AML1/Runx1 is important for the development of hindbrain cholinergic branchiovisceral motor neurons and selected cranial sensory neurons

Francesca M. Theriault, Priscillia Roy, and Stefano Stifani*

Center for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, QC, Canada H3A 2B4

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The mechanisms that regulate the acquisition of distinctive neuronal traits in the developing nervous system are poorly defined. It is shown here that the mammalian *runt*-related gene *Runx1* is expressed in selected populations of postmitotic neurons of the embryonic central and peripheral nervous systems. These include cholinergic branchial and visceral motor neurons in the hindbrain, restricted populations of somatic motor neurons of the median and lateral motor columns in the spinal cord, as well as nociceptive and mechanoreceptor neurons in trigeminal and vestibulocochlear ganglia. In mouse embryos lacking *Runx1* activity, hindbrain branchiovisceral motor neuron precursors of the cholinergic lineage are correctly specified but then fail to progress to a more differentiated state and undergo increased cell death, resulting in a neuronal loss in the mantle layer. In contrast, the development of cholinergic somatic motor neurons is unaffected. *Runx1* inactivation also leads to a loss of selected sensory neurons in trigeminal and vestibulocochlear ganglia. These findings uncover previously unrecognized roles for *Runx1* in the regulation of mammalian neuronal subtype development.

One of the critical events during nervous system development is the generation of distinct subclasses of neurons at precise locations and at defined times. In the developing mammalian CNS, cell cycle exit and activation of general neuronal traits act in concert. In addition, a coordinated acquisition of both general and subtype-specific neuronal features must be achieved to ensure the differentiation of different neuronal populations. Studies in the developing neural tube have identified several homeodomain (HD) transcriptional repressors that act in concert with specific basic helix-loop-helix transcription factors to regulate the differentiation and survival of individual neuronal subtypes (1–4). Much remains to be learned, however, about the identity of other proteins involved in regulating the differentiation of distinct neuronal subtypes in correct numbers and at different locations in the nervous system.

In this study, we have characterized the neural expression and function of the mammalian transcription factor AML1 (acute myeloid leukemia 1)/Cbfa2 (core binding factor α 2)/Runx1 (Runt-related transcription factor 1) (hereafter referred to as Runx1). The Runx protein family includes three members, Runx1–3, structurally and functionally related to the *Drosophila* protein Runt (5–9). In mice, individual Runx proteins act in mostly nonredundant manners to regulate a variety of cell differentiation events (6–8). In particular, *Runx1* is required for fetal liver-derived hematopoiesis, and its human homolog is frequently targeted by chromosomal translocations that lead to acute myeloid leukemia (6, 7). In *Drosophila*, *runt* is involved in a number of developmental mechanisms, including neuronal development (5, 9). During insect embryonic neurogenesis, *runt* promotes the specification of a particular subset of CNS neurons, and its inactivation leads to a selective loss of those cells (5, 9). These findings, combined with the previous observation that murine *Runx1* is expressed in embryonic neural tissues (10, 11), suggested that *Runx1* may participate in mammalian neurogen-

esis. To date, this possibility had not been tested. Here we demonstrate for the first time that *Runx1* activity is required for the development of selected populations of central and peripheral neurons, including cholinergic branchial and visceral motor neurons in the hindbrain and sensory neuron subtypes in trigeminal and vestibular ganglia. These results identify RUNX1 as a regulator of the development of particular motor and sensory neurons in the mammalian CNS and peripheral nervous system (PNS).

Materials and Methods

Embryological Analysis. *Runx1*^{lacZ/+} mice were generated and genotyped as described (12). For staging of the embryos, the day of appearance of the vaginal plug was considered as embryonic day (E) 0.5. Embryos were dissected at various gestational stages; fixed in 2% paraformaldehyde, 10 mM sodium periodate, and 70 mM L-lysine; transferred to 30% sucrose; embedded in OCT compound (TissueTek); and cryostat sectioned (14 μ m). β -Galactosidase (β -gal) activity was assessed as described (12), and counterstaining with eosin followed. Double-labeling immunofluorescence experiments (13) were performed with the following antibodies: mouse monoclonals against Nkx2.2 (1:15), Islet1 (1:75), Pax6 (1:15), HB9 (“Mnr2”, 1:5), Lim3 (1:5), or Lim1 + 2 (1:2) (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA), β -gal (Promega; 1:250), Ki67 (BD Pharmingen; 1:100), or NeuN (Chemicon; 1:50); rabbit polyclonals against β -gal (Cappel; 1:5,000), calbindin D-28k (Chemicon; 1:250), tyrosine hydroxylase (TH) (Chemicon; 1:75), or retinaldehyde dehydrogenase 2 (RALDH2) (a kind gift of P. McCaffery, University of Massachusetts Medical School, Waltham, MA; 1:200); and goat polyclonal against choline acetyltransferase (ChAT) (Chemicon; 1:10). Double-labeling histochemical studies were performed by first subjecting the sections to incubation with rabbit antibodies against either Phox2b (1:700; ref. 14) or TrkA (1:200; ref. 15), followed by visualization with either the DAB or NovaRed substrate kits for peroxidase (Vector) and detection of β -gal activity.

Cell Counting. To count the numbers of Hoechst-stained nuclei, as well as the numbers of β -gal⁺, NeuN⁺, and TrkA⁺ cells in cranial ganglia from *Runx1*^{lacZ/+} or *Runx1*^{lacZ/rd} embryos, >35

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Abbreviations: β -gal, β -galactosidase; bm, branchiomotor; ChAT, choline acetyltransferase; En, embryonic day *n*; HD, homeodomain; LMC, lateral motor column; MMC, median motor column; PNS, peripheral nervous system; RALDH2, retinaldehyde dehydrogenase 2; RUNX1, runt-related transcription factor 1; sm, somatic motor; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; vm, visceromotor; VZ, ventricular zone.

*To whom correspondence should be addressed. E-mail: stefano.stifani@mcgill.ca.

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serial sections for each genotype ($n = 4$ embryos per genotype) were used. To count the numbers of Phox2b⁺ and β -gal⁺ cells in the hindbrain of the same embryos, >30 sections for each genotype ($n = 3$ embryos per genotype) were used. The numbers of Phox2b⁺ cells were counted in three separate domains of the hindbrain defined as follows: (i) ventromedial (a 35- $\times$ 40- μ m area extending from the ventral ventricular zone (VZ) to the lateral mantle layer adjacent to the VZ), (ii) lateral (a 70- $\times$ 70- μ m area comprising the mantle layer lateral to the ventromedial domain), or (iii) dorsolateral (a 30- $\times$ 50- μ m area located dorsal to the lateral domain and extending above the dorsoventral boundary). The numbers of β -gal⁺ cells were counted in the ventromedial and lateral quadrants but not in the dorsolateral quadrant where β -gal is not expressed. Values are shown as mean $\pm$ SD.

Results

Expression of *Runx1* in Selected Cranial Ganglion Sensory Neurons. To determine the neural cell types in which *Runx1* is expressed, we used *Runx1*^{lacZ/+} mice in which the β -gal gene was recombined into the *Runx1* locus to produce a fusion protein of the N-terminal 242 aa of RUNX1 (containing a nuclear localization sequence) and β -gal (12). The expression of this nuclear fusion protein in heterozygous animals faithfully reproduces the expression of *Runx1* transcripts (10–12). In agreement with previous *in situ* hybridization studies (10), we found that *Runx1* was expressed in neither the CNS nor the PNS at E9.5 even though, as reported (10, 12), its expression was detected in the wall of the dorsal aorta (Fig. 1*A* and data not shown). Expression of β -gal in the PNS was first observed in a few trigeminal ganglion cells at approximately E10.5 (Fig. 1*B* and *C*) and became robust in trigeminal ganglia by E12 (Fig. 1*E*). Expression was also observed in the vestibular (dorsolateral), but not cochlear (ventromedial), portion of vestibulocochlear ganglia at E12 (Fig. 1*F* and *G*). In mice, vestibular neurons differentiate earlier (E10–E12) than cochlear neurons (E12–E14) (16, 17). These combined findings suggest that *Runx1* expression is correlated with differentiated neurons and not neuronal progenitors. In agreement with this possibility, essentially all β -gal⁺ cells in vestibular ganglia also expressed calbindin D-28k, a marker of bipolar mechanoreceptor neurons (16) (Fig. 1*G*). Interestingly, not all calbindin D-28k⁺ cells were β -gal⁺, suggesting that *Runx1* is not expressed in all vestibular neurons. Similarly, essentially all of the β -gal⁺ cells in trigeminal ganglia expressed the TrkA neurotrophin receptor, a marker of postmitotic nociceptive and thermoreceptive neurons (18) (Fig. 1*H*). No β -gal expression was observed in proliferating cells expressing the mitotic cell marker protein Ki67 (data not shown). Together, these findings show that *Runx1* is expressed in certain specific populations of postmitotic trigeminal and vestibular ganglion neurons.

Involvement of *Runx1* in the Development of Selected Trigeminal and Vestibular Sensory Neurons. *Runx1*^{lacZ/+} mice were crossed to mice heterozygous for a disrupted *Runx1* allele lacking coding sequences for the DNA-binding Runt domain (*Runx1*^{rd/+} mice) (8). Similar to *Runx1*^{rd/rd} embryos, doubly heterozygous *Runx1*^{lacZ/rd} embryos lack *Runx1* activity and die at $\approx$ E12.5 because of impaired fetal liver-derived hematopoiesis (12). At E10.5, *Runx1*^{lacZ/rd} and *Runx1*^{lacZ/+} embryos showed no differences in the number of trigeminal ganglion neurons expressing the general neuronal marker protein NeuN (Fig. 1*I* and *J*) or the HD protein Islet1, which is expressed in essentially all ganglionic neurons at this stage (19) (Fig. 1*K* and *L*). Further, we observed no differences in either TrkA or β -gal expression in mutant or control ganglia at E10.5 (data not shown). These results suggest that *Runx1* inactivation does not perturb the differentiation of cranial sensory neurons at E10.5.

In contrast, at E11.5 we observed a significant decrease in both

Fig. 1. *Runx1* expression in selected cranial ganglion sensory neurons. (A–F) Histochemical detection of β -gal activity (blue) in near transverse sections through E9.5 (A), E10.5 (B and C), and E12 (E and F) *Runx1*^{lacZ/+} embryos. (D) Schematic representation of the planes of the sections shown in E and F. (G) Combined double-label immunofluorescence analysis of β -gal (green) and calbindin D-28k (red) expression in vestibular ganglia. (H) Combined double-label analysis of β -gal activity (blue) and TrkA immunoreactivity (brown) in trigeminal ganglia at E12.5. (I–L) Expression of either NeuN (I and J) or Islet1 (K and L) in trigeminal ganglia of *Runx1*^{lacZ/+} (I and K) or *Runx1*^{lacZ/rd} (J and L) embryos at E10.5. Fv, fourth ventricle; mye, myelencephalon; Rp, Rathke's pouch; trn, trigeminal nerve rootlets; V, trigeminal ganglion; VIII, vestibulocochlear ganglion; vz, ventricular zone. [Scale bars = 1 nm (D), 130 μ m (E and F), and 30 μ m (H).]

Islet1 (Fig. 2*A* and *B*) and NeuN (Fig. 2*D*) expression in the vestibular ganglia of *Runx1*^{lacZ/rd} embryos compared with *Runx1*^{lacZ/+} littermates. These changes were correlated with a considerable reduction in the number of β -gal⁺ cells in the same ganglia (Fig. 2*C*). *Runx1*^{lacZ/rd} embryos also displayed a significant decrease in the number of β -gal⁺ cells in trigeminal ganglia (Fig. 2*E*), and this decrease was correlated with a reduced number of TrkA⁺ cells (Fig. 2*F*). These combined findings strongly suggest that *Runx1* inactivation causes a selected loss of the vestibular and trigeminal ganglion neurons in which *Runx1* would normally be expressed. In agreement with this, *Runx1*-deficient embryos displayed a proportional decrease in both the total number of trigeminal ganglion cells (Fig. 2*G*) and the expression of the general neuronal marker NeuN (Fig. 2*H*). In contrast, no changes in Ki67 expression were observed in mutant trigeminal ganglia (Fig. 2*I* and *J*), suggesting that *Runx1* inactivation does not perturb the proliferation of ganglionic progenitors or their transition into sensory neurons.

To examine these phenotypes further, we asked whether the observed neuronal losses were correlated with increased numbers of dying cells. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) labeling showed a significant increase in cell death in cranial ganglia of *Runx1*-deficient embryos (Fig. 2*K* and *L*). To determine the specificity of these effects, we examined geniculate ganglia, where *Runx1* is not expressed at E11.5 (data not shown). The expression of the

Fig. 4. Expression of *Runx1* in selected hindbrain cholinergic motor neurons. (A–I) Double-label immunofluorescence analysis of β -gal, ChAT, and TH expression in the hindbrain of E11.5 *Runx1*^{lacZ/+} embryos. (C and D) Combined β -gal and ChAT staining. (D) Higher magnification view of the area boxed in C. (G–I) Combined β -gal and TH staining. (H and I) Higher magnification views of the areas boxed in G.

observations suggested that *Runx1* might be expressed in hindbrain branchiomotor (bm) and visceromotor (vm) neurons. These cells derive from ventral progenitors that express the Nkx2.2 HD protein. bm/vm neurons initially appear in the ventral mantle layer and then migrate to more dorsolateral positions from where they innervate either muscles derived from the branchial arches or parasympathetic targets. bm/vm neurons express Phox2b as well as the Islet1, but not Islet2, HB9, or Lim3 HD proteins (2, 14, 20). In contrast, somatic motor (sm) neurons of the hindbrain derive from progenitor cells that express Pax6 and are located dorsal to Nkx2.2⁺ cells. These neurons occupy more ventral locations than bm/vm neurons and are characterized by the expression of Islet1, Islet2, HB9, and Lim3, but not Phox2b (2, 14, 20).

Double-labeling studies showed that β -gal expression did not overlap with, but was immediately lateral to, the expression of Nkx2.2 at both E10.5 (Fig. 3C) and E11.5 (Fig. 3H), suggesting that *Runx1* is expressed in postmitotic motor neurons derived from those progenitors. In agreement with this, essentially all β -gal⁺ cells in the hindbrain mantle layer expressed the general motor neuron marker protein Islet1 (Fig. 3D–F) and the bm/vm neuron marker Phox2b (Fig. 3I–L) at both E10.5 and E11.5. Importantly, not all Phox2b⁺ cells expressed β -gal, suggesting that *Runx1* is expressed in a subset of hindbrain bm/vm neurons. In contrast, β -gal expression did not overlap with that of sm neuron markers like HB9 (Fig. 3M–R) or Lim3 (data not shown) at either E10.5 or E11.5. Together, these results demonstrate that *Runx1* is expressed in selected hindbrain bm/vm neurons.

To identify the specific bm/vm neuron subtypes in which *Runx1* is expressed, double-labeling studies were performed by using antibodies against either ChAT, a marker of cholinergic neurons, or TH, a general marker of noradrenergic and dopaminergic neurons (21). Virtually all of the β -gal⁺ cells in the hindbrain mantle layer also expressed ChAT, indicating that they corresponded to cholinergic bm/vm neurons (Fig. 4A–D, arrow in A). In contrast, β -gal was not expressed in ventral ChAT⁺ cells likely corresponding to sm neurons based on their location, ventral projections, and expression of sm neuron markers (Fig. 4B and C, and data not shown). In addition, no evident overlap of β -gal and TH expression was detected, further suggesting that *Runx1* is specifically expressed in cholinergic bm/vm neurons (Fig. 4E–I). Consistent with these observations, at later gestational stages β -gal expression was observed in hindbrain motor nuclei that contain cholinergic bm/vm neurons, including the bm nucleus of the facial nerve, the vm dorsal motor nucleus of the vagal nerve, and the nucleus ambiguus (data not shown). To-

gether, these findings show that in the hindbrain *Runx1* is selectively expressed in postmitotic cholinergic bm/vm neurons.

Restricted Expression of *Runx1* in Spinal Cord Motor Neurons. The second site of robust *Runx1* expression in the CNS was at the brachial (C5–T1) level of the spinal cord, where two separate populations of β -gal⁺ cells were observed at E11.5. One group was located medioventrally (Fig. 6B, arrows, which is published as supporting information on the PNAS web site) and comprised cells that also expressed Islet1, HB9 (Fig. 6C–J), and Lim3 (data not shown), indicating that they correspond to spinal cord motor neurons. Based on these immunological properties, their cell body position, and the fact that they did not express the RALDH2 protein (Fig. 6K–N), these *Runx1*⁺ motor neurons likely correspond to medial constituents of the median motor column (MMC) (1, 2, 22). It is unlikely that these cells correspond to lateral MMC neurons because the latter are found only at thoracic levels (22) where *Runx1* is not expressed (ref. 10 and data not shown). The second population of β -gal⁺ cells was located dorsolaterally (Fig. 6B, arrowheads) and was positive for both Islet1 and RALDH2 expression (Fig. 6C–F and K–N), suggesting that these particular *Runx1*⁺ cells comprise lateral motor column (LMC) neurons (1, 22). Further, because we observed no overlapping immunoreactivity of β -gal and Lim1 + 2, a marker of lateral LMC neurons (1, 22) (Fig. 6P), it is likely that these cells correspond to medial LMC neurons. These findings show that *Runx1* is expressed in restricted types of spinal cord motor neurons.

Comparison of the brachial spinal cord of E11.5 *Runx1*^{lacZ/+} and *Runx1*^{lacZ/rd} littermates revealed no significant differences in the number of cells expressing β -gal, showing that *Runx1* inactivation causes neither reduced β -gal expression nor a change in the number of β -gal⁺ cells (Fig. 6O). HB9 and RALDH2 expression also was not affected by *Runx1* inactivation (data not shown). Further, we failed to detect any obvious differences in Lim1 + 2 immunoreactivity (Fig. 6P and Q). Moreover, the number of TUNEL⁺ cells in the spinal cord of these embryos was essentially the same (Fig. 6R and S). These combined findings suggest that *Runx1* activity is not required for the development of the MMC and LMC neuron subtypes in which it is expressed at E11.5. Moreover, its inactivation does not appear to cause changes in cell fate choices in the spinal cord. Although it remains possible that *Runx1* may play important roles during later stages of spinal motor neuron development, the early embryonic death of *Runx1*-deficient embryos has thus far precluded the analysis of this possibility.

Involvement of *Runx1* in the Development of Hindbrain Cholinergic Branchial and Visceral Motor Neurons. At E10.5, no differences in the expression of β -gal (Fig. 5A and B), Phox2b (which is expressed in both bm/vm progenitors and neurons at this stage) (Fig. 5C and D), and Nkx2.2 (Fig. 5E and F) were observed in the hindbrain of *Runx1*-deficient and control littermates, suggesting that *Runx1* is not important for early phases of bm/vm neuron differentiation.

At E11.5, *Runx1*^{lacZ/rd} embryos continued to exhibit no statistically significant difference in Phox2b expression in a ventromedial quadrant of the hindbrain encompassing the ventral VZ and the mantle layer immediately lateral to the VZ and containing mostly neuron progenitors and premigratory precursors (Fig. 5H, I, and L). Moreover, we observed essentially equivalent numbers of Nkx2.2⁺ cells in this ventromedial quadrant in *Runx1*^{lacZ/+} (219 ± 47 per section; >15 sections; $n = 3$ embryos) and *Runx1*^{lacZ/rd} (227 ± 46 per section; >15 sections; $n = 3$ embryos) littermates. These combined observations strongly suggest that the inactivation of *Runx1* does not perturb the initial generation of those Phox2b⁺ neurons in which *Runx1* is expressed.

In contrast, E11.5 *Runx1*^{lacZ/rd} embryos displayed a significant

Fig. 5. Loss of cholinergic branchiovisceral motor neurons in the hindbrain of *Runx1*-deficient embryos. (A–F) Expression of β -gal (A and B), Phox2b (C and D), or Nkx2.2 (E and F) in the hindbrain of either *Runx1*^{lacZ/+} (A, C, and E) or *Runx1*^{lacZ/rd} (B, D, and F) embryos at E10.5. (G) Schematic representation of the planes of the sections shown in the indicated panels. (H–K) Expression of Phox2b (H and I) or β -gal (J and K) in the hindbrain of either *Runx1*^{lacZ/+} (H and J) or *Runx1*^{lacZ/rd} (I and K) embryos at E11.5. Notice the decreased β -gal expression in both the hindbrain mantle layer and the vestibular ganglia (VIII) of *Runx1*^{lacZ/rd} embryos. (L) Quantitative analysis of Phox2b expression in the ventromedial (VMQ), lateral (LQ), or dorsolateral (DLQ) quadrants of the hindbrain of *Runx1*^{lacZ/+} (bars 1, 3, and 5) or *Runx1*^{lacZ/rd} (bars 2, 4, and 6) embryos. (M) Quantitative analysis of β -gal expression in the VMQ and LQ of the hindbrain of *Runx1*^{lacZ/+} (bars 1 and 3) or *Runx1*^{lacZ/rd} (bars 2 and 4) embryos. (L and M) Results of cell counts in each quadrant are depicted as mean $\pm$ SD; *, $P < 0.01$. (N–Q) Analysis of the hindbrain of *Runx1*^{lacZ/+} (N and P) or *Runx1*^{lacZ/rd} (O and Q) embryos for expression of β -gal (N and O) or ChAT (P and Q). (P) Arrow points to ChAT⁺ cells that are lost in *Runx1*-deficient embryos; arrowhead points to ChAT⁺ cells that are not affected by *Runx1* inactivation. (R–U) Analysis of TUNEL⁺ cells in either the hindbrain (R and S) or the forebrain (T and U) of *Runx1*^{lacZ/+} (R and T) or *Runx1*^{lacZ/rd} (S and U) embryos. The areas stained in R and S correspond to the boxed regions in N and O. (U) * indicates intraventricular blood cells. tv, telencephalic vesicle; VIII, vestibular ganglion; vz, ventricular zone. [Scale bars = 1 mm (G), 90 μ m (H–K), 80 μ m (N–Q), and 25 μ m (T and U).]

reduction in the number of Phox2b⁺ cells in a lateral quadrant of the mantle layer that contains more developmentally mature bm/vm neurons that have migrated from their place of birth

(Fig. 5 H, I, and L). The decrease in Phox2b expression was specifically observed in this lateral region of the hindbrain, where *Runx1* and Phox2b expression overlaps (Fig. 3). In contrast, we detected no differences in Phox2b expression in a more dorsolateral quadrant where *Runx1* is not expressed (Fig. 5 H, I, and L). We therefore examined whether this specific loss of Phox2b⁺ cells was correlated with a loss of *Runx1*⁺ bm/vm neurons. Cell counting studies in the ventromedial (where small numbers of β -gal⁺ cells are found) and lateral (where the majority of *Runx1*⁺ cells are located) quadrants of the hindbrain of E11.5 embryos revealed a significant decrease in the number of β -gal⁺ cells in *Runx1*-deficient embryos (Fig. 5 J, K, and M). These findings suggest that *Runx1* inactivation causes a specific loss of Phox2b⁺/*Runx1*⁺ bm/vm neurons. To test this possibility further, we compared ChAT expression in mutant and control embryos. A significant decrease in both ChAT (Fig. 5 P and Q, arrow) and β -gal (Fig. 5 N and O) expression was detected in the lateral quadrant of the hindbrain, consistent with a loss of *Runx1*⁺/ChAT⁺ bm/vm neurons. In contrast, no significant changes in ChAT expression were detected at more ventral locations of the hindbrain (Fig. 5 P and Q, arrowhead), where ChAT⁺ cells do not express *Runx1* (Fig. 4).

The decreased expression of β -gal in the lateral quadrant of the hindbrain of *Runx1*-deficient embryos was not correlated with ectopic β -gal expression in ventral territories occupied by sm neurons (Fig. 5 J, K, N, and O). Similarly, neither an ectopic expression of Phox2b (Fig. 5 H and I) nor an increased expression of ChAT (Fig. 5 P and Q) were observed in the same ventral regions. These combined observations strongly suggest that *Runx1* inactivation causes neither a rerouting of bm/vm neurons to ventral domains nor a conversion to an alternative neuron cell fate. Instead, *Runx1*^{lacZ/rd} embryos displayed an increase in the number of TUNEL⁺ cells near the boundary between the ventromedial and lateral quadrants (Fig. 5 R and S), suggesting a correlation between increased rate of cell death and the loss of β -gal⁺ cells. This increase in TUNEL⁺ cells was specific to this particular region because we did not detect changes in TUNEL staining in either the spinal cord (Fig. 6 R and S) or the forebrain (Fig. 5 T and U). Together, these results show that *Runx1* plays an important role in the postmitotic development and/or survival of hindbrain cholinergic bm/vm neurons.

Discussion

In this study, we have demonstrated that *Runx1* function is important for the postmitotic development of specific neuronal subtypes in the CNS and PNS. In both the hindbrain and spinal cord, *Runx1* is expressed in selected postmitotic neurons of the mantle layer and not in neural progenitors of the VZ. Within the mantle layer, *Runx1* expression is restricted to cholinergic bm/vm neurons in the hindbrain and certain MMC and LMC motor neurons in the spinal cord. Similarly, in both trigeminal and vestibular ganglia, *Runx1* expression is correlated with phases of active neuronal differentiation and coincides with nociceptive/thermoceptive and mechanoreceptor neurons, respectively. Apart from these cells, *Runx1* expression was observed in only a few other neuron types, including nociceptive, but not proprioceptive, sensory neurons in dorsal root ganglia and restricted neuron subtypes in the retina (refs. 10 and 12 and data not shown).

Our studies have shown further that *Runx1* expression only becomes detectable in hindbrain bm/vm neurons at approximately E10.5, even though these neurons already begin to be generated at roughly E9.5 (20, 21). At E11.5, when hindbrain cholinergic bm/vm neuron production has essentially ceased (20, 21), only small numbers of *Runx1*⁺ cells are found in the most ventral aspect of the hindbrain and most of them are located dorsolaterally, likely as a result of the migration of the earlier-born cells. These combined observations show that *Runx1* ex-

pression is activated after the initial generation of specific bm/vm neurons and suggest that this gene is involved in the developmental maturation of these cells. In agreement with this possibility, we have shown that although *Runx1* inactivation does not cause a detectable loss of hindbrain bm/vm neurons at E10.5, a significant loss of bm/vm neurons in which *Runx1* is normally expressed is observed in the hindbrain mantle layer at E11.5. This neuronal loss is specific because a number of dorsolateral Phox2b⁺ neurons that do not express *Runx1* are not affected by the disruption of *Runx1* function. Moreover, ChAT⁺/*Runx1*⁻ neurons located in the ventral hindbrain, likely corresponding to sm neurons, are also spared. In addition, no detectable differences in the numbers of neurons expressing TH are observed among *Runx1*-deficient and control embryos (data not shown), suggesting further that *Runx1* inactivation perturbs only the development of those bm/vm neurons in which it is expressed. A similar situation was observed in trigeminal and vestibular ganglia, where disruption of *Runx1* function also causes no detectable effect at E10.5 but results in a significant loss of sensory neurons at E11.5. Importantly, no loss of sensory neurons was observed in geniculate ganglia, which are adjacent to vestibulocochlear ganglia but do not express *Runx1* at E11.5.

The observed decrease in *Runx1*⁺ bm/vm neurons in the lateral hindbrain does not appear to be the consequence of their abnormal migration to ventral regions because we detected neither an ectopic expression of β -gal and Phox2b nor an increased number of ChAT⁺ neurons in ventral territories where sm neurons are located. Further, we failed to detect an increase of either TH⁺ bm/vm neurons or ventral sm neurons in the hindbrain of *Runx1*-deficient embryos. Similarly, we found no evidence that *Runx1*-deficient cells in trigeminal and vestibular ganglia adopt alternative neuronal fates and instead observed that the expression of a number of different neuronal markers is reduced in the mutant ganglia. Together, these results strongly suggest that in both the neural tube and cranial ganglia *Runx1* is involved in mechanisms important for the postmitotic development of the restricted neuronal populations in which it is expressed. They suggest further that *Runx1*-inactivation is not correlated with changes in neuronal fates but rather with a reduced development/survival of those cells.

Runx1 may be directly involved in promoting cell survival. This possibility is in agreement with our finding that in both cranial ganglia and hindbrain, the neuronal losses associated with *Runx1*-inactivation are correlated with increased cell death. These effects are specific and not simply a consequence of the hematopoietic deficiency of *Runx1*-deficient embryos, because no differences in TUNEL labeling were observed in other regions of the neural tube where either *Runx1* expression is not detected, like the forebrain, or *Runx1* is expressed but its inactivation does not cause a detectable phenotype, like the spinal cord. A prosurvival role for *Runx1* would also be consistent with previous studies showing that its overexpression in T hybridoma cells results in an up-regulation of the antiapoptotic gene *Bcl-2* and renders those cells resistant to apoptosis mediated by the T cell receptor (23). Alternatively, or in addition, *Runx1* may be involved in regulating the expression of genes that define the particular phenotypic traits of those neuron subtypes, and its inactivation may cause a developmental arrest eventually resulting in the elimination of those cells through apoptosis. An involvement of *Runx1* in cranial sensory neuron differentiation is consistent with recent studies showing that *Runx1* and the related gene *Runx3* are expressed in separate dorsal root ganglion neuron subtypes. *Runx1* is expressed in nociceptive neurons whereas *Runx3* is found in proprioceptive neurons (12, 15, 24). Importantly, *Runx3* inactivation causes a perturbation of the development of dorsal root ganglion proprioceptive, but not nociceptive, neurons (15, 24). Future studies aimed at further elucidating the involvement of *Runx1* in cell survival and/or differentiation events in selected sensory and motor neurons will provide important information that will help clarify mechanisms underlying the development of neuronal subtypes in the mammalian CNS and PNS.

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