Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1

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Trigeminal nuclei and the dorsal spinal cord are first-order relay stations for processing somatic sensory information such as touch, pain, and temperature. The origins and development of these neurons are poorly understood. Here we show that relay somatic sensory neurons and D2/D4 dorsal interneurons likely derive from Mash1-positive neural precursors, and depend on two related homeobox genes, Rnx and Tlx-1, for proper formation. Rnx and Tlx-1 maintain expression of Drg11, a homeobox gene critical for the development of pain circuitry, and are essential for the ingrowth of trkA+ nociceptive/thermoceptive sensory afferents to their central targets. We showed previously that Rnx is necessary for proper formation of the nucleus of solitary tract, the target for visceral sensory afferents. Together, our studies demonstrate a central role for Rnx and Tlx-1 in the development of two major classes of relay sensory neurons, somatic and visceral.

[Key Words: Rnx; Tlx-1; MASH1; DRG11; trkA; somatic sensory; D2; D4 interneurons]

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the developing hindbrain and spinal cord, *Rnx* and *Tlx-1* are initially expressed in longitudinal columns of cells, extending through the hindbrain and spinal cord (Fig. 1B; Logan et al. 1998), but the expression pattern becomes quite complex at later developmental stages [Logan et al. 1998; Qian et al. 2001; see below].

Here we show that *Rnx* and *Tlx-1* are associated with sequential development of multiple classes of neurons throughout the developing hindbrain and spinal cord. In addition to giving rise to the nTS and noradrenergic centers [Qian et al. 2001], we show that early born *Rnx*-expressing cells, derived from a subset of Mash1-positive precursor cells, are fated to form D2 and D4 interneurons. We then show that late born *Rnx*-expressing cells, derived from the entire dorsoventral extent of Mash1-positive domain, form first-order relay somatic sensory neurons, including the trigeminal nuclei and the superficial laminae of the dorsal spinal cord. Analyses of *Rnx* and *Tlx-1* single and double mutants show that the proper development of D2/D4 interneurons and relay somatic sensory neurons is dependent on *Rnx* or *Tlx-1*. Our studies provide a molecular model for the sequential formation of distinct classes of relay sensory neurons in the developing hindbrain and spinal cord.

**Results**

*Rnx*-expressing cells likely develop from MASH1-positive neural precursors

In the developing hindbrain and spinal cord, *Rnx* is initially expressed in two longitudinal columns of cells in E10.5–E11.5 mouse embryos (Figs. 1B, 2B; Qian et al. 2001), and the expression then becomes continuous in E12.5 embryos (Fig. 2E). We showed previously that dorsally derived *Rnx*-expressing cells in E10.5–E11.5 medulla likely develop from MASH1-positive precursors [Qian et al. 2001]. To determine the origins of the remaining *Rnx*-expressing cells, we again compared *Rnx* expression with that of two neural precursor markers, NGN1 and MASH1. Double staining of *Rnx* mRNA and MASH1 protein shows that in the spinal cord (Fig. 2B–D) of E11.5 wild-type embryos, the dorsal and ventral stripes of *Rnx*-expressing cells are located precisely at the lateral edges of the most dorsal and most ventral portions of the MASH1-positive domain, respectively (Fig. 2B, arrowheads). A few cells show codetection of *Rnx* mRNA and MASH1 protein (Fig. 2C,D, arrows), whereas no NGN1-positive cells coexpress *Rnx* (data not shown).

In E12.5 spinal cord, the dorsoventral limits of *Rnx*-expressing cells are located precisely at the lateral edges of the most dorsal and most ventral portions of the MASH1-positive domain, respectively (Fig. 2B, arrowheads). A few cells show codetection of *Rnx* mRNA and MASH1 protein (Fig. 2C,D, arrows), whereas no NGN1-positive cells, which are located dorsal to MASH1-positive territory, coexpress *Rnx* (data not shown).

In E12.5 spinal cord, the dorsoventral limits of *Rnx*-expressing cells again coincide perfectly with that of MASH1-positive territory (Fig. 2F, arrowheads). At this stage, multiple layers of cells at the lateral edge of MASH1-positive territory show double staining of MASH1 protein and *Rnx* mRNA (Fig. 2F,G, arrows). Note that instead of the columnar organization seen in E10.5–E11.5 embryos (Fig. 2A,B), the entire dorsoventral extent of MASH1-positive territory forms *Rnx*-expressing cells in E12.5 embryos (Fig. 2F). Again, no adjacent NGN1-positive cells coexpress *Rnx* in E12.5 embryos (Fig. 2H, arrows). Since Math1-positive precursors are located further dorsal to NGN1-positive cells [Helms and Johnson 1998; Gowan et al. 2001], we reasoned that they...
should also not coexpress \textit{Rnx}. Identical results are seen in the caudal developing medulla (data not shown). These studies suggest that both early and late born \textit{Rnx}-positive cells may develop exclusively from \textit{Mash1}-positive precursors.

**Development of D2 interneurons in the spinal cord is dependent on \textit{Rnx}**

In E10.5–E11.5 mouse embryos, the dorsal column of \textit{Rnx}-expressing cells spans from the medulla to the caudal spinal cord in E10.5–E11.5 mouse embryos [Fig. 1B]. In chick spinal cord, dorsal \textit{Rnx}-expressing cells correspond to D2 interneurons, as indicated by coexpression of \textit{Rnx/Tlx-3} and \textit{Isl1} [Logan et al. 1998]. \textit{Isl1} is a marker for D2 interneurons [Tsuchida et al. 1994]. Consistently, we found that \textit{Isl1} expression is lost in the dorsal spinal cord of E11.5 \textit{Rnx} mutant mouse embryos [Fig. 3, cf. B and C, arrows], suggesting that \textit{Rnx} function is necessary for D2 interneuron development. In contrast, \textit{Isl1} expression is lost in dorsally derived cells (cf. D, and G) and in the ventral motor neurons (cf. B and C, “mn”).

**The ventral column of \textit{Rnx}-expressing cells corresponds to D4 interneurons**

In the medulla of E11.5 wild-type embryos, both \textit{Lmx1b} and \textit{Rnx} are expressed in two stripes of cells along the dorsoventral axis [Fig. 4A,B, red and black arrows]. The following data indicate that ventrally derived \textit{Rnx}-expressing cells in the medulla and spinal cord correspond to D4 interneurons, which are previously defined by expression of the lim-homeobox gene, \textit{Lmx1b} [Tsuchida et al. 1994]. In the medulla of E11.5 wild-type embryos, both \textit{Lmx1b} and \textit{Rnx} are expressed in two stripes of cells along the dorsoventral axis [Fig. 4A,B, red and black arrows]. Within the ventral column, expression of \textit{Rnx} and \textit{Lmx1b} is detected in a cluster of cells adjacent to the ventricular zone [Fig. 4A,B, black arrowheads], and in a string of cells that appear to be migrating laterally and ventrally [Fig. 4A,B, black arrows]. Two-color in situ hybridization shows that \textit{Rnx} and \textit{Lmx1b} are coexpressed.
in these cells (Fig. 4E, arrows), indicating that these cells correspond to the previously described D4 interneurons. Expression of another Tlx family member, Tlx-1, is restricted exclusively to the medial area where Rnx appears to be expressed (Fig. 4, cf. A and D, arrowheads). Panels E-G and M-N show double staining of Rnx or Lmx1b mRNA in the cytoplasm (purple) and Phox2a protein in the nucleus (brown). [O-X] Loss of Lmx1b and Phox2a expression in prospective D4 interneurons in Rnx and Tlx-1 single or double mutants. Transverse sections through medulla [O-T] and spinal cord (U-X) of E11.5 wild-type (O,R,U,W), Rnx single mutant (P,S,V,/,X), and Rnx/Tlx-1 double mutant (Q,T) embryos. Panels O-T and panels U-X are equivalent to the boxed regions shown in A and H, respectively. In situ hybridization was performed with indicated probes.

Rnx and Tlx-1 are required for proper formation of D4 interneurons

To determine the roles of Rnx and Tlx-1 during development of D4 interneurons, we analyzed Rnx and Tlx-1 single or double mutants (Roberts et al. 1994; Shirasawa et al. 2000). In the medulla of E11.5 Rnx/Tlx-1 double
mutants, expression of both *Lmx1b* and *Phox2a* is eliminated in the lateral area (Fig. 4, cf. O and Q; R and T, arrows). In contrast, *Lmx1b* expression in the dorsal medial area is normal in double mutants [Fig. 4, O and Q, arrowheads], and no defect is detected in *Rnx* or *Tlx-1* single mutants [Fig. 4, cf. O and P; R and S, data not shown]. In the caudal spinal cord, expression of *Lmx1b* and *Phox2a* in ventrally migrating D4 interneurons is already eliminated in *Rnx* single mutants [Fig. 4, cf. U and V; W and X, arrows], consistent with a lack of strong *Tlx-1* expression at this axial level [data not shown]. Again, the most dorsal *Lmx1b* expression is unperturbed in *Rnx* single [Fig. 4V, arrowhead] or *Rnx/Tlx-1* double mutants [data not shown]. As no cell death was detected by TUNEL assay [see below, Fig. 7], we conclude that initiation of *Lmx1b* expression in prospective D4 interneurons is independent of *Rnx* or *Tlx-1*, but late expression of both *Lmx1b* and *Phox2a* requires either *Rnx* or *Tlx-1*.

Late born *Rnx*-expressing cells are fated to form the trigeminal nuclei and the dorsal horn of the spinal cord

As described in Figure 2E, *Rnx*-expressing cells in E12.5 embryos no longer show a columnar pattern. Our data, detailed in the following section, show that the late born cells are fated to form first-order relay somatic sensory stations, including the trigeminal nuclei and the superficial laminae of the dorsal spinal cord.

In the developing pons, the earliest born *Rnx*-expressing cells seen in E10.5 mouse embryos coexpress *Phox2a* and/or *Phox2b* and develop into noradrenergic neurons [Qian et al. 2001]. By E11.5, while *Rnx* expression has been down-regulated in prospective noradrenergic neurons [Qian et al. 2001], a large number of new *Rnx*-expressing cells emerge, most of which do not express either *Phox2b* or *Phox2a* [Fig. 5A, arrow, data not shown]. At E14.5 and E16.5, the *Rnx*-positive, *Phox2*-negative cells are located in a ventral lateral area of the developing pons corresponding to the principal trigeminal nucleus [Pr5] [Fig. 5B,C, arrows]. Interestingly, most neurons in the Pr5 express *Drg11* [Fig. 5D, arrow]. Note that *Drg11* is also expressed in regions surrounding the Pr5, where *Rnx* is not expressed [Fig. 5, cf. C and D, arrowheads].

Similarly, in the caudal medulla at E12.5, a new group of *Rnx*-positive, *Phox2*-negative cells is observed that spans most of the dorsal medulla [Fig. 5E]. By E14.5, these neurons are restricted to the dorsal lateral region corresponding to the spinal trigeminal nucleus [Sp5] [Fig.
5F, arrow]. At E16.5, Rnx expression in the caudal Sp5 shows a lamina-specific pattern, with predominant expression detected in the superficial laminae (Fig. 5G, arrow) and two deep laminae (Fig. 5G, red arrowheads). In contrast, Drg11 expression is restricted to the superficial portion of the Sp5 [Fig. 5H, arrow], and extends to a deeper zone in comparison with the superficial Rnx expression [Fig. 5, cf. G and H, black arrowheads]. Because Drg11 expression in the Sp5 is dependent on Rnx (see below), Rnx expression is likely to have been down-regulated in Drg11-positive, Rnx-negative zone. No Phox2b expression is detected in the Sp5 (data not shown).

In E14.5 developing spinal cord, late born Rnx-expressing cells are restricted to the dorsal horn of the spinal cord [Fig. 5J, arrow]. By E19, Rnx expression is primarily observed in the superficial laminae [Fig. 4K, arrow]. Again, Drg11 [Fig. 4L, arrows], but not Phox2b [Pattyn et al. 1997; data not shown], is expressed in the dorsal spinal cord.

Rnx and Tlx-1 are required for proper differentiation of relay somatic sensory neurons

To determine the role of Rnx and Tlx-1 in the development of relay somatic sensory neurons, we further analyzed single and double mutant embryos. We found that Drg11 expression is eliminated in E14.5 Sp5 and E16.5 dorsal horn of the spinal cord in Rnx/Tlx-1 double mutants [Fig. 6, cf. A and C, D and F, arrows]. Partial loss of Drg11 expression is observed in Rnx single mutants [Fig. 6, cf. A and B, D and E, arrows], whereas no defect is detected in Tlx-1 single mutants [data not shown]. The situation in the developing Pr5 is a bit different. Drg11 expression in this nucleus is not affected in Rnx or Tlx-1 single mutants [Fig. 6, cf. G and H, arrows, data not shown], and is only partially reduced in Rnx/Tlx-1 double mutants [Fig. 6, cf. G and I, arrows]. The partial dependence of Pr5 development on Rnx/Tlx-1 is reminiscent of the partial requirement of Rnx for formation of another pons structure, the locus ceruleus [Qian et al. 2001].

Expression of two additional markers, Ebf3 and Lmx1b, was also affected in Rnx/Tlx-1-deficient mice. Ebf3 is a basic helix-loop-helix gene expressed in the superficial laminae of the spinal cord [Fig. 6], arrow) and the Sp5 [Garel et al. 1997; Wang et al. 1997]. Ebf3 expression in these two structures is absent in Rnx/Tlx-1 double mutants [Fig. 6, cf. J and K, arrows; data not shown], and it is greatly reduced in Rnx single mutants [data not shown] at E14.5 or later stages. Lmx1b is initially expressed in D4 interneurons [Fig. 4], but the expression is expanded to the dorsal horn of the spinal cord at later stages [Fig. 6L, arrow]. In E14.5 Rnx/Tlx-1 double mutants, lmx1b expression is still restricted to the dorsal spinal cord [Fig. 6M, arrow]. However, instead of the nearly uniform expression seen in wild-type embryos, many cells within the superficial laminae show a

Figure 6. Loss or reduction of Drg11, Ebf3, and Lmx1b expression in relay somatic sensory neurons in Rnx and Tlx-1 single or double mutants. Transverse sections through the spinal trigeminal nucleus (Sp5) [A–C, arrows], the spinal cord [D–F, and J–O], and the principle trigeminal nucleus [Pr5] [G–I, arrows] of embryos with indicated genotypes and developmental stages. In situ hybridization was performed with indicated probes. N and O are higher magnification of the dorsal horn shown in panels L and M, respectively.
reduction of Lmx1b expression in Rnx/Tlx-1 double mutants [Fig. 6, cf. N and O, arrows].

Examination of embryos at earlier stages shows that initiation of Drg11 and Ebf3 expression is independent of Rnx or Tlx-1, as indicated by their normal expression in E12.5 Rnx/Tlx-1 double mutants [Fig. 7, cf. A and B, arrows; data not shown]. Significant loss of Drg11 and Ebf3 expression is first seen in E13.5 mutant embryos, and by E14, the expression is nearly completely lost [data not shown]. To determine whether the eventual absence of Drg11 and Ebf3 expression was caused by loss of mutant cells, we examined cell death by the TUNEL labeling method [Gavrieli et al. 1992] in embryos from E11.5 to E14.5, at 12-h intervals. At every embryonic stage examined, no increase of cell death was detected in Rnx/Tlx-1 double mutants in comparison with wild-type embryos [Fig. 7, cf. C and D, arrows; data not shown]. Thus, the mutant cells likely survived in Rnx/Tlx-1 double-deficient mice.

To address possible fates of the mutant cells, we examined the expression of a series of additional molecular markers. First, the mutant cells in the superficial laminae of the spinal cord apparently continued to differentiate into neuronal cells, indicated by expression of SCG10, a pan-neuronal marker [Stein et al. 1988], in E14.5 mutant embryos [Fig. 7, cf. E and F, arrows]. Second, the mutant cells do retain certain normal features, as demonstrated by the normal expression of Hoxb8 [Fig. 7, cf. G and H, arrows] and neuropilin-1 [data not shown], two dorsal horn neuron markers [Graham et al. 1991; He and Tessier-Lavigne 1997; Kolodkin et al. 1997]. Third, none of the deep laminar markers, including Lh2A [Fig. 7, cf. I and J, arrows], Slit2 (see Fig. 9 below), and Sema3A [see Fig. 9 below], show ectopic expression in the superficial laminae of Rnx/Tlx double mutants. Thus, there is no evidence indicating a fate switch from the superficial to the deep layers of neurons.

**Projection defect of trkA+ afferents in Rnx and Tlx-1 double mutants**

The superficial laminae of the dorsal spinal cord and Sp5 are the primary targets of trkA+ nociceptive/thermoceptive sensory afferents. The loss of expression of Drg11 and Ebf3 in these structures led us to examine whether central projections of trkA+ afferents are affected in Rnx and Tlx-1 double mutants. Since trkA mRNA is expressed in the DRG and nTG [Huang et al. 1999; Huang and Reichardt 2001], but not in the spinal cord or Sp5 [data not shown], trkA immunostaining is indicative of the central projections of the nociceptive/thermoceptive sensory afferents. In E14.5 wild-type embryos, trkA+ afferents have reached the dorsal entry zone [Fig. 8A, arrowhead], and have started to make collateral branches into the spinal cord [Fig. 8A, arrow]. In E14.5 Rnx/Tlx-1 double mutants, trkA+ afferents are able to reach the dorsal entry zone [Fig. 8B, arrowhead], but fail to make any entry to the dorsal horn [Fig. 8B, arrow]. At E18.5, ingrowth of trkA+ afferents in double mutants is much shallower in comparison with the projections seen in wild-type embryos [Fig. 8, cf. C and D, arrows]. Ingrowth of cranial trkA+ afferents to the spinal-trigeminal nucleus is also compromised in Rnx/Tlx-1 double mutants [Fig. 8, cf. E and F, arrows]. In contrast, the projection of a subset of trkA+ afferents to the deep laminae of the spinal cord appears not to be affected in mutant embryos [Fig. 8, cf. C and D, arrowheads].

Previous in vitro studies suggest that bovine Slit2 protein is able to stimulate collateral branching of rat trkA+ sensory afferents [Wang et al. 1999]. However, in E14.5 dorsal horn, Slit2 is not expressed in the superficial laminae in wild-type embryos [Fig. 9A, asterisk], where predominant Rnx expression is detected [Fig. 9J]. Slit2 expression in other areas is also unperturbed in Rnx/Tlx-1 double mutants, including the deep laminae [Fig. 9, cf. A and B, arrows] and the dorsal middle line [Fig. 9, cf. A and B, arrowheads]. Expression of two additional Slit family members, Slit-1 and Slit-3 [Holmes et al. 1998; Itoh et al. 1998; Brose et al. 1999; Li et al. 1999; Yuan et al. 1999].
is also largely normal in Rnx/Tlx-1 double mutants (Fig. 9, cf. C and D; data not shown). Furthermore, expression of Robo-1 and Robo-2 (Kidd et al. 1998), encoding the receptors for the Slit proteins (Brose et al. 1999; Li et al. 1999), is normal in the peripheral sensory ganglia in Rnx/Tlx-1 double mutants (data not shown).

In chick embryos, it has been hypothesized that dorsal restriction of trkA+ afferent projections is controlled by the repulsive signal, Sema-3A, which is expressed in the ventral two-thirds of the developing spinal cord (Shepherd et al. 1996, 1997; Fu et al. 2000). According to this hypothesis, entry failure of trkA+ afferents in Rnx and Tlx double mutants could be caused by an ectopic expression of Sema-3A in the dorsal horn, or by an overexpression of neuropilin-1, encoding the Sema-3A receptor, in the primary sensory afferent neurons. However, no abnormal expression of Sema-3A or Slits, or the genes encoding their receptors. To examine whether the projection defect was caused by major morphological change in the dorsal horn, we examined the gross pattern of neuronal migration by BrdU pulse-chase labeling. We injected BrdU at E10.5, E11.5, E12.5, or E13.5, and collected and analyzed the embryos at E14.5 or E16.5. Cells going through the final cell cycle at the time when BrdU was injected will show strongest staining with anti-BrdU antibody, while those cells going through additional rounds of cell division will show weaker staining, thus providing cell birthdating and cell migration information. In wild-type embryos (at the lumbar level), cells born at E10.5 and E11.5 are primarily restricted to ventral or deep layers of the dorsal horn (Fig. 9G, arrow; data not shown). In embryos chase-labeled at E12.5, strong BrdU staining is located in the superficial laminae (Fig. 9I, arrows). Note that a weaker staining is detected in the deep medial areas (Fig. 9I, asterisk), suggesting that these cells may have gone through additional rounds of cell division. Consistently, cells born at E13.5 are primarily located in the same deep medial area (Fig. 9K, arrow). No dramatic change of BrdU-labeling is observed in Rnx/Tlx-1 double mutants (Fig. 9, cf. G and H; I and J; L and K), suggesting that the gross pattern of neuronal migration might not be affected in Rnx/Tlx-1-deficient embryos.

Discussion

Possible fates of dorsal MASH1-positive precursors and origins of first-order relay sensory neurons and D2/D4 interneurons

The origins of various relay sensory neurons in the hindbrain and spinal cord is only beginning to be understood, partly due to the lack of prospective molecular markers for these neurons. Earlier studies have shown that the dorsal ventricular zone of the hindbrain and spinal cord is subdivided into three compartments, distinguished by complementary expression of the proneural proteins, Math1, NGN1/2, and MASH1 (Ma et al. 1997; Helms
and Johnson 1998; Lee et al. 1998; Gowan et al. 2001).

Math1-positive precursors form multiple components of the proprioceptive pathway, including the pontine nucleus, cerebellum granule cells, and spinal D1 interneurons (Ben-Arie et al. 2000; Bermingham et al. 2001; Gowan et al. 2001). Dorsal NGN1/2-positive precursors form D3A interneurons in the spinal cord (Gowan et al. 2001). Our present and previous (Qian et al. 2001) studies show that the broad MASH1-positive domain appears to form a variety of Rnx-expressing cells, including (1) three major relay sensory stations, the nTS, the nTG, and the superficial laminae of the dorsal spinal cord; (2) two classes of dorsal interneurons, D2 and D4; and (3) brainstem (nor)adrenergic centers [summarized in Fig. 10A].

All these neurons show unique molecular identities, and are formed at distinct positions along the dorsoventral or rostrocaudal axes, or at distinct embryonic stages [Fig. 10]. For example, spinal D2 interneurons, coexpressing Rnx and Isl1 [Logan et al. 1998], may be formed from the most dorsal MASH1-positive domain in E10.5–E11.5 embryos [Figs. 2,3]. D4 interneurons, which first express Rnx plus Lmx1b and then Phox2a, likely develop from the most ventral portion of MASH1-positive precursors in E10.5–E11.5 embryos [Figs. 2,4]. In contrast, late born Rnx-positive cells appear to form from the entire dorsal MASH1-positive domain, and are fated to form the trigeminal nuclei and the superficial laminae of the dorsal spinal cord [Figs. 2,5]. The fate of the intermediate portion of the MASH1-positive domain in E10.5–E11.5 embryos (Fig. 2B, arrow) remains to be determined.

Figure 9. Expression of Slit1/2 and Sema-3A, and BrdU pulse-chase labeling, in wild-type and Rnx/Tlx-1 double mutants. Transverse sections through the spinal cord of E14.5 A–F and E16.5 G–L embryos with indicated genotypes. In situ hybridization was performed with indicated probes [A–F]. Panels G–L show BrdU pulse-chase labeling. Panels G and H show embryos pulse labeled at E11.5 and analyzed at E14.5. Note, cells born at this stage are located in the ventral two-thirds of the spinal cord in both wild-type and mutant embryos [cf. G and H, arrows]. Panels I and J show embryos pulse-labeled at E12.5 and analyzed at E14.5. Note, strong BrdU staining is detected in the superficial laminae in both wild-type and mutant embryos [cf. I and J, arrows]. A weaker staining is observed in the deep dorsal medial area in both wild-type and mutant embryos [cf. I and J, asterisks]. In embryos pulse-labeled at E13.5, BrdU staining is predominantly located in the deep medial area [K, arrows]. Again, no gross defect was detected in Rnx/Tlx-1 double mutants [cf. K and L, arrows].
cently identified as a prospective marker for the nTS neurons and possibly the [nor]adrenergic neurons [Qian et al. 2001], is absent in Mash1-deficient mice [Hirsch et al. 1998]. Spinal expression of Phox2a, which we show here as a comarker for D4 interneurons [Fig. 4], is also absent in Mash1 mutants [Hirsch et al. 1998]. Furthermore, genetic fate mapping studies do not show any progeny cells from NGN1- and Math1-positive precursors that coexpress the D2 interneuron marker, Isl1, and D2 cell development is not affected in ngn1/2- or Math1-deficient mice [Bermingham et al. 2001; Gowan et al. 2001]. These studies strongly suggest that Rnx-expressing cells develop from MASH1-positive precursors, although our data have not ruled out the formal possibility that NGN1- and Math1-positive cells might give rise to a subset of Rnx-expressing cells.

Our studies reveal an ontogenetic relationship between D2 interneurons and the nTS [Fig. 10]. Both of them develop from the dorsal column of Rnx-expressing cells in E10.5–E11.5 embryos, and both appear to migrate ventrally [Fig. 3; Qian et al. 2001]. The nTS is the target for trkB+ visceral sensory afferents. Interestingly, D2 interneurons are located in the deep laminae of the dorsal horn [Ozaki and Snider 1997], to which trkB+ mechanoceptive afferents are thought to project [Brown et al. 1977; Ralston et al. 1984; Shortland et al. 1989; Snider 1994]. Actually, many visceral sensory neurons that project to the caudal nTS [Qian et al. 2001], portions of the Pr5 and Sp5, and the superficial laminae of the dorsal spinal cord [Fig. 5]. The molecular identities of a subset of cells in the Sp5 and the dh are unclear [marked with “?”].

A central role of Rnx and Tlx-1 in the formation of first-order relay somatic and rely visceral sensory neurons

We showed previously that Rnx is required for proper development of the nTS, the major relay station for visceral sensory afferents [Qian et al. 2001]. The defect of the nTS provides a plausible explanation for why Rnx-deficient mice die from a defect of breathing control
Here we demonstrate that Rnx and Tlx-1 together are also required for proper development of most relay somatic sensory neurons, including the spinal-trigeminal nucleus [Sp5], the dorsal horn of the spinal cord, and a portion of the principle trigeminal nucleus [Pr5]. The defects in Rnx/Tlx-1 double mutants are indicated by the loss of reduction of expression of Drg11, Ebf3, and Lmx1b (Fig. 6), and by the failure of ingrowth of trkA+ nociceptive and thermoceptive afferents to the nTG and the dorsal horn of the spinal cord (Fig. 8; also see below). In addition, Rnx and Tlx-1 are required for the proper formation of two classes of dorsal interneurons, D2 and D4, as indicated by the loss of Isl1 and Lmx1b expression in Rnx/Tlx-1 double mutants (Figs. 3,4). Thus, the Tlx family of homeobox proteins emerges as the central regulators for the development of the relay somatic and the relay visceral sensory neurons.

In the dorsal spinal cord, the mutant cells appear to migrate properly, based on the comparable BrdU pulse-chase labeling patterns in wild-type and double mutant embryos (Fig. 9), although this methodology does not chase labeling patterns in wild-type and double mutant embryos (Figs. 3,4). Hence, the Tlx family of homeobox proteins may have a unique function in maintaining expression of a variety of transcription regulators. We found previously that initiation of Phox2b expression in the developing nTS is independent of Rnx (Qian et al. 2001). Here we show that initiation of Drg11 and Ebf3 expression in the developing nTG and the dorsal spinal cord, and the initial Lmx1b expression in the prospective D4 interneurons, are not affected in Rnx/Tlx-1 double mutants (Figs. 4,7; data not shown). However, expression of all these genes is lost during a period when no cell death is detected by the TUNEL method (Figs. 4,6,7; Qian et al. 2001). The mechanisms by which Rnx and Tlx-1 maintain expression of a variety of molecular targets remain to be determined.

Rnx and Tlx-1 apparently share functional redundancy. We have not observed any neuronal defects in Tlx-1 single mutants, and the severity of abnormality in Rnx single mutants inversely correlates with the degree of Tlx-1 expression in that structure. For instance, Tlx-1 is expressed in medullary but not spinal D4 interneurons. Consequently, spinal D4 cell development is compromised in Rnx single mutants, while defect of medullary D4 interneuron formation only occurs in Rnx/Tlx-1 double mutants (Fig. 4).

Central ingrowth of the trkA+ afferents is dependent on Rnx and Tlx-1

Rnx and Tlx-1 represent the very few transcription factors identified thus far that are required for proper entry of trkA+ sensory afferents to the Sp5 and the dorsal horn of the spinal cord. The failure of ingrowth of trkA+ afferents observed in Rnx/Tlx-1 double mutants is different from the projection phenotype seen in Drg11-deficient mice. In Drg11 mutants, trkA+ afferents are able to make collateral entry to the dorsal spinal cord, although the projections are rerouted to the medial area (Chen et al. 2001). The difference is possibly due to the fact that besides Drg11, Rnx and Tlx-1 control additional targets, such as Ebf3 and Lmx1b.

The cause of the trkA+ afferent ingrowth defect is still unclear. Although we have not ruled out a defect of projection neurons in the DRG and the trigeminal ganglia, several observations are more consistent with the target field defect model. Of the three Tlx family members, Tlx-2 is expressed exclusively in peripheral sensory ganglia (Tang et al. 1998; data not shown), and might compensate the loss of Tlx-1 and Rnx. Indeed, no abnormal neuronal differentiation is detected in the DRG and trigeminal ganglia in Rnx/Tlx-1 double mutants (data not shown). This is best exemplified by the finding that Drg11 expression is lost in the mutant dorsal spinal cord and spinal trigeminal nuclei (Fig. 6), but is normal in the DRG and trigeminal ganglia (data not shown). In addition, Isl1 expression is absent in the spinal D2 interneurons, but normal in the DRG in Rnx/Tlx-1 double mutants (Fig. 3). As mentioned above, we have no evidence indicating aberrant neuronal migration of the dorsal horn neurons. We think that the simplest interpretation is that Rnx and Tlx-1 might regulate the expression of molecules that are crucial for collateral entry of sensory afferents.

Previous in vitro studies suggested that Sema-3A and members of the Slit family may control trkA+ afferent projections in the dorsal spinal cord (Puschel et al. 1996; Shepherd et al. 1997; Wang et al. 1999; Fu et al. 2000). However, expression of these signaling molecules and their receptors is largely unperturbed in Rnx/Tlx-1 double mutants (Fig. 9). Thus, our studies raise the possibility that in vivo, trkA+ afferent ingrowth might be controlled by molecules other than Sema-3A or Slits. Alternatively, Rnx and Tlx-1 might regulate expression of molecules required for Sema-3A or Slit protein functions.

**Are the relay somatic and the relay visceral sensory neurons phylogenetically related?**

Our studies reveal significant parallels between brainstem relay somatic (nTG) and relay visceral (nTS) sensory nuclei. First, both the nTS (Qian et al. 2001) and the nTG (Figs. 4–6) likely develop from MASH1-positive neural precursors, and depend on members of the Tlx family of homeobox genes for proper formation. Second, Drg11 and Phox2b, which are expressed in the nTG and nTS, respectively (Fig. 5; Qian et al. 2001), are closely related members of paired class homeobox genes (Saito et al. 1995; Patten et al. 1997). Third, regulation of Drg11 and Phox2b shows remarkable similarities. As mentioned above, initiation of Drg11 (Fig. 6) and Phox2b (Qian et al. 2001) expression is independent of Rnx. However, once the cells are in the migration pathways, expression of both genes becomes dependent on Rnx or Tlx-1 (Qian et al. 2001; data not shown). These parallels
raise the possibility that brainstem relay visceral and relay somatic sensory nuclei might have evolved from a common ancestor.

Materials and methods

Animals

The generation of Tlx-1 and Rax mutant mice has been described (Roberts et al. 1994; Shirasawa et al. 2000). Tlx-1 and Rax heterozygous mice were intercrossed to generate double heterozygous mice, which were then intercrossed to generate double mutant embryos. The following primers that produce a 0.13-kb product: HOX11P1, 5′-CTTCCGTATCAGAA CAGATCC-3′ and HOX11P8, 5′-CGGAGCCCCC CGGCCGCTTAAGAG-3′. Mutant Tlx-1 allele was amplified with the following primers that produce a 0.2-kb fragment: Tlx1P1, 5′- CCTCCATCCAGGATGGAGACTATGGCC-3′ and Tlx1P2, 5′-GG GAAATCTCTGACTAGGGGAG-3′. Wild-type and mutant Rax alleles were genotyped with HOX1L2P1, 5′-AGCGGCGAC TGCTCCATCCAGG-3′ and Hox11P2 (see above), which produces 0.33-kb and 0.4-kb products, respectively.

In situ hybridization and immunostaining

Section in situ hybridization was performed as described (Ma et al. 1998). Anti-trkA immunostaining was performed as described by Huang et al. (1999). In situ hybridization combined with antibody immunostaining was performed essentially as described (Tiveron et al. 1996). The following primers were used: Tlx1, Enx and Rnx (Qian et al. 2001), Phox2a (Tiveron et al. 1996), Phox2b (Pattyn et al. 1997), trkA (Birren et al. 1993), Lmx1b (Chen et al. 2001), Drg11 (Saito et al. 1995), Slt1 (Yuan et al. 1999), Slt2 (Yuan et al. 1999), Slt3 (Yuan et al. 1999), Robo1 (Brose et al. 1999; Li et al. 1999), Robo2 (Brose et al. 1999; Li et al. 1999), Sema-3A (Messersmith et al. 1995), neuropilin1 (He and Tessier-Lavigne 1997), neuropilin2 (Kolodkin et al. 1997), and Hoxb8 (Graham et al. 1991).

BrdU pulse-chase labeling

Pregnant wild-type mice carrying E10.5, E11.5, E12.5, and E13.5 embryos were injected with 5-bromo-2-deoxy-uridine (BrdU) (Sigma) 50 mg/kg body weight, and embryos were collected at E14.5 or E16.5, fixed overnight in 4% paraformaldehyde, sunk in 20% sucrose, and embedded in OCT (Tissue-Tek). For immunostaining on frozen sections with anti-BrdU antibody (Boehringer Mannheim) [1:5 dilution], sections were dried at 50°C for 15 min; fixed in 4% paraformaldehyde for 10 min; incubated for 2 h at 70°C in 0.2% formamide, 1× SSC, plus 0.1% Tween-20, 1 h at 70°C in 0.2x SSC, washed with PBS, treated with 2 N HCl in PBS for 30 min at 37°C, rinsed in PBS five times; and washed with PBS (3 × 5 min). Subsequent immunostaining with anti-BrdU antibody (Boehringer Mannheim) [1:50 dilution] was performed with a Vectastain detection kit (Vector Labs) according to the manufacturer’s instructions, except that 0.001% rather than 0.003% H2O2 was used in the final enzymatic reaction. The slower rate of reaction prevented overstaining and made it easier to distinguish cells that have gone through the final round, or additional rounds, of cell cycle.

Formation of relay somatic sensory neurons

TUNEL staining on cryostat sections

Two pairs of E10.5–E14.5 wild-type and mutant embryos were used. The TUNEL assay on frozen sections was performed according to the ApopTag method (Intergen).

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