The Lbx1 lineage differentially contributes to inhibitory cell types of the dorsal cochlear nucleus, a cerebellum-like structure, and the cerebellum

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The dorsal cochlear nucleus (DCN) is a mammalian-specific nucleus of the auditory system. Anatomically, it is classified as a cerebellum-like structure. Previous analyses demonstrated that inhibitory serial sister cell types (SCTs) of the DCN and cerebellum are derived from the pancreatic transcription factor 1α (Ptf1α) lineage. Postmitotic neurons of the Ptf1α lineage often express the transcription factor Ladybird homeobox protein homolog 1 (Lbx1) which is involved in neuronal cell fate determination. Lbx1 is therefore an attractive candidate for a further component of the genetic program shared between the DCN and cerebellum. Here, we used cell-type specific marker analysis in combination with an Lbx1 reporter mouse line to analyze in both tissues which cell types of the Ptf1α lineage express Lbx1. In the DCN, stellate cells and Purkinje-like cartwheel cells were part of the Lbx1 lineage and Golgi cells were not, as determined by cell counts. In contrast, in the cerebellum, stellate cells and Golgi cells were part of the Lbx1 lineage and Purkinje cells were not. Hence, two out of three phenotypically similar cell types differed with respect to their Lbx1 expression. Our study demonstrates that Lbx1 is differentially recruited to the developmental genetic program of inhibitory neurons both within a given tissue and between the DCN and cerebellum. The differential expression of Lbx1 within the DCN and the cerebellum might contribute to the genetic individualization of the inhibitory SCTs to adapt to circuit specific tasks.

KEYWORDS
transcription factor, auditory system, cell type, development, evolution, inhibitory neuron, mouse, RRID: AB_11212597, RRID: AB_2685195, RRID: AB_2749901, RRID: AB_304362, RRID: AB_309787, RRID: AB_477329, RRID: AB_514500, RRID: AB_591279, RRID: AB_91937, RRID: IMSR_JAX:007908, RRID: SCR_003070

INTRODUCTION

The nuclei of the mammalian auditory hindbrain represent evolutionary novelties, as they are not present in other vertebrates (Carr & Soares, 2002; Grothe et al., 2004; Nothwang, 2016). This makes the auditory system a model system to study evolutionary developmental processes associated with novel brain structures. A conspicuous structure among auditory nuclei is the dorsal cochlear nucleus (DCN). This second order nucleus receives auditory nerve-mediated cochlear input as well as multimodal input from various sources in the brain...
(Trussell & Oertel, 2018). The DCN consists of a superficial molecular layer (ML), which contains granule cell axons that form a system of parallel fibers, a fusiform layer (FL) with fusiform projection neurons, and a deep layer (DL) (Figure 1(a)). This three-layered structure that is surrounded by a domain of granule cells is unique among auditory hindbrain nuclei.

With its sensory input, layered organization, parallel fibers, and cell types, the DCN resembles the cerebellum and is classified as a

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**FIGURE 1** The cerebellum-like histology of the dorsal cochlear nucleus (DCN), and the serial sister cell types (SCTs) of the DCN and cerebellar cortex (CC). (a) Left side: Main circuitry of the DCN. The granule cells, which reside in an external granule cell domain, innervate the dendrites of fusiform, giant, and cartwheel cells via a system of parallel fibers within the molecular layer (ML). In addition, fusiform and giant cells receive auditory nerve input in the deep layer (DL). Right side: The shared features between the DCN and the CC are a ML that includes parallel fibers derived from granule cells, a principal cell layer, and the interneuron types stellate cells, Golgi cells, and unipolar brush cells. Only the shared neuron types are color-coded. (b) Left side: The pancreatic transcription factor 1a (Ptf1a) lineal SCTs of the DCN are serial SCTs of the Ptf1a lineal SCTs of the CC. The asterisk marks cells of the CC that are derived from Pax2+ intermediate progenitors. Only the cartwheel/Purkinje cells, Golgi cells and stellate cells are phenotypically highly similar SCTs and thus considered shared among the two structures. Right side: The Atoh1 lineal SCTs of the DCN are serial SCTs of the Atoh1 lineal SCTs of the CC. Only the granule cells and unipolar brush cells are phenotypically similar serial SCTs and thus considered shared among the two structures. an, auditory nerve input; AVCN, anterior ventral cochlear nucleus; b, basket cell; c, cartwheel cell; cf, climbing fiber input; cn, candelabrum cell; f, fusiform cell; G, Golgi cell; gi, giant cell; gr, granule cell; grd, granule cell domain input; L, Lugaro cell; P, Purkinje cell; pf, parallel fibers; st, stellate cell; t, tuberculo-ventral cell; u, unipolar brush cell [Color figure can be viewed at wileyonlinelibrary.com]
cerebellum-like structure (Figure 1(a)) (Bell, 2002; Oertel & Young, 2004; Sawtell & Bell, 2013). This term encompasses various vertebrate structures with a common histology including an ML with parallel fibers and topographically organized sensory input in a DL. They are present in many fish, amphibians, and mammals with some vertebrate groups harboring several such structures, whereas mammals possess a single one, the DCN. One explanation for their common histological architecture is a shared genetic developmental program (Bell, 2002).

In support of a shared genetic developmental program, the excitatory and inhibitory neurons of both, the DCN and the cerebellum, belong to the Atoh1 and Ptf1a lineages, respectively (Arie et al., 1997; Fujiyama et al., 2009; Hoshino et al., 2005; Pascual et al., 2007). The excitatory cell types as well as the inhibitory cell types in the DCN and cerebellum were therefore classified as serial sister cell types (SCTs; Figure 1(b)) (Arendt et al., 2016). SCTs arise by the splitting of an ancestral cell type in descendant cell types via genetic individualization that is differential use of genetic information for phenotypic specialization (Arendt et al., 2016). Serial SCTs are SCTs born in different regions (Arendt et al., 2016), such as those of the DCN and the cerebellum. Neurons of the DCN originate in rhombomeres (r) 4 and 5 (di Bonito et al., 2013; Farago et al., 2006; Nichols & Bruce, 2006), whereas those of the cerebellum, originate in r1 (Chizhikov & Millen, 2003; Millet et al., 1996; Wingate & Hatten, 1999; Zervas et al., 2004).

Here, we further addressed the existence of a shared genetic developmental program between the two regions. We focused on the phenotypically similar cell types of the pancreatic transcription factor Ptf1a (Ptf1a) lineage in DCN and cerebellum, that is, the stellate and Golgi cells, as well as the Purkinje and Purkinje-like cartwheel cells (Figure 1(b)). The stellate cells have straight dendrites radiating in a star-like manner (Zhang & Oertel, 1993), the Golgi cells exhibit extensive axonal arborization (Ferragamo et al., 1998), and cartwheel cells are considered Purkinje-like due to their spine-covered dendrites that extend into the ML (Sawtell & Bell, 2013).

Whereas the stellate and Golgi cells of the cerebellum undergo an intermediate progenitor stage that involves migration into a secondary neurogenic niche, the cerebellar Purkinje cells and the inhibitory cell types of the DCN do not. Cartwheel cells of the DCN express the molecular marker Bhlhb5 as soon as they have completed final mitosis (Cai et al., 2016). This indicates that they directly differentiate rather than first becoming an intermediate progenitor that eventually produces cartwheel cells and other inhibitory neuronal subtypes of the DCN. Still, Pax2 expression, which is a hallmark of the intermediate progenitor stage in the CC (Maricich & Herrup, 1999; Weisheit et al., 2006), is also observed in cartwheel cells and another cell population of the DCN (Cai et al., 2016).

Many Ptf1a lineal cells in the hindbrain and spinal cord express the transcription factor Ladybird homeobox protein homolog 1 (Lbx1) (Hernandez-Miranda et al., 2017), which is a postmitotic determinant of cell fate by promoting specification of inhibitory neurons in the medulla (Pagliardini et al., 2008) and the spinal cord (Cheng et al., 2005; Gross et al., 2002; Huang et al., 2008; Kruger et al., 2002; Müller et al., 2002). The purpose of the present study was therefore to address the question whether Lbx1 expression is shared between the phenotypically similar Ptf1a lineal serial SCTs of the DCN and cerebellum. To this end, we analyzed a Lbx1 reporter mouse line in the DCN and the cerebellar cortex (CC). Our results demonstrate that the stellate cells of both tissues express Lbx1, whereas the Golgi and Purkinje/cartwheel cells differ in their Lbx1 expression pattern between both tissues. Our study thus reveals shared and distinct deployment of a transcription factor in serial SCTs of a cerebellum-like structure and the cerebellum.

2 | MATERIALS AND METHODS

2.1 | Animals

All protocols were in accordance with the German Animal Protection Law and approved by the local animal care and use committee (LAVES, Oldenburg) and followed the NIH guide for the care and use of laboratory animals. Mice of both sexes were used. Lbx1<Cre/+> mice carry an allele in which the Cre open reading frame replaces the first exon of the Lbx1 gene (Sieber et al., 2007). These mice were kept on a C57Bl/6N genetic background. TdTomato reporter mice (Jackson Laboratory, JAX Stock #007908, RRID: IMSR_JAX:007908) express the tdTomato fluorescent protein following Cre-mediated recombination (Madisen et al., 2010). They are of mixed genetic background (129S6 × C57Bl/6). Lbx1<Cre/+>; tdTomato mice permanently express tdTomato in the Lbx1 lineage. Primers for genotyping the Lbx1<Cre> allele are as follows: for, CCCTTCTCTCGACCGTC, and rev, ACACTCGGTGGGGCGACT. Genotyping of the tdTomato reporter line was performed according to the protocol published on www.jax.org.

2.2 | Antibody characterization

The rabbit anti-Bhlhb5 antibody (Atlas Antibodies, HP064128, RRID: AB_2685195, used at 1:500) was raised against recombinant protein epitope signature tag antigen sequence AAAAAALHPAL GAYEQAGYPFSAGLPPAASCPKCALFNSVSSSLCKQCTE. It was validated immunohistochemically in human tissue by comparing independent antibodies targeting different epitopes of the protein. The murine sequence is 100% identical to the human sequence.

The mouse anti-CamKII antibody (Merck Millipore, 05-532, RRID: AB_309787, used at 1:500) was raised against partially purified rat CamKII. Its quality was assured by immunoblot on bovine brain cytosol or rat brain microsome preparations (technical information by Merck). This antibody reacts with a single band of ~50 kDa in homogenates of various regions of the rat brain (Erondu & Kennedy, 1985). This band disappears upon reaction with a different antibody against CamKII (Erondu & Kennedy, 1985).

The in situ hybridization results (Figures 2 and 3(c)) we obtained by using the mouse anti-digoxigenin antibody (Abcam, ab420, RRID:
AB_304362, used at 1:200) are in agreement with those of the Allen Brain Atlas who used the same probes, but a different antibody for detection (Lein et al., 2007).

The sheep anti-digoxigenin antibody conjugated with horseradish peroxidase (Roche, 11207733910, RRID: AB_514500, used at 1:1000) specifically recognizes digoxigenin and digoxin and does not cross-react with other steroids (technical information by Roche).

The mouse anti-GFAP antibody (Merck Millipore, MAB360, RRID: AB_11212597, used at 1:500) was raised against purified GFAP from porcine spinal cord and was verified by detecting a band of ~51 kDa by Western Blot (technical information by Roche).

The mouse anti-Neurogranin antibody (Merck Millipore, AB5620, RRID: AB_91937, used at 1:500) was raised against rat recombinant Neurogranin. It reacted in cells of the murine cerebellum that were also positive for Neurogranin mRNA, as revealed by a combination of immunocytochemistry and in situ hybridization (Singec et al., 2003).

The rabbit anti-Parvalbumin (Parv) antibody (Merck Millipore, P3088, RRID: AB_477329, used at 1:1000) was raised against frog muscle Parv (technical information by Merck). It shows no reactivity in Parv−/− mice (Burette et al., 2009).

The purified rabbit anti-Pax6 antibody (BioLegend, 901302, RRID: AB_2749901, used at 1:150) was raised against the peptide QVPGSEPDMSQYWPRLQ derived from the C-terminus of the murine Pax6 protein (technical information by BioLegend). Western blot analysis showed two closely migrating bands of ~50 kDa in tissue extracts from adult brain, olfactory bulb, eye, and olfactory turbinates, but not in liver (Davis & Reed, 1996). In situ hybridization to target Pax6 mRNA showed the same tissue expression pattern (Walther & Gruss, 1991). The two bands resulting from the Western blot correspond to the predicted sizes of alternative spliced variants of Pax6 (Walther & Gruss, 1991).

The rabbit anti-red fluorescent protein antibody (MBL, PM005, RRID: AB_591279, used at 1:250) reacts with red fluorescent protein-

**Figure 2** The Ladybird homeobox protein homolog 1 (Lbx1) lineage contributes many inhibitory and few excitatory neurons of the dorsal cochlear nucleus (DCN). (a1–a3) Cells of the Lbx1 lineage were mostly located in molecular layer (ML) and fusiform layer (FL), and to a lesser extend in deep layer (DL). (b1–d3) In situ hybridization to target neurotransmitter-related molecules. (b1–b3) Many Viaat+ inhibitory neurons in the ML and FL were tdTomato− (arrows). Some Viaat+ in the FL and most in the DL were tdTomato− (arrowheads). Some Lbx1 lineal cells did not express Viaat (empty arrows). (c1–c3) Some Vglut1+ excitatory neurons were tdTomato+ (arrows), and some were tdTomato− (arrowheads). Many cells of the Lbx1 lineage were Vglut1− (empty arrows). (d1–d3) No Vglut2+ excitatory neurons expressed tdTomato. Cells were Vglut2− and tdTomato− (arrowheads) or Vglut2− and tdTomato− (empty arrows). Scale bars, in Panel (a), 100 μm, in Panels (b–d), 10 μm [Color figure can be viewed at wileyonlinelibrary.com]
tagged fusion proteins on Western blot and in immunocytochemistry (technical information by MBL).

The affinity-purified rabbit anti-Tbr2 antibody was a generous gift from Dr Robert F. Hevner (Englund et al., 2005).

2.3 | Immunohistochemistry

Postnatal Day 25 mice were transcardially perfused with Zamboni’s fixative solution (15% picric acid, 2% paraformaldehyde in phosphate-
buffered saline [PBS, pH 7.4]. Brains were removed, postfixed for 1–4 h, and kept in 30% sucrose in PBS at 4°C overnight. They were then embedded in 30% sucrose in PBS at −30°C, and 30 μm thin sections were produced using a sliding microtome (Microm HM 430, Thermo Fisher Scientific, Waltham, MA). Heads of E14.5 or E17.5 were immersion-fixed with 4% paraformaldehyde in PBS for 4 h at room temperature or overnight at 4°C. Afterward, they were cryoprotected in 30% sucrose in PBS at 4°C overnight. Sections of 30 μm thickness were cut on a CM-1950 cryostat (Leica, Wetzlar, Germany). Fluorescent immunohistochemistry was performed as previously published (Ebbers et al., 2015; Ebbers et al., 2016). Primary antibodies used were: rabbit anti-Bhlhb5 (1:500; Atlas Antibodies, HPA064128, RRID: AB_2685195), mouse anti-CamKII (1:250, Merck Millipore, 05-532, RRID: AB_309787), mouse anti-GFAP (1:500, Merck Millipore, MAB360, RRID: AB_11212597), rabbit anti-Neurogranin (1:500, Merck Millipore, AB5620, RRID: AB_91937) mouse anti-Pav (1:1000, Merck Millipore, P3088, RRID: AB_477329), rabbit anti-Pax6 (1:150, Biologend, 901302, RRID: AB_2749901), and rabbit anti-Tbr2 (1:2000, generous gift from Robert Hevner (Englund et al., 2005)). We made use of the following secondary antibodies coupled to Alexa Fluor dyes (AF): goat anti-mouse IgG AF 647 (1:1000, Thermo Fisher Scientific, A21235, RRID: AB_263282) and goat anti-rabbit IgG AF 594 (1:1000, Thermo Fisher Scientific, A32733, RRID: AB_2762827).

2.4 | Fluorescent in situ hybridization

In situ hybridization probes were generated from the cDNAs of mouse Vglut1 (nucleotides 1015–1996, GenBank accession number NM_182993.1), Vglut2 (nucleotides 2190–2769, NM_080853.2), Viat (nucleotides 178–1157, NM_009508.1), and Gad1 (nucleotides 1099–2080, NM_008077.2). The corresponding primers were taken from the Allen Brain Atlas (Lein et al., 2007).

Mice of postnatal Day 25 were transcardially perfused using 4% paraformaldehyde in PBS. Brains were dissected, postfixed for 1–4 h, and equilibrated in 30% sucrose in PBS at 4°C overnight. Then, 20 μm thin sections cut on Superfrost Plus slides (Thermo Fisher Scientific) using a CM-1950 cryostat (Leica). In situ hybridization was conducted as previously published until blocking (Schlüter et al., 2018). After blocking, sections were incubated with an anti-digoxigenin antibody conjugated with horseradish peroxidase (1:1000, Roche, 11207733910, RRID: AB_514500) for 1 h at room temperature. Simultaneously, we made use of an antibody against red fluorescent protein (1:250, MBL, PM005, RRID: AB_591279) at 4°C overnight to recover the weakened tdTomato-signal. Secondary antibodies used were donkey anti-rabbit IgG AF 594 (1:1000, Thermo Fisher Scientific, A32754, RRID: AB_2762827) and goat anti-mouse IgG AF 647 (1:1000, Thermo Fisher Scientific, A21235, RRID: AB_2535804). Slides were mounted using Mowiol (0713, Roth, Karlsruhe, Germany), supplemented with DABCO (0718, Roth) and DAPI (D8417, Merck, Darmstadt, Germany). Sense probes corresponding to the reverse-complementary sequence of the antisense probes, were used as negative controls.

2.5 | Cell counting

For cell counting, three Lbx1Cre;tdTomato reporter mice at P25 were used. For the DCN, regions of interest in its dorsal, middle, and ventral part were imaged using a ×40 objective (Leica) with 0.75–×1.5 zoom. Cells belonging to a given cell type was identified by their molecular marker: Pav for the stellate and cartwheel cells (Caicedo et al., 1996; Fujiyama et al., 2009) and Pax6 for the granule cells (Fujiyama et al., 2009). More than 30 cells per cell type were counted along the rostrocaudal axis of the DCN per animal and analyzed for tdTomato expression, thus in total at least 100 cells per cell type.

A similar procedure was used for the nodulus of the cerebellum. Regions of interest were imaged to represent its left, middle and right part. Molecular markers were Pav for the stellate and basket cells (Stichel et al., 1986) and Neurogranin for the Golgi cells (Simat et al., 2007). Cerebellar stellate cells were distinguished from basket cells by dividing the ML into two equal halves, the upper half harboring stellate cells and the lower half basket cells (Sudarov et al., 2011).

2.6 | Imaging and software

Microscopic analysis was performed using a TCS SP8 confocal laser scanning microscope with HC PL FLUOTAR ×10 and HC PL APO ×40 objectives (Leica). Images were processed using the software Fiji (https://fiji.sc/, RRID: SCR_003070).

3 | RESULTS

3.1 | The Lbx1 lineage contributes many inhibitory and few excitatory neurons to the DCN

The DCN shows a complex cerebellum-like histological architecture (Figure 1a). The ML contains the parallel fibers that innervate the dendrites of fusiform and giant cells, the principal neurons of the DCN, as well as local interneurons. The FL lies the DL with the dendrites of fusiform cells and giant cells, which receive auditory nerve input. In addition, this layer harbors the cell bodies of the giant cells, and local interneurons. In total, the DCN contains four inhibitory SCTs: stellate cells in the ML, cartwheel cells in the ML and FL, Golgi cells in the FL and DL, and tuberculo-ventral cells in the DL (Figure 1a). It further possesses four excitatory SCTs: granule cells in the FL, unipolar brush cells in the FL and DL, fusiform cells in the FL and giant cells in the DL (Figure 1a).
To identify the cell types in the DCN and CC that are derived from the Lbx1 lineage, we generated an Lbx1<sup>Cre<sup>+</sup></sup>:tdTomato reporter mouse line to stably label these cells with the red fluorescent protein. Fluorescence microscopy revealed that tdTomato<sup>+</sup> cells reside in all three layers of the DCN. They were predominantly distributed in the FL along the border to the ML, and more loosely in the ML and DL (Figure 2(a)).

To determine the neurotransmitter phenotype of Lbx1 lineal cells, we used fluorescent in situ hybridization against neurotransmitter-related molecules. Viao<sub>a</sub> encodes a transporter common to both GABAergic and glycinergic inhibitory neurons (Chaudhry et al., 1998; Wang et al., 2009) and marks inhibitory neurons of the DCN (Ito et al., 2011; Lein et al., 2007). A combination of fluorescent in situ hybridization with reporter fluorescence demonstrated that many Lbx1 lineal neurons in the ML and FL expressed Viao<sub>a</sub> (Figure 2(b)). Hence, most Lbx1 lineal cells are inhibitory neurons. However, we also noted inhibitory neurons in the FL and DL that were not part of the Lbx1 lineage. Furthermore, some neurons with a history of Lbx1 expression were not inhibitory, as they were Viao<sub>a</sub> negative.

The latter might represent excitatory neurons. To test this hypothesis, we analyzed the co-expression of tdTomato with Vglut1 and Vglut2. These two genes encode different types of vesicular glutamate transporters (Belloccchio et al., 2000; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2000). Previous studies had demonstrated that they are markers of excitatory neurons in the DCN where they are expressed in a mutually exclusive manner (Ito et al., 2011; Lein et al., 2007). We detected some tdTomato<sup>+</sup> neurons that expressed Vglut1 in the FL and DL, but also observed many Vglut1<sup>+</sup> neurons that did not express tdTomato (Figure 2(c)). No neurons co-expressed tdTomato and Vglut2 (Figure 2(d)). In line with our observation of Viao<sub>a</sub> expressing tdTomato<sup>+</sup> cells, we observed many tdTomato<sup>+</sup> cells without Vglut1- or Vglut2 signals (Figure 2(c)). Thus, we concluded that the Lbx1 lineage generates inhibitory interneurons of the DCN. In addition, the Lbx1 lineage contributes a less populous population that is excitatory and of the DCN. Parv<sub>b</sub> is a molecular marker for the small stellate cells in the ML (Caicedo et al., 1996), and all Parv<sub>b</sub> cells of small size in the ML were derived from the Lbx1 lineage (Figure 3(a)). Parv immunoreactivity activity was also observed in cartwheel cells in the ML and FL (Fujiyama et al., 2009; Fujiyama et al., 2009). Cartwheel cells are larger than stellate cells and round-shaped. Parv<sub>b</sub> cartwheel cells in the ML and FL were tdTomato<sup>+</sup> (Figure 3(a)). Another way to probe for cartwheel cells is to use CamKII<sub>x</sub> as a marker (Ochiishi et al., 1998; Takaoka et al., 2005). Immunohistochemical analysis revealed that all CamKII<sub>x</sub> cells co-expressed tdTomato (Figure 3(b)). Golgi cells are the only Gad1 expressing cells in the DL, and one of the two Gad1 expressing types in the FL besides the cartwheel cells (Fujiyama et al., 2009). Fluorescent in situ hybridization in combination with immunohistochemistry revealed that no Gad1<sup>+</sup> cells in the DL co-expressed tdTomato, and only some of the Gad1<sup>+</sup> cells in the FL co-expressed tdTomato (Figure 3(c)). Because all cartwheel cells expressed tdTomato (Figure 3(b)), the tdTomato<sup>+</sup> Gad1 expressing cells in the FL were presumably cartwheel cells. We concluded that Golgi cells did not belong to the Lbx1 lineage. Moreover, we did not probe for the tuberculo-ventral cells, as they do not have a cellular counterpart in the CC.

Furthermore, the DCN contains four types of excitatory neurons. The Vglut2<sup>+</sup> fusiform cells and giant cells were already shown not to contribute to the Lbx1 lineage (Figure 2(d)). However, some Vglut1<sup>+</sup> neurons belong to the Lbx1 lineage (Figure 2(c)). Expression of Vglut1 is restricted to unipolar brush cells and granule cells in the adult DCN (Ito et al., 2011). Unipolar brush cells are marked by Tbr2 (Diño & Mugnaini, 2008), and granule cells are characterized by the expression of Pax6 (Fujiyama et al., 2009). No tdTomato<sup>+</sup> neurons were positive for Tbr2 (Figure 3(d)). By contrast, some tdTomato<sup>+</sup> neurons were positive for Pax6, although we noted that not all Pax6<sup>+</sup> neurons were tdTomato<sup>+</sup> (Figure 3(e)). This indicates that the Lbx1 lineage contributes only a subpopulation of the granule cell.

In combination with the neurotransmitter phenotype, the marker analysis revealed that the Lbx1 lineage contributes cartwheel, stellate, as well as a subpopulation of granule cells to the DCN. It further shows that Golgi, fusiform, giant, unipolar brush, and many granule cells are not derived from the Lbx1 lineage.

Finally, we quantified the relative number of Lbx1 lineal cells within a given neuronal population. Cell counting revealed that 88.0% of the stellate and 90.5% of the cartwheel cells express tdTomato in the adult DCN of Lbx1<sup>Cre<sup>+</sup></sup>:tdTomato reporter mice (Table 1). The ~10% cells that do not express tdTomato likely represent cells with too low expression of Cre to drive the reporter system (Cre-escapers). Similarly, Cre escapers were previously reported for the Pt<sub>10</sub>Cre reporter mouse line in the cerebellum (Hoshino et al., 2005). Thus, likely the entire populations of cartwheel and stellate cells in the DCN belong to the Lbx1 lineage. Moreover, around half of the population of the granule cells within the FL and DL express Lbx1 (Table 1).

### Table 1. Relative number of tdTomato<sup>+</sup> cells per cell type

|                |                  |            |
|----------------|------------------|------------|
| DCN            | Stellate cells   | 88.0 ± 4.3%|
|                | Cartwheel cells  | 90.5 ± 1.8%|
|                | Granule cells    | 48.1 ± 0.9%|
| Cerebellum     | Stellate cells   | 93.9 ± 1.6%|
|                | Basket cells     | 96.1 ± 2.5%|
|                | Golgi cells      | 93.0 ± 1.6%|

Note: Data shown as mean ± SEM. Abbreviation: DCN, dorsal cochlear nucleus.
3.3 | The \textit{Lbx1} lineage contributes stellate, basket, and Golgi cells to the CC

To gain insight into commonalities and differences in the genetic program of the DCN and the cerebellum, we analyzed the contribution of the \textit{Lbx1} lineage to the CC as well. We focused on the CC, because its organization is resembled by cerebellum-like structures. The distribution of \textit{Lbx1} lineal cells was consistent across all subdivisions of the cerebellum (Figure 4(a)). We focused our analysis on the 10th vermal lobule, also called the nodulus. Due to its high number of unipolar brush cells (Englund et al., 2006), this lobule resembles most closely the DCN. Within the nodulus, \textit{tdTomato}+ cells were predominantly in the ML and scattered across the granule cell layer (GL) (Figure 4(b)). The ML of the CC harbors two types of interneurons, the stellate cells that reside in the outer ML, and the basket cells that reside in the inner ML (Voogd & Glickstein, 1998). Both types were \textit{tdTomato}+.
(Figure 4(c)). Moreover, Purkinje cells, which are the large principal cells of the Purkinje cell layer, did not express tdTomato (Figure 4(c)). Parv is a marker for both stellate and basket cells, as well as for Purkinje cells (Stichel et al., 1986). Parv+ neurons in the outer ML and the inner ML were tdTomato+ and thus belong to the Lbx1 lineage, whereas the large Purkinje cells did not (data not shown). To probe for Golgi cells, we used Neurogranin as a marker (Simat age, whereas the large Purkinje cells did not (data not shown). To

Moreover, cerebellar unipolar brush cells are marked by Tbr2 (Englund et al., 2006). No Tbr2+ cells in the cerebellum expressed tdTomato (Figure 4(e)). As the number of tdTomato+ cells in the GL was small, we concluded that the numerous granule cells, which densely populate this layer, do not belong to the Lbx1 lineage. Furthermore, due to the absence of molecular markers, we did not probe for candelabrum and Lugaro cells. Because GFAP+ astrocytes may belong to the Ptf1a lineage of the cerebellum (Hoshino et al., 2005; Pascual et al., 2007), we also probed for astrocytes in the Lbx1 lineage. No GFAP+ cells expressed tdTomato (data not shown). Taken together, the stellate, basket and Golgi cells of the CC are part of the Lbx1 lineage, whereas the Purkinje, granule, and unipolar brush cells are not.

Moreover, cell counting revealed that 93.9% of the stellate, 96.1% of the basket, and 93.0% of the Golgi cell population in the CC belong to the Lbx1 lineage (Table 1). These data suggest that the entire populations of the stellate, basket and Golgi cells of the CC are Lbx1 lineal cells.

3.4 | Embryonic expression pattern of Lbx1 in stellate and cartwheel cells of the DCN

Finally, we wished to compare the embryonic expression pattern of Lbx1 between the tissues and their SCTs. We focused on the DCN, as the embryonic expression pattern of Lbx1 in the CC has been reported (Glassmann et al., 2009). To distinguish between embryonic cartwheel cells and stellate cells, we made use of Bhlhb5 as a marker. The basic helix loop helix transcription factor Bhlhb5 was previously shown to be an embryonal marker of cartwheel cells and unipolar brush cells (Cai et al., 2016). In the DCN primordium of E14.5 embryos, we observed tdTomato+ cells that express Bhlhb5 as well as cells that do not (Figure 5). The Bhlhb5+, tdTomato+ cells represent cartwheel precursor cells, as unipolar brush cells, the only other Bhlhb5 expressing cell type, are not part of the Lbx1 lineage (Figure 3). Lbx1 lineal cells with no Bhlhb5 expression can either represent stellate cells or granule cells. However, Lbx1 is only expressed in postmitotic cells (Sieber et al., 2007) and the majority of the granule cell population is born after E14.5 (Ivanova & Yuasa, 1998; Martin & Rickets, 1981; Pierce, 1967). In contrast, stellate and cartwheel cells are born between E12-E13 (Martin & Rickets, 1981; Pierce, 1967). We therefore conclude that the Bhlhb5+ Lbx1 lineal cells at E14.5 represent stellate cells. Our embryonic expression analysis thus reveals that both cartwheel cells and stellate cells of the DCN express Lbx1 as early as E14.5, shortly after becoming postmitotic.

4 | DISCUSSION

Our Lbx1 lineage analysis provides novel insights into the developmental program underlying formation of the DCN. Knowledge of this genetic program is a prerequisite for understanding the evolutionary developmental process resulting in the emergence of this brain structure in mammals. In addition, our data provide insight into the genetic relationship between the cerebellum and cerebellum-like structures and into the genetic individuation process of Ptf1a lineal inhibitory serial SCTs.

4.1 | Lbx1 expression genetically subdivides both inhibitory and excitatory neurons in the DCN

Co-expression studies with marker genes in the DCN identified a contribution of the Lbx1 lineage to the inhibitory stellate cells and cartwheel cells (Figure 3, Table 1). This is in agreement with the previously reported Lbx1 expression in Ptf1a lineal cells in the developing hindbrain (Pagliardini et al., 2008) and spinal cord (Cheng et al., 2005; Gross et al., 2002; Huang et al., 2008; Kruger et al., 2002; Müller et al., 2002). We noted, however, that Golgi cells, another major Ptf1a lineal inhibitory cell type of the DCN, are not part of the Lbx1 lineage (Figure 3). Thus, our data demonstrate that at least two populations of inhibitory neurons exist in the DCN with respect to their developmental program: cells with a history of Lbx1 expression, and cells without one (Table 2). This heterogeneity may be part of the genetic individuation by which
the four phenotypically different inhibitory subtypes of the DCN are diversified.

In contrast to Ptf1a, the Lbx1 lineage also contributes excitatory neurons in the DCN, as we observed a tdTomato+ subpopulation of granule cells. A similar observation has been reported in the spinal cord where the Lbx1 lineage contributed excitatory cells, as its function becomes antagonized by the transcription factor Tlx3 (Cheng et al., 2005). It will be interesting to analyze whether a similar mechanism is involved in the specification of granule cells in the DCN. The contribution of the Lbx1 lineage to a subpopulation of the granule cells identifies a genetic heterogeneity previously not reported for this cell population. Granule cells receive information from widespread areas of the brain that are associated with multiple sensory modalities. One possibility is that the genetic heterogeneity reflects different sources of input to granule cells. The use of the Lbx1 reporter mouse line will pave the way to solve this issue by detailed studies of the Lbx1 lineage subpopulation of granule cells with respect to their physiological properties and connectivity.

4.2 Lbx1 expression genetically subdivides inhibitory neurons in the CC

Our study further demonstrates that Lbx1 is expressed in the stellate, basket and Golgi cells of the CC, but not in the Purkinje cells (Figure 4, Table 2). Two previous studies are in line with our results. First, in situ hybridization reveals that Lbx1 is expressed in the proliferative white matter of the cerebellum at postnatal Day 8 (Glassmann et al., 2009). This is where and when the stellate and basket cells are born from intermediate progenitors (Leto et al., 2009), before they migrate into the ML. By using genetic lineage tracing, we corroborate that these cell types are part of the Lbx1 lineage (Figure 4(c)). Second, a recent single-cell transcriptomic study has separated the inhibitory lineage into a GABAergic interneuron lineage and a Purkinje cell lineage (Carter et al., 2018). The analysis revealed that Lbx1 is only expressed in the GABAergic interneuron lineage, which agrees with our study. Thus, similar to the DCN, the CC harbors inhibitory cells with and without a history of Lbx1 expression (Table 2). This genetic heterogeneity may contribute to the phenotypic heterogeneity of inhibitory neurons of the CC.

4.3 Similar patterns of Ptf1a and Lbx1 expression in the Lbx1 lineal cells of the DCN and CC

The Lbx1 lineal cartwheel and stellate cells of the DCN are born at E12-E13 (Ivanova & Yuasa, 1998; Martin & Rickets, 1981; Pierce, 1967) and genetic lineage tracing reveals that they express Lbx1 by E14.5 (Figure 5). Hence, these cell types express Lbx1 shortly after birth. In the CC, Lbx1 is expressed in stellate and basket cells of early postnatal mice (Glassmann et al., 2009), which overlaps with the time window during which they are born, that is, mainly between P0 and P7 (Leto et al., 2006; Sudarov et al., 2011; Weisheit et al., 2006). The Lbx1 lineal Golgi cells of the CC are born during late embryonic life. Analysis of the Lbx1Cre/+; tdTomato reporter mice at E17.5 uncovered that cells of the prospective CC, among them likely the Golgi cells, express Lbx1 (data not shown). Thus, all Lbx1 lineal cells of the DCN and CC likely express Lbx1 shortly after birth.

Previous studies in r4/5 which contribute the prospective inhibitory cells of the DCN (Fujiyama et al., 2009; Sieber et al., 2007) showed Ptf1a expression in mitotically active progenitors and Lbx1 expression in postmitotic neurons. In the CC, Ptf1a is expressed in mitotically active progenitors of the stellate, basket, and Golgi cells (Fleming et al., 2013; Hoshino et al., 2005; Pascual et al., 2007) Thus, Ptf1a expression in the progenitor state, and Lbx1 expression in the early postmitotic state are commonalities between Lbx1 lineal cells of the DCN and CC and suggest a similar context for cell specification in both regions.

4.4 Evolutionary development of a cerebellum-like auditory structure

The mammalian auditory hindbrain structures represent evolutionary novelties, as they are not present in other vertebrates. Insight into their developmental program therefore also sheds light on evolutionary processes. The DCN belongs to the cerebellum-like structures that all receive sensory input in the periphery and parallel fiber input in the ML. The distribution of a given cerebellum-like structure in the different vertebrate groups indicates that these structures arose multiple times independently. The cerebellum-like rostrolateral nucleus, for instance, sporadically occurs in few neoterygian fish, with absence in sister groups of taxa in which it is present (Bell, 2002; Saidel & Butler, 1997). It was therefore suggested that the similarities among different cerebellum-like structures, as well as their similarities to the cerebellum itself, are due to a shared developmental gene regulatory network module, active in each of these structures (Bell, 2002).

Various observations support this hypothesis. All cerebellum-like structures are derived from the somatosensory portion of the alar plate (Bell, 2002). Ectopic cerebellum-like structures can be induced in the prospective caudal diencephalon or midbrain of chickens, when

| TABLE 2 Lbx1 lineages among the phenotypically similar Ptf1a lineal serial SCTs of the CC and DCN |
|-------------------|-------------------|
| Cerebellar stellate cells | Lbx1 lineage |
| DCN stellate cells | Lbx1 lineage |
| Cerebellar Golgi cells | Lbx1 lineage |
| DCN Golgi cells | not Lbx1 lineage |
| Cerebellar Purkinje cells | not Lbx1 lineage |
| DCN cartwheel cells | Lbx1 lineage |

Abbreviations: CC, cerebellar cortex; DCN, dorsal cochlear nucleus; Lbx1, Ladybird homeobox protein homolog 1; Ptf1a, pancreatic transcription factor 1a; SCTs, sister cell types.
beads containing Fgf8 are implanted nearby (Martinez et al., 1999). Molecular analysis demonstrated that the factor caused reduced expression of the transcription factor Otx2 and induced expression of En1, Wnt1, and Fgf8 itself (Martinez et al., 1999). Furthermore, spontaneous mutations in three different genes, that is, in the gene encoding 82 glutamate receptor subunit (Lurcher mouse), the gene encoding the retinoic acid receptor-related orphan receptor alpha (Staggerer mouse), and the gene encoding cytosolic carboxypeptidase 1 (Purkinje cell degeneration mouse) cause postnatal degeneration of Purkinje cells as well as cartwheel cells in the DCN (Berrebi et al., 1990). This has led to the conclusion that both cells form a genetically related cell family (Berrebi et al., 1990). Finally, Atoh1 and Ptf1a lineages contribute excitatory and inhibitory neurons to both the cerebellum and the DCN (Arie et al., 1997; Fujiyama et al., 2009; Hoshino et al., 2005; Pascual et al., 2007) and the respective excitatory cell types and the inhibitory cell types represent thus serial SCTs (Figure 1(b)) (Arendt et al., 2016). Together, these data support a scenario where a genomically hardwired gene regulatory module installed in r1 to generate the complex cerebellum, was activated during mammalian evolution in r4/5 to generate the cerebellum-like structure of the DCN.

Our observation that stellate cells of both the DCN and the cerebellum are part of the Lbx1 lineage and exhibit a similar sequential expression pattern of Ptf1a and Lbx1 support a shared genetic developmental program between these cell types. In contrast, the observed differences in Lbx1 expression in Golgi and Purkinje/cartwheel cells of both tissues reveal also differences in genetic developmental program of serial SCTs. We note, however, that differences in recruitment of Lbx1 or any other gene regulatory element cannot easily be taken as evidence against a shared genetic developmental program between the cerebellum and cerebellum-like structures. Clearly, more data regarding gene regulatory elements involved in cell specification are needed to draw any conclusion on a shared genetic developmental program between cerebellum and related structures. For example, inhibitory interneurons of the CC undergo an intermediate progenitor stage that is associated with Pax2 expression (Marichich & Herrup, 1999; Weisheit et al., 2006). As newly born cartwheel cells immediately start expressing the marker Bhlhb5 (Cai et al., 2016), they seem to differentiate directly suggesting that inhibitory neurons of the DCN do not undergo an intermediate progenitor stage. Still, like the intermediate progenitors in the CC, cartwheel cells and a Bhlhb5-negative population express Pax2 (Cai et al., 2016). It will be interesting to analyze whether Pax2 has similar functions in DCN and CC, even though the developmental program of the inhibitory neurons of the DCN does not seem to involve an intermediate progenitor stage.

### 4.5 Genetic individuation of Ptf1a lineal inhibitory serial SCTs

The scenario outlined above makes predictions for the process of genetic individuation of the serial SCTs in the DCN and cerebellum. The process of genetic individuation of Golgi cells, stellate cells, and Purkinje/cartwheel cells should be highly similar between the DCN and cerebellum. However, our comparative Lbx1 lineage analysis uncovered marked differences in the genetic individuation of phenotypically similar SCTs of the DCN and cerebellum (Table 2). Cartwheel cells of the DCN and Golgi cells of the CC belong to the Lbx1 lineage, whereas their counterparts, the Purkinje cells in the CC and the Golgi cells in the DCN, do not. Thus, phenotypically highly similar serial SCTs share genetic components such as Ptf1a, but also display distinct factors. This indicates that the emergence of a neuromorphologically closely related serial SCT is not simply based on recapitulation of the process of genetic individuation. Instead, this evolutionary process might recruit different transcription factors. Confirmation of this reasoning will, of course, depend on detailed functional analysis of the role of Lbx1 in the inhibitory serial sister cells of the DCN and cerebellum. In general, Lbx1 acts as a cell autonomous selector to establish the identity of neurons and its loss results in respecification or cell death. In the hindbrain, for instance, Lbx1 controls the choice between the specifications of somatosensory versus viscero sensory fates of relay neurons (Sieber et al., 2007). In the dorsal spinal cord, knockout of Lbx1, transforms GABAergic neurons into glutamatergic neurons (Cheng et al., 2005). Interestingly, also glycine transporter 2 expression, a marker of a glycinergic transmitter phenotype, depends on Lbx1 expression (Huang et al., 2008). Therefore, Lbx1 in cartwheel cells might contribute to their use of glycine as neurotransmitter. In general, deviations between serial SCTs from a strict recapitulation of a gene regulatory module for differentiation might provide the flexibility to tailor each cell type to circuit specific tasks.

Genetic differences between serial SCTs are likely the consequences of the fundamental organizational principle of the developing nervous system. The early neural tube is essentially composed of transverse neural segments, the so-called neuromeres, which intersect with longitudinally arranged zones. This results in a checkerboard pattern of distinct progenitor domains (Nieuwenhuys, 2017; Puelles, 2013), where the major histogenetic events such as cell proliferation and differentiation take place. This “Bauplan” facilitates the generation of serial SCTs and at the same time lays the basis for differences in their genetic individuation.

### 4.6 Summary

In summary, our analysis revealed a complex and differential contribution of the Lbx1 lineage to the DCN and the cerebellum. In both tissues, Lbx1 genetically differentiates between distinct types of inhibitory neurons. Unexpectedly, the Lbx1 lineal cell types differ largely between the DCN and the cerebellum. Future work should therefore be directed toward the functional role of Lbx1 in the DCN and CC. It will, for instance, be important to analyze whether Lbx1 acts as a selector gene (Ho bert, 2008) by contributing neuronal identity features to Golgi cells and cartwheel/Purkinje cells, and whether its function is similar in stellate cells in the DCN and CC. In addition, more comparative gene expression studies are required for understanding similarities and differences in the genetic program underlying the anatomical resemblance of the CC and the DCN and more general,
between the cerebellum and cerebellum-like structures. A rich selection of candidate genes is already provided by the currently published single-cell transcriptomic atlas of the developing cerebellum (Carter et al., 2018). The differentially expressed transcription factors listed therein are likely to play a role in genetic individuation of cerebellar SCTs and the serial SCTs present in cerebellum-like structures.

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