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

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Accepted 10 December 2003

Development 131, 1259-1266
Published by The Company of Biologists 2004
doi:10.1242/dev.01029

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 preparation, 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...
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 (Gaudo 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). In contrast to Hoxb1–/– embryos, however, the appearance of Phox2b protein expression was intact in Hoxb1–/– 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). 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 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. 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.](image)
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 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.

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.

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...
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 Hox1–/– 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
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; Gauf 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,
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 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.

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 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.

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