Hox Paralog Group 2 Genes Control the Migration of Mouse Pontine Neurons through Slit-Robo Signaling

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The pontine neurons (PN) represent a major source of mossy fiber projections to the cerebellum. During mouse hindbrain development, PN migrate tangentially and sequentially along both the anteroposterior (AP) and dorsoventral (DV) axes. Unlike DV migration, which is controlled by the Netrin-1/Dcc attractive pathway, little is known about the molecular mechanisms guiding PN migration along the AP axis. Here, we show that Hoxa2 and Hoxb2 are required both intrinsically and extrinsically to maintain normal AP migration of subsets of PN, by preventing their premature ventral attraction towards the midline. Moreover, the migration defects observed in Hoxa2 and Hoxb2 mutant mice were phenocopied in compound Robo1;Robo2, Slit1;Slit2, and Robo2;Slit2 knockout animals, indicating that these guidance molecules act downstream of Hox genes to control PN migration. Indeed, using chromatin immunoprecipitation assays, we further demonstrated that Robo2 is a direct target of Hoxa2 in vivo and that maintenance of high Robo and Slit expression levels was impaired in Hoxa2 mutant mice. Lastly, the analysis of Phox2b-deficient mice indicated that the facial motor nucleus is a major Slit signaling source required to prevent premature ventral migration of PN. These findings provide novel insights into the molecular control of neuronal migration from transcription factor to regulation of guidance receptor and ligand expression. Specifically, they address the question of how exposure to multiple guidance cues along the AP and DV axes is regulated at the transcriptional level and in turn translated into stereotyped migratory responses during tangential migration of neurons in the developing mammalian brain.

Citation: Geisen MJ, Di Meglio T, Pasqualetti M, Ducret S, Brunet J-F, et al. (2008) Hox paralog group 2 genes control the migration of mouse pontine neurons through Slit-Robo signaling. PLoS Biol 6(6): e142. doi:10.1371/journal.pbio.0060142

Introduction

In the developing central nervous system (CNS), neurons migrate sometimes over long distances from their birthplace to their final location, where they condense in specific nuclei. As neuronal function depends upon precise connectivity with their targets, the final positioning of migrating neurons is critical to the building of ordered connectivity between presynaptic and postsynaptic partners. In many brain regions, neurons migrate in a tangential direction, orthogonal to the radial axis and independently of radial glia, resulting in mixing of cells that originated from distinct ventricular regions [1]. Mounting evidence suggests that tangentially migrating neurons are guided during their journey by the same set of attractive and repulsive guidance cues that regulate axonal pathfinding and topographical mapping [2]. However, little is known about how exposure of migrating neurons to several simultaneous extrinsic inputs along the orthogonal axes of the brain may be integrated at the transcriptional level and in turn translated into directional migratory responses specific for each neuronal population.

In the mouse hindbrain, the precerebellar system is a suitable model to study the molecular mechanisms controlling long-distance tangential migration. Precerebellar nuclei are essential for coordinated motor activity and provide the principal input to the cerebellum [3]. They convey information to the cerebellum through the climbing and mossy fiber projection systems. Although the only source of climbing fibers is the inferior olivary nucleus (ION), mossy fibers have multiple origins, such as the lateral reticular (LRN) and external cuneate (ECN) nuclei in the posterior hindbrain, and the pontine gray (PGN) and reticulotegmental (RTN) nuclei, collectively referred thereafter as pontine neurons (PN), in the anteroverentral hindbrain [4].

The early patterning of the mouse hindbrain along the anteroposterior (AP) axis is characterized by a segmentation...
Author Summary

In the developing central nervous system, neurons migrate sometimes over long distances from their birthplace to their final location, where they condense in specific nuclei. The precise positioning of migrating neurons is critical to the building of ordered connectivity with their target partners. Little is known about how exposure of migrating neurons to simultaneous attractive and repulsive guidance cues may be integrated at the transcriptional level and in turn translated into directional migratory responses specific for each neuronal population. Here, we focus on the molecular mechanisms regulating the directionality of long-distance migration of pontine neurons in the mouse brainstem. Such neurons belong to the so-called precerebellar system, which is essential for coordinated motor activity, and provide the principal input to the cerebellum. We provide evidence for the implication of homeodomain transcription factors of the *Hox* gene family in the control of pontine neuron migration along the brain rostrocaudal axis. We identify the guidance receptor Robo2 as a direct target gene of the *Hoxa2* gene. We further show that repulsive signaling mediated through the Robo2 receptor expressed in migrating neurons and its ligand Slit2 secreted from the facial motor nucleus are key components of the molecular guidance system that maintains caudorostral migration and prevents premature attraction towards the brainstem ventral midline. Our data provide a conceptual framework to understand how transcriptional regulation of the response to environmental guidance cues controls stereotyped neuronal migratory behavior in the developing mammalian brain.

caudal patterning information to developing neurons [5,17]. More recently, it has been shown that *Hox* gene expression is maintained up to late stages of development in specific neuronal subpopulations in the hindbrain and spinal cord, where they may be important for the establishment of topographically organized sensory and motor circuits [18–20]. However, the potential role of *Hox* genes in orienting directional neuronal migration through regulation of guidance molecules remain largely unknown.

Herein, we found that the paralog group (PG)2 *Hox* genes, *Hoxa2* and *Hoxb2*, are required to maintain normal migration of PN along the rostral pathway. In *Hoxa2* and *Hoxb2* mutants, subsets of PN prematurely migrated ventrally, settling at ectopic posterior locations. Interestingly, the PN migratory defects observed in PG2 *Hox* mutants phenocopied those found in *Robo1*;*Robo2*, *Slit1*;*Slit2*, and *Robo2*;*Slit2* compound mutants. Furthermore, in PG2 *Hox* mutants the expression levels of *Robo2* and *Slit2* were decreased and chromatin immunoprecipitation assays demonstrated the direct binding of *Hoxa2* on the *Robo2* locus. In addition, we identified the facial motor nucleus (FMN), which is located in ventrolateral r6, as an important source of Slit ligands for Robo receptor–expressing PN. This is supported by the finding that in the *Phox2b* knockout mice, which completely lack the FMN, the PN undergo the same ectopic and premature ventral migratory defects as observed in *Hox* PG2, *Robo1*;*Robo2*, *Slit1*;*Slit2*, or *Robo2*;*Slit2* compound mutants. Altogether, our data provide important novel insights into the intrinsic and extrinsic molecular determinants involved in tangential migration of PN neurons along the AP axis.

Results

Rhombomeric Mapping of Migration Pathway and *Hox* Gene Expression of Pontine Neurons

Tangentially migrating PN originate in the r6–r8 rhombic lip between embryonic day (E)13.5 and E17.5 [10]. They migrate first ventrally and then rostrally until they reach a final anteroventral position in the pontine primordium (Figure 1A–1C; [6]). During their migration, PN navigate across distinct rhombomeric-derived territories and express several molecular markers, including the homeobox-containing gene Barhl1 (Figure 1A, 1B, 1E, and 1H; [21]). To map the migratory route of PN in relationship to rhombomeric territories, we simultaneously performed lacZ staining and in situ hybridization with *Barhl1* on whole-mount brains or tissue sections from *Krox20::Cre;ROSA26R* (Figure 1A and 1B), *Krox20::Cre;Z/AP* (Figure 1G–1I), or R4::Cre;Z/AP (Figure 1D–1F) mouse lines [20,22–24] in which lacZ or alkaline phosphatase expression is permanently activated in r3 and r5 or r4 progenies, respectively. The migratory pathway of *Barhl1*+ PN neurons can be divided into three distinct phases (Figure 1A–1C). First, PN undertake a short ventral migration upon leaving the r6–r8 rhombic lip (phase 1). In the next step (phase 2), PN turn rostrally, parallel to the AP axis. They travel through r5 and r4, where they pass between the vestibulocochlear (VIIIth, dorsally) and the facial (VIIth, ventrally) nerve roots (Figure 1C–1F; [25]) and continue migrating through r3 until they reach the trigeminal (Vth) nerve root located in the caudal aspect of r2 (Figure 1A–1C). Interestingly, the PN stream never enters r2 but makes an abrupt change of direction and undertakes a final ventral process into distinct morphological segments called rhombomes, resulting in spatial segregation of the neuroepithelium contributing to each segment [5]. Distinct precerebellar neuronal populations are contributed by rhombomeric portions of the rhombic lip, a stripe of neuroepithelium that arises dorsally at the interface with the roof plate and runs throughout the rostrocaudal extent of the hindbrain, giving rise to spatiotemporally defined sequences of migratory populations [4,6–12]. Indeed, rhombomere (r)1 rhombic lip derivatives specifically contribute to the external granular layer (EGL) of the cerebellum [12], whereas a recent fate mapping study subdivided the mouse r2–r8 rhombic lip into several rhombomeric domains before turning neuronal populations are contributed by rhombomeric territories, we simultaneously performed lacZ staining and in situ hybridization with *Barhl1* in situ hybridization with *Barhl1* in
Figure 1. Rhombomeric Mapping of Migration Pathway and Hox Gene Expression of Pontine Neurons

(A–C) Lateral (A) and ventral (B) views of E15.5 Krox20::Cre;ROSA26R whole-mount brain. The progenies of r3 and r5 are traced through lacZ activity. Subsequently, the brain was hybridized with a Barhl1 antisense probe. Insets in (A) and (B) show whole-mount brains after staining for lacZ activity and before in situ hybridization with Barhl1.

Barhl1+ neurons stream from a dorsoposterior to an anteroventral location, crossing the r5- and r4-derived territory before entering the r3-derived domain where they turn to migrate ventrally.
In (B), PN finally settle in the r4-derived territory, just abutting r3- and r5-derived domains. The migration is subdivided into three distinct phases (1–3) as indicated in the summary diagram in (C). (D–F) Adjacent coronal cryostat sections of E15.5 R4::Cre;Z/AP brain at the level of the rostral phase (phase 2) of PN migration, stained for alkaline phosphatase activity (D) or hybridized with a Barhl1 probe (E). (F) Merge of (D) and (E). nVII and nVIII (arrows) exit the brain at the level of the r4-derived domain (D). During phase 2, PN migrate in a corridor below the nVIII and above the nVII (F). (G and H) Adjacent coronal cryostat sections of E15.5 Krox20::Cre;Z/AP brain at the level of the final ventral phase (phase 3) of PN migration stained for alkaline phosphatase activity (AP) (G) or hybridized with a Barhl1 probe (H). The black dashed line in (G) delimits the outline of the brain, the red dashed lines delimit the r3-derived territory. (I) Merge of (G) and (H). PN neurons migrate through the r3-derived territory before they reenter and settle in the r4-derived domain ventrally (I). PN never enter the r2-derived territory (I). The black dashed line in (I) delimits the outline of the brain; the red dashed lines delimit the r3-derived territory. Note that the nV exits the brain at the level of the r2-derived domain (G and I). (J–P) E14.5 cryostat hindbrain sections hybridized with Hoxa2 (J and L), Hoxb2 (K and K), Hoxa3 (L and L’), Hoxb3 (M and M’), Hoxb4 (N and N’), Hoxd4 (O and O’), and Hoxb5 (P and P’) probes. The arrows show expression in migrating PN. (J–P) correspond to sections at the level of nVII (phase 2), only the right side of the section is shown. (J–P’) correspond to sections posterior to nVII (phase 1). (Q and R) Cryostat sections of P0 (Q), and P4 (R) brains hybridized with a Hoxa2 probe. The section level is indicated by the red box in the left diagram. Arrows show Hoxa2 expression in the PGN and RTN nuclei. nV, trigeminal nerve; nVII, facial nerve; r, hindbrain patterning [20,26–29], and their expression is maintained throughout migration, nucleogenesis, and establishment of connectivity to cerebellum of PN (both RTN and PGN) up to postnatal stages (Figure 1J–1R; and unpublished data). Such expression patterns suggested that PN may be endowed with molecular information as to their origin and relative position along the AP axis throughout their migration.

Hoxa2 and Hoxb2 Are Required for Migration of Subsets of Pontine Neurons along the Anteroposterior Axis

To address the potential involvement of Hox genes in PN rostral migration, we focused on the PG2 genes, Hoxa2 and Hoxb2. These genes perform important roles in rostral hindbrain patterning [20,26–29], and their expression is maintained throughout migration, nucleogenesis, and establishment of connectivity to cerebellum of PN (both RTN and PGN) up to postnatal stages (Figure 1J–1K, 1Q, and 1R; and unpublished data).

In Hoxa2 heterozygous mutants [30], PN displayed a normal migratory behavior as assessed by whole-mount in situ hybridization on E14.5 and E15.5 hindbrains with antisense probes for known markers of migrating PN, such as Barhl1 (Figure 2A–2C; [21]), Pax6, and Tag1 ([31,32]; unpublished data). In contrast, in Hoxa2+/− homozygous mutant mice, we observed defects of PN navigation along the rostral pathway (Figure 2D–2F). Although migratory abnormalities could be observed in all Hoxa2+/− mutants (n = 15), they were variably penetrant. In some cases (n = 10), small cohorts of neurons left the stream prematurely and migrated ventrally towards the midline (Figure 2D–2F). Ectopic neurons expressed Barhl1, Pax6, and Tag1, and often condensed in small ectopic nuclear formations close to the midline, posterior to the normal location of pontine nuclei (Figure 2D and 2E; and unpublished data). In these mutants, only a subset of PN migrated ectopically, whereas the bulk of the stream maintained a caudorostral migratory path. In order to investigate whether this subset was a random or specific subpopulation of PN, we analyzed the status of the RTN and PGN by in situ hybridization with Barhl1 and Pax6 antisense probes on Hoxa2−/− brains at E17.5 days postcoition (dpc) (Figure S3, and unpublished data). A general slight reduction of both PGN and RTN was apparent (Figure S3K and S3L), suggesting that the Hoxa2 inactivation randomly induced the ectopic migration of subsets of neurons contributing to both nuclei. In further support of this idea, in a number of mutant fetuses (n = 5), we observed more-severe defects in which almost the entire PN stream underwent a premature ventral migration (Figure 2G and 2H). It is also noteworthy that the abnormal migratory phenotypes were often asymmetrically distributed among the two sides (Figure 2H and 2I; and unpublished data). Finally, by in situ hybridization with Barhl1, Pax6, Robo3, and Tag1 antisense probes on E13.5, E14.0, E14.5, and E17.5 whole-mount brains, we found ectopic migrations throughout development of PN, arguing that the Hoxa2 inactivation did not selectively affect neurons migrating within a particular time window (Figures 2D–2I and S3A–S3J).

Altogether, these data indicated stochastic compensation for the loss of Hoxa2 along the AP migratory path of PN. This may result from partial functional redundancy with other Hox genes expressed in migrating PN (Figure 1). Indeed, premature ventral migrations of PN could also be observed in some Hoxb2−/− (Figure 2J–2L; n = 2 out of 6). Moreover, Hoxa2+/−/Hoxb2+/− mutant specimen (n = 3; Figure 2M–2O) appeared to display more ectopic PN as compared to single mutants (compare Figure 2D–2L and 2M–2O), thus indicating some degree of functional redundancy and genetic interaction among Hox PG2 genes.

In summary, these results indicated that Hoxa2, and to a lesser extent Hoxb2, may regulate the response of subsets of PN to environmental cues to precisely maintain their rostral migratory route, thus ultimately contributing to control the final location of pontine nuclei along the AP axis.

Autonomous and Nonautonomous Roles of Hoxa2 in Regulating Rostral Migration of Pontine Neurons

Hox PG2 may be intrinsically required in PN throughout migration. Alternatively, or in addition, they may be required nonautonomously to pattern the environment through which PN migrate and to which PN respond in order to direct their rostral migration.
To address this question, we focused on Hoxa2 function. To achieve Hoxa2 inactivation in PN, we mated a mouse carrying a Hoxa2 floxed allele, Hoxa2\textsuperscript{flox} [33] with the \textit{Wnt1::Cre} transgenic mouse line that allows Cre-mediated deletion in rhombic lip progenitors [6,9,25,34]. In whole-mount brains from \textit{Wnt1::Cre};Hoxa2\textsuperscript{flox/flox} fetuses, we found scattered ectopic neurons that appeared to migrate prematurely from the PN stream (Figure 3E–3H). At E15.5, the ectopic neurons had reached the ventral midline as assessed by \textit{Tagl} expression (Figure 3G). These results supported an intrinsic requirement of Hoxa2 expression in PN to maintain rostral migration (see also below). However, the phenotype was less pronounced than in Hoxa2 null mutants (compare Figure 3E–3H with Figure 2D–2I). Such a difference may not be explained by an incomplete deletion of Hoxa2 in PN precursors, as we have previously shown that the \textit{Wnt1::Cre} driver is able to induce a complete excision of Hoxa2 [35]. Thus, these results may rather indicate that Hoxa2 is required both in a cell autonomous and a nonautonomous manner to regulate the response of PN to guidance cues during their rostral migration (see below; see also Figure 4).

Figure 2. Migration Defects of Pontine Neurons in Single Hoxa2, Hoxb2, and Compound Hoxa2;Hoxb2 Mutant Mice

(A–I) Lateral (A, D, and G) and ventral (B, E, and H) views of Hoxa2\textsuperscript{+/-} (A and B) and Hoxa2\textsuperscript{-/-} (D, E, G, and H) whole-mount brains at E14.5 hybridized with a \textit{Barhl1} probe to visualize migrating PN. In Hoxa2\textsuperscript{-/-} specimen (D, E, G, and H), the black arrows show ectopic \textit{Barhl1}\textsuperscript{+} cells prematurely migrating towards the midline. The white arrows indicate PN migrating along the normal pathway. Note that the migration phenotype is asymmetrically and variably distributed when comparing distinct mutants or both sides of a given specimen (D, E, G, and H).

(C, F, and I) Diagrams summarizing the normal (C) and abnormal (F and I) PN migratory phenotypes shown in (A and B) and (D, E, G, and H), respectively.

(J–L) Lateral (J) and ventral (K) views of Hoxb2\textsuperscript{+/-} mutant brain hybridized with \textit{Barhl1}. Similar to Hoxa2\textsuperscript{+/-} mutants, ectopic \textit{Barhl1}\textsuperscript{+} cells leave the stream of PN and prematurely migrate ventrally (black arrows). (L) Summary diagram of the Hoxb2 deficient phenotype.

(M–O) Lateral (M) and ventral (N) views of Hoxa2\textsuperscript{+/-};Hoxb2\textsuperscript{-/-} double mutant brain hybridized with \textit{Barhl1}. (O) Summary diagram of the Hoxa2\textsuperscript{+/-};Hoxb2\textsuperscript{-/-} mutant phenotype. Note that Hoxa2\textsuperscript{+/-};Hoxb2\textsuperscript{-/-} mutants appear to display more ectopic PN as compared to single mutants.

nV, trigeminal nerve; nVIII, vestibulocochlear nerve.

doi:10.1371/journal.pbio.0060142.g002
Hoxa2-Dependent Maintenance of Robo2 and Slit2 Expression during Pontine Neuron Migration

We next determined whether the PN migration defects detected in Hoxa2−/− mutants could be explained by a perturbed expression of ligands or receptors known to control their migration. Chemoattraction of tangentially migrating PN along the DV axis involves the Netrin-1 ligand/Dcc receptor guidance system [13–15,36]. Netrin-1 is secreted by the floor plate, whereas its receptor Dcc is expressed in PN throughout their migration (Figure 4A). In Hoxa2−/− mutant brains, we found that Netrin-1 expression in the floor plate was unaffected (unpublished data). Dcc was also normally detected both in the PN stream and the ectopically migrating neurons, as shown both by in situ hybridization and antibody staining (Figure 4E and 4S; and unpublished data).

It is noteworthy that PN are not immediately attracted towards the floor plate, but undertake their long rostral migration before finally turning ventrally towards the floor plate. This suggests that, in wild-type mice, the Netrin-1/Dcc attractive signaling system may be antagonized during the rostral phase of PN migration (phase 2; Figure 1C). The chemotropic molecules of the Slit family and their Robo receptors are major repellents for developing neurons and have been shown to antagonize Netrin-1 activity on axonal growth in a dose-dependent manner [37,38]. Thus, we first investigated the spatial distribution of the Robo1–3 receptors and Slit1–3 ligands during PN migration. In addition to Robo3/Tag1 (Figure 4B; [16]), Robo1 and Robo2 were also found to be expressed during PN migration as shown by Robo2 in situ hybridization and by anti-Robo1 whole-mount immunostaining on E14.5 hindbrains (Figures 4K, 4Q, S1A, and S1B). The presence of Robo receptors in migrating PN was further supported by the binding on whole-mount hindbrains of a Slit2 fragment genetically fused to alkaline phosphatase (LRR-hSlit2-AP; [39]) (Figure S1C). Slit1–3 were all expressed in the floor plate and rhombic lip, though not in PN (Figures 4C, 4D, 4U, and 4M; see also Figure S3D and S3H; and unpublished data). Interestingly, from E13.0, Slit2 and Slit3, but not Slit1, were also expressed in neurons of the FMN (Figures 4C, 4U, and 4M; and unpublished data; see below).

We next asked whether Hoxa2 may regulate Robo and/or Slit expression during PN migration. In E14.5 Hoxa2−/− fetuses, Robo3 and Dcc were expressed at normal levels in migrating PN (Figure 4E, 4F, and 4M; see also Figure S3D and S3H; and unpublished data). In contrast, Robo2 transcript levels were significantly lower in migrating PN of Hoxa2−/− and Hoxa2+/−/Hoxb2−/− compound null mutants than in control mice (Figure 4N and 4T). Interestingly, down-regulation of Robo2 expression was particularly evident in ventrally migrating ectopic cells that nonetheless maintained normal expression of Dcc, Robo3, and Barhl1 as assessed by in situ hybridization on adjacent sections in both single Hoxa2−/− and compound Hoxa2−/−/Hoxb2−/− null mutants (compare Figure 4I–4L with 4M–4P, and 4O–4Q with 4R–4T). Moreover, we found a notable down-regulation of Slit2 expression in the FMN of Hoxa2−/− mutants (n = 6), whereas normal Slit2 expression levels were detected in rhombic lip and floor plate (compare Figure 4C and 4D with 4G and 4H).

Thus, the PN migratory defects observed in the absence of PG2 Hox function may be mediated, at least partially, through decreased Slit-Robo signaling due to defective maintenance of Robo2- and Slit2-sustained expression during PN migration along the AP axis.

Slit-Robo–Mediated Signaling Is Required to Maintain Normal Rostral Pontine Neuron Migration

In Robo3-deficient mice, PN can still migrate along the rostral pathway (phase 2) but are unable to undergo their
Hox-Dependent Pontine Neuron Migration

(A and H) Analysis of Dcc, Robo3, and Slit2 expression in wild-type (WT) (A–D) and Hoxa2\(^{-/-}\) (E–H) E14.5 whole-mount hindbrains. (A and E) Anti-DCC antibody immunohistochemistry on WT (A) and Hoxa2\(^{-/-}\) (E) hindbrains. Dcc expression is present both in normally and ectopically (arrow in [E]) migrating PN. (B and F) Lateral views of WT (B) and a Hoxa2\(^{-/-}\) (F) brain hybridized with a Robo3 antisense probe. In (F), Robo3 is expressed both in normally and ectopically (arrows) migrating PN. (C, D, G, and H) Ventral (C and G) and dorsal (D and H) views of WT (C and D) and Hoxa2\(^{-/-}\) (G and H) brains hybridized with a Slit2 probe. Slit2 is expressed in the FMN (arrows in [C]), floor plate (FP), and rhombic lip (RL) (white and black arrows in [D], respectively). In Hoxa2\(^{-/-}\) mutants, Slit2 is down-regulated in the FMN (arrows in [G]), though not in FP and RL (white and black arrows in [H], respectively).

(1–N) Adjacent cryostat sections of WT (1–K) and Hoxa2\(^{-/-}\) (L–N) E14.5 hindbrains hybridized with Barhl1 (I and L), Robo3 (J and M), and Robo2 (K and N) probes. Arrows on each panel show expression in migrating PN. In Hoxa2\(^{-/-}\) mutants, Barhl1 (L) and Robo3 (M) expression is unaffected, whereas Robo2 (N) is down-regulated (arrow; compare K and N).

(O–T) Adjacent cryostat sections of E14.5 Hoxa2\(^{-/-}\);Hoxb2\(^{-/-}\) (O–Q) and Hoxa2\(^{-/-}\);Hoxb2\(^{-/-}\) (R–T) hindbrains hybridized with Barhl1 (O and R), Dcc (P and S), and Robo2 (Q and T) probes. In Hoxa2\(^{-/-}\);Hoxb2\(^{-/-}\) mutants, Robo2 expression in the main PN stream is down-regulated (arrow in [T]). Note also that ectopically migrating neurons do express Barhl1 and Dcc (brackets in [R] and [S]), whereas no expression of Robo2 is detected in the ectopic stream (bracket in [T], compare with [R] and [S] adjacent sections). Summaries on the right show the distinct migratory phenotypes and planes of section shown in (1–K), (L–N), (O–Q), and (R–T).

(U) Lateral view of a E14.5 WT whole-mount brain doubly hybridized with anti-Robo3 (green) and anti-Islet1 (red) antibodies.

(V) E15.5 WT hindbrain doubly immunostained using anti-Robo3 (green) and anti-Isl1 (red) antibodies.

(W) Drawing summarizing the expression of Robo2 (green) in migrating PN and Slit2 (brown) in the FMN and their relative positions in respect to one another.

nV, trigeminal nerve; nVIII, vestibulocochlear nerve.

doi:10.1371/journal.pbio.0060142.g004

Figure 4. Hoxa2-Dependent Expression of Guidance Molecules during Pontine Neuron Migration.

Figure 5A, 5B, and S2A–S2E). PN migratory abnormalities (Figures 5A, 5B, and S2A–S2E). PN migratory abnormalities (Figures 5A, 5B, and S2A–S2E).

final ventral migration (phase 3) to reach the floor plate (Figure S2I; [16]). To address the potential involvement of Robo1–2 and their ligands Slit1–2 in PN migration, we next analyzed single and compound knockout mice. None of the single or compound heterozygous mutants showed significant PN migratory abnormalities (Figures 5A, 5B, and S2A–S2E). In contrast, similar migration defects of PN were observed at E15.5 in all Robo1\(^{-/-}\);Robo2\(^{-/-}\) (n = 19) and Slit1\(^{-/-}\);Slit2\(^{-/-}\) (n = 18) compound mutant mice, following whole-mount labeling with antisense Barhl1 or Robo3 riboprobes or immunostaining with anti-Robo3 (Figures 5E, 5F, 5L, 5J, S2F, and S2G; and unpublished data). Although PN normally left the rhombic lip (phase 1), during phase 2, cohorts of PN left the stream and prematurely migrated ventrally, condensing in small ectopic clusters adjacent to the midline. The leading processes of Robo3-expressing PN neurons were still oriented toward the floor plate and crossed it normally (unpublished data). Moreover, DiI tracing performed on E18-postnatal day (P0) mutants showed that PN axon projections to the cerebellum were normal (unpublished data), thereby suggesting that migration, but not axon guidance, was selectively affected in these mutants. The ectopic nuclei expressed PN markers including Barhl1, Pax6, Robo3, and Tag1, and were observed at least until E17.5 (Figures 5E, 5F, 5L, 5J, S2F, and S2G; and unpublished data), as double knockouts were not viable and died a few hours after birth. The ectopic nuclei were also immunoreactive for Dcc (unpublished data). However, many PN still reached a normal location in ventral r4 (Figure 5E, 5F, 5I, and 5J). Such phenotypes were fully penetrant although the size and position of the ectopic PN clusters varied between mutant embryos and brain stem side (Figures 5F, S2F, and S2G). Such PN migratory defects strikingly phenocopied the abnormalities observed in Hox PG2 knockout animals (compare Figure 5E, 5F, 5I, and 5J with Figure 2D, 2E, 2G, and 2H). Strikingly, similar ectopic PN migrations were also observed in compound Robo2\(^{-/-}\);Slit2\(^{-/-}\) fetuses in which only one dose of Robo2 and Slit2 was simultaneously deleted (Figure 5M), thus providing strong genetic evidence supporting their dosage-dependent interaction in regulating PN migration. This latter result further supports the idea that the abnormal neuronal migration observed in Hoxa2 knockout mice may be at least partly due to the simultaneous reduction of both Robo2 and Slit2 levels (Figure 4), further underscoring the intrinsic and extrinsic requirements of Hoxa2 in regulating rostral PN migration.

In summary, Robo1–2 and Slit1–2 molecules control in a redundant manner the horizontal, rostrally oriented migration of PN (phase 2), similar to Hox PG2 genes (summary in Figure 5N). However, as Slits are expressed in the floor plate throughout the AP extent of the hindbrain, the lack of expression at this location was unlikely to explain the rostrocaudal specificity of the migration defects.

The Facial Motor Nucleus Prevents Premature Ventral Pontine Neuron Migration

The FMN, which is located in ventrolateral r6, expresses high levels of Slit2, and to a lesser extent Slit3 (Figures 4C and 4D). Double immunostaining for Islet1 and Robo3 (Figure 4V) further revealed that the rostral turn between phase 1 and 2 of PN migration coincides with the AP level of the FMN, and that PN initiate their final movement toward the floor plate (phase 3) only after they have migrated over the FMN (Figure 4U–4W). These observations, together with the results of the Slit knockouts and the reduced expression of Slit2 in the FMN of Hoxa2-deficient mice, strongly suggested that the FMN may play a major role in maintaining normal horizontal migration of PN through expression of Slits.

To further support the potential involvement of the FMN in PN horizontal migration, we analyzed a mouse mutant devoid of cranial branchiomotor nuclei. The paired homeodomain-containing Phox2b gene is required for the generation of all branchiomotor neurons [40]. Phox2b inactivation resulted in the lack of all cranial branchiomotor nuclei, including the absence of the FMN as confirmed by the lack of Islet1 and Slit2 staining on whole-mount E14.5 Phox2b\(^{-/-}\) specimen (Figure 5L; [40]; and unpublished data). In contrast, Slit2 was normally expressed in the rhombic lip and floor plate of Phox2b\(^{-/-}\) mutants (Figure 5L; and unpublished data). In E14.5 Phox2b\(^{-/-}\) embryos, cohorts of PN prematurely migrated ventrally and generated small supernumerary nuclei in ectopic posterior locations (Figures 5G, 5H, and S2H; n = 4) as in Hox PG2, Robo1\(^{-/-}\);Robo2\(^{-/-}\), Slit1\(^{-/-}\);Slit2\(^{-/-}\), and Robo2\(^{-/-}\);Slit2\(^{-/-}\) mutant mice (compare Figures 2 and 5).

Overall, these results strongly indicated that the FMN is an
important extrinsic source of Slit signaling for rostrally migrating PN to prevent their premature ventral migration (summary model in Figure 5O).

**Robo2 Is a Direct Target of Hoxa2**

In *Drosophila* embryo, *Robo2* expression in the mesoderm is likely to be controlled by Hox cofactor genes such as *homothorax* [41]. In addition, a putative Hox binding site has been described in the *Drosophila Robo2* locus, although evidence of direct regulation is lacking [41]. To investigate whether Hox PG2 factors may directly regulate some aspects of the Slit-Robo signaling system in the mouse, we tested the ability of Hoxa2 to bind the *Robo2* regulatory genomic region in vivo. As Hox proteins preferentially bind their target genes through heterodimerization with Pbx cofactors (e.g., [42,43]), we screened in silico about 500 kb of genomic sequence containing the entire *Robo2* locus for the presence of potential NGATNATNN Pbx/Hox consensus binding sites (Figure 6E; [44–47]). We only considered potential sites that were embedded within 150–500 base pair–long DNA stretches, displaying more than 90% nucleotide conservation at the nucleotide level in mammals and other vertebrates (Figure 6B; and unpublished data). By applying such constraints, we selected four putative Pbx/Hox binding sites (Figure 6E; [44–47]).
located within 10 kb upstream of the Robo2 transcription start site, as well as within the first and second introns (Figure 6A and 6B; and unpublished data).

To test the potential of Hoxa2 to bind Robo2 in vivo, we performed chromatin immunoprecipitation (ChIP) analysis [48] on the selected sites from P19 teratocarcinoma cells, a suitable cell culture system to study Hox-regulated targets (e.g., [43]). Indeed, P19 cells expressed significant levels of Robo2 and Hoxa2, as detected by reverse transcriptase PCR (RT-PCR) (Figure 6C and 6D). To perform ChIP on putative Hoxa2 binding sites, we generated a specific polyclonal antibody raised against a unique peptide of the Hoxa2 protein.
protein (see Materials and Methods). Nonquantitative PCR amplification of DNA fragments containing the four putative binding sites was carried out on anti-Hoxa2 immunoprecipitated chromatin. As shown in Figure 6F, the immunoprecipitated chromatin showed a substantial enrichment selective for the sequence, including the site located in the second intron. No enrichment was detected for the remaining sites, as well as for the control Gapdh gene (Figure 6H; and unpublished data). To further support these data, we carried out real-time quantitative PCR (qPCR) assays on immunoprecipitated chromatin with the anti-Hoxa2 antibody. A strong enrichment of the fragment containing the putative Pbx/Hox binding site in the second intron was confirmed, as compared to controls (Figure 6G).

Altogether, these results demonstrated that Hoxa2 can directly bind Robo2 genomic sequences in vivo and, together with the results in knockout animals, strongly suggested that Hoxa2 may directly regulate sustained Robo2 expression during PN migration.

**Discussion**

**Hox Gene–Dependent Control of Pontine Neuron Migration along the Anteroposterior Axis**

The LRN, ECN, RTN, and PGN constitute the major sources of cerebellar mossy fibers [3]. These nuclei originate from the same stripe of rhombic lip neuroepithelium in the posterior hindbrain, and their generation periods partially overlap [4,10,49,50]. Neurons of these nuclei also express a similar set of transcription factors before leaving the rhombic lip, including Pax6 and Math1 [51], and during their migration and settling, such as Pax6 and Barhl1 [21,32]. Expression of such transcription factors may provide cells with information about their specification as mossy fiber precerebellar neurons and/or to acquire a general migratory behavior upon exiting the rhombic lip. Accordingly, precerebellar neurons migrate abnormally in Pax6 and Barhl1 knockout mice [21,32]. Yet, neurons contributing to distinct nuclei migrate following specific pathways and settle at stereotypic AP and DV positional coordinates in the brain stem. Thus, other sets of transcription factors must regulate the responsiveness of migrating precerebellar neurons to environmental guidance cues and drive their distinct migratory routes.

Hox genes are prime candidates to regulate the directionality of cell migration along the AP axis, although so far little evidence has been available in the mammalian central nervous system. We found that PN contributing to RTN and PGN expressed Hox PG2–5 genes throughout their AP migration and settling, thus expressing a code characteristic of their axial origin posterior to r5. Segmental specification and the Hox expression program of precerebellar neurons may select which migratory direction to take upon leaving the rhombic lip. In Caenorhabditis elegans, the rostral or caudal migratory choices of the QR or QL neuroblasts are regulated by the expression of the lin-39 and mab-5 Hox genes, respectively [52]. In the chick embryo, overexpression of Hoxa2 in the r1 rhombic lip, normally devoid of Hox expression, induced neuronal derivatives to migrate ventrally instead of rostrally, thereby adopting a migratory route reminiscent of more-posterior rhombic lip derivatives [53]. Here, we show that mouse PG2 Hox genes are involved in the maintenance of the rostral migration of PN by preventing premature ventral migration and settling of PN at posterior locations. Similarly, specific Hox programs might control the directionality of migration of other precerebellar nuclei along the AP axis. Analysis of the specific Hox expression codes of all precerebellar nuclei will be required to support such an hypothesis.

An interesting finding is the variability in the penetrance and/or severity of the migratory phenotypes in Hoxa2 or Hoxb2 mutants. In Hoxa2 mutants, the fraction of PN displaying migration errors varied both in spatial distribution and number among individuals, whereas the bulk of PN followed a normal rostral migration pathway (Figure 2). We also often observed asymmetric phenotypes between the two sides of the same brain stem. Furthermore, in Hoxb2 mutants, only one third of the specimen displayed an abnormal phenotype. The lack of specific molecular markers did not allow us to distinguish between reticulotegmental or pontine gray neuron identities within the ectopic PN subpopulation. Nonetheless, overall, our results strongly suggested that the ectopic neurons belonged to a random subpopulation due to insufficient redundant functional gene effects. On the one hand, such differences in phenotypes indicate locally random variations of threshold levels of guidance cues and/or of responses of PN in mutants. On the other hand, the limited extent of the abnormal migratory phenotype indicates that such a molecular guidance system is quite robust and buffered against a certain degree of variation, such as loss of function of one or two Hox genes. In fact, even in double PG2 Hox mutants, many PN still migrated normally (Figure 2).

Thus, the loss of PG2 Hox function may be stochastically compensated by other members of the Hox family during PN migration through rhombomeric domains. In a strict interpretation of the “posterior prevalence” model of Hox function [54], only the most “posterior” (i.e., 5’ located in the cluster) PG Hox gene expressed, i.e., PG5, would be expected to select the migratory behavior of PN. The results of PG2 Hox gene inactivations indicated that such a strict model is unlikely to be operating in migrating PN. Rather, a partially quantitative aspect may be added in which local guidance responses of PN may also rely on overall Hox protein distribution and/or levels. Alternatively, a “dynamic” posterior prevalence model might be at work in which the preponderant role of PG5 to PG2 may be sequentially switched while PN are progressing rostrally across interrhombomeric domains. The program of Hox gene expression in migrating neurons may continuously integrate extrinsic segment-specific cues with intrinsic regulation of relevant target gene expression, instructing guidance information to PN about the progression of their positional coordinates along the AP axis. Discriminating between such possibilities will need to await the analysis of PN migration in single and compound knockouts for PG3–5 Hox genes.

**Autonomous and Nonautonomous Requirements of PG2 Hox Genes to Regulate Responsiveness of Migrating Pontine Neurons to Guidance Cues**

We show that the integrity of the Hox expression program of PN during migration is required to regulate their responsiveness to guidance cues and that Hox PG2 genes are important components of such a molecular guidance system. Specifically, the analysis of Hoxa2 knockout animals supported that Hoxa2 is required both intrinsically in PN and extrinsically to define
a local environment permissive to their rostral migration. This assumption is based on the following observations: (1) *Hoxa2* is expressed throughout migration and settling of PN (Figure 1); (2) in *Hoxa2* mutants, early steps in migration appear unaffected; this suggests that migration itself, rather than an early event such as the generation of precursors fated to form the pontine nucleus, is being affected; (3) the inactivation of *Hoxa2* in *Wnt1*+ precerebellar precursors resulted in migration errors of PN (Figure 3), although at a lower penetrance and severity than in the null mutants, suggesting that *Hoxa2* may be additionally required to pattern the environment through which PN migrate; and (4) in *Hoxa2* mutants, the expression of Robo2 in migrating PN and Slit2 in FMN are down-regulated, and compound Robo2+/−;Slit2+/− mutants showed that normal expression levels of such molecules are required for PN guidance (Figures 4 and 5).

Thus, the PN migratory phenotype observed in *Hoxa2*+/− mutants is likely to result from an impairment of *Hoxa2* function both in migrating neurons and in the local environment through which PN migrate.

**Maintenance of Pontine Neuron Migration along the Anteroposterior Pathway Requires Slit-Robo Signaling**

Little is known about how signaling mediated by distinct guidance molecules distributed along the DV and AP axes is integrated in PN during their tangential migration. Our data suggest that specific molecules control PN migration behavior at precisely defined choice points and that Slit/Robo signaling plays a key role in this process.

Although numerous studies have involved Slit-Robo ligand–receptor interaction in axon guidance and branching [2,39,55–61], much less is known about their involvement in neuronal migration, in particular in vivo (e.g., [59,62–64]). For instance, it was previously shown in mice that the Robo3 receptor is required for the last phase of ventral PN migration (Figure S2; [16]). In mice lacking Robo3, PN still leave the rhombic lip and migrate rostrally, but are unable to turn towards the ventral midline, despite normal expression of Netrin-1 and Dcc (Figure S2; [16]; and unpublished data). Robo3 was proposed to function as a negative regulator of Slit responsiveness, somehow repressing Slit repulsive activity from the floor plate and thus interfering with Robo1/Robo2 receptor activation [65]. Robo3 is coexpressed with Robo1 and Robo2 during PN migration until they reach the floor plate (Figures 4B and S1), when Robo3 expression is down-regulated. Thus, one possibility is that in Robo3-deficient mice, Robo1/Robo2 repulsive activity would be activated too early, unmasking Slit repulsive activity from the floor plate and forcing PN to remain in the dorsal hindbrain. According to this model, midline-derived Slit would be the main repulsive source for PN neurons (but see below).

In addition to the floor plate source, Slits are expressed at the rhombic lip. Although migrants from the rhombic lip have been shown to be repelled by exogenous sources of Slit2 in coculture [66], our present data indicated that Slit-mediated repulsion may not control the phase 1 of PN migration from the rhombic lip, as PN still leave the rhombic lip in compound Slit1;Slit2−/−, Robo1+/−;Robo2−/−, and Robo2+/−;Slit2−/− deficient mice. However, Slit3 might still compensate for the loss of Slit1/Slit2 expression at the rhombic lip. Instead, our analysis revealed a major role for Slit1/Slit2 and Robo1/Robo2 during the phase 2 of PN migration along the AP axis and identified the FMN as another important source of Slits for migrating PN, in addition to the floor plate (see also below). Impaired Slit/Robo signaling resulted in strands of PN migrating out from the stream along the DV pathway in ectopic posterior positions above the FMN (Figure 5). Thus, Slit2-mediated signaling from the FMN and Slit1/Slit2 from the floor plate are among the main driving forces that prevent PN from reaching the ventral midline upon leaving the rhombic lip, forcing them to migrate rostrally towards r3. The fact that in compound Slit1;Slit2 and Robo1;Robo2 mutants, the leading process of PN still cross the floor plate and then project into the cerebellum (unpublished data) strongly suggests that in this system, Slit/Robo signaling primarily controls cell migration and not axon guidance.

The DV PN migration requires floor plate-derived Netrin-1 and its receptor Dcc [14,15]. However, even in the absence of Netrin-1 or Dcc, PN manage to undertake phase 1 and phase 2 of migration, leaving the rhombic lip and navigating rostrally before aggregating in an ectopic dorsal position [15]. Thus, attraction by Netrin-1 towards the midline appears to be essential only during phase 3 of migration, once PN turn ventrally towards the floor plate. As a corollary, PN must be partially insensitive to the Netrin-1/Dcc attraction before reaching ventral r4, despite their continued expression of Dcc (Figure 4A) and the presence of Netrin-1 all along the floor plate [15].

Our results show that in the absence of Slit1−2- or Robo1−2-mediated signaling, many PN migrate prematurely towards the midline. This suggests that Slit repulsive activity may counterbalance and prevail over Netrin-1/Dcc-mediated attraction. Slit-Robo signaling may negatively regulate the responsiveness of PN to Netrin-1/Dcc through several possible mechanism(s). For instance, the activation of Robo1/Robo2 receptors in migrating PN by secreted Slit ligands might lead to dimerization of the intracellular domains of Robo and Dcc, resulting in a partial silencing of Netrin-1 attraction on PN, as described in *Xenopus* spinal axons [38]. Thus, in such a scenario, the activity of Slit-activated Robo receptors would be required to inhibit Dcc activity in target neurons. However, Slit/Robo and Netrin-1/Dcc guidance systems could also be acting independently, and PN migratory behavior could result from a balanced integration of attractive and repulsive responses within target neurons. Such possibilities are not mutually exclusive and remain speculative at this point.

It is noteworthy that single Robo1, Robo2, Slit1, or Slit2 homozygous mutant mice did not show significant PN migration defects (Figure S2). This argues for redundant functional roles among Robo receptors or Slit ligands. However, the PN migratory defects in compound Robo1;Robo2 mutants phenocopied those of compound Slit1;Slit2 mutant mice, showing that both Robos and Slits are required for PN migration. This strongly favors a direct Slit/Robo interaction in migrating PN. Strong support for this idea also came from the analysis of compound Robo2+/−;Slit2+/− heterozygotes in which the deletion of only one dose of Robo2 or Slit2 was sufficient to induce migration defects similar to those observed in double Robo1−2- or Slit1−2-deficient mice, demonstrating Slit/Robo dose-dependent interactions. Moreover, from the results of Slit1;Slit2 compound mutants and the analysis of *Phox2b* knockout mice lacking the FMN (Figure 5), it can be inferred that the Slit2 source diffusing from the
FMN is necessary to maintain the normal rostral migration of PN neurons (models in Figures 5O and 7A). At r3 level, PN neurons may turn ventrally because Slits diffusing from the FMN in ventral r6 may become limiting and fall below a threshold level. Notably, it is also at this axial level that Robo3 function becomes preponderant and interferes with Slit repulsive activity.

Finally, our results indicate that neither Robos nor Slits are essential for the anterior progression towards r3 of PN, but rather that they prevent migrating neurons from entering the wrong territories. Additional signals must be involved in attracting PN anteriorly and/or repelling them from the posterior brain stem. Attractive signals from trigeminal branchiomotor neurons (MN) can be ruled out because PN still migrate in Phox2b knockout animals, which lack all branchiomotor nuclei, including trigeminal MN (Figure 5G and 5H). Another possibility is that PN might use the adjacent trigeminal nerve tract as a migration substrate to orientate themselves. Neurons from the posterior migratory stream have been shown to adopt such an axonophilic migration [67]. Chemoattraction of PN could also be provided by the meninges that overlay the migrating stream. Signalizing from the meninges has been implicated in the tangential migration of cortical hem-derived Cajal-Retzius cells in the cerebral cortex. The meninges secrete the chemokine CXCL12 and enhance the migration of CXCR4 expressing Cajal-Retzius cells [68]. Interestingly, migrating PN express CXCR4, and their migration is disrupted in CXCR4-deficient animals [69]. Moreover, CXCR4/CXCL12 signaling can be modulated by Slit upon Robo binding to CXCR4 [70]. Lastly, the meninges over the migrating PN have been shown to be a localized source of retinoic acid, and treatment of the fetus with exogenous sources of retinoic acid has been shown to result in migratory abnormalities of precerebellar neurons [71].

The Role of the Facial Motor Nucleus in Pontine Neuron Migration

Our data indicate a novel role for the FMN in maintaining the rostral migration of PN. In mammals, facial motor neurons migrate tangentially from the ventricular region of r4 across r5 to colonize r6, where they undergo a radial migration to finally condense into the FMN, next to the pial side in ventral r6 [72–74]. Such a stereotyped migration takes place between E10.0 and E14.0, at which stage most of the facial motor neurons have reached their final destination in ventral r6 [73]. The FMN is an important signaling source required to maintain the caudorostral PN migratory pathway (red bar), partly through Slit2–3 expression.

In Hox PG2 mutant mice, Netrin-1 and Slit1–3 expression at the FP level as well as Dcc expression within migrating PN are not affected. However, Hoxa2 is required to maintain sustained Robo2 expression within migrating PN, and normal Slit2–3 expression within the FMN (dashed circle). Insufficient Slit-Robo signaling in turn led to the inability of some PN to maintain their normal rostral pathway, resulting in ectopic neurons prematurely migrating ventrally at posterior locations. Such behavior of migrating PN is phenocopied in compound Robo1;Robo2-, Slit1;Slit2-, Robo2;Slit2-deficient mice as well as in FMN-deficient Phox2b mutant mice.

doi:10.1371/journal.pbio.0060142.g007
signaling from the FMN. Specifically, by the time most of the facial motor neurons have reached their final location in ventral r6, i.e., around E14.0, the amount of Slit secreted from the FMN could become quantitatively sufficient to antagonize the Netrin-1/Dcc-mediated attraction. This may prevent further ventral progression of neurons along the ECN/LRN pathway and allow later-born neurons, at least those closer to the source, to maintain a rostral PN pathway. An additional role might be played by the Slit-expressing facial motor axons exiting the hindbrain at the level of r4. Indeed, the PN stream has to navigate in a “corridor” delimited dorsally by the VIIIth and ventrally by the VIIth nerve roots (Figure 1; [25]). Thus, decrease of Slit-mediated repulsion at the level of the VIIth nerve root might also contribute to the PN navigation errors observed in the various mutants.

Notably, the migratory behavior of the FMN varies in different vertebrate species due to specific signaling cues in r5 and r6 (e.g., [74]). Sharks, lizards, or salamanders have similar organization and location of FMN to mammals, whereas in zebrafish, facial motor neurons migrate into r6 and r7 [75]. Chick embryos are peculiar in that facial motor neurons migrate dorsally within r4, similarly to trigeminal branchiomotor neurons in r2. Interestingly, rhombomere contributions and migratory pathways of pontine nuclei also vary among vertebrates (e.g., [8]). Thus, one possibility is that the distinct migratory behavior of the FMN in different vertebrates may in turn contribute to explain distinct species-specific migratory routes of PN.

Finally, based on our results, it is likely that structure(s) other than the FMN are involved in signaling to PN. Indeed, in Phox2b mutants, the migration errors induced by the lack of FMN are only partial, whereas the bulk of PN follow their normal pathway of migration (Figure 5G and 5H), thus indicating the influence of additional structure(s). Also, in compound Slt1/Slt2 mutant mice, the expression of Slt3 in FMN is not sufficient to rescue the absence of Slt2, also resulting in a partially penetrant migration phenotype similar to the Phox2b mutant phenotype (Figure 5I and 5J). Hence, whereas our analysis does not allow us to determine the exact location of the Slt1 source, a combinatorial activity of Slt molecules from the FMN and other sources such as the floor plate appears necessary to maintain the rostral migration of PN.

Hoxa2 Controls Pontine Neuron Migration through Direct Regulation of Robo2

To date, only a handful of direct targets of Hox genes have been identified in vertebrates [42,43]. In particular, despite the increasing evidence for Hox gene expression during late phases of mammalian nervous system development (e.g., [20]), the nature of Hox direct targets remains largely elusive. Thus, it is unclear how Hox genes may contribute to the molecular regulation of complex aspects of neural circuit assembly, such as neuronal migration and/or topographic axon pathfinding.

In the case of Hox PG2 genes, we show that their main role is to maintain the migration of the PN along a defined AP pathway. Interestingly, this is achieved not by direct negative regulation of Netrin-1/Dcc, but via the local modulation of Slit-Robo signaling levels while PN migrate through distinct rhombomeric territories. Another important finding is that the regulation of genes encoding for transcription factors involved in general aspects of PN differentiation or migration such as Barhl1 or Pax6 was not affected by the lack of Hox PG2 genes, indicating that regulation of PN directional migration by Hox genes act in parallel with or downstream from such transcription factor activities (Figure 2; and unpublished data).

As for Hoxa2, our ChIP assays in P19 cells and in situ hybridization data on mutant fetuses strongly support direct regulation of Robo2 expression levels in migrating PN, whereas regulation of Slit2 expression in the FMN appears to be indirect. In fact, at the time when Slit2 down-regulation is observed, Hoxa2 is not expressed in facial branchiomotor neurons (unpublished data). Generation, migration, and initial differentiation of facial branchiomotor neurons appear to take place normally in Hoxa2 mutants (unpublished data), although an early patterning defect of r4- and r5-derived motor neurons cannot be formally ruled out. Down-regulation of Slit2 in the absence of Hoxa2 might be more likely due to abnormalities in r4 neural crest-derived glia resulting in a late impairment of FMN maintenance and connectivity. Indeed, lack of Hoxb1 in r4 neural crest-derived glia resulted in late FMN defects similar to in Hoxa2 knockout animals [76]. In addition, in Hoxa2+/− mutants, the identity of branchial arch-derived muscles targeted by the facial nerve is altered, potentially resulting in a late degeneration of the FMN [30].

Conclusions

In conclusion, we provide for the first time, to the best of our knowledge, evidence for the implication of Hox PG2 genes in tangential migration of PN and identify the guidance receptor Robo2 as a direct target gene of Hoxa2. We further show that Slit-Robo signaling involving sources of Slit2 from the facial branchiomotor nucleus and Robo2 expression in PN are key components of the molecular guidance system maintaining PN rostral migration. Our data provide a conceptual framework to understand how the PN response to multiple guidance cues along the AP and DV axes is regulated at the transcriptional level and in turn translated into coherent neuronal migratory behavior (see model in Figure 7).

Materials and Methods

Mouse line generation and genotyping. Slit- and Robo-deficient mice were previously described and genotyped by PCR [39,56,60]. Hoxa2 null and Hoxa2lox/lox lines were previously described and genotyped by PCR [30,33]. The day of the vaginal plug was counted as E0.5. All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture.

Rescue of Phox2b−/−embryo lethality. Phox2b−/− homozygous mutant embryos die at E10.5–E13.5 [40]. However, treatment with noradrenergic agonists allows the rescue of embryo lethality through later stages. To obtain Phox2b−/− fetuses, drinking water of pregnant Phox2b−/− females was supplemented with 1 mg/ml of t-phenylephrine, 1 mg/ml of isoproterenol, and 2 mg/ml of ascorbic acid, from E8.5 onwards. Mutant fetuses were genotyped as described in [40].

Binding with LRR2-Slit2-AP. To generate the human Leucine Rich Repeat (LRR) Slit2-alkaline phosphatase fusion protein (LRR2-Slit2-AP), the second LRR of Slit2 (amino acids 341–505) was amplified by PCR and cloned between the XhoI and XbaI sites of AP-Tag5 vector (Genhunter). Binding was performed as described in [39].

Immunohistochemistry. After dissection, brains were fixed by immersion in 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4) (PFA). Brains were blocked in 0.2% gelatin in PBS containing 0.2% Triton-X100 and incubated overnight at room temperature with goat anti-Robo1 (R&D Systems), goat anti-human DCC (Santa Cruz Biotechnology), goat anti-human ROBO3 (R&D Systems), and rabbit anti-Islet 1 (Abcam), followed by species-specific biotin-
coupled secondary antibodies (Jackson Laboratories) or fluoro- 
colour-coded secondary antibodies donkey anti-goat CY3 (Jackson 
Laboratories) and donkey anti-goat A488 (Invitrogen). Detection was 
performed using Vectastain Elite ABC Kit following manufacturer 
instructions.

**In situ hybridization.** In situ hybridization was performed as 
described in [16]. Briefly, brains were dissected, fixed in 4% 
paraformaldehyde (PFA) overnight, cryoprotected in 20% sucrose, 
and then embedded in Shandon Cryomatrix (Thermo Electron 
Corporation) before freezing at −80 °C. The 20-μm cryostat sections 
were cut in a coronal plane. For whole-mount staining, brains 
were fixed in 4% PFA overnight, dehydrated, and then stored at −20 °C 
in 100% methanol. For double in situ hybridization analyses, antisense 
riboprobes labeled with fluorescein-11-d-UTP were additionally used.

Whole-mount hindbrains were processed as for single in situ 
hybridization [16], but the two antisense riboprobes labeled with 
digoxigenin or fluorescein-11-d-UTP (Sigma) or fluorescein-11-d-UTP 
(Invitrogen) were mixed (200 ng/ml) in the hybridization buffer. The Dig-UTP 
probes were recognized by an anti-DIG antibody conjugated to alkaline phosphate, 
which was detected first by NBT-BCIP reaction. Next, hindbrains were 
rinsed in a solution of Tris (pH 7.6) 100 mM, NaCl 150 mM, Tween 0.1%, 
and stored at −20 °C in 100% methanol. The following probes were used:

Pax6

\[ \text{Pax6 (forward)} = 5'-\text{AAAGGCAGTTCCGGAACTCT-3'} \]

Pax6

\[ \text{Pax6 (reverse)} = 5'-\text{CTATGGGTTTTGCTTTATCTGTCCC-3'} \]

\[ \text{Hoxa2} \]

\[ \text{Hoxa2 (forward)} = 5'-\text{GATATACGC-3'} \]

\[ \text{Hoxa2 (reverse)} = 5'-\text{CTGTCGGCCCTTGGACTAGTAGCCTG-3'} \]

\[ \text{RTN} \]

\[ \text{RTN (forward)} = 5'-\text{GGCCTACACCTGACACGT-3'} \]

\[ \text{RTN (reverse)} = 5'-\text{TGCACCGATCACTCTGCTG-3'} \]

\[ \text{PGN} \]

\[ \text{PGN (forward)} = 5'-\text{TGCACCGATCACTCTGCTG-3'} \]

\[ \text{PGN (reverse)} = 5'-\text{TGTGTTATGAGTCCTCAGATG-3'} \]

\[ \text{Barhl1} \]

\[ \text{Barhl1 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Barhl1 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Barhl2} \]

\[ \text{Barhl2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Barhl2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Robo2} \]

\[ \text{Robo2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Robo2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit1–2} \]

\[ \text{Slit1–2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit1–2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Dcc} \]

\[ \text{Dcc (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Dcc (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Hoxa2} \]

\[ \text{Hoxa2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Hoxa2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Hoxa2} \]

\[ \text{Hoxa2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Hoxa2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Robo2} \]

\[ \text{Robo2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Robo2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit1} \]

\[ \text{Slit1 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit1 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit2} \]

\[ \text{Slit2 (forward)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

\[ \text{Slit2 (reverse)} = 5'-\text{TGATAAGTTGACCAGT-3'} \]

**Supporting Information**

**Figure S1.** Expression Patterns of Robo1, Robo2, and Slit3

(A) Lateral view of a E14.5 wild-type (WT) whole-mount brain, 
immunostained with anti-Robo1 antibody. The black arrow shows 
the presence of Robo1 in migrating PN.

(B) Ventral view of a E15.5 WT whole-mount brain hybridized with 
a Robo2 probe. Robo2 is expressed within PN throughout their 
migration and maintained within the nascent PGN (red arrows).

(C) LRR2-hSlit2-AP binding on a E14.5 WT whole-mount brain 
lateral view. The arrow indicates the presence of Slit2 receptors 
in the basal lamina.

(D) Expression of Slit3 in the FM (white arrow) of a E14.5 whole-

**Figure S2.** Pontine Nuclei Development in Single and Compound 
Robo1–2, Slit3–1, and Phox2B Mutant Mice

(A–H) Ventral views of whole-mount brains at the level of pontine 
nuclei (PN) of E17.5 wild-type (WT) (A), Slit1–2 (B), Slit2–3 (C), Robo1–2–3 (D), Robo2–3 (E), Robo4–5 (F), Slit1–2 (G), and Phox2B–/– (H), hybridized with a Barhl1 probe. No abnormalities are observed in single 
Slit1–2 or Robo1–2 mutants (B–E), whereas ectopic Barhl1 cells 
present posterior to PN are similarly observed near the ventral midline 
of compound Robo1,Robo2 (F) and Slit1 Slit2 (G) mutant brains (arrows).

**Figure S3.** Ectopic Migration of Subsets of PN in 
Hoxa2 Mutant Mice

(A–J) Analysis of PN migration defects by in situ hybridization of 
Hoxa2 at E13.0 (A and B, and E and F), Hoxa2 at E14.0 (C and D, and G and H), and Tag1 at E17.5 (I and J). 
Lateral (E–J) and ventral (A–D) views of Hoxa2–/– (A, E, I, C, and G) and 
Hoxa2+/– (B, F, J, D, and H) whole-mount brains. Note that 
etocopically migrating streams of PN are observed at all stages (arrows 
in [B, F, D, H, and J]) suggesting a random affection of PN migration 
induced by the loss of Hoxa2. (K and L) Coronal cryostat sections of 
E17.5 brains hybridized with Barhl1. The section level is indicated 
by the red box in the left diagram. The picture highlighted the region 
depicted by the red box in the left diagram. The dashed lines show 
approximately the limits of the RTN and PGN, respectively. Note 
that a slight decrease of both the RTN and the PGN is observed in 
Hoxa2–/– brains. Arrows show the PGN and RTN.

**Acknowledgments**

We wish to thank M. Wassef and R. Wingate for critical reading of the manuscript. We would like to acknowledge the following colleagues for the generous gift of plasmids or mouse lines: M. Tessier-Lavigne (Robo and Slit knockouts and Dcc cDNA), P. Charnay (Krox20:Cre 

**Found at doi:10.1371/journal.pbio.0060142.sg003 (5.1 MB JPG).**

**Found at doi:10.1371/journal.pbio.0060142.sg002 (4 MB JPG).**

**Found at doi:10.1371/journal.pbio.0060142.sg001 (3.2 MB JPG).**

**Found at doi:10.1371/journal.pbio.0060142.sg000 (4 MB JPG).**

**Found at doi:10.1371/journal.pbio.0060142.sg003 (3.1 MB JPG).**
and J-FB contributed reagents/materials/analysis tools. FMR wrote the designed the experiments. MJG, TDM, MP, and SD performed the MJG, TDM, MP, SD, and AC, and FMR analyzed the data. MP and J-FB contributed reagents/materials/analysis tools. FMR wrote the paper.

Funding. MJG was supported by La Ligue Nationale Contre Le Cancer and Association pour la Recherche sur le Cancer (ARC), and the Luxembourg Ministère de la Culture, de l'Enseignement Supérieur et de la Recherche. AC was supported by grants from the Association pour la Recherche sur le Cancer (ARC) and the Fondation pour la Recherche Médicale (programme EFRM). Work in FMR laboratory was supported by the Agence Nationale pour la Recherche (ANR), the Fondation pour la Recherche Médicale (Equipe FRM), the Association pour la Recherche contre le Cancer (ARC), the Association Française contre les Myopathies (AFM), the Ministère pour la Recherche (ACI program), and by institutional funds from CNRS and INSERM.

Competing interests. The authors have declared that no competing interests exist.

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