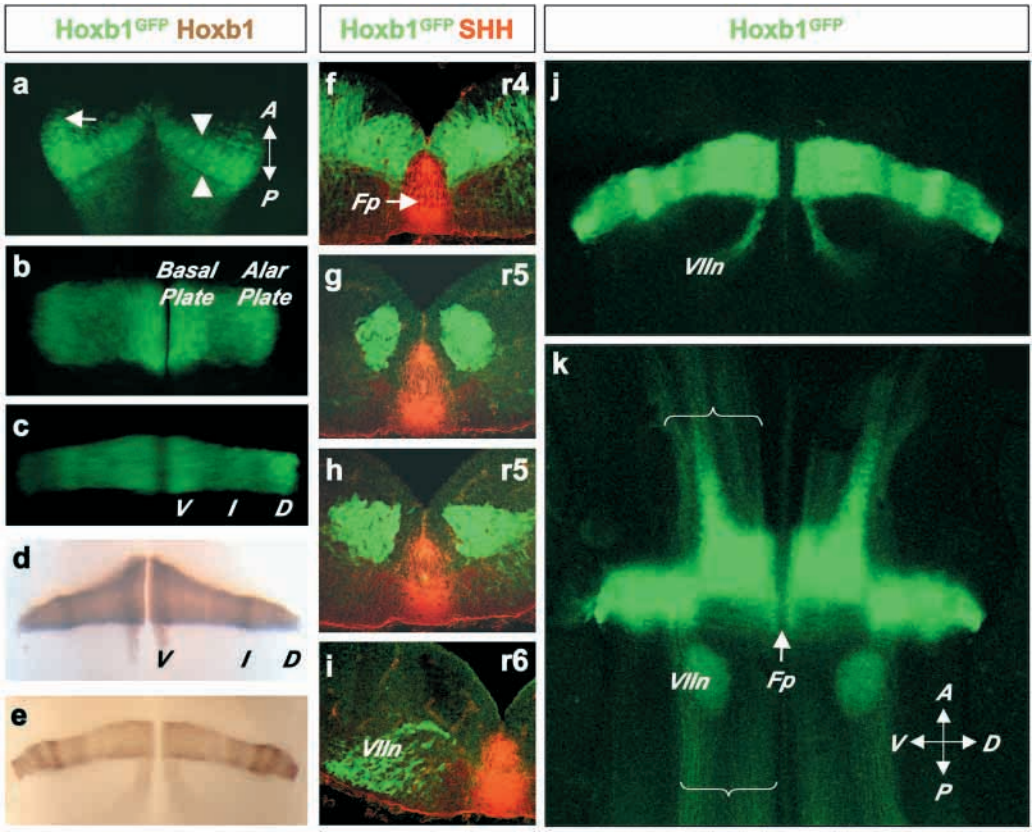

Fig. 2. Spatiotemporal appearance of Hoxb1-positive neuronal columns in r4. (a) Dorsal view E.8.5, six-somite embryo expressing Hoxb1^{GFP} uniformly in the presumptive r4 (between arrowheads). Hoxb1^{GFP} is also expressed in more anterior domains in the dorsal region of r3 (arrow). The Hoxb1^{GFP}-negative stripe below r4 (lower arrowhead) indicates the downregulation of Hoxb1^{GFP} expression in the posterior hindbrain region. (b) Ventricular view of E9.5, 21-somite embryo expressing Hoxb1^{GFP} in the ventricular neuroepithelium of r4. The hindbrain was split dorsally and splayed with the ventricular surface exposed to show Hoxb1^{GFP}-expressing ventral and dorsal columns corresponding to regions of the basal and alar plates, respectively. (c) A slightly older embryo (E9.5, 27 somites) exposing the ventricular neuroepithelium to show the appearance of a Hoxb1^{GFP}-expressing intermediate column. (d) Hoxb1-immunolabeling of E10.5 embryo shows three distinct ventral, intermediate and dorsal columns. Hoxb1-immunolabeling can also be seen in the anterior and posterior boundaries of r4 and in the posterior migrating branchiomotor neurons of the facial nucleus in ventral r5. (e) Hoxb1-immunolabeling of E11.5 embryo shows the appearance of more Hoxb1-expressing columns. (f-i) Transverse sections from AP levels spanning r4 to r6 of E11.5 *Hoxb1^{GFP}* heterozygous embryo demonstrating the developing facial nucleus. The sections were immunolabeled with anti-Shh (red) to distinguish the floor plate from the developing Hoxb1^{GFP}-expressing cells. Hoxb1^{GFP}-expressing progenitor and early differentiating cells in r4 are located immediately dorsolateral to the Shh-expressing floor plate (f). The posteriorly migrating Hoxb1^{GFP}-expressing neurons in r5 have taken a position lateral to the ventral progenitor domain that juxtaposes the floor plate (g,h). In r6, the prospective upper medulla, Hoxb1^{GFP}-positive neurons of the facial nucleus have taken a ventrolateral position in the marginal layer of the neuroepithelium (i). (j,k**)** Ventricular and pial views of Hoxb1^{GFP}-labeled live, E12.5 embryonic tissue. Ventricular view of the hindbrain shows r4-specific neuronal columns and the posterior migrating neurons of the facial nucleus (j). Pial view of the same embryo in j shows extensive migration along the AP and DV axes of r4-derived Hoxb1^{GFP}-labeled neurons (k). The facial nucleus (VIIn) and the axonal projections (brackets) of r4-derived neurons are also labeled by Hoxb1^{GFP}. Hoxb1^{GFP}-labeled, commissural axons are also visible traversing at the level of the floor plate (arrow). A, anterior; D, dorsal; Fp, Floor plate; I, intermediate; P, posterior; r, rhombomere; V, ventral; VIIn, facial nucleus.

apparent. The segregation of increasing numbers of *Hoxb1*-expressing columns parallels the concomitant formation of increasing numbers of neuronal subtypes within r4 (Taber Pierce, 1973; Marin and Puelles, 1995).

Migration pattern of the facial branchiomotor neurons

A very prominent population of neurons that are specified within r4 consist of those that innervate the muscles of facial expression, the facial branchiomotor (FBM) neurons (Goddard et al., 1996; Studer et al., 1996). However, in newborn animals these motoneurons are located within the ventrolateral region of the r6-derived upper medulla. The migration pattern of these motoneurons from r4 to the ventrolateral region of r6 is shown in Fig. 2f-i by a series of transverse sections of the hindbrain of E11.5 heterozygous *Hoxb1^{GFP}* embryos. Sections from levels spanning r4 to r6 were immunostained with anti-Shh to distinguish Hoxb1^{GFP}-expressing cells (green) from the Shh-expressing floor plate (red). In ventral-r4, Hoxb1^{GFP} expression was primarily observed in the ventricular and mantle layers of the neuroepithelium, juxtaposed to the floor plate (Fig. 2f). This region contains proliferating progenitor and early differentiating neurons, respectively. However, a small population of Hoxb^{GFP}-expressing neurons was visible in the marginal layer that contained more differentiated neurons. As Hoxb1^{GFP}-expressing neurons migrated posteriorly into r5, they formed a distinct cluster in the mantle layer immediately lateral to the ventral progenitor domain (VPD). The VPD is a region containing progenitor cells juxtaposed to the Shh-expressing floor plate (Fig. 2g,h; defined molecularly in the next section). At the level of r6, the Hoxb^{GFP}-expressing FBM neurons took a lateral course into the marginal layer of the neuroepithelium to contribute to the formation of the facial nucleus (VIIn, Fig. 2i).

At E12.5, the expression of Hoxb1^{GFP} persisted in columns

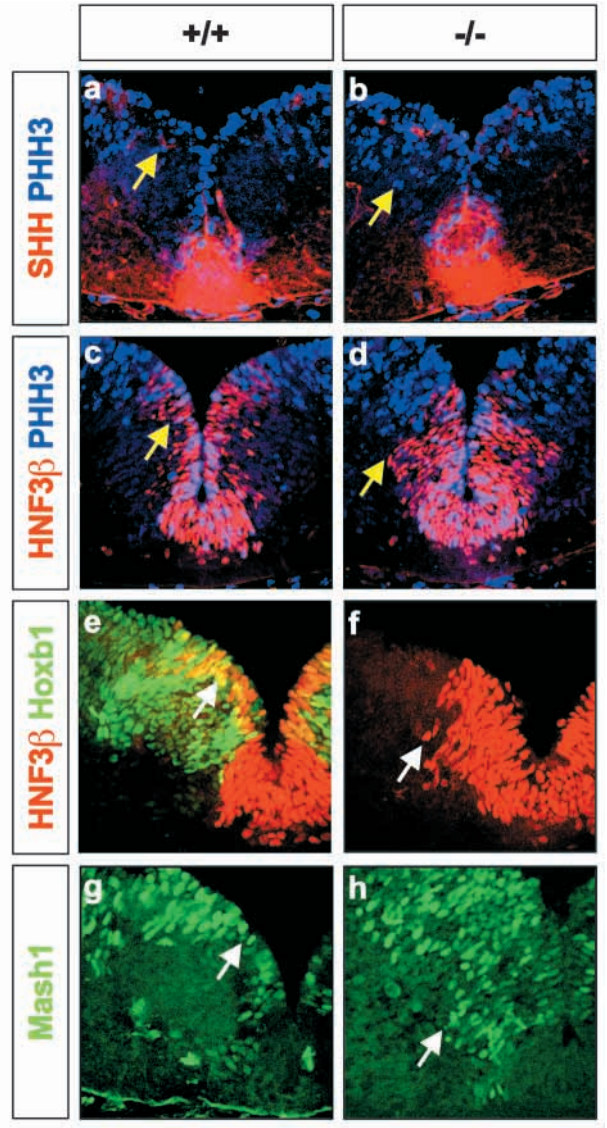

Fig. 3. Cytoarchitecture of r4-specific ventral progenitor domain is disrupted in *Hoxb1* mutant mice. (a,b) In transverse sections of E11.5 embryos, the expression of phosphorylated histone H3 (Phh3, blue) is observed among actively dividing progenitor cells in ventral r4 and in the inner layers of Shh-expressing floor plate cells (red). In control mice, Phh3 is restricted to the innermost neuroepithelial layer of the of the ventral progenitor domain (VPD); whereas in the dorsal region, Phh3 is expressed in cells extending into the mantle layer (compare similarities with Mash1 expression in g). In *Hoxb1* mutant mice, Phh3-expressing progenitor cells in the VPD cells have expanded into the mantle layer. At this level of analysis, it is difficult to detect appreciable changes in the expression of Shh in the floor plate between control and *Hoxb1* mutant mice. (c,d**)** In E11.5 control embryos, HNF3β-immunolabeling (red) shows two distinct, expression domains: a ventral domain corresponding to the floor plate and a more dorsal region corresponding to the VPD, which co-expresses PHH3 (blue). In *Hoxb1* mutant mice, HNF3β-expressing progenitor cells in the VPD are reduced and have expanded into the mantle layer of the neuroepithelium. (e,f**)** In E11.5 control embryos, Hoxb1-positive progenitor cells (green) are co-expressed with the dorsal domain of HNF3β (red). In *Hoxb1* mutant mice, HNF3β-expressing progenitor cells have expanded into the mantle layer formerly occupied by Hoxb1-expressing FBM neurons. (g,h**)** In E11.5 control embryos, Mash1 (green) expression is restricted to the inner ventricular neuroepithelium of the VPD. In *Hoxb1* mutant mice, Mash1-positive progenitor cells have expanded into the mantle layer.

arrayed along the DV axis of the ventricular neuroepithelium (Fig. 2j). Furthermore, Hoxb1^{GFP} expression could still be seen among posteriorly migrating FBM neurons located in the deeper mantle layer. The morphology of migrating neurons in r5 was pyramidal, with their apical dendritic ends facing in the direction of their migratory path (data not shown). The extensive migration patterns of neurons derived from r4 can be visualized by exposing the pial surface of the hindbrain of E12.5, *Hoxb1^{GFP}* heterozygous embryos (Fig. 2k). In contrast to the ventricular layer, where Hoxb1^{GFP}-positive progenitor cells were restricted to an r4 region, the marginal layer contained differentiated Hoxb1^{GFP}-positive neurons, which were seen in r4 as well as in more anterior and posterior regions of the hindbrain. The migration pattern of the FBM neurons and their lack of migration in *Hoxb1* mutant homozygotes, has been well documented (Goddard et al., 1996; Studer et al., 1996; Pata et al., 2000). Here, we show that in addition to the FBM neurons, there was also extensive migration of non-branchiomotor, *Hoxb1*-expressing neurons rostral to r4. This observation provides an explanation of how disruption of *Hoxb1* results in perturbation of the organization of neurons in r3, a region that is rostral to the majority of *Hoxb1*-expressing cells (data not shown). By E14.5, the expression of *Hoxb1*, as detected by Hoxb1^{GFP} and confirmed by Hoxb1 immunostaining, continues to be observed in the developing hindbrain (data not shown). This continued expression suggests functions for Hoxb1 during later periods of hindbrain development.

Hoxb1 is required for specification of the ventral progenitor domain in r4

The most prominent feature of *Hoxb1* mutant homozygous mice is a failure to specify at least two pools of motoneurons: the FBM and contralateral vestibuloacoustic efferent (CVA) neurons (Goddard et al., 1996; Studer et al., 1996; Pata et al., 2000). In the spinal cord, specification of motoneurons is crucially dependent on Shh signaling (Chiang et al., 1996; Ericson et al., 1996, 1997; Briscoe et al., 1999; Pierani et al., 1999). To determine whether this signaling pathway is affected by the *Hoxb1* mutation, we examined the cellular organization of the floor plate in r4, using two floor plate-specific markers Shh and HNF3β (Foxa2 – Mouse Genome Informatics; Ruiz i Altaba et al., 1993; Weinstein et al., 1994; Chiang, 1996; Ericson et al., 1996). At all stages examined, the pattern of Shh expression in the r4 floorplate of homozygous mutants was indistinguishable from that of wild-type or heterozygous controls (Fig. 3a,b and data not shown). This is in contrast to what was observed for HNF3β expression. In the spinal cord and most regions of the hindbrain, HNF3β expression was restricted to the floor plate. In ventral r4, however, HNF3β was detected in two distinct domains (Fig. 3c-f and data not shown): the most ventral domain, corresponding to the Shh-expressing floor plate, and a more dorsal domain that co-expresses Hoxb1. We will refer to the more dorsal of the two HNF3β-expressing domains as VPD. This domain appears to be unique to r4 and may represent an important signaling center for this

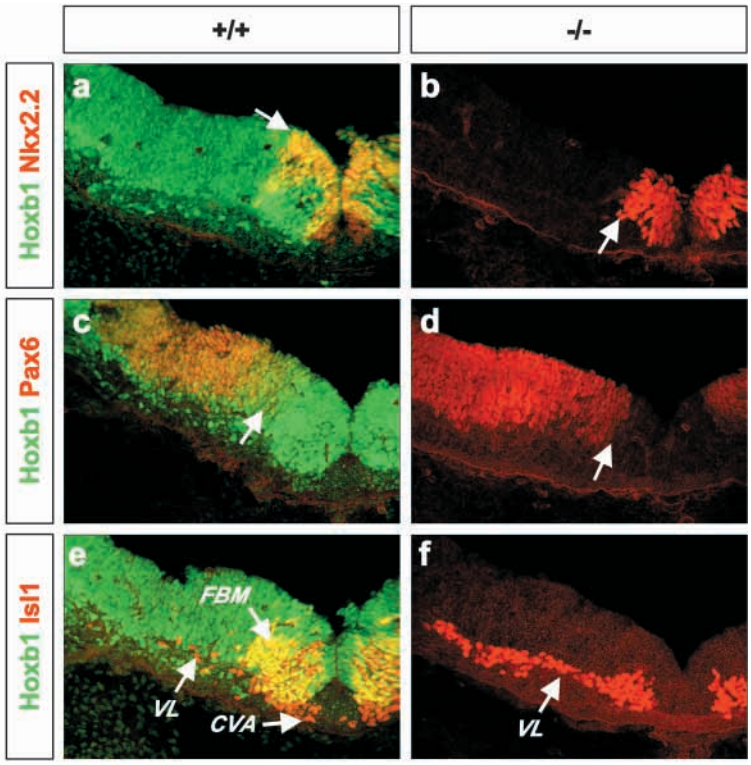

Fig. 4. Effectors of SHH-mediated DV patterning is defective in *Hoxb1* mutant mice. (a,b) Nkx2.2-positive progenitor cells (red) co-express Hoxb1 (green) in transverse sections of E11.5 control embryos. In r4 of control tissues, Nkx2.2-expressing progenitor cells (red) are confined to the inner layers of the ventral neuroepithelium juxtaposed to the floor plate. In *Hoxb1* mutant mice, Nkx2.2-expressing progenitors are reduced dorsally and have expanded into the mantle layer formerly occupied by postmitotic, Hoxb1-expressing neurons (arrows). (c,d) Pax6-positive progenitor cells (red) co-express Hoxb1 (green) in E11.5 control embryos. The Pax6-positive progenitor domain complements the more ventral Nkx2.2-positive VPD. In *Hoxb1* mutant mice, the progenitor domains of both Pax6 and Nkx2.2 are shifted ventrally (arrows). (e,f) In E11.5 control embryos, Isl1 (red) is expressed in at least three populations of cells in ventral r4: the facial branchiomotor motor (FBM) neurons, the contralateral vestibuloacoustic efferent (CVA) neurons, and a population of ventrolateral (VL) neurons. At this stage, Hoxb1 (green) is primarily co-expressed with Isl1-positive FBM. While the FBM and CVA are missing in *Hoxb1* mutant mice, the VL population has expanded dorsolaterally in the ventral region of r4.

rhombomere. Fig. 3c,g shows that this domain also expresses phosphorylated histone H3 (Phh3) and Mash1. Mash1 labels early neuronal progenitor cells, whereas Phh3 is a marker for actively dividing cells (Johnson et al., 1990; Gradwohl et al., 1996; Lee, 1997; Ma et al., 1997; Wei et al., 1999). As expected in wild-type embryos, Mash1 and Phh3-expressing cells were found close to the inner ventricular layer (i.e., the proliferative layer of the developing neural tube). Fig. 3d shows that the integrity of the VPD, the more dorsal HNF3β expression domain, requires Hoxb1 function, since in the absence of Hoxb1, the cytoarchitecture of this zone was disorganized. In *Hoxb1* mutant homozygous embryos, cells expressing Mash1 and Phh3, which are normally restricted to the ventricular layer, had expanded into the mantle layer, a region normally occupied by postmitotic, early differentiating neurons (Fig. 3b,d,f,h). This aberrant behavior of cells in *Hoxb1* mutant homozygotes suggests a deficiency in neuronal specification, such that cells leaving the ventricular layer have not appropriately turned off expression of these genes. Furthermore, as indicated by the continued expression of Phh3, these cells continue to divide aberrantly.

Changes in Nkx2.2, Pax6 and Isl1 expression in r4 of *Hoxb1* mutant mice

In the ventral spinal cord, the activities of the transcription factors Nkx2.2, Pax6 and Isl1 are required for interpretation of the floor plate intercellular signal, Shh (Ericson et al., 1996, 1997; Briscoe et al., 1999, 2000). These molecules are required for proper formation of motoneurons and ventral interneurons. As a further indication that the motoneuron population in r4 is not properly specified in *Hoxb1* mutant embryos, the normal expression pattern of these transcription factors is markedly perturbed by this mutation (Fig. 4).

In normal embryos at E11.5, approximately 18-20 layers of cells expressing Nkx2.2 could be viewed in transverse section (Fig. 4a). This figure shows such a section through the ventral region of r4. In *Hoxb1* mutant homozygotes, there was a loss of Nkx2.2 expression in the most dorsal aspect of this expression domain, which now occupies only approx. 12 cell layers (Fig. 4b). As described for Mash1 and Phh3, this transcription factor also remained active in cells that were leaving the ventricular surface and progressing towards the mantle layer. Moreover, the Pax6-expression domain expanded ventrally in *Hoxb1* mutant embryos into the region that formerly expressed Nkx2.2 (Fig. 4c,d). This observation is consistent with the reported role of Nkx2.2 as a negative regulator of Pax6 expression (Briscoe et al., 1999, 2000).

In the r4-region of E11.5 mouse embryos, Isl1 prominently labelled both the FBM and CVA (contralateral vestibuloacoustic) neurons, and a population of ventrolateral (VL) neurons scattered along a ventral-dorsal region of the mantle layer (Fig. 4e). Expression of this protein is one of the earliest postmitotic markers for motoneurons along the entire extent of the neural tube (Ericson et al., 1996, 1997; Pfaff, 1996). In *Hoxb1* mutant homozygous embryos, the two Isl1-labeled motor pools, FBM and CVA, were not observed, whereas the Isl1-labeled VL neuron population had increased in number and was displaced more dorsally (Fig. 4f and data not shown).

Increased cell death in *Hoxb1* mutant embryos

To determine how the two motoneuron pools (FBM and CVA) are lost in *Hoxb1* mutant mice, we performed TUNEL assays to assess possible loss via programmed cell death. In *Hoxb1* mutant mice, we observed a dramatic increase in apoptosis in r4 (Fig. 5a,b). This wave of ectopic cell death began at E9.5

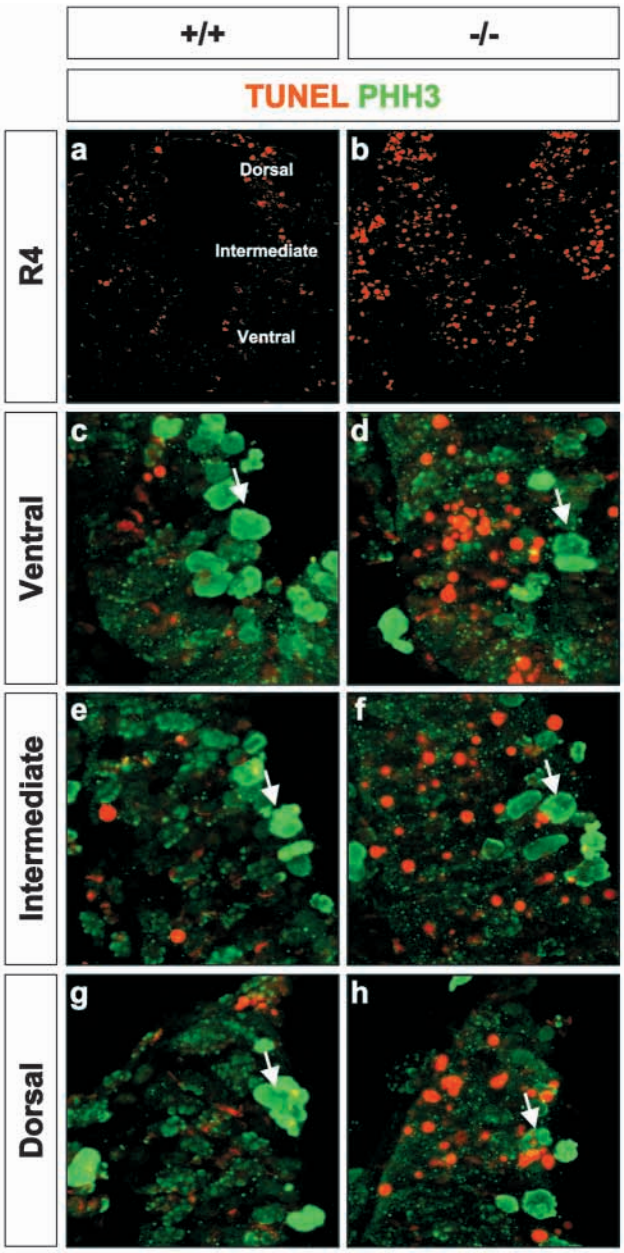

Fig. 5. Mutation of *Hoxb1* results in programmed cell death during early neurogenesis of r4. (a,b) The TUNEL assay was used to detect apoptotic cells (red) in transverse sections through r4 of E9.5 embryos. Apoptosis was observed sporadically in control tissues, whereas in *Hoxb1* mutant mice, programmed cell death was observed extensively throughout the entire DV region of the neuroepithelium in r4. (c-h) Detection of actively dividing cells with Phh3 (green) and apoptotic, TUNEL-positive cells (red) in the ventral, intermediate and dorsal regions through r4 of E9.5 embryos. In control tissue (c,e,g), Phh3-expressing cells are restricted to the inner ventricular neuroepithelium with few detectable TUNEL-positive cells. In *Hoxb1* mutant mice (d,f,h), apoptotic cells are detected throughout the neuroepithelium; more specifically, from the ventricular layer (arrows) to the marginal layer.

and was finished by E10.5. This increase in apoptosis in r4 of *Hoxb1* mutant embryos was not restricted to just the ventral aspect of the neural tube, where motoneurons are normally formed, it was also seen in intermediate and dorsal zones that coincide with the three zones normally occupied at this stage by cells expressing high levels of Hoxb1 (Fig. 2c,d). Fig. 5c-h illustrate parts of transverse sections through r4 at higher magnifications. These figures highlight the cellular detail of apoptosis occurring in these three zones in *Hoxb1* control and mutant embryos. The sections were also immunolabeled for Phh3 to delineate the distribution of dividing cells within these regions. From these figures, it is apparent that in *Hoxb1* mutants, cells are aberrantly dying in r4 throughout the extent of the neuroepithelium, from the inner ventricular to the outer pial surfaces. In particular, note that cells, at the position of the very early, still proliferating neural progenitors, are dying. This may explain the absence of Isl1-positive FBM and CVA neurons normally seen at later stages (Fig. 4e,f).

Hoxb1 is epistatic to *Phox2b* in r4

Pattyn et al. (2000) have recently demonstrated that the formation of the branchial and visceral motoneurons in the hindbrain is crucially dependent on the function of the paired-like homeodomain transcription factor, Phox2b. In mouse embryos homozygous for *Phox2b* loss-of-function mutations, the branchial and visceral motoneurons of the brainstem are not properly specified. These neurons do not express early postmitotic molecular markers common to motoneurons, such as *Isl1* and *Phox2a*. Moreover, they do not turn off early neuronal progenitor markers such as *Mash1* and *Nkx2.2*. Progenitors continue to divide as they migrate from the ventricular to the mantle layer, and they are programmed for cell death at E10.5. The aberrant cellular phenotype of the branchial motor neurons in *Phox2b* mutants is very similar to the mutant cellular phenotype described above for the r4-component of this motoneuron system in *Hoxb1* mutant embryos, suggesting possible involvement of these two transcription factors in a common genetic pathway.

Fig. 6 shows that Hoxb1 is epistatic to Phox2b for the formation of the FBM neurons. At the onset of Phox2b expression, E9.5 (Pattyn et al., 1997), the dorsal region of the r4 VPD domain was significantly depleted in mutant compared with control embryos, whereas the more ventral VPD maintained the expression of Phox2b (Fig. 6a,b). At this stage, the dorsal VPD was occupied by a significant number of TUNEL-positive cells (Fig. 6b). At a slightly later time point, E10.0, the entire VPD was almost completely devoid of Phox2b-expressing progenitor cells with significant numbers of TUNEL-positive cells occupying this region (Fig. 6c,d). After E10.5, few TUNEL-positive cells were detected in r4 of control and mutant embryos (data not shown). By E11.0-E11.5, the expression of Phox2b in the r4 VPD of mutant embryos was completely absent compared with control embryos (Fig. 6e-h). However, an expanded population of cells expressing Isl1 and/or Phox2b was observed in the marginal layer of ventral r4. The loss of Phox2b expression in the dorsal VPD was supported by a similar loss of Nkx2.2 expression in this progenitor pool (Fig. 6g,h). The expanded population of Nkx2.2-expressing progenitor cells in the ventral region of the VPD corresponded to an increase of Isl1- and Phox2b-expressing VL neurons (Fig. 6e-h). The overall requirement along the DV axis of r4 neuronal columns expressing Phox2b and its effector, Phox2a, on Hoxb1 is demonstrated in Fig. 6i-l. From these flatmount preparations, it is apparent that by

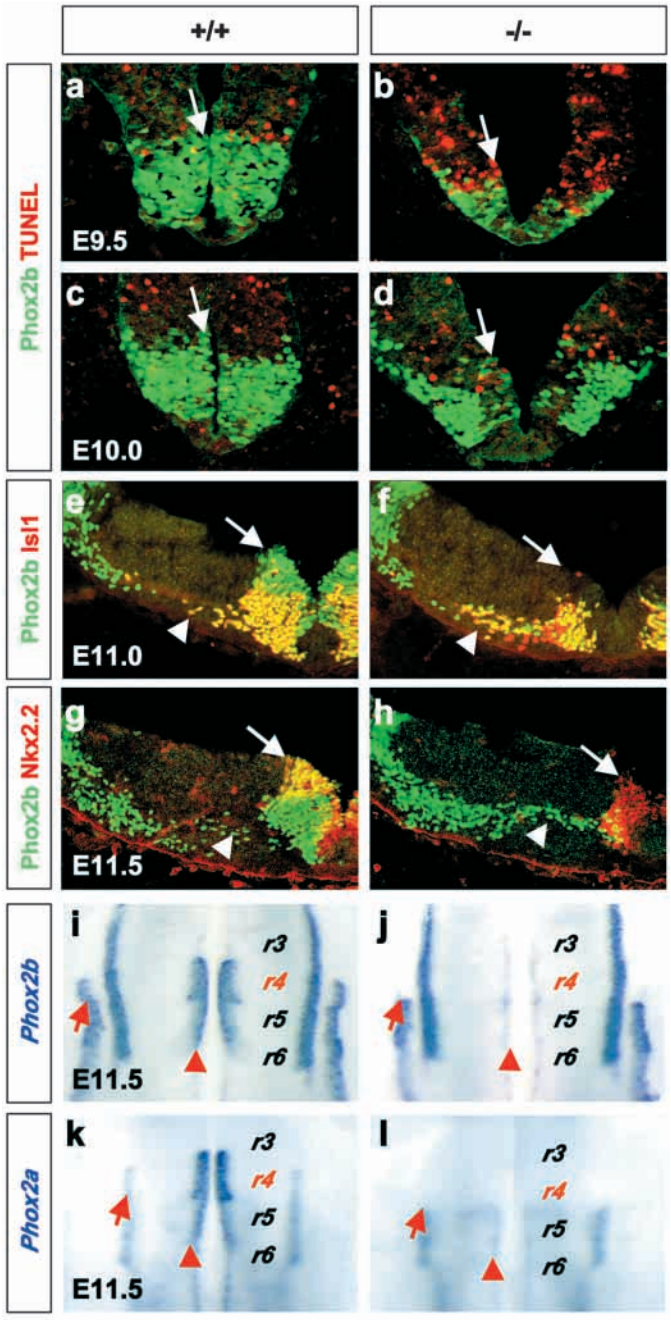

Fig. 6. *Hoxb1* is required early in the specification of progenitors of the facial branchiomotor neurons. (a-d)Transverse sections through the r4 ventral progenitor domain (VPD) immunolabeled with Phox2b (green) and the TUNEL assay to detect apoptotic cell death (red). (a) In control E9.5 embryo, Phox2b is expressed in the ventral neural tube throughout the neuroepithelium from the ventricular layer (arrow) to the outer marginal layer. Few detectable TUNEL-positive cells are observed within the Phox2b-expressing domain of control embryos. (b) In a Hoxb1-mutant embryo, Phox2b-expressing cells are dramatically reduced in the ventricular layer with significant numbers of TUNEL-positive cells throughout the ventricular and marginal neuroepithelium. (c) In a slightly older, control embryo (E10.0), Phox2b expression remains throughout the ventral neuroepithelium with few, sporadic TUNEL-positive cells. (d) In an E10.0 *Hoxb1*-mutant embryo, few Phox2b-expressing cells are detected in the ventricular layer (arrow) of the neuroepithelium with an appreciable amount of TUNEL-positive cells. (e-h)In an E11.0 control embryo, Phox2b expression (green) remains in two distinct regions in the ventricular layer (arrow) of the r4 VPD: a dorsal and ventral region (e). Postmitotic cells representing the FBM and CVA neurons emerging from the r4 VPD co-express Phox2b and Isl1 (yellow). Another population of Phox2b- and Isl1-doubled labeled cells is seen in the ventrolateral region of r4 (arrowhead), which appear to emerge from the more ventral region of the r4 VPD. In an E11.0 *Hoxb1*-mutant, the r4 VPD is devoid of Phox2b-expressing progenitor cells (arrow, f). A mixed population of cells expressing Phox2b and/or Isl are observed in the ventromedial and ventrolateral (arrowhead) region of r4. In an E11.5 control embryo, the r4 VPD (arrow) maintains its distinct cytoarchitecture, expressing both (yellow), Phox2b (green) and Nkx2.2 (red) with a cluster of postmitotic Phox2b-expressing cells outside of the VPD. A population of Phox2b-expressing cells is also observed in the ventrolateral region of the r4 VPD (arrowhead, g). In an E11.5 *Hoxb1*-mutant embryo, the dorsal Nkx2.2-positive region of the r4 VPD is absent and the more ventral region has expanded from the ventricular to the marginal layer of the neuroepithelium (arrowhead, h). Moreover, the population of Phox2b-expressing cells in the ventrolateral region have expanded (arrowhead). Note that between E11.0 and E12.0, no significant cell death were detected in either control or Hoxb1-mutant embryos in r4 (data) not shown. (i,j) In a flat-mount of E11.5 control embryo, *Phox2b* is expressed in three distinct, longitudinal columns containing progenitor and early postmitotic neurons (i). In an E11.5 *Hoxb1* mutant embryo, the ventral columns in r4 and r5 representing the developing and migrating neurons of the FBM are missing (panel b, arrowhead). The dorsal-most *Phox2b*-expresssing columns are also absent (arrows). (k,l) In a flat-mount E11.5 *Hoxb1*-mutant, *Phox2a* is expressed in two distinct columns containing early, postmitotic neurons (k). In an E11.5 *Hoxb1* mutant embryo, the r4-component of the intermediate column is missing. In the ventral region, the columns in r4 and r5 representing early differentiating and migrating FBM neurons, respectively, are also absent (arrowhead).

E11.5 the entire Phox2b and Phox2a expressing, FMB neural population has gone in the Hoxb1 mutant embryos (Fig. 6j and l), as well as the r4 components of restricted, more dorsal neural columns. Taken together, these experiments demonstrate that Hoxb1 function is required to activate and maintain cells expressing one of the earliest known transcription factors needed for specification of the r4 components of the branchiomotor system.

Hoxb1 is required for specification of neurons along the entire dorsoventral extent of r4

Examination of Fig. 6 shows that the *Hoxb1* mutation not only affects the formation of the r4-component of the branchiomotor system, but also the r4 component of intermediate and dorsal neural columns that express *Phox2a* and *Phox2b* (arrow), respectively. As an example, the r4-component of the most dorsal *Phox2b*-expressing column is absent in *Hoxb1* mutant homozygous embryos (Fig. 6j). Similarly, the r4 component of the intermediate column that expresses *Phox2a* is also absent in *Hoxb1* mutant embryos (Fig. 6l). These results show that *Hoxb1* is not only involved in the specification of motoneurons within r4, but in the specification of neurons throughout the DV extent of r4. These results are entirely consistent with the report recently published by Davenne et al. (1999) that demonstrated a role for *Hoxa2* in the specification of neurons along DV extent of r2 and r3. Consistent with the early mis-specification of neurons throughout the DV extent of r4, *Hoxb1* mutants showed

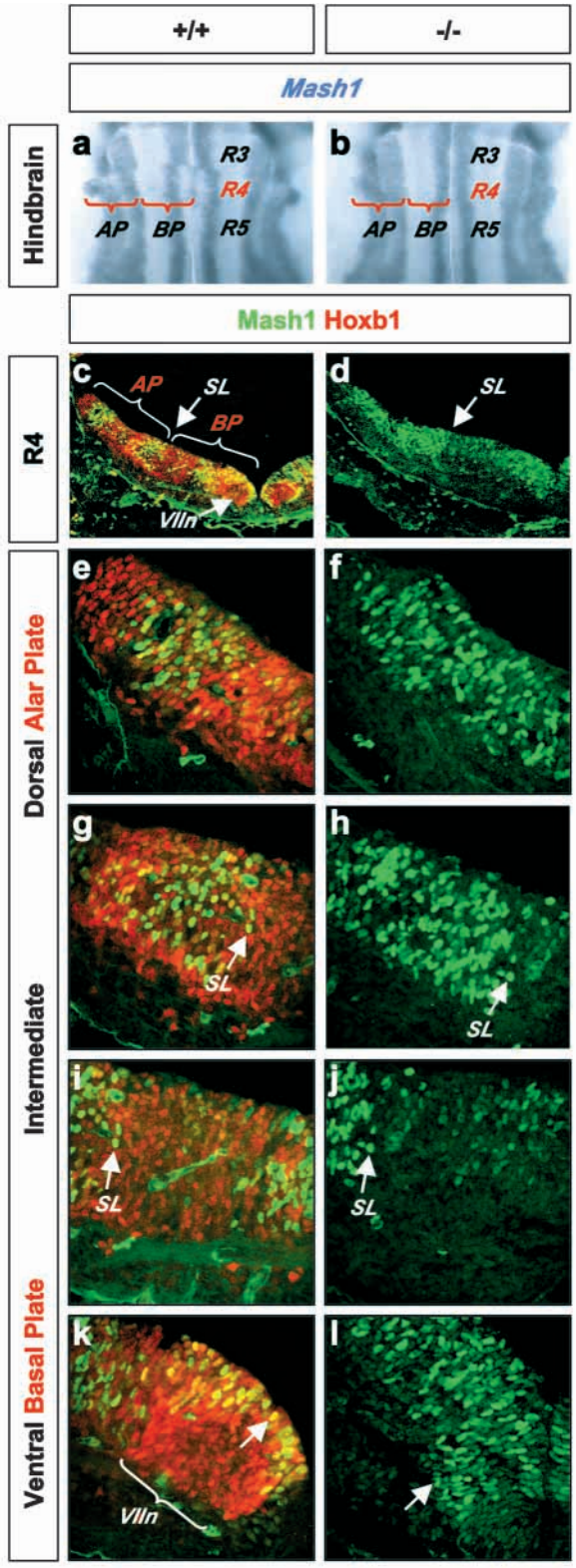

Fig. 7. Hoxb1 is required for restricting Mash1-expressing progenitor columns along the DV axis of r4. **(**a,b**)** *Mash1* RNA in situ expression in splayed, flat-mount E11.5 embryos (preparations as in Fig. 6). In r4 control embryos, *Mash1* is highly expressed in four broad, longitudinal columns in the alar and basal plates: AP and BP, respectively (a). In *Hoxb1* mutant mice, the column in the BP has shifted ventrally and the columns in the AP appear as one, broad column (b). The boundary between the AP and BP represents the hypothetical sulcus limitans (SL). This boundary also appears to have shifted ventrally in *Hoxb1* mutant mice. The abnormal ventral shift of the SL was also observed for the expression of *Pax7*, *Ngn1* and *Ngn2* (data not shown). (c-l**)** Immunolocalization of Mash1 (green) and Hoxb1 (red) in transverse sections through r4 of E11.5 embryos. In control tissues, Hoxb1-positive columns appear to complement the neuronal columns expressing Mash1 (c). In *Hoxb1* mutant, the dorsal, Mash1-expressing columns have merged and the ventral column has shifted toward the ventral midline (d). Higher magnification of c and d shows in detail the relationship of Hoxb1- and Mash1-expressing progenitor and early differentiating neurons (e-l). In control tissues, Mash1-expressing columns are bordered by Hoxb1-positive columns. However, the expression of Mash1 and Hoxb1 appear to be co-localized in a subset of progenitor cells in the ventricular layer (e,g,i,k). In *Hoxb1* mutant mice, the expression of Mash1 has expanded into the domain formerly occupied by Hoxb1-positive postmitotic neurons (d,f,h,i,j). The expansion of Mash1-progenitor cells into the region once occupied by Hoxb1-postmitotic FBM neurons is most apparent in the ventral-most region of r4 in *Hoxb1* mutant mice (l, arrow).

extensive aberrant cell death in E9.5 mutant embryos in the dorsal, intermediate and ventral regions of r4 (Fig. 5).

Hoxb1 restricts the extent of *Mash1* expression within r4

We have shown that the pattern of *Hoxb1* expression in r4 is very dynamic. Initially *Hoxb1* is expressed uniformly in r4. Subsequently, its expression becomes confined to more and more refined columns within r4. The early neural specification defects seen in *Hoxb1* mutants suggest interactions of *Hoxb1* with homologues of the *Drosophila* proneural genes *achaete-scute* and *atonal*, the bHLH transcription factors *Mash1*, *Ngn1* and *Ngn2* (Jan and Jan, 1994; Anderson and Jan, 1997; Fode et al., 1998; Ma et al., 1996, 1997, 1998, 1999). These genes are expressed in broad columns along the length of the AP axis in proliferating and early differentiating neurons as early as E8.5 in the mouse, approx. 1 day after Hoxb1 expression is first detected in r4 (Guillemot and Joyner, 1993; Lee, 1997).

Comparison of the columnar expression pattern of Mash1 in *Hoxb1* mutant and control embryos suggests that one role of Hoxb1 is to restrict the boundaries of these columns within r4. This is illustrated in Fig. 7. Fig. 7a,b show flatmount preparations of *Hoxb1* control and mutant hindbrains labeled with a *Mash1* RNA probe. Figs 7c-l show transverse sections through r4 immunostained with Mash1 and Hoxb1 antibodies. It is apparent from these figures that in control embryos the Mash1-expressing, early neural progenitor columns are juxtaposed by columns of cells expressing high levels of Hoxb1. In *Hoxb1* mutant homozygotes, the widths of the Mash1-expressing columns in r4 have expanded. Very similar changes in the widths of the r4-component of the *Ngn1* and *Ngn2* neuronal expression columns are observed in *Hoxb1* mutant embryos (data not shown).

DISCUSSION

Individual members of the *Hox* complex are differentially expressed along the embryonic AP axis. As such, they are ideally suited to provide the positional cues to reiterated cell

types and structures, such as neurons, vertebrae and muscles, that are needed to guide their distinction along this major axis. Indeed, it appears that a major role for Hox genes during embryogenesis is to provide such positional values to these cells (Chisaka and Capecchi, 1991; Krumlauf, 1994; Lumsden and Krumlauf, 1996; Duboule, 1998). The question then arises, when and through which molecular circuits are Hox positional values contributed to these differentiating cells? From the studies described here, we would argue that with respect to *Hoxb1* and the neurons derived from r4, this specification occurs very early during hindbrain neurogenesis. Specifically, Hoxb1-mediated neuronal specification appears to occur at the ventricular or proliferative layer of the r4 neuroepithelium, working in parallel with molecules required for early DV patterning, Shh and HNF3β, and those involved in neural determination, Mash1, Ngn1 and Ngn2.

Fate of the facial branchiomotor neurons in *Hoxb1* mutant embryos

The most prominent mutant phenotype associated with disruption of *Hoxb1* is the absence of a functional FBM nucleus (Goddard et al., 1996; Studer et al., 1996; Pata et al., 2000). As a consequence, *Hoxb1* mutant homozygous adults show complete paralysis of the muscles of facial expression, which are normally innervated by this nerve (Goddard et al., 1996). Consistent with the hypothesis that the absence of a FBM nucleus results from a failure to specify FBM neurons, early postmitotic molecular markers that normally label these neurons, such as Isl1 and *Phox2a*, fail to do so in r4 and r5 of these mutant embryos. Even more informative, one of the earliest transcription factors known to be required for specification of all branchial and visceral motor neurons of the brainstem, Phox2b, is not expressed in a distinct pool of progenitor cells in the ventral progenitor domain of r4 of *Hoxb1* mutant embryos, at any stages that we have examined. Instead, we observe that cells expressing effector molecules of Shh, such as HNF3β and Nkx2.2, that are normally associated with early, dividing neural progenitors, are reduced and continue to be expressed ectopically in the mantle layer normally occupied by postmitotic neurons. This aberrant cellular phenotype is very similar to that described by Brunet and his colleagues for mis-specified FBM neurons resulting from disruption of Phox2b (Pattyn et al., 2000). This is accompanied in *Hoxb1* mutant embryos by induction of a wave of ectopic apoptosis that begins at E9.5, and corresponds directly to the time of normal onset of FBM neuron generation and *Phox2b* expression (Taber Pierce, 1973; Pattyn et al., 1997). The ectopic apoptosis is, however, not restricted to the ventral region of r4 in *Hoxb1* mutant embryos, but extends across the three regions of high Hoxb1 expression. And indeed, failure to specify the r4-component of specific neuronal columns is observed throughout the DV extent of the neural tube.

An alternative to the hypothesis that failure to specify the FBM neurons leads to their aberrant death in *Hoxb1* mutants, is that as a consequence of this mutation, these neurons acquire a different fate. For example, in the absence of *Hoxb1* gene product, these neurons could now behave as r2-like, trigeminal-branchiomotor neurons. This hypothesis would predict that these mis-specified neurons should still express Phox2b, Phox2a and Isl1, reflecting their motoneuron character. However, the failure to detect populations of such cells in *Hoxb1* mutant embryos that express these markers, either in the ventral or in ectopic regions of r4, argues against this alternative hypothesis. There still remains the possibility that in *Hoxb1* mutant mice, later-born neurons derived from the ventral r4 VPD, such as the VL neuron population, may be mis-specified, owing to their dependence on interactions with earlier-born motoneurons (McConnell, 1995; Sockanathan and Jessell, 1998).

Although the fate of cells within r4 is affected by the *Hoxb1* mutation, the overall cytoarchitecture of this rhombomere is not dramatically altered by this mutation (Goddard et al., 1996; Studer et al., 1996). This observation suggests that the *Hoxb1* mutation affects selective cell populations within r4 and that the wave of ectopic apoptosis observed in r4 does not dramatically alter the final cell number within this rhombomere. Interestingly, in *Hoxb1* mutant embryos we also observe ectopic expansion of cell proliferation identified by Phh3 expression, extending from the ventricular to the mantle neuroepithelial layers. This increase in cell proliferation may, in part, compensate for the loss of cells via aberrant apoptosis early in neurogenesis.

Dorsoventral patterning of neurons in the hindbrain

Davenne et al. (1999) have recently shown that *Hoxa2* plays an important role in the DV patterning of neurons within r2 and r3. As observed for the *Hoxb1* mutation, disruption of *Hoxa2* selectively affects the formation of the r2/r3-component of neuronal columns that express transcription factors, such as *Pax6* and *Phox2b*, that are in turn involved in the specification of neuronal subtypes. Together, these observations emphasize that the neuronal columns that extend longitudinally across multiple rhombomeres and even into the spinal cord, are built in modules, with different *Hox* genes being responsible for the formation of the separate modules. Concomitant with this early role of *Hox* genes in neuronal specification, the progenitor cells automatically acquire a positional value along the AP axis, that allows them to be distinguished from similar cells within a contiguous longitudinal functional column. These observations also emphasize that these *Hox* genes are epistatic to the set of transcription factors that are used to specify neuronal subtype differentiation. The obvious advantage of this strategy is that positional value can be assigned to multiple neuronal subtypes within an AP region, rather than having to ascribe positional cues individually to each subtype subsequent to its specification.

Interestingly, *Hoxb1* mutant mice also show defects in the function of the lacrimal and salivary glands (Goddard et al., 1996). These glands are innervated by postganglionic parasympathetic neurons of the pterygopalatine and submandibular ganglia, respectively (Carpenter and Smith, 1988). These ganglia are in turn innervated by preganglionic parasympathetic neurons of the superior salivatory nucleus. The source of these visceral efferent neurons has not been well established. They may arise in r5, or they may be born in r4 and migrate into r5. In either case, their function is affected by the *Hoxb1* mutation. However, the expressivity of this defect in *Hoxb1* is variable. Generally, variability in expressivity of a mutant phenotype in Hox mutants is associated with the participation of more than one Hox gene in that function. Therefore, it may be possible that Hox genes expressed in r5,

such as members of the *Hox3* paralogous family, are good candidates for such a shared function with *Hoxb1*.

A potential role for *Hoxb1* in the restriction and/or reinforcement of neuronal subtypes

To examine the role of *Hoxb1* during early neural differentiation, the effects of the *Hoxb1* mutation on cells expressing the neural-specific bHLH transcription factors were studied. From an examination of in situ hybridization and immunohistochemical patterns of flat-mount and transverse section preparations, respectively, it is apparent that the broad longitudinal columns expressing *Mash1, Ngn1* and *Ngn2* are juxtaposed in r4 with columns of cells expressing high levels of Hoxb1. In the absence of *Hoxb1*, the width of these columns expands in r4, suggesting that Hoxb1 normally restricts the domains of these early neural progenitor cells. Since it has been shown in multiple laboratories that in both *Drosophila* and vertebrates the early neural progenitor domains are restricted and reinforced by the Delta/Serrate/Notch signaling pathways (Jan and Jan, 1994; Heitzler et al., 1996; Anderson and Jan, 1997; Panin et al., 1997), it is attractive to consider that the restriction of the *Mash1, Ngn1* and *Ngn2* domains within r4 by *Hoxb1* is also mediated by the same signaling pathway. On the basis of this hypothesis, it will be of interest to determine whether the production of successively more refined *Hoxb1*-expressing columns within r4 is involved in restricting and/or reinforcing increasing numbers of neuronal subtype columns within this rhombomere.

In *Hoxb1* mutant mice, there is a loss of r4-dorsal and intermediate columns expressing *Phox2b* and *Phox2a*, respectively. Interestingly, in *Mash1* mutant mice, there is also a loss of the same *Phox2b*-expressing column, but in contiguous dorsal columns along the hindbrain (Hirsch et al., 1998). Together, these data suggest that these two transcriptional systems work in parallel with each other during neural determination of common progenitors, and provide further support for *Hoxb1* contributing the AP-specific information to progenitor cells that may otherwise be similar along the length of the hindbrain.

In conclusion, the present study provides evidence that Hoxb1 is required for the formation of multiple neuronal subtypes along the full extent of the DV axis of r4. The role of Hoxb1 appears to be required very early during hindbrain neurogenesis in parallel with molecules required for DV patterning and neural determination, and prior to the activation of the transcription factors such as Nkx2.2, Isl1, Phox2b and Phox2a, which are used to specify neuronal subtypes. In the absence of *Hoxb1*, the r4-component of multiple neuronal subtypes fails to be properly specified and is then destined for aberrant programmed cell death.

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