Formation of brainstem (nor)adrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3

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Brainstem visceral sensory and (nor)adrenergic neurons play crucial roles in modulating cardiovascular and respiratory functions. The origins and formation of these neurons are poorly understood. Here we show that these two classes of neurons are derived from *Mash1*-positive precursor cells, and can be prospectively identified by combinatorial expression of two homeobox genes, *Rnx* and *Phox2* (*Phox2a* or *Phox2b*). It was previously shown that *Rnx*-deficient mice die from respiratory failure. Here we show that Rnx function is required for formation of first-order relay visceral sensory neurons in the brainstem. In addition, as in *Phox2b*-deficient mice, the development of most (nor)adrenergic centers is compromised in *Rnx* mutants. We also provide genetic evidence to show that Rnx and Phox2 proteins may function independently to specify the (nor)adrenergic phenotype. Our studies reveal a surprising ontogenetic relationship between relay visceral sensory and (nor)adrenergic neurons, and suggest that it may be a common theme in the developing nervous system that the same set of transcriptional regulators is associated with formation of multiple components within a neuronal network.

Physiological control of cardiovascular and respiratory systems involves complex neuronal circuits. Respiration patterns are generated by rhythmic neurons in the ventral medulla and modulated by input from other groups of neurons in the brainstem, including visceral sensory and (nor)adrenergic neurons (i.e., adrenergic and noradrenergic neurons) (Fig. 1; Blessing 1997; Rekling and Feldman 1998). For instance, activation of peripheral chemoreceptors and pulmonary stretch receptors, carried via first-order relay visceral sensory neurons in the nucleus of the solitary tract (nTS), leads to an increase of respiration rate (Fig. 1; Blessing 1997). In contrast, input from (nor)adrenergic neurons predominantly causes a depression of respiratory frequency (Fig. 1; Bolme and Fuxe 1973; Champagnat et al. 1979; Errchidi et al. 1991; Al-Zubaidy et al. 1996).

Despite the pivotal roles played by visceral sensory and (nor)adrenergic neurons in modulating cardiorespiratory activities, the development of these two classes of neurons is poorly understood. At the molecular level, the most important progress made so far is the identification and characterization of two closely related genes *Phox2a* and *Phox2b*, which encode a paired-like class of homeodomain proteins (Valarché et al. 1993; Yokoyama et al. 1996; Pattyn et al. 1997). Phox2 proteins are required for formation of the (nor)adrenergic neurons throughout the nervous system (Morin et al. 1997; Pattyn et al. 1999, 2000a). However, *Phox2* genes are also associated with non-(nor)adrenergic structures, including hindbrain parasympathetic preganglion motor neurons and branchial motor neurons (Valarché et al. 1993; Tiveron et al. 1996; Pattyn et al. 1997, 2000b), suggesting that they are necessary but not sufficient to specify the (nor)adrenergic phenotype. Consistent with this, ectopic expression of *Phox2a* in Zebrafish embryos induces (nor)adrenergic neurons only in highly restricted locations (Guo et al. 1999). Neural crest cell culture studies also show that Phox2a requires additional signals to induce the (nor)adrenergic feature (Lo et al. 1999).

Thus far, the factors that work cooperatively with Phox2 proteins in the specification of (nor)adrenergic neurons remain to be identified. The proneural protein MASH1 has been shown to be required for the formation
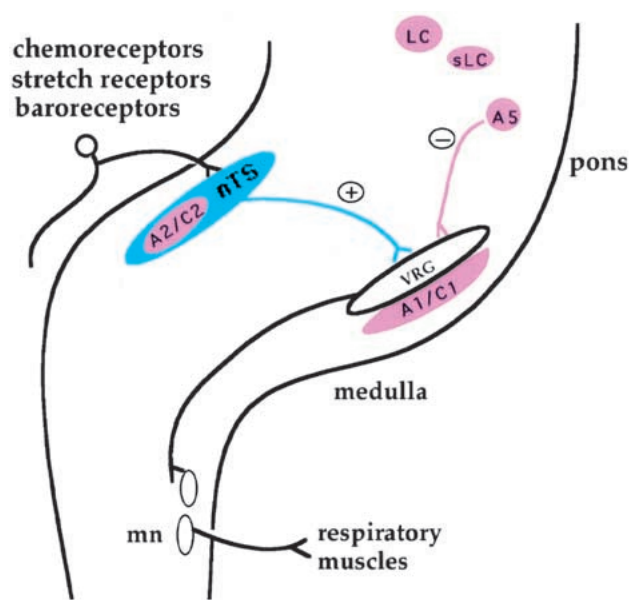
Figure 1. Schematic of respiratory neuronal circuitry. The basic breathing rhythm is generated by respiratory neurons located in the ventral medulla (VRG). The activation of peripheral chemoreceptors by a high carbon dioxide level in the blood flow, that of stretch receptors by air inflation in the lung, or that of baroreceptors by high blood pressure, provides stimulation of respiration rates. The peripheral stimulatory signals are carried via first-order relay visceral sensory neurons in the nucleus of the solitary tract (nTS). The nTS, in turn, makes connections to the VRG. Via further relays through spinal respiratory motor neurons (mn), the activities of respiratory muscles will be modulated. In contrast, inputs from (nor)adrenergic centers (in pink) cause a depression of respiration frequency. (Nor)adrenergic neurons are divided into two classes, namely adrenergic (C1 and C2) and noradrenergic (A1, A2, A5, sLC, and LC). sLC, Locus subcoeruleus; LC, locus coeruleus.

of most (nor)adrenergic neurons (Hirsch et al. 1998), but MASH1 function is primarily mediated by regulating the expression of *Phox2* genes (Hirsch et al. 1998; Pattyn et al. 2000a). In vitro promoter analyses show that the retinoic acid inducible transcription factor, AP2, may be involved in regulation of the (nor)adrenergic phenotype (Kim et al. 2001), but its in vivo requirement is unknown.

The molecular basis of the formation of relay visceral sensory neurons is even less understood. *Phox2b* is expressed in the nTS (Pattyn et al. 1997), but its function in these neurons has not been characterized. *Tlx* is another family of homeobox genes expressed in restricted regions of the developing hindbrain. There are three family members, *Hox11/Tlx1*, *Hox11-L1/Tlx2/Enx*, and *Hox11-L2/Tlx3/Rnx* (Dube et al. 1991; Hatano et al. 1991, 1997; Kennedy et al. 1991; Dear et al. 1993, 1995; Raju et al. 1993; Roberts et al. 1994, 1995; Shirasawa et al. 1997, 2000; Logan et al. 1998; Tang et al. 1998). For simplicity, these three genes will be referred to here as *Tlx1*, *Enx*, and *Rnx*, respectively. In both chick and mouse embryos, *Rnx* is expressed in two longitudinal stripes of postmitotic neurons in the developing hindbrain and spinal

cord (Logan et al. 1998; Shirasawa et al. 2000). It was hypothesized that part of the ventral stripe may give rise to the nTS in chick embryos (Logan et al. 1998). However, because of extensive cell migration and complex morphogenesis in the developing hindbrain (Altman and Bayer 1987), it has been difficult to definitely assign cell fates if prospective markers for the cells of interest have not been established.

Rnx-deficient mice show a phenotype resembling the congenital hypoventilation syndrome in humans, and all die within 24 h after birth from respiratory failure (Shirasawa et al. 2000). Here we show that *Rnx* is expressed in, and required for proper formation of, first-order relay visceral sensory neurons and most of the (nor)adrenergic centers in the brainstem. All of these neurons can be prospectively defined by a combinatorial expression of *Rnx* and *Phox2b/Phox2a* at a time when they are formed. Furthermore, we show that *Rnx* and *Phox2* genes are required independently for specification of the (nor)adrenergic phenotype. These findings establish a central role of *Rnx* in the formation of two major classes of modulator neurons in the brainstem that are crucial for cardiorespiratory control.

Results

Coexpression of Rnx and Phox2b in developing nTS

Rnx-deficient mice die from a hypothesized central defect in respiratory control (Shirasawa et al. 2000). Since the *Phox2* genes are expressed in and required for proper development of many visceral sensory and autonomic neurons (Morin et al. 1997; Pattyn et al. 1999, 2000a), we performed a detailed examination of *Rnx* expression in relation to that of *Phox2a* and *Phox2b*. As in chick embryos (Logan et al. 1998), whole mount in situ hybridization of embryonic day (E)9.5–E11 mouse embryos reveals that *Rnx* is initially expressed in two longitudinal stripes (Fig. 2A,B; data not shown). This can be seen best in *neurogenin1* mutants due to the absence of a subset of peripheral ganglia (Fig. 2B, arrows and arrowheads; Ma et al. 1998). The dorsal stripe of *Rnx* expression is first detected in E10.5 mouse embryos (data not shown), and by E11, it appears to overlap that of *Phox2b* (Fig. 2B,C, arrowheads; also see below). The rostral extent of *Phox2b* and *Rnx* expression is at the axial level where the geniculate ganglion is located (Fig. 2B,C, g). Caudally, *Phox2b* expression ends around the obex, the junction between the spinal cord and the hindbrain. Thus, the anterior posterior extent of *Phox2b* expression in the dorsal hindbrain appears to correspond to the location of the nTS (Saper 2000).

By E11.5, neurons derived from the *Rnx/Phox2b* dorsal stripe appear to migrate in a ventral direction, as shown by in situ hybridization on adjacent transverse sections (Fig. 2D,E, black arrows). Coexpression of *Rnx* and *Phox2b* in these cells is demonstrated by double labeling, with *Rnx* mRNA detected by in situ hybridization and Phox2b protein detected by immunostaining (Fig. 2F,G). In contrast, the ventral stripe of *Rnx*-expressing
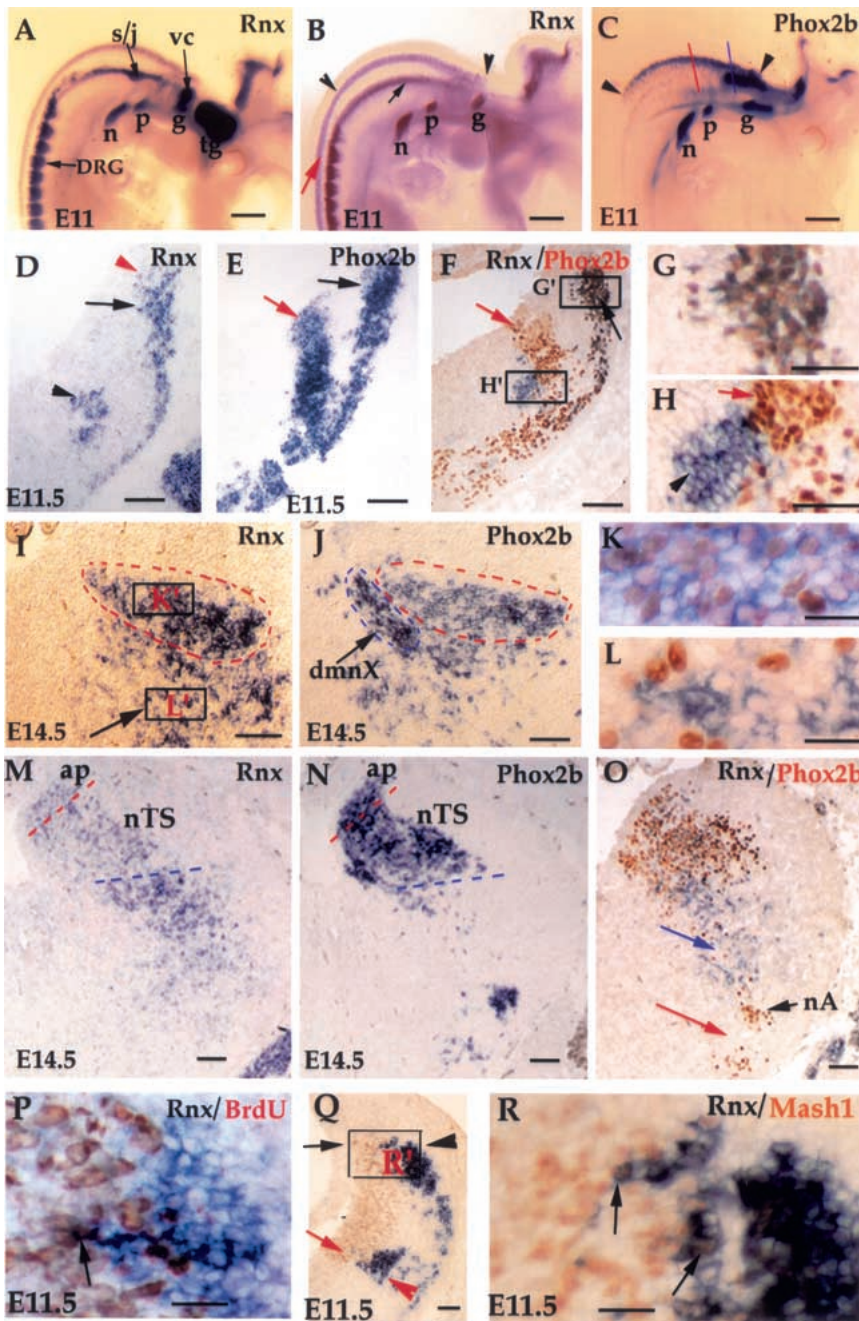
Figure 2. Coexpression of *Rnx* and *Phox2b* in, and developmental origin of, the nTS. (*A–C*) Whole mount in situ hybridization with indicated probes. E11 wild-type (*A,C*) and *ngn1* mutant (*B*) embryos. *Rnx* is expressed in every sensory ganglion (*A*), including trigeminal (tg), geniculate (g), pertrosal (p), nodose (n), vestibular-cochlear (vc), and superior/jugular ganglia (s/j). *Rnx* expression in the central nervous system is seen best in *ngn1* null mutants because of the absence of sensory ganglia (cf. *A* and *B*). The dorsal expression of *Rnx* and *Phox2b* seems to overlap (*B,C*, arrowheads) (see below). The fates of the ventral stripe of *Rnx*-expressing cells in the brainstem (*B*, arrow) as well as the longitudinal stripes in the spinal cord (*B*, red arrow) will be reported elsewhere. The blue and red lines in panel *C* represent the axial levels for the sections shown in panels *D–L* and *M–O*, respectively. (*D–O*) Adjacent transverse sections through E11.5 embryos (*D–L*) and E14.5 embryos (*I–O*). Dorsal is up and lateral is to the right. In situ hybridization was performed with indicated probes. Double labeling was performed on sections *F–H*, *K*, *L*, and *O*, with *Rnx* mRNA detected by in situ hybridization (purple staining in the cytoplasm), and Phox2b protein detected by immunostaining with Phox2b antibody (brown staining in the nucleus). Expression of *Rnx* and *Phox2b* in the dorsal position (*D,E*, black arrows) overlaps, as demonstrated by double labeling (*F,G*). (Panel *G* corresponds to box *G'* shown in panel *F*). In contrast, the ventral *Rnx* stripe (*D*, arrowhead) and the intermediate *Phox2b* stripe (*E*, red arrow) do not overlap (*F,H*). (Panel *H* is a higher magnification of box *H'* shown in panel *F*). By E14.5, neurons coexpressing *Rnx* and *Phox2b* are located in a region that corresponds to the nTS (*I,J*, dashed red circles), which is demonstrated by double staining (*K*). The nTS is distinct from the adjacent location of the dorsal motor nucleus of the vagus (*H*, dmnX), which itself is labeled by in situ hybridization on an adjacent section with choline acetyl transferase as the probe (Tiveron et al., 1996; data not shown). Again in the more ventral area (*I*, arrow), the expression of *Rnx* and *Phox2b* does not overlap (*L*). (Panel *L* corresponds to box *L'* in panel *I*). *Rnx* expression is reduced significantly in caudal nTS (*M*, nTS), starting at the axial level of area postrema (*M*, ap). Weak *Rnx* expression is also detected in the ap (*M*, ap). In contrast, strong *Phox2b* expression is detected at any axial level examined (*J,N*). Panel *O* shows double staining of *Rnx* and *Phox2b*. The most ventral *Rnx* expression (*O*, blue arrow) is located in the area dorsal to the nucleus ambiguus (nA), which is a dense cluster of Phox2b-expressing branchial motor neurons (*O*, nA) (Pattyn et al. 2000b). No *Rnx* expression is detected in the area ventral to the nA (*O*, red arrow). (*P–R*) Transverse sections through the developing medulla of E11.5 wild-type embryos. Panel *P* shows *Rnx* in situ hybridization (purple) followed by anti-BrdU immunostaining (brown) (the section roughly corresponds to the boxed region shown in panel *Q*). No double staining is detected (*P*), except for a single cell (*P*, arrow). Panels *Q* and *R* show double staining of *Rnx* mRNA (purple) and MASH1 protein (brown). Panel *R* is a high magnification of the boxed region shown in panel *Q*. MASH1 is expressed in precursor cells located in the ventricular zone (*Q,R*, brown staining). The dorsal (*Q*, black arrowhead) and ventral (*Q*, red arrowhead) stripes of *Rnx*-expressing cells are located lateral to the most dorsal (*Q*, black arrow) and the most ventral (*Q*, red arrow) parts of the MASH1-expressing domain, respectively. A few double-stained cells were detected (*R*, arrows). Bars: *A–C*, 200 µm; *K,L,P,R*, 50 µm; all remaining panels, 100 µm.

cells (Fig. 2D, arrowhead) and the intermediate stripe of *Phox2b*-expressing cells (Fig. 2E, red arrow) do not overlap (Fig. 2F,H).

By E14.5, cells coexpressing *Rnx* and *Phox2b* are located in a region corresponding to the nTS (Fig. 2I–O). At this stage, *Rnx* expression in the caudal part of the nTS is significantly reduced (Fig. 2, cf. M and I, nTS). Weak *Rnx* expression is also detected in the area postrema (Fig. 2M, ap). (The area postrema, which is located dorsal-medial to the nTS, is innervated by a subset of primary visceral sensory afferents important for cardiovascular control; Blessing 1997). In contrast, strong *Phox2b* expression is detected throughout the entire nTS (Fig. 2J,N). In summary, the dorsal stripe of *Rnx*-expressing cells seen in E11 mouse embryos (Fig. 2B, arrowheads) is fated to form the relay visceral sensory neurons in the nTS, and possibly the area postrema, rather than the nearby vestibular nucleus proposed in chick embryos (Logan et al. 1998).

It has been hypothesized that the breathing problem in *Rnx*-deficient mice is caused by a functional disorder of ventral respiratory neurons (Shirasawa et al. 2000). The rhythmic respiratory neurons are normally located in a region ventral to the nucleus ambiguus (nA) (Rekling and Feldman 1998). The nA is a dense cluster of branchial motor neurons that can be recognized by the expression of *Phox2b* (Fig. 2O, nA) (Pattyn et al. 2000b). We find that persistent *Rnx* expression is not detected in the area ventral to the nA (Fig. 2O, red arrow). This result suggests that *Rnx* is either not expressed, or expressed transiently in ventral respiratory neurons.

The nTS develops from Mash1-expressing neural precursors

We note that in E11.5 mouse embryos, a few *Rnx*-expressing cells are located within the ventricular zone (Fig. 2D, red arrowhead). Double labeling, with *Rnx* mRNA detected by in situ hybridization and dividing cells detected by anti-BrdU immunostaining, shows that *Rnx* is predominantly expressed in postmitotic cells (Fig. 2P). However, occasionally we do observe double-labeled cells (Fig. 2P, arrow), suggesting that *Rnx* expression is initiated right after cells exit from the cell cycle. Thus, the nTS neurons are likely derived from the ventricular zone medial to the most dorsal *Rnx*-expressing cells.

The ventricular zone in the developing neural tube is subdivided to distinct territories that express, from dorsal to ventral, *Math1*, *ngn1*, and *Mash1*, respectively (Gradwohl et al. 1996; Ma et al. 1997; Gowan et al. 2001). We find that the dorsal stripe of *Rnx*-expressing cells is located lateral to the MASH1-expressing territory (Fig. 2J, black arrow). Moreover, coexpression of MASH1 protein and *Rnx* mRNA is detected in a few cells within the subventricular zone (Fig. 2K, arrows). We conclude that dorsally derived *Rnx*-expressing cells, which are fated to form the nTS, develop from the most dorsal MASH1-positive territory. Interestingly, the ventral stripe of *Rnx*-expressing cells apparently develops from the most

ventral part of the MASH1-positive domain (Fig. 2Q, red arrow).

Development of the nTS is compromised in Rnx mutant mice

The nTS is a key component of the respiratory neuronal circuitry (Fig. 1). Indeed, we find that development of the nTS is compromised in *Rnx*-deficient mice. In E19 *Rnx* mutant mice, *Phox2b* expression in the prospective nTS area is nearly completely eliminated (Fig. 3A–F). This is true at all axial levels examined (Fig. 3A–F). In addition, *Phox2b* expression in the area postrema is also missing (Fig. 3, cf. C and D, ap). In contrast, expression of *Phox2b* in other brainstem structures is not affected, including
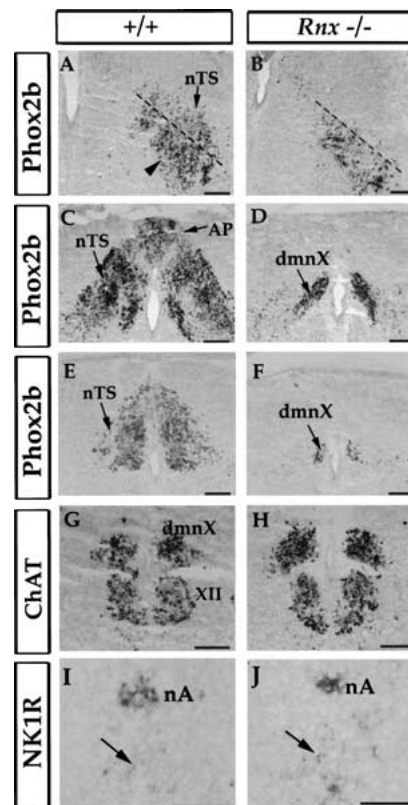

Figure 3. Loss of *Phox2b* expression in the nTS of E19 *Rnx* mutant embryos. Adjacent transverse sections through E19 wild-type and mutant embryos. In situ hybridization was performed with indicated probes. *Phox2b* expression is lost throughout the entire nTS (*A–F*, nTS), including the rostral part of the nTS (*A,B*), medial part of the nTS (at the axial level of area postrema) (*C,D*), and caudal commissural nTS (*E,F*). No defects were detected in other *Phox2b*-expressing structures, including a group of neurons located ventral to the rostral nTS (*A*, arrowhead), and the dorsal motor nucleus of the vagus (*C–F*, dmnX). The normal formation of the dmnX and hypoglossal motor nucleus (*G*, XII) is also indicated by expression of *ChAT* (*G* and *H*, dmnX). The expression of neurokinin-1 receptor (NK1R) in the nucleus ambiguus (*I,J*, nA) and in the prospective respiratory pacemaker neurons (*I,J*, arrows) is not affected in *Rnx*-deficient mice (cf. *J* and *I*). Bars, 200 µm.

the dorsal motor nucleus of vagus (dmnX) (Fig. 3D,F, dmnX) and a group of neurons located ventral to the rostral part of the nTS (Fig. 3A, arrowhead). The proper development of dmnX is further indicated by normal *ChAT* expression (Fig. 3G,H). Expression of neurokinin-1 receptor, a possible marker for ventral respiratory pacemaker neurons (Gray et al. 1999), is also not affected in *Rnx*-deficient mice (Fig. 3J,I, arrows).

To determine the developmental stages when the nTS defect occurs, we examined *Phox2b* expression in E11–E16.5 *Rnx* mutant embryos. As described above, neurons in the nTS are formed in a dorsal position and then migrate ventrally (Fig. 2). In both E11 (data not shown) and E12.5 *Rnx* mutants, *Phox2b* expression in the most dorsal position (Fig. 4B, arrowhead) and in early migratory neurons (Fig. 4B, red arrow) is normal. However, in the area corresponding to the cells that have migrated to the more ventral position, *Phox2b* expression is eliminated (Fig. 4A,B, arrows). Consistently, BrdU pulse-chase labeling shows that by E12.5, *Phox2b* expression is no longer detected in the cells born at E10.5 (Fig. 4, cf. C and D, red arrows). Nonetheless, the detection of early *Phox2b* expression suggests that the nTS neurons are formed in

Rnx mutants, consistent with the finding that *Rnx* is predominantly expressed in postmitotic cells (Fig. 2).

The eventual loss of *Phox2b* expression in *Rnx* mutants could be explained by two scenarios: loss of mutant cells by cell death or a later role of Rnx protein in maintaining *Phox2b* expression. To help distinguish these two possibilities, we took an indirect approach by examining molecular markers expressed in regions surrounding the nTS. *Ebf2*, a basic helix-loop-helix gene (Garel et al. 1997), is expressed in the region dorsal lateral to the nTS (Fig. 4E, arrowhead), whereas the dorsal motor nucleus of vagus (dmnX), which expresses *Phox2b*, is ventral to the nTS (Fig. 4E, dmnX). Strikingly, in both E15 and E19 *Rnx* mutant embryos, the dmnX approximates *Ebf2*-expressing cells (Fig. 4F,H), suggesting that the intervening nTS structure is absent. To determine whether the mutant neurons die by apoptosis, we performed TUNEL labeling experiments (Gavrieli et al. 1992). Surprisingly, increased cell death was not detected in the prospective mutant nTS area at any stage examined, from E10.5 to P0 (Fig. 4I–L; data not shown). Thus, mutant neurons in the prospective nTS may either die through a pathway not detected by TUNEL labeling, or
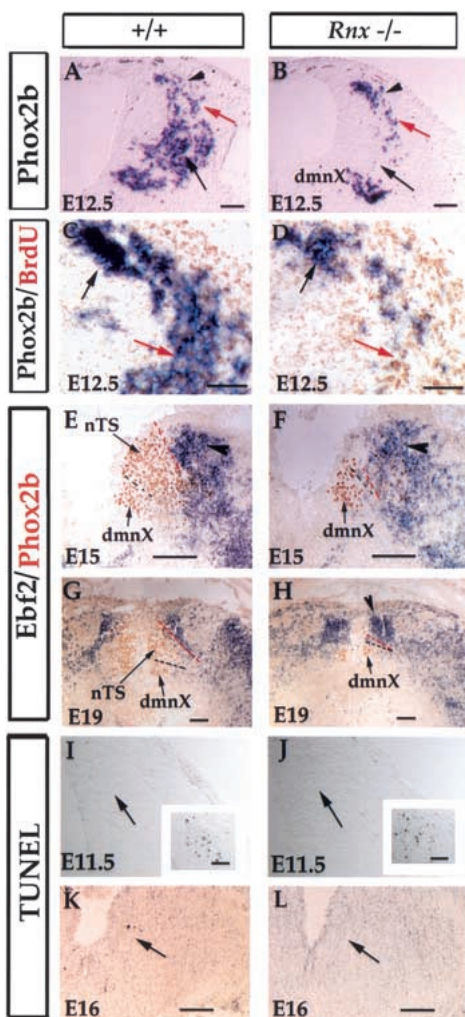

Figure 4. The nTS neurons are generated in *Rnx* mutants, but are apparently lost at later stages. (*A,B*) In situ hybridization on transverse sections through developing medulla of E12.5 *Rnx* wild-type (*A*) and mutant (*B*) embryos. Initiation of *Phox2b* expression in the most dorsal part of the developing hindbrain is not affected in *Rnx* mutants (cf. *A* and *B*, arrowheads). Nor is its expression in early migratory neurons affected (cf. *A* and *B*, red arrows). However, *Phox2b* expression is absent in a more ventral area (cf. *A* and *B*, black arrows). (*C,D*) Pulse-chase BrdU labeling in *Phox2b*-positive neurons. Transverse sections through E12.5 wild-type (*C*) and *Rnx* mutant (*D*) embryos. BrdU was injected at E10.5 and embryos were collected at E12.5. In situ hybridization with *Phox2b* probe (purple) followed by anti-BrdU immunostaining (brown). *Phox2b*-positive cells born at E10.5 are recognized by strong BrdU staining, and are located in the ventral position in wild-type embryos (*C*, red arrow), but are absent in *Rnx* mutants (*D*, red arrow). In contrast, cells born after E10.5 are located in the dorsal position and show either no or weak BrdU staining (*C,D*, black arrows). (*E–H*) The nTS structure may be missing in *Rnx* mutants. Double staining of *Ebf2* mRNA (purple) and Phox2b protein (brown). Transverse sections through medulla of E15 and E19 wild-type (*E,G*) and *Rnx* mutant (*F,G*) embryos. *Ebf2* expression (*E*, arrowhead) is located dorsal lateral to the nTS in wild-type embryos (*E,G*, redlines). The dmnX, as indicated by Phox2b expression in *Rnx* mutants (*F,H*, dmnX), is located ventral to the nTS in wild-type embryos (*E,G*, dmnX). In E15 and E19 mutants, the dmnX abuts *Ebf2*-expressing cells (*F,H*, dmnX). At later stages, *Ebf2*-expressing cells appear reorganized in *Rnx* mutants, as indicated by an ectopic aggregation in the dorsal medial region (*F*, arrowhead). (*I–L*) TUNEL labeling was performed on transverse sections through E11.5 and E16 wild-type (*I,K*) and *Rnx* mutants (*J,L*). No increased cell death was detected in the prospective nTS areas of *Rnx* mutants (cf. *I,K* and *J,L*, arrows). The detection of extensive cell death in the accessory ganglia (*I,J*, boxed regions) serves as the positive control. No differential cell death was detected in other developmental stages (data not shown). Bars: *A–D,I,J*, 100 µm; *E–H*, 400 µm; *K,L*, 200 µm.

be converted to other cell fates. Potential support for the latter scenario is that there is an ectopic aggregation of *Ebf2*-expressing cells in the dorsal medial region of the medulla (Fig. 4H, arrowhead), although this could be simply due to a relocation of *Ebf2*-expressing cells.

Aberrant central innervation of primary visceral sensory afferents in Rnx mutants

Given the dramatic absence or reorganization of the nTS structure, we asked whether central innervation of the primary visceral sensory neurons is affected in *Rnx* mutants. The innervation pattern was analyzed by DiI-labeling. In wild-type embryos, after entering the hindbrain, the visceral sensory afferents are bundled together to form the descending solitary tract (ST), and then make

collateral projections to the nTS. We found that formation of the ST is not affected in E12.5 *Rnx* mutants (Fig. 5A,B). At E16, the initial collateral projection from the ST to the prospective nTS region is also normal in *Rnx* mutants (Fig. 5C,D, nTS). However, in marked contrast to the pattern seen in wild-type embryos (Fig. 5C,E), *Rnx* mutant afferents do not terminate in the prospective nTS area, but instead continue to grow dorsal-laterally into the adjacent territory (Fig. 5D,F, white arrows). Higher magnification shows that mutant axons directly cross the prospective target, rather than change the growth cone morphology and send side branches to ectopic sites (data not shown). The "overshooting" phenotype is fully consistent with a loss or a defect of the normal targets in the nTS. Moreover, no defect of neuronal differentiation in the primary sensory afferent neurons was detected, as indicated by normal expression of
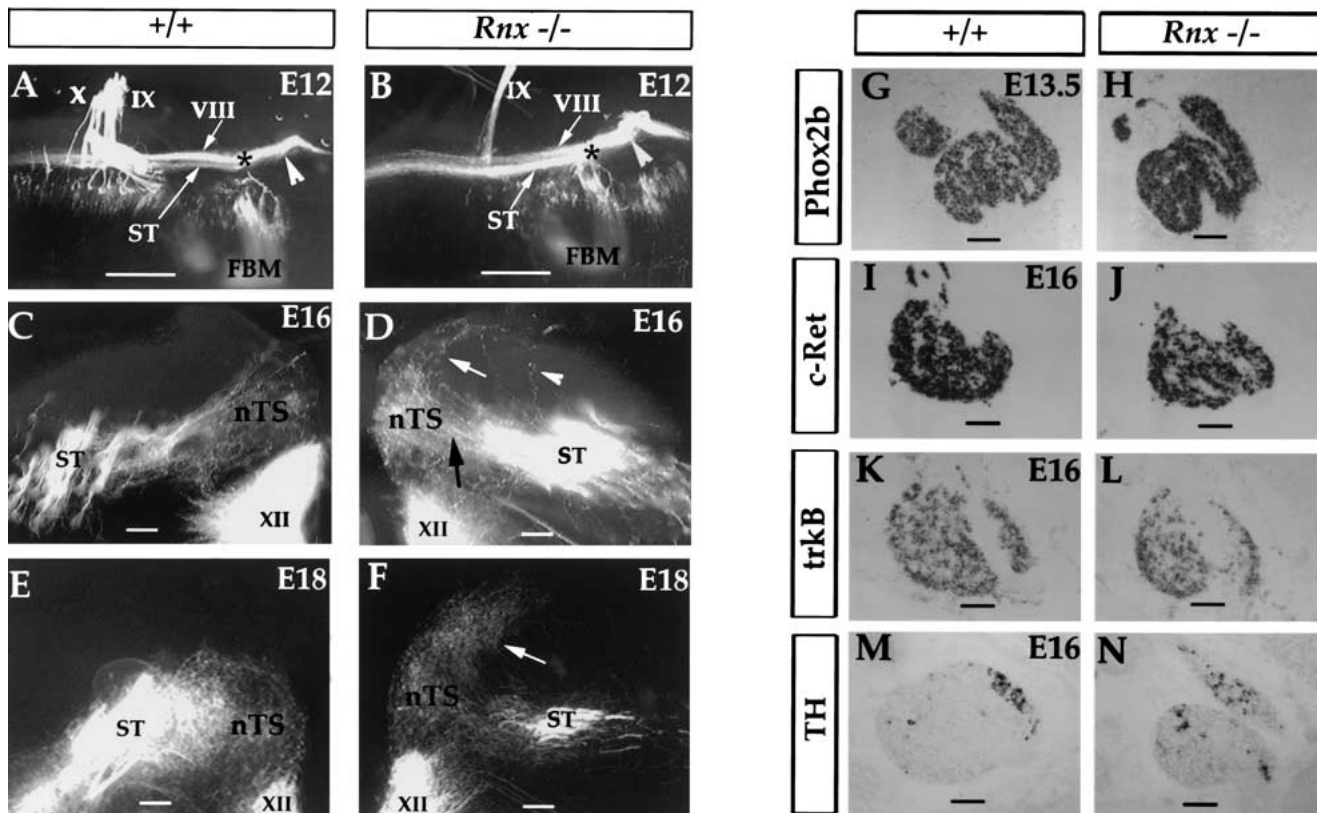

Figure 5. Aberrant central projection of visceral sensory afferents in *Rnx* mutants and normal development of peripheral visceral sensory ganglia. (*A,B*) Flat-mounted E12 wild-type (*A*) and *Rnx* mutant (*B*) embryos. Dorsal is up and rostral is to the right. Central projections of the VIIth, VIIIth, IXth, and Xth nerves are labeled with DiI. (FBM) facial branchial motor nucleus. The axial position of FBM corresponds to the rostral limit of the descending solitary tract (*A,B*, ⋆). Rostral to the FBM is the ascending VIIIth afferents (*A,B*, white arrowheads). Caudal to the FBM is the descending solitary tract (ST), which is located ventral to the descending VIII afferents (*A,B*, VIII). Clearly, the formation of the ST is not affected in *Rnx* mutants (*B*, ST). (*C–F*) Transverse sections through the medulla of E16 and E18 wild-type (*C,E*) and *Rnx* mutant embryos (*D,F*), which are shown to mirror each other. Initial collateral projections from the ST to the prospective nTS area are not affected in *Rnx* mutants (*D*, black arrow). Only a very few aberrant projections from the ST are seen in *Rnx* mutants (*D*, arrowhead). However, *Rnx* mutant afferents do not terminate in the area that normally constitutes the nTS. Instead they continue to grow dorsal-laterally to adjacent territory (*D*, white arrow). By E18, most axons have passed through the prospective nTS area (*F*, arrow). (*G–N*) Transverse sections through the nodose and pertrosal complex. In situ hybridization was performed with indicated probes and developmental stages. Note absence of any apparent alterations in expression patterns. Bars: *A,B*, 500 μm; *C–F*, 100 μm; *G–N*, 200 μm.

a panel of markers including *Phox2b* (Pattyn et al. 1997), *TH* (Helke and Niederer 1990), *DBH* (Morin et al. 1997), *trkB* (Ernfors et al. 1992), and *c-Ret* (Morin et al. 1997; Pattyn et al. 1999) (Fig. 5G–N; data not shown).

Rnx *is expressed transiently in, and required for proper formation of, the brainstem (nor)adrenergic centers*

Phox2b is essential for formation of the (nor)adrenergic neurons throughout the nervous system (Pattyn et al. 2000a). Consistent with the loss of *Phox2b* expression in the nTS at late embryonic stages, the development of the A2/C2 cluster of the (nor)adrenergic neurons, which is located within the nTS, is compromised in *Rnx* mutants (Fig. 6C,D). The defect is indicated by a complete absence of expression of tyrosine hydroxylase (*TH*) (data not shown) and dopamine-beta-hydroxylase (*DBH*) (Fig. 6; data not shown), two key enzymes for catecholamine biosynthesis (Nagatsu et al. 1964; Friedman and Kaufman 1965). Surprisingly, formation of other (nor)adrenergic centers is also compromised in *Rnx* mutants, including A1/C1 (Fig. 6, cf. A and B), A5 (Fig. 6, cf. E and F), the locus subcoeruleus (sLC) (Fig. 6, cf. E and F), and the caudal part of the locus coeruleus (LC) (Fig. 6, cf. E and F, arrowhead). Only the most rostral part of the LC is still formed (Fig. 6, cf. E and F, LC).
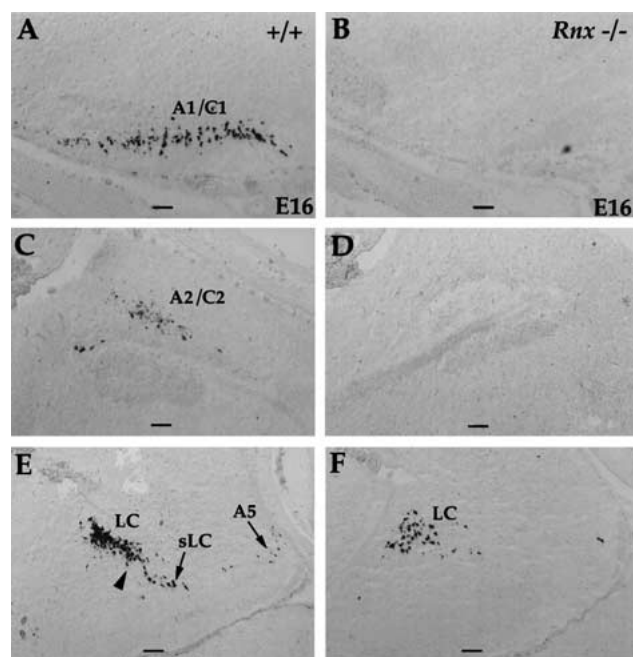

Figure 6. Formation of (nor)adrenergic neurons is compromised in *Rnx* mutants. Sagittal sections through E16 wild-type (*A,C,E*) and *Rnx* mutant (*B,D,F*) embryos. In situ hybridization was performed with *DBH* probe. In *Rnx* mutants, expression of *DBH* is absent in most of (nor)adrenergic centers, including A1/C1 (cf. *A* and *B*), A2/C2 (cf. *C* and *D*), A5 (cf. *E* and *F*), locus subcoeruleus (sLC) (cf. *E* and *F*), and caudal locus coeruleus (cf. *E* and *F*, arrowhead). Only the rostral locus coeruleus is still formed in *Rnx* mutants (*F*, LC). Bars, 200 μm.

At E14.5 or later embryonic stages, no *Rnx* expression was detected in the (nor)adrenergic centers (data not shown). To determine whether *Rnx* is expressed transiently in these neurons, we examined *Rnx* expression at earlier embryonic stages. In the developing pons, (nor)adrenergic neurons are subdivided into two groups: (1) the LC, which is dependent on both *Phox2a* and *Phox2b* and develops from rhombomere 1 (Morin et al. 1997; Pattyn et al. 2000a, and (2) the A5 and the sLC, which require *Phox2b* function for the formation and develop from caudal developing pons (Morin et al. 1997; Pattyn et al. 2000a). Double staining shows that *Rnx* and *Phox2a* are coexpressed in the caudal but not the rostral part of developing pons of E10.5 wild-type embryos (Fig. 7A–E). Meanwhile, extensive coexpression of *Phox2b* and *Rnx* is detected in the most caudal part of developing pons (Fig. 7G,H). By E11.5, *Rnx* expression has been turned off in these *Phox2a*- or *Phox2b*-expressing (nor)adrenergic neurons (Fig. 7F,I). Since expression of *Phox2* genes is associated predominantly with (nor)adrenergic cells in developing pons (Pattyn et al. 2000a; also see below, Fig. 8), we conclude that *Rnx* is expressed transiently in subsets of prospective (nor)adrenergic neurons. Consistent with this, *DBH* expression is eliminated in the caudal but not the rostral part of developing pons of E10.5 *Rnx* mutant embryos (Fig. 7J–O), which corresponds precisely to the partial defect seen in the pons area of E16.5 *Rnx* mutants (Fig. 6E,F).

The development of medullary (nor)adrenergic neurons (the A1/C1 and A2/C2 clusters) occurs at later embryonic stages. *DBH* expression in this area is first detected around E12.5, but is absent in *Rnx* mutants at all stages examined (data not shown). Therefore, *Rnx* is required for proper specification of (nor)adrenergic neurons throughout the developing hindbrain (except rostral locus coeruleus).

Expression of Rnx *and* Phox2b *may be independently regulated*

The dual genetic requirement of *Rnx* and *Phox2* genes in controlling *DBH* expression raises two possible scenarios: (1) they function in a cascade, or (2) they function independently. Support for the latter scenario is that expression of *Phox2* genes in prospective (nor)adrenergic neurons is independent of *Rnx*. In caudal developing pons of both E10.5 and E11.5 wild-type embryos, all of the *Phox2b*-expressing neurons have already turned on the expression of *DBH* (Fig. 8E,G). Strikingly, while expression of *DBH* is completely eliminated in E10.5 *Rnx* mutant embryos (Fig. 8A,B), neither *Phox2b* nor *Phox2a* expression is affected (Fig. 8, cf. C and D; data not shown). Selective elimination of *DBH* expression is also observed in E11.5 mutant embryos (Fig. 8, cf. G and H).

Several observations also argue against the model that *Rnx* is a downstream target of Phox2b. First, in the most dorsal part of developing medulla of E10.5 (data not shown) and E11.5 wild type embryos, expression of *Rnx* but not *Phox2b* is detected in cells that are close to the ventricular zone (Fig. 8I, arrows). In contrast, in
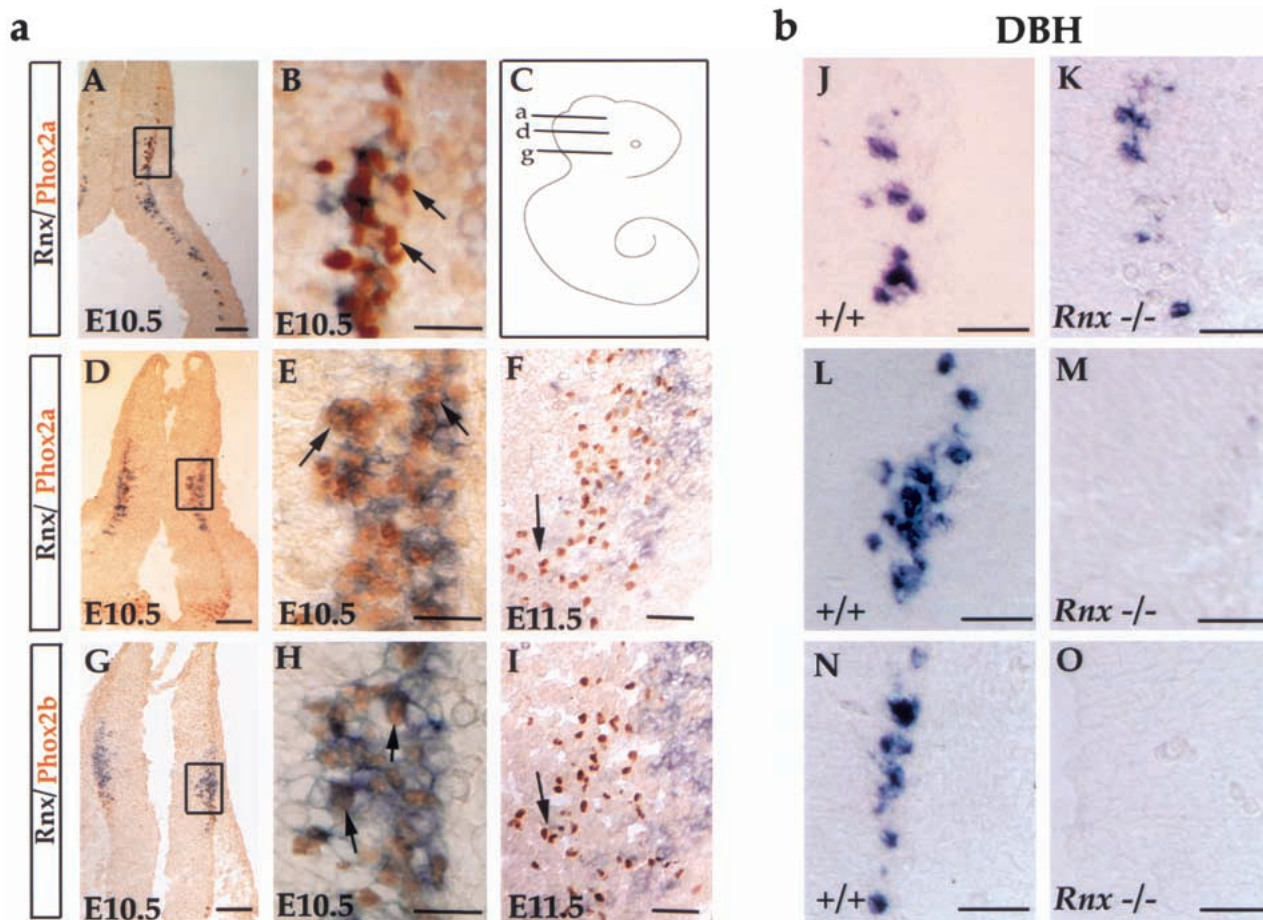
Figure 7. Overlapping expression of *Rnx* and *Phox2a/Phox2b* in wild-type, and loss of *DBH* expression in *Rnx* mutant developing pons. (*a*) Transverse sections through developing pons of wild-type embryos with indicated embryonic stages. Sections *A*, *D*, and *G* are from three different axial levels of developing pons, corresponding to lines a, d, and g of the cartoon shown in panel *C*, respectively. Lines a and d may correspond to rostral and caudal parts of rhombomere 1, respectively, whereas line g may correspond to rhombomere 2. Panels *B*, *E*, and *H* are higher magnifications of the boxed regions shown in panels *A*, *D*, and *G*, respectively. Double staining (*Rnx* mRNA, purple; Phox2 proteins, brown) shows that *Rnx* coexpresses with *Phox2a* in the caudal (*E*, arrows) but not rostral (*B*, arrows) part of the developing pons. In the most caudal part of the developing pons, *Rnx* expression overlaps with that of *Phox2b* (*H*, arrows). By E11.5, *Rnx* expression is turned off in both Phox2a-expressing cells (*F*, arrow) and Phox2b-expressing cells (*I*, arrow). (*b*) Transverse sections through developing pons of E10.5 wild-type (*J,L,N*) and *Rnx* mutant embryos (*K,M,O*). Panels *J*, *L*, and *N* are roughly equivalent to panels *B*, *E*, and *H*, respectively. In situ hybridization was performed with *DBH* probe. Expression of *DBH* in the rostralmost part of developing pons was not affected (cf. *J* and *K*), but is absent in the caudal parts of developing pons of E10.5 *Rnx* mutant embryos (cf. *L,N* and *M,O*). Bars: *A,D,G*, 200 μm; *B,E,H*, 50 μm; *F,I*, 100 μm.; *J–O*, 100 μm.

more matured marginal zone, all cells coexpress *Rnx* and *Phox2b* (Fig. 8I, arrowheads). Thus, *Rnx* expression precedes that of *Phox2b* in the cells that eventually coexpress both genes. Second, *Rnx* expression is not detected in many *Phox2b*-expressing cells (see Fig. 2). Third, no *Phox2b* expression is detected in the trigeminal nuclei where *Rnx* is expressed (Y. Qian et al., unpubl.).

Besides Phox2b and Rnx, MASH1 is another transcription factor required for formation of brainstem (nor)adrenergic centers (Hirsch et al. 1998). Consistently, in E10.5 developing pons, *Rnx*-expressing neurons are located precisely lateral to the portion of the ventricular zone that expresses MASH1 protein (Fig. 8J), suggesting

that like the nTS, all (nor)adrenergic neurons develop from *Mash1*-positive precursors.

Discussion

Both relay visceral sensory and (nor)adrenergic neurons in the brainstem are critically important for cardiovascular and respiratory control. Prior to this study, formation of these neurons was poorly understood. Here we show that these two classes of neurons are ontogenetically related. Genetic analysis demonstrates that *Rnx* plays a central role in formation of these neurons (Fig. 9a). Importantly, we show that *Rnx* and *Phox2b* are independently required for specification of the (nor)adrenergic phenotype. Our findings suggest that network-spe-
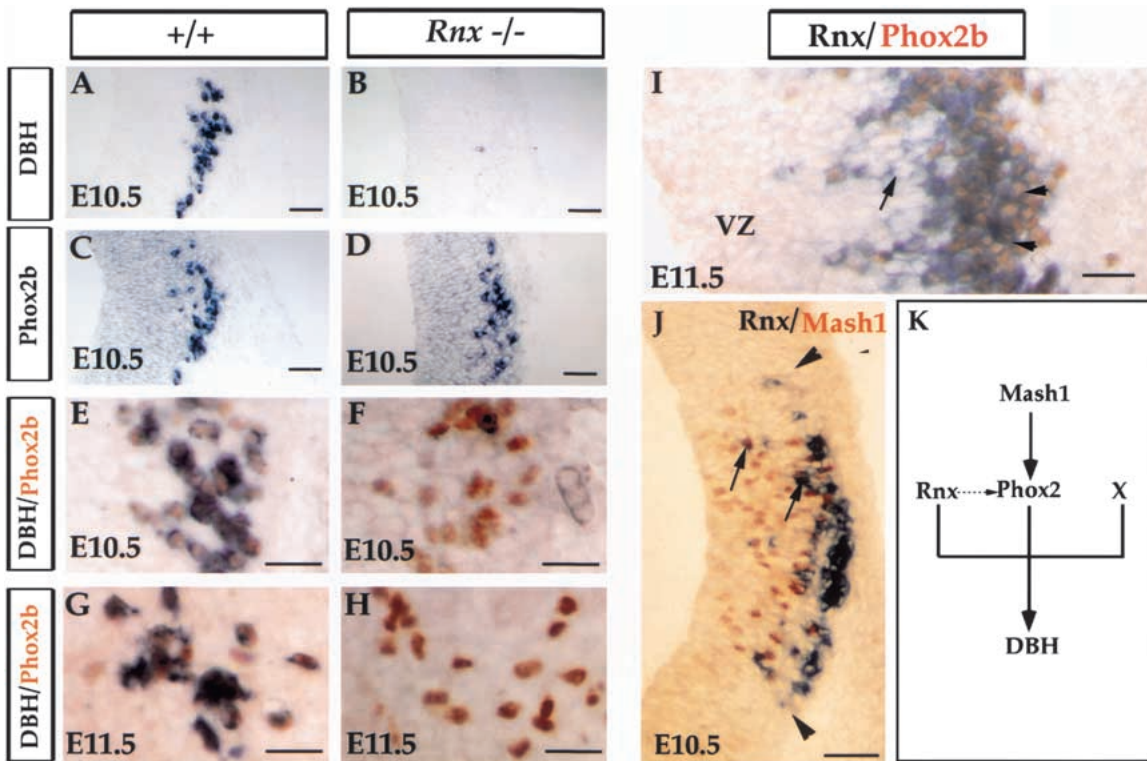
Figure 8. Independent regulation of *Phox2b* and *Rnx* expression. (*A–H*) Transverse sections through caudal developing pons of E10.5 (*A–F*) and E11.5 (*G,H*) embryos. In situ hybridization was performed with indicated probes (*A–D*). Panels *E–H* show double labeling of *DBH* mRNA (purple) and Phox2b protein (brown). *DBH* and Phox2b are coexpressed in both E10.5 and E11.5 wild-type embryos (*E,G*). Although *DBH* expression is absent in *Rnx* mutants (cf. *A* and *B*), *Phox2b* expression is still detected in both E10.5 (cf. *D,F* and *C,E*) and E11.5 (cf. *H* and *G*) *Rnx* mutants. (*I*) Longitudinal section through the dorsal stripe of *Rnx*-expressing cells of E11.5 wild-type embryos. Double staining of *Rnx* mRNA (purple) and Phox2b protein (brown). In a region closer to the ventricular zone (VZ), many cells express *Rnx* but not Phox2b (*I*, arrows). In contrast, coexpression of these two genes was detected throughout the marginal zone (*I*, arrowheads). (*J*) Double staining of *Rnx* mRNA (purple) and MASH1 protein (brown) on transverse sections through developing pons of E10.5 wild-type embryos. The axial level corresponds to the sections shown in panels *A–D*. *Rnx*-expressing cells are located lateral to the Mash1-expressing domain, and they share identical boundaries along the dorsal ventral axis (*J*, arrowheads). A few double-stained cells were detected (*J*, arrows). (*K*) Schematic summary diagram regarding *DBH* regulation in brainstem (nor)adrenergic neurons. MASH1 and Phox2 proteins function in a cascade, whereas Rnx and Phox2b may function independently in regulating *DBH* expression. The dashed arrow is a hypothetical function of Rnx in maintaining *Phox2* expression at later developmental stages. X is a hypothetical regulator. See text for detailed discussion. Bars: *A–D*, 100 μm; *E–I*, 50 μm; *J*, 100 μm.

cific transcription factors may be a common theme in developing nervous system.

The developmental origin, and a requirement of Rnx *for formation, of first-order relay visceral sensory neurons*

The studies reported here establish *Rnx* as the first gene characterized thus far that is required for proper formation of first-order relay visceral sensory neurons in the medulla (the nTS and area postrema). The defect of the nTS is first indicated by a complete loss of *Phox2b* expression at late embryonic stages. Examination of markers (*Phox2b* and *Ebf2*) expressed in the surrounding region shows that the whole nTS structure seems to be missing. The defect is further supported by aberrant central projections of the primary visceral sensory afferents, since no abnormality of neuronal differentiation is de-

tected in primary visceral sensory ganglia. The restriction of defect to central visceral sensory neurons is consistent with the finding that among the three *Tlx* family members, only *Rnx* is expressed in the nTS, whereas both *Enx* and *Rnx* are expressed in the peripheral ganglia and might therefore have redundant or compensating functions there (data not shown).

Our studies reveal a possible embryological origin for the nTS neurons. We find that of the two longitudinal stripes of *Rnx*-expressing cells in the developing medulla, only dorsally derived neurons coexpress *Rnx* and *Phox2b*. These double-positive cells migrate ventrally, and aggregate together to form the nTS (and part of (nor)adrenergic neurons; see below; Fig. 9b). Furthermore, the nTS neurons are apparently derived from the ventricular zone corresponding to the most dorsal part of the *Mash1*-expressing domain (Fig. 9b). Our conclusion is in contrast to the previous hypothesis that the nTS devel-
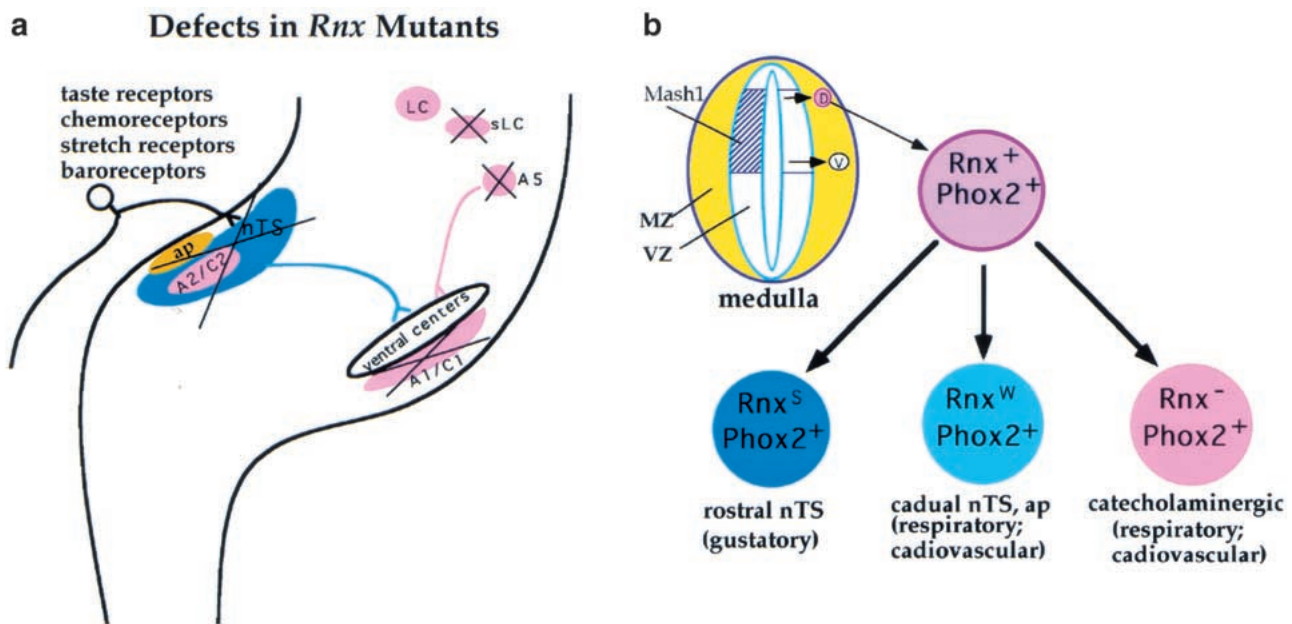
Figure 9. (*a*) Summary of developmental defects in *Rnx* mutants. The formation of the first-order relay visceral sensory neurons in the nTS and the area postrema (ap), and most of the (nor)adrenergic centers (pink), is compromised in *Rnx* mutants. Only a subset of (nor)adrenergic neurons in the locus coeruleus (LC) still forms. The nTS and the ap receive innervation of various visceral sensory afferents, including taste receptors, baroreceptors, chemoreceptors, and pulmonary stretch receptors. The defects of these autonomic neuronal structures not only explain why *Rnx* mutant mice suffer from breathing problems, but also indicate that the mutant mice may have additional problems in the regulation of cardiovascular and gustatory functions. (*b*) Ontogeny of relay visceral sensory and (nor)adrenergic neurons. Both groups of neurons develop from the precursors expressing *Mash1*, and they coexpress *Rnx* and *Phox2b* (or *Phox2a*) when they are formed. In the case of the developing medulla, like the nTS, the (nor)adrenergic neurons may develop from dorsally derived *Rnx*-expressing cells (pink circle, D), although we have not excluded the possibility that ventrally derived *Rnx*-expressing cells (white circle, V) may give rise to the ventral A1/C1 cluster of (nor)adrenergic neurons (see text for detailed discussion). It is interesting to note that strong *Rnx* expression (Rnx^S) is sustained in rostral nTS, which is required for gustatory function. In contrast, *Rnx* expression is either reduced (Rnx^W) in caudal nTS and the area postrema (ap), or is turned off completely in (nor)adrenergic neurons, both of which are associated with cardiovascular and respiratory control. VZ, the ventricular zone where dividing precursors are located; MZ, the marginal zone where postmitotic neurons are located.

ops from the ventral stripe of *Rnx*-expressing cells in chick embryos, which is primarily based on *Rnx* expression at early embryonic stages (Logan et al. 1998).

Rnx *is required for specification of (nor)adrenergic neurons*

Besides MASH1 and Phox2 proteins, Rnx is the third transcription factor required for formation of the majority of brainstem (nor)adrenergic neurons, as indicated by the absence of *DBH* expression in *Rnx*-deficient mice. Our studies suggest possible origins of the (nor)adrenergic neurons. In developing pons, (nor)adrenergic neurons, including sublocus coeruleus and the A5/A7 clusters, can be prospectively defined by combinatorial expression of *Rnx* and *Phox2a*/*Phox2b*, and these neurons develop from an intermediate portion of the ventricular zone along the dorsal-ventral axis, in which *Mash1* is expressed (Fig. 8J). The formation of medullary (nor)adrenergic neurons (the A1/C1 and A2/C2 clusters) depends on *Phox2b* (Pattyn et al. 2000a), but not on *Phox2a* (Morin et al. 1997). There are two stripes of *Rnx*-expressing cells in the developing medulla. Since only dorsally

derived cells coexpress *Rnx* and *Phox2b* (Fig. 2), the dual genetic requirement of these two genes for the formation of (nor)adrenergic neurons suggests that these neurons develop from dorsally derived *Rnx*-expressing cells, as the neurons in the nTS do (Fig. 9b). Support for this is that the A2/C2 cluster is located within the nTS (Fig. 1). We have not yet excluded the possibility that ventrally derived *Rnx*-expressing cells might give rise to the ventral A1/C1 cluster of (nor)adrenergic neurons. However, this possibility assumes that *Phox2b* expression is initiated after *Rnx* expression has been turned off, because no coexpression of *Rnx* and *Phox2b* is detected in ventrally derived cells (Fig. 2).

Rnx might be the missing factor that functions cooperatively with Phox2b in specification of the (nor)adrenergic phenotype, because these two genes are apparently independently regulated. First, *Rnx* expression precedes that of *Phox2b* in cells that eventually coexpress both genes (Fig. 8I). Indeed, *Rnx* expression in these double-positive cells is not affected in the *Phox2b* mutant (J.F. Brunet, pers. comm.). Second, initial expression of *Phox2b* in prospective (nor)adrenergic neurons (and relay visceral sensory neurons) is not affected in *Rnx*-deficient

mice (Figs. 4,8). However, it should be pointed out that *Rnx* and *Phox2b* are coexpressed in non-(nor)adrenergic neurons in the nTS, suggesting that specification of the (nor)adrenergic phenotype may require additional factors (Fig. 8K). It is also important to note that *Phox2b* expression in prospective (nor)adrenergic neurons and relay visceral sensory neurons is eventually lost in *Rnx*-deficient mice, suggesting that either the mutant cells die or Rnx has a later role in maintaining *Phox2* expression (Fig. 8J, dashed arrow). Support for a cell loss model is that the nTS structure is apparently missing in *Rnx* mutants (Fig. 4E–H).

Rnx *deficiency and failure of respiratory control*

Rnx-deficient mice die from respiratory failure (Shirasawa et al. 2000). Electrophysiological recordings show a rapid respiratory rate and frequent apneas in *Rnx* mutant mice (Shirasawa et al. 2000). The observation that *Rnx* is expressed in the area dorsal to the nucleus ambiguus (nA) whereas respiratory neurons are located ventral to the nA (Rekling and Feldman 1998) suggests that the development of rhythmic respiratory neurons per se may not be affected in *Rnx*-deficient mice. Consistent with this, expression of neurokinin-1 receptor, a possible anatomical marker for pacemaker respiratory neurons (Gray et al. 1999), is not affected in *Rnx*-deficient mice.

The developmental defects of the nTS, the area postrema, and the (nor)adrenergic neurons offer an alternative explanation. In wild-type mice, input from noradrenergic neurons causes a depression of respiratory frequency (Errchidi et al. 1991; Al-Zubaidy et al. 1996). Therefore, the improper formation of noradrenergic neurons might contribute to the rapid shallow breathing symptom observed in *Rnx*-deficient mice (Shirasawa et al. 2000). Transection of the chemoreceptor afferents in neonatal rat (Hofer 1986), or a loss of peripheral visceral sensory neurons as in *BDNF*-deficient mice (Erickson et al. 1996; Brady et al. 1999), causes irregular respiratory rhythms. Because the nTS and the area postrema contain first-order relay neurons for all of the visceral sensory afferents, the apparent loss of these two structures in *Rnx*-deficient mice should cause a disorder in respiratory control. Moreover, the mutant mice may suffer additional physiological problems, such as defect of taste sensation and abnormality of cardiovascular control (Fig. 9a).

Network-specific transcription factors and formation of neuronal circuitry

Recent studies show that *Math1*, which encodes a basic helix-loop-helix type of transcription factor, is required for the formation of most components of the proprioceptive neuronal circuitry (Bermingham et al. 2001). The concept of network-specific transcription factors was first suggested by Brunet and his colleagues, based on *Phox2* gene expression in and requirement for the formation of many components within autonomic neuronal

circuitry (Tiveron et al. 1996; Morin et al. 1997; Pattyn 1997, 1999, 2000a). However, *Phox2b* is also associated with several nonautonomic structures, such as the branchial motor neurons (Pattyn et al. 2000b) and a large group of uncharacterized neurons in the medulla (Fig. 2F, red arrow).

Thus, it is important to emphasize that the combinatorial expression of *Rnx* and *Phox2b/Phox2a* is restricted exclusively to autonomic components, including the nTS, the area postrema, the (nor)adrenergic centers, the peripheral visceral sensory ganglia (Fig. 2A,C, g, p, and n), and the sympathetic and parasympathetic ganglia (data not shown). Most strikingly, most of these autonomic components develop from *Mash1*-positive precursors (Fig. 9b; Anderson et al. 1997). (The visceral sensory ganglia are the only exception, and are derived from *neurogenin2*-positive placodal neural precursors; Fode et al. 1998). Thus, it appears to be an emerging common theme that the same set of regulatory molecules is repeatedly involved in the development of multiple components within a physiological neuronal network. Future studies will be directed to determine whether or not network-specific transcription factors control the expression of network-specific molecules, such as members of the cadherin superfamily (Suzuki et al. 1997; Inoue et al. 1998; Yagi and Takeichi 2000).

Materials and methods

Animals

The generation of *ngn1* and *Rnx* mutant mice has been described (Ma et al. 1998; Shirasawa et al. 2000). The morning that vaginal plugs were observed was considered E0.5. PCR-based genotyping of *ngn1* mutant mice was performed as described (Ma et al. 2000). The mutant *Rnx* allele was detected by PCR amplification with primers derived from the *neo* gene (Ma et al. 2000), which produces a 0.6 kb product. The wild-type *Rnx* allele was detected by the following PCR primers: 5′-AGCG GCGACTGCTCTCCATCCAGG-3′ and 5′-GCATCGACCAA ATCCTCAACAGCC-3′, which produces a 0.3 kb product.

In situ hybridization

Section and whole-mount in situ hybridizations were performed as described (Ma et al. 1998). In situ hybridization combined with *Phox2b* antibody immunostaining was performed essentially as described (Tiveron et al. 1996). The *Tlx1* probe (0.7 kb), the *Enx* probe (1.0 kb), and the *Rnx* probe (1.0 kb) were amplified by RT-PCR using a cDNA template prepared from the trigeminal ganglia of E12.5 mouse embryos. Other probes used in this study were *Phox2a* (Tiveron et al. 1996), *Phox2b* (Pattyn et al. 1997), *TH* (Pattyn et al. 2000), *DBH* (Morin et al. 1997), *trkB* (Birren et al. 1993), and *c-Ret* (Morin et al. 1997).

TUNEL staining on cryostat sections

Three pairs of E10.5–E19 wild-type and mutant embryos were used. TUNEL staining on frozen sections was performed by the ApopTag method according to the manufacturer's instructions (Intergen).

In situ hybridization combined with Brdu immunostaining

For transient labeling, pregnant wild-type mice carrying E11.5 embryos were injected with 5-bromo-2-deoxy-uridine (BrdU) (Sigma) (50 mg/kg body weight) 2 h before they were sacrificed. For pulse-chase labeling, BrdU was injected at E10.5 and collected at E12.5. In situ hybridization was performed first, followed by immunostaining with anti-BrdU antibody. After in situ hybridization, sections were first washed with PBS (3 × 5 min), fixed in 4% paraformaldehyde for 15 min, washed with PBS (3 × 5 min), treated with 2N HCl in PBS for 30 min at 37°C, rinsed in PBS five times, and washed with PBS (3 × 5 min). The subsequent immunostaining with anti-BrdU antibody (Boehringer Mannheim) (1:50 dilution) was performed with a Vectastain detection kit (Vector labs).

DiI tracing

E12 embryos for DiI-labeling were directly fixed by immersing them in 4% paraformaldehyde. E14–E18 embryos were first perfused with and then immersed in 4% paraformaldehyde. Two to four pairs of wild-type and mutant embryos were used. For E12 wild-type and *Rnx* mutant embryos, DiI was injected into the facial and vestibular-cochlear complex as well as the IXth and Xth nerves. For each of the embryos at E14 or later stages, DiI was applied to the facial and vestibular-cochlear complex on one side of the embryos, and to the IXth and Xth nerves on the contralateral side. DiI was soaked in filter (Fritzsch and Nichols 1993) and diffused for three (E12 embryos), four (E14–E16 embryos), or five days (E17 and E18 embryos) at 37°C. Brains of E12 mice were dissected, mounted flat, and viewed with an epifluorescence microscope. Brains of the E14–E18 embryos and newborn mice were embedded in 12% gelatin and cut with a vibratome at 80 μm thickness. Sections were mounted and viewed with an epifluorescence microscope and a Biorad Radiance 2000 confocal microscope.

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