Contribution of Hox genes to the diversity of the hindbrain sensory system

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Summary

The perception of environmental stimuli is mediated through a diverse group of first-order sensory relay interneurons located in stereotypic positions along the dorsoventral (DV) axis of the neural tube. These interneurons form contiguous columns along the anteroposterior (AP) axis. Like neural crest cells and motoneurons, first-order sensory relay interneurons also require specification along the AP axis. Hox genes are prime candidates for providing this information. In support of this hypothesis, we show that distinct combinations of Hox genes in rhombomeres (r) 4 and 5 of the hindbrain are required for the generation of precursors for visceral sensory interneurons. As *Hoxa2* is the only Hox gene expressed in the anterior hindbrain (r2), disruption of this gene allowed us to also demonstrate that the precursors for somatic sensory interneurons are under the control of Hox genes. Surprisingly, the Hox genes examined are not required for the generation of proprioceptive sensory interneurons. Furthermore, the persistence of some normal rhombomere characteristics in Hox mutant embryos suggests that the loss of visceral and somatic sensory interneurons cannot be explained solely by changes in rhombomere identity. Hox genes may thus directly regulate the specification of distinct first-order sensory relay interneurons within individual rhombomeres. More generally, these findings contribute to our understanding of how Hox genes specifically control cellular diversity in the developing organism

Key words: Somatosensory, Viscerosensory, Proprioceptive, Dorsal interneurons, Rhombomeres, Hindbrain, Homeodomain proteins

Introduction

Information from different sensory modalities is conveyed with spatial precision from peripheral sensory ganglia to contiguous nuclei within the central nervous system (CNS) (Carpenter and Sutin, 1983). In the spinal cord and hindbrain, for example, distinct nuclei located in stereotypic positions along the dorsoventral (DV) axis are dedicated to the perception of multiple sensations, such as proprioception, pain, temperature, touch, hearing, balance, taste and respiratory control. Within these nuclei, first-order sensory interneurons relay information to various spinal cord and hindbrain neurons that are required for simple reflexes or ultimately, to higher brain centers involved in cognition. Recent work has unveiled some of the genes that have helped identify the precursors for these diverse groups of first-order sensory interneurons. In the spinal cord, for example, the homeodomain proteins LH2A/B and Lbx1 specifically label proprioceptive and somatic sensory interneurons, respectively (Gross et al., 2002; Muller et al., 2002). In the hindbrain, the bHLH protein Mash1 (Ascl1 – Mouse Genome Informatics) and the homedomain proteins Phox2b and Rnx (Tlx3 – Mouse Genome Informatics) characterize the progenitors and precursors for visceral sensory interneurons of the solitary tract nucleus – a structure essential for gustatory and respiratory control (Amiel et al., 2003; Qian et al., 2001; Shirasawa et al., 2000). These studies have significantly advanced our knowledge of the possible molecular determinants necessary for producing the great diversity of sensory interneurons along the DV axis. However, an issue that remains to be explored is how sensory interneurons in one segment of the body acquire their distinction amongst multiple segments along the anteroposterior (AP) axis.

Hox genes have become prime molecular candidates for providing AP-positional information to all cells at a given axial level. Together with other investigators, we have characterized the AP-restricted function of Hox genes in the developing spinal cord and hindbrain through gain- and loss-of-function analyses. These studies have focused primarily on the specification of motoneurons. In the spinal cord, for example, *Hoxc8* and *Hoxd10* are required for the normal development of motoneurons controlling movement of the forelimbs and hindlimbs, respectively (Carpenter et al., 1997; Tiret et al., 1998). In the hindbrain, *Hoxb1* and *Hox3* genes are necessary and sufficient for the specification of rhombomere (r) 4-branchial and r5-somatic motoneurons, respectively (Bell et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003; Goddard et al., 1996; Guidato et al., 2003; Studer et al., 1996). These examples of motoneuron specification illustrate the phenomenon of spatial colinearity, whereby expression and function of Hox genes along the AP axis of the organism is correlated with their chromosomal location (Lewis, 1978; McGinnis and Krumlauf, 1992).

In this study, we examined the role of Hox genes on the specification of interneurons in the sensory system of the

developing hindbrain. In the early developing hindbrain, Hox genes are generally expressed throughout the neuroepithelium, from the ventricular to the pial layers, suggesting multiple roles in neuronal differentiation (Gaufo et al., 2003). Moreover, the reinforcement of later Hox gene expression in multiple longitudinal columns that correspond to the positions of various neuronal lineages suggests the potential dependence of many neuronal subtypes on Hox gene expression along the DV axis (Davenne et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003; Pattyn et al., 2003). To begin to identify the neuronal subtypes that are dependent on Hox genes, we analyzed the development of three-distinct first-order sensory interneurons arranged in non-overlapping domains along the DV axis. These interneurons include first-order proprioceptive, visceral and somatic sensory relay interneurons that form contiguous columns along the AP axis (Bermingham et al., 2001; Gross et al., 2002; Lee et al., 2000; Muller et al., 2002). Analysis of *Hoxb1*, *Hoxa3*, *Hoxb3* and *Hoxa2* loss-of-function mutations in embryonic mice reveal that these Hox genes are required for the specification of visceral and somatic sensory interneurons via the regulation of *Phox2b* and *Lbx1*, respectively. However, formation of proprioceptive sensory interneurons expressing *LH2A/B* appears to be independent of Hox gene function. Taken together, these findings suggest that Hox genes contribute to the diversity of the sensory system by regulating the differentiation of specific subsets of first-order sensory relay interneurons along the AP axis of the developing hindbrain.

Materials and methods

Mice

The generation of *Hoxb1*, *Hoxa3* and *Hoxb3* mutant mice have been previously described (Gaufo et al., 2003; Manley and Capecchi, 1998). Briefly, the *Hoxa2* mutant mouse was generated by replacing the coding sequence of the first exon with the *Cre recombinase* gene followed by a *Neo* cassette flanked by two FRT sites (detailed protocol available upon request). Hox mutant embryos were generated by single- or compound-heterozygote crossings and compared with littermate or age-matched controls.

In situ hybridization and immunohistochemistry

Embryonic days 10.5-11.5 whole-embryos were dissected along the dorsal midline and processed for in situ hybridization using digoxigenin-labeled *Dbh*, *Mash1*, *Phox2b* and *Rnx* probes as previously described (Gaufo et al., 2000; Pattyn et al., 1997; Qian et al., 2001). Transverse sections (10 μm) through r2 to r6 of E10.5-11.5 embryos were processed for immunohistochemistry using Phox2b (Pattyn et al., 1997), Lbx1 (Gross et al., 2002) and LH2A/B (Lee et al., 2000) rabbit polyclonal antibodies, Lmx1b guinea pig polyclonal antibody, and Lim1/2 and Isl1/2 mouse monoclonal antibodies (Developmental Studies Hybridoma Bank). Primary antibodies were detected using various fluorochrome-conjugated secondary antibodies (Molecular Probes; Jackson Immunoresearch). Fluorescent images were captured on a BioRad 1024 confocal microscope and processed in Adobe Photoshop and Powerpoint.

Results

Identification of noradrenergic precursor interneurons in the caudal hindbrain

In the caudal hindbrain, a population of noradrenergic interneurons contribute to the formation of the solitary tract nucleus (STN), a structure critical for regulating cardiovascular, respiratory and gustatory functions (Carpenter and Sutin, 1983; Qian et al., 2001; Saper, 2000). The early interneurons contributing to the STN can be identified by the expression of dopamine β-hydroxylase (Dbh), the gene encoding the enzyme necessary for the biosynthesis of norepinephrine (Qian et al., 2001). Dbh is initially expressed at about E10.5, starting in r4 and spreading more caudally to r5 and r6 (data not shown). The pattern of Dbh expression is consistent with the rostrocaudal progression of neurogenesis in the developing hindbrain (Lumsden and Keynes, 1989). By E11.5, the expression of Dbh forms a contiguous column that spans multiple rhombomeres on both sides of the dorsal hindbrain (Fig. 1A). The homeobox-containing genes Phox2b and Rnx and the bHLH gene Mash1 demarcate the domain that gives rise to noradrenergic interneurons (Hirsch et al., 1998; Pattyn et al., 1997; Qian et al., 2001). In a flat-mount prepartion, Phox2b and Rnx RNA expression are seen as restricted columns of postmitotic interneurons in the marginal layer of the neuroepithelium, whereas *Mash1* RNA is expressed in broad columns of dividing neural progenitors in the ventricular layer of the neuroepithelium (Fig. 1B-D, boxed) (Gaufo et al., 2000; Qian et al., 2001).

Hoxb1 regulates precursors of noradrenergic interneurons in r4

The early appearance of *Dbh* RNA expressing interneurons

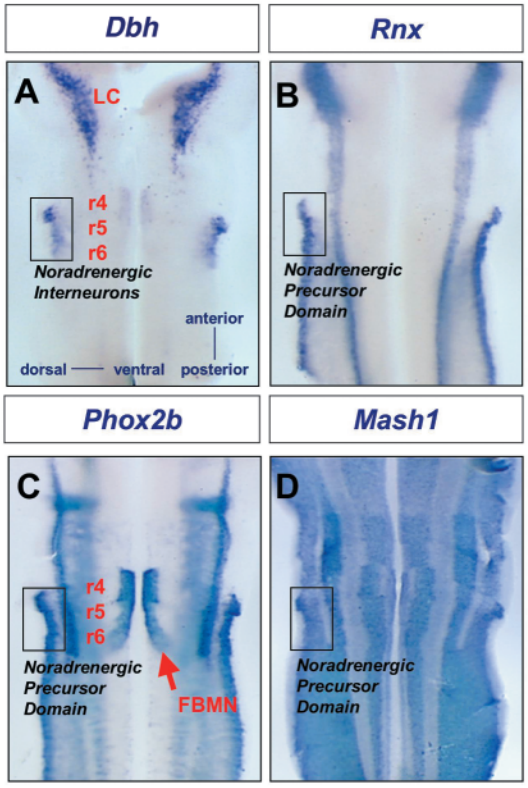


Fig. 1. Characterization of noradrenergic interneuron precursors in the caudal hindbrain. Hindbrain flat-mount preparations of E11.5 control embryos showing the mRNA expression of (A) *Dbh*, (B) *Rnx*, (C) *Phox2b* and (D) *Mash1*. The box outlines the domain that gives rise to noradrenergic visceral sensory interneurons of the solitary tract nucleus (STN). LC, locus ceruleus; FBMN, facial branchiomotoneurons.

within individual rhombomeres in the caudal hindbrain suggests that their formation may be independently regulated along the AP axis in a segmental manner. The initial appearance of *Dbh* RNA expression in r4 of the caudal hindbrain suggests *Hoxb1*, the expression of which is restricted to r4, as one of these potential regulatory transcription factors (Gaufo et al., 2000; Goddard et al., 1996; Studer et al., 1996). Indeed, examination of *Dbh* RNA expression in dorsal r4 shows its complete absence in E11.5 *Hoxb1*$^{-/-}$ embryos compared with controls (Fig. 2A,B). The expression of *Rnx* RNA, a gene whose expression is known to be required for production of *Dbh* interneurons, was also absent in E11.5 *Hoxb1*$^{-/-}$ embryos compared with controls (Fig. 2C,D). By contrast, the expression of *Phox2b* RNA was largely intact in *Hoxb1*$^{-/-}$ embryos compared with controls, although in greatly reduced amounts (Fig. 2E,F). Analysis of younger E10.5 *Hoxb1*$^{-/-}$ embryos shows that *Phox2b* RNA is detectable at appreciable levels nearly comparable with controls (Fig. 2E,F, inset). The presence of *Phox2b* RNA during these developmental periods is worth noting for it suggests that the identity of r4, with respect to this marker, is intact in early *Hoxb1*$^{-/-}$ embryos. However, the expression of Phox2b protein in transverse sections through dorsal r4 of E10.5 *Hoxb1*$^{-/-}$ embryos was undetectable compared with controls (Fig. 3A,B).

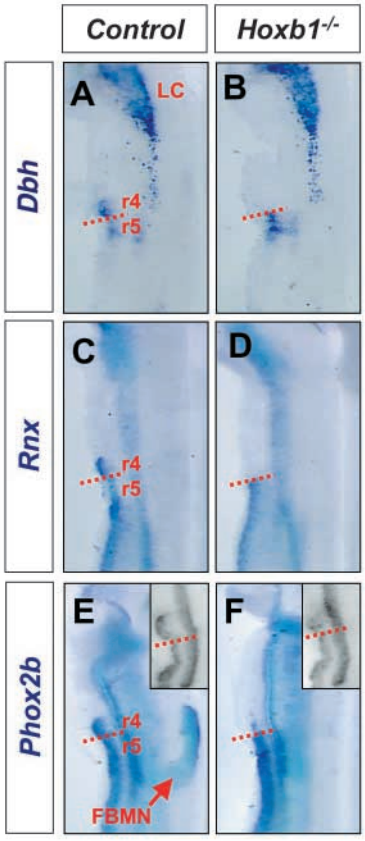


Fig. 2. Hoxb1 regulates early differentiation of noradrenergic visceral sensory interneurons in r4. (A-D) Hindbrain flat-mount preparations showing *Dbh* and *Rnx* expression are missing in dorsal r4 of E11.5 *Hoxb1*$^{-/-}$ embryos compared with control littermates. (E,F) Expression of *Phox2b* mRNA is significantly reduced in dorsal r4 of E10.5 (insets) and E11.5 *Hoxb1*$^{-/-}$ embryos compared with control littermates.

As *Mash1* RNA and protein, which precede the expression of *Phox2b*, are both intact in *Hoxb1*$^{-/-}$ embryos (data not shown) (Gaufo et al., 2000), the induction of *Phox2b* RNA concurrent with the loss of Phox2b protein defines the earliest detectable defect in the differentiation of noradrenergic interneurons observed in this study.

Combined actions of *Hoxa3* and *Hoxb3* are required for the specification of Phox2b-expressing noradrenergic interneuron precursors in r5

The loss of r4 Phox2b protein expression in *Hoxb1*$^{-/-}$ embryos suggests that noradrenergic precursors in the more caudal rhombomeres may have similar requirements for Hox genes. We therefore investigated the functions of the *Hox3* paralogous genes, *Hoxa3*, *Hoxb3* and *Hoxd3*, which are expressed in overlapping rhombomeres posterior to r4 (Gaufo et al., 2003). In contrast to *Hoxb1*$^{-/-}$ embryos, however, the appearance of Phox2b protein expression was intact in *Hox3* single mutant embryos, suggesting possible redundant functions amongst the *Hox3* paralogous genes (data not shown). Consistent with this hypothesis, analysis of combined mutations for *Hoxa3* and *Hoxb3* in E10.5 embryos exhibited dorsal r5-specific loss of Phox2b protein expression (Fig. 3E,G). The dependence of Phox2b protein on the Hox3 genes appears to be restricted to r5, as Phox2b protein was expressed in more caudal rhombomeres in various Hox3-double mutation combinations (data not shown). However, we cannot rule out the possible redundant functions amongst the three Hox3 genes or other members of the Hox gene family in more caudal rhombomeres. As a control for noradrenergic interneuron specification, we used *Mash1*$^{-/-}$ embryos to illustrate the complete loss of Phox2b protein expression in the dorsal region of all rhombomeres (Fig. 3A,D,E,H, data not shown). The latter finding confirms the role of *Mash1* as a global determinant of noradrenergic neurons (Hirsch et al., 1998), as well as demonstrating that *Mash1* and Hox genes converge on the regulation of a distinct neuronal subtype program.

Loss of precursors for noradrenergic interneurons results in the expansion of neighboring interneurons

The absence of Phox2b protein expression among the precursors of noradrenergic interneurons in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos suggests an early regulatory role for Hox genes. To examine a cellular consequence of this defect, we examined for the presence of neighboring interneurons in the surrounding environment. Consistent with our observation in younger embryos (Fig. 3), examination of Phox2b protein together with Lmx1b, a homeodomain protein that also detects noradrenergic precursors, is eliminated from the dorsal region of r4 and r5 in E11.5 *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos, respectively (Fig. 4A-D). Analysis of Lim1/2 protein expression, which delineates an interneuron population ventral to noradrenergic interneurons, shows an expanded domain in r4 and r5 of E11.5 *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos, respectively (Fig. 4A-D, bracket). The expanded domain of Lim1/2 was independently confirmed by Pax2-immunolabeling (data not shown). As will be shown in the next section, the expression of Lim1/2 is co-expressed with a population of Lbx1-expressing somatic sensory interneuron precursors (Fig. 5). As no significant cell death was observed

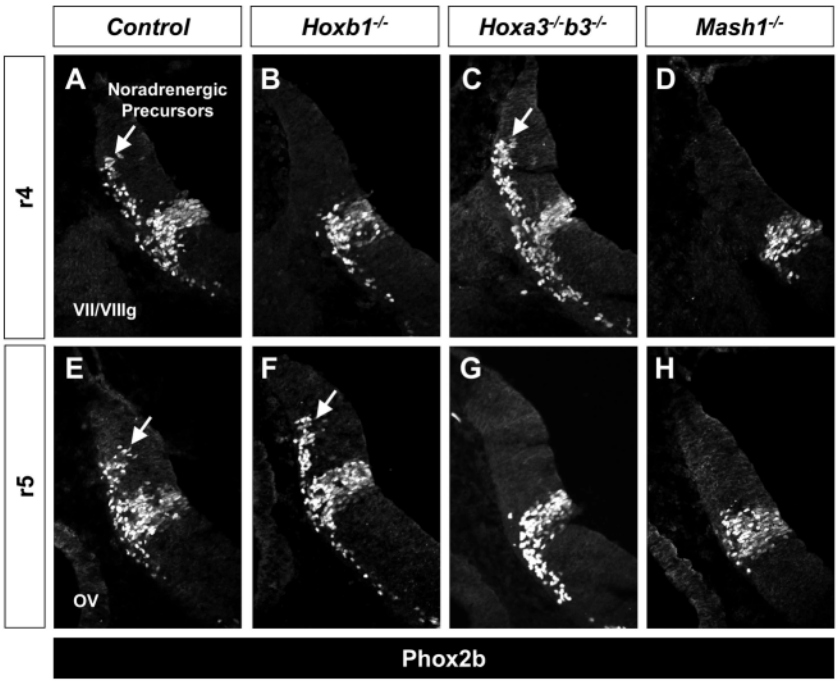
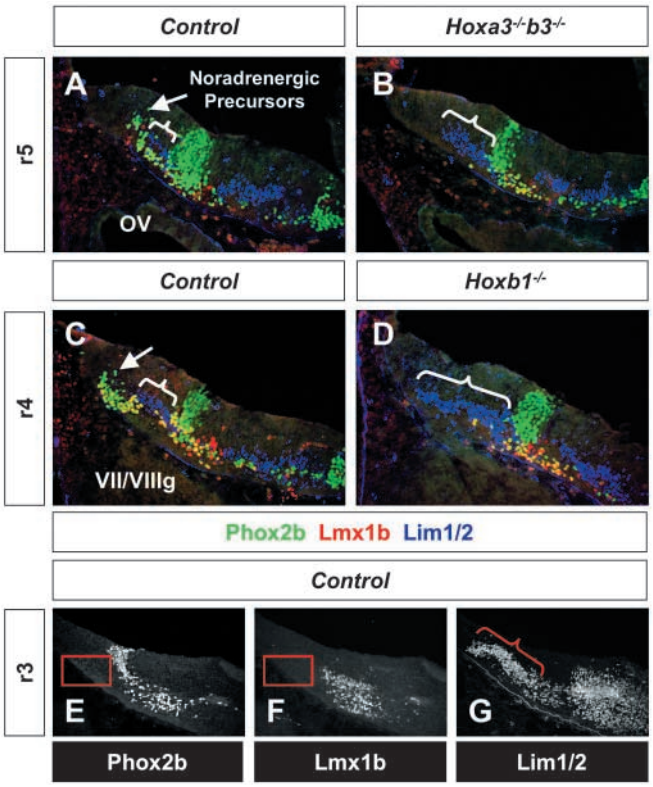


Fig. 3. Distinct combination of Hox genes is required for *Mash1*-dependent Phox2b protein expression in r4 and r5. Transverse sections through r4 and r5 of E10.5 control (A,E), *Hoxb1*$^{-/-}$ (B,F), *Hoxa3*$^{-/-}$*b3*$^{-/-}$ double (C,G) and *Mash1*$^{-/-}$ (D,H) embryos. The expression of *Phox2b* among precursors of noradrenergic visceral interneurons is missing (compare with arrows in A,C,E,F) in dorsal r4 of *Hoxb1*$^{-/-}$ and dorsal r5 of *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos compared with controls. In the *Mash1*$^{-/-}$ embryo, Phox2b expression is completely eliminated in the dorsal region of r4 and r5. VII/VIIIg, ganglia; OV, otic vesicle.

in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos between E10.5 and E11.5 (data not shown), the molecular and cellular alterations in these mutants suggest a change in cell identity such that the mutant segment resembles the identity of a more anterior segment, a phenomenon that is characteristic of many Hox gene loss-of-function mutations (Gaufo et al., 2003; Hafen et al., 1984; Lewis, 1978; McGinnis and Krumlauf, 1992; Rozowski and Akam, 2002; Struhl, 1981; Studer et al., 1996; Weatherbee et al., 1998).



To examine the possibility of a change in rhombomere identity, we compared r4 and r5 of *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos with the more anterior r3 of normal embryos. In r3, the DV axial level comparable with r4 and r5 is devoid of Phox2b and Lmx1b protein (Fig. 4E,F, boxed region). Furthermore, this dorsal region of r3 is occupied by a broad column of Lim1/2-expressing interneurons (Fig. 4G, bracket). The similarity in the protein expression profile of normal r3 to that of r4 and r5 in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos provides evidence for a change in rhombomere identity. However, it is important to note that the RNA for *Phox2b* is largely intact during this time period in r4 and r5 of *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos, respectively (Fig. 2E,F, data not shown), arguing for incomplete transformation to a more anterior identity.

Presence of proprioceptive and somatosensory precursors in Hox mutant embryos suggest independent or redundant roles for Hox genes in r4 and r5

The expression of *Hoxb1*, *Hoxa3* and *Hoxb3* throughout the neuroepithelium of r4 and r5 suggests that they may regulate

Fig. 4. Loss of precursors of noradrenergic visceral sensory interneurons is associated with the expansion of neighboring interneurons in r5 and r4. Transverse sections through r5 and r4 of E11.5 control (A,C), *Hoxa3*$^{-/-}$*b3*$^{-/-}$ (B) and *Hoxb1*$^{-/-}$ (D) embryos. The co-expression (yellow) of Phox2b (green) and Lmx1b (red) among noradrenergic precursors is eliminated in the dorsal hindbrain of *Hoxa3*$^{-/-}$*b3*$^{-/-}$ and *Hoxb1*$^{-/-}$ embryos. The intermediate column of Phox2b and the more ventral population of Lmx1b-expressing cells appear unaffected in the Hox mutant embryos. The domain of Lim1/2 (blue, bracket) expression has expanded in both Hox mutant embryos compared with controls. Comparison of transverse sections at comparable DV axial levels through r3 of an E11.5 control embryo (E-G) with r5 and r4 *Hoxa3*$^{-/-}$*b3*$^{-/-}$ *and Hoxb1*$^{-/-}$ mutant embryos (B,D) show similar expression patterns of Phox2b, Lmx1b and Lim1/2.

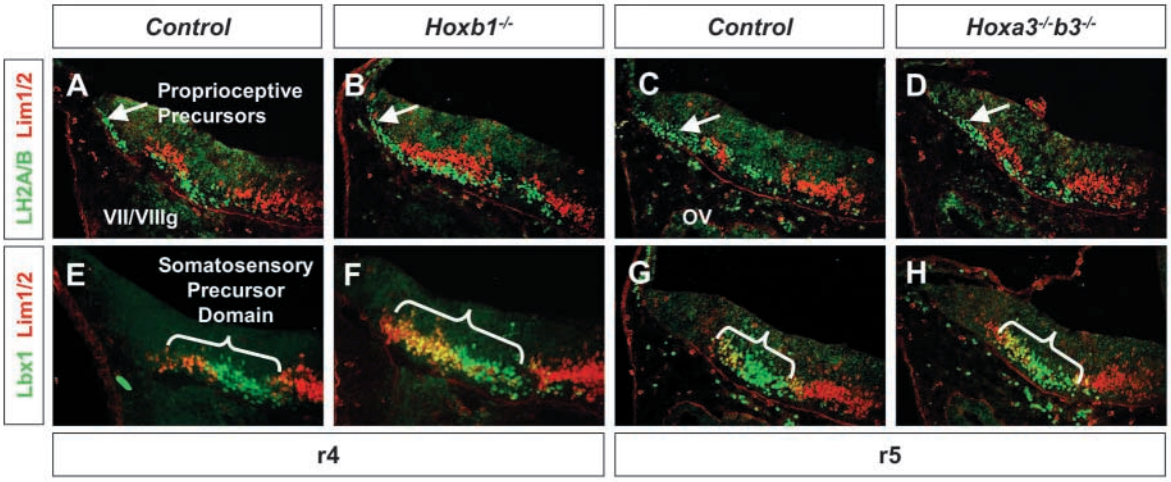


Fig. 5. Regulation of proprioceptive and somatic sensory interneurons is independent of *Hoxb1*, *Hoxa3* and *Hoxb3* functions in r4 and r5. Expression of LH2A/B (green, arrow) and Lim1/2 (red) in r4 and r5 of controls (A,C) compared with *Hoxb1*$^{-/-}$ (B) and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ (D) embryos. Lim1/2 expression is expanded in both Hox mutant embryos compared with their controls. Expression of Lbx1 (green) and Lim1/2 (red) in E11 control (E) and *Hoxb1*$^{-/-}$ (F) embryos and E11.5 control (G) and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ (H) embryos is intact. The co-expression of Lim1/2 and Lbx1 suggests that the Lbx1 population is also expanded in Hox mutant embryos compared with controls (bracket).

other first-order sensory relay interneurons. We therefore assessed for the presence of precursors for proprioceptive and somatosensory interneurons. Unlike noradrenergic interneurons, proprioceptive and somatosensory interneurons have homologous interneurons in the spinal cord. As in the spinal cord, hindbrain *LH2A/B*-expressing precursors for proprioceptive interneurons derive from progenitors expressing the bHLH gene *Math1* (Lee et al., 2000; Lee et al., 1998). In both *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos, the expression of LH2A/B appears normal compared with controls (Fig. 5A-D, arrow). We next examined for the presence of precursors for somatic sensory interneurons by assaying the expression of the homeodomain protein Lbx1, a regulator of somatic sensory interneurons in the spinal cord (Gross et al., 2002; Muller et al., 2002). Like the precursors for proprioceptive interneurons, the precursors for somatic sensory interneurons were intact in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos (Fig. 5E-H, bracket). However, the domain of Lbx1 and Lim1/2 expression appear expanded in the various Hox mutant embryos.

Redundant functions of Hox genes in somatic sensory interneuron specification

Several possibilities may explain the loss of noradrenergic visceral sensory interneurons and the sparing of proprioceptive and somatic sensory interneurons in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos in r4 and r5, respectively. The simplest explanation is that the specification of proprioceptive and somatic sensory interneurons is independent of Hox gene function. Alternatively, redundant functions with other Hox genes in r4 and r5 may compensate for the loss of *Hoxb1*, *Hoxa3* and *Hoxb3* functions. Another possibility may be that different combinations of Hox genes are required for the specification of proprioceptive and somatic sensory interneurons. To address these issues, we analyzed for the presence of proprioceptive and somatic sensory interneurons in r2 of *Hoxa2*$^{-/-}$ embryos. In r2, *Hoxa2* is the only Hox gene expressed and, thus, the function of a single Hox gene can be addressed (Davenne et al., 1999). Analysis of LH2A/B expression in r2 showed no dramatic differences between E11.5 control and *Hoxa2*$^{-/-}$ embryos (Fig. 6A-B). Together with the observations in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ embryos, these data suggest that the specification of precursors for proprioceptive interneurons is independent of Hox gene function.

We next turned our analysis to the identification of precursors for somatic sensory interneurons in r2 of *Hoxa2*$^{-/-}$ embryos. Lbx1-expressing precursors for somatic sensory interneurons, based on their location in the anterior hindbrain and molecular homology to interneurons in spinal cord, suggest that they give rise to the main trigeminal sensory nucleus (Carpenter and Sutin, 1983; Qian et al., 2002). In contrast to precursors for proprioceptive interneurons in r2, the presence of Lbx1-expressing precursors was completely eliminated in E11.5 *Hoxa2*$^{-/-}$ embryos (Fig. 6C,D). The presence of trigeminal branchiomotoneurons, as identified by co-labeling of the homeodomain proteins Phox2b and Isl1/2, suggest that the identity of r2 in *Hoxa2*$^{-/-}$ embryos is intact (Fig. 6E,F, box). This observation therefore rules out the possibility that the absence of somatic sensory interneurons in *Hoxa2*$^{-/-}$ embryos results from a complete change in r2 identity. Moreover, the significant reduction in Lbx1 expression in r3 of *Hoxa2*$^{-/-}$ embryos provides further support that multiple Hox genes cooperate to specify precursors of somatic sensory interneurons in more caudal segments of the neural tube (Fig. 5; Fig. 6G,H).

Discussion

Hox genes are required for the generation of cellular diversity along the AP axis of developing organisms. In the hindbrain, for example, this function is clearly reflected in the early ubiquitous expression of Hox genes in rhombomeres followed by strengthened expression of these genes in longitudinal columns corresponding to the position of various neuronal lineages (Davenne et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003; Pattyn et al., 2003). Like in many tissues, however, little

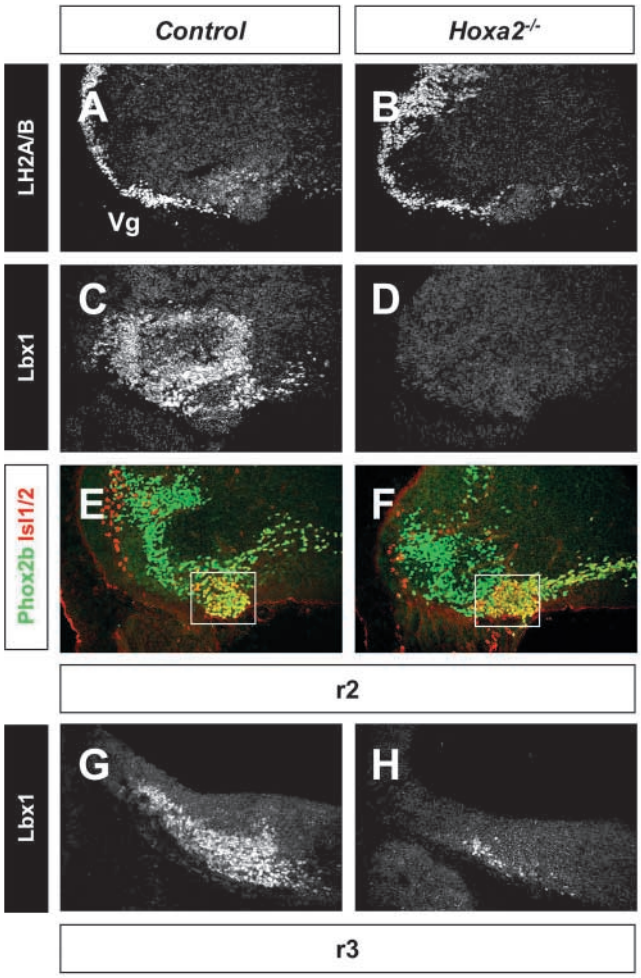


Fig. 6. Differential regulation of precursors for proprioceptive and somatic sensory interneurons in r2. Expression of LH2A/B is intact in r2 of E11.5 control (A) and *Hoxa2*$^{-/-}$ (B) embryos. Labeling of Lbx1 in r2 of E11.5 control (C) and *Hoxa2*$^{-/-}$ (D) embryos shows complete loss of Lbx1 expression in the *Hoxa2*$^{-/-}$ embryo. The trigeminal branchiomotoneurons (box), co-labeled with Phox2b (green) and Isl1/2 (red), are intact in the *Hoxa2*$^{-/-}$ embryo compared with the littermate control (E,F). Lbx1 expression in r3 of E11.5 control (G) and *Hoxa2*$^{-/-}$ (H) embryos shows a significant reduction in the *Hoxa2*$^{-/-}$ embryo.

is known about the specific cell types that are dependent on Hox gene function. In this report, we have addressed this issue by analyzing the contributions of Hox genes to the development of first-order sensory interneurons in the developing hindbrain (Fig. 7). Contrary to a general role for Hox genes, as would be expected based on their more ubiquitous expression pattern, the requirement for Hox genes appear to be specific to distinct neuronal cell lineages.

Hox gene control of sensory structures is evolutionary conserved

In the present study, we show that *Hoxb1*, *Hoxa3* and *Hoxb3*, are required for the segment-specific formation of *Mash1*-dependent noradrenergic visceral sensory interneurons. By analogy to the sensory system of *Drosophila*, the Hox gene *Ubx* appears to control the segmental pattern of *achaete-scute* complex-dependent sensory bristles (Rozowski and Akam, 2002). In contrast to mouse, where Hox genes positively influence the specification of *Mash1*-dependent noradrenergic interneurons, *Ubx* suppresses the differentiation of progenitors or proneural clusters into sensory bristles. The regulation of analogous sensory structures in the mouse is therefore opposite to that observed in *Drosophila*. However, the stage by which the mouse and *Drosophila* Hox genes regulate this differentiation process appears to be similar. In both mouse and *Drosophila*, the formation of progenitors appears to be independent of Hox gene function. However, subsequent activation or suppression of noradrenergic visceral sensory interneuron or sensory bristle formation, respectively, is dependent on Hox genes (Gaufo et al., 2000; Rozowski and Akam, 2002). Our study in the mouse suggests that Hox genes regulate noradrenergic visceral sensory interneurons at the level of *Phox2b* expression. However, direct evidence for this hypothesis will require testing the functional relevance of a conserved Hox/*Pbx* regulatory element in the promoter/enhancer region of *Phox2b* (data not shown). Nevertheless, the similarities in the segmental regulation of sensory structures by *Mash1* and *achaete-scute* complex in mouse and *Drosophila*, respectively, supports an evolutionarily conserved regulatory process in neuronal subtype specification.

The present study also shows that the expression of *Rnx*, a known determinant of noradrenergic visceral sensory interneurons important for gustatory, cardiovascular and respiratory control (Carpenter and Sutin, 1983; Qian et al., 2001), is also subject to Hox gene regulation. In contrast to *Phox2b* RNA, however, the expression of *Rnx* RNA appears to be completely eliminated in *Hoxb1*$^{-/-}$ embryos. The loss of *Rnx* RNA expression is consistent with the absence of *Dbh* RNA. The presence of *Phox2b* RNA in *Hoxb1*$^{-/-}$ embryos, however, suggests that the identity of r4 is initially intact and therefore, the loss of noradrenergic visceral sensory interneurons is not solely due to a secondary effect resulting from changes in rhombomere identity. From these observations, Hox, *Phox2b* and *Rnx* genes may be placed in a hierarchical order to broadly define a regulatory cascade in the specification of noradrenergic visceral sensory interneurons within a hindbrain segment. Furthermore, the convergence of these genes on a common function is supported by central respiratory defects in mice with targeted mutations for *Hoxa3* and *Rnx* and in humans with heterozygous mutations for *PHOX2B* (Amiel et al., 2003; Chisaka and Capecchi, 1991; Shirasawa et al., 2000). Altogether, these observations showing the segment-specific control of sensory structures and the convergence of genes on a common physiological function provides evidence for an evolutionary conserved pathway.

Maintenance of complementary gene expression ensures cellular diversity

An established function of Hox genes is to generate cellular diversity within multiple tissue types. In the mouse, for example, Hox genes are known to be essential for the specification of tissues that contribute to the musculoskeletal, urogenital, hematopoietic and nervous systems (Alvares et al., 2003; Arenkiel et al., 2003; Bell et al., 1999; Davenne et al., 1999; Davidson et al., 2003; Gaufo et al., 2000; Gaufo et al., 2003; Goddard et al., 1996; Guidato et al., 2003; Ivanova et al., 2002; Manley and Capecchi, 1998; Patterson and Potter,

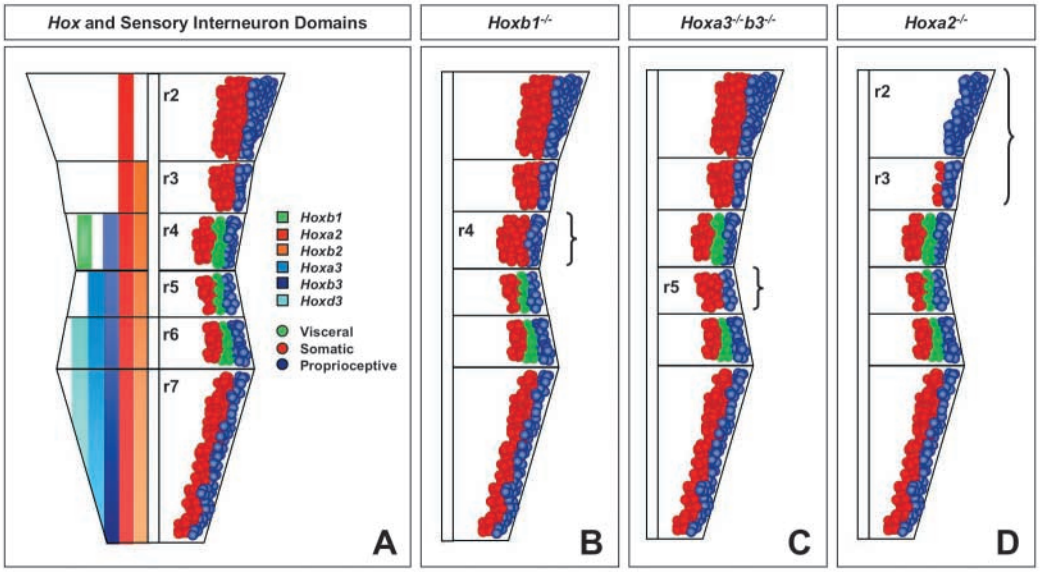


Fig. 7. Summary. (A) Schematic view of a flat-mount hindbrain showing Hox gene expression on the left (bars) and first-order visceral (noradrenergic), somatic and proprioceptive sensory relay interneurons (circles) on the right. The loss of *Hoxb1* (B) and the combination of *Hoxa3* and *Hoxb3* (C) result in a specific loss of visceral interneurons in r4 and r5, respectively. The loss of visceral sensory interneurons in these Hox mutant embryos is associated with the expansion of the somatic sensory interneuron domain. In *Hoxa2* loss-of-function (D), somatic sensory interneurons are completely eliminated in r2 and significantly reduced in r3, presumably through the redundant role of *Hoxb2* in this rhombomere (see A). Although Hox genes are expressed throughout the early neuroepithelium, the present finding suggests a specific role for Hox genes in the generation of cellular diversity in the developing hindbrain.

2003; Rossel and Capecchi, 1999; Studer et al., 1998; Studer et al., 1996; Watari et al., 2001; Wellik and Capecchi, 2003). How Hox genes regulate cellular diversity within these varied tissues remains to be determined. Owing to the well-characterized expression patterns of genes in the neural tube (Briscoe et al., 2000; Hirsch et al., 1998; Qian et al., 2001; Qian et al., 2002), it is possible to assess a detailed role of Hox genes in this complex tissue. In the present study, we demonstrate that Hox genes are required for the specification of visceral and somatic sensory interneurons. However, proprioceptive sensory interneurons appear to be independent of Hox gene function. The latter observation suggests that although Hox genes are ubiquitously expressed in the neuroepithelium, their effects are neuronal subtype specific. It also suggests that the proprioceptive sensory system is ancient relative to the use of Hox genes to specify AP identity of the visceral and somatic sensory systems in the hindbrain region (r2-r5) examined.

The present study also reveals a duality of Hox gene function in the regulation of various neuronal subtypes in the hindbrain. For example, the loss of *Phox2b*-expressing noradrenergic visceral interneurons in *Hoxb1*$^{-/-}$ and *Hoxa3*$^{-/-}$*b3*$^{-/-}$ mutant embryos is associated with the expansion of neighboring *Lim1/2*- and *Lbx1*-expressing interneurons into the region normally occupied by *Phox2b*. This observation suggests that Hox genes can act either as an activator or repressor depending on their location along the DV axis. This appears to be a general Hox mechanism, as we have observed the same phenomenon in the specification of hindbrain motoneurons in various Hox mutant embryos (Gaufo et al., 2000; Gaufo et al., 2003). How can this apparent regulatory paradox be rectified? A clue may arise from what has been observed in the spinal cord. Along the DV axis of the spinal cord, distinct homeodomain and bHLH proteins show complementary expression domains and cross-repressive interactions. In the ventral spinal cord, for example, the loss of *Pax6* is associated with the dorsal expansion of the more ventral Nkx2.2 into the domain normally occupied by Pax6 (Ericson et al., 1997). In the dorsal spinal cord, Math1 and Ngn1 also show an inverse regulatory relationship (Gowan et al., 2001). As the Hox proteins are co-expressed with these DV-restricted homedomain and bHLH proteins, it is possible that the duality of Hox protein function arise from interactions with either the proteins themselves or co-factors associated with their pathways. Future work to identify potential Hox protein binding partners with DV-restricted expression patterns, may give insight into this dual nature of Hox gene function. Short of these experiments, the present study shows that Hox genes are required to maintain the normal complement of gene expression necessary to generate a diverse group of cells.

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