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Neurod1 regulates survival and formation of connections in mouse ear and brain

Israt Jahan, **Jennifer Kersigo**, **Ning Pan**, and **Bernd Fritzsch**
Department of Biology, College of Liberal Arts and Sciences, University of Iowa, 143 BB, Iowa City IA 52242, USA

Abstract

The developing sensory neurons of the mammalian ear require two sequentially activated bHLH genes, *Neurog1* and *Neurod1*, for their development. Neurons never develop in *Neurog1* null mice, and most neurons die in *Neurod1* null mutants, a gene upregulated by *Neurog1*. The surviving neurons of *Neurod1* null mice are incompletely characterized in postnatal mice because of the early lethality of mutants and the possible compromising effect of the absence of insulin on peripheral neuropathies. Using *Tg (Pax2-cre)*, we have generated a conditional deletion of floxed *Neurod1* for the ear; this mouse is viable and allows us to investigate ear innervation defects of *Neurod1* absence only in the ear. We have compared the defects in embryos and show an ear phenotype in conditional *Neurod1* null mice comparable with the systemic *Neurod1* null mouse. By studying postnatal animals, we show that *Neurod1* not only is necessary for the survival of most spiral and many vestibular neurons, but is also essential for a segregated central projection of vestibular and cochlear afferents. In the absence of *Neurod1* in the ear, vestibular and cochlear afferents enter the cochlear nucleus as a single mixed nerve. Neurites coming from vestibular and cochlear sensory epithelia project centrally to both cochlear and vestibular nuclei, in addition to their designated target projections. The peripheral innervation of the remaining sensory neurons is disorganized and shows collaterals of single neurons projecting to multiple endorgans, displaying no tonotopic organization of the organ of Corti or the nucleus. Pending elucidation of the molecular details these *Neurod1* functions, these data demonstrate *Neurod1* is not only a major factor for the survival neurons but is crucial for the development of normal connections, both in the ear and in the central system.

Keywords

Ear development; Neuronal development; Afferent connections; Cochlea; Vestibular ganglion; Organ of Corti; Mouse (*Neurod1* conditional knockout)

Introduction

Basic helix-loop-helix (bHLH) genes contribute to proliferation, cell cycle exit, cell fate acquisition, and differentiation of neuron and glia cell types (Guillemot 2007; Ohsawa and Kageyama 2008) by interacting with various suppressors (*Hes, Id*), by combining in various ways (*Neurog2/Neurod1*), and by cooperating to regulate downstream genes (Kageyama et al. 2007). The absence of bHLH genes can result in phenotype switch (Ma et al. 1999; Shroyer et al. 2007), lack of differentiation (Bermingham et al. 1999), or cell death (Chen et

Correspondence to: Bernd Fritzsch.
bernd-fritzsch@uiowa.edu.

al. 2002). Acquisition of cellular identity depends on proneural bHLH genes, whereas inhibitors of their function (*Hes, Id*) retain cells in an unspecified proliferative stage or promote the differentiation of glia/supporting cells.

Three bHLH genes are important for the ear: *Neurog1* (Ma et al. 1998), *Atoh1* (Bermingham et al. 1999), and *Neurod1* (Kim et al. 2001). *Neurog1* null mice lack inner ear neurons, *Atoh1* null mice lack differentiated hair cells, and *Neurod1* null mice lack most spiral and many vestibular neurons. In addition, lack of *Neurog1* results in massive hair cell loss in the saccule and cochlea (Ma et al. 2000; Matei et al. 2005), and the absence of *Atoh1* results not only in hair cell death (Chen et al. 2002), but also the remaining *Atoh1*-lacZ-positive cells remain epithelial (Dabdoub et al. 2008; Fritzsch et al. 2005a). Other than the initial description of neuronal loss (Kim et al. 2001; Liu et al. 2000) and the more recent verification that the deafness in these mice is related to the disorganization of the cochlea (Xia et al. 2007), no detailed analysis has been performed on the remaining neurons and their peripheral and central innervations in *Neurod1* null mice. The lack of further studies is in large measure related to the limited viability of these mice, as they also lack pancreatic beta cells and thus are insulin-deficient.

We have generated a conditional *Neurod1* null mouse by crossing a floxed *Neurod1* line (Goebbels et al. 2005; Pan et al. 2009) with a *Tg(Pax2-cre)* line (Ohyama and Groves 2004) thus avoiding insulin deficiency. We show that this conditional deletion of *Neurod1* has embryonic ear defects comparable with the simple *Neurod1* null mouse, but that these mice can be used to investigate postnatal effects in an otherwise uncompromised animal. Our data suggest that *Neurod1* is essential for the proper migration and segregation of spiral and vestibular neurons and for the differential peripheral and central projections of vestibular and cochlear afferents.

Materials and methods

Mice and genotyping for generation of conditional *Neurod1* knockout mice

Neurod1 systemic null (KO) mice die within a few days after birth because of severe hyperglycemia. To overcome this problem, we extended our analysis in the inner ear by using *Neurod1* conditional knockout (CKO) mice [$Neurod1^{f/f}$,*Tg(Pax2-cre)*].

To generate the *Neurod1* CKO mice, we crossed a *Pax2*-cre line (Ohyama and Groves 2004) with the floxed *Neurod1* line (Goebbels et al. 2005). For this study, we crossed homozygotic floxed *Neurod1* mice ($Neurod1^{f/f}$) with heterozygous $Neurod1^{f/+}$,*Tg(Pax2-cre)* mice. The resulting $Neurod1^{f/f}$,*Tg(Pax2-cre)* mice were CKO mutants, and the $Neurod1^{f/+}$,*Tg(Pax2-cre)* heterozygous siblings served as controls, here referred to as wildtypes. To monitor endogenous *Neurod1* expression, we used $Neurod1^{f/z}$,*Tg(Pax2-cre)* mice as mutants and $Neurod1^{+/z}$ mice as controls, in which a LacZ reporter replaced the *Neurod1* coding region (Kim et al. 2001). Expression of the lacZ reporter was monitored by β-galactosidase staining. To compare the *Neurod1* CKO effect with the systemic *Neurod1* null mice (*Neurod1* KO mice), we examined the $Neurod1^{z/z}$ mice. Offspring was genotyped by polymerase chain reaction analysis of tail DNA by using *cre*-specific primers, which produce a 280-bp product, and *Neurod1*-specific primers, which produce a 400-bp product from the *Neurod1*-coding region and a 600-bp product from the floxed allele. Embryos were collected from timed pregnant females at embryonic day 9.5 (E9.5), E11.5, E12.5, E13.5, and E14.5, counting noon of the day the vaginal plug was found as E0.5. We also analyzed mice at postnatal day 0 (P0), P7, P14, and P30. Pregnant mothers or juvenile mice were anesthetized with a lethal dose of Avertin (1.25% 2.2.2-tribromoethanol at a dose of 0.025 ml/g body weight). Embryos were dissected from the uterus and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) by using a peristaltic pump.

Heads were isolated and fixed in 4% PFA for further analysis. All animal procedures were approved by the University of Iowa Animal Care and Use Committee (IACUC) (ACURF #0804066).

X-gal staining

Heads of mice perfused with 4% PFA were hemisected. Ears were dissected and then briefly washed with 0.1 M phosphate buffer. The samples were stained in a solution containing 0.1 M phosphate buffer, 0.01% deoxycholic acid, 0.02% NP40, 2 mM magnesium chloride, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for about 24 h at room temperature (Matei et al. 2006).

In situ hybridization

In situ hybridization was performed by using an RNA probe labeled with digoxigenin. The plasmids containing the cDNAs were used to generate the RNA probe by in vitro transcription. The dissected ears were dehydrated in 100% methanol and rehydrated in graded methanol and digested briefly with 20 μg/ml proteinase K (Ambion, Austin, Tex., USA) for 15–20 min. The samples were then hybridized overnight at 60°C to the riboprobe in hybridization solution containing 50% (v/v) formamide, 50% (v/v) 2× saline sodium citrate (Roche), and 6% (w/v) dextran sulfate. After the unbound probe had been washed off, the samples were incubated overnight with an anti-digoxigenin antibody (Roche Diagnostics, Mannheim, Germany) conjugated with alkaline phosphatase. Following a series of washes, the samples were reacted with nitroblue phosphate/5-bromo, 4-chloro, 3-indolyl phosphate (BM purple substrate; Roche Diagnostics, Germany) which is enzymatically converted to a purple-colored product. The ears were mounted flat in glycerol and viewed with a Nikon Eclipse 800 microscope by differential interference contrast microscopy, and images were captured with MetaMorph software. All probes used in this study are listed in Table 1.

Immunofluorescence

For immunofluorescence staining, the ears were dehydrated in 100% ethanol overnight and rehydrated and blocked with 0.25% normal goat serum in phosphate-buffered saline (PBS) containing 0.01% Triton X-100 for 1 h. The primary antibodies for tubulin (Sigma), Myo VII (Myosin VIIa, Proteus Biosciences), and caspase 3 (Cell Signaling Technology) were used at dilutions of 1:800, 1:200, and 1:100, respectively. Ears were incubated with antibodies for 48 h at 4°C. After several washes with PBS, corresponding secondary antibodies (Alexa fluor molecular probe 647 or 532 or 488; Invitrogen) were added at a dilution of 1:500 and incubated overnight at 4°C. The ears were washed with PBS and mounted in glycerol. Images were taken with a Leica TCS SP5 confocal microscope.

Lipophilic dye tracing

The heads of the mice were cut sagittally along the midline, and three different colored dyes were inserted to label the afferent and efferent fibers from and to the inner ear. Lipophilic-dye-soaked filter strips (Fritzsch et al. 2005b) were inserted into the alar plate of the brainstem to label the afferent fibers of the eighth cranial nerve. Efferent fibers were labeled by applying dye into the olivo-cochlear efferent bundle as it crosses the floor plate in rhombomere 4. Disregarding the type of dye inserted into the brainstem, the afferents were shown with a green color and efferents with a red color. Half heads were kept in an oven at 60°C for about 3–7 days depending on the age of the mice to allow proper diffusion. In the E14.5 mice the dyes were injected into rhombomeres 2, 5, and 7 to label the afferent and efferent fibers. The ears, vestibular ganglia, and brains were subsequently dissected out for

analysis, and images were taken via the Leica TCS SP5 confocal microscope. Afferent tracing from the ear was performed by selectively placing the dye-soaked filter strips into the vestibular and cochlear sensory epithelia. For double- and triple-color tracing from the ear, NeuroVue red dye inserted into the cochlear apex was coded as red and NeuroVue maroon, a blue color dye inserted into the base was coded as blue. NeuroVue Jade dye, which gives a yellow color with white light, was applied to the anterior crista/horizontal crista or utricle and was shown with a false color code as green in epifluorescent light. Brains were viewed as whole-mounts or as coronal sections.

Results

KO and CKO *Neurod1* null mice have a similar ear phenotype

The near complete early fatality of the *Neurod1* KO mice (Kim et al. 2001) combined with the possibility of additional ear-specific phenotypes attributable to insulin deficiency (Sanchez-Calderon et al. 2007) has arrested analysis in postnatal mice as proinsulin plays a neuro-protective role (Sanchez-Calderon et al. 2007). Moreover, one characteristic of the lack of insulin is peripheral neuropathy, a problem that could compromise later stage analysis of innervation in *Neurod1* null mice. We have sidestepped this problem by crossing a *Tg(Pax2-cre)* line (Ohyama and Groves 2004) with a conditional deletion of *Neurod1* (Goebbels et al. 2005) to generate a new line that is viable and fertile. *Pax2*-cre is expressed in the early somite stage in the ear before and during *Neurod1* expression (Kim et al. 2001; Ohyama and Groves 2004). In contrast to late-expressing *Tg(Atoh1-cre)* that had no effect on inner ear neurons (Pan et al. 2009), *Tg(Pax2-cre)* has been used to effectively recombine floxed genes such as *Dicer* (Soukup et al. 2009) in the ear.

To ascertain the effectiveness of conditionally deleted *Neurod1* with *Tg(Pax2-cre)*, we compared the phenotypes of *Neurod1* CKO mice with those of *Neurod1* KO mice. The loss of spiral ganglia was remarkable and indistinguishable for *Neurod1* CKO and KO mice (Fig. 1) with only a few scattered surviving ganglion neurons near the middle turn of the cochlea (Fig. 1a–c). *Neurod1*-lacZ showed a massive loss of spiral ganglia with some vestibular ganglia in both types of mutants, compared with heterozygotic control mice (Fig. 1a–c). In addition to sensory neurons, *Neurod1*-lacZ was expressed in many hair cells of all sensory epithelia of both CKO and KO mice (Fig. 1a, c). In control mice, *Neurod1*-lacZ expression was also detected in hair cells but with a more prominent expression in the spiral and vestibular ganglia (Fig. 1b).

Absence of *Neurod1* causes massive reduction and disorganization of innervations

Consistent with the previous study of *Neurod1* KO mice (Kim et al. 2001), we found distorted and diminished innervation of the inner ear in *Neurod1* CKO mice by using lipophilic dye tracing (Figs. 1d, f, 2c–h) and nerve fiber labeling with anti-tubulin (Figs. 1g–i, 2i–n). Only a few afferent fibers extended to the cochlea of *Neurod1* CKO mice (Figs. 1f–i, 2e–j) compared with the dense innervation in control littermates (Figs. 1e, 2a, b). Whereas radial fibers were tightly spaced in control mice, both KO and CKO *Neurod1* mice had severely reduced radial fibers (Figs. 1d, f–i, 2c–n). Cochlear innervation was restricted to type I radial fibers to inner hair cells in the apex and base of the *Neurod1* KO mice without any recognizable type II fiber projections to outer hair cells. Type II fibers preserved some normal orientation, including the innervation to three rows of outer hair cells, only in the middle turn of this mutant cochlea (Fig. 2d). In newborn *Neurod1* CKO mice, we observed a similar reduction of afferent fibers and incomplete formation of radial fibers (Figs. 1f–i, 2e–n). In both mutants, afferent fibers were comparatively more in the middle turn of the cochlea consistent with the topology of remaining spiral ganglion neurons. The basal part of the middle turn showed some type II fibers, which extended randomly toward the base and

apex of the cochlea (Fig. 2f, j, m). The fibers near the basal and apical tip were extremely reduced with only a few collaterals from the middle turn extending along inner hair cells (Figs. 1g–i, 2d, g, h, k). In contrast, the apical part of the middle turn (Fig. 2c, e, i, l, n) showed numerous disorganized projections to inner and outer hair cells and a disorganization of type II fibers, as recently described in various mutants affecting supporting cell development (Fritzsch et al. 2010; Puligilla et al. 2007).

To further assess the correlation between the defects in innervations to the reduced number of spiral neurons, we injected dyes into the apex of the cochlea (Fig. 2h). In wildtype control mice, such injections led at best to efferent fibers going to different parts of the same epithelium and, only in the vestibular system efferent fibers reached nearby sensory epithelia (data not shown). Our injections into the apex labeled unusually projecting type I and type II afferent fibers in the middle turn (Fig. 2h). Although type II fibers showed some extent of normal trajectory in the upper middle turn, type I fibers had multiple terminal branches in the lower middle turn and apex of the cochlea (Fig. 2h), rather having single type I fibers innervating single inner hair cells, as in the wildtype. Moreover, in the lower middle turn, type II fibers seemed to be branches of type I fibers, which finally ran as a single fiber to the apical tip (Fig. 2g, k). Combined, these data suggest that the remaining innervation of the ear in *Neurod1* CKO mice comes about through extensive branching of individual neurons so that type II neurons branch to both the apex and the base, whereas type I neurons reach multiple inner hair cells within the same area and between different parts of the cochlea.

We have previously shown that the efferent system is severely reduced in the *Neurod1* KO mouse (Kim et al. 2001); we obtained similar information in *Neurod1* CKO mice (Fig. 3). The reduction and disorganization of efferent fibers were closely matching that of afferent fibers consistent with previous data suggesting that the efferent fibers navigate along afferents (Fritzsch et al. 1999). We showed that in control mice, the efferent fibers from the lateral superior olivocochlear nucleus projected to the inner hair cells, which exclusively make contacts with the afferent fibers in the inner hair cells, and that the fibers from the medial olivocochlear nucleus innervate the outer hair cells after crossing the tunnel of Corti (Fig. 3a, d, insert in d, f, h). Only wildtype efferent fibers exhibited the formation of the intraganglionic spiral bundle (IGSB in Fig. 3a). The efferent fibers in the middle turn of *Neurod1* CKO mice projected directly to the inner and outer hair cells following the afferent fibers (Fig. 3g, g’). However, the base and the apex were innervated by fibers emanating from inner spiral bundles that extended along the inner hair cells or toward the outer hair cells (Fig. 3b, c, e, i).

Consistent with the retention of the vestibular ganglia, we found a persistence of more profound projections to the vestibular sensory epithelia, which we analyzed by both tubulin immunofluorescence labeling and dye tracing (Fig. 4). We found some variability among those remaining fibers. Among the five vestibular sensory epithelia, the saccule was the most affected in terms of fiber reduction (Fig. 4b, d). We demonstrated the size reduction of vestibular ganglia and of their survival near adulthood in *Neurod1* CKO mice (Fig. 4i–k). Canal crista and utricular fibers were also severely disorganized in contrast to those of control littermates (Fig. 4a–d). Dye injections in the anterior canal labeled many fibers to the utricle displaying an unusual trajectory parallel to the striola region with both bouton and calyceal endings (Fig. 4f). Few fibers in the saccule were labeled from the injection in the apex of the cochlea (Fig. 4g). This mixed peripheral innervation was consistent with the mixed neuron labeling from both vestibular and cochlear nucleus injections (Fig. 4i–k). As previously shown in *Neurod1* KO mice (Kim et al. 2001), the surviving vestibular and spiral ganglia were displaced from their original position. Our observation in CKO mice revealed that these ganglia were not only displaced, but also remained as mixed ganglia of both

vestibular and spiral neurons (Fig. 4i–k); this correlates with the lost specificity of topological fiber projection.

In summary, our data suggest that *Tg(Pax2-cre)* can effectively recombine floxed *Neurod1* early enough in ear development to generate a viable *Neurod1* null mouse with an ear phenotype that is nearly identical in terms of loss of spiral afferents presumably through neuronal apoptosis. The *Neurod1* CKO mouse can thus be used to assess in depth the phenotype of ear-specific *Neurod1* loss after birth without any additional systemic problems related to the absence of insulin. In both KO and CKO of *Neurod1*, we cannot rule out that additional effects through the local production of *Neurod1* expression-mediated proinsulin (Sanchez-Calderon et al. 2007) adds to the *Neurod1* phenotype.

Loss of inner ear sensory neurons correlates with expression changes in other bHLH genes

To assess further the timeline of neuronal loss, we studied the onset of apoptosis in delaminating sensory neurons of E9.5 otocysts with a conditional deletion of *Neurod1* (Fig. 5). We showed a rapid and complete elimination of the floxed *Neurod1* via Cre-mediated recombination by demonstrating absence of any in situ signal for *Neurod1* in the *Neurod1*$^{f/f}$*,Tg(Pax2-cre)* mice (Fig. 5a, a’). Consistent with this early and complete loss of *Neurod1*, we found an enhanced upregulation of *Neurog1* nearly throughout the otocyst (Fig. 5b’). *Neurog1* is expressed in the proliferating precursor neuronal cells and is responsible for the formation and delamination of inner ear sensory neurons (Ma et al. 1998) but is rapidly downregulated in differentiating neurons. We observed prominent *Neurog1* gene expression in the ventral region of the otic vesicle in E9.5 control mice (Fig. 5b). We found less intense *Neurog1* expression in the ventral region of the *Neurod1* CKO otocyst, whereas in the postero-dorsal region, there was an aberrant upregulation of *Neurog1* expression, suggesting a negative feedback loop of *Neurod1* on *Neurog1* (compare Fig. 5b, b’). The reduction in the ventral part of the otocyst could indicate a loss of neuronal precursors attributable to apoptosis in *Neurod1* CKO mice. We tested the latter assumption by using an antibody against activated Caspase 3 and found additional labeled cells inside and outside the otocyst in the *Neurod1* CKO mice (Fig. 5c, c’), suggesting an increased cell death of delaminating and intra-otocyst cells in the absence of *Neurod1*. These data were consistent with a progressive loss of sensory neurons in *Neurod1* CKO mice, resulting in a much reduced number of *Neurod1*-positive neurons (Fig. 5).

The bHLH genes *Nhlh1* and *Nhlh2* are expressed in all cranial ganglia including the vestibulo-cochlear ganglia and also in cochlear and vestibular hair cells (Kruger et al. 2006). Previous work has demonstrated that *Neurog1, Neurod1*, and *Nhlh1* are involved as a cascade in sensory neurogenesis (Ma et al. 1997). Moreover, *Neurod1* and *Nhlh1* have been suggested to play redundant roles in the development of vestibular and spiral ganglion neurons (Kruger et al. 2006). To improve our understanding of this aspect of possibly multiple interacting bHLH genes being involved in the differentiation of inner ear sensory neurons, we investigated *Nhlh1* and *Nhlh2* gene expression in *Neurod1* CKO mice by in situ hybridization. In control mice, both *Nhlh1* and *Nhlh2* were massively expressed in the vestibular and spiral ganglia in the early embryonic stage (Fig. 5d, e). *Nhlh2* expression remained exclusively in the sensory neurons and was greatly reduced in the mutant spiral ganglion and, to a lesser extent, in the vestibular ganglion (Fig. 5d, d’). At later stages, *Nhlh2* was almost absent in the region of the spiral ganglion but continued to be expressed in a reduced set of vestibular ganglion neurons in *Neurod1* CKO mice (data not shown). Likewise, *Nhlh1* expression was progressively reduced in these ganglia in the absence of *Neurod1* as early as E11.5 (Fig. 5e’). The reduction in *Nhlh1* and *Nhlh2* expression in sensory neurons of the ear closely followed the progressive reduction of sensory neurons as demonstrated by *Neurod1* expression with the β-galactosidase reporter system (Fig. 5f’).

In combination, these data indicate that a rapid loss of sensory neurons occurs in *Neurod1* CKO mice with minor expression changes in *Neurog1*, indicating a possible feedback loop of *Neurod1* on *Neurog1*, as suggested in the development of the olfactory system (Kawauchi et al. 2004). Both *Nhlh1* and *Nhlh2* genes might be able to rescue the remaining sensory neurons as previously proposed (Kruger et al. 2006), but additional double-null mutants are required to determine which of the two *Nhlh* genes is more important for this possible rescue of inner ear sensory neurons in the absence of *Neurod1*.

In addition to *Nhlh* genes, we also observed the expression of *Prox1* in *Neurod1* CKO mice. *Prox1* is expressed in inner ear supporting cells and spiral neurons (Bermingham-McDonogh et al. 2006) and is required for the differentiation of type II spiral neurons, hair cells, and supporting cells (Fritzsch et al. 2010; Kirjavainen et al. 2008). With in situ hybridization, we showed dominant *Prox1* expression in the spiral ganglia in wildtype newborn mice (Fig. 5g), whereas in the mutant, only a few *Prox1*-positive neurons remained near the modiolus (Fig. 5g', g"). The downregulation of *Prox1* may be involved in the disorganization of cochlear afferents in *Neurod1* CKO mice, as recent evidence supports the role of *Prox1* in guiding at least the extension of type II spiral fibers (Fritzsch et al. 2010).

Absence of *Neurod1* results in overlapping projections of vestibular and cochlear afferents

Using lipophilic dye tracing, we have previously demonstrated that vestibular and cochlear afferents develop segregated central projections into the vestibular and cochlear nuclei, respectively, as early as E12.5 (Maklad and Fritzsch 2003). We injected lipophilic dyes in the cochlea and vestibular organs (Fig. 6a–a") of *Neurod1* CKO mutant mice and showed here, for the first time, that the absence of *Neurod1* resulted in overlapping vestibular and cochlear afferent projections to the vestibular and cochlear nuclei (Fig. 7). This overlapping central and peripheral projection was consistent with the finding of mixed vestibular and cochlear neurons in the surviving ganglia of *Neurod1* CKO mice (Figs. 4j, 6c). Lipophilic dyes with different chromatic properties were applied to the cochlea and utricle and showed that, as early as E14.5, the vestibular and cochlear fibers were segregated and reached their target nuclei in the brain in control littermates (Fig. 7a). In contrast, in *Neurod1* CKO mice, fibers from both end organs reached the brain as a single nerve that entered through the cochlear nucleus instead of having two divisions (Figs. 7b, d, f, 9b). In E16.5 (data not shown), newborn (P0), and P7 control mice, we demonstrated not only the complete segregation between vestibular and cochlear fibers, but also fibers from the basal and apical region of the cochlea reaching distinct aspects of the dorsal and ventral cochlear nuclei (Fig. 7c, e). In the absence of *Neurod1*, these fibers displayed no tonotopic organization in the target nuclei, and fibers labeled from vestibular injections overlapped with those from cochlear injections (Fig. 7b, d, f). Moreover, the efferent bundles were also misguided in *Neurod1* CKO mice in which some efferent fibers were rerouted and exited with the facial nerve or cochlear nerve, instead of exiting only with the vestibular division (insert in Fig. 7d). This suggests that the absence of a vestibular division results in a rerouting of efferent fibers, which sometimes reach the ear after having exited through the facial nerve (Fig. 6b)

In *Neurod1* CKO mutant mice, vestibular and cochlear afferents not only entered as a single nerve root, but their fibers also projected to both cochlear and vestibular nuclei, respectively, in addition to their original target nuclei projections (Figs. 7, 8). For example, at E14.5, some cochlear fibers were found projecting not only to the DCN and AVCN, but also caudally to the vestibular nuclei and rostrally to the cerebellum (Fig. 7b). Some vestibular fibers reached the cochlear nuclei, and some projected through the cochlear nuclei to reach vestibular nuclei (Figs. 7d, f–f', 8b, c'). These differential projections of cochlear and vestibular afferents were most apparent in later stages and in coronal sections (Fig. 8). Vestibular fibers projected to both vestibular and cochlear nuclei in *Neurod1* CKO mice

(Figs. 7f, f', 8b, c', c", e), whereas these were limited entirely to the vestibular nuclei in control animals (Fig. 8a). Likewise, cochlear afferents projected exclusively to the cochlear nuclei in wildtype mice (Fig. 8a), whereas many cochlear afferents projected to vestibular nuclei in *Neurod1* CKO mice (Figs. 7f", 8b, c, c", d, d'). This altered central projection of the vestibulo-cochlear fibers was confirmed by applying dye only to the anterior crista of the inner ear. This labeling showed that the vestibular afferent fibers projected to both the vestibular and cochlear nucleus, rather than being restricted to the vestibular nucleus (Fig. 8e).

In summary, these data demonstrate that, in the absence of *Neurod1*, the cochlear and vestibular fibers fail to segregate and reach distinct parts of their target nuclei (Fig. 9b). *Neurod1* is therefore not only necessary for the survival of most cochlear and many vestibular neurons, but is also required to regulate the expression of genes that allow organ-specific fiber projections such as Brn3a (Huang et al. 2001). Further work with restricted injections to all six sensory organs is in progress to show the lack of segregation among vestibular fibers in more detail.

Discussion

Neurod1 is essential for neuronal differentiation (Pan et al. 2009) and can convert non-neuronal cells into neurons (Lee et al. 1995) through the regulation of over 500 downstream genes (Seo et al. 2007). We have analyzed the role of *Neurod1* in inner ear neurosensory cell development by using a newly generated *Neurod1* CKO mouse. Consistent with our previous report (Kim et al. 2001), we have found substantial loss of inner ear sensory neurons and the remaining afferents and efferents are disorganized in *Neurod1* CKO mice but not completely absent as previously suggested (Liu et al. 2000). In addition, we have identified a novel role: *Neurod1* is required for segregating and organizing central and peripheral projections in the surviving sensory neurons.

Neuron formation of the inner ear depends on a number of bHLH genes (*Neurog1, Neurod1, Nhlh1*, and *Nhlh2*). *Neurog1* null mice never form neurons and show a reduction of hair cells, suggesting a lineage and some clonal neurosensory relationship (Satoh and Fekete 2005). *Neurod1* CKO mutants show loss of spiral and vestibular neurons and truncation of sensory epithelia. *Nhlh1* and *Neurod1* play a redundant role in the development of sensory neurons in *Nhlh1-Neurod1* compound null mice (Kruger et al. 2006). We suggest that residual neurons might survive by compensatory gene activation of *Nhlh1* and/or *Nhlh2*. Crossing our viable *Neurod1* CKO mice with existing mutants of both *Nhlh1* and *Nhlh2* might show that these three genes are necessary for the survival of all inner ear sensory neurons. Clearly, neither *Nhlh1* nor *Nhlh2* can substitute for *Neurod1* to upregulate the program needed for nerve guidance. *Neurog1* null mice show no neuronal migration and can be compared with *Neurod1* null mice to establish the expression profiles of genes necessary for migration.

Neurod1 is required for segregated projections in the peripheral ear and for proper afferent and efferent organization within sensory epithelia

Previous work has identified several genes relevant for some aspects of pathfinding of nerve fibers, such as neurotrophins (Tessarollo et al. 2004), POU domain factors (Huang et al. 2001), semaphorins (Gu et al. 2003), *Erbb2* (Morris et al. 2006), *Slitrk6* (Katayama et al. 2009), *Foxg1* (Pauley et al. 2006), and *Prox1* (Fritzsch et al. 2010). In addition, defects in supporting cell development affect sensory neuron projections (Puligilla et al. 2007; Shim et al. 2005). *Neurod1* is known to lie upstream to *Prox1* in the developing hippocampus and is involved in directed growth of neuronal processes (Roybon et al. 2009). Consistent with a possible regulation of *Prox1* by *Neurod1* in the ear, we have found little *Prox1* expression

left in only a few sensory neurons in the mutant mouse (Fig. 5g'). The unusual and random fiber growth to the area of outer hair cells (Fig. 2i, l, n) is therefore possibly in part mediated by the downregulation of *Prox1*, since the *Prox1* null phenotype has derailed innervation of outer hair cells (Fritzsch et al. 2010). We have previously shown that *Neurod1* regulates the neurotrophin receptor tyrosine kinase *Ntrk3*, and that the reduction in innervation closely mimics the loss of spiral neurons in *Ntf3* and *Ntrk3* null mutant mice (Fritzsch et al. 2004). The derailment of peripheral innervation including the unusual branching of what appears to be single fibers will allow us to correlate the *Neurod1* phenotype with specific genes with similar defects and ultimately to establish causality comparable with that of *Ntrk3* and *Prox1*.

Neurod1 promoter analysis has revealed many downstream target genes including Prox1, Ntrk3, semaphorin 3a, neuropilin2, plexin A2, Ephb2, Robo1, and *Slit2/3*, which are directly involved in fiber guidance (Seo et al. 2007). In addition to the role of chemoattractants for fiber growth, the repellents play an equally important role in generating the correct innervation pattern. A class of axon repellents, the *semaphorins*, prevent the growth of neurons in the dorsal part of otocyst, if the *neuropilin* receptor is present (Gu et al. 2003). *Semaphorin* signaling of axon repulsion requires the *plexin A2* co-receptor which is expressed in the vestibular and spiral ganglia of developing inner ear (Murakami et al. 2001). Moreover, *Slit/Robo* signaling can affect axon guidance, axon branching, and neuronal migration (Wong et al. 2002). *Ephb2* guides the contralateral efferent growth cones, which show inappropriate pathway selection in the midline with a defect in the vestibular efferent in the absence of *Ephb2* (Cowan et al. 2000). The way in which these known and suspected (Fekete and Campero 2007) guidance molecules in the ear depend on *Neurod1* requires further investigation. A comparison of *Neurod1* with wildtype ears by using deep sequencing might allow to elucidate, in more detail, the expression differences related to the aberrant projections reported here for *Neurod1* null mice.

Neurod1 is required for segregated central projection of cochlear and vestibular neurons

We describe here, for the first time, the disorganization of centrally and peripherally projecting afferents in *Neurod1* null mice. In the ear of *Neurod1* null mice, individual afferents branch profusely to reach most hair cells despite their highly reduced numbers. This expansion is comparable to the only other case of severe reduction of afferents, the *Ntf3* null mouse (Farinas et al. 2001). The exuberant branching of remaining afferents implies that competition with other afferents may normally block such expansion, but a genuine direct effect of *Neurod1* is also possible. The efferents are also reduced in *Neurod1* CKO mice; the remaining efferents never form the normal intra-ganglionic spiral bundle, and the inner spiral bundle provides branches that run along the inner hair cells.

In *Neurod1* CKO mice, cochlear and vestibular fibers enter the cochlear nucleus as a single bundle, and both cochlear and vestibular fibers project to both cochlear and vestibular nuclei. *Neurod1* therefore regulates aspects of central and peripheral branching and pathfinding in sensory neurons. This effect is related to the nerve fibers, not to the *Neurod1* expression in the dorsal cochlear nucleus (DCN) (Fritzsch et al. 2006) as *Tg (Pax2-cre)* do not express in the DCN, thereby cannot eliminate *Neurod1* in the DCN. However, even a severe reduction of cochlear nuclei in mutants with conditional deletion of *Atoh1* has only minor effects on afferent projections (Maricich et al. 2009). Conditional deletions of *Neurod1* at a later stage are now needed to uncouple neuronal survival in the ear from the proposed pathfinding regulation. We demonstrate here that *Neurod1* is responsible not only for survival, but also for neuronal differentiation such as the segregation of cochlear and vestibular afferent projections. Indeed, *Neurod1* expression in cochlear spiral neurons is, in addition to *Gata3* (Karis et al. 2001) and *Prox1* (Fritzsch et al. 2010), the only approximately specific marker for spiral neurons.

In summary, our findings show a more sophisticated action of *Neurod1* in the developing ear than the previously suggested simple role in neuronal survival. These data are in line with the 500 genes directly regulated by Neurod1 (Seo et al. 2007), most of which have a known or suspected function in neuronal guidance. More work on putative neuronal guidance genes is now needed in these viable mutants in order to understand the precise way in which *Neurod1* can regulate features of inner ear neuronal development including targeted peripheral and central projections that are so disorganized in *Neurod1* null mice.

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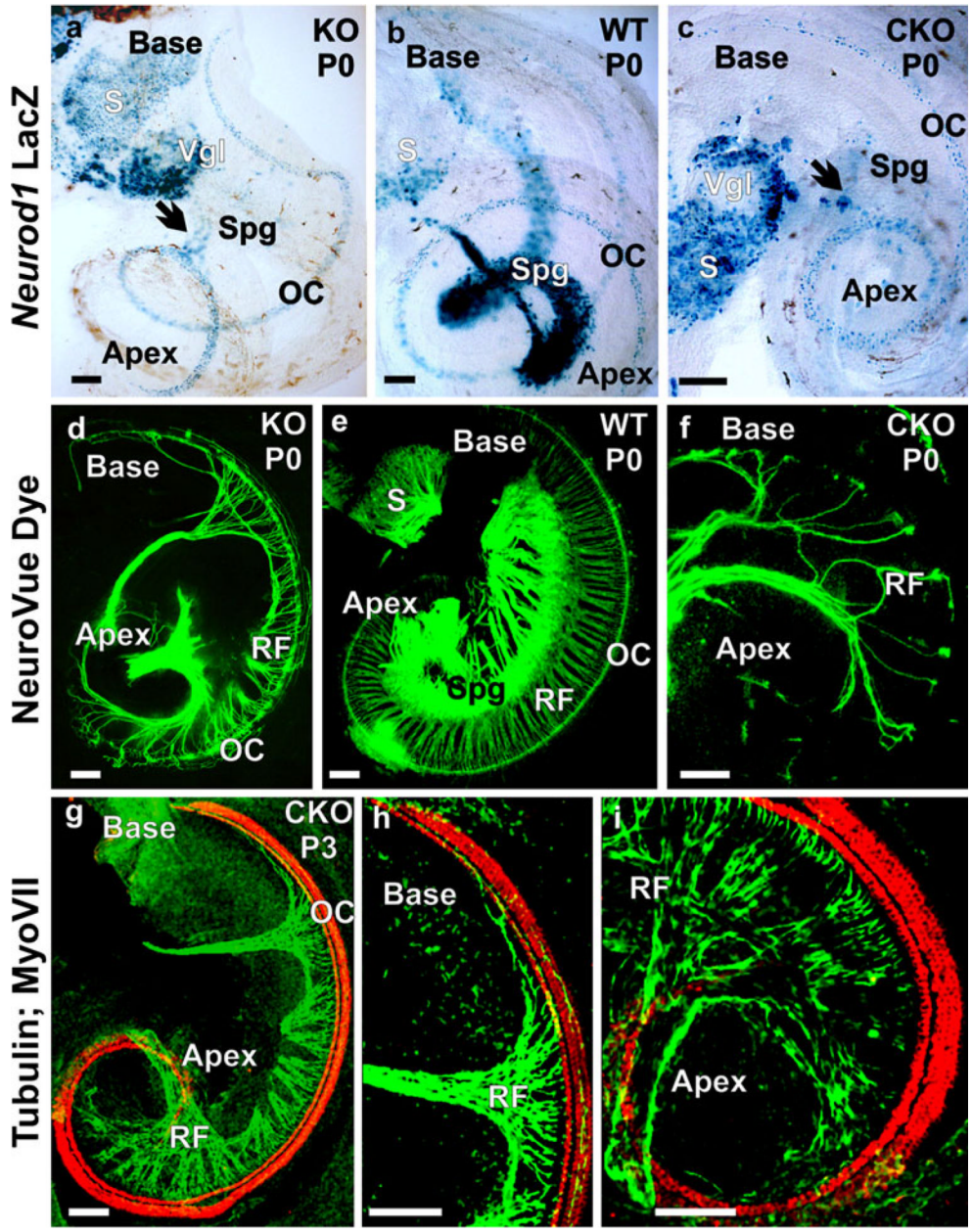

Fig. 1.

Neurod1 conditional knockout (*CKO*) and knockout (*KO*) mice have a similar phenotype. In newborn mice, *Neurod1*-LacZ reaction shows expression in spiral ganglion (*Spg*) in wildtype (*WT*) mice (**b**), whereas only a few scattered spiral ganglion neurons are positive for *Neurod1*-LacZ in the *Neurod1* KO and CKO (*arrows* in **a, c**). Additional expression of *Neurod1*-LacZ is seen in the hair cells of the organ of Corti (*OC*). The near complete loss of spiral ganglion neurons results in alterations of the projections of afferents in both mutants (**d, f**). The wildtype (**e**) has a well-developed spiral ganglion, and lipophilic dyes show regularly spaced radial fibers that carry both afferent and efferent fibers to the OC. In contrast, fewer and disorganized afferents reach the OC in both *Neurod1* KO and CKO mice. The fiber reduction in the CKO mice is more severe in the apex and base, with some retained innervation in the middle turn, consistent with the region of remaining spiral

ganglion neurons shown with tubulin (*green*) and Myo VII (*red*) immunofluorescence staining (**g–i**). (*RF* radial fibers, *S* saccule, *Vgl* vestibular ganglion). *Bars* 100 μm

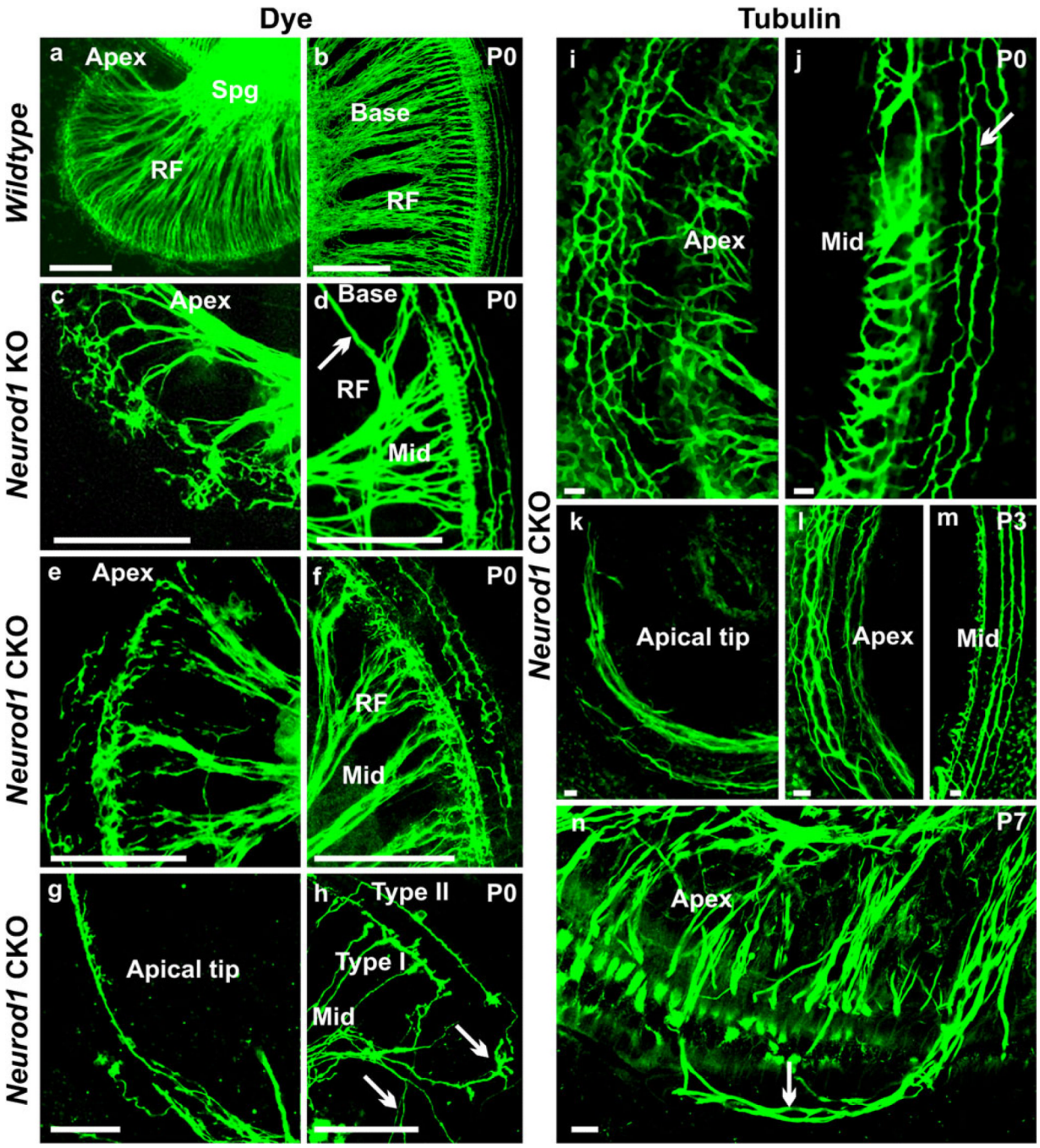

Fig. 2.

Afferents are disorganized in *Neurod1* mutant mice. Afferents form tightly spaced radial fiber bundles to the organ of Corti (**a, b**) in wildtype animals (*RF* radial fibers, *Spg* spiral ganglion). In *Neurod1* KO and CKO mice, radial fibers are severely reduced and more widely spaced with profuse collateral branching (**c–n**). Both mutants have few recognizable type II fibers in the middle turn of the cochlea (**d, m**), but most of these fibers near the apical region are reduced or disorganized with random extension toward the base (*arrow* in **d**) or apex (**f, i**, *arrow* in **j, l**). The type I fibers have extensive branching to the inner hair cells near the lower middle turn (**c, e, h, i, n**). The basal and apical tips are innervated by a single

fiber projecting from the inner spiral bundles with progressive abolition of type II fibers (**g, k**). Lipophilic dye applications to the apex of the *Neurod1* CKO mice shows fibers in the middle turn bearing characteristics of type II fibers extending along the rows of outer hair cells (**h**). In addition, some fibers to the inner hair cells are labeled and resemble normal type I afferents, but many other fibers branch profusely (*arrows* in **h**) as they reach inner hair cells near the apical middle turn (**h**). This abnormal branching and overshooting of type I fibers in the apical region is observed more clearly in later stages in which they appear to innervate hair cells in the rows of outer hair cells (*arrow* in **n**). *Bars* 100 μm (**a–h**), 10 μm (**i–n**)

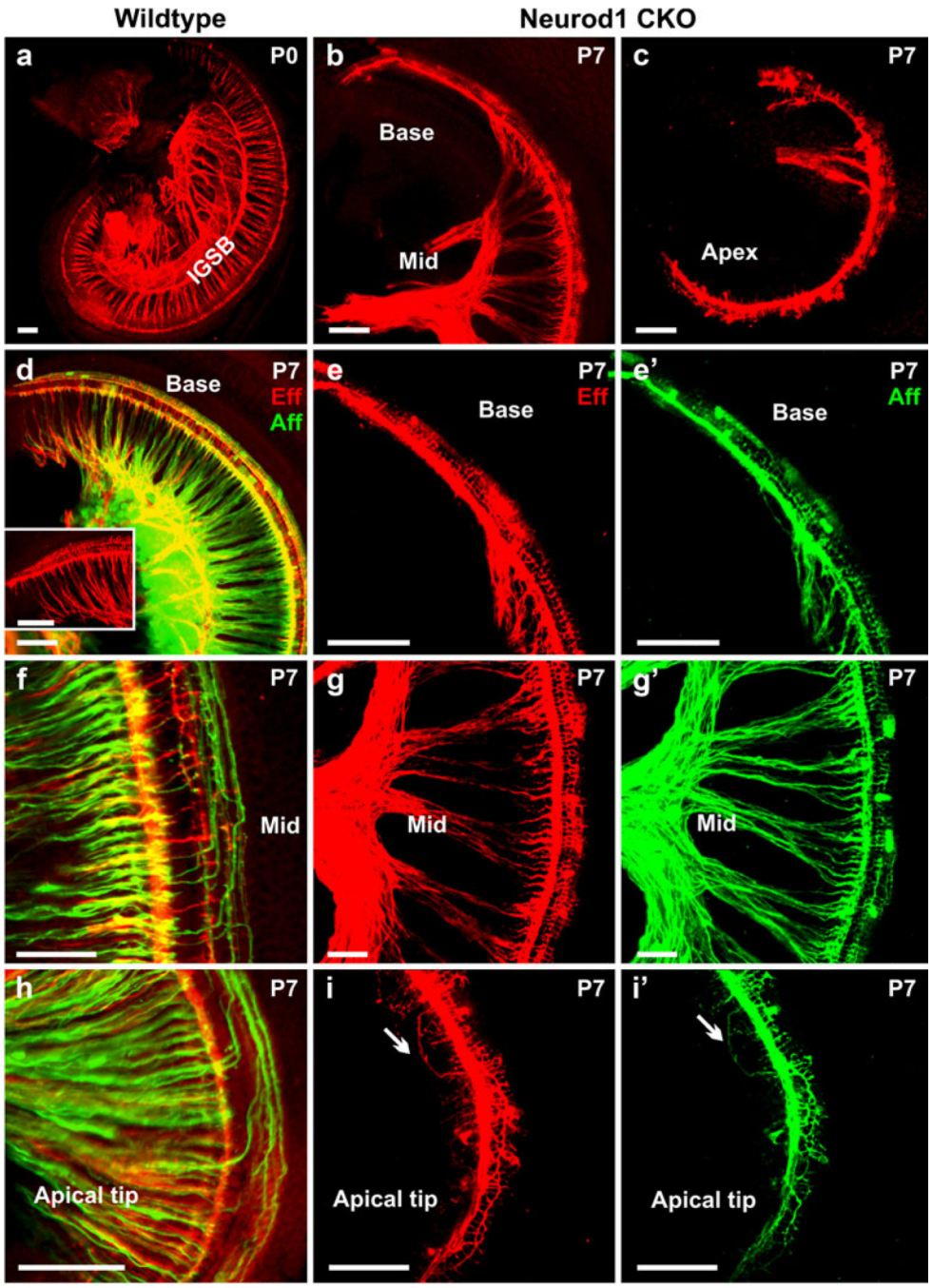

Fig. 3.

Defects in efferent innervation are comparable with those in afferents in *Neurod1* CKO mice. Efferent fibers (*red, Eff*) in the *Neurod1* CKO mice are massively reduced and disorganized, comparable with the afferent fibers (*green, Aff*) shown with dye application in the olivo-cochlear efferent bundles and cochlear nucleus. Only the wildtype mice show the formation of the intraganglionic spiral bundle (*IGSB*) together with the efferents to inner and outer hair cells (**a, d,** inset in **d, f, h**). The efferent organization is disrupted in the absence of *Neurod1*. Near the middle turn of *Neurod1* CKO mice, some efferent fibers innervate the inner hair cells along with the afferent fibers (**g, g'**). A collateral branch from this efferent extends as a single fiber along type I afferent fiber to the inner hair cells without

any formation of radial fibers (**b, c, e, e'**). In the apical tip, both efferents and afferents are reduced, with random overshooting of type II fibers, which extend toward the apex (*arrows* in **i, i'**). Some efferent fibers cross the tunnel of Corti innervating only the first row of outer hair cells rather than forming three parallel bundles along three rows of outer hair cells (**b, c, e, g, i**). The disorganization of afferents in the cochlea is even more severe in the later stage, with progressive reduction of type II neurons (**e', g', i'**). *Bars* 100 μm (**a–e', h–i'**), 50 μm (**f–g'**)

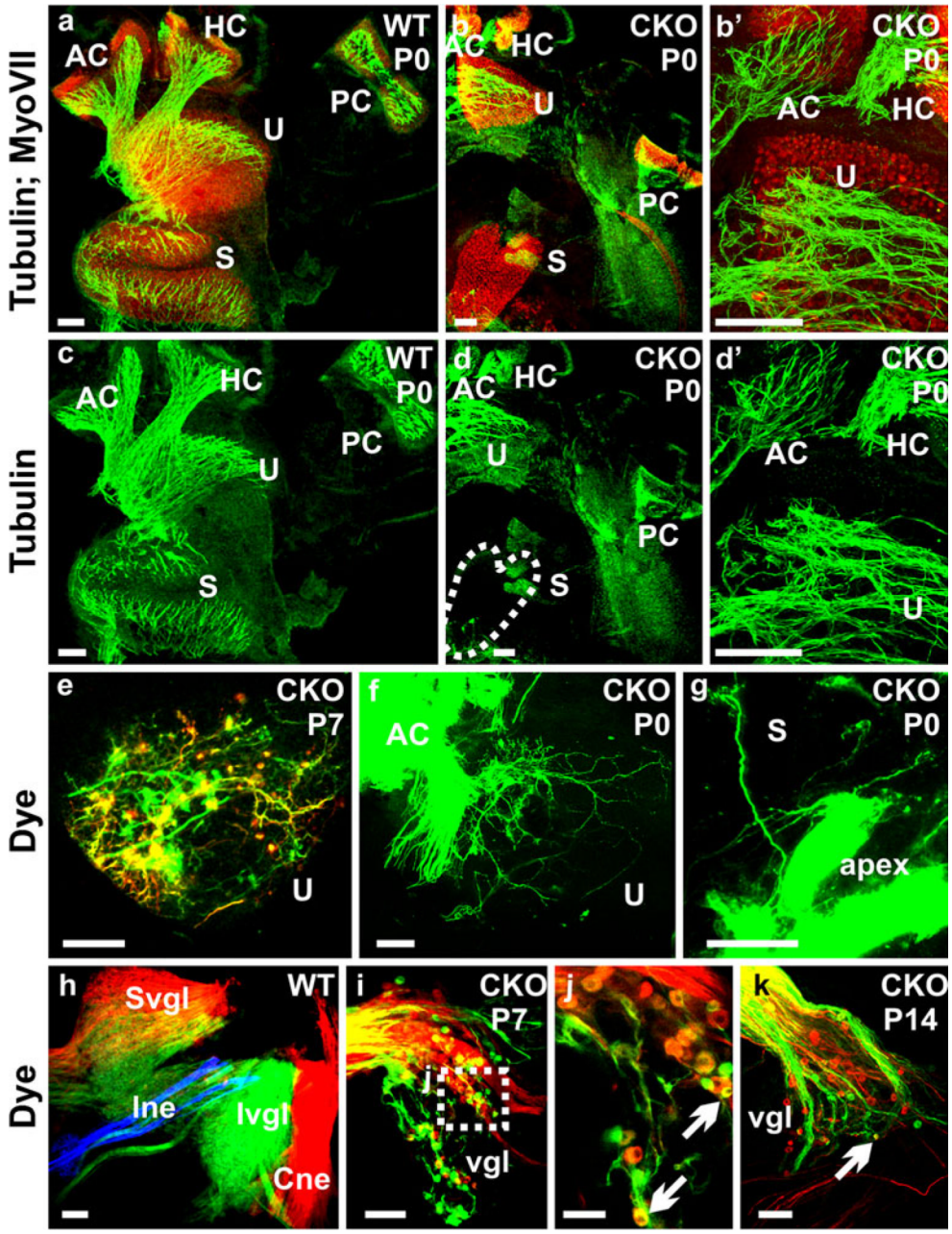

Fig. 4.

Absence of *Neurod1* causes reduction of innervation to vestibular end organs with more profound loss in the saccule (*S*). The innervation of vestibular end organs in *Neurod1* CKO mice are reduced and disorganized (**b, b', d, d', e**) in comparison with the control littermate (**a, c**) shown with the merged tubulin (*green*) and Myo VII (*red*) immunofluorescence staining (*HC* horizontal crista, *PC* posterior crista). In addition, the abnormal branching of fibers to reach other sensory epithelia is observed by dye application to the anterior crista (*AC*); this results in fibers reaching the utricle (**f**, *U*). These fibers take unusual trajectories and form both calyceal and bouton endings on hair cells (**f**). Such branching is also obvious in dye applications in the apex of the cochlea which labels few fibers to the saccule (**g**). The vestibular ganglia (*vgl*) in *Neurod1* CKO mice (**i–k**) are reduced in comparison with the control ganglia (**h**), which are labeled from the brainstem dye injection (*Cne* cochlear nerve,

Ine intermediate nerve, *Ivgl* inferior vestibular ganglion, *Svgl* superior vestibular ganglion). Moreover, these ganglia represent mixed types of neurons, which label both the cochlear and vestibular nuclei (*arrows* in **j, k**). These mixed ganglia may therefore be responsible for fiber projection in between the different sensory epithelia (**f, g**). *Bars* 100 μm (**a–i, k**), 50 μm (**j**)

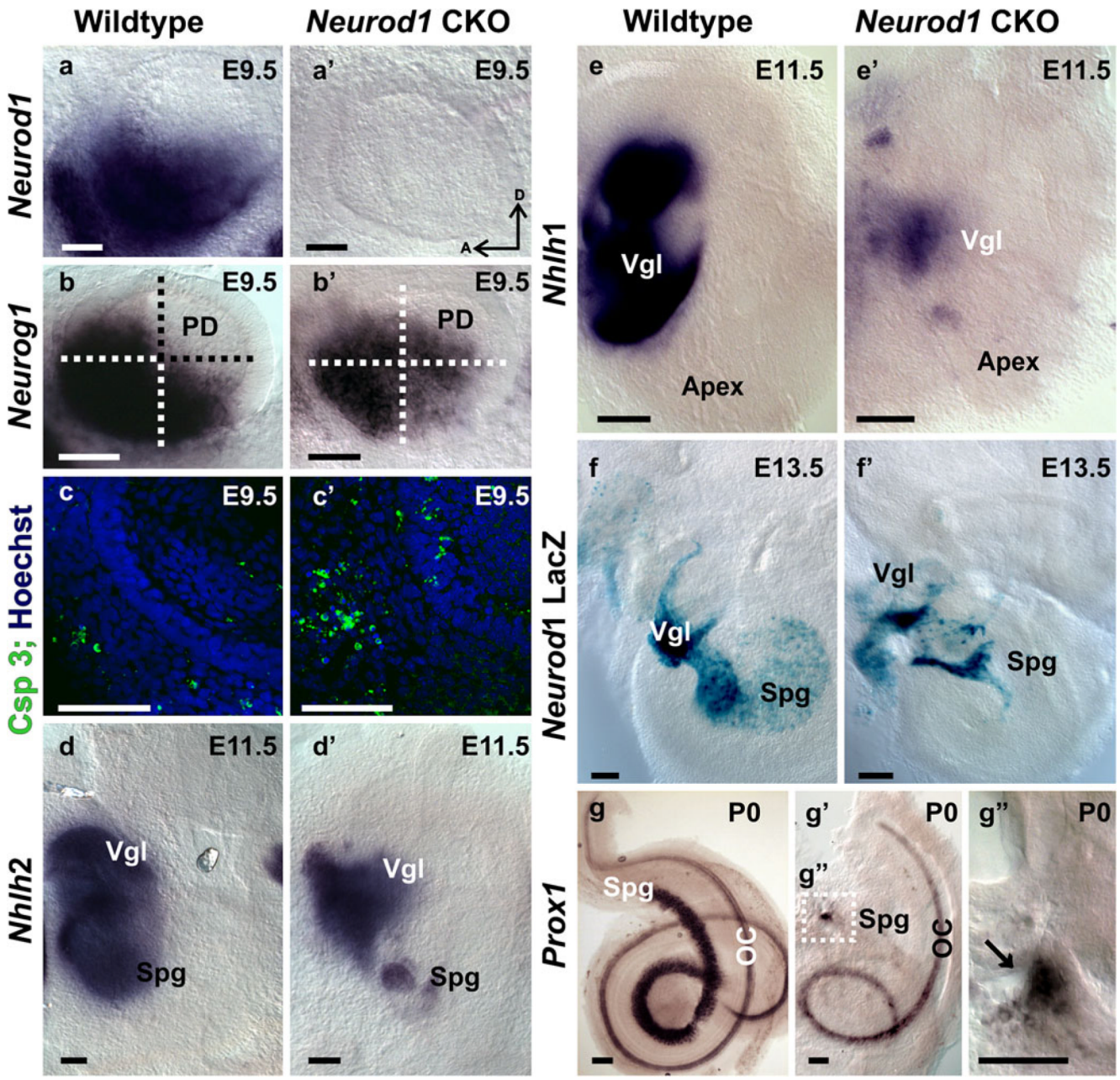

Fig. 5.

Conditional deletion of *Neurod1* is early and complete and induces gene expression changes. In situ hybridization with a *Neurod1* probe results in massive labeling in the otocyst as early as E9.5 in wildtype mice (**a**), whereas no *Neurod1* in situ hybridization signal can be seen in *Neurod1* CKO mice (**a'**). The bHLH gene *Neurog1* shows changes in expression with upregulation in the postero-dorsal quadrant and a reduction in the ventral half (**b, b'**) in the absence of *Neurod1* (*PD* postero-dorsal). These changes imply that *Neurod1* has been effectively recombined at least a few hours before our investigation of these expression changes. The distribution of activated Caspase 3, a marker for apoptotic cell death, shows greater apoptotic cells both inside and even more profoundly outside the otocyst in the forming vestibular ganglion (**c, c'**). These data suggest that *Neurod1* absence leads to a rapid loss of neuronal precursors through apoptosis and could be related to the signal reduction of

Neurog1 in the ventral half (**b'**). *Nhlh2* is another early marker of developing sensory neurons. This gene shows expression changes with a reduction in spiral ganglion (*Spg*) neurons as early as E11.5 (**d, d'**; *Vgl* vestibular ganglion). Similar changes are also seen in another early neuronal marker, *Nhlh1*, which makes the loss of sensory neurons expression more obvious (**e, e'**). A direct comparison of wildtype and *Neurod1* CKO or KO (shown here) mice shows that, by E13.5, the loss of spiral ganglion neurons is completed (**f, f'**) validating the observations of an early onset of apoptosis. A specific marker for spiral ganglion neurons, *Prox1*, confirms the loss of spiral ganglia in a later stage (**g', g"**; *OC* organ of Corti). *Bars* 100 μm

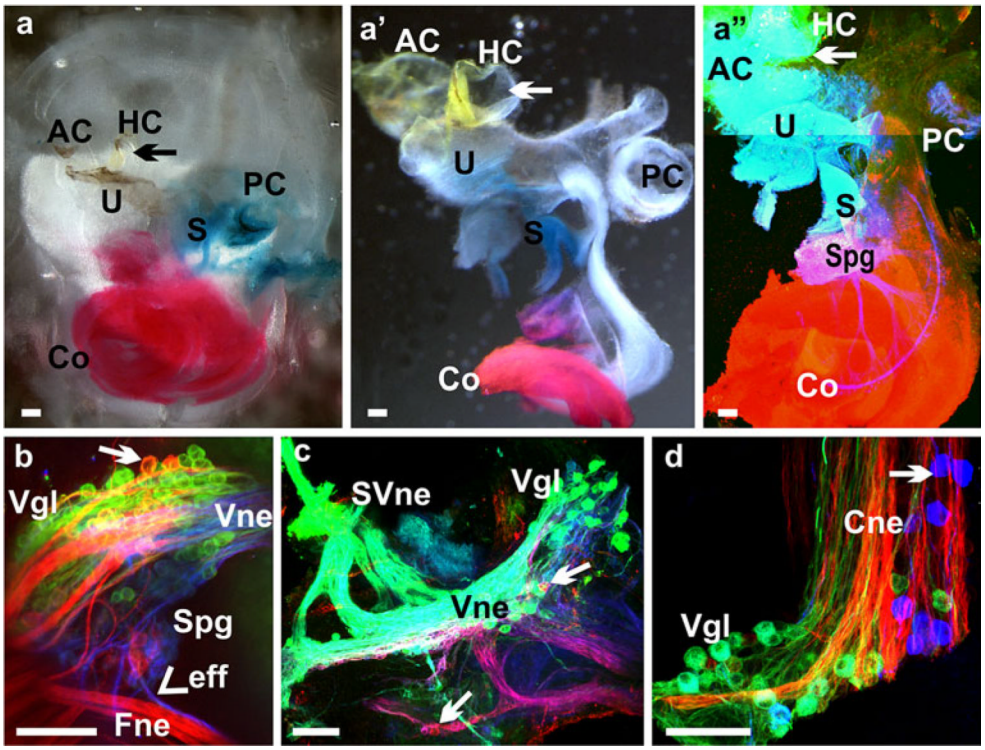

Fig. 6.

Afferent disorganization may correlate with the mixed ganglia in *Neurod1* CKO mice. Afferents of the inner ear were labeled by using filter strips that were soaked with chromatically different lipophilic dye tracers and that were inserted into the various sensory epithelia of inner ear (*AC* anterior crista, *HC* horizontal crista, *PC* posterior crista, *U* utricle, *S* saccule, *Co* cochlea, *Spg* spiral ganglion, *Vgl* vestibular ganglion, *Cne* cochlear nerve, *Vne* vestibular nerve, *Fne* facial nerve, *Eff* efferent fibers, *SVne* superior vestibular nerve). NeuroVue red (direct and false-colored *red*) dye was applied into the apex of cochlea, with NeuroVue jade (direct color *yellow*, false-colored *green*; *arrows* in **a–a"**) dye into the anterior canal crista/horizontal canal crista/utricle and NeuroVue maroon (direct and false-colored *blue*) into the base of cochlea or into the saccule (**a-a"**). Vestibular ganglion neurons with vestibular nerve fibers were labeled by the dye inserted into the canal crista and were shown to branch unusually from the vestibular nerve to innervate the canal crista and utricle (**c**). In addition, these ganglion neurons were labeled not only from the vestibular end organs, but also from the dye injected into the cochlea (*red* neurons, *arrow* in **b, c**). These mixed ganglion neurons migrated unusually near the nerve entry site to the brainstem (*arrow* in **d**). Moreover, efferents entered into the ear together with the facial nerve (*arrowhead* in **b**). *Bars* 100 μm

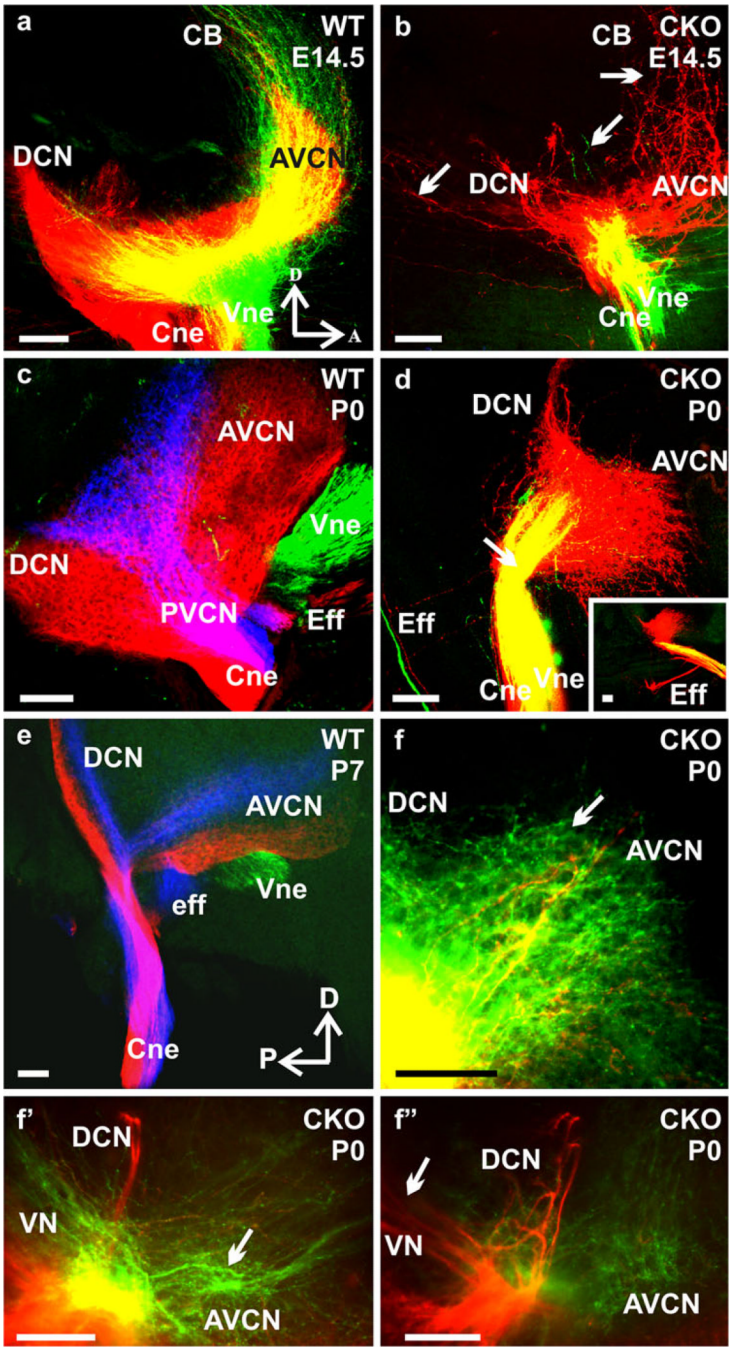

Fig. 7.

Loss of *Neurod1* results in overlapping central projections. Various dyes were applied into the different sensory epithelia of the inner ear to label their central projections. This approach showed segregated central projections of vestibular and cochlear afferents to the vestibular and cochlear nuclei and other targets such as the cerebellum as early as E14.5 (**a**). In contrast, afferent fibers entered into the brain as a single root in *Neurod1* CKO mice (**b, d, f-f"**), and afferents labeled from the cochlea projected to the cerebellum instead of stopping at the antero-ventral cochlear nucleus (**b**). In newborn and P7 wildtype mice, central projections from the base (*blue*), apex (*red*), and vestibular endorgans (*green*) and all

afferents entered the hindbrain as discrete fascicles, terminating exclusively in cochlear and vestibular nuclei (**c, e**). In *Neurod1* CKO mice, afferents to the cochlear nuclei were greatly reduced, showing overlapping projections from both the apex and base, with little extension toward the dorsal cochlear nucleus, and both vestibular and cochlear afferents entered through the same root (*arrow* in **d**). Vestibular afferents not only entered through the cochlear nucleus, but also projected variably to the antero-ventral cochlear nucleus (*arrow* in **f**) in addition to the vestibular nucleus (**f'**). Similarly cochlear afferents projected to vestibular nucleus (*arrow* in **f"**) in addition to cochlear nucleus.. Efferent fibers to the ear normally exited with the vestibular nerve root (**c**) but instead exited through the facial nerve in the mutant (**d**, *insert* in **d**) and were rerouted outside the brain to the vestibulo-cochlear nerve or the "cochlear nerve root" (*AVCN* antero-ventral cochlear nucleus, *PVCN* postero-ventral cochlear nucleus, *DCN* dorsal cochlear nucleus, *CB* cerebellum, *Cne* cochlear nerve, *Vne* vestibular nerve, *Eff* efferent nerve fibers, *VN* vestibular nucleus, *A* anterior, *D* dorsal, *P* posterior). *Bars* 100 μm

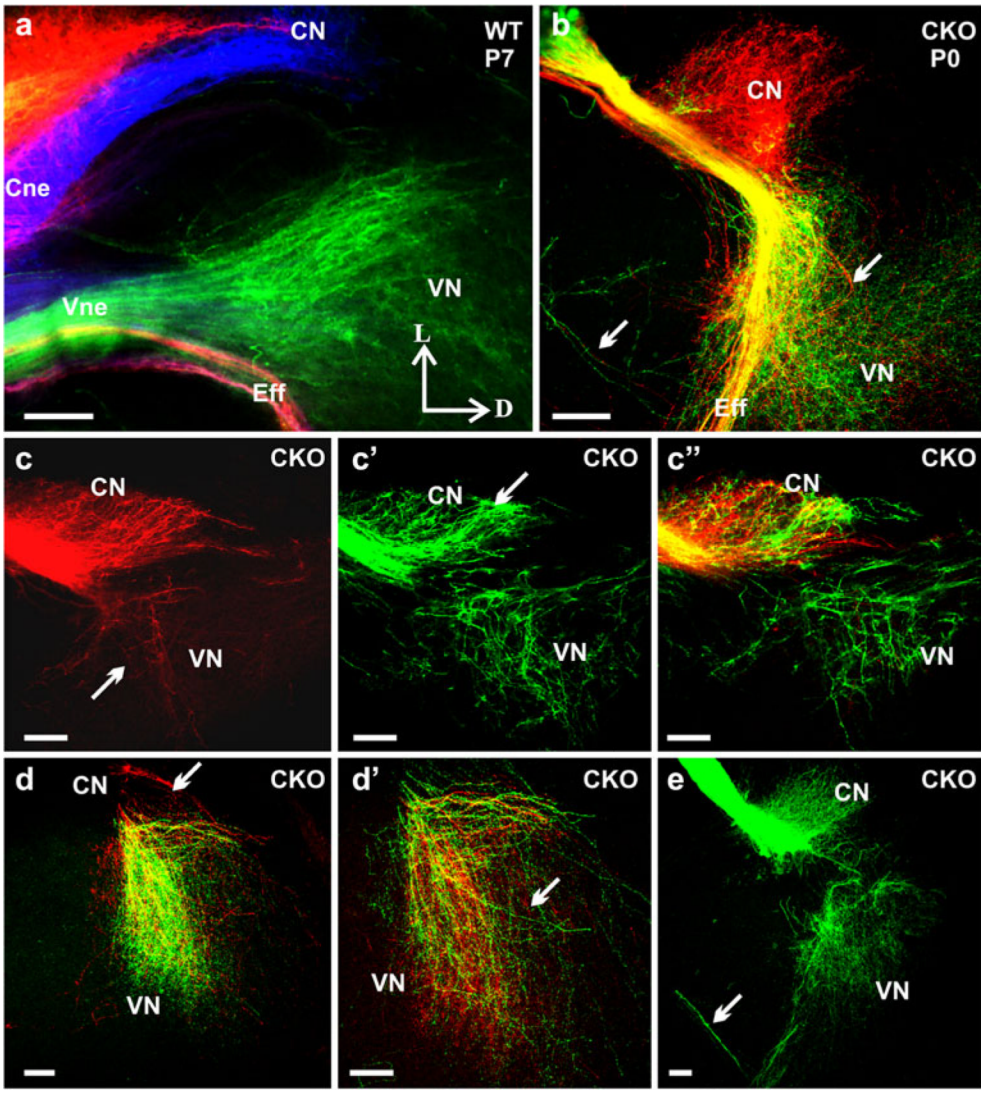

Fig. 8.

Vestibular and cochlear fibers project to both nuclei in *Neurod1* CKO mice. Coronal sections from the cochlear and vestibular nuclei with same dye injections as shown in Fig. 7 reveal that afferents labeled from the cochlea (*red*) target mostly the cochlear nucleus but extend also to the vestibular nuclei (*right arrow* in **b**, *arrows* in **c, d, d'**) in the absence of *Neurod1*. Likewise, afferents labeled from vestibular endorgans (*green*) project into the cochlear nucleus in addition to their original vestibular nuclei projections (**b, c', c", d, d', e**). This altered and overlapping projection is confirmed by specific dye application only in vestibular or cochlear epithelia showing that the fibers from vestibular end organs reach to the cochlear nucleus and vice versa (vestibular afferents labeling shown in **e**). The sections from wildtypes demonstrate the distinct topology of afferents from vestibular epithelia (*green*), cochlear apex (*red*), and cochlear base (*blue*) and the efferents that exit with the vestibular nerve root (**a**), whereas they exit through the cochlear nerve root (**b**) or through the facial nerve (*left arrows* in **b**, *arrow* in **e**) in *Neurod1* CKO mice (*CN* cochlear nucleus, *Cne* cochlear nerve, *Eff* efferent nerve fibers, *VN* vestibular nucleus, *Vne* vestibular nerve, *L* lateral, *D* dorsal). *Bars* 100 μm

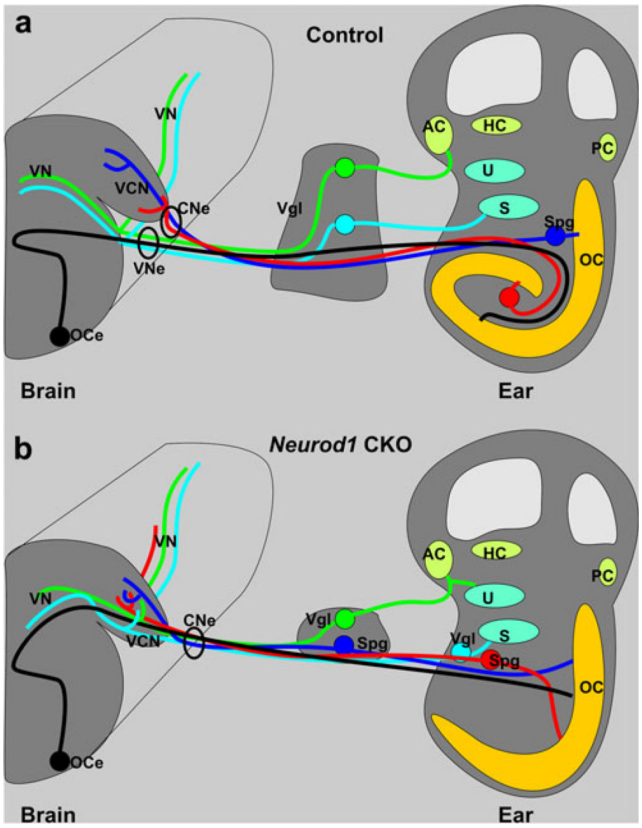

Fig. 9.

Illustration comparing the neuronal connection of the wildtype ear with the brain (**a**) with that of the *Neurod1* conditional null (*CKO*) mouse (**b**). All major features can be distinguished, but the cochlea is shortened and misshapen in *Neurod1* CKO mice. Whereas the normal organ of Corti (*OC*) has multiple turns and an accompanying spiral ganglion (*Spg*), there is typically no spiral ganglion found near the shortened organ of Corti in *Neurod1* CKO mice. However, spiral ganglion neurons as verified by their backfilling from the organ of Corti can be found near the saccule (*S*) or in the much reduced vestibular ganglion. A comparable but less severe disorganization is found in the vestibular ganglia (*Vgl*), which normally lie outside the ear but are scattered both inside and outside the ear in *Neurod1* CKO mice. As a consequence, nearby neurons may project to both vestibular and cochlear sensory epithelia. As fibers approach the brain, spiral afferents normally segregate from vestibular afferents, so that each fascicle enters as distinct vestibular and cochlear nerves into the vestibular and cochlear nuclei (**a**). In contrast, in *Neurod1* CKO mice, all afferents enter the brain together as a single root into the ventral cochlear nucleus (*VCN*). Despite this common entry point, fibers nevertheless project to the vestibular nuclei (*VN*) but also end up without any apparent topology in the cochlear nuclei. These data show that *Neurod1* expression is needed for proper migration of peripheral and central projection of all spiral neurons and to a lesser extent of vestibular neurons. Rerouting to the periphery is also observed in olivocochlear and vestibular efferents, which normally exit the brain via the vestibular nerve to reroute to the cochlea but instead exit the brain via the cochlear nerve or, less often, via the facial nerve to enter into the ear (*AC* anterior crista, *HC* horizontal crista, *PC* posterior crista, *U* utricle, *CNe* cochlear nerve, *OCe* olivocochlear efferent, *VNe* vestibular nerve)

Table 1

List of probes

Probe	Source
Neurod1	Lee et al. 1995
Neurog1	Ma et al. 1998
Nhlh1 and *Nhlh2*	Gift from Dr. Thomas Braun, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany
Prox1	Oliver et al. 1993