Neurod1 Is Essential for the Primary Tonotopic Organization and Related Auditory Information Processing in the Midbrain

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Hearing depends on extracting frequency, intensity, and temporal properties from sound to generate an auditory map for acoustical signal processing. How physiology intersects with molecular specification to fine tune the developing properties of the auditory system that enable these aspects remains unclear. We made a novel conditional deletion model that eliminates the transcription factor *NEUROD1* exclusively in the ear. These mice (both sexes) develop a truncated frequency range with no neuroanatomically recognizable mapping of spiral ganglion neurons onto distinct locations in the cochlea nor a cochleotopic map presenting topographically discrete projections to the cochlear nuclei. The disorganized primary cochleotopic map alters tuning properties of the inferior colliculus units, which display abnormal frequency, intensity, and temporal sound coding. At the behavioral level, animals show alterations in the acoustic startle response, consistent with altered neuroanatomical and physiological properties. We demonstrate that absence of the primary afferent topology during embryonic development leads to dysfunctional tonotopy of the auditory system. Such effects have never been investigated in other sensory systems because of the lack of comparable single gene mutation models.

Key words: auditory pathway; cochlear nucleus; inferior colliculus; *Neurod1* mutation; plasticity; sensory topographical map

Significance Statement

All sensory systems form a topographical map of neuronal projections from peripheral sensory organs to the brain. Neuronal projections in the auditory pathway are cochleotopically organized, providing a tonotopic map of sound frequencies. Primary sensory maps typically arise by molecular cues, requiring physiological refinements. Past work has demonstrated physiologic plasticity in many senses without ever molecularly undoing the specific mapping of an entire primary sensory projection. We genetically manipulated primary auditory neurons to generate a scrambled cochleotopic projection. Eliminating tonotopic representation to auditory nuclei demonstrates the inability of physiological processes to restore a tonotopic presentation of sound in the midbrain. Our data provide the first insights into the limits of physiology-mediated brainstem plasticity during the development of the auditory system.

Introduction

Sensory systems develop topographic maps of sensory inputs in nervous systems: the somatosensory system projects topograph-

ical maps of the skin to the brainstem, midbrain, and cortex (Penfield and Boldrey, 1937; Renier et al., 2017), the olfactory system projects odor properties to a given olfactory glomerulus (Mombaerts, 1999; Gogos et al., 2000), the retina develops a topographical map in the midbrain (Sperry, 1963; Goodhill, 2007; Huberman et al., 2008), and vestibular, lateral line, and electroreceptive organs project to specific nuclei of the hindbrain and midbrain to establish computational maps (Krahe and Maler, 2014; Chagnaud et al., 2017). In the auditory system, cochlear sensory hair cells are connected to the brain by spiral ganglion (SG) neurons that are organized within the cochlea in an orderly fashion according to frequency, so called tonotopic organization, with high frequencies at the base and low frequencies at the apex

Received Oct. 10, 2018; revised Nov. 17, 2018; accepted Dec. 5, 2018.

Author contributions: I.M. and K.P. wrote the first draft of the paper; J.S., B.F., and G.P. edited the paper; R.B., J.S., B.F., and G.P. designed research; I.M., K.P., T.C., M.D., R.B., and B.F. performed research; I.M., K.P., T.C., M.D., R.B., B.F., and G.P. analyzed data; B.F. and G.P. wrote the paper.

This work was supported by the Czech Science Foundation (17-04719S to G.P.), by BIOCEV CZ.1.05/1.1.00/02.0109 from the ERDF, by the Czech Academy of Sciences RVO: 86652036, by Charles University (GA UK 324615 to I.M. and GAUK 780216 to M.D.), and by the NIH (R01 AG060504 to B.F.). We thank Dr. K. Kandler for helpful comments on an earlier version of the paper, Dr. D. Šuta for help processing single-unit data, and A. Pavlinek for editing the paper.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.2557-18.2018>
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(Rubel and Fritzsch, 2002; Muniak et al., 2016). This tonotopic (or cochleotopic) organization is maintained throughout the auditory pathways (Kandler et al., 2009). The formation of a tonotopic map requires the precise projection of the SG neuron afferents of the cochlea onto the first auditory nuclei of the hind-brain, the cochlear nuclei (CN). In contrast to the better-characterized visual system or olfactory system, only some molecular mechanisms are known to lead to the cochleotopic mapping of spiral ganglion afferents onto the CN (Cramer and Gabriele, 2014; Goodrich, 2016; Yang et al., 2017) but it is unknown how much the initial cochleotopic map is physiologically refined (Marrs and Spirou, 2012). Developing second-order maps are highly plastic (Hubel et al., 1977; Renier et al., 2017) and convergence of multiple sensory organs can plastically reshape primary sensory maps as a compromise between activity and molecular cues (Constantine-Paton and Law, 1978; Elliott et al., 2015). Indeed, the auditory system is well known for having a high level of plastic changes throughout life (Syka, 2002; Eggermont, 2017) but how embryonic development affects and possibly limits these later plastic changes remains unclear because of the lack of models (Kral et al., 2016) beyond simply removing parts of the cochlea (Harrison, 2016).

In the formation of the primary tonotopic map, several factors apparently define the precision of some SG neuron projections (Cramer and Gabriele, 2014; Lu et al., 2014; Goodrich, 2016; Yang et al., 2017). The basic helix-loop-helix (bHLH) gene *Neurod1* (Liu et al., 2000) was shown to be essential for inner ear neuronal development as well as normal growth of the cochlea. Subsequent work on mutants null for *Neurod1* showed retention of some sensory neurons using specific neuronal tracing techniques (Kim et al., 2001). *NEUROD1* cross-regulates other transcription factors in neurons and hair cells, leading to the transformation of some neurons to intraganglionic hair cells, and transformation of some outer hair cells to inner hair cells (Jahan et al., 2010b). In addition, deletion of *Neurod1* leads to gross projection mapping errors of the few remaining neurons (Jahan et al., 2010a) that go beyond those described in other primary sensory system (Huberman et al., 2008). In previously generated mutants, *Neurod1* deletion occurs both in the ear and the central auditory nuclei, which limits SG neuronal viability and hampers physiological assessment of the wiring defects (Gurung and Fritzsch, 2004; Fritzsch et al., 2006; Jahan et al., 2010a).

We therefore generated a novel mutant with a conditional deletion of *Neurod1* only in the ear to spare many SG neurons and to retain *Neurod1* expression in the auditory nuclei and auditory midbrain. We show here how a shortened and nearly overlapping cochleotopic projection from SG neurons to the CN is expanded across the entire inferior colliculus (IC), affecting the frequency, intensity, and temporal processing of the central auditory system of adult mice at the physiological and behavioral level. Unique to our study are the consequences of compressing the unsegregated and disorganized peripheral projection map of SG neurons onto the tonotopic organization of the central auditory pathways. This type of disorganization of a neural map of the sensory periphery is nearly impossible to achieve with other sensory systems that would require, for example, trigeminal neurons to the face to also innervate the foot or retina ganglion neurons to connect to both eyes and the brain.

Materials and Methods

Animals

All experiments using animals were performed according to protocols approved by the Animal Care and Use Ethics Committee of the Institute of Molecular Genetics, Czech Academy of Sciences. The experimental mice were housed in a controlled environment (12 h light/dark cycles) with *ad libitum* access to food and water. All experiments were performed with littermates (males and females) crossbred from two transgenic mouse lines: floxed *Neurod1* (*Neurod1*^{loxP/loxP}; Goebbels et al., 2005) and *Isl1*-cre (*Isl1*-cre; *Isl1*^{tm1(cre)Sev}) from The Jackson Laboratory. Breeding pairs contain a mouse with two floxed *Neurod1* alleles (*Neurod1*^{loxP/loxP}) and a mouse with one floxed *Neurod1* allele together with one *Isl1*-cre allele (*Isl1*^{cre/+}; *Neurod1*^{loxP/+}). Genotyping was performed by PCR on tail DNA. The specific primers used were the following: *Isl1*-cre F 5'-GCC TGC ATT ACC GGT CGA TGC AAC GA-3' and *Isl1*-cre R 5'-GTG GCA GAT GGC GCG GCA ACA CCA TT-3' with a 700 bp product; *Neurod1* F 5'-ACC ATG CAC TCT GTA CGC ATT-3' and *Neurod1* R 5'-GAG AAC TGA GAC ACT CAT CTG-3' with a 400 bp product for the WT allele or 600 bp for the floxed allele. Heterozygous mice *Isl1*^{cre/+}; *Neurod1*^{loxP/+} (*HET*) were comparable to the control mice (*Isl1*^{+/+}; *Neurod1*^{loxP/loxP} or *Isl1*^{+/+}; *Neurod1*^{loxP/+}) without any detectable morphological and functional differences. *Neurod1*CKO offspring were recovered at expected Mendelian ratios from E9.5 to P0 birth (123 litters collected and genotyped: 234 *Neurod1*CKO; 260 *HET*; 534 control offspring). The sex ratio of *Neurod1*CKO mice at weaning showed the same representation of males and females as the control mice.

Morphology of the cochlea, cochlear nuclei, and IC

X-gal staining. The mouse line *Isl1*-cre was bred with R26R-*lacZ* (*Gt(ROSA)26Sor^{tm1Sor}*; The Jackson Laboratory) and animals carrying both loci were subjected to X-gal staining (Dvorakova et al., 2016).

Lipophilic dye tracing. We studied the pattern of innervation in whole or dissected ears using lipophilic dye tracing in aldehyde fixed tissue as previously described (Fritzsch et al., 2016a). At least three mutants and similar numbers of control littermates of both sexes were shipped to the University of Iowa and used for different stage (E13.5, E14.5, E16.5, 6 at E18.5, P0, P3 and 6 at P7). We inserted filter strips loaded with different colored lipophilic dyes into the cochlear apex, base, vestibular end organs, cochlear/vestibular nuclei of the brainstem around rhombomere 5 to label afferents, and into rhombomere 4 near the midline to label facial motoneurons/efferents to the ear (Simmons et al., 2011). After allowing appropriate time for diffusion of the lipophilic tracer (between 48 and 120 h), we prepared the ears as whole mounts, mounted with glycerol on a glass slide, using appropriate spacers to avoid distortion, and imaged them using a Leica SP8 confocal microscope. Image stacks were collected and single images or sets of stacks were obtained to provide detailed information about the progressive development and loss of ear innervation over time. Selected ears were further dissected to reveal the detailed innervation of the flat mounted sensory epithelia. Images were compiled into plates to show the most pertinent details using Corel Draw. Only general image modifications, such as contrast or brightness adjustments, were used to enhance the visual appeal without affecting the scientific content.

Immunohistochemistry and morphological evaluations. For whole mounts, dissected tissues were fixed in 4% paraformaldehyde (PFA). For vibratome sections, 4% PFA-fixed samples were embedded in 4% agarose gel and sectioned at 80 μ m on a Leica VT1000S vibratome. The primary antibodies used were rabbit anti-Myo7a (1:500; 25-6790, Proteus BioSciences), rabbit or mouse anti-NeuN (1:500, ab177487, Abcam; or 1:100, MAB377, Merck), mouse anti-acetylated α -tubulin (1:400; T6793, Sigma-Aldrich), mouse anti-tubulin β 3 (Tuj1; 1:500; 801202, BioLegend), rabbit anti-neurofilament 200 (1:200; N4142, Sigma-Aldrich), rabbit anti-Prox1 (1:500; 925201, BioLegend), mouse anti-VGLUT1 (1:200; MAB5502, Merck), rabbit anti-parvalbumin (1:2000; ab11427, Abcam), rabbit anti-calretinin (1:100; sc-50453, Santa Cruz Biotechnology), goat anti-prestin (1:50; sc-22692, Santa Cruz Biotechnology), goat anti-Neurod1 (1:100; sc-1084, Santa Cruz Biotechnology),

mouse anti-Islet1 (1:50; Developmental Studies Hybridoma Bank 39.3F7 was deposited to the DSHB by T. M. Jessell/S. Brenner-Morton), and mouse anti-C-terminal binding protein 2 (CtBP2; 1:200; 612044, BD Biosciences). The secondary antibodies used were AlexaFluor 488 AffiniPure Goat Anti-Mouse IgG (1:500; 115-545-146, Jackson ImmunoResearch), AlexaFluor 594 AffiniPure Goat Anti-Rabbit (1:500; 111-585-144, Jackson ImmunoResearch), and DyLight488-conjugated AffiniPure Mouse Anti-Goat IgG (1:500; 205-485-108, Jackson ImmunoResearch). Nuclei were stained by Hoechst 33258 (1:2000; 861405, Sigma-Aldrich). Samples were mounted in Aqua-Poly/Mount (18606, Polysciences) or in prepared Antifade medium and images were taken on Zeiss LSM 5 DUO, Zeiss LSM 880, or Leica SPE confocal microscopes. ImageJ and ZEN software were used for image processing.

For neuron quantification, neurons were counted in all NeuN/Tuj1 stained vibratome sections containing a SG using the “Cell Counter” ImageJ plugin, as described previously (Bohuslavova et al., 2017). Briefly, the total number of neurons from all sections per individual cochlea was determined ($n = 3/\text{genotype/age}$). The mean number of neurons in control mice represented 100% of the SG neurons. To determine the length of the cochlea, individual adult cochleae were flat-mounted with the sensory epithelium facing up and the entire length of the cochlear duct from the hook region along the basilar membrane was measured using the “Measure line” ImageJ plugin ($n = 3/\text{genotype}$). The volume of the CN and IC was established by analyzing parallel serial equally spaced sections through the brain. Eighty micrometer coronal vibratome sections were prepared by sectioning five brains of control and five brains of mutant mice. The areas of the left and right CN and IC were determined in each section using ImageJ, and the volume of the organs was calculated. Volumes of paired organs were normalized to body weight. The quantification of neuron soma size was done using the lipophilic tracer dye labeled samples. The area of neuron somas (80 neurons/2 mutants) was determined in image stacks using ImageJ. Data are provided as mean $\pm$ SD.

c-Fos staining. Auditory stimulation was described previously (Karmakar et al., 2017). For each experiment, two 2-month-old mice (control and *Neurod1*CKO pair) were placed in a small wire cage under a speaker in a soundproof room. These animals were kept in silence for 1 h and then 15 kHz tone pips at 75 dB sound pressure level (SPL) followed for 90 min. Immediately after sound exposure, mice were transcardially perfused by 4% PFA and brains were dissected and fixed in 4% PFA for 1 h. Tissues were mounted into 4% agarose in PBS and 80 μm coronal sections were cut on a Leica VT1000S vibratome. Sections were blocked in 2.5% normal goat serum, 0.5% Tween and 0.1% Triton in PBS. To detect activated neurons in the CN, we used primary rabbit anti-c-Fos antibody (1:5000; PC38, Calbiochem) and secondary goat anti-rabbit antibody conjugated by AlexaFluor 594 (1:500; 111-585-144, Jackson ImmunoResearch). Stained sections were mounted in Antifade medium and pictures were taken on a Zeiss LSM 880. c-Fos⁺ neurons in the CN were counted using Cell Counter (ImageJ) and statistics were made using GraphPad software (unpaired *t* test, $n = 4/\text{genotype}$).

Hearing function evaluation

Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) tests were performed on mice as described previously (Chumak et al., 2016). Briefly, for ABR recording, responses to tone bursts (3 ms duration, 1 ms rise/fall times, frequencies of 2, 4, 8, 16, 32, and 40 kHz) and clicks of different intensity were recorded. The response threshold to each frequency was determined as the minimal tone intensity that still evoked a noticeable potential peak in the expected time window of the recorded signal. Click-evoked ABR responses were used to analyze the amplitude of single ABR waves and the latency of positive ABR peaks I to IV. For comparison of amplitude-intensity functions, the amplitude of a single ABR wave was calculated as the peak to peak interval and normalized in relation to the wave amplitude at 90 dB SPL because of significant differences in ABR wave amplitude between the groups. For DPOAE tests, cubic (2 F1–F2) distortion product otoacoustic emissions over a F2 frequency range from 4 to 38 kHz were recorded (control, $n = 12$; *Neurod1*CKO, $n = 8$).

Table 1. Number of animals used

Experimental approach	No. of animals	
	<i>WT</i>	<i>Neurod1</i> CKO
Histology of the inner ear	39	38
Histology of the brain	20	17
Dye tracing	27	27
Physiology	30	25

Behavioral tests

In the behavioral tests, eight mice were used from both experimental groups (control and *Neurod1*CKO) at the age of 2–3 months. All behavioral tests were performed in a sound-attenuated chamber (Coulbourn Habitest, model E10–E21) located in a soundproof room. During the testing procedure, the mouse was confined to a small wire mesh cage on a motion-sensitive platform. The animal's reflex movements were detected and transduced by a piezoelectric accelerometer. The amplified voltage signal was acquired and processed using a TDT system III with Enhanced Real-Time Processor RP2.1 (Tucker Davis Technologies) and custom-made software programmed in MATLAB. The startle responses were evaluated in a 100 ms window beginning at the onset of the startle stimulus. The magnitude of the acoustic startle reflex was given by the maximal peak-to-peak amplitude of transient voltage occurring in the response window. Acoustic startle stimuli (tone pips or noise bursts) and prepulse stimuli were generated by the TDT system and presented via a loudspeaker (SEAS, 29AF/W) placed 12 cm above the platform inside the chamber. Stimulus presentation and data acquisition were controlled by a custom-made application in MATLAB. The acoustic startle reflex (ASR; a transient motor response to an intense, unexpected stimulus) was used as an indicator of the behavioral responsiveness to sound stimuli. The ASRs to 4, 8, 16, and 32 kHz tone pips and white noise (WN) bursts (50 ms duration, 3 ms rise/fall times, varying intensity levels) were recorded. Each test session contained the following: a baseline trial (–10 dB SPL stimulus intensity) and 13 startle stimuli of different intensities (50, 55, 60, 65, 70, 75, 80, 85, 90, 100, 110, 115, and 120 dB SPL). The intertrial interval varied from 15 to 50 s.

In the prepulse inhibition procedure, three different trial types were used: a baseline trial without any stimulus, an acoustic startle pulse alone (WN at 110 dB SPL, 50 ms, 3 ms rise/fall times), and a combination of the prepulse and startle pulse. The interstimulus interval between the prepulse and the startle stimulus was set to 50 ms; each of the trial types was presented three times. The intertrial interval was randomized and varied from 15 to 30 s. The efficacy of the prepulse inhibition (PPI) of the ASR was expressed as an ASR ratio in percentage: 100% corresponds to the amplitude of the ASR without a prepulse; smaller values of the ASR ratio indicate greater PPI. As a prepulse we used: either (1) WN bursts or tone pips (50 ms duration, 3 ms rise/fall time) at frequencies of 4, 8, and 16 kHz at increasing intensities; or (2) a gap in the background WN of low intensity (65 dB SPL). Both, when presented before the startle stimulus, are expected to decrease the amplitude of the startle response that followed.

Vestibular tests

To evaluate the motor coordination of the mice from both strains (control, $n = 4$; *Neurod1*CKO, $n = 3$), we used three stable rods of varying thickness and length (rod 1: 25 mm diameter, 50 cm distance from the open end to the end line; rod 2: 20 mm, 35 cm to the end line; and rod 3: 15 mm diameter, 35 cm to the end line). Two times were measured: orientation time, time taken to turn 180° from the starting position toward the shelf; and transit time, the time taken to travel to the end line (Deacon, 2013). For statistical purposes, we set the maximal test time at 300 s. If the mouse fell off a rod, the maximal score (300 s) was assigned for that particular rod. All tests were performed in an anechoic soundproof room and acoustic stimuli were delivered in free-field conditions via a two-driver loudspeaker system (Selenium 6W4P woofer and RAAL70–20 tweeter). Four types of trials were used: silence, the presence of a continuous broad band noise with intensity 80 dB SPL and presence of a 600 ms series of 100 μs clicks at an intensity of 70 dB SPL with

Figure 1. Morphology of the *Neurod1*cKO inner ear is altered. **A–D**, Whole-mount immunostaining with anti-Myo7a [a marker of hair cells (HCs)] and anti-acetylated α -tubulin (nerve fibers) antibodies shows subtle changes in a number of radial fibers (RFs), lack of the intraganglionic spiral bundle (IGSB; arrow), noticeable disorganization of RFs, and overshooting fibers in the apex of *Neurod1*cKO cochlea (**C, D**). Scale bar, 50 μ m. **E–H**, Whole-mount immunostaining of SG neurons (anti-NeuN, a neuronal soma marker) and innervation shows reduction and disorganization of SG neurons in mutants. Scale bar, 50 μ m. **E'–H'**, Higher-magnification images demonstrate a decreased number and altered distribution of SG neurons. Scale bar, 20 μ m. **I, J**, Immunohistochemistry for Myo7a and for β tubulin (a marker of inner pillar supporting cells) shows the disorganization of OHCs (red) and inner pillar cells (IPs; green) in the apex in *Neurod1* cKO compared with the organ of Corti of control littermates at P7. Scale bar, 20 μ m. HS, Hoechst nuclear staining. **K, L**, Immunohistochemistry for prestin (a marker for OHC) and calretinin (Figure legend continues.)

interstimulus intervals linearly decreasing and increasing from 25 to 6.6 ms and back to 25 ms.

Extracellular recording of the neuronal activity in the IC

For extracellular recording, *Neurod1* cKO ($n = 9$; 432 units from the IC) and control mice ($n = 10$; 480 units) were tested. The surgery and extracellular recording in the IC were performed in mice anesthetized with 35 mg/kg ketamine (Calypsol, 50 mg/ml) and 6 mg/kg xylazine (Xylapan, 20 mg/ml) in saline via intraperitoneal injection. Approximately every hour, additional subcutaneous injections of one-half of the original dose of the anesthetics were administered to keep a sufficient level of anesthesia. Basic reflexes (pedal reflex or eye blink reflex), respiratory rate, and heart rate, were monitored. For access to the IC, an incision was made through the skin of the skull and underlying muscles were retracted to expose the dorsal cranium. A holder was glued to the skull and small holes were drilled over both ICs. Neuronal activity (multiple units) in the IC was recorded using a 16-channel, single shank probe (NeuroNexus Technologies) with 50 μ m between the electrode spots. The signal obtained from the electrode was amplified 10000 times, bandpass filtered over the range of 300 Hz to 10 kHz and processed by a TDT System III (Tucker Davis Technologies) using an RX5-2 Pentusa Base Station. The data were recorded and processed in BrainWare software (Jan Schnupp, Tucker Davis Technologies) for artifact rejection and separation of single units on the basis of spike-shape clustering and then analyzed with custom software based on MATLAB (MathWorks). The recorded data were processed and analyzed with custom software based on MATLAB. The stimulation signals were generated using a TDT System III with the RP 2.1 Enhanced Real-Time Processor. Acoustic stimuli were delivered in free-field conditions via a two-driver loudspeaker system (Selenium 6W4P woofer and RAAL70-20 tweeter) placed 70 cm in front of the animal's head.

Frequency-intensity mapping. To determine the neuronal receptive fields, pure tones (frequency 2–40 kHz with 1/8 octave step, 60 ms duration, 5 ms rise/fall times, various intensity with 5 dB step) were presented in a random order, each stimulus appearing three times. A discrete matrix corresponding to the response magnitude evoked by each of the frequency-intensity combinations was thereby obtained, smoothed using cubic spline interpolation and used for extraction of the basic parameters: the excitatory response threshold (the lowest stimulus intensity that excited the neuron, measured in dB SPL), the characteristic frequency (CF), the frequency with the minimal response threshold (measured in Hz), and the bandwidth of the excitatory area 10, 20, and 30 dB above the excitatory threshold, expressed by quality factor Q ($Q = \text{CF}/\text{bandwidth}$).

Two-tone stimulation. To detect inhibitory areas, a two-tone stimulation was used. Pure tone at the neuron's CF fixed 10 dB above the threshold at CF and pure tone pips of variable frequency and intensity, analogous to those used for the excitatory area mapping, were simultaneously presented. Similar to frequency-intensity mapping, a two-dimensional matrix was obtained, and the presence of the low- and high-frequency sideband inhibitory areas was evaluated.

Rate intensity function of the IC neurons. Neuronal responses to broadband noise (BBN) bursts of variable intensity (10 dB steps, 50 repetitions) were used to construct the rate intensity function (RIF). On each RIF, two points of interest were defined: R10, describing the starting

point of the RIF's rise, and R90, describing the RIF's transition to the saturated region. A 100% scale was assigned to the neuron's total range of response amplitudes, with 0% corresponding to spontaneous activity and 100% corresponding to its maximum response magnitude. The two points of interest, R10 and R90, correspond to 10 and 90% of this scale, respectively. On each RIF, two points of interest were defined: R10, describing the starting point of the RIF's rise, and R90, describing the RIF's transition to the saturated region (Bures et al., 2010). The RIF was qualified as saturating if the response magnitude within the top 10 dB interval of the highest stimulus intensities was flat ($\pm 10\%$). The RIF was qualified as non-monotonic if the response magnitude at the highest stimulus intensity was smaller than the maximum response magnitude by $>20\%$. The remaining RIFs were qualified as strictly monotonic. RIFs were further used for evaluating the following parameters: the percentage of saturating, non-monotonic, and strictly monotonic RIFs; the sound pressure level (S10) corresponding to point R10; the relative response at R10; the dynamic range (DR) of the RIF: $\text{DR} = \text{S90} - \text{S10}$; and the maximum response magnitude. Spontaneous activity of the IC neurons was determined at the 0 dB SPL BBN stimulation. A Fisher's exact test was used to examine the relationship between mutant and control neurons with a certain RIF.

Temporal properties of the IC neurons. Two types of stimuli were used: (1) a 600 ms series of 100 μ s clicks at an intensity of 70 dB SPL for control and 80 dB SPL for *Neurod1* cKO mice with interstimulus intervals linearly decreasing and increasing from 25 to 6.6 ms and back to 25 ms, and (2) trains of five clicks at an intensity of 70 dB SPL for control and 80 dB SPL for *Neurod1* cKO mice with various interclick intervals (100, 50, 30, 20, 15, 10, and 5 ms). In the case of the long changing clicks stimulus, we calculated the percentage of the clicks in the train to which the neurons responded in the time window that started 5 ms after the click and lasted 5 ms. For trains of five clicks with different interspike intervals we computed the vector strength (VS) values along with the Rayleigh statistics that were computed for each spike pattern; only responses with Rayleigh statistics of least 5.991 were considered as significantly phase-locking (Zhou and Merzenich, 2008). The VS quantifies how well the individual spikes are synchronized (phase-locked) with a periodic signal.

Experimental design and statistical analysis

All comparisons were made between animals with the same genetic background, typically littermates. The number of mice used for different analyses was as follows:

The number of samples (n) for each comparison can be found in the individual method descriptions and are given in the corresponding figures. Note that for histology and dye tracing we used the left and right ears as independent samples, doubling the total given in Table 1. Phenotyping and data analysis were performed blind to the genotype of the mice. All values are presented either as the mean $\pm$ SD or SEM. For statistical analysis, GraphPad Prism software was used. To assess differences in the mean, two-sided Student's t tests, one-way or two-way ANOVA with Bonferroni's multiple-comparison test, multiple t test with Holm-Sidak comparison method, χ^2 test, and unpaired two-tailed t tests were used. Significance was determined as $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. Complete results of the statistical analyses, including exact p values are included in the figure legends.

Results

Neurod1-deficient mice retain many SG neurons

Neurod1 was eliminated specifically in the inner ear by crossing *Neurod1*^{loxP/loxP} mice (Goebbels et al., 2005) with *Islet1*^{cre} mice (Dvorakova et al., 2016; *Neurod1* cKO). *Neurod1* cKO mice are viable without any obvious abnormal motor activity behavior that would indicate major defects in the vestibular system. To evaluate the cochlear phenotype of *Neurod1* cKO mutants, we stained cochlear whole mounts with antibody to Myo7a, a hair cell marker. The organization of the organ of Corti of mutants was comparable to controls, with three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs; Fig. 1A–D). In contrast to previous work showing that all (Liu et al., 2000) or

←

(Figure legend continued.) (a marker for IHCs) shows trans-differentiated IHCs instead of OHCs (arrows) in the apex of the mutant cochlea at 1 month of age (M1). Scale bar, 20 μ m. **M**, Confocal analysis of immunostaining for a synaptic ribbon protein (CtBP2) in the IHC area shows a reduction of ribbons in mutant adult mice compared with the controls. Phalloidin labels F-actin in stereocilia of hair cells. HS, Nuclear staining Hoechst. Scale bar, 10 μ m. **O**, Quantification of ribbon synapses per IHC area along the tonotopic axis. Data are expressed as the mean $\pm$ SEM; $n = 3$; two-way ANOVA. $***p < 0.0001$. **P**, **Q**, Immunohistochemistry for NeuN in SG neurons at M1, the dotted line indicates the boundaries of SG in the vibratome sections of the cochlea. Scale bar, 50 μ m. **R**, Quantification of SG neurons in the control and *Neurod1* cKO cochlea at P0 and M1. Data are represented as the mean $\pm$ SEM; $n = 3/\text{genotype}/\text{age}$. Two-tailed unpaired t test. $***p < 0.001$.

Figure 2. Deletion of *Neurod1* affects the size of the components of the auditory pathways. **A, B**, Representative image of a whole-mount of control and *Neurod1*cKO cochlea at P3. Hair cells labeled with anti-Myo7a (green) and neuronal fibers with anti- β -tubulin (magenta). Note a reduced density of radial fibers in *Neurod1*cKO cochlea. Scale bar, 100 μ m. Quantification of cochlear length at 4 weeks of age ($n = 3$). **C**, Sections of the CN immunostained with anti-NeuN (red) of adult control and *Neurod1*cKO, showing the DCN and PVCN. The line indicates the boundaries of the CN. HS, Nuclear staining Hoechst. Scale bar, 200 μ m. **D**, Quantification of the volume of the adult CN normalized to body weight ($n = 5$). **E, F**, Immunostaining of coronal brain sections for NeuN and quantification of the volume of the adult control and *Neurod1*cKO IC, normalized to body weight ($n = 5$). Scale bar, 200 μ m. Data (**B, D, F**) are the mean $\pm$ SEM. Two-tailed unpaired t test. *** $p < 0.001$, **** $p < 0.0001$. **G**, In the adult cochlea, decreased density and increased length of radial fibers (RFs) stained by OsO₄ in *Neurod1*cKO are noticeable compared with the control. Scale bar, 100 μ m. **H**, Using a LacZ reporter (R26R mouse line), *Isl1*-cre-mediated β -galactosidase reporter expression shows no Cre recombination in the CN in the brain sections. The dotted line indicates the boundaries of the CN. Cre recombination is detected by the LacZ reporter in the facial motor nucleus (FMN). Scale bar, 200 μ m.

nearly all SG neurons degenerate in different *Neurod1* mutants (Kim et al., 2001; Jahan et al., 2010a), we detected dense radial fibers and SG neurons in the cochlea of *Neurod1*cKO (Fig. 1A–D, E–H, E'–H'). However, there were noticeable abnormalities associated with aberrant innervation and migration of neurons in

the cochlea, e.g., elongated and reduced radial fibers, increased spacing between radial fiber bundles, no intraganglionic spiral bundle formed by efferent axons, spread out and missing SG neurons, disorganized central axons, and complete absence of SG at the apical end. In the apex of *Neurod1*cKO, radial fiber bundles were noticeably disarranged with crossing fibers (Fig. 1D) and the epithelium was disorganized with missing OHCs, trans-differentiated OHCs into IHCs, and disarranged β -tubulin⁺ supporting pillar cells (Fig. 1I–L). This sensory epithelium phenotype of trans-differentiation of some OHCs into IHCs was comparable to previous reports on different *Neurod1* deletion mutants (Liu et al., 2000; Kim et al., 2001; Jahan et al., 2010a). We also directly assessed the synapses between auditory nerve terminals and IHCs by immunostaining the cochlear sensory epithelium for CtBP2, a major component of presynaptic ribbons in the adult animals (Chumak et al., 2016). Ribbon counts showed an average reduction of 58% in *Neurod1*cKO compared with controls, indicating a reduction of IHC afferent ribbon synapses (Fig. 1M–O). The total number of SG neurons positioned inside the *Neurod1*cKO cochlea was reduced at P0 by 80% compared with control littermates but the number of surviving SG neurons was maintained during postnatal development up to adulthood (Fig. 1P–R).

***Neurod1*cKO mice have a shortened cochlea, smaller cochlear nuclei, normal size of the IC, and altered ABR**

The dissected cochleae of control and *Neurod1*cKO mice were mounted, imaged in a confocal microscope and the length of the organ of Corti was measured (Fig. 2A) and found to be on average $\sim 40\%$ shorter in the adult mutant (Fig. 2B). Note that the density of radial fibers in *Neurod1*cKO is reduced compared with littermate control. Although their density was evidently lower than in controls, radial fibers were preserved in adult mutant cochlea (Fig. 2G). We next measured the volume of the CN at the entry of the auditory nerve in coronally sectioned adult brains. The volume of the CN was reduced by $\sim 39\%$ (Fig. 2C,D). Because *Isl1*^{cre} is not expressed in the CN (Fig. 2H), the size reduction is not likely because of *Neurod1*

deletion in the CN (Fritzsche et al., 2006) but is exclusively a secondary effect of reduced afferent input consistent with the effects of neonatal cochlear ablation previously reported (Rubel and Fritzsche, 2002). In contrast to the CN, sections of the IC showed no significant reduction in the adult mutant mice, indi-

Figure 3. DPOAEs and ABRs are altered in *Neurod1cKO*. **A**, OHC function assessed by DPOAE shows significantly reduced levels in the low-frequency range. Data are the mean $\pm$ SEM; $n = 12$ /control, $n = 8$ /*Neurod1cKO*; two-way ANOVA with Bonferroni *post hoc* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$. **B**, The average ABR thresholds of control ($n = 12$) and *Neurod1cKO* mice ($n = 8$). Five of the 8 *Neurod1cKO* animals did not have any response to the highest measured intensity at 2 or 40 kHz, indicated by the dotted line. Data are the mean $\pm$ SD; two-way ANOVA. $***p < 0.0001$. **C**, Averaged ABR response curves evoked by an 80 dB SPL click; control in black, *Neurod1cKO* in red. The solid line shows the average and the shading indicates $\pm$ SEM. The ABR wave amplitudes of the mutant were five times lower than the control. Averaged individual ABR wave latency values are shown by the corresponding peaks. **D**, Averaged click-evoked ABR amplitude-intensity functions normalized to the wave amplitude at 90 dB SPL for ABR waves I and IV, and the ratio of ABR wave IV amplitude to wave I amplitude. Data are represented as the mean $\pm$ SD; two-way ANOVA. $**p < 0.01$, $***p < 0.001$.

cating that the reduction of the CN does not result in a matching reduction of the IC (Fig. 2*E,F*).

We evaluated DPOAE, as an objective measure of the function of the cochlear OHCs and cochlear amplification, using frequency range from 4 to 38 kHz (Fig. 3*A*). Based on the base-apical gradient from high to low frequencies (Müller et al., 2005), we detected significant DPOAE changes in the frequency range between 4 and 18 kHz corresponding to the locations of the OHCs in the mid-apex and the apex of the cochlea. These data indicate that OHC dysfunction mostly correlates with the morphological disorganization of the epithelium detected in the apex (Fig. 1*I–L*). Hearing of mice was assessed with ABRs, which measure electrical activity associated with the propagation of acoustic information through auditory nerve fibers to higher auditory centers. The ABR thresholds of mutant mice were elevated compared with the thresholds of control animals throughout the entire measured frequency range, with a relatively even threshold shift in all frequencies averaging ~ 35 dB of SPL (Fig. 3*B*). Using click-evoked ABR, we evaluated waveform characteristics (Fig. 3*C*). ABR wave amplitudes were five times lower, but the absolute latency of peak I was shorter in *Neurod1cKO* mice. Wave I reflects the synchronous

firing of the auditory nerve, whereas waves II–IV are attributed to the electrical activity of downstream circuits in the CN, superior olivary complex, and IC, respectively (Martin and Rickets, 1981). We expected that CN responses should be more affected compared with IC responses because of the overall size reductions. The shorter wave I latency in *Neurod1cKO* indicates altered properties of SG neurons in information processing. The relative interpeak latency between peaks I and II was retained within normal limits, whereas the interval between peaks II and III was prolonged, most likely because of the reduced size of the CN of *Neurod1cKO*. The timing and distribution of ABR III and IV peaks were comparable between control and mutant mice. After normalization and direct comparison of wave I with wave IV, we found that the 3:1 ratio of controls changed to a 1:1 ratio in the *Neurod1cKO* mice (Fig. 3*D*). Moreover, normalization showed that although attenuated, the overall intensity function of wave IV was near normal, whereas wave I was offset even more with higher intensities. These data indicate that morphological changes in the cochlea and CN translate to ABR differences.

Figure 4. Translocated SG neurons form an aberrant, unsegregated spiro-vestibular ganglion in *Neurod1*cKO. **A–F**, Representative images of triple-dye labeling from the anterior vestibular end organs [labeled as utricle (U); magenta], apex (green), and base (red) of the cochlea shows the distribution of vestibular and SG neurons in whole-mounted collapsed stacks (**A–D**) and optical confocal sections (**E, F**). Dye injection sites are indicated by arrowheads and labeled correspondingly “apex”, “base”, “U” (**A, B**). In the control (**A, C**), neurons located in the vestibular ganglion (VG) of the control are only labeled by utricular dye applications (**A, C**, magenta; basal turn, red; apex, green) injections label only fibers in the AN and utricular injection labels fibers of the vestibular nerve (VN). In *Neurod1*cKO (**B–F**), neurons labeled by dyes injected into the cochlear base and apex, and the utricle combine to form an aberrant enlarged ganglion, the SVG, (*Figure legend continues.*)

Spiral ganglion neurons form an aberrant “spiro-vestibular” ganglion

The application of different colored lipophilic dyes into the apex (green) and base of the cochlea (red), and anterior vestibular end organs (utricle, anterior and horizontal canal cristae; magenta) showed an aberrant distribution of spiral and vestibular ganglion neurons into a “spiro-vestibular” ganglion (SVG) complex in *Neurod1*CKO (Fig. 4*B,D*) in contrast to the vestibular ganglion of control mice with neurons exclusively labeled by anterior vestibular dye applications (Fig. 4*A,C*). Additionally, apex-dye application labeled fibers were detected in the base of the *Neurod1*CKO cochlea (Fig. 4*B*, arrows). In controls, the segregated central axons of neurons labeled by dyes injected into the base and apex in the auditory nerve (AN) were separated from the vestibular nerve (labeled by the dye injection into vestibular end organs; Fig. 4*A,C*). In contrast, the segregation of central axons was lost as basal and apical afferents completely overlapped with vestibular axons in the auditory-vestibular nerve (AVN) in *Neurod1*CKO (Fig. 4*B*, in detail *D,E*). Note that double-labeled neurons (from base/apex or base/utricular injections) were detected only in the *Neurod1*CKO at E18.5 and P7 (Fig. 4*D–D'''*, *E–E'''*, arrowheads), indicating multiple branches of some single SG neurons and abnormal innervation within the cochlea and between the cochlea and vestibular organs. Soma size of SVG neurons labeled from dye injections into the apex of the cochlea (green) was noticeable smaller in mutants (Fig. 4*F*; area: $253 \pm 80 \mu\text{m}^2$) compared with adjacent vestibular neurons labeled from anterior vestibular organ applications (magenta; area: $364 \pm 74 \mu\text{m}^2$; $n = 80$ neurons/2 mutants, t test, $p < 0.001$). We verified the tracing data using PROX1 immunocytochemistry that labels only SG neurons in wild-type (Fritzsche et al., 2010). In *Neurod1*CKO, PROX1⁺ translocated SG neurons were detected in the area of the vestibular ganglion, thus forming a mammalian SVG (Fig. 4*H*) instead of the normally distinct SG in Rosenthal’s canal and a vestibular ganglion between the ear and the brain of controls (Fig. 4*G*). The adult SVG of *Neurod1*CKO mice was enlarged and deformed compared with control animals (Fig. 4*I,J*). Our data confirm previous suggestions of such unusual migration of some SG neurons to colocalize with vestibular neurons in *Pax2^{cre}Neurod1^{loxP/loxP}* (Jahan et al., 2010b) or *Wnt1^{cre}Sox10^{loxP/loxP}* mice (Mao et al., 2014). We showed not only translocated SG neurons and the formation of the aberrant SVG but also single ganglion neurons having multiple

branches within the cochlea (double-labeled by base/apex injections) and between the cochlea and vestibular end organs (double-labeled by cochlear/vestibular injections) in *Neurod1*CKO.

Spiral ganglion neurons are miswired in the cochlea of *Neurod1*CKO

Having recognized the major effects of *Neurod1* deletion on the cochlea and auditory nucleus size, overall physiology, and abnormal arrangement of SG neurons together with vestibular ganglion neurons in the SVG complex (Fig. 4), we next wanted to establish the details of the SG connection between the organ of Corti and vestibular organs as well as connection between the cochlea and the CN. We first investigated whether SG neuron projections are restricted to the CN by backfilling afferents in the ear through dye tracing injections into the CN (red) and cerebellum (green). These dye-tracing data showed that afferents in the cochlea of controls were strictly labeled through dye injected into the CN (Fig. 5*A*, red). In contrast, some SG neurons in the developing cochlea of *Neurod1*CKO mutants projected to the cerebellum, because their afferents were colabeled through dye injected into the cerebellum (Fig. 5*B*, overlapping yellow fibers in the *Neurod1*CKO cochlea). Note that the location of the vestibular ganglion (green dye tracing injection into the cerebellum) was anterior to the auditory nerve in controls (Fig. 5*A*) but posterior to the aberrant SVG ganglion in *Neurod1*CKO (Fig. 5*B*) consistent with the reorganization of anterior posterior fibers and cell distributions in the SVG (Fig. 4*B,D*). We next wanted to establish whether the branches of cochlear projecting neurons also reach vestibular organs in the ear by injecting dye into the cochlea, posterior canal crista and utricle ($n = 9$ at E14.5, E16.5, E18.5). Applying dye to the posterior vertical canal crista consistently labeled fibers in the cochlea in a pattern reminiscent of backfilling from the cerebellum in *Neurod1*CKO (Fig. 5*C,D*). Neurons labeled with posterior canal injections had multiple branches toward the developing organ of Corti (Fig. 5*D'*, in detail *D''*) that overlapped with fibers to the base and to the posterior canal crista after dye was applied to the apex (Fig. 5*D'*, inset, green), indicating the presence of neurons projecting to both the cochlea and the posterior canal crista. No such labeled intertwined innervation was found in the control cochlea at E14.5 nor were fibers labeled from the cerebellum (Fig. 5*A*). Later at E16.5, posterior vertical canal injections labeled at most a few efferent collaterals in control animals (Fig. 5*E*), similar to previous descriptions of branching of the efferent axons in the ear (Simmons et al., 2011; Sienknecht et al., 2014). In contrast, posterior vertical canal injection labeled SG fibers in the base, middle, and apex of the shortened cochlea of *Neurod1*CKO (Fig. 5*F,F'*). Not only translocated SG neurons but also vestibular ganglion neurons in the SVG innervated the cochlear hair cells, as indicated by an unusual innervation pattern near inner hair cells in *Neurod1*CKO (Fig. 5*F'*, arrow). These fiber terminations are comparable to rerouted vestibular fibers in neurotrophin mutants (Tessarollo et al., 2004; Fig. 5*F'*, inset), implying that some neurons reach both the organ of Corti as well as vestibular organs.

We next investigated in more detail the branching of SG neurons within the cochlea and between the cochlea and vestibular organs. In controls, dye injections into the cochlear apex (green) and base (red) resulted in a spatially restricted labeling of SG neurons and fibers in the cochlea according to the injection site without any labeling detected from injections to anterior vestibular organs (Fig. 6*A*). In addition to the projection of afferents from the ear to the brain, the ear is also innervated by efferents

(Figure legend continued.) as shown in whole mounts (*B, D*) or optical sections (*E, F*). In mutants, there is a tendency of fibers labeled from the apex (green) to be more anterior in the combined SVG and the combined AVN (*B*), whereas apical fibers run in the most posterior part of the AN in control animals (*A, C*). Overlapping fibers (yellow) labeled by the basal and apical dye applications are present in the mutant cochlear base (*B*, arrows). Note that double-labeled SVG neurons can be seen after basal and apical injections (*D, E*, yellow cells). The double-labeled cells after basal and anterior vestibular end organ injections can best be visualized when the dye is false colored in blue (*E*, double-labeled cells in magenta). *D'–D'''*, *E'–E'''*, Images of individual colors of the separate channels shown as a merged image in *D* and *E* (arrowheads indicate double-labeled cells from merged images). *F*, A merged image shows only apical turn dye labeled SG neurons (green, dotted circles) near magenta labeled vestibular neurons in the SVG. Note that green cells are substantially smaller compared with the VG neurons labeled from anterior vestibular end organ dye application. Scale bars, 100 μm ; dorsal is up and posterior is to the right in all dye-tracing images. *G, H*, Representative vibratome sections show translocated spiral ganglion PROX1⁺ neurons in the SVG as detected by immunohistochemistry for PROX1 (a marker for SG neurons) and NeuN (a marker for differentiated neurons) in *Neurod1*CKO at P0. Scale bar, 100 μm . HS, Nuclear staining Hoechst. *I, J*, Immunohistochemistry for NeuN and β -tubulin in the adult control VG and *Neurod1*CKO SVG. Scale bar, 50 μm . Dotted lines indicate the boundaries of the SG, VG, and SVG.

Figure 5. Loss of *Neurod1* causes projection defects of SG neurons. **A, B**, Only afferents to the cochlea are labeled by the lipophilic dye injected into the CN (red) but not by dye injected into the cerebellum (green) in the control at E13.5. In *Neurod1cKO* littermates, some SG neurons and fibers are labeled from cerebellar injections (**B**, yellow, representing green overlapping with red). Note that afferents in the cochlea are distributed irregularly in mutants compared with controls (**A, B**, red). **C–F'**, The cyan arrowheads show the approximate application site of the dye [posterior canal crista (PC)] that results in labeling of multiple cells and branches projecting to the developing organ of Corti in *Neurod1cKO*. Injection of three different colored dyes [utricle, magenta; PC, cyan; apex, green] into the E14.5 *Neurod1cKO* ear shows overlap of spiral and vestibular ganglion neurons in the spiro-vestibular ganglion and magenta fibers to the cochlea (**C, D, D'**). Higher-magnification of fibers in the cochlea labeled from PC injections shows multiple neuronal branches toward the developing organ of Corti hair cells (**D'**). The dye injected into the apex of *Neurod1cKO* (**D'**, inset, green) labels fibers reaching the base as well as fibers to the posterior canal crista in a pattern reminiscent of fibers labeled from PC injections. These neurons have branched axons that reach not only the brain but also the PC nerve. **E, F, F'**, After PC dye application at E16.5, only a few efferent collaterals are labeled in controls that contribute to the intraganglionic spiral bundle (IGSB). In *Neurod1cKO* at E16.5, PC dye application labels SG neurons near the apex that terminate in an unusual pattern along IHC (**F'**, arrow). This pattern is comparable to the reported pattern of vestibular fibers in a replacement model of neurotrophins (**F'**, inset; data from Tessarollo et al., 2004), indicating that some SG neurons project like vestibular neurons in the brain, reach vestibular end organs (such as the canal crista) and terminate at the organ of Corti in a pattern comparable to rerouted vestibular neurons. Scale bars, 100 μ m. PC, Posterior crista; U, utricle; VG, vestibular ganglion.

that are rerouted facial branchial motor neurons that end on hair cells instead of muscle fibers (Simmons et al., 2011; Fritsch and Elliott, 2017). Typically, dye applications into the cochlea label efferent branches as the dye diffuse close to the radial fibers into the organ of Corti and efferent fibers can be distinguished by a recognizable intraganglionic spiral bundle, formed exclusively by efferents. Accordingly, in our controls, most efferent fibers were strictly either labeled by the dye applications to the apex or base in controls (Fig. 6A', red or green). Note that fibers formed evenly spaced radial bundles. Overlapping (yellow) fibers represented efferents forming the intraganglionic spiral bundle and branching to reach multiple points along the cochlea in controls (Fig. 6A'). In contrast similar dye applications (into the base, apex, and anterior vestibular organs) labeled neurons and branches throughout the inner ear in *Neurod1cKO*, showing mingled bundles of fibers from the apex and base (Fig. 6B). Tracing data revealed at least three different groups of "SG neurons" within the cochlea: those restricted to base or apex, those overlapping between base and apex and those that have in addition branches to vestibular organs (Fig. 6B–B''). A few SG neurons labeled from vestibular injections revealed multiple branches to reach different parts of the cochlea (Fig. 6B'). Thus, our tracing experiments show that both the branching properties and migration properties of inner ear sensory neurons are affected by the deletion of *Neurod1*, generating neurons that are colocalized with vestibular neurons (Fig. 4) and reach multiple parts of the organ of Corti as well as different vestibular organs (Figs. 5, 6).

To fully evaluate efferent innervation in the cochlea, we applied lipophilic dyes into the olivocochlear bundle (green) to label efferents and into the CN (red) to label SG neurons and afferent radial fibers reaching the organ of Corti. In control animals, efferents formed the intraganglionic spiral bundle along SG neurons and together with afferents formed evenly spaced radial bundles (Fig. 7A, in detail A'). In contrast, *Neurod1cKO* efferents reached outer hair cells like in control littermates but did not form the intraganglionic spiral bundle (Fig. 7B, B'). Accordingly, the function of outer hair cells of control and *Neurod1cKO* was comparable to the exception of the mid-apex, as shown by DPOAE tests (Fig. 3A). These data support the notion that much of the intracochlear trajectory of

efficients depends on the distribution of SG neurons but efferents can reach hair cells no matter the deviation from their normal trajectory.

Neurod1 deletion results in a loss of the tonotopic organization of the CN

Next, we evaluated the auditory nerve fiber projections to the brainstem cochlear nucleus complex, composed of the anterior ventral CN (AVCN), posterior ventral CN (PVCN), and dorsal CN (DCN; Muniak et al., 2013, 2016). In control animals, application of different colored lipophilic dyes into the apex (green) and base of the cochlea (red), and vestibular end organs (magenta) showed the segregation of central axons of SG neurons from the base and apex in the auditory nerve and that these discrete, non-overlapping bundles were separated from the vestibular nerve (Fig. 8*A,E*). In contrast, the segregation of central axons of SG neurons was lost as basal and apical afferents completely overlapped in the AVN (Fig. 8*B,F*). Dye applications in the control cochlea showed the tonotopic organization of the CN subdivisions, with low-frequency-encoding fibers from the apex terminating dorsally and high-frequency-encoding fibers from the base terminating ventrally in both the VCN and DCN (Fig. 8*A,E*). In the CN of *Neurod1*CKO, fibers labeled with lipophilic dye tracing from the entire cochlea were restricted to the VCN with just a few fibers occasionally expanding to the DCN (Fig. 8*B,F–F'''*). Very large dye injections covering the basal half and apical half resulted in segregated projections with almost no space between apical and basal afferents in control animals (Fig. 8*G*). In contrast, comparable injections in *Neurod1*CKO mutants showed the entering fibers overlapped in the AVN and were mostly restricted to the VCN with just a few fibers occasionally expanding to the DCN (Fig. 8*H,I*). In essence, the DCN remained virtually non-innervated by cochlear afferents and the dorsal part of both AVCN and PVCN likewise received only sporadic and variable individual fibers, indicating a relocation of high-frequency-encoding basal turn fibers. In controls, cochlear afferents formed parallel fibers, the isofrequency bands (Fig. 8*C,G*, inset), whereas *Neurod1*CKO afferents neither expanded across the entire CN nor showed a parallel fiber organization (Fig. 8*D,H,I*). As expected from overlapping peripheral connections of neurons in the SVG (Fig. 4) as well as neurons in cochlea reaching vestibular organs (Figs. 5, 6), we also found fibers in the CN of *Neurod1*CKO labeled by the application of lipophilic dye into vestibular end organs (Fig. 8*F''*). Thus, *Neurod1*CKO mutants have both the peripheral and central connections of spiral and vestibular neurons incompletely segregated and miswired with overlapping central ax-

Figure 6. Distribution and multiple branches of SG neurons reveal disorganization in the *Neurod1*CKO cochlea. *A, B*, The application of different colored dyes into the apex (green), base (red), and utricle (magenta) shows a spatially restricted labeling of SG neurons and fibers based on the injection site in controls (*A*), whereas both fibers and neurons are labeled throughout the *Neurod1*CKO cochlea by the utricle, base and apex injections (*B*). *A'*, In controls, overlapping (yellow) efferent fibers superimpose in the intraganglionic bundles (IGSB). Some efferent fibers are labeled either by the dye application to the apex or base in controls (red or green) and together with afferents form evenly spaced radial bundles. *B'–B'''*, images of individual colors of the separate channels shown as a merged image in *B*. Note that SG neurons near the apex are double labeled with magenta (utricle) and green (apex) as indicated by arrowheads, and show in addition two or more local branches to different parts of the cochlea (*B'*). Scale bars, 100 μ m.

ons of SG neurons in the auditory-vestibular nerve and limited projections of widely ramifying fibers to the ventral part of CN that showed variable and inconsistent segregation (Fig. 8*J*, summary).

Since the size of the CN depends on the full complement of afferents and the survival of CN neurons depends on innervation (Rubel and Fritzsch, 2002; Syka, 2002), we next investigated the survival of one class of neurons that receive input from auditory nerve fibers through the large end bulbs of Held, the bushy cells in the AVCN (Muniak et al., 2016). Although the size of the AVCN in *Neurod1*CKO was reduced, SG afferents of *Neurod1*CKO and

Figure 7. Disorganized inner ear efferents reach the organ of Corti in the *Neurod1cKO* cochlea. **A, A'**, In control animals, the injections of lipophilic dye into the olivocochlear bundle (OCB; green) labels inner ear efferent fibers forming the intraganglionic bundle (IGSB) and dye application into the CN (red) labels SG neurons and afferent radial fibers reaching the organ of Corti (OC). **B, B'**, In *Neurod1cKO*, the same dye applications label the scattered SG neurons (red dye-labeled; arrowheads) in the cochlea and show strong efferent innervation that lacks an IGSB formation. Note also the profound innervation of the OHC region of the OC only in the apex of the *Neurod1cKO* mutant (**B**, arrow). Scale bars, 100 μ m.

control formed morphologically comparable clusters of boutons that wrap the somas of their targets with the end bulbs of Held, as shown by excitatory VGLUT1 synaptic marker labeling (Fig. 9A–B'). However, innervation patterns of afferents outside the overlapping area in AVCN of *Neurod1cKO* (Fig. 8D,H,I) suggest an unusual distribution that does not conform to the regular pattern of stratified afferent projections in controls (the isofrequency bands in Fig. 8C,G, inset), which represent the tonotopic input of the organ of Corti onto the CN (Muniak et al., 2013, 2016).

c-Fos shows a distorted frequency map representation in the CN

We next wanted to investigate whether SG afferents formed functional synapses onto AVCN bushy cells and how distinct frequencies are mapped throughout the system in terms of the activation of the immediate early response of c-Fos, a technique shown to allow such mapping (Tomková et al., 2015; Karmakar et al., 2017). We subjected freely moving adult mice (2 months old) to pure tone auditory stimulation with 15 kHz pips at 75 dB for 90 min in a sound-proof chamber. We used a 15 kHz tone that drives cochlear activation near the middle in controls and likely also in *Neurod1cKO* mutants, assuming the apex and base shorten equally. Consistent with previous reports (Karmakar et al., 2017), in control mice, we found a narrow band of c-Fos⁺ cells near the center of the AVCN, corresponding with the known isofrequency representation (Fig. 9C). In contrast, in *Neurod1cKO*, 15 kHz-stimulation activated AVCN neurons but the c-Fos activation area was spread all over the AVCN and the number of activated

neurons was doubled (*Neurod1cKO* 20.7 ± 2.5 ; control 9.8 ± 1.4 , $p = 0.0099$; Fig. 9D). These data indicate that the pure tone stimulation of 15 kHz activated AVCN neurons but the tonotopic precision of SG axon targeting was degraded with a spread of activation to many surrounding frequencies.

Frequency tuning, intensity, and temporal coding properties of IC units are distorted in *Neurod1cKO*

With this background on neuroanatomical, quantitative and frequency related c-Fos activity changes in mind, we next investigated the tuning properties of IC neurons at various frequencies as well as other parameters of their function i.e., two-tone suppression, intensity, and temporal resolution as the IC is the first level of auditory space map projection and shows some amelioration of primary afferent dysfunction (Buran et al., 2010; Pelgrim et al., 2018).

The study of the tuning characteristics of IC neurons revealed striking differences in the shape of the excitatory receptive fields between the *Neurod1cKO* and control mice. Instead of simple narrow V-shape receptive fields (a mono-peak response) seen in the controls, we recorded mostly wide receptive fields with two or more peaks in clusters of IC neurons of *Neurod1cKO* mice (Fig. 10A), suggesting multiple inputs from the lower levels of the auditory system. Two-tone stimulation, used to detect inhibitory areas surrounding the excitatory tuning curves, showed the presence of low- and high-frequency sideband inhibitory areas in controls, and small and disorganized inhibitory areas in *Neurod1cKO* (Fig. 10B). Because we mostly recorded the multiple-unit activity of neuronal clusters in the IC, we wanted to establish whether the multi-peaked broad tuning curves in mutants are formed by the integration of responses of several neurons with different best frequencies or whether they reflect a miswiring of auditory fibers occurring below the IC. We found that the multi-peaked broad tuning curves in the responses of neuronal clusters were also observed in the tuning curves of isolated single units recorded at the same electrode (Fig. 10C–H).

The investigation of responsiveness of IC units of the mutant mice to different sound frequencies revealed a frequency range with a limited presence of CFs from 9 up to 28 kHz (Fig. 11A). These features are consistent with the observed shortening of the cochlea, and the reduced amplitude of the ABR. In addition, the excitatory thresholds of IC units were higher in mutants than in control animals in all measured frequencies (Fig. 11A). One commonly used metric of auditory tuning is the “quality factor”, or Q, defined as the CF divided by the bandwidth, typically measured 10 dB above the minimum threshold. Comparing the data of control and mutant mice, the tuning curves recorded at individual electrodes in the IC of mutant mice had a significantly lower quality factor Q_{10} , indicating that their frequency selectivity was worse (Fig. 11B).

To assess the responsiveness of neurons to sound intensity, we recorded the responses of IC multiunits to WN bursts of variable

Figure 8. *Neurod1*^{cKO} SG neurons project unsegregated widely ramifying central axons to the CN. **A, C, E,** Injections of different colored dyes (magenta, vestibular organs; red, base; green, apex) label distinct bundles of neuronal fibers (AN) projecting to the CN and vestibular nuclei in controls. The tonotopic organization of the CN subdivisions in controls is shown in the AVCN and the DCN with low-frequency fibers labeled from the apex and high-frequency from the base and no labeling between (the middle cochlea; **A, E, G**). The only mixed bundle (yellow) are inner ear efferents (IEEs; **A**). In *Neurod1*^{cKO}, neuronal fibers completely overlap forming a mixed-labeled AVN (yellow) and are restricted to the ventral part of the CN with just a few fibers occasionally expanding to the DCN (**B, D, F**). The overlapping fibers in the AVCN indicate a loss of the tonotopic organization of the central projections (**B**, yellow fibers). **C, D,** Higher-magnification shows central afferents as parallel fibers (isofrequency bands) in control AVCN and DCN, whereas central projections of afferents in mutants branch in a restricted and erratic pattern in the CN (green dye injection into the apex). **E, F,** The tonotopic organization of the CN subdivisions with low-frequency-encoding fibers from the apex (green) terminating ventrally and high-frequency-encoding fibers from the base (red) terminating dorsally in the control AVCN and DCN. In contrast, disorganized fibers labeled by dye injected in the apex of *Neurod1*^{cKO} project into the ventral part of the AVCN, whereas virtually only sporadic fibers labeled by basal dye injection reach only PVCN subdivisions. **F'–F''',** Single color images show the incomplete cochlear projection segregation as well as some “vestibular” fibers projecting to the CN of *Neurod1*^{cKO} (**F''**) that never happens in control animals (**A, C, E**). **G,** Very large lipophilic dye injections result in barely segregated projection with almost no space between apical and basal afferents in controls. Inset, Cochlear afferents organized as parallel fibers in isofrequency bands. **H, I,** Comparable large dye injections label sporadic and divergent fibers mostly in the ventral part of the CN of *Neurod1*^{cKO} at P7 and E18.5. Note that single afferents project widely ramifying across the CN complex (**H, I**, arrowheads). Scale bars, 100 μ m. **J,** Schematics of changes in *Neurod1*^{cKO}: (1) SG neurons are miswired and misplaced into an aberrant SVG, (2) overlapped central auditory and vestibular afferents form the AVN, and (3) the CN has reduced projections without tonotopic organization both in terms of branch distribution within the cochlea and to the CN. The triangles indicate the application sites of the dyes in the base, apex, utricle (U), and posterior canal crista (PC). AC, Anterior crista; HC, horizontal crista; VG, vestibular ganglion; VN, vestibular nerve.

Figure 9. The formation of isofrequency bands in the anteroventral CN of *Neurod1cKO* is altered. **A, A', B, B',** Auditory nerve synaptic terminals of control and mutant mice are comparable, as anti-VGLUT1 labels the endbulb of Held synapses and anti-parvalbumin labels the bushy cell soma in the AVCN of the adult control and *Neurod1cKO*. **C, C',** Activated neurons are labeled by anti-c-Fos antibody in response to 15 kHz pure tone stimulation in the AVCN of 2 months old controls and *Neurod1cKO*. Dotted line indicates boundaries of the AVCN. A, Anterior; L, lateral; M, medial; P, posterior. **D,** The total number of c-Fos⁺ neurons determined in the sections of the AVCN of control ($n = 4$) and *Neurod1cKO* mice ($n = 4$). Two sections were evaluated per each individual mouse. Values are mean $\pm$ SEM. ** $p = 0.0099$, t test. Scale bars: 200 μ m; **A', B',** 10 μ m. HS, Nuclear staining Hoechst.

intensity and used them to construct RIFs. The percentage of multiunits with non-monotonic responses (i.e., encoding the increasing intensity initially by increasing spike rate, and then decreasing spike rate as sound intensity is further increased) was doubled in the IC of *Neurod1cKO* (35%) compared with controls (16%; $p < 0.0001$; *Neurod1cKO* $n = 9$; 432 IC recording sites and control $n = 10$; 480 IC recording sites; Fig. 11C). Saturated rate intensity functions were present only in 7% of the measured IC multiunits in *Neurod1cKO* compared with 37% in the controls ($p < 0.0001$), whereas monotonic rate-intensity functions represented 55% of the recorded neuronal clusters in *Neurod1cKO* compared with 47% in the controls ($p = 0.0007$). As a result, the IC multiunits in *Neurod1cKO* had a narrower dynamic range (23.38 ± 0.31 dB vs control 24.23 ± 0.29 dB, $p < 0.05$) as well as significantly lower maximum response magnitudes (*Neurod1cKO* 16.96 ± 0.44 spikes/s; control 25.26 ± 0.51 spikes/s, $p < 0.0001$; Fig. 11D). Additionally, it was necessary to use significantly higher WN intensity in *Neurod1cKO* to reach the R10, representing 10% of the RIF response magnitude (53.8 ± 0.5 dB SPL; control 34.3 ± 0.4 dB SPL, $p < 0.0001$; Fig. 11D), as a consequence of a higher excitatory threshold. However, at this low suprathreshold noise level, *Neurod1cKO* IC units showed facilitated evoked activity (0.15 ± 0.0029 vs control 0.12 ± 0.00091 , $p < 0.0001$). We also observed significantly higher spontaneous activity in the *Neurod1cKO* IC units than in controls (*Neurod1cKO* 6.21 ± 0.34 spikes/s; control 1.5 ± 0.11 spikes/s, $p < 0.0001$; Fig. 11D).

We performed acoustical stimulation of the IC units with two types of click trains, a long one with changing frequency of clicks in the train and a train of five clicks with different interclick intervals from 100 up to 5 ms. In *Neurod1cKO*, this revealed a significantly lower efficiency in recognizing individual clicks in

the complex train (*Neurod1cKO* $23.96 \pm 1.07\%$; control $49.86 \pm 1.47\%$, $p < 0.0001$; Fig. 11E); and a lower degree of response synchronization with clicks in the train for the whole range of interclick intervals, suggesting a reduction in the precise response timing in the *Neurod1cKO* (Fig. 11F).

Auditory behavior of *Neurod1cKO* is altered

Previous work has demonstrated that even minor frequency presentation mistakes in the CN can lead to system effects, revealed by altered auditory behavior (Karmakar et al., 2017; Cruces-Solis et al., 2018). The thresholds and growth of the ASR were measured for tonal and WN stimuli in a continuous-noise and in a noiseless background. In mutants, ASR thresholds were significantly reduced for startle tone stimuli of 8 kHz (*Neurod1cKO* 72.14 ± 2.67 dB SPL; control 81.43 ± 3.78 dB SPL, $p < 0.001$; $n = 8$ /group) and 16 kHz (*Neurod1cKO* 66.43 ± 6.27 dB SPL; control 83.57 ± 3.78 dB SPL, $p < 0.0001$), suggesting that instead of perceiving a pure tone, mutants perceive noise-like stimuli. This is further supported by the fact that the startle thresholds of mutants in response to pure

tone stimulation were similar to those for WN (Fig. 12A). The differences in thresholds in response to WN between control and mutant mice were not significant. Consistent with the lower dynamic range, lower maximum response and higher percentage of non-monotonic responses of IC multiunits (Fig. 11C,D), we found that *Neurod1cKO* mice had a reduced amplitude of the startle response at higher intensities of noise startle stimuli (Fig. 12B). However, the 16 kHz stimulation resulted in significantly larger amplitudes of the response at low and middle intensities in mutants (Fig. 12C). To further assess complex auditory discrimination behavior in *Neurod1cKO* mutants, we subjected control and mutant adult mice to a PPI paradigm, which is an operational measure of sensory-motor gating. We used either WN or pure tone pips at increasing intensities as a non-startling acoustic stimulus (prepulse) that preceded the startle stimulus in a quiet background. PPI with WN as the prepulse resulted in mutants with a larger inhibition of the startle response than in controls (Fig. 12D). PPI growth functions demonstrated that the prepulse WN bursts can significantly modulate the startle response at sound pressures as low as 10 dB SPL in mutant mice, indicating heightened sensitivity of the central auditory system. Similarly, subsequent comparisons revealed that the PPI of *Neurod1cKO* for 16 kHz prepulses (a preserved audible frequency in the mutant IC) was enhanced starting at 40 dB SPL compared with control mice (Fig. 12E). Additionally, mutant mice showed higher sensitivity to increasing intensities of the continuous background WN than controls (Fig. 12F). Thus, *Neurod1cKO* displayed hyperacusis-like behavioral ASR and PPI responses parallel to animal models with noise-induced cochlear neuropathy, which is also associated with increased behavioral salience of still-audible sounds (Hickox and Liberman, 2014).

Figure 10. Deletion of *Neurod1* affects the IC tuning curves of multiple and isolated single units. **A**, Representative examples of tuning curves recorded in the IC in control and *Neurod1cKO* mice. **B**, Response map to two-tone stimulation (fixed tone of 13 kHz), shows low- and high-frequency sideband inhibitory areas in control (the areas are outlined by white lines) and small and disorganized inhibitory areas in *Neurod1cKO* mice. **C**, Feature space window (in which each action potential is represented by a single point) with a delineated cluster of action potentials (purple dashed box) belonging to isolated single units recorded at the electrode channel. Action potentials corresponding to multiple unit activity are within the larger box (*Figure legend continues.*)

Figure 11. Characteristics of IC neurons are affected by acoustic information processing in the periphery. **A**, The dependence of IC excitatory thresholds on the CF of neuronal clusters (multiunits) in control and *Neurod1cKO* mice. Excitatory thresholds are shown as individual units and as averages in 0.3 octave bins. Data are mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test. **** p < 0.0001. **B**, Sharpness of the neuronal tuning expressed by quality factor Q_{10} (the ratio between the CF and bandwidth at 10 dB above the minimum threshold) averaged in 0.3 octave bins. Data are represented as the mean $\pm$ SEM; two-way ANOVA with Bonferroni *post hoc* test. **** p < 0.0001, ** p < 0.01. **C**, Percentages of strictly monotonic, saturating and non-monotonic RIFs. Fisher's exact test. *** p < 0.001, **** p < 0.0001. **D**, Comparison of the RIF parameters in control and *Neurod1cKO* mice: dynamic range, relative response at the RIFs point R10 (10% of the RIF response magnitude), maximum response magnitude and spontaneous activity. Data are the mean $\pm$ SD; unpaired *t* test. * p < 0.05, **** p < 0.0001. For all extracellular recordings *Neurod1cKO* (n = 9; 432 units from the IC) and control mice (n = 10; 480 units) were used. **E**, The average probability of a response to individual clicks in the series of clicks. Inset, Diagram of the clicks train, where the rate continuously speeds up and then slows down again (mean $\pm$ SEM; n = 10/control, n = 9/*Neurod1cKO*; **** p < 0.0001, unpaired *t* test). **F**, Degree of synchronization for different interstimulus intervals (ISIs) expressed using the vector strength computed for responses to click trains with different interclick intervals (mean $\pm$ SD; n = 10/control, n = 9/*Neurod1cKO*; **** p < 0.0001, two-way ANOVA with Bonferroni *post hoc* test).

(Figure legend continued.) (indicated by solid blue line). **D**, **E**, Examples of the tuning curves representing multiple unit activity and the activity of a single isolated unit from five different recordings in *Neurod1cKO* mice. **F**, Tuning curve of a multiple unit in *Neurod1cKO* mice; for the same recording, frequency profile of the same response to tonal stimulation at different frequencies with bimodal frequency tuning (at 30 dB sound attenuation) is calculated from single-unit activity (**G**). **H**, Action potential curves, recorded at two different points marked with the corresponding color in the frequency profile at (**E**), demonstrating very similar spike shapes and therefore classified as generated by the same neuron (single unit).

Neurod1 deletion results in aberrant vestibular function

Finally, we investigated the possibility of interference of vestibular function of *Neurod1cKO* with auditory stimuli. This would be expected given the distribution of SG neurons in the aberrant SVG, shared central projections after labeling vestibular and cochlear afferents, and labeled neurons in the SG by dye injected into vestibular organs (Figs. 4, 6, 8). Thus, these results demonstrate interconnections of afferents between cochlear and vestib-

Figure 12. ASR and PPI responses are altered in *Neurod1cKO*. **A**, Thresholds of ASRs for WN, and tone pips at 8 and 16 kHz in control and *Neurod1cKO* mice. Data are the mean $\pm$ SEM, $n = 8$ /group. Statistical significance determined by the Holm–Sidak method, t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Amplitude–intensity ASR functions for WN (**B**), and for tone pips of 16 kHz (**C**) in control and *Neurod1cKO* mice. Data are the mean $\pm$ SEM, $n = 8$ /group. **D**, Efficacy of the WN and (**E**) 16 kHz tone prepulse intensity on the relative ASR amplitude; 100% corresponds to the ASR amplitude without a prepulse (uninhibited ASR). Data are the mean $\pm$ SEM, $n = 8$ /group. **F**, Impact of a continuous background WN with increasing intensity on the relative ASR amplitude; 100% corresponds to the amplitude of uninhibited ASR (response in silence). Data are the mean $\pm$ SEM, $n = 8$ /group. Two-way ANOVA with Bonferroni *post hoc* tests for **B–F**. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. **G**, Ratio of the orientation (orient) and transition (trans) time in the presence of background WN (BWN) or series of clicks to the time in silence on rods of 20 or 15 mm diameters. The green line depicts the point when the ratio is 1 (no change in measured times with/without sound). Data are the mean $\pm$ SEM (control, $n = 4$; *Neurod1cKO*, $n = 3$). Statistical significance determined using two-way ANOVA with Sidak’s multiple-comparison test. $*p < 0.05$, $**p < 0.01$.

ular sensory epithelia previously only shown in certain neurotrophin mutants (Tessarollo et al., 2004) and *Foxg1* mutant mice (Pauley et al., 2006). We tested whether the motor coordination of *Neurod1cKO* can be affected by acoustic stimulation. We conducted two different experiments, one with continuous

WN at an 80 dB SPL intensity, and another with a 600 ms series of clicks with a changing frequency of spike occurrence at an intensity of 70 dB SPL. We measured turning time (time taken to perform an 180° turn from the starting position) and transition time on static rods of two different diameters. WN had a negative

Figure 13. Model of tonotopic phenotype in *Neurod1cKO*. In control mice, spiral ganglion afferent neurons (SGN) are radially connected with a single ending to IHCs (10–20 neurons/1 IHC). IHCs at different parts of the cochlea are stimulated by different frequencies, forming a tonotopic map with high frequencies at the base and low frequencies at the apex. The auditory system is tonotopically organized at all levels including the auditory nerve. The CN is the first target of the auditory nerve. SGN axons project centrally into the AVCN, relaying distinct frequencies within their corresponding isofrequency band. The IC is a major center for the integration of auditory sensory information from ascending pathways, including the pathway from the CN. The tuning curve of each IC unit represents a combination of the frequency and amplitude that evokes a response. At one frequency, the best frequency, sensitivity is highest. In *Neurod1cKO* mutants, the SG is disorganized, with aberrant innervation and a reduced number of neurons, many of which are displaced in the spiro-vestibular ganglion. There is little cochleotopic organization of afferents to the organ of Corti, within the auditory nerve and in the CN. SG afferents relaying specific frequencies target more AVCN cells outside the isofrequency band. Characteristics of IC neurons are altered with an enlarged frequency range and higher excitatory thresholds. The tuning characteristics of IC neurons are broad with multiple peaks of “best frequency”. AC, Anterior crista; AN, auditory nerve; HC, horizontal crista; PC, posterior crista; RF, radial fibers; VG, vestibular ganglion; VN, vestibular nerve.

effect on the transition time of *Neurod1cKO* mice on both rods (Fig. 12G). However, the train of clicks with changing frequency of occurrence had a lower impact on the mutant mice than on the controls. These results demonstrate that *Neurod1* deletion affected the processing of vestibular information during sound exposure. Although we cannot exclude the possibility that the altered motor behavior of *Neurod1cKO* mutants was the result of their increased sound sensitivity to WN exposure, our data indicate that the vestibular and auditory systems may be functionally interconnected. The possibility of an interconnection is supported by the observed shared central projections and distribution of SG neurons in the vestibular ganglion and their projection to both the cochlea and vestibular organs.

Discussion

The formation of a neural map of the sensory periphery is an essential feature of all sensory systems. Understanding the mechanisms of formation, plasticity, and wiring of the sensory maps in both neonatal and adult states is a major endeavor in neuroscience. Sensory maps of the retina, somatosensory space, and cochlea show plastic reorganization of central map properties as a result of loss or modifications of specific features, for example filling in lost fingers or expanding size of somatosensory maps according to their use (Buonomano and Merzenich, 1998; Zhou and Merzenich, 2008), filling in lost areas of the retina (Baker et al., 2005; Keck et al., 2008), and even providing some hearing after destruction of SG neurons (Chambers et al., 2016; Wang, 2016). In contrast to all these data on ontogenetic and adult map effects, for the first time we succeeded to eliminate auditory primary map formation during development altogether. We genetically engineered a new mouse model lacking cochleotopic projections to the CN and tonotopic segregation in the ear. Using this new model, we have explored the ability of second-order

neurons projecting from the CN to the IC to self-organize a secondary frequency map in the absence of a refined cochleotopic map. To the best of our knowledge, this is the first time that a single gene mutation results in physiologically uncorrectable primary map mistakes of both the peripheral and central innervation.

We show that genetically induced abnormal inner ear development results in a shorter cochlea, miswired and displaced SG neurons, and reduced spatially unsegregated central axons of SG neurons. The SG-to-CN afferent projections are represented by widely ramifying fibers that mostly reach the ventral part of the CN (Fig. 8). The tonotopic order of SG projections to all three CN subdivisions and their precise parallel fiber organization into isofrequency bands are largely absent in *Neurod1cKO*. The processing of the limited frequency range of the periphery and CN is expanded across the entire IC (Fig. 13, schematic). These alterations of cochlear mapping onto the CN and CN onto the IC result in altered tuning characteristics of IC neurons in *Neurod1cKO* mutants, including an enlarged frequency range, higher excitatory thresholds, and worsened tuning capabilities. In association with the broad and multi-peaked tuning curves of IC units, lower thresholds of startle responses to pure tones in mutants were measured, suggesting that mutants apparently perceive noise-like stimuli rather than pure tones. Modifications in intensity coding in the IC of *Neurod1cKO* mutants indicate a central reorganization of the tuning properties of the auditory system with changes in the balance of inhibition and excitation as a response to the peripheral auditory deficiency. Furthermore, increased spontaneous activity of IC units in *Neurod1cKO* suggests hypersensitivity to sound in the central auditory pathways. Thus, our data provide evidence that a disorganized primary tonotopic auditory map leads to higher-order tonotopic

topic information processing problems in the IC that are not self-correcting as seems to be the case for simple intensity distortions (Pelgrim et al., 2018).

Central reorganization of the auditory system has been previously demonstrated in studies using noise exposure or other cochlear damage protocols (Kandler et al., 2009; Harrison, 2016). However, unique to our study are the consequences of simultaneously disorganizing and compressing the primary sensory projection map onto the precise tonotopic organization of the central auditory pathways. Our data are in line with recent evidence from the CN and primary sound localization circuits that precise tonotopy depends on refinement at the subcellular and circuit level (summarized by Kandler et al., 2009; Clause et al., 2014). The smaller size of the CN and reduced cochlear nuclear circuits have been reported in genetically produced mutations, blocking synaptic transmission from hair cells and/or activation of SG neurons, and in cochlear ablation studies (Rubel and Fritzsch, 2002; Seal et al., 2008; Tritsch and Bergles, 2010; Tritsch et al., 2010); however, although reduced, the cochlear nuclear circuits that are established in these animals maintain their normal topographic organization patterns (Cao et al., 2008; Wright et al., 2014). This indicates that the reduction in the size of the CN of our *Neurod1*CKO mutant might only have a minimal contribution to the observed differences in the topographic disorganization of the CN and the responses of IC neurons, including wide and multi-peaked tuning curves. Minor distortions of neuroanatomical mapping to the CN have been reported before, such as distortions in the patterning of the SG axons (Yang et al., 2017), and distortions of cochleotopic projections in *Npr2* mutants (Lu et al., 2014). Analysis of *Hoxa2* mutants imply that even relatively minor alterations in isofrequency mapping have behavioral consequences (Karmakar et al., 2017). Compared with these minor changes of topological projections, our profoundly disorganized and truncated cochleotopic mapping implies that NEUROD1 may specify some navigational cues in SG neurons that read diffusible gradients mediated by specific Wnt signals in the dorsal hindbrain (Yang et al., 2017), substrate cues (Gu et al., 2003; Lu et al., 2014), and physiological activity (Kandler et al., 2009; Marrs and Spirou, 2012; Elliott et al., 2015) to establish the cochleotopic map onto the CN. NEUROD1 variably regulates, directly or indirectly, ~3000 genes, including nearly all frizzled class receptor (*Fzd*) genes (Pataskar et al., 2016) relevant for Wnt signaling (Dijksterhuis et al., 2014). Without NEUROD1, spiral ganglia afferent fibers will experience expression changes in many genes possibly involved in neurite guidance. For example, alterations in local *Fzd* receptors could provide further mechanistic insights into the distorted mapping of *Neurod1*CKO SG neurons. In addition, bHLH proteins interact with Gata zinc-finger proteins (Rawnsley et al., 2013). There is an interesting resemblance of phenotypes between a delayed *Gata3* conditional KO (Duncan and Fritzsch, 2013) and our *Neurod1*CKO mutant. Both mutants have decreased number and misplaced SG neurons, and display incorrect patterning within the CN. However, the blurring of the boundaries between spiral and vestibular ganglion neurons with SVG formation and multiple branches within the inner ear represents a unique feature of our *Neurod1*CKO mutant. Although some afferents can still reach the CN, their inability to navigate properly results in reduced and disorganized coverage of the CN in the *Neurod1*CKO mutant. In particular, the well known regulation of neurotrophin receptors *Ntrk2* (TrkB) and *Ntrk3* (TrkC) by NEUROD1 (Kim et al., 2001) may regulate the branching of afferents previously described using neurotrophin and neurotrophin receptor deletions (Fariñas et al., 2001; Fritzsch et al.,

2016b), and misexpression (Tessarollo et al., 2004; Yang et al., 2011). *Neurod1* mutation might also affect the central projections, as previously described in *Neurotrophin3* mutants, which use BDNF/Ntrk2 signaling to expand into the CN after a partial loss of basal turn SG neurons (Fritzsch et al., 1997). The expanded branching of afferents along the cochlea in our mutant, with basal turn SG neurons projecting to the apex (and vice versa; Fig. 6), generates broad mapping of large areas of the cochlea to only parts of the ventral CN (Fig. 13, summary) instead of a point to point connection as in control animals (Muniak et al., 2016).

The connectational reasons for the occurrence of the unusual multi-peak broad tuning curves in the IC of our *Neurod1*CKO mutants are yet to be specified. Nevertheless, our data show that the peripheral deficit in sound encoding cannot be compensated up to the level of the IC. The tuning curves of single units in the IC of control mice have a characteristic V-shape with one peak and thus one best frequency. In the *Neurod1*CKO, there are either two-peak or multi-peak tuning curves, suggesting multiple frequency inputs. Where this integration of inputs occurs must be shown in further experiments. Clearly, at the level of the cochlea, neurons of the SG are connected to more than one IHC as revealed by our dye tracing data, which show that single SG neurons reach multiple areas of the organ of Corti, for example a given neuron can reach both the base and apex (Fig. 6). This would explain the multi-peaked tuning curves in the IC. However, we also cannot exclude more SG neurons with different frequency tuning being connected to several CN neurons as afferents are not segregated into isofrequency bands in the CN (Fig. 8).

In conclusion, our data demonstrate that the physiological tonotopic properties of the *Neurod1*CKO auditory system are changed at all levels investigated. It remains unclear how exactly the frequency presentation distortion of the cochlea onto the cochlear nuclei relates to these property changes and other well known plastic changes in the cortical auditory system (Harrison, 2016). Our behavioral data show that the startle response is affected and that the cross-innervation of vestibular end-organs affects complex vestibular functions after sound disturbance. Future work is needed to establish how tonotopic distortions affect space map formation (Syka, 2002; Eggermont, 2017) and cortical information processing (Kral et al., 2016) that could benefit cochlear implant treatment (Harrison, 2016). Using ouabain in our mouse model should allow to demonstrate that the reported recovery after afferent ablation (Chambers et al., 2016) relies on a developmental stabilization of tonotopic map features which we have disrupted here.

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