Control of Axon Guidance and Neurotransmitter Phenotype of dB1 Hindbrain Interneurons by Lim-HD Code

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Hindbrain dorsal interneurons (HDIs) are implicated in receiving, processing, integrating, and transmitting sensory inputs from the periphery and spinal cord, including the vestibular, auditory, and proprioceptive systems. During development, multiple molecularly defined HDI types are set in columns along the dorsoventral axis, before migrating along well-defined trajectories to generate various brainstem nuclei. Major brainstem functions rely on the precise assembly of different interneuron groups and higher brain domains into common circuitries. Yet, knowledge regarding interneuron axonal patterns, synaptic targets, and the transcriptional control that govern their connectivity is sparse. The dB1 class of HDIs is formed in a distinct dorsomedial position along the hindbrain and gives rise to the inferior olive nuclei, dorsal cochlear nuclei, and vestibular nuclei. dB1 interneurons express various transcription factors (TFs): the pancreatic transcription factor 1a (Ptf1a), the homeobox TF-Lbx1 and the Lim-homeodomain (Lim-HD), and TF Lhx1 and Lhx5. To decipher the axonal and synaptic connectivity of dB1 cells, we have used advanced enhancer tools combined with conditional expression systems and the PiggyBac-mediated DNA transposition system in avian embryos. Multiple ipsilateral and contralateral axonal projections were identified ascending toward higher brain centers, where they formed synapses in the Purkinje cerebellar layer as well as at discrete midbrain auditory and vestibular centers. Decoding the mechanisms that instruct dB1 circuit formation revealed a fundamental role for Lim-HD proteins in regulating their axonal patterns, synaptic targets, and neurotransmitter choice. Together, this study provides new insights into the assembly and heterogeneity of HDIs connectivity and its establishment through the central action of Lim-HD governed programs.

Key words: axons; hindbrain; interneurons; Lim-HD proteins; neurotransmitter; synapses

Introduction

During early development, the hindbrain is subdivided along the anteroposterior (AP) axis into rhombomeres. Individual rhombomeres give rise to distinct neuronal columns, depending on the position of neural progenitors along the dorsoventral (DV) and AP axes (Lumsden and Krumlauf, 1996; Moens and Prince, 2002). In the dorsal hindbrain, interneurons receive, integrate, process, and transmit sensory inputs from the vestibular, auditory, and proprioceptive systems to higher brain regions (Altman and Bayer, 1977; Wang and Zoghbi, 2001; Maklad et al., 2003;

Ryugo and Parks, 2003; Oertel and Young, 2004; Guthrie, 2007). The development of hindbrain dorsal interneuron (HDIs) occurs in sync with establishment of peripheral circuitries and is accompanied by axonal projections in specific tracts and somal migration to form distinct brainstem nuclei (Bourrat and Sotelo, 1990; Oertel and Young, 2004; Rubel et al., 2004; Landsberg et al., 2005; Farago et al., 2006; Pasqualetti et al., 2007). Knowledge on how the generation of HDIs is registered with the formation of networks with other brain regions is sparse.

HDIs are divided into six subclasses based on their DV positions and molecular profiles (Liu et al., 2008; Storm et al., 2009; Kohl et al., 2012). For instance, the most dorsal group of neurons, termed dA1, expresses the basic helix-loop-helix transcription factor (TF) Atoh1 and the Lim-homeodomain (HD) proteins Lhx2 and Lhx9 (Ben-Arie et al., 1997; Wang et al., 2005), whereas the more ventral dB1 neurons expresses the pancreatic transcription factor 1a (Ptf1a) and Lhx1 and Lhx5 (Glasgow et al., 2005; Hori et al., 2008; Meredith et al., 2009; Storm et al., 2009). Genetic studies revealed the contribution of dB1 inhibitory neurons from rhombomeres 2–6 to the cochlear nuclei (CN), which act together with dA1-excitatory interneurons to transmit auditory information to the inferior colliculi (Farago et al., 2006; Fujiyama et al., 2009). More caudally, dB1 generates the inferior olive nuclei (ION), which projects excitatory inputs through climbing

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fibers to Purkinje cerebellar cells (Yamada et al., 2007; Renier et al., 2010). dB1/Ptf1a⁺ cells were also found in vestibular nuclei (Yamada et al., 2007). Albeit this information, the precise *en route* axonal patterns and synaptic targets of dB1/Ptf1a⁺ neurons is unclear as well as the molecular cues that govern their connectivity.

Using an advanced enhancer-based conditional expression system in the chick (Kohl et al., 2012, 2013; Hadas et al., 2014), combined with a Ptf1a enhancer element (Meredith et al., 2009), in this study we targeted dB1 interneurons to perform spatiotemporal tracing of this molecularly defined HDI population. We found dB1 axons to extend in five specific tracts and to form synapses in the Purkinje cerebellar layer, midbrain vestibular, and auditory nuclei and in the medulla, in correspondence with their cell-body migration to form cochlear, vestibular, and ION hindbrain nuclei centers. Genetic manipulations revealed a fundamental role for the Lim-HD TFs in instructing the axonal patterns, synaptic targets, and neurotransmitter profile of dB1 interneurons.

This study provides new understanding on how HDIs generate brainstem circuitries in the developing embryo and on the fundamental role of the Lim-HD code in these processes.

Materials and Methods

In ovo electroporations. Fertile Loman chick eggs were incubated at 38°C until reaching the desired stages. DNA solution of 5 µg/µl was injected into the lumen of the hindbrain of E2.5 chick embryos (Stage 15 Hamburger Hamilton). Electroporation was performed using a BTX ECM 830 electroporator with four 45 ms pulses of 25 V and pulse intervals of 300 ms. Embryos were incubated for 1–14 d before analysis.

Plasmids. A plasmid containing an insert of 2.3 Ptf1a-enhancer element (Meredith et al., 2009) was cloned upstream to Cre-recombinase and electroporated along with a conditional reporter plasmid, in which a transcriptional STOP cassette was inserted between the CAG enhancer/promoter module and nuclear GFP gene (pCAG-LoxP-STOP-LoxP-nGFP) or membranous GFP gene (pCAG-LoxP-STOP-LoxP-mGFP) (Avraham et al., 2009, 2010a, b; Kohl et al., 2012, 2013). In some cases, an alternating reporter plasmid was used in which a floxed *mCherry* gene was inserted between the CAG enhancer/promoter module and the GFP sequence (pCAG-LoxP-mCherry-LoxP-GFP) (Avraham et al., 2009). For ectopic expression of Lhx2/9 in dB1 or Lhx1 in dA1 neurons, *Lhx2/9/1* genes were inserted between the floxed STOP module and a taumyc reporter (pCAG-LoxP-STOP-LoxP-Lhx2/9/1-IRES-taumyc) (Avraham et al., 2009). To target dA1 and dB1 neurons in the same embryo, we also used the Flp/FRT conditional expression system under the control of Ed11 enhancer and an FRT conditional GFP reporter (Park et al., 2011). For integration of the reporter gene into the chick genome, the PiggyBac (PB) DNA-integration method was applied (Wang et al., 2010), in which a Cre-conditional reporter cassette (mGFP) was cloned between the two PB arms (PB-CAG-LoxP-STOP-LoxP-mGFP-PB) and electroporated along with the Ptf1a enhancer plasmid and the Pbase transposase. For tracing synaptic connections, a reporter plasmid containing SV2-GFP synaptic reporter cassette was cloned between the two PB arms (PB-CAG-LoxP-STOP-LoxP-SV2-GFP-PB) (Kohl et al., 2012; Hadas et al., 2014) and electroporated into the embryonic hindbrain along with the Ptf1a enhancer and the Pbase transposase. To trace synaptic targets in dB1 or dA1 following modification of Lim-HD expression, the Ptf1a enhancer or Ed11 enhancer was cloned upstream to PhiC31 recombinase and electroporated along with a PhiC31-Lhx1/2-conditional Cre cassette (pCAG-AttB-STOP-AttP-Lhx1/2-IRES-Cre), Cre-conditional SV2-GFP cassette, and Pbase transposase. For restricted labeling of ION-derived dB1 interneurons, Pdx1::Cre plasmid (Gu et al., 2002; Song et al., 2010) was electroporated along with the Ptf1a::FLPo recombinase plasmid and a dual-conditional reporter system (PB-CAG-Frt-STOP-Frt-LoxP-Stop-LoxP-n/cGFP-PB) (Hadas et al., 2014). Schematic description of the plasmids is provided in each figure.

Immunofluorescence. Immunofluorescence staining was performed on whole mounts and frozen section as previously described (Kohl et al.,

2012). Briefly, for whole-mount preparations, the c-Myc antibody (1:500, Santa Cruz Biotechnology) was applied. For frozen sections, embryos were incubated in 30% sucrose/PBS for ON, and embedded in Optimal Cutting Temperature solution (Leica Microsystems). Cryostat sections (12 µm) were collected and incubated for ON with the following antibodies: mouse monoclonal Lmx1b, Brn3a, Pax6, Lhx1/5, En-1, Ben, and synaptotagmin (1:100, Developmental Studies Hybridoma Bank), rabbit polyclonal Pax2 (1:100, Abcam), rabbit polyclonal VGlut2 (1:150, Synaptic Systems), rabbit polyclonal GABA (1:100, Sigma-Aldrich), rabbit polyclonal GFP (1:500, Invitrogen), rat polyclonal Olig3 (1:400, provided by H. Takebayashi), rabbit polyclonal Lhx2/9 (1:100, provided by T. Jessell), rabbit polyclonal Axonin-1 (1:500, provided by E. Stoeckli), rabbit polyclonal Calbindin 28KD (1:100, Swant), and rabbit polyclonal Zic1 (1:400, provided by R. Segal). Phalloidin staining was used to visualize F-actin filaments (1:300, Invitrogen). Secondary goat anti-rabbit or anti-mouse Alexa-488 or Alexa-594 antibodies (all 1:400, Invitrogen) were added for 2 h. All embryos were visualized under Nikon E400 microscope with DP70 CCD camera (Olympus).

Cell counts. Quantification of dB1/dA1-GFP⁺ cells was performed by counting the number of Ptf1a/Ed11-GFP-expressing cells, which coexpress a neuronal marker (Pax2, Lhx1/5, GABA, Brn3a, Lhx2/9, Olig3, Lmx1b, VGlut2), of the total GFP⁺ cells. Quantification of dB1/dA1 cells that ectopically express Lhx1/2/9-taumyc was performed by counting green (Alexa-488)/red (Alexa-594) cells coexpressing Lhx1/2/9-taumyc and various neuronal markers, of the total Lhx1/2/9-taumyc-expressing cells. Quantification of SV2-GFP presynapses derived from normal or Lim-code modified dB1/dA1 neurons was performed by counting the number of SV2-GFP-expressing synapses that coexpress cerebellar markers (Zic1, calbindin) of the total SV2-GFP presynapses. Percentages represent one section of seven different sections from three independent embryos demonstrating similar electroporation levels.

Results

Identification of dB1-derived hindbrain neurons and nuclei

Sensory dorsal interneurons in the hindbrain are divided into different types based on their specific DV positions, genetic profile, and neuronal roles (Liu et al., 2008; Storm et al., 2009; Kohl et al., 2012). We have previously developed an enhancer-based molecular approach in the chick CNS that enables targeting of genetically distinct interneurons, thereby providing means for labeling neuronal soma, axons, and presynaptic terminals (Liu et al., 2008; Avraham et al., 2009, 2010b; Storm et al., 2009; Kohl et al., 2012, 2013; Hadas et al., 2013, 2014). Using these tools, we here aimed to decode the axonal projection patterns, somal position, and presynaptic sites of the dB1 class of interneurons in the chick hindbrain.

The transcriptional code of dB1 neuron is similar to that of spinal dI4 neurons (Glasgow et al., 2005; Liu et al., 2008; Storm et al., 2009; Kohl et al., 2012). We have recently used the enhancer of Ptf1a (Meredith et al., 2009) that drives expression of GFP in mice spinal dI4 neurons (Ptf1a::GFP), in the chick hindbrain. Ptf1a::GFP reliably labeled dB1 (Lhx1/5/⁺Pax2⁺) interneurons (Kohl et al., 2012). For studying the wiring of dB1 at late developmental stages, a dB1-constitutive expression system, based on Cre-mediated activation of expression downstream of ubiquitous enhancer/promoter, was used (Fig. 1K). To delineate the identity of the labeled cells, a plasmid mixture that includes Ptf1a::Cre and a conditional nuclear GFP (nGFP) was electroporated into the hindbrain of E2.5 (Stage 15 Hamburger Hamilton) chick embryos. Immunofluorescence was performed at E3.5 (stage Stage 18 Hamburger Hamilton) on hindbrain sections at the levels of rhombomere (r) r4–r5 using different markers to reveal the identity of nGFP⁺ cells. Approximately 90% of nGFP-expressing cells coexpressed Lhx1/5 and Pax2 and were located at a medial domain within the alar plate, ventrally to Olig3-expressing dA1 and dA3 neurons and dorsally to Pax6-expressing motoneurons (Fig. 1A,B,D,F; Table 1). The labeled cells were flanked by Lmx1b⁺/

Figure 1. Labeling dB1 neuronal subgroup using Ptf1a enhancer element. **A–F**, Cross-section views of E3.5 hindbrains at the level of r4–r5 that were electroporated at E2.5 with Ptf1a::Cre plasmid, along with a conditional nGFP plasmid and immunostained with interneuronal markers. Ptf1a enhancer-derived nGFP⁺ neurons express Lhx1/5 (**A**) and Pax2 (**B**) yet are segregated from neurons expressing Lmx1b (**C**), Olig3 (**D**), Brn3a (**E**), and Pax6 (**F**). Higher-magnification views of the boxed areas in **A–F** are represented at the right panel in different channels. Arrowheads indicate the same GFP⁺ cell in all channels. Arrows indicate representative neurons that do not express GFP. **G, H**, Cross-section views of E7 hindbrains at the level of r7 that were electroporated at E2.5 at the level of r7–r8 with the Ptf1a::Cre plasmid along with a conditional nGFP plasmid (**G**) or with Pdx1::Cre and Ptf1a::FLPo plasmids along with a dual-conditional nGFP plasmid (**H**), and immunostained with the ION marker BEN. GFP⁺ neurons are seen in the ventral hindbrain and express BEN. Higher-magnification views of the boxed areas in **G, H** are represented at the right panel in different channels. Arrowheads indicate the same GFP⁺ cell in all channels. **I, J**, Flat-mounted and cross-section views of hindbrains that were electroporated at E2.5 with conditional alternating mCherry/GFP plasmid for testing the electroporation specificity. Ptf1a-derived GFP expression is restricted to the location of dB1 (**I**, boxed area), whereas CAG derived mCherry-expressing neurons are widespread along the entire dorsoventral axis. **K–K''**, Schematic representations of the plasmids used for labeling dB1 neurons. In all images, scale bars, interneuron subgroups, plasmids, and antibodies are indicated. FP, Floor plate; r, rhombomere.

Brn3a⁺ neurons: dorsally dA3 and ventrally dB3 groups (Fig. 1C,E). These results provide support for the validity of using the Ptf1a enhancer element in conjunction with Cre/Lox system for directing expression to dB1 hindbrain interneurons.

To further verify the specificity of the Ptf1a-conditional expression system, we used mCherry/GFP alternating plasmid in conjunction with the Ptf1a::Cre construct to simultaneously detect Ptf1a::Cre conditional GFP⁺-expressing cells and noncondi-

Table 1. Quantification of the results obtained on neuronal markers by expression of the multiple constructs in dB1 cells

Reporter plasmid	Marker	No. of plasmid-expressing cells	No. of marker-expressing cells	Overlapping, no. (%)
a. Ptf1a::Cre-nGFP	Pax2	76	387	69/76 (90.7)
b. Ptf1a::Cre-nGFP	Lhx1/5	71	269	64/71 (90.1)
c. Ptf1a::Cre-nGFP	GABA	65	126	56/65 (86.1)
d. Ptf1a::Cre-nGFP	Brn3a	44	146	0
e. Ptf1a::Cre-nGFP	Olig3	68	189	0
f. Ptf1a::Cre-nGFP	Lmx1b	57	155	0
g. Ptf1a::Cre-nGFP	VGlut2	39	89	0
h. Ptf1a::Cre-nGFP	Ben	50	187	43/50 (86)
i. Ptf1a::Cre-nGFP	Ben	71	201	62/71 (87.3)
j. edl1::Cre-nGFP	GABA	65	123	0
k. edl1::Cre-nGFP	VGlut2	63	193	56/63 (88.8)
l. Lhx2-IRES-nGFP	Lhx2/9	45	93	41/45 (91.1)
m. Lhx2-IRES-nGFP	Olig3	32	80	1/32 (3.1)
n. Lhx2-IRES-nGFP	Pax2	51	116	3/51 (5.8)
o. Lhx2-IRES-nGFP	Lhx1/5	49	108	4/49 (8.1)
p. Lhx2-IRES-nGFP	GABA	79	125	3/79 (3.7)
q. Lhx2-IRES-nGFP	VGlut2	42	89	17/42 (40.4)
r. Lhx9-IRES-nGFP	Lhx2/9	58	99	52/58 (89.6)
s. Lhx9-IRES-nGFP	Olig3	47	121	0
t. Lhx9-IRES-nGFP	Pax2	56	165	4/56 (7.1)
u. Lhx9-IRES-nGFP	Lhx1/5	65	107	3/65 (4.6)
v. Lhx1-IRES-nGFP	VGlut2	58	196	3/58 (5.1)
w. Lhx1-IRES-nGFP	GABA	72	101	6/72 (8.3)

tional mCherry-expressing cells (Fig. 1K') (Avraham et al., 2009; Kohl et al., 2012). A restricted column of GFP expression in dB1 neurons is evidenced in the hindbrain, whereas mCherry is expressed in a broad domain along the hindbrain DV axis (Fig. 1I,J). Together, these data demonstrate the specificity of the Ptf1a::Cre enhancer tool for selected labeling of dB1 interneurons in the chick hindbrain.

Studies in rodents have demonstrated that dB1 neurons give rise to multiple brainstem nuclei (Yamada et al., 2007). For restricting expression of reporter genes to a specific dB1 derivative, the ION, we designed two strategies: topographic and genetic. Topographically, the expression was constrained to IO neurons by directing the Ptf1a reporter/driver plasmids (Fig. 1K) to r7 via the usage of pointed electrodes. For genetic targeting, enhancer-intersection between Ptf1a and Pdx1 enhancers was used. The Pdx1 enhancer was shown previously to label IO and serotonergic neurons in the mice hindbrain (Song et al., 2010). Cre and FLP recombinases under the control of Pdx1 and Ptf1a enhancers, respectively, and a Cre/FLP dual-conditional reporter plasmid (Fig. 1K'') were used. Embryos were analyzed at E7.5 by transverse sections and stained with the ION-specific marker Ben (Chédotal et al., 1996). In both strategies, GFP⁺-dB1 neurons were found at a ventral position adjacent to the midline and coexpressed Ben, as expected from IO neurons (Fig. 1G,H). Together, these results demonstrate the tagging of the ION-specific dB1 lineage as well as confirm the normal somal translocation of genetically labeled dB1 interneurons in our system.

Lineage analysis in the mice hindbrain revealed the contribution of Ptf1a⁺ neurons to the dorsal CN and vestibular nuclei, in addition to the ION (Yamada et al., 2007; Fujiyama et al., 2009). We set to explore whether dB1 neurons reside in similar hindbrain nuclei in the chick. Embryos were electroporated with the Ptf1a::Cre and nGFP reporter and analyzed by sagittal sections at E9.5, when hindbrain nuclei centers can be clearly recognized (Fig. 2D). GFP⁺ neurons were evident within the auditory, vestibular, and precerebellar centers in the medulla. These include

nucleus angularis (NA, the chick homolog of the mammalian dorsal CN; Fig. 2A–A''), nucleus medialis vestibularis (MVE; Fig. 2B–B''), and ION (Fig. 2C–C''). Hence, the evolutionary conservation between birds and rodents in dB1 transcriptional code (Storm et al., 2009; Kohl et al., 2012) is also manifested in the neuronal fate exemplified in the contribution of dB1 interneurons to different hindbrain nuclei.

Axonal pathways and synaptic targets of dB1 interneurons

To delineate the spatial and temporal growth patterns of dB1 axons, E2.5 embryos were electroporated with the Ptf1a::Cre enhancer along with an mGFP at the level of r3–r7 (Fig. 3I). At E5, dB1 axons crossed the floor-plate and turned rostrally adjacent to the midline along the contralateral medial longitudinal funiculus (cMLF) (Fig. 3A,A', arrow). In addition, contralateral axons that extended dorsally away from the cMLF were visible (Fig. 3A,A'', arrowhead). At E6, the dorsally projecting commissural axons converged into a longitudinal bundle that ascended along the contralateral dorsal funiculus (cDF) (Fig. 3B,B'', arrowhead). The cMLF was also clearly apparent (Fig. 3B, arrow), as well as additional ascending ipsilateral axons that projected adjacent to the midline, forming the ipsilateral MLF (iMLF) (Fig. 3B,B', dotted arrow). Notably, GFP⁺ axons in iMLF and cMLF were less fasciculated than the cDF bundle. At E7, two more ascending ipsilateral projections were evident: one emerged from a medial position forming an ipsilateral funiculus (iLF) (Fig. 3C,C', asterisk), whereas the other elongated in a dorsal position forming an ipsilateral funiculus (iDF) (Fig. 3C,C'', asterisk). These results demonstrate five axonal tracts that are projected by dB1 neurons in defined spatial and temporal patterns (Fig. 3E,F). These findings confirm previous classical retrograde/anterograde labeling studies in the hindbrain, which presented axonal projections that originate, for example, from vestibular nuclei into the MLF and from the CN to the DF (Rubel and Parks, 1975; Takahashi, 1988; Marín and Puelles, 1995; Díaz et al., 1998, 2003; Sugihara et al., 1999; Díaz and Glover, 2002; Barmack, 2003; Gottesman-Davis and Peusner, 2010; Krützfeldt et al., 2010). Our observations (Figs. 1, 2, and 3) add to these descriptive studies by linking the genetic identity of these neurons with their multiple hindbrain funiculi and distribution in different nuclei centers.

To label dB1 axons at more advanced stages of embryonic development, the PB transposition system, which facilitates integration into the genome, was applied (Fig. 3I') (Wang et al., 2009, 2010; Kohl et al., 2012; Schecterson et al., 2012). Five ascending ipsilateral and contralateral trajectories were evident at E7.5 (Fig. 3D), similar to those detected with the transient Cre/Lox expression system (Fig. 3C), confirming the suitability of the PB system for specific labeling of dB1 neurons. Notably, additional descending c/iMLF were seen at this stage (Fig. 3D). Such tracts were also detected using the transient plasmid system at similar stages (Fig. 3C; and data not shown), suggesting that later-born dB1 neurons may provide additional descending projections.

Using topographic and genetic approaches, we managed to exclusively tag the ION-derived dB1 lineage (Fig. 1G,H). We next followed their axonal projection by electroporating r7–r8 exclusively with the Ptf1a-mediated PB-mGFP system (Fig. 3I'). Analysis of E7 hindbrains clearly revealed an ascending cDF (Fig. 3G). A similar trajectory was found using Pdx1/Ptf1a enhancer intersection (Fig. 3H,I''). Notably, in both experiments, GFP⁺ cell bodies were located at the ventral hindbrain (Fig. 3G,H), in agreement with our previous finding (Fig. 1G,H). The observed cDF axonal tract is similar to the climbing fiber projection of IO

Figure 2. dB1 somas are located at auditory, vestibular, and ION hindbrain centers. **A–C**, Sagittal section views of brightfield and immunofluorescent E9.5 brainstems that were electroporated at E2.5 with Ptf1a enhancer-derived nGFP plasmid (**E**) to label dB1 neurons. nGFP⁺ neurons are located in the NA (**A–A''**, arrows), MVE (**B–B''**, arrows), and ION (**C–C''**, arrows). High-magnification views of the boxed areas in **A–C'** are represented at the right panel of each image. **D**, Illustration of a brainstem sagittal section representing the AP positions of the nuclei centers demonstrated in **A–C'**. The illustration does not reflect the actual lateral–medial location of each nucleus. **E**, Schematic representations of the plasmids used for labeling dB1 neurons. In all images, scale bars, AP, anterior; P, posterior; Phallo, phalloidin.

neurons (Renier et al., 2010), supporting further our specific tracing of the developing ION-derived dB1 interneurons.

To decipher the axonal connectivity of dB1 interneurons at more advanced stages of development, E2.5 embryos were electroporated using the PB system (Fig. 4*H*) and analyzed at E8.5–E15.5 in sagittal sections. GFP⁺ axons elongated in the medulla and pons at E8.5 and turned toward the cerebellar primordium (Fig. 4*A–A''*). The ION, as well as minor subsets of cochlear and vestibular neurons, were previously demonstrated to project to the cerebellum (Huang et al., 1982; Barmack et al., 1993; Sens and de Almeida, 2007; Shin et al., 2011). Hence, we next addressed whether dB1 interneurons target the cerebellum. Analyzing the

cerebellum at E13.5 and E15.5 revealed GFP⁺ axons reaching the Purkinje layer, as evident by staining with calbindin (Fig. 4*C–C''*), but not the external granular layer (EGL) (Fig. 4*B–B''*, *D, D''*). Usage of the Pdx1/Ptf1a intersection (Fig. 4*H''*) further demonstrated the targeting of GFP⁺ axons to the Purkinje layer (Fig. 4*E–E''*), validating the mapping of IO-derived dB1 axonal projections to the precise cerebellar layer.

An important question raised by these observations is whether the ascending dB1 axons merely extend in the cerebellum or whether they form synaptic connections at the Purkinje layer. E2.5 embryos were electroporated with the conditional Cre-dependent reporter plasmid containing the synaptic reporter cas-

Figure 3. Axonal projections of dB1 neurons at E5–E7.5. **A–D**, Flat-mounted views of hindbrains that were electroporated at E2.5 with Ptf1a enhancer-derived mGFP plasmids (**A–C**) or with the constitutive PB conditional system (**D**). mGFP⁺ axonal projections are shown at E5–E7.5. High-magnification views of each of the axonal tracts are represented (**A'–C'**). **A**, At E5, dB1 axons extend longitudinally at the cMLF and cDF. **B**, At E6, an additional ipsilateral longitudinal fascicle is growing at the iMLF. **C**, At E7, two additional longitudinal tracts are evidenced at the iDF and iLF. **D**, At E7.5, the five tracts that are present at E7 are also labeled using the PB system. **E, F**, A schematic summary of dB1 axonal projections along the hindbrain at E5 and E7. **G, H**, Flat-mounted views of hindbrains that were electroporated at E2.5 at the level of r7–r8 with the Ptf1a-enhancer derived mGFP plasmids (**G**) or with Pdx1::Cre and Ptf1a::FLPo plasmids along with a dual-conditional mGFP plasmid (**H**). An axonal tract extends longitudinally at the cDF. **I–I''**, Schematic representations of the plasmids used for labeling dB1 axons. In all images, white arrows indicate the cMLF, arrowheads indicate the cDF, dashed arrows indicate the iMLF, and asterisks indicate the iDF and iLF.

sette SV2-GFP (Alsina et al., 2001; Leal-Ortiz et al., 2008), along with Ptf1a enhancer and PB vectors (Fig. 4H'), and analyzed at E13.5 and E15.5. This method enables the specific expression of GFP at dB1 presynaptic vesicles, as we previously demonstrated (Kohl et al., 2012; Hadas et al., 2014). Cross sections were stained with the general presynaptic marker synaptotagmin (Gardzinski et al., 2007; Nowack et al., 2010) as well as with cerebellar markers, to allow the detection of dB1-synaptic termini. The expression of the reporter cassette displayed a punctuated and succeeding staining of GFP⁺ particles (Fig. 4F'–G'), which is typical of presynaptic vesicles (Alsina et al., 2001; Leal-Ortiz et al., 2008; Kohl et al., 2012). In many cases, the SV2-GFP⁺ synapses were contained with synaptotagmin (Fig. 4F', G', arrows). Staining with the Purkinje-specific markers calbindin and Lhx1/5 (Baimbridge et al., 1992; Morales and Hatten, 2006) revealed close association between SV2-GFP⁺ synaptic vesicles and calbindin⁺/neurons (Fig. 4F', G'). No SV2-GFP⁺ vesicles were found in other cerebellar layers (data not shown). These results demonstrate that hindbrain-dB1 axons project to and synapse at the Purkinje cerebellar layer.

Previous axonal labeling studies have delineated the ascending circuitry of hindbrain auditory and vestibular nuclei; the CN

project primarily through the DF into the nucleus mesencephalicus lateralis pars dorsalis (MLD, the avian analog of the mammalian inferior colliculi) in the anterior dorsal midbrain, which transmits inputs to the medial geniculate body of the thalamus (Semple and Aitkin, 1980; Oliver, 1984; Winer et al., 1996; Logerot et al., 2011; Schecterson et al., 2012). The vestibular nuclei, through the MLF, project to the Edinger–Westphal nuclei in the midbrain, which is further connected to the ciliary ganglion in the orbit (Akert et al., 1980; Semple and Aitkin, 1980; Oliver, 1984; Sekiya et al., 1984; Wathey, 1988; Balaban, 2003; Kozicz et al., 2011). As we described the localization of dB1-soma in the NA and MVE (Fig. 2) and dB1 axons in the respective DF and MLF (Fig. 3), we next investigated their connectivity with these midbrain auditory and vestibular centers (Fig. 5B). Electroporated embryos were analyzed at E13.5 by costaining with the hindbrain-midbrain boundary marker, En-1 (Wurst et al., 1994), clearly demonstrating GFP⁺ axons that cross the hindbrain-midbrain boundary and extend into the anterior midbrain (Fig. 5A–A'). Assessment of synaptic termini at E15.5 revealed accumulation of GFP⁺ synapses in the anterior midbrain (Fig. 5C–C'), in the Edinger–Westphal nuclei (Fig. 5D–D') MLD nuclei (Fig. 5E–E'), as identified by anatomical landmarks. These find-

ings show the connectivity between hindbrain dB1 interneurons and central auditory and vestibular target sites within the midbrain. In addition, dB1-derived presynapses were also observed in a dorsal position within the medulla (Fig. 5*F–F'*), a domain that consists of hindbrain auditory nuclei (Altman and Bayer, 1980; Marin and Puelles, 1995; Cramer et al., 2000). This finding suggests the existence of local dB1 circuitry within the medulla, in agreement with previous cellular-labeling studies, which showed NA neurons that project locally to the superior olive nuclei of the hindbrain auditory center (Conlee and Parks, 1986; Yang et al., 1999; Cramer et al., 2000).

Lim-HD code regulates dB1 axonal patterns

Lim-HD transcription factors are central regulators of axon pathfinding in central and peripheral neurons (Hobert and Westphal, 2000; Kania and Jessell, 2003; Avraham et al., 2009, 2010b; Green and Wingate, 2014). We have previously demonstrated their similar role in axonal patterning of hindbrain dA1 interneurons. Alteration of the Lim-HD code of dA1 into that of dB1 shifted dA1 axonal projections into dB1 patterns (Kohl et al., 2012). Here we aimed to address whether Lim-HD proteins are also implicated in governing dB1 axonal paths. *Lhx2* or *Lhx9*, the two dA1-specific Lim-HD TFs, were ectopically expressed in dB1 cells (*dB1^{Lhx2/9}*), along with the reporter gene *taumyc* (Fig. 6*I*). Cross sections of E3.5 embryos confirmed that ~90% of the *taumyc⁺*-*dB1^{Lhx2/9}* cells coexpressed *Lhx2/9* proteins and downregulated the expression of the endogenous *Lhx1/5* (Fig. 6*A–D*; Table 1). Thus, the reciprocal cross-repression between *Lhx1/5* and *Lhx2/9*, which was shown in spinal cord interneurons (Avraham et al., 2009) and hindbrain dA1 cells (Kohl et al., 2012), is also valid for dB1 interneurons. Assessment of additional cell-fate markers revealed that ~95% of *dB1^{Lhx2/9}* cells downregulated the dB1 marker *Pax2* but did not upregulate the dA1 marker *Olig3* (Fig. 6*E–H*; Table 1). Moreover, *dB1^{Lhx2/9}* cells remained in their medial location, without shifting dorsally into dA1 (*Lhx2/9⁺*/*Olig3⁺*) typical domains (Fig. 6*A–H*). The loss of *Pax2* in *dB1^{Lhx2/9}* neurons supports earlier spinal cord studies showing that *Lhx1/5* is upstream of *Pax2* in interneurons (Pillai et al., 2007). Yet, the typical position of *dB1^{Lhx2/9}*, together with their lack of expressing dA1-specific markers, suggests that switching the Lim-code of dB1 into dA1 did not confer a full change of their cell fate acquisition.

We next asked whether *dB1^{Lhx2/9}* neurons shift their axonal projections into dA1-related patterns. To compare between axonal patterns of intact and modified dB1 interneurons, we first simultaneously labeled intact dA1 and dB1 axonal subpopula-

tions. E2.5 hindbrains were coelectroporated with the following: (1) *Ptf1a::Cre* and Cre-conditional mCherry plasmids to trace dB1 axons, and (2) *Ed11::FLPo* and FLP-conditional GFP plasmids to label dA1 axons (Fig. 6*Q*). Flat-mounted hindbrains of E5 embryos revealed the expected distinct axonal tracts for each neuronal type; dA1 neurons projected via the cLF and the cDF, in relation to rostral or caudal hindbrain axes, respectively (Fig. 6*K*) (Kohl et al., 2012), whereas dB1 axons extended into the cMLF and cDF (Fig. 6*J*; see also Fig. 3*A*). No overlapping was evident between the tracts of each axonal class (Fig. 6*L*). In contrast, analysis of *dB1^{Lhx2}* or *dB1^{Lhx9}* neurons revealed a substantial change in their axonal trajectories; *dB1^{Lhx2}* and *dB1^{Lhx9}* axons projected in two main commissural tracts that ascended in the cLF and cDF (Fig. 6*M, M', O, O'*), which are similar to those generated by normal dA1 neurons. To further validate these results, embryos were coelectroporated with *Ed11::Flp* conditional-GFP plasmids to trace dA1 axons and *Ptf1a::Cre + Lhx2/9-taumyc* conditional plasmids to trace *dB1^{Lhx2}/dB1^{Lhx9}* axons (Fig. 6*I, Q*). E4.5 embryos clearly showed colocalization of *dB1^{Lhx2}* and *dB1^{Lhx9}* axons with that of dA1 axons (Fig. 6*N, N', P, P'*). Noticeably, *dB1^{Lhx9}* also projected into an ipsilateral route (Fig. 6*O, P*, dotted arrow), which seemed similar to the iLF of normal dB1 (Fig. 3*C*), suggesting a variance in the efficiency of *Lhx2* versus *Lhx9* to confer total shift in dB1 axonal growth. These findings demonstrate that modifying the Lim-code of dB1 interneurons into that of dA1 is accompanied with shift of dB1 into dA1 axonal patterns, indicating the regulatory role of specific Lim-HD proteins in instructing axonal projection of dB1 interneurons.

Lim-HD code is determining neurotransmitter profile of hindbrain interneurons

Along with a role in axonal guidance, Lim-HD proteins are implicated in neurotransmitter phenotype (Pillai et al., 2007; Hori et al., 2008; Serrano-Saiz et al., 2013). For example, *Lhx1* and *Lhx5* function together with *Pax2* in governing the inhibitory (GABAergic) profile of dorsal spinal cord interneurons (Pillai et al., 2007). *Lhx6* and *Lhx7* control the GABAergic and cholinergic fate of cortical interneurons and forebrain neurons, respectively (Lopes et al., 2012). Hence, we asked whether Lim-HD proteins also play a role in determining the neurotransmitter profile of hindbrain interneurons. We first determined the neurotransmitter profile of intact dB1 and dA1 neurons by electroporating E2.5 embryos with *Ptf1a::Cre* or *Ed11::Cre* conditional-GFP plasmids, respectively (Fig. 7*I, I'*). Embryos were analyzed one day later by staining with GABA and VGlut2 at the level of r4–r5. As expected from previous studies (Maricich and Herrup, 1999; Glasgow et al., 2005; Hoshino et al., 2005; Fujitani et al., 2006; Rose et al., 2009), 86% of *dB1-GFP⁺* neurons coexpressed the inhibitory neurotransmitter GABA, whereas none expressed the excitatory neurotransmitter VGlut2 (Fig. 7*A, C*; Table 1). Concomitantly, dA1-GFP⁺ neurons were colabeled with VGlut2, but not with GABA (Fig. 7*E, G*).

Next, we tested whether Lim-HD proteins play a role in determining the inhibitory or excitatory profile of dB1 and dA1 interneurons, by switching their Lim-HD code. E2.5 embryos were electroporated with the relevant combination of vectors (Fig. 7*I'–I''*) (Kohl et al., 2012) and examined at E4.5. Ectopic expression of *Lhx2* in dB1 (*dB1^{Lhx2}*) neurons abolished GABA expression in ~97% of cells (Fig. 7*B*; Table 1) and upregulated VGlut2 in ~50% of *dB1^{Lhx2}* neurons (Fig. 7*D*; Table 1). In the reciprocal experiment, ectopic expression of *Lhx1* in dA1 neurons (*dA1^{Lhx1}*) led to downregulation in VGlut2 expression in 97% of the cells (Fig. 7*F*;

Figure 4. dB1 interneurons project to and synapse at Purkinje cerebellar layer. Sagittal section views of brightfield (*A*) and immunofluorescent (*B–G*) embryos electroporated at E2.5 with *Ptf1a* enhancer-derived myristolated GFP (mGFP) reporter (*A–D*), *Pdx1 + Ptf1a* enhancer-derived mGFP reporter (*E*), or *Ptf1a* enhancer-derived synaptic (SV2-GFP) reporter (*F, G*) and the PB plasmids. dB1-derived GFP⁺ axons (*A–E*) and presynapses (*F, G*) are shown from E8.5–E15.5, following staining with cerebellar and presynaptic markers. *A–A'*, GFP⁺ axons ascend in the medulla and turn toward the cerebellar primordium at E8.5 (arrows). *B, D'*, GFP⁺ axons extend in the cerebellum and reach the Purkinje/Calb⁺ layer (*C*, arrow) but not the EGL/Ax-1⁺ domain (*B, D*, arrow) at E13.5–E15.5. *E*, ION-derived GFP⁺ axons reach the Purkinje/Calb⁺ layer (arrow). *F–G'*, SV-GFP⁺ presynapses are seen in the Purkinje/Calb⁺ layer at E13.5–E15.5. Colabeling of SV-GFP⁺/Syn-Tag⁺ synapses is evident (*F', G'*, arrows). High-magnification views of the boxed areas in *A–G'* are represented at the right panel of each image. Scale bars, plasmids, staining, and embryonic days are indicated. *H–H'*, A scheme representing the constructs used for labeling dB1 axons and presynapses. Cb, Cerebellum; Ax-1, axonin-1; Syn-Tag, synaptotagmin; Calb, calbindin.

Figure 5. dB1 interneurons form synaptic connections in auditory and vestibular nuclei. Sagittal section views of brightfield (*A–F*) and immunofluorescent (*C'–F'*) brainstems electroporated at E2.5 with Ptf1a enhancer-derived mGFP (*A–A'*) or synaptic (SV2-GFP) (*C–F'*) reporters and the PB plasmids. dB1-derived GFP⁺ axons (*A–A'*) and presynapses (*C–F'*) are shown at E13.5 and E15.5, following staining with presynaptic and midbrain markers. *A*, GFP⁺ axons are evident ascending beyond the midbrain–hindbrain border, marked by the En-1 (red). *B*, A schematic illustration of a brainstem sagittal section representing the AP and DV positions of the auditory and vestibular nuclei in the midbrain. The nuclei positions do not reflect the actual lateral–medial locations of each nuclei. *C–E'*, SV2-GFP⁺ presynapses are found in the dorsal midbrain (*C–C'*), at the EW (*D–D'*, arrowheads) and MLD (*E–E'*, arrowhead). *C'*, Arrows indicate SV2-GFP-labeled synapses coexpressing the synaptic marker Syn-Tag. *F–F'*, SV-GFP⁺ presynapses are found in the dorsal medulla. *F'*, Arrows indicate SV2-GFP-labeled synapses coexpressing the synaptic marker Syn-Tag. In all images, higher-magnification views of the boxed areas in the left panels are presented at their respective right panels. Scale bars, plasmids, staining, and embryonic days are indicated. Cb, Cerebellum; MB, midbrain; Syn-Tag, synaptotagmin; Phallo, phalloidin; EW, Edinger–Westphal; MG, medial geniculate nucleus; DLA, dorsolateralis anterior nucleus; r, rhombomere.

Table 1). Yet, in this case, almost no upregulation in GABA was evident (Fig. 7*H*). Hence, ectopic expression of Lhx2/9 in dB1 neurons, which led to downregulation of Lhx1/5, Pax2, and GABA, supports a role of Lhx1 in upregulation of GABA phenotype via Pax2 (Pillai et al., 2007). Similarly, ectopic expression of

Lhx1 in dA1 was sufficient to repress Lhx2/9 and VGlut2, suggesting a regulatory link between Lhx2/9 and excitatory phenotype. Together, these results demonstrate a central role of the Lim-HD code in setting the neurotransmitter phenotype of hindbrain interneurons, as well as indicating for differences in dA1

Figure 6. Alterations in dB1 axonal projection by modification of the Lim-HD code. **A–H**, Cross sections of E3.5 hindbrains that were electroporated at E2.5 with Ptf1a enhancer-derived ectopic Lhx2 (dB1^{Lhx2}; **A, C, E, G**) or Lhx9 (dB1^{Lhx9}; **B, D, F, H**) plasmids. Sections were stained with different interneuron markers. The ectopic expression of Lhx2/9 is accompanied with expression of taumyc (**I**). **A, B**, Ectopic expression of Lhx2 (**A**) or Lhx9 (**B**) in dB1/taumyc⁺ neurons. **C, H**, Ectopic expression of Lhx2 (**C, E, G**) or Lhx9 (**D, F, H**) in dB1 neurons downregulates expression of Lhx1/5 (**C, D**) and Pax2 (**G, H**) but does not activate expression of Olig3 (**E, F**). **A–H**, Boxed areas are represented in their different channels in magnified views to the right of each panel. Arrowheads indicate representative neurons that coexpress taumyc and the examined interneuron marker. Arrows indicate representative neurons that do not express taumyc. **I**, A schematic representation of the plasmids used for ectopic expression of Lhx2 or Lhx9 in dB1 neurons. **J–L**, Flat-mounted views of E5 hindbrains coelectroporated at E2.5 with Ptf1a::Cre-derived mCherry (**J**, red) and Edl1::Flp-derived GFP (**K**, green) plasmids (**Q**). **J, K**, Axonal patterns of dB1 and dA1 are shown. **L**, A merged image demonstrating the distinct axonal patterns of dB1 or dA1 subgroups. Arrowheads and arrows indicate cDF and cMLF, respectively. Dashed arrow and asterisks indicate cLF and cDF, respectively. **M, O**, Flat-mounted views of E4.5 hindbrains that were electroporated at E2.5 with Ptf1a enhancer-derived ectopic Lhx2 (Ptf1a^{Lhx2}; **M**) or Lhx9 (Ptf1a^{Lhx9}; **O**)-taumyc expression plasmids (**I**). Altered dB1 axonal trajectories are shown. **N, P**, Flat-mounted hindbrains of E4.5 embryos coelectroporated at E2.5 with Ptf1a enhancer-derived ectopic Lhx2 (**N**) or Lhx9 (**P**)-taumyc expression plasmids in dB1 neurons (red) and Edl1::Flp-derived-GFP expression plasmid in dA1 neurons (green). Colocalization of Ptf1a^{Lhx2/9} and dA1 is shown. **M–P**, Boxed areas represent higher-magnification views in **M'–P'**, respectively. Arrows and arrowheads indicate cDF and cLF, respectively. Dashed arrows indicate iLF. **Q**, A schematic illustration of the plasmids used in **J–P**. In all images, plasmids, antibodies, and scale bars are indicated.

versus dB1 groups in their switching between inhibitory and excitatory neurotransmitter properties.

Lim-HD code controls dB1 synaptic targeting

The change of dB1 axonal and neurotransmitter patterns into dA1-like patterns upon the switch of dB1-to-dA1 Lim-code was evidenced at early stages of CNS development: E5 and E6. Next, we aimed to test whether such a Lim-code alternation may also affect later stages of CNS development: targeting and synaptogenesis. Because Lim-HD genes are transiently expressed during early development (Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002), we designed a two-step ectopic expression strategy that directs short-term expression of Lim-HD and Cre recombinase followed by a long-range expression of Cre-dependent reporter gene (Fig. 8*D, D'*). The transitory ectopic expression of Lim-HD genes is a consequence of the episomal state of plasmids within chick cells that leads to a temporal loss of plasmids, whereas the stable expression of reporters is attained via PB-mediated DNA transposition.

To follow the cerebellar connectivity of dB1^{Lhx2/9}, transient expression of Lhx2 and Cre was activated by Ptf1a enhancer, and subsequently stable expression of SV2-GFP reporter was attained in dB1 neurons (Fig. 8*D*). Embryos were coelectroporated at E2.5 and analyzed at E14.5 to identify cerebellar presynaptic sites of dB1^{Lhx2}. Cerebellar sections were stained with Zic1 and calbindin to label the EGL/internal granular layer (IGL) or Purkinje cells, respectively (Kohl et al., 2012). SV2-GFP⁺ synapses were distributed at the EGL,

IGL, and Purkinje layers in a similar ratio of ~30% in each layer (Fig. 8*A–C', E*). This is contrast to control dB1, in which ~85% of SV2-GFP⁺ synapses are restricted to the Purkinje cells (Figs. 4 and 8*E*). The change in the presynaptic targets of dB1^{Lhx2} neurons resembles the normal presynaptic sites of dA1 (Kohl et al., 2012), indicating that the early modification in dB1 Lim-code affects their presynaptic targeting at much advanced stages.

In a complementary experiment, we determined whether the reciprocal modification of the dA1-Lim code into dB1 shifts their synaptic targets accordingly. Lhx1 and Cre were ectopically expressed in E2.5 hindbrains under Edl1 enhancer to drive constitutive expression of SV2-GFP reporter in dA1 neurons (Fig. 8*D'*). dA1^{Lhx1}-derived SV2-GFP⁺ synapses were found at all cerebellar layers and not restricted to Purkinje cells (Fig. 8*E*), similar to intact dA1 interneurons (Kohl et al., 2012). Hence, the transient expression of Lhx1 in dA1 neurons does not affect their cerebellar targeting. Together, these experiments reveal the impact of the Lim-code in conferring the formation of dB1-cerebellum circuitry on top of governing their axonal patterns and GABA expression, as well as suggest varied roles of the Lim-code in dB1 versus dA1 circuit formation.

Discussion

The heterogeneity of dB1 interneurons

Studies in mice revealed the indispensable role of dB1/Ptf1a⁺ interneurons in the establishment of hindbrain somatosensory centers (Hoshino et al., 2005; Fujitani et al., 2006; Yamada et al.,

Figure 7. Alterations in dA1 and dB1 neurotransmitter profile by modification of the Lim-HD code. **A–H**, Cross-section views of E3.5 hindbrains at the level of r4–r5 that were electroporated at E2.5 with Ptf1a::Cre-derived GFP plasmid (**A**, **C**), Ptf1a::Cre-derived ectopic Lhx2-taumyc plasmid (**B**, **D**), Edl1::Cre-derived GFP plasmid (**E**, **G**), and Edl1::Cre-derived ectopic Lhx1-taumyc plasmid (**F**, **H**). Sections were stained with GABA and VGlut2 antibodies. Higher-magnification views of the boxed areas in **A–H** are represented at the right panels in different channels. Arrowheads indicate representative axons that coexpress GFP/taumyc and the neurotransmitter. Arrows indicate representative neurons that do not express GFP/taumyc. **A**, **B**, Intact dB1-GFP⁺ neurons express GABA, whereas dB1^{Lhx2}-taumyc⁺ neurons downregulate GABA. **C**, **D**, Intact dB1-GFP⁺ neurons do not express VGlut2, whereas part of dB1^{Lhx2}-taumyc⁺ neurons upregulate VGlut2. **E**, **F**, Intact dA1-GFP⁺ neurons express VGlut2, whereas dA1^{Lhx1}-taumyc⁺ neurons downregulate VGlut2. **G**, **H**, dA1-GFP⁺ neurons and dA1^{Lhx1}-taumyc⁺ neurons do not express GABA. In all images, scale bars, plasmids, antibodies, and interneuron subgroups are indicated. **I–I''**, Schematic representation of plasmids used for labeling intact dB1 (Ptf1a::Cre) and dA1 (Edl1::Cre) neurons (**A**, **C**, **E**, **G**). Schematic representation of plasmids used for ectopic expression of Lim-HD transcription factors, Lhx2 in dB1 (Ptf1a::Cre) neurons, and Lhx1 in dA1 (Edl1::Cre) neurons (**B**, **D**, **F**, **H**).

2007; Hori et al., 2008). However, knowledge regarding the transcriptional network that controls their connectivity formation is sparse. In this study, dB1 neurons were genetically traced in the chick hindbrain, revealing their contribution to different brainstem nuclei, projection into discrete tracts, and synaptic formation at multiple target sites. A possible explanation for the different fates of dB1 is their division to several subpopulations, each with its specific TFs and fate.

Support for this hypothesis comes from studies on dorsal spinal dI1 interneurons, which give rise to two subpopulations: dI1_{comm} neurons, which express Lhx2 and project commissural axons; and dI1_{ipsi}, which express Lhx9 and project ipsilaterally (Gowan et al., 2001; Wilson et al., 2008; Avraham et al., 2009). An alternative possibility is diverged time-births of dB1 subgroups. The gradual appearance of dB1 axonal trajectories supports such a scenario. Further

Figure 8. Alteration in dB1 synaptic targets by modification of the Lim-code. **A–C**, Sagittal section views of E14 cerebellums from embryos electroporated at E2.5 with a combination of Ptf1a::Phic31o, Phic31o-conditional Lhx2-Cre, Cre-conditional SV2-GFP, and the PBase plasmids. Sections were stained with cerebellar (Zic1, Calb) and presynaptic (Syn-Tag) markers.

support comes from the cerebellum in which dA1 progenitors that derive from r1-rhombic lip give rise to four subpopulations that are born in a consecutive temporal cascade (Green and Wingate, 2014). Axonal branching and collaterals are another possible mechanism for the multiple dB1 trajectories. Such a mechanism was shown in mice where spinal sensory axons invade the spinal cord at E14 mouse bifurcate to send rostral and caudal branches, whereas three days later collaterals sprout from the longitudinal tract and project toward their targets (Sharma et al., 2000). Future experiments will be required to reveal the underlying mechanism/s of dB1 heterogeneity.

Role of Lim-HD code in the circuitry of hindbrain interneurons

The deterministic role of the Lim-HD code in fate determination and axon guidance was shown in various species and in different regions of the nervous system (Shirasaki and Pfaff, 2002), including the hindbrain (Kohl et al., 2012). Our data demonstrate a fundamental role for Lim-HD proteins in regulating the circuitry and neurotransmitter phenotypes of two genetically distinct hindbrain interneurons: dB1 and dA1. The role of the Lim-code was investigated throughout the development of hindbrain circuitry, from cell fate acquisition, to axonal trajectory and neurotransmitter phenotype, up to targeting and synaptogenesis. We found that Lhx1 and Lhx2/9 do not determine the fate of dB1 and dA1 neuron, respectively (see also Kohl et al., 2012). Yet, Lhx1 determines the axonal patterning

←
dB1^{Lhx2}-derived SV2-GFP⁺ synapses are evident in the EGL (**A–A''**), Purkinje (**B–B''**), and IGL (**C–C''**). Higher-magnification views of the boxed areas in each panel are presented at their respective right panel. Arrowheads indicate SV2-GFP⁺ synapses. Arrows indicate SV2-GFP⁺/Syn-Tag⁺ synapses. In all images, scale bars, plasmids, and antibodies are indicated. **D–D'**, Schematic representation of plasmids used for transient expression of Lhx2 and constitutive expression of SV2-GFP in dB1 neurons (**D**) or Lhx1 and SV2-GFP in dA1 neurons (**D'**). **E**, Quantification of the number of SV2-GFP⁺ synapses derived from normal and Lim-code modifies dA1 and dB1 neurons at different cerebellar layers. **F**, A model illustrating the distinct roles of the Lim-code in conferring dA1 and dB1 circuit formation. Left panel, Typical axonal patterns, neurotransmitter phenotype, and cerebellar synaptic targets of dA1 (red) and dB1 (yellow) interneurons. Middle panel, dA1^{Lhx1} neurons that switched their axonal patterns into dB1-like routes, downregulated VGlut expression, but retained their synaptic targets at the EGL, IGL, and Purkinje layers. Right panel, dB1^{Lhx2} neurons that switched their axonal projections into dA1-like interneurons. Syn-Tag, synaptotagmin; Calb, calbindin.

and suppression of excitatory neurotransmitter phenotype of dB1 neurons, whereas Lhx2/Lhx9 direct the axonal projection, excitatory neurotransmitter phenotype, and targeting within the cerebellum of dA1 neurons (Fig. 8F).

Role of Lim-HD TFs in axonal patterning

The Lim-HD switch, generated by ectopic Lhx2/9 expression in dB1 neurons, demonstrated an axonal shift from dB1 to dA1, providing evidence for the regulation of dB1 axonal patterns via the Lim-HD code (Fig. 8F). Concomitantly, Lhx1 in dA1 neurons shifted their axonal projections to dB1 patterns (Kohl et al., 2012). A similar role of Lhx1/5 was shown in other neurons; Lhx5-null mice have defects in hippocampal axon crossing (Zhao et al., 1999). Lhx1 is required for spinal neurons of the lateral motor column (LMC1) for guiding their axons to the dorsal limb via upregulation of EphA4 receptor (Kania et al., 2000). Lhx1⁺/EphA4⁺ LMC1 axons are repelled from the ventral limb through their interaction with EphrinA5, and guided to the dorsal limb (Kania and Jessell, 2003; Krawchuk and Kania, 2008; Luria et al., 2008). Interestingly, EphA4 and EphrinB1–3 are also implicated in prevention of midline crossing of spinal ipsilaterally projecting neurons and decussation of the corticospinal tract (Kullander et al., 2001, 2003). Hence, the emergence of dB1 ipsilateral tracts may account for elevations of EphA4 levels. Further studies are required to reveal whether Eph-ephrin signaling acts in controlling the diverse paths of dB1 neurons, as previously shown for hindbrain CN and cC-VC neurons (Zhu et al., 2006; Hsieh et al., 2010).

Several other guidance cues are downstream of Lim-HD proteins; Lhx2/9 induce the expression of Robo1–3 receptors to regulate axonal growth of thalamocortical neurons and spinal interneurons and repress protocadherin10 to direct forebrain axons (Wilson et al., 2008; Chatterjee et al., 2012; Marcos-Mondéjar et al., 2012). Lhx1 regulates the extracellular matrix protein Reelin that participates in the LMC axonal choice (Kania and Jessell, 2003; Shirasaki et al., 2006; Palmesino et al., 2010). As some of these genes are expressed in the hindbrain (Hammond et al., 2005; Howell et al., 2007; Neudert and Redies, 2008; Sela-Donenfeld et al., 2009), it will be interesting to address their role in mediating dB1 axonal patterns.

Role of Lim-HD TFs in neurotransmitter phenotype

The choice of neurotransmitter expression is fundamental for neuronal activity. We revealed that Lim-HD proteins are required for GABA or VGLut2 expression in dB1 and dA1 interneurons, respectively, together with their roles in axonal patterning. A dual role for Lim-HD proteins in axonal navigation and neurotransmitter identity was described before. In the *Drosophila* nerve cord, Isl1 regulates axonal pathfinding and production of serotonin and dopamine in ventral interneurons (Thor and Thomas, 1997). Likewise, in vertebrates, it patterns axonal growth (Kania and Jessell, 2003; Tanaka et al., 2011) and cholinergic fate in spinal motoneurons and forebrain cholinergic neurons, by forming complexes with Lhx3/8 that drive expression of acetylcholine-synthesizing enzymes (Cho et al., 2014). Furthermore, Lhx1- and Lhx5-null mice displayed loss of GABAergic markers in spinal interneurons, together with axonal guidance errors (Pillai et al., 2007). Yet, these interneurons did not switch into glutamatergic fates. Contrary to this study, we find that a switch from Lhx1/5 to Lhx2 in dB1 neurons led to repression of Pax2 and GABA, together with partial upregulation of VGLut2. Pax2 is a central regulator of GABAergic fate in different types of inhibitory neurons (Maricich and Herrup, 1999; Gross et al.,

2002; Cheng et al., 2004; Glasgow et al., 2005; Hoshino et al., 2005; Mizuguchi et al., 2006; Wildner et al., 2006; Pillai et al., 2007; Hori et al., 2008). Hence, the loss of Pax2 observed in the dB1^{Lhx2/9} neurons is consistent with their decrease in GABA, suggesting a genetic network downstream of Lhx1/5 that governs the inhibitory-neurotransmitter program of dB1 neurons. The plasticity in dB1-neurotransmitter choice is further supported in Ptf1a-null mice, where spinal dI4 interneurons downregulated Pax2 and switched from GABAergic to glutamatergic fate (Glasgow et al., 2005), and precerebellar glutamatergic climbing fiber/Ptf1a⁺ neurons trans-differentiated into mossy-fiber neurons (Fujiyama et al., 2009). Notably, the molecular pathways that induce VGLut2 in dB1^{Lhx2} interneurons remain to be elucidated. Moreover, as the neurotransmitter shift in dB1^{Lhx2} is observed at early developmental stages, whether this affects functioning of dB1 circuits is not known.

Intriguingly, in the precerebellar hindbrain, dB1 gives rise to the excitatory ION neurons (Yamada et al., 2007). A role for Lim-HD transcription factors in direct regulation of excitatory phenotype was demonstrated in *C. elegans*. Moreover, Lhx1 was found necessary in adult mice for the maintenance of excitatory ION neurons (Serrano-Saiz et al., 2013). The dual role of Lhx1 in governing inhibitory or excitatory fates may arise from temporal differences of its expression in dB1 subclasses. A transient expression that upregulates Pax2 may govern inhibitory phenotype, whereas a persistent expression may instruct excitatory phenotype via upregulation of VGLut2.

In the corresponding experiment, the forced expression of Lhx1 in dA1 interneurons resulted in repression of VGLut2 without induction of GABA. The inability of dA1^{Lhx1} neurons to induce GABA is compatible with their inability to upregulate Pax2 (Kohl et al., 2012), arguing that forced Lhx1 expression in dA1, which leads to repression of Lhx2/9, is not sufficient to initiate the GABAergic cascade. Concomitantly, Atoh1^{-/-} mice displayed loss of excitatory dA1 interneurons without switching into dB1-like fates (Fujiyama et al., 2009; Maricich et al., 2009; Rose et al., 2009). This is in contrast to Tlx1/3^{-/-} mutants, where Pax2 and GABAergic markers were upregulated in excitatory spinal interneurons (Cheng et al., 2004; Hori et al., 2008; Guo et al., 2012), further arguing for distinct molecular machineries that govern excitatory-to-inhibitory and vice versa fates in different neurons. Our findings are in agreement with such a model, demonstrating that the initial choice of neurotransmitter phenotype of hindbrain interneurons is tied to the Lim code. However, for dB1, rather than dA1, this choice seems revocable.

Role of Lim-HD TFs in synaptic targeting

The possible role of Lim-HD in final targeting was addressed previously in spinal motor neurons (Sharma et al., 2000). Ectopic expression of Lhx3, which is specific to motor neurons that innervate axial muscles, into motor neurons that innervate intercostal muscle, resulted in their shift of innervation from intercostal to axial muscles. Similar alternations in Lim code of motor neurons that innervate limb musculature caused rerouting of the ventral-limb projecting axons to the dorsal limb, and vice versa (Kania and Jessell, 2003). Notably, these studies have used constitutive ectopic expression of Lim-HD genes, and the projection of motor neurons to peripheral targets was analyzed at mid developmental stages E13.5 mouse or E6 chick, respectively (Sharma et al., 2000; Kania and Jessell, 2003). In the current study, we have applied a novel neuronal-specific experimental paradigm that enables transient ectopic expression of Lim-HD

followed by a sustained expression of presynaptic reporter. By that, we preserved the temporal cascade of *Lhx*'s expression. Targeting within the CNS and the formation of presynaptic terminals were studied at advanced developmental stages: chick E14.5. We found that switching Lim-HD code in dB1, but not in dA1, is sufficient to alter dB1 cerebellar target sites, albeit the similar effect on axonal growth in both subpopulations (Fig. 8F).

Our cumulative results (Kohl et al., 2012; and current study) demonstrate that in the hindbrain Lim-HD TFs differ in their instructive capacity. *Lhx2/9* directs axon projection, neurotransmitter phenotype, and targeting, whereas *Lhx1* determined only axon patterning and partial neurotransmitter phenotype (Fig. 8F). Further studies will be required to decipher the pathways downstream of Lim-HD proteins that govern these different responses.

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