Early Deletion of Neurod1 Alters Neuronal Lineage Potential and Diminishes Neurogenesis in the Inner Ear

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Neuronal development in the inner ear is initiated by expression of the proneural basic Helix-Loop-Helix (bHLH) transcription factor Neurogenin1 that specifies neuronal precursors in the otocyst. The initial specification of the neuroblasts within the otic epithelium is followed by the expression of an additional bHLH factor, Neurod1. Although NEUROD1 is essential for inner ear neuronal development, the different aspects of the temporal and spatial requirements of NEUROD1 for the inner ear and, mainly, for auditory neuron development are not fully understood. In this study, using Foxg1²Cre for the early elimination of Neurod1 in the mouse otocyst, we showed that Neurod1 deletion results in a massive reduction of differentiating neurons in the otic ganglion at E10.5, and in the diminished vestibular and rudimental spiral ganglia at E13.5. Attenuated neuronal development was associated with reduced and disorganized sensory epithelia, formation of ectopic hair cells, and the shortened cochlea in the inner ear. Central projections of inner ear neurons with conditional Neurod1 deletion are reduced, unsegregated, disorganized, and interconnecting the vestibular and auditory systems. In line with decreased afferent input from auditory neurons, the volume of cochlear nuclei was reduced by 60% in Neurod1 mutant mice. Finally, our data demonstrate that early elimination of Neurod1 affects the neuronal lineage potential and alters the generation of inner ear neurons and cochlear afferents with a profound effect on the first auditory nuclei, the cochlear nuclei.

Keywords: Foxg1, cochlear nuclei, Neurod1, vestibular system, auditory system, neurons, hair cells, projections

INTRODUCTION

The inner ear is a highly organized structure of interlinked channels and chambers built to encode sound, space orientation, and motion (Goodrich, 2016; Elliott et al., 2021). The sensory organs are represented by five vestibular epithelia (the maculae of utricle and saccule, and the cristae of the three semicircular canal ampullae), and, in mammals, by the auditory sensory organ of the cochlea, the organ of Corti (Rubel and Fritzsch, 2002; Fritzsch et al., 2013). The sensory receptors for hearing and balance are mechanotransducing hair cells (Elliott et al., 2018). Apart from sensory hair cells, the sensory epithelium consists of non-sensory supporting cells necessary for hair cells’ development,
function, and maintenance. The sensory epithelia are innervated by neurons of the inner ear of two different ganglia, the vestibular and spiral ganglia. The vestibular ganglion is located in the lateral portion of the internal auditory meatus, with the superior division innervating the utricle, the superior semi-circular canal, and the lateral semi-circular canal, while the inferior region of the vestibular ganglion innervates the saccule and the posterior semi-circular canal (Khan and Chang, 2013). The auditory spiral ganglion twists along the length of the cochlear duct with peripheral neuronal processes innervating hair cells within the auditory sensory epithelium and central axons merging into the cochlear nerve (Pavlinkova, 2020). These inner ear neurons transmit received information from the sensory cells to the central nervous system.

All sensory organs of the inner ear and its associated sensory ganglia derive from a single embryonic source, the otic placode. Neurons seem to be the first differentiated cells in the developing inner ear in all species examined (Fritzsch and Straka, 2014). The initial specification of the neuroblasts within the otic epithelium is followed by the delamination of neuroblasts as early as embryonic day nine (E9) in the mouse embryo. The neuronal cells form the inner ear ganglion, later separating into the vestibular and spiral ganglia between E9.5 and E14.5 (Ma et al., 2000). Cells fated to develop as sensory hair cells and supporting cells of the sensory epithelia arise from the prosensory domain of the otocyst (Wu and Kelley, 2012). Deciphering the developmental mechanisms of these specialized neurosensory cells of the inner ear is a central focus of cell-based therapy strategies.

The development of sensory hair cells, neurons, and non-sensory cells in the inner ear is regulated by a network of signaling pathways and transcription factors. Proneural atonal-related basic helix-loop-helix (bHLH) transcription factors, ATOH1, NEUROGENIN1 (NEUROG1), and NEUROD1, are critical early regulators of inner ear neurogenesis (Bermingham et al., 1999). DE afford the first bHLH factor upregulated in the otocyst to initiate the specification and differentiation of inner ear neurons (Ma et al., 2000). Neurog1 null mice lack inner ear neurons and have a massive loss of sensory epithelia through the alteration of Atoh1 expression (Ma et al., 2000; Matei et al., 2005; Kim et al., 2001).

Additionally, NEUROG1 activates the expression of the downstream bHLH gene, Neurod1, which is essential for neuronal differentiation (Kim et al., 2001). Neurod1 null mice exhibit severely impaired differentiation of auditory and vestibular neurons (Liu et al., 2000a; Kim et al., 2001) but they also suffer from other neuronal developmental defects (Miyata et al., 1999; Liu et al., 2000b) and a severe diabetic phenotype (Kim et al., 2001). Therefore, conditional Neurod1 deletion mutants were generated to investigate more specifically its role in inner ear neuronal development and hearing function. Conditional Pax2loxP; Neurod1f/f deletion mice showed abnormalities in the formation of inner ear ganglia, disorganized cochlear innervation, and unsegmented vestibular and spiral ganglion afferents (Jahan et al., 2010a). Unfortunately, Pax2loxP; Neurod1f/f mice have Neurod1 deletion in the ear and the central auditory nuclei, limiting the evaluation of spiral ganglion neuronal viability and central projections and hampering physiological assessment of the wiring defects. Furthermore, Pax2loxP activity decreases with age, starting at E10.5 and producing uneven deletion effects (Duncan and Fritzsch, 2013). A delayed conditional Neurod1 deletion using Isl1Cre eliminates Neurod1 from differentiating inner ear neurons and retains Neurod1 expression in the auditory nuclei and midbrain (Macova et al., 2019). The Isl1Cre; Neurod1f/f mutants form the unsegregated and disorganized peripheral projection map of spiral ganglion neurons with altered sensory information processing in the central auditory pathway (Macova et al., 2019; Filova et al., 2020). However, detailed insights into the effects of an early absence of Neurod1 in otic neuroblasts are lacking.

To go beyond existing data and reveal the dependency of early neuronal development on the otocyst-expressed NEUROD1 protein, we chose the Foxg1loxP transgene to eliminate Neurod1. Foxg1loxP has been demonstrated to lead to earlier and more profound recombination than other Cre drivers expressed in the ear placode and the developing ear (Duncan and Fritzsch, 2013; Dvorakova et al., 2020). Foxg1loxP is not expressed in the auditory and vestibular nuclei to possibly affect neuronal viability in the inner ear (Hébert and McConnell, 2000; Bérubé et al., 2005; Kasberg et al., 2013; Abrams and Reiter, 2021). In this study, we revisited the embryonic phenotype of Neurod1 deletion mice to determine NEUROD1 requirements for the generation and survival of neuroblasts and, overall, for early inner ear development.

MATERIAL AND METHODS

Experimental Animals

All experiments with mice were approved by the Animal Care and Use Committee of the Institute of Molecular Genetics, Czech Academy of Sciences. Animals were housed in a controlled environment with 12-h light/dark cycles and free access to water and food. For obtaining the mouse model with conditional deletion of Neurod1, we crossed Foxg1loxP knock-in/knock-out mice (129Cg-Foxg1tm(cre)Skf/1; #004337 The Jackson Laboratory) with mice carrying the Neurod1 gene flanked by loxP sites (Neurod1loxP/loxP) (Goebbels et al., 2005). Mice were genotyped using DNA isolated from the tail. The following PCR primers were used: Cre Forward 5′-GCC TGC ATT ACC GTG CGA TGC AAC GA-3′ and Cre Reverse 5′-GTG GCA GAT GGC 3′ with a 700 bp product; Neurod1 Forward 5′-ACC ATG CAC TCT GTA CGC TGC GCA ACG CCA CCA TT-3′ with a 700 bp product; Neurod1 Forward 5′-ACC ATG CAC TCT GTA CGC ATT-3′ and Neurod1 Reverse 5′-GAG AAC TGA GAC ACT CAT CTC-3′ with a 400 bp product for the WT allele or 600 bp for the allele containing loxP sites. The noon of the day the vaginal plug was found was defined as embryonic day 0.5 (E0.5). Mice of both sexes were used for experiments.

Morphological Evaluation of the Inner Ear, Cochlear Nucleus, and Inferior Colliculus

Pregnant mice were sacrificed at noon, and embryos or dissected embryonic tissues were then fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Neuronal nuclei were transcardially perfused with PBS followed by 4% PFA. Brains and inner ear were dissected from the skull and fixed in 4% PFA. For长时间
storage, tissues were kept in methanol at −20°C. Embryos or tissues were stained as whole mounts or 80-μm vibratome sections (Leica VT1000 S). Samples were blocked in a blocking solution consisting of 2.5% donkey or goat serum, 0.5% Tween20, and 0.1% Triton X-100 for at least 1 h at room temperature. Next, samples were incubated with primary antibodies (Supplementary Table S1) diluted in blocking solution for 72 h at 4°C. After several washes with PBS, samples were incubated with secondary antibodies (Supplementary Table S1) for 24 h at 4°C. Finally, cell nuclei were labeled with Hoechst 33258 (Merck 861405; always showed as blue staining) diluted 1:2000 in PBS. After staining, samples were mounted in Aqua-Poly/Mount (Polysciences 18606) or prepared an anti-fade medium. Images were taken on Zeiss LSM 880 NLO inverted confocal microscope and Nikon CSU-W1 spinning disk confocal microscope. NIS-Elements, ImageJ, and ZEN software were used for image processing.

The area of the inner ear ganglion at E10.5 was determined as ISL1+ area in 80-μm vibratome transverse sections of the embryo (dotted area). For quantification, sections with the two largest areas containing the inner ear ganglion were picked for each embryo, and outliers were excluded (n = 10 embryos/genotype; 16

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**FIGURE 1** | Brain abnormalities are detected in Neurod1CKO mutants. **(A,D)** Adult brain size was reduced in Foxg1Cre+/+, Neurod1loxP/loxP heterozygotes (+/−) and Foxg1Cre+/+, Neurod1loxP/loxP homozygous (−/−) mutants compared to controls. **(B)** The Neurod1CKO cerebellum was smaller with many Purkinje cells (Calbindin+) but with fewer granule cells (Pax6+). **(C,E,F)** The cochlear nucleus’s size and inferior colliculus were significantly reduced in Neurod1CKO mutants compared to controls and heterozygotes. The dotted line indicates the boundaries of the cochlear nucleus. The values represent means ± SD, one-way ANOVA. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Scale bars: 1,000 μm (A,B); 200 μm (C). CN, cochlear nucleus; DCN, dorsal cochlear nucleus; HS, Hoechst nuclear staining; IC, inferior colliculus; VCN, ventral cochlear nucleus.
sections/genotype). In the area of inner ear ganglion, ISL1+ cells were counted in the same sections using the Cell Counter ImageJ plugin. For quantifying proliferation and apoptosis, anti-Phospho-Histone H3 and Cleaved Caspase3 antibodies were used, respectively. Phospho-Histone H3 or Cleaved Caspase3 positive cells were counted in the ISL1+ ganglion area in vibratome sections at E10.5. For each genotype two sections per embryo were selected (n = 5 embryos/genotype). We compared the size of E18.5 auditory and vestibular organs between controls and mutants. Inner ear whole mounts were stained with an antibody against hair cell marker, MyosinVIIa. The cochlear length was established as a line between inner and outer hair cells from the apical tip to the base. The length of the organ of Corti was measured using the "Measure line" ImageJ plugin. The size of the vestibular organ epithelium was measured using immunolabeling of the MyosinVIIa+ area using "Polygon selection," and "Measure" functions of ImageJ. Volumes of the cochlear nucleus (CN) and inferior colliculus (IC) were determined by using 80-μm coronal brain sections (n = 5 brains/genotype). The areas of the left and right CNs and ICs were measured in all sections, and the total volume of the brain structures was calculated. Parvalbumin+ bushy cells were counted.
in 80-μm vibratome sections (image view 134.95 × 134.95 μm) of adult VCN (two sections/one animal and four animals/genotype). Number of parvalbumin+ bushy cells per measured area of VCN was determined. To quantify endbulbs of Held, we used vibratome sections of the adult VCN. Three cells with the visually largest VGLUT1+ area were selected and measured in each section (two sections/one animal and four animals/genotype). The VGLUT1+ area of the synapse was determined per the bushy cell area.

**Lipophilic Dye Tracing**

Heads of P0 pups were fixed in 4% PFA in 0.1 M phosphate buffer with 300 mM sucrose (to minimize neuronal swelling) for 48 h at 4°C and processed for dye tracing. To trace projections of the ear to the brain, we used NeuroVue dyes (NV Jade, NV; NV Red, NVR) as previously described (Fritzsch et al., 2016; Schmidt and Fritzsch, 2019). Lipophilic dyes were applied using dye-soaked filter strips, which provide a more precise and reliable dye application than crystals or dye-injections (Schmidt and Fritzsch, 2019). For tracing inner ear central projections to the brain, the dye was placed into the inner ear to label the spiral ganglion, organ of Corti, and vestibular neurons. The dye was allowed to diffuse for 24–72 h, depending on the embryonic age and dye. Mutant and control samples were always processed in parallel, with an identical dye placement and dye diffusion time. Brains and ears were subsequently microdissected, mounted in glycerol, and imaged using a Leica SP8 confocal microscope. After imaging, some brains and ears were post-fixed in 4% PFA and either processed for immunohistochemistry (see above) or sectioned at 100-μm, mounted in glycerol, and imaged to reveal the distribution of projections in sections.

**Scanning Electron Microscopy**

Inner ears devoid of cartilage were fixed in 2.5% glutaraldehyde and 2% formaldehyde in 1×PHEM buffer at °C overnight. After that, tissues were washed with 1×PHEM buffer a few times, dehydrated in graded ethanol series, and finally transferred into 100% acetone and dried to a critical point in Leica CPD300 with CO2. The dried samples were mounted on regular SEM stubs using conductive carbon and coated with 7 nm of platinum in Leica ACE600. The images were taken using a dual-beam system FEI Helios NanoLab 660 G3 UC scanning electron microscope at 2 kV and 0.2 nA with an Everhart-Thornley secondary electron detector.

**Experimental Design and Statistical Analysis**

All comparisons were made between animals with the same genetic background, typically littermates, and we used male and female mice. The number of samples (n) for each comparison can be found in the individual method descriptions and in the corresponding figures. Phenotyping and data analysis was performed blind to the genotype of the mice. All values are presented either as the mean ± standard deviation (SD) or standard error of the mean (SEM). For statistical analysis, GraphPad Prism software was used. The differences in the mean were compared using unpaired two-tailed t-test or one-way ANOVA tests for statistical evaluation. Significance was determined as p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), or p < 0.0001 (****). Complete results of the statistical analyses are included in the figure legends.

**RESULTS**

**Neurod1 Mutants Have Reduced Body Size and Vestibular Dysfunction**

Neurod1 was eliminated by crossing Neurod1loxPloxP mice (Goebbels et al., 2005) with Foxg1Cre line (Hébert and McConnell, 2000), which has efficient Cre activity during early ear development, starting in the otic placode (Dvorakova et al., 2020). We confirmed Foxg1Cre expression in sensory cells and neurons in the inner ear using tdTomatoAi14 reporter mice (Supplementary Figures S1A,B). Foxg1Cre activity was undetectable in the cochlear nucleus, showing no tdTomato expression in neurons of the cochlear nucleus (Supplementary Figures S1C,D). Homozygous mutant embryos (Neurod1CKO; genotype Foxg1Cre+/+, Neurod1loxPloxP) were recovered at the expected Mendelian ratios at all embryonic days examined in utero (Supplementary Figure S2A), suggesting no effect of Neurod1 elimination on embryonic survival. However, postnatal development of homozygous Neurod1CKO and heterozygous (Foxg1Cre+, Neurod1loxPlox+) mutants was reduced (Supplementary Figure S2B), with fewer mice identified at the time of genotyping (3 weeks of age) compared to control mice (without Cre allele, genotypes: Foxg1+/+, Neurod1loxPloxP). Neurod1CKO mice were born significantly smaller than their littermates (Supplementary Figure S2C), indicating abnormalities during embryonic development. Neurod1CKO failed to thrive from birth to weaning with significant deficits in body size and body weight compared to controls at postnatal day P21. Neurod1CKO mice suffered from severe ataxia (Supplementary Movie), and due to rapid deterioration, had to be sacrificed 3–4 weeks after birth. Surprisingly, the decreased gene dose of Neurod1 in heterozygous mutant mice negatively affected their postnatal development. Although these mice did not present any signs of ataxia, they were significantly smaller than controls.

To investigate the reduced postnatal survival of homozygous and heterozygous Neurod1 mutants, we first evaluated changes in the brain since Foxg1Cre is highly expressed in the forebrain. Correspondingly, the size of the forebrain was visibly more petite, and the weight of the brain was significantly reduced in both homozygous and heterozygous Neurod1 mutants compared to controls (Figures 1A,D). The size and morphology of the cerebellum and the size of the cochlear nucleus were exclusively affected only in Neurod1CKO mutants (Figures 1B,C,E,F). Analysis of the gross external morphology of born pups showed no abnormalities in an eye formation, in a shape of the forehead or the snout (Supplementary Figure S2D). Foxg1Cre is expressed in the olfactory system and eye (Dvorakova et al., 2020), but the aspects of the olfactory epithelium and retina of the visual system were comparable between control and Neurod1CKO (Supplementary Figure S3). Our results confirmed the previous study's conclusions that NEUROD1 is
not required for eye (Penniset al., 2003) and olfactory embryonic development (Packard et al., 2011).

Reduced Sensory Epithelia and a Severe Loss of Spiral Ganglion Neurons are Found in the Neurod1CKO Inner Ear

Next, we evaluated the inner ear morphology of heterozygous and homozygous Neurod1 mutants. Radial fibers, spiral ganglion, and the organ of Corti of heterozygous Foxg1<sup>Cre</sup>/+<br>Neurod1<sup>loxP/+</sup> mice were comparable to the morphology of the control cochlea (Supplementary Figure S4). The formation and size of sensory epithelia of the vestibular-end organs and innervation of Foxg1<sup>Cre</sup>/+<br>Neurod1<sup>loxP/+</sup> were also similar to control mice. Thus, the normal inner ear morphology and the normal vestibular behavior indicate that eliminating one Neurod1 allele did not affect the inner ear development of the heterozygous Neurod1 mutant. In contrast, the cochlea of Neurod1CKO was severely shortened, with the length of the organ of Corti reaching only 47% of the littermate control at E18.5 (Figures 2A–D,K). Besides the length of the cochlea, the most noticeable deficiency in Neurod1CKO was a reduction of radial fibers. Higher-magnification images showed substantially decreased and disorganized innervation of the sensory epithelium with a few fibers of outer spiral bundles turning randomly toward the base or apex (Figure 2B') compared to dense parallel outer spiral bundles directed toward the base in the control cochlea (Figure 2A'). Additionally, abnormalities in the apical epithelium of Neurod1CKO with multiple hair cell rows were noticeable compared to the control cochlea (Figures 2C,D). Correspondingly, all sensory epithelia of the vestibular end-organs of Neurod1CKO were significantly reduced compared to controls (Supplementary Figure S5).

In line with severe reduction of innervation in the Neurod1CKO cochlea, we found only a rudiment of the spiral
ganglion at P0, represented by a clump of NeuN+ neurons (Figures 2F,H), compared to the spiral ganglion neurons located in the Rosenthal’s spiral canal in controls (Figures 2E,G). In contrast to controls, some NeuN+ neurons were entangled with fibers in the Neurod1CKO cochlea (Figures 2E,F). Some of these fibers were type Ia neuron afferents, as shown by anti-calretinin labeling of the cochlea in adult mice (Supplementary Figure S6). Thus, these results indicate a significant loss of neurons, severe abnormalities in the formation of the spiral ganglion and innervation.

Early Inner Ear Neuronal Development Is Altered in Neurod1CKO

Having recognized a severe loss of neurons of Neurod1CKO, we wanted to determine the effects of Neurod1 elimination on early inner ear development. First, we determined the efficiency and relative onset of Neurod1 deletion in the otocyst of Neurod1CKO. We could not detect any NEUROD1 expressing cells in the ear epithelium or any NEUROD1+ delaminating neuroblasts in Neurod1CKO at E9.5 and E10.5 (Figure 3). The elimination of NEUROD1 protein was consistent with the regional expression of Cre recombinase under Foxg1. ISL1 expressing neurons were detected in the otic ganglion of Neurod1CKO. However, the number of ISL1+ cells was noticeably reduced as early as E9.5, indicating a negative effect of Neurod1 elimination on early neurogenesis in the inner ear. Note the decreased ISL1+ neuroblasts in the epithelium of the Neurod1CKO otocyst compared to the control. Second, we measured the size of the inner ear ganglion. The Neurod1CKO ganglion was reduced by 53% compared to control embryos at E10.5 (Figures 4A–D). The number of neurons expressing ISL1 in Neurod1CKO was decreased on average by about 80% (96 ± 5, n = 10 embryos) compared to controls (390 ± 20, n = 10 embryos). Interestingly, the number of proliferating cells in the ganglion was similar between the control and Neurod1CKO inner ear (Figures 4E,F,I). However, we detected massive apoptosis in neurons of the Neurod1CKO ganglion. The number of apoptotic cells was six times higher in the mutant than in the control otic ganglion (Figures 4G,H,J). These results indicate that NEUROD1 is important for neurogenesis and neuronal survival in the early stages of inner ear neuronal development.
Depleted Inner Ear Neurons of Neurod1CKO Form an Aberrant Cochlear and Vestibular Ganglia

After a period of proliferation, all inner ear neurons undergo terminal mitosis, begin to differentiate and extend their processes to the peripheral and central targets (Pavlinkova, 2020). By E13.5, sensory neurons cease to proliferate as the last spiral ganglion neurons exit the cell cycle in the apex, and thus, the final number of neurons of the mouse inner ear is established (Matei et al., 2005). The superior and inferior vestibular ganglia in the control inner ear were segregated, and the cochlear neurons formed a spiral along the sensory epithelia at E13.5 (Figure 5A). In contrast, the number of neurons in Neurod1CKO was severely reduced, forming a diminished vestibular ganglion and a rudiment of the cochlear ganglion near the saccule and cochlear base (Figure 5B). The abnormalities in neuronal development were even more apparent in the Neurod1CKO cochlea at E14.5. The spiral ganglion neurons were in the Rosenthal’s canal, extending projections, and located near the SOX2+ sensory epithelium in the control cochlea (Figures 5C,C'). At E14.5, cochlear neurons form the SG along with the sensory cells of the organ of Corti in the control cochlea (Continued).

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smaller size soma in Neurod1CKO (Figures 6E,F) in contrast to the vestibular ganglion of control mice with neurons exclusively labeled by utricle-dye applications (Figure 6B).

**Elimination of Neurod1 Affects Central Projections of Inner Ear Neurons**

The cochlear nucleus is the first auditory nucleus in the brain, where the auditory nerve fibers project. Although Foxg1Cre is not expressed in the cochlear nucleus (Supplementary Figure S1), a substantial size reduction of the cochlear nucleus in Neurod1CKO indicates a secondary effect of reduced afferent input from the cochlea (Figure 1E). In line with reduced auditory afferents, we found significantly attenuated clusters of auditory-nerve endbulbs of Held that wrap the somas of spherical bushy cells in the Neurod1CKO cochlear nucleus (Figure 7). A critical step in inner ear neurogenesis is the segregation of auditory and vestibular neurons into a spiral ganglion and vestibular ganglion and the segregation of the central projections (Dvorakova et al., 2020). Dye tracing analyses demonstrated disorganized and reduced central projections in the cochlear nucleus of Neurod1CKO compared to the segregated basal and apical projections in control mice (Figures 8A,B). Note the unusual projections from the apex near the cochlear nucleus entry area. Coronal sections of control mice showed clear segregation of the base and apex with minimal overlap (Figure 8C) in contrast to primarily overlapping and diminished basal and apical projections in the Neurod1CKO AVCN (Figures 8D,D'). Lipophilic NeuroVue dyes applied to the vestibular end-organs showed projections to the vestibular nuclei in the control brain (Figure 8E). Using single dye tracing, we showed that fibers labeled by dye application in the utricle reach both the cochlear nucleus (AVCN) and vestibular nuclei (LVN, SVN, MVN) in Neurod1CKO mice (Figure 8F), indicating an interconnection between the vestibular and auditory system.

**Ectopic Hair Cells Are Formed in the Neurod1CKO Inner Ear**

The development of sensory cells was disrupted by the elimination of Neurod1, indicated by significantly smaller vestibular end-organ sensory epithelia and a shorter organ of Corti in the cochlea (Figure 2 and Supplementary Figure S5). Additionally, the cochlear sensory epithelium was disorganized. Immunolabeling with the inner hair cell (IHC) marker calretinin and prestin, a marker of outer hair cells (OHC), showed multiple OHC rows, transdifferentiated (ectopic) IHCs among OHCs, and two rows of IHCs in the adult apex of Neurod1CKO compared to three rows of OHCs and a single row of IHCs in control (Figures 9C,D). Missing OHCs were found in the Neurod1CKO base (Figures 9A,B). Scanning electron microscopy images demonstrated cellular abnormalities in the sensory epithelium.
of Neurod1CKO, indicating disrupted planar cell polarity (Figures 9E,F). An additional effect associated with Neurod1 deficiency was a noticeable presence of ectopic hair cells located outside of sensory epithelia. Besides regular sensory epithelia containing MyosinVIIa expressing hair cells in the saccule, and IHCs and OHCs in the organ of Corti Neurod1CKO cochlea, extra ectopic MyosinVIIa positive cells were found near the saccule (Figure 10A and Supplementary Figure S5B) and in the cochlea, near the rudimental spiral ganglion (Figures 10A,B). Detailed images showed the distribution of ectopic hair cells intermingled with neuronal fibers, and some cells were double labeled by tubulin and MyosinVIIa (Figures 10A’,B’). These topologically inappropriate "trans-fated" ectopic hair cells form a unique organization in the area of the two neuronal aggregations of vestibular and cochlear neurons, independent of known inner ear structures.

DISCUSSION

The focus of this study was to determine the requirements for NEUROD1 in the early neuronal development of the inner ear and processes that are affected by Neurod1 elimination (Figure 11). NEUROD1, a bHLH transcription factor, is an essential factor for the lineage commitment and differentiation in various developmental systems, including gastrointestinal cells (Cherry et al., 2011), pancreas (Romer et al., 2019; Bohuslavova et al., 2021), brain (Miyata et al., 1999; Liu et al., 2000b; Hevner et al., 2006), and neurosensory organs (Liu et al., 2000a; Cherry et al., 2011). The main function of NEUROD1 in neurogenesis and the promotion of neuronal fate is supported by its ability to reprogram other somatic cells into neurons. For example, forced expression of Neurod1 enhances neuronal conversion from human fibroblasts (Pang et al., 2011), and exogenous Neurod1 expression converts mouse astrocytes to neurons (Guo et al., 2014). In the inner ear, neuronal development is initiated by the upregulation of Neurog1 in the anteroventral quadrant of the otocyst. Neurog1 is followed by the upregulation of Neurod1 in the proneural domain of the otocyst, resulting in the delamination and migration of primary neuroblasts to the forming otic ganglion. Delaminating neuronal progenitors proliferate and differentiate to produce vestibular and auditory neurons between E9.5 to E13.5 (Matei et al., 2005).

In contrast to the Neurog1 deletion mutant with no neurons found in the E10.5 otic ganglion (Ma et al., 1998) or any later stages of inner ear development (Liberman, 1991), some inner ear neurons differentiate and form peripheral and central projections in Neurod1 null mice (Liu et al., 2000a; Kim et al., 2001). Previously, we generated a delayed Neurod1 conditional deletion using Isl1cre that spares many cochlear and vestibular neurons, resulting in the near-normal vestibular behavior and partially preserved auditory system function (Macova et al., 2019). Similarly, Pax2cre is delayed and leaves many more neurons in conditional Neurod1 deletion mice (Jahan et al., 2010a) relative to Foxg1cre, used in this study. Consequently, these phenotypes indicate a temporal effect of Neurod1 elimination for the neuronal loss in the developing inner ear. However, mechanisms of neuronal loss due to the Neurod1 elimination are not fully understood. Different mechanisms have been implicated in determining neuron quantities, including neuronal delamination, migration, proliferation, and survival. Our study revisited the major events of early neuronal development of the inner ear to address different regulatory functions and temporal requirements for NEUROD1.

Foxg1cre recombination occurs efficiently and invariably in the ear placode, forebrain, olfactory and pre-lens placodes, anterior optic vesicle, pharyngeal endoderm, and midhindbrain junction, matching the normal pattern of Foxg1 expression (Dvorakova et al., 2020; Hébert and McConnell, 2000; Dastidar et al., 2011; Duggan et al., 2008; Shen et al., 2018; Panalippan et al., 2018). A caveat must be considered in using Foxg1cre line. First, haploinsufficiency of the Foxg1 gene due to a partial replacement of Foxg1 coding sequences with the cre gene (Hébert and McConnell, 2000) is associated with impaired development of the telencephalon and microcephaly in heterozygous Foxg1cre/+ transgenic mice similar to the phenotype of heterozygous Foxg1+/− null mice (Shen et al., 2018; Frullanti et al., 2016; Eagleson et al., 2007; Kawaguchi et al., 2016). Correspondingly, a similar neurodevelopmental phenotype of a reduced size of the forebrain was found in our heterozygous Foxg1cre/+; Neurod1loxP/+ mutants (Figure 1). Second, ectopic low levels of Cre activity have been reported due to genetic background effects (Hébert and McConnell, 2000;...
Disorganized central projections of Neurod1CKO inner ear neurons show aberrant interconnections between the auditory and vestibular systems. (A,B) Lipophilic differently colored NeuroVue dyes were applied to the apex and base to label cochlear afferents. In the lateral view of the cochlear nucleus of the control, dye tracing shows the normal segregated basal and apical cochlear afferents of the auditory nerve and segregated projections to the AVCN and DCN. In contrast, reduced, disorganized, and overlapping central projections are shown in the cochlear nucleus of Neurod1CKO mice. Note unusual fibers from the apex projecting away from the cochlear nucleus and auditory nerve (arrowhead). (C) Coronal sections of control show the segregation of basal and apical afferents. (D,D′) Sections of Neurod1CKO show an overlap of disorganized fibers from the apex and base. Dotted lines in corresponding figures indicate the section planes in (A,B). (E,F) Dye tracing from the utricle and the posterior canal crista (PCC) shows vestibular afferents innervating the lateral, medial, and superior vestibular nuclei that are immediately adjacent to the AVCN in the control mice. A single dye tracing from the utricle shows vestibular afferents reach vestibular nuclei and form a profound projection to the AVCN in Neurod1CKO mice. The area of the AVCN is indicated by dotted circle. Scale bars: 100 µm. AN, auditory nerve; AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; LVN, MVN, SVN, lateral, medial, and superior vestibular nuclei; VN, vestibular nerve.
Ma et al., 2002). To confirm the scope of Cre activity in our model, we crossed Foxg1Cre mice to a tdTomatoAi14 reporter mouse strain. We verified Foxg1Cre-mediated loxP recombination in the organ of Corti, spiral ganglion neurons, and the cerebellum of adult progeny recapitulating endogenous Foxg1 expression (Supplementary Figure S1). We did not detect any Foxg1Cre activity in the cochlear nucleus, showing no tdTomato expression in neurons of the cochlear nucleus. Because Foxg1Cre is not expressed in the cochlear nucleus, the reduced size of the Neurod1CKO cochlear nucleus is exclusively an effect of diminished afferent input from spiral ganglion type I neurons, similar to cochlear ablation studies (Mostafapour et al., 2000).

We showed that early elimination of Neurod1 affected delaminating and migrating ISL1+ neurons in the E9.5 and E10.5, resulting in a significantly smaller inner ear ganglion at E10.5 (Figure 4). One mechanism contributing to the diminished number of inner ear neurons in Neurod1CKO was massive apoptosis at E10.5, confirming that Neurod1 is required for early neuronal survival (Liu et al., 2000a). In contrast, neoblasts lacking Neurod1 proliferate at a similar rate as neuronal precursors in control embryos, suggesting that NEUROD1 is not needed for the proliferation of neurons, at least in the E10.5 inner ear. Intriguingly, our data confirmed the initial finding in the global Neurod1 deletion mutant (Liu et al., 2000a) that some inner ear neurons survive without Neurod1. One could speculate that the neurogenic fate commitment is predefined early in some Neurog1+ specified neuronal precursors ensuring terminal differentiation of these Neurod1 null neurons. Alternatively, residual neurons might survive by compensatory activation of different transcription factor(s), such as GATA3 (Duncan and Fritzsche, 2013) or NHLH1/NHLH2 bHLH factors (Krüger et al., 2006).

Despite diminished neurogenesis, these Neurod1 lacking neurons form inner ear ganglia, grow neuritic processes, establish bipolar connections to their targets, and persist up to the adulthood of Neurod1CKO mice. The formation of inner ear ganglia was considerably disrupted when the last sensory neurons undergo terminal mitosis, at E13.5 (Matei et al., 2005). The vestibular ganglion was diminished, and the spiral ganglion was represented by a small clump of neurons near the saccule in the Neurod1CKO inner ear (Figure 5). In addition to a significant loss of neurons, the rudiment of the spiral ganglion was misplaced away from the sensory epithelium in the Neurod1CKO cochlea, indicating migration defects. Cochlear neurons found in the aberrant vestibular ganglion (“spiro-vestibular” ganlion) of Neurod1CKO further corroborate abnormalities in inner ear neurons’ migration (Figure 6). The remaining neurons projected disorganized peripheral fibers, with some neurons forming unusual peripheral projections connecting the cochlea and the vestibular end-organs. The central projections of our Neurod1CKO mutant are primarily identical to central projection abnormalities in various Neurod1 deletion mutants (Kim et al., 2001; Jahan et al., 2010a; Macova et al., 2019; Filova et al., 2020). Mainly, central projections of cochlear and vestibular neurons are reduced, unsegregated, and disorganized. Using our Neurod1 mutant, we showed, for the first time, central projections from vestibular neurons reached the cochlear nucleus, the first processing station in the central auditory system (Figure 8). Consistently with the aberrant migration and disorganized innervation phenotypes of Neurod1 mutants, NEUROD1...
orchestrates transcriptional networks regulating axon guidance, neurite growth, cell migration, and adhesion in post-mitotic inner ear neurons (Filova et al., 2020).

In addition to neuronal phenotypes, Neurod1 deletion affects the development of inner ear sensory organs (Kim et al., 2001; Macova et al., 2019; Filova et al., 2020; Jahan et al., 2010b). Like other Neurod1 deletion mutants, due to Foxg1Cre activity in the sensory precursors, we were unable to uncouple secondary effects of Neurod1 deletion in inner ear neurons on sensory cell development. The elimination of Neurod1 affects the size of inner ear sensory epithelia and the differentiation and organization of apical epithelium in the cochlea. Nevertheless, some effects are more pronounced in our Neurod1CKO than in delayed Neurod1 mutants suggesting a temporal dependency of Neurod1 elimination. Particularly, the truncated cochlea of Neurod1CKO reached 47% length of the control cochlea compared to 60% of the cochlea of mice with delayed Neurod1 mutation (Macova et al., 2019). We also found a more profound formation of ectopic myosin VIIa positive hair cells near the two remaining neuronal aggregations of vestibular and cochlear sensory neurons (Figure 10) compared to the Neurod1 conditional mutation generated by Pax2Cre (Jahan et al., 2010b). Previous work identified a set of upregulated genes in the absence of Neurod1, particularly Atoh1, Fgf8, and Nhlh2 (Jahan et al., 2010b), suggesting that some neuronal precursors adopt a hair cell fate and differentiate as hair cells (Fritzsch and Elliott, 2017; Elliott et al., 2021). Taken together, NEUROD1 mediates a neuronal program and promotes neuronal fate by the upregulation of downstream targets and through suppression of other bHLH genes such as Atoh1. Another possible role of NEUROD1 in neurogenesis and neurogenic competence may be reprogramming of the epigenome in the developing inner ear (Matsuda et al., 2019). Further experiments will be necessary to investigate how NEUROD1 mediates transcriptional and possibly epigenetic networks during neuronal development in the inner ear. By understanding the interplay of NEUROD1 regulatory

**FIGURE 10** | Ectopic hair cells differentiate outside the sensory epithelia in the Neurod1CKO inner ear. Representative images of whole mount immunolabeling with MyosinVIIa (MyoVII, a marker of hair cells) and tubulin (neuronal fibers) show hair cells in the saccule and rows of hair cells forming the organ of Corti in the Neurod1CKO cochlea. Ectopic MyoVIIa positive cells (eHCs) are found near the saccule and the aberrant cochlear ganglion (a dotted line delineates the area). Larger magnification images show eHCs entangled with neuronal fibers, and some cells are double-labeled for tubulin and MyoVIIa. Scale bars: 100 µm (A, B); 20 µm (A', B').
networks capable of initiating cell-fate changes, we hope to develop efficient therapeutic strategies to restore and regenerate neurons for clinical applications.

A crucial role of NEUROD1 in neurogenesis and neuronal differentiation in the inner ear is conserved across vertebrates, as shown by studies in chicken (Evens et al., 2013), Xenopus laevis (Schlosser and Northcutt, 2000), and zebrafish (Schwarzer et al., 2020). These studies also provide significant insights into the role of NEUROD1 as a neurogenic programing factor. For example, ectopic expression of Neurod1 is sufficient to mediate neurogenesis in the chicken developing inner ear (Evens et al., 2013). In zebrafish, the expression of Neurod1 is maintained in progenitor cells in a neurogenic niche that are expected to contribute to neuronal regeneration in the adult zebrafish statoacoustic ganglion (Schwarzer et al., 2020). Understanding a role of NEUROD1 in neurogenesis of species that can produced neurons throughout life and regenerate damaged neurons can provide significant insights into the rekindling of regeneration in the mammalian inner ear. An importance of NEUROD1 as a neurogenic reprograming factor is supported by in vivo studies utilizing Neurod1 to produce functional neurons by reprogramming astrocytes (Guo et al., 2014; Brulet et al., 2017). A reprogramming cells within the auditory system is considered a promising way to cure hearing loss (Meas et al., 2018).

CONCLUDING REMARKS

Our findings provide insights into the temporal requirements for NEUROD1 during inner ear development. In early neuronal development, we demonstrated that NEUROD1 is necessary for the generation and survival of neuronal precursors, as the absence of Neurod1 results in massive apoptosis of E10.5 neuroblasts. In later neuronal development of post-mitotic neurons, Neurod1 is critical for segregating neurons of the vestibular and auditory systems, migration, and formation of peripheral and central projections of these neurons. Understanding how neuronal fate is promoted within the nervous system and how neural circuit assemblies are organized has essential implications for cell-based therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the Institute of Molecular Genetics, Czech Academy of Sciences.

AUTHOR CONTRIBUTIONS

GP and BF designed and supervised the experiments. IF, RB, MT, EY, and BF performed experiments and analyzed the data. IF prepared the first draft of the manuscript. GP, EY, and BF...
reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.845461/full#supplementary-material

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