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Molecular mechanisms in the development and function of auditory neurons
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Abbreviations

ABR	Auditory brainstem response
AC	Anterior semicircular canal crista
AVCN	antero-ventral cochlear nucleus
AVN	auditory-vestibular nerve
ATOH1	Atonal homologue 1
bHLH	Basic helix-loop-helix
CN	Cochlear nucleus
CPLX2	Complexin 2
CRE	Cre recombinase
CVG	Cochleovestibular ganglion
DCN	dorsal cochlear nucleus
E	Embryonic day
EYA1	Eyes absent homolog 1
FGF8	Fibroblast growth factor 8
FOXG1	Forkhead box protein G1
HC	Horizontal semicircular canal crista
GATA3	GATA binding protein 3
GRM8	Glutamate receptor 8
IC	Inferior colliculus
IEE	Inner ear efferents
IGSB	Intraganglionic spiral bundle
IHC	Inner hair cell
ISL1	Insulin gene enhancer protein ISL-1
iVG	Inferior vestibular ganglion
LCC	Light coat and circling mutant mice
LMX1a/b	LIM homeobox transcription factor 1 alpha/beta
LOC	Lateral olivocochlear neurons
MOC	Medial olivocochlear neurons

MYO7a	Myosin VIIA
NeuN	Neuronal nuclei antigen
NEUROD1	Neurogenic differentiation factor 1
NEUROG1	Neurogenin 1
NGFR	Nerve growth factor receptor
NHLH2	Nescient helix-loop-helix 2
NTNG1	Netrin G1
OC	Organ of Corti
OHC	Outer hair cell
PAX2	Paired domain gene 2
PC	Posterior semicircular canal crista
PCDH15	Protocadherin 15
RF	Radial fibers
RIF	Rate-intensity function
RUNX1	Runt related transcription factor 1
SA	Saccule
SG	Spiral ganglion
SGNs	Spiral ganglion neurons
SIX1	Sine oculis-related homeobox 1
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin 4
SOX2	Sex determining region Y-box factor 2
SR	Spontaneous firing rate
SVG	Spiro-vestibular ganglion
sVG	Superior vestibular ganglion
TBX1/2/3	T-box 1/2/3
TMIE	Transmembrane inner ear (gene)
UT	Utricle
VG	Vestibular ganglion

Summary

Neurosensory hearing loss has been a growing problem in aging societies. It has been estimated that by 2050 the world might face a pandemic of around 900 million hearing impaired people. Two basic forms of hearing loss include external or middle ear-related (conductive) hearing loss and inner ear related (neurosensory) hearing loss. Help for neurosensory hearing loss is currently beyond direct therapeutic intervention. Recent data suggest that the prevention of neuronal loss and enhancement of long-term maintenance of neurons are the most feasible targets for the foreseeable future.

As the Head of the Laboratory of Molecular Pathogenetics at the Institute of Biotechnology CAS, my research has primarily focused on the intricate field of transcriptional regulation during embryonic development. It has aimed to uncover and understand the molecular mechanisms underpinning developmental programming and identifying the molecular triggers responsible for abnormal embryonic development, pathophysiological conditions, and disease predispositions. I have led several research programs focusing on the networks governed by key transcription factors such as HIF-1, ISL1, SOX2, and NEUROD1. My research team investigates how the dysfunction of these transcription factors impacts embryonic development and increases an individual's susceptibility to diseases like diabetes, heart disease, and hearing loss. Using mouse models, advanced microscopic techniques, both single-cell and bulk transcriptome analyses, as well as epigenetic analyses, we seek to identify potential molecular targets for the development of preventive and diagnostic strategies.

In this thesis, I have summarized seven years of research carried out by my research team under my leadership and supervision, and in collaboration with my colleagues. Together, we aim to pinpoint the molecular cues responsible for specifying cellular phenotypes in the inner ear, the intricacies of molecular regulation crucial for neurosensory development and maintenance, and ultimately apply this knowledge for devising novel therapeutic strategies and preventive measures against hearing impairment.

1. Introduction

(*The references highlighted in blue are our publications, representing the basis of this thesis.*)

The partial or total inability to hear sounds, is among the top 10 disabilities of today's society and approximately 5% of the world's population or 430 million people, including 34 million children, require hearing rehabilitation (WHO, 2023). Neurosensory hearing loss is permanent and results from the death of neurons or sensory cells that have no ability to regenerate. Auditory neurons are a critical component of the auditory pathway, as they transmit auditory information from the mechanosensory hair cells to the cochlear nucleus in the brainstem. The prevalence of hearing impairment increases with age, because of loss of sensory hair cells in combination with the loss of auditory neurons and neurons of cochlear nuclei. The current effective therapies for hearing impairment utilize either hearing aids to increase hair cell stimulation or cochlear implants as a substitute for hair cells. These medical devices require the presence of functional auditory neurons in the inner ear. Therefore, recent studies focus on possibilities for neuronal replacement, including exogenous stem cell transplantation and endogenous cell source replacement. It is also important to resolve the dependency of sensory hair cells, sensory neurons, and cochlear nucleus neurons. Transcriptional networks are key in controlling the regeneration or replacement of sensory cells and neurons from stem cells. Understanding and identifying individual transcription factors involved in the development and survival of auditory neurons are crucial for future more effective treatments for hearing loss (Shi and Edge, 2013; Meas, Zhang and Dabdoub, 2018). This work focuses on the research on auditory neurons, particularly on molecular networks important for the development, maintenance, and function of auditory neurons.

1.1. Overview of cellular composition in the inner ear

The inner ear is a structure consist of intricate ducts and canals that facilitates the perception of auditory signals, gravitational forces, and head/body movements. The primary mechanosensory transducers for auditory and vestibular systems are sensory hair cells, responsible for the mechano-electrical transduction of signals into neuronal signals (Dabdoub et al., 2015; Fritzsch, 2022). The inner ear hair cells represent modified epithelial cells with stereocilia on the apical end and the afferent and efferent

nerve fibers forming synapses on the basal end. The vestibular sensory epithelium is represented by the maculae of the saccule, utricle and the semi-circular canals expands into an ampulla with sensory epithelium forming the crista ampullaris (Figure 1A). Within all vestibular end organs, type I and type II hair cells are the peripheral receptor cells (Figure 1B). Transduction of auditory signals occurs in the sensory epithelium of the cochlea, the organ of Corti (Driver and Kelley, 2020). The cochlear outer and inner hair cells (OHCs and IHCs) are the primary mechanosensory transducers of acoustic signals (Figure 1C). OHCs are sound amplifiers, whereas IHCs

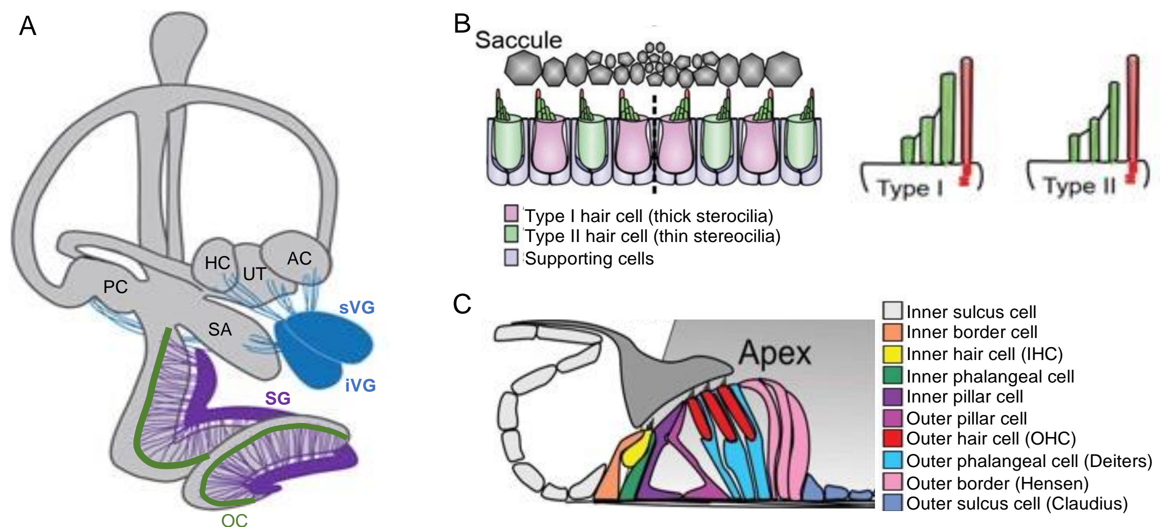

Fig. 1. Inner ear sensory organs. (**A**) Schematic of a mammalian inner ear showing the location of the auditory epithelium, the organ of Corti (OC) in the cochlea and the five vestibular epithelia: anterior (AC), horizontal (HC) and posterior semicircular canal crista (PC), the utricle (UT), and saccule (SA). The superior (sVG) and inferior vestibular (iVG), and spiral (SG) ganglia innervate the vestibular and auditory sensory epithelia, respectively. (**B**) Vestibular hair cells (example of the saccule organization): Type I with thick stereocilia; Type II with thin stereocilia. (**C**) The organ of Corti is formed by two rows of sensory hair cells, and numerous supporting cells. Inner phalangeal cells and Dieters' cells surround the sensory hair cells, providing mechanical support. Pillar cells separate the outer hair cells (OHCs) and the inner hair cells (IHCs) while forming the tunnel of Corti. Claudius cells and Boettcher cells (base only) are not associated with sensory hair cells.

are primary sensory cells. Both vestibular and cochlear epithelia contain, in addition to

sensory hair cells, highly ordered rows of non-sensory supporting cells (Driver and Kelley, 2020; Fritzsch, 2022). Hair cells synapse on afferent sensory neurons, which project centrally to the cochlear or vestibular nuclei within the hindbrain. The central processes of these neurons form the VIIIth cranial nerve (the vestibulocochlear nerve). Inner ear neurons are the first neurons of the afferent (ascending) pathways that relay

information to the cortex (Figure 1D, E). Additionally, the efficacy of the hair cell-afferent neuron transmission is controlled by the central nervous system through the efferent descending pathways from the cortex (Fritzsch and Elliott, 2017; Frank and Goodrich, 2018). At the lowest levels, efferent neurons from the brainstem project back into the inner ear to modulate both neurons and hair cells. This intricate network of ascending, descending, and reciprocal connections is responsible for the encoding, transmitting, and interpreting of sounds and motions.

1.2. Tonotopic organization of the auditory system

In the auditory system, cochlear sensory hair cells are connected to the brain by SGNs 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 (Rubel and Fritzsch, 2002; Muniak et al., 2016). The position of SGNs along the tonotopic axis of the cochlea correlates with the input frequency received from IHCs (Rubel and Fritzsch, 2002) (Figure 2). Because of the specialized innervation within

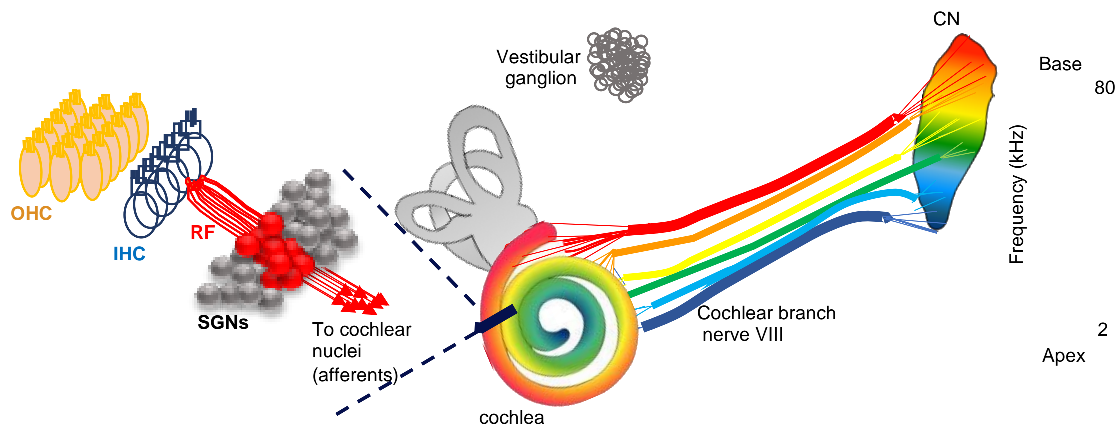

Fig. 2. Schematics of the tonotopic organization. The position of spiral ganglion neurons (SGNs) correlates with the input frequency received from inner hair cells (IHCs), high frequencies at the base and low frequencies at the apex, so called the tonotopic organization. The formation of a tonotopic map requires the precise projection of the SGN afferents of the cochlea onto the first auditory nuclei of the hindbrain, the cochlear nuclei (CN). The tonotopy is maintained throughout the auditory pathway. RF, radial fibers

the cochlea, auditory processes respond to a narrow range of frequencies, with the maximal response at one particular frequency. It is defined as the sound frequency at which an individual auditory nerve fiber is most sensitive, the characteristic frequency. Detection of complex sounds depends on the properties of auditory neuronal subtypes,

particularly on spontaneous firing rate as well as on the proportions of these neuronal subtypes along the tonotopic axis. The distribution of these subtypes varies based on species (Schmiedt, 1989). This tonotopic (or cochleotopic) organization is maintained throughout the auditory pathways (Kandler, Clause and Noh, 2009). The formation of a tonotopic map requires the precise projection of the spiral ganglion neuron afferents of the cochlea onto the first auditory nuclei of the hindbrain, the cochlear nuclei. Tonotopy of the cochlear nuclei is imprinted via the precise distribution of auditory axons with respect to their position of origin in the spiral ganglion (Muniak et al., 2013).

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 due to the lack of models (Kral et al., 2016) beyond simply removing parts of the cochlea (Harrison, 2016).

1.3. Auditory pathways and neurons in the inner ear

Spiral ganglion neurons (SGNs), forming the ascending auditory pathway, innervate the sensory epithelia of the organ of Corti in the cochlea and transmit auditory information in the form of electrical signals to the brain (Figure 3). The somata of auditory neurons form the spiral ganglion that twists within a coiled cochlear duct.

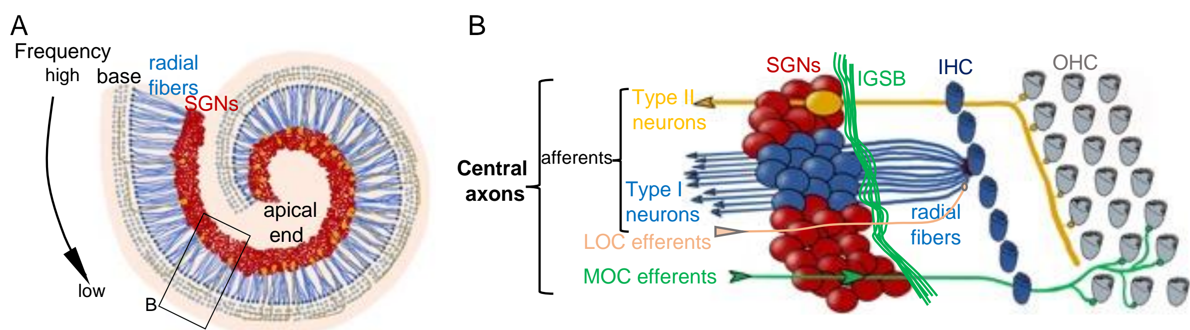

Fig. 3. Organization of the adult cochlea. (A) Three rows of OHCs and a row of IHCs connected to spiral ganglion neurons (SGNs) by radial fibers. (**B**) Diagram shows innervation of the organ of Corti. Each bipolar spiral ganglion neuron sends a long central axon (afferents) to the cochlear nucleus. Type I neurons extend radial fibers toward the inner hair cells (5-30 type I neurons innervate one inner hair cell); type II neurons provide diffuse innervations to the outer hair cells and supporting cells within the cochlea. Medial (MOC) and lateral (LOC) olivocochlear neurons of the auditory brainstem project to the sensory epithelium (efferents). The LOCs form synapses with type I spiral ganglion neurons. The MOCs terminate on OHCs and form the intraganglionic spiral bundle (IGSB).

Peripheral neuronal processes innervating hair cells of the organ of Corti are referred to as the dendrites, whereas the central processes are the axons (Dabdoub et al.,

2015; Fritzsch, 2022). Most of them are myelinated, which is considered to be a characteristic of the axons. The axons of the afferent neurons merge into the vestibulocochlear nerve, also known as the VIII. cranial nerve, relaying acoustic information further to the central nervous system. Two types of auditory neurons have been described in the cochlea (Figure 3B, (Perkins and Morest, 1975)). Large, myelinated, bipolar type I neurons connect IHCs in the organ of Corti to the cochlear nuclei in the brain. Smaller, pseudounipolar type II neurons connect OHCs to the cochlear nuclei and their peripheral processes remain unmyelinated and thin. Type I SGNs represent the majority of all neurons in the spiral ganglion (approximately 95%), and a total of 5-30 type I neurons innervate one inner hair cell in the cochlea (Ryugo, 1992). In contrast, type II spiral ganglion neurons receive input from dozens of outer hair cells as well as supporting cells, representing the remaining 5% of the population of SGNs (Fechner et al., 2001; Reid, Flores-Otero and Davis, 2004). Type II auditory neurons appear to play a role in pain signaling and in damage perception (Flores et al., 2015; Liu, Glowatzki and Fuchs, 2015). Type I neurons can be further divided into three genetically distinct subtypes, known as type Ia, Ib, and Ic (Petitpre et al., 2018; Shrestha et al., 2018; Sun et al., 2018). The subclasses exhibit significant variations, such as differences in expression of transcription factors, neurotransmitter receptors, channel subunits, or cell adhesion molecules. Type Ia, Ib, and Ic neurons show different spatial arrangements as well as high selectivity for a limited range of frequencies. Additionally, type I SGNs exhibit differences in spontaneous firing rates (SRs). Thus, they can be classified as low-SR, medium-SR, and high-SR fibers and single inner hair cells appear to be innervated by fibers with different SRs (Liberman, 1982; Liberman, Wang and Liberman, 2011; Wu, Young and Glowatzki, 2016). Although the total number of auditory neurons decreases with age, type Ic neurons seem to be particularly vulnerable to noise or aging (Shrestha et al., 2018).

The auditory pathway descending from the cortex is called the efferent system or the olivocochlear system (Fritzsch and Elliott, 2017; Frank and Goodrich, 2018; Elgoyhen, Wedemeyer and Guilmi, 2019). While being unique to the auditory region, it consists of efferent neurons subdivided into medial olivocochlear efferents and lateral olivocochlear efferents (Figure 3B). The efferents are derived from facial branchial motor neurons. The groups differ in the neuron bodies' location and degree of

myelination. Thicker myelinated medial efferents form synapses with OHCs of the organ of Corti in the cochlea. Thin unmyelinated lateral efferents innervate the dendrites of nerve fibers connecting IHCs (Elgoyhen, Wedemeyer and Guilmi, 2019). The pathway uses acetylcholine as the major neurotransmitter (Elgoyhen et al., 2001; Lustig et al., 2001). The efferent system is involved in the improvement of signal detection, the functioning of outer hair cells, and protection of the cochlea from acoustic damage. The difference in the olivocochlear system efficiency is an essential factor of the vulnerability to permanent acoustic injury (Maison and Liberman, 2000).

1.4. Molecular diversity of auditory neurons in the cochlea

Transcriptomic profiling of individual cells has emerged as a powerful way to investigate cellular diversity in the cochlea and decipher molecular characteristics for the maintenance and function of neurosensory cells (Burns et al., 2015; Jean et al., 2023; van der Valk et al., 2023). Transcriptome analysis of matured auditory neurons in the cochlea characterized molecular differences between type I and type II neurons, and three subtypes of type I spiral ganglion neurons (Petitpre et al., 2018; Shrestha et al., 2018; Sun et al., 2018; Petitpre et al., 2022). Unique and combinatorial molecular profiles discriminate four distinct types of matured spiral ganglion neurons, correspondingly to the classification of auditory neurons based on their functional properties. For example, differences in the expression of the *Ngfr* gene encoding p75 neurotrophin receptor between type I and type II spiral ganglion neurons correlate with their differences in the response to neurotrophins and to injury (Sun et al., 2018). Additionally, differential expression patterns indicate tonotopic heterogeneity within the type II neurons, indicating that apical and basal type II neurons may have distinct functions (Vyas et al., 2019). Based on the transcriptome profiling, type I spiral ganglion neurons are separated into three distinct subtypes Ia, Ib, and Ic that compose 35%, 40%, and 25%, respectively, of the total population of neurons in the mouse cochlea (Shrestha et al., 2018). Additionally, the proportions of these subtypes of neurons differ along the tonotopic axis of the mouse cochlea with a larger proportion of Ia neurons and smaller proportion of Ib neurons in the cochlear base compared to the rest of the cochlea (Shrestha et al., 2018). Each neuronal subtype expresses unique set of genes encoding Ca^{2+} binding proteins, and K^{+} channel and Na^{+} channel subunits that affect

their response properties based on the sensitivity to sound and spontaneous firing rate. Spontaneous and sensory inputs promote the maturation and connectivity of type I and type II, and type I subclasses of spiral ganglion neurons. These processes are negatively affected by mutations in genes encoding the mechanotransduction components of hair cells. For example, mutations in *Tmie* (Zhao et al., 2014) and *Pcdh15* (Alagramam et al., 2011) interrupt transduction and the specification of Ia, Ib, and Ic neuron subtypes (Sun et al., 2018). All four types of auditory neurons exist at birth, suggesting that initial neuronal diversification in the cochlea is independent of activity patterns and the postnatal maturation of the organ of Corti (Petitpre et al., 2018).

Potential differences in the physiological properties and molecular expression profiles of spiral ganglion neurons may also affect how these neurons transmit signals to their targets in the cochlear nucleus subdivisions in the brainstem. Single cell transcriptome profiling showed molecular differences in the expression genes encoding axonal proteins, including glutamate receptor *Grm8*, the exocytosis regulator *Cplx2,* and netrin family gene *Ntng1*. Differences in the expression of axonal proteins may affect synaptic properties and connectivity for different subtypes of spiral ganglion neurons (Shrestha et al., 2018). For example, mutations of transcription factors *Neurod1* and *Isl1* affected axon guidance, migration of neurons, and targeting cochlear nucleus neurons (Macova et al., 2019; Filova et al., 2022b). Transcription factor RUNX1 controls SGN subtype composition in the cochlea (Shrestha, Wu and Goodrich, 2023). These findings demonstrate that diverse neuronal identities are essential for normal auditory stimulus coding and that SGN identities are plastic during embryonal and postnatal development.

1.5. Neuronal development in the inner ear

Neuronal development proceeds in parallel with the patterning and morphogenesis of the inner ear (Figure 4). All sensory organs of the inner ear and its associated sensory ganglia derive from a single embryonic source, the otic placode. The induction and morphogenesis of the inner ear from the otic placode represent highly orchestrated processes regulated by transcription factors and signaling molecules (Barald and Kelley, 2004; Groves and Fekete, 2012; Dvorakova et al., 2020). Neurons seem to be

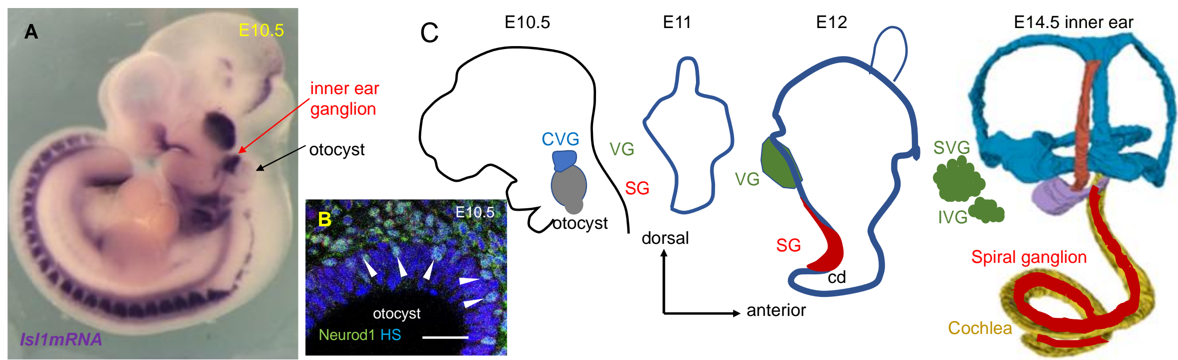

Fig. 4. Morphogenesis of inner ear ganglia. (**A**) Neurons forming the cochleovestibular ganglion in the mouse embryo at E10.5 are visualized by *in situ* hybridization. Image taken from (Chumak et al., 2016). (**B**) NEUROD1^{+} delaminating neuroblasts detected in the proneurosensory epithelium of the otocyst. Image taken from (Filova et al., 2022a). (**C**) The stages of inner ear morphogenesis are shown schematically from the otocyst to the mature three-dimensional structure. In parallel, the neurons delaminate to form first a cochleovestibular ganglion (CVG) followed by the gradual separation of the vestibular (VG, green) and spiral (SG, red) ganglia, which eventually innervate the vestibular and auditory sensory epithelia. cd, cochlear duct; HS, Hoechst nuclear staining; SVG, superior vestibular ganglion; IVG, inferior vestibular ganglion.

the first differentiated cells in the developing inner ear in all species examined; however, the inner ear structures, including the structures for hearing, vary among species (Fritzsch and Straka, 2014; Manley, 2017). Neurogenesis is confined to spatially restricted regions of the otocyst, partially overlapping with areas producing sensory cell precursors. The induction and morphogenesis of the inner ear from the otic placode represent highly orchestrated processes regulated by transcription factors and signaling molecules (reviewed by (Barald and Kelley, 2004; Groves and Fekete, 2012; Elliott et al., 2021)). As the otic placode invaginates and forms the otocyst, neurogenesis is initiated by the expression of proneural bHLH transcription factor Neurogenin 1 (*Neurog1*), which specifies neuronal precursors (Ma et al., 1998; Ma, Anderson and Fritzsch, 2000), followed by the expression of another bHLH transcription factor, *Neurod1* (Liu et al., 2000b; Jahan et al., 2010a; Filova et al., 2022a). Initial specification of neuroblasts within the otic epithelium is followed by the delamination of neuroblasts from the anteroventral region of the otocyst as early as embryonic day 9 (E9) in the mouse embryo. Soon after delamination, neuroblasts robustly express the bHLH gene *Neurod1*, proliferate, and form an inner ear ganglion (Figure 4A, B). A critical step in neurogenesis is the segregation of auditory and vestibular neurons, as the vestibuloacoustic ganglion segregates into a medial spiral ganglion and a lateral vestibular ganglion. The molecular cues regulating the

specification and segregation of auditory and vestibular neurons are not fully understood. Current evidence indicates that the development of auditory and vestibular neurons is spatially and temporally segregated before or shortly after *Neurog1* expression (reviewed by (Appler and Goodrich, 2011)). All neurons after a period of proliferation undergo their final cell divisions and begin to differentiate (Matei et al., 2005). As neurons mature, post-mitotic auditory and vestibular neurons extend their processes to their peripheral targets (the organ of Corti and the five vestibular sensory epithelia) and to central targets (the cochlear and vestibular nuclei of the brain stem). The central projections of inner ear neurons reach the hindbrain as early as E11.5 in the mouse (Fritzsch et al., 2015; Dvorakova et al., 2020). E12.5 is the earliest embryonic day to detect segregated central projections of auditory neurons to the cochlear nucleus from the vestibular nerve in the mouse (Fritzsch et al., 2015; Dvorakova et al., 2020; Filova et al., 2022a). This is a time before peripheral projections reach their targets. Auditory neurons mature and extend their peripheral neurites, starting in the base of the cochlea around E12.5 in the mouse (Fariñas et al., 2001; Appler et al., 2013; Dvorakova et al., 2020; Filova et al., 2022a).

The development of the three primary components of the peripheral auditory system, namely SGNs, cochlear hair cells, and the cochlear nuclei, is intricately interconnected. The proliferation patterns of these auditory components significantly influence their spatial and temporal differentiation processes. Notably, SGNs initiate this developmental cascade by being the first cells to exit the cell cycle, progressing from base-to-apex from ~E10.5 - 12.5, followed by hair cells, which exit the cell cycle opposite direction, from apex-to-base between ~E12.5 - 14.5, and neurons of the cochlear nuclei exit the cell cycle between E10.5 - 14.5 (Elliott et al., 2021). Transcription factors EYA1 and SOX2, both play a critical role in the proliferation and generation of neurosensory cells from the otic epithelium (Zou et al., 2004; Kiernan et al., 2005; Ahmed, Xu and Xu, 2012; Dvorakova et al., 2016; Dvorakova et al., 2020). The development of SGNs requires the transcription factor basic helix-loop-helix *Neurog1*, followed by its downstream target *Neurod1*. In contrast, hair cells and cochlear nuclei critically depend on *Atoh1* and require *Neurod1* expression for interactions with *Atoh1*. However, the landscape of interactions and crosstalk between these pivotal transcription factors, including NEUROG1, NEUROD1, ATOH1, and an

array of other contributing transcription factors, remains somewhat enigmatic and has not been fully elucidated.

2. Characteristics of main published findings

The primary focus of my research is the field of transcriptional regulation in both developmental processes and disease pathways. Understanding transcriptional networks may be the key to controlling the regeneration or replacement of neurons for future more effective treatments for hearing loss. This thesis summarizes research findings that have been published in high quality international scientific journals over the past decade. The references to my published articles are highlighted in blue throughout this document. This research has demonstrated that three key transcription factors play pivotal roles not only in the developmental processes of auditory neurons but also in the intricate orchestration of the formation and function of the auditory pathway.

The detailed descriptions of all methods and materials can be found in the corresponding publications. The analyses of hearing function, auditory behavior, and acoustic information processing in the inferior colliculus were conducted in collaboration with Prof. J. Syka at the Institute of Experimental Medicine CAS. The lipophilic dye tracing experiments were carried out in collaboration with Prof. B. Fritzsch at the University of Iowa and University of Nebraska Medical Center, USA.

<u>This thesis is organized into two distinct thematic areas:</u>

1) Early development of the inner ear
2) The limits of neural plasticity during the development of the auditory system

The first research area summarizes our investigation into the roles of the SOX2 and NEUROD1 transcription factors in the development of neuronal and sensory progenitors. The second research area focuses on the formation of tonotopic maps and the impact of developmental defects in the periphery on auditory information processing in the central auditory system of *Isl1* and *Neurod1* conditional deletion mutants. Each subchapter provides a concise summary and discussion of the main results obtained. Furthermore, the conclusions of each subchapter include proposals for future studies and experiments.

2.1. An early development of the inner ear: role of SOX2 and NEUROD1

All sensory organs of the inner ear and its associated sensory ganglia are derived from *Eya1/Six1/Brg1*-expressing otic progenitors. *Eya1/Six1/Smarca4* and *Sox2* define the neurosensory domain of the otocyst, whereas *Neurog1* is needed to initiate the proliferation and differentiation of the SGNs (Ma, Anderson and Fritzsch, 2000; Kiernan et al., 2005; Dvorakova et al., 2020; Kersigo et al., 2021; Zine and Fritzsch, 2023). A unique trio of transcription factors, *Tbx1/2/3* in concert with *Neurog1*, regulate otocyst processing and otic neurogenesis (Kaiser et al., 2021). Conditional deletions of *Gata3, Lmx1a/b,* and *Dicer* and deletion of *Pax2* results in complete loss of SGNs (Bouchard et al., 2010; Kersigo et al., 2011; Duncan and Fritzsch, 2013; Chizhikov et al., 2021). Deletions of the transcription factor *Neurod1* result in abnormal neurogenesis and axonogenesis (Liu et al., 2000b; Jahan et al., 2010a; Macova et al., 2019; Filova et al., 2020; Filova et al., 2022a).

2.1.1. *SOX2 is necessary for the development of neurogenic and sensory progenitors in the inner ear.*

SRY-box 2 (SOX2) is an essential factor able to generate induced pluripotent stem cells (Takahashi and Yamanaka, 2006). SOX2 is necessary for embryonic development in a variety of organs, in particular the brain and various sensory organs (Kondoh and Lovell-Badge, 2016), including the olfactory (Panaliappan et al., 2018), visual (Taranova et al., 2006), and otic systems (Kiernan et al., 2005; Puligilla et al., 2010). SOX2 acts by maintaining an undifferentiated pro-neurosensory cell state able to proliferate in various developmental systems (Wegner, 1999; Reiprich and Wegner, 2015; Hagey et al., 2018; Kageyama, Shimojo and Ohtsuka, 2019). SOX2 is highly expressed in proliferative progenitors but is downregulated in differentiating cells (Dabdoub et al., 2008; Nishimura, Noda and Dabdoub, 2017). In the brain, Sox2 and other Sox genes dynamically specify the proliferating neuronal lineage, upregulate differentiating transcription of basic helix-loop-helix (bHLH) factors and are ultimately downregulated by bHLH transcription factors to allow the differentiation of neurons and glia cells (Reiprich and Wegner, 2015) in sequential transcriptional waves (Telley et al., 2016). This process is conserved across neurosensory evolution (Fritzsch et al., 2015).

In the ear, SOX2 expression is in precursors with the ability to proliferate and differentiate as hair cells or neurons. SOX2 is needed to maintain hair cell precursors but is downregulated in differentiating hair cells upon upregulation of the basic Helix-Loop-Helix (bHLH) transcription factor ATOH1 (Kiernan et al., 2005; Dabdoub et al., 2008). The Sox2 dependent processes regulate the formation of HCs and supporting cells of the six mammalian sensory epithelia (the three cristae of the semicircular canals, the two maculae of the saccule and utricle, and the organ of Corti of the cochlea) out of sensory precursors (Fekete and Wu, 2002; Fritzsch et al., 2002) but may also be involved in non-sensory development (Gu et al., 2016). In analogy to SOX2 role in hair cell development, it has been proposed that SOX2 is necessary for inner ear neurogenesis. A lack of *Sox2* expression in *Lcc* mutant [light coat and circling; a mutant with an X-ray-irradiation-induced mutation in an enhancer of *Sox2* expression in the developing inner ear (Kiernan et al., 2005)] has been correlated with late absence of neurons at E15.5 (Puligilla et al., 2010).

To go beyond existing data, we generated a new mouse model of *Sox2* conditional deletion, *Isl1-cre;Sox2$^{f/f}$* (*Isl1-cre;Sox2$^{f/f}$*) (Dvorakova et al., 2016). We chose *Isl1-cre* to achieve an early and near complete overlap of *Cre* and *Sox2* expression in the sensory epithelium and delaminating neurons in the inner ear (Radde-Gallwitz et al., 2004; Huang et al., 2008; Mak et al., 2009; Chumak et al., 2016; Gu et al., 2016). Our conditional deletion of *Sox2* using *Isl1-cre* provided the first mouse model that tests *in vivo* the function of SOX2 in inner ear sensory neuron formation, inner ear sensory epithelia formation, and HC/supporting cell differentiation. Previous works have only provided indirect evidence for the role of SOX2, using a partially uncharacterized model with limited or no expression of *Sox2* in the ear (Kiernan et al., 2005; Dabdoub et al., 2008) or induced delayed loss of *Sox2* on HCs using inducible Sox2-creER mediated recombination (Kempfle, Turban and Edge, 2016).

Computer assisted 3D-reconstruction revealed profound differences of the ear of our *Isl1-cre;Sox2$^{f/f}$* mutant at E12.5 and E14.5 (Figure 5A) (Dvorakova et al., 2016). The mutant inner ears had no semicircular ampullae, and only rudiments of the

posterior and anterior semicircular canals with the horizontal canal being the only canal

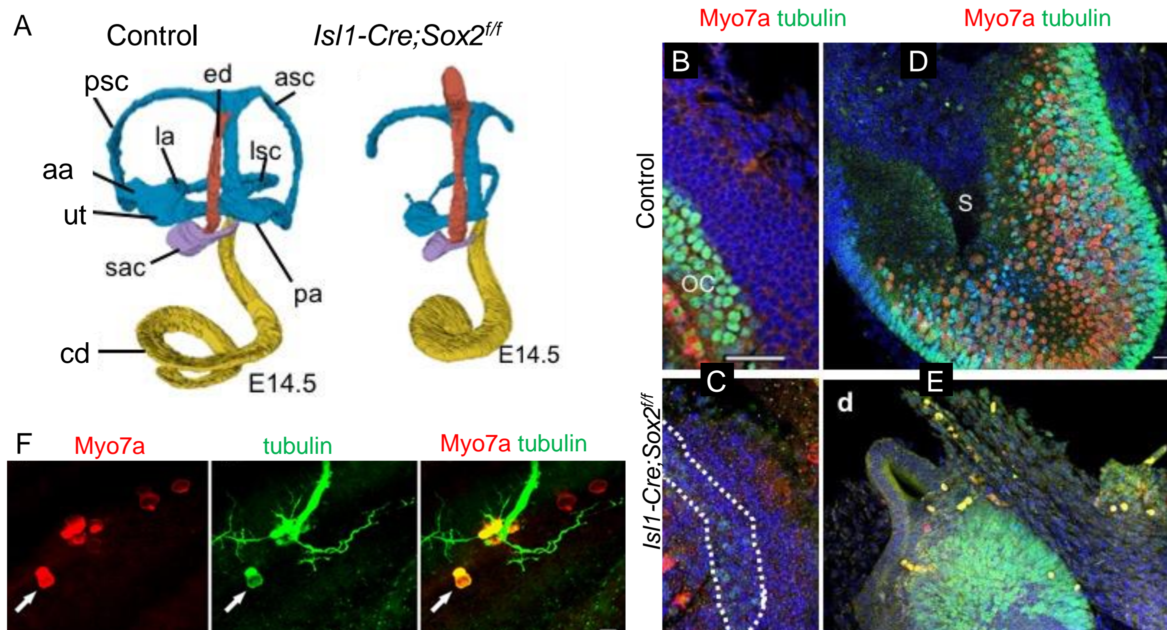

Fig. 5. Deletion of *Sox2* alters the inner ear morphology, and the differentiation and survival of neurosensory cells. (**A**) 3D-reconstruction reveals severe changes in the developing inner ear of *Isl1-Cre;Sox2*$^{f/f}$ at E14.5, including no ampullae of semicircular canals, only rudiments of the posterior and anterior semicircular canals, the utricle and saccule are smaller, and the cochlear duct (cd) has decreased coiling and is shorter compared to controls. aa, anterior ampulla; asc, anterior semicircular canal; cd, cochlear duct; ed, endolymphatic duct; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; sac, saccule; ut, utricle (**B-E**) At E18.5, the innervation of mutant cochlea, saccule (S) and utricle (U) is severely reduced and shows an unusual pattern compared to controls. Fibers show mostly directional growth toward remaining hair cells (Myo7a labeled cells). Scale bars: 100 µm. (**F**) Some hair cells in the cochlea are positive for both Myo7a (marker of hair cells) and tubulin (marker of neurons) with a patchy distribution and unusual pattern of innervation. Scale bars: 10 µm.

ever forming. The utricle and saccule were smaller, and the cochlear duct was 20% shorter compared to controls at E14.5. This phenotype was comparable to previous reports on the *Lcc* mutant with X-ray-irradiation-induced mutation localized to chromosome 3 and associated with a severe inner ear malformation due to the absence of *Sox2* in the developing inner ear (Kiernan et al., 2005).

The earliest events in the developing ear is the upregulation of the transcription factor Neurogenin1 and neuronal delamination (Ma et al., 1998; Ma, Anderson and Fritzsch, 2000), and this early event was unaffected in *Isl1-cre;Sox2*$^{f/f}$ mutant (Dvorakova et al., 2016). These data suggest that many vestibular neurons form, develop, and project but are only later eliminated, likely due to the lack of neurotrophins (Fritzsch et al., 2016) and/or HCs (Pan et al., 2011). In contrast, neurons of the spiral ganglion are formed only in the base, although the population of these neurons

becomes smaller after E15.5, and by E18.5 only a few neurons are left projecting to the base.

Interestingly, our data demonstrate that different sensory epithelia vary remarkably in hair cell formation in our *Isl1-cre;Sox2$^{f/f}$* mutant: some epithelia never form (canal cristae, the organ of Corti in the apex), while others are variably reduced (utricle, saccule, cochlear basal turn) (Dvorakova et al., 2016). These hair cells receive innervation from the few surviving neurons, indicating that some HCs are refractory to the delayed loss of Sox2. Such differential effects are well known for other factors. For example, Pax2 and Gata3 predominantly affect the cochlea (Bouchard et al., 2010; Duncan and Fritzsch, 2013). The loss of *Neurod1* affects HC type differentiation in the apex but not in the base (Jahan et al., 2010a; Macova et al., 2019). How these factors compound each other to cause the differential effects of *Sox2* loss in our *Sox2* conditional deletion mutant remain to be investigated. Alternatively, since only epithelia known or suspected to have common neuronal/hair cell progenitors retain HCs (Ma, Anderson and Fritzsch, 2000; Matei et al., 2005; Raft and Groves, 2014), it might be possible that only HCs derived from such common neurosensory progenitors form, whereas epithelia without such common precursors develop no HCs at all (canal crista, apex). The expression of neuronal markers in some of these HCs supports this notion.

However, the precise role of SOX2 in neuronal development has remained somewhat ambiguous. In light of this, in our subsequent research, we shifted our focus to early otic neuronal development to address the concern related to the delayed Cre activity associated with *Isl1-Cre*. To achieve this, we opted for *Foxg1-Cre*, a promoter known for its expression in the otic placode. *Foxg1-Cre* has previously proven effective in the study of olfactory placode development, which involves a sequential activation of bHLH transcription factors (Panaliappan et al., 2018). In contrast to the massive effects on neurosensory development in the olfactory system, otic development continues without SOX2 protein to generate vestibular neurons with a transient near normal development of central and peripheral processes in our *Foxg1-Cre;Sox2$^{f/f}$* mutant (Figure 6) (Dvorakova et al., 2020). Despite this early development, all neurons eventually die by apoptosis due to the failure of sensory epithelia differentiation and the resulting absence of neurotrophic support. Following apoptosis of these neurons, some efferent fibers remain for an unknown duration as signs of previous development.

3D-reconstruction revealed profound differences in the morphogenesis of the inner ear of mutants (Figure 6), such as cochlear agenesis, rudimental saccule development but all other structures were missing or undiscernible at E14.5. Inner ear malformations in our *Foxg1-Cre;Sox2*$^{f/f}$ were more pronounced compared to the previously described *Lcc* mutant (Kiernan et al., 2005). Our data confirm that late forming spiral ganglion

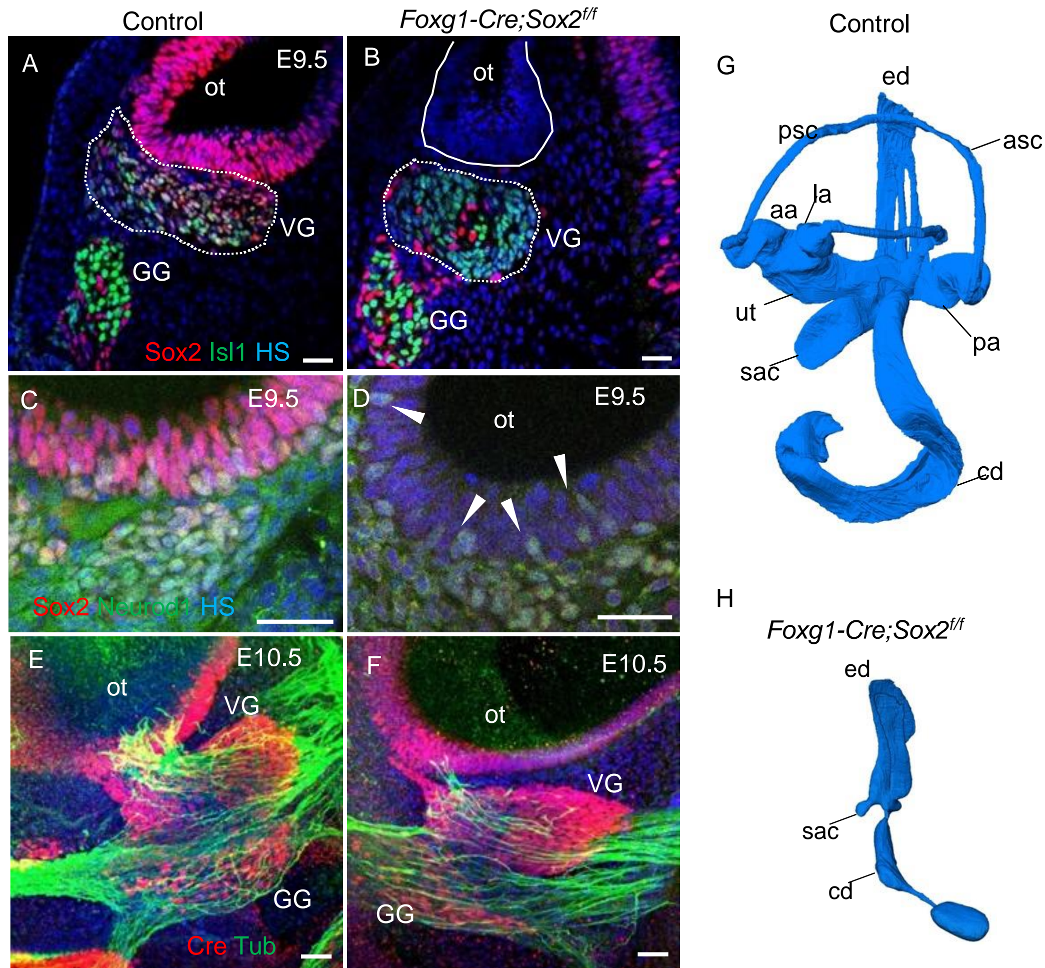

Fig. 6. Changes in inner ear development in *Foxg1-Cre;Sox2*$^{f/f}$ mutant. (**A, B**) In E9.5 controls, SOX2 is expressed in the otocyst (ot) and in developing neurons of the vestibular ganglion (VG), whereas SOX2 is not detectable in the ear epithelium or neurons in *Foxg1-Cre;Sox2*$^{f/f}$. (**C, D**) NEUROD1^{+} neuroblasts (arrowheads) delaminate from the SOX2+ otic epithelium in controls and SOX2 negative epithelium in mutant. (**E, F**) Whole-mount immunostaining with anti-acetylated α-tubulin (nerve fibers) and anti-CRE shows apparently diminished innervation of the ear epithelium in mutant compared to littermate heterozygous *Foxg1-Cre;Sox2*$^{+/f}$ (arrowheads), indicating abnormalities in inner ear development at E10.5. Inner ear structural malformations in Sox2cKO are revealed by computer-assisted 3D-reconstruction. (**G, H**) The inner ear of mutant shows cochlear agenesis and a rudimental saccule but all other structures are missing or undiscernible compared to the control inner ear at E14.5. aa, anterior ampulla; asc, anterior semicircular canal; cd, cochlear duct; ed, endolymphatic duct; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; sac, saccule; ut, utricle. Note the enlargement of the apex. Scale bars: 50 μm

neurons depend on SOX2⁺ sensory epithelia and never develop, as previously suggested (Dvorakova et al., 2016; Kempfle, Turban and Edge, 2016).

Future study should determine what members of the *Sox* gene family members play a role in differentiation of the neuronal lineage and early neurogenesis in the developing inner ear. We confirm previous work on the lack of sensory development in the ear in the absence of *Sox2* but find that transient inner ear neuronal development does occur, with surprisingly developed central and peripheral connections, indistinguishable from control littermates. However, the fact that early but not late neurons can form in the inner ear of *Foxg1-Cre;Sox2$^{f/f}$* (Dvorakova et al., 2020) and *Isl1-cre;Sox2$^{f/f}$* (Dvorakova et al., 2016) indicates different levels of *Sox2* dependency that requires further work to be fully mechanistically explained.

2.1.2. *Neurod1 is necessary for the generation and survival of neuroblasts, and neuronal cell fate promotion.*

The development of sensory hair cells, neurons, and non-sensory cells in the inner ear is regulated by a network of signaling pathways and transcription factors. Proneural atonal-related basic helix-loop-helix (bHLH) transcription factors, ATOH1, NEUROG1, and NEUROD1, are critical players in inner ear development (Elliott et al., 2021). NEUROG1*,* the first bHLH factor upregulated in the otocyst, initiates the specification and differentiation of inner ear neurons (Ma, Anderson and Fritzsch, 2000). NEUROG1 activates the expression of the downstream bHLH gene, *Neurod1*, which is essential for neuronal differentiation (Kim et al., 2001). *Neurod1* null mice exhibit severely impaired differentiation of auditory and vestibular neurons (Liu et al., 2000b; Kim et al., 2001) but they also suffer from other neuronal developmental defects (Miyata, Maeda and Lee, 1999; Liu et al., 2000a) and a severe diabetic phenotype (Kim et al., 2001). Therefore, conditional *Neurod1* deletion mutants were generated to investigate more specifically its role in inner ear neuronal development and hearing function. Conditional *Pax2^{Cre}; Neurod1$^{f/f}$* deletion mice showed abnormalities in the formation of inner ear ganglia, disorganized cochlear innervation, and unsegregated vestibular and spiral ganglion afferents (Jahan et al., 2010b). Unfortunately, *Pax2^{Cre}; Neurod1$^{f/f}$* mice have *Neurod1* deletion in the ear and the central auditory nuclei, limiting the evaluation of spiral ganglion neuronal viability and central projections and hampering physiological

assessment of the wiring defects. Furthermore, Pax2^{Cre} activity decreases with age, starting at E10.5 and producing uneven deletion effects (Duncan and Fritzsch, 2013). A delayed conditional *Neurod1* deletion using *Isl1^{Cre}* eliminates *Neurod1* from differentiating inner ear neurons and retains *Neurod1* expression in the auditory nuclei and midbrain (Macova et al., 2019). The *Isl1^{Cre}; Neurod1$^{f/f}$* mutants form the unsegregated and disorganized peripheral projection map of spiral ganglion neurons with altered sensory information processing in the central auditory pathway (Macova et al., 2019; Filova et al., 2020). However, detailed insights into the effects of an early absence of *Neurod1* in otic neuroblasts were lacking. Therefore, we chose the *Foxg1^{Cre}* transgene to eliminate *Neurod1.* We and others have demonstrated that *Foxg1^{Cre}* lead to earlier and more profound recombination than other Cre drivers expressed in the ear placode and the developing ear (Duncan and Fritzsch, 2013; Dvorakova et al., 2020). *Foxg1^{Cre}* is not expressed in the auditory and vestibular nuclei to possibly affect neuronal viability in the inner ear (Hebert and McConnell, 2000; Berube et al., 2005; Kasberg, Brunskill and Steven Potter, 2013; Abrams and Reiter, 2021). In this study, we revisited the embryonic phenotype of *Neurod1* deletion mice to determine NEUROD1 requirements for the generation and survival of neuroblasts and, overall, for early inner ear development.

In our study, we demonstrated that early elimination of *Neurod1* affected early delaminating and migrating ISL1^{+} neurons, resulting in a significantly smaller inner ear ganglion at E10.5 (Figure 7A, B). One mechanism contributing to the diminished number of inner ear neurons in *Foxg1-Cre;Neurod1$^{f/f}$* was massive apoptosis at E10.5, confirming that *Neurod1* is required for early neuronal survival (Liu et al., 2000b). In contrast, neuroblasts lacking *Neurod1* proliferate at a similar rate as neuronal precursors in control embryos, suggesting that NEUROD1 is not needed for the proliferation of neurons, at least in the E10.5 inner ear. Intriguingly, our data confirmed the initial finding in the global *Neurod1* deletion mutant (Liu et al., 2000b) that some inner ear neurons survive without *Neurod1*. One could speculate that the neurogenic fate commitment is predefined early in some *Neurog1^{+}* specified neuronal precursors ensuring terminal differentiation of these *Neurod1* null neurons. Alternatively, residual neurons might survive by compensatory activation of different transcription factor(s),

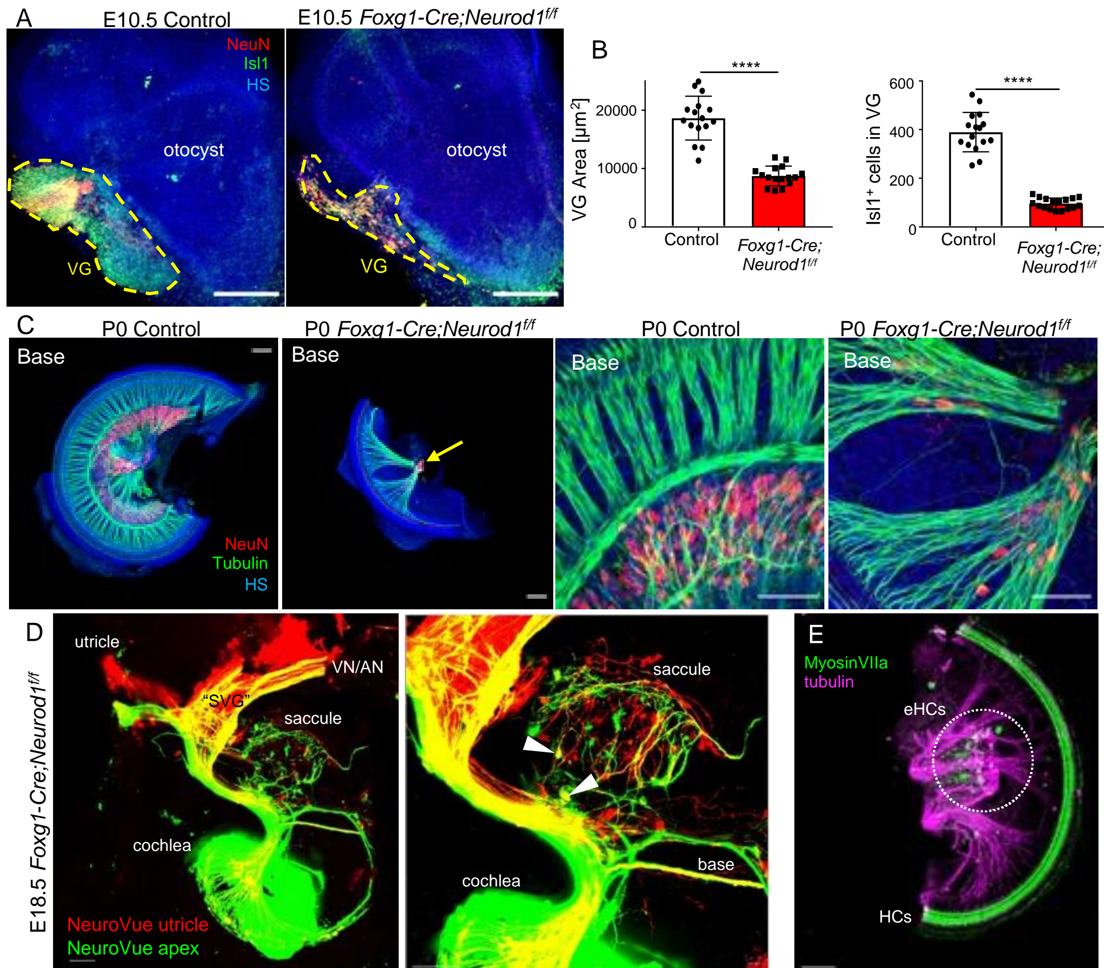

Fig. 7: Diminished inner ear ganglion and altered fate of cochlear neurons in *Foxg1-Cre;Neurod1$^{f/f}$*. (A) Representative whole-mount immunolabeling of the otocyst shows the size of vestibular ganglia (dotted line area) with anti-Isl1 and anti-NeuN (a nuclear marker of neurons). (**B**) Quantification of the vestibular ganglion size and number of ISL1^{+} neurons in the inner ear ganglion at E10.5. The values represent mean ± SD, *t*-test, ****P≤0.0001 (n =10 embryos/genotype, 16 vibratome sections/embryo). (**C**) The whole-mounted basal half of the cochlea immunolabeled with anti-NeuN (a nuclear marker of differentiated neurons) and with anti-tubulin (nerve fibers) shows NeuN^{+} neurons forming spiral ganglion in control at P0. In contrast, only a small cluster of NeuN^{+} neurons is found in the mutant cochlea (arrow). Note the reduced size of the cochlea and massive reduction of innervation. Higher-magnification images of the base show aberrant distribution of NeuN^{+} neurons that are entangled with radial fibers in mutant compared to the spiral ganglion neurons restricted to the Rosenthal's canal in the control cochlea. (D) A NeuroVue dye labeling from the utricle (red) and apex (green) shows a unique pattern of double labeled bilateral projections to reach saccule, base and apex in *Foxg1-Cre;Neurod1$^{f/f}$*. Note double labeled neurons (arrowheads) and a clutter of larger-size vestibular neurons (VGNs) with smaller cochlear neurons (SGNs) forming the aberrant "spiro-vestibular" ganglion ("SVG"). AN, auditory nerve; VN, vestibular nerve. (E) The mutant cochlea contains a large number of ectopic hair cells (eHCs) labeled my MyosinVIIa, a marker of hair cells, entangled with neuronal fibers labeled by tubulin. Scale bars: 200 µm (A), 100 µm (C-E).

such as GATA3 (Duncan and Fritzsch, 2013) or NHLH1/NHLH2 bHLH factors (Kruger et al., 2006).

Despite diminished neurogenesis, these *Neurod1* lacking neurons form inner ear ganglia, grow neuritic processes, establish bipolar connections to their targets, and persist up to the adulthood of these mutant mice. The vestibular ganglion was diminished, and the spiral ganglion was represented by a small clump of neurons in the *Foxg1-Cre;Neurod1*$^{f/f}$ inner ear (Figure 7C, D). In addition to a significant loss of neurons, the rudiment of the spiral ganglion was misplaced away from the sensory epithelium in the *Foxg1-Cre;Neurod1*$^{f/f}$ cochlea, indicating migration defects (Fritzsch et al., 2019). The remaining neurons projected disorganized peripheral fibers, with some neurons forming unusual peripheral projections connecting the cochlea and the vestibular end-organs. The central projections of our *Foxg1-Cre;Neurod1*$^{f/f}$ mutant are primarily identical to central projection abnormalities in various *Neurod1* deletion mutants (Kim et al., 2001; Jahan et al., 2010b; Macova et al., 2019; Filova et al., 2020). Central projections of cochlear and vestibular neurons are reduced, unsegregated, and disorganized.

In addition to neuronal phenotypes, *Neurod1* deletion affects the development of inner ear sensory organs (Kim et al., 2001; Jahan et al., 2010a; Macova et al., 2019; Filova et al., 2020). Like other *Neurod1* deletion mutants, due to *Foxg1*^{Cre} activity in the sensory precursors, we were unable to uncouple secondary effects of *Neurod1* deletion in inner ear neurons on sensory cell development. The elimination of *Neurod1* affects the size of inner ear sensory epithelia and the differentiation and organization of apical epithelium in the cochlea. We also found a profound formation of ectopic myosin VIIa positive hair cells near the two remaining neuronal aggregations of vestibular and cochlear sensory neurons (Figure 7E). Previous work identified a set of upregulated genes in the absence of *Neurod1*, particularly *Atoh1*, *Fgf8,* and *Nhlh2* (Jahan et al., 2010a; Filova et al., 2020), suggesting that some neuronal precursors adopt a hair cell fate and differentiate as hair cells (Bohuslavova et al., 2017). Taken together, NEUROD1 mediates a neuronal program and promotes neuronal fate by the upregulation of downstream targets and through suppression of other bHLH genes such as *Atoh1*. Another possible role of NEUROD1 in neurogenesis and neurogenic competence may be reprograming of the epigenome in the developing inner ear (Matsuda et al., 2019). Further experiments will be necessary to investigate how NEUROD1 mediates transcriptional and possibly epigenetic networks during neuronal

development in the inner ear. By understanding the interplay of NEUROD1 regulatory networks capable of initiating cell-fate changes, we hope to develop efficient therapeutic strategies to restore and regenerate neurons for clinical applications.

2.2. The limits of physiology-mediated brainstem plasticity during the development of the auditory system

Cochlear sensory hair cells are connected to the brain by SGNs that are organized within the cochlea in an orderly fashion according to frequency, with high frequencies at the base and low frequencies at the apex (Muniak et al., 2016). The tonotopic organization of type I SGNs corresponds to multiple diversities in their molecular profiles, connectivity patterns, and physiological features along the tonotopic axis (Petitpre et al., 2018; Shrestha et al., 2018; Sun et al., 2018; Petitpre et al., 2022). The cochleotopic or tonotopic pattern is maintained throughout the auditory pathways in the brain (Kandler, Clause and Noh, 2009). The central auditory pathway transmits ascending acoustic information from the cochlear nucleus through the lateral lemniscus complex, the inferior colliculus in the midbrain, and the medial geniculate nucleus of the thalamus to the auditory cortex (Di Bonito and Studer, 2017). 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 cochlear nuclei (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). 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 has been unclear beyond simply removing parts of the cochlea (Harrison, 2016).

To investigate the consequences of the disorganized peripheral projection maps of cochlear neurons for the tonotopic organization of the central auditory pathways, we generated two novel conditional deletion mutans with either deletion of the transcription factor *Neurod1* (Macova et al., 2019) or *Isl1* (Filova et al., 2022b). These deletions occur specifically in the inner ear but not in the central auditory nuclei, and therefore, physiological assessments of the defects are not hampered. Our analyses have showed how peripheral defects in the development of SGNs alters sound information

processing of the central auditory system of adult mice at the physiological and behavioral level. Surprisingly, auditory processing features are preserved despite the significant hearing impairment and abnormalities in the formation of spiral ganglia and projections, revealing central auditory pathway resilience and plasticity.

2.2.1. Neurod1 elimination alters the formation of the tonotopy and auditory information processing.

Neurod1 was eliminated specifically in the inner ear by crossing *Neurod1*$^{f/f}$ mice (Goebbels et al., 2005) with *Isl1*^{cre} mice (Dvorakova et al., 2016) (*Isl1-Cre;Neurod1*$^{f/f}$). *Isl1-Cre;Neurod1*$^{f/f}$ mice are viable without any obvious abnormal motor activity behavior that would indicate major defects in the vestibular system. However, the elimination of *Neurod1* results in a shorter cochlea with a reduced number of SGNs, and disorganized innervation (Figure 8A). The application of different colored lipophilic dyes into the apex (green) and base of the cochlea (red), and vestibular end organs (magenta) showed an aberrant distribution of spiral and vestibular ganglion neurons into a “spiro-vestibular” ganglion (SVG) complex and unsegregated-overlapping central axons of the auditory nerve and the vestibular nerve in *Isl1-Cre;Neurod1*$^{f/f}$ in contrast to control mice with neurons and fibers segregated (Figure 8B). Afferent projections from the cochlea to the first central nuclei, the cochlear nuclei, were also evaluated by lipophilic dye labeling (Figure 8C). The spiral ganglion to cochlear nucleus afferent projections are represented by reduced and widely ramifying fibers that mostly reach the ventral part of the cochlear nucleus (Figure 8C). The tonotopic order of SG projections to all three cochlear nucleus subdivisions and their precise parallel fiber organization into isofrequency bands are largely absent in the *Isl1-Cre;Neurod1*$^{f/f}$ mutant. Hearing of mutant mice was significantly impaired, as assessed with auditory brainstem responses (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 to the thresholds of control animals throughout the entire measured frequency range (Figure 8D).

Reduced and disorganized afferent projections of SGNs onto the cochlear nucleus resulted in altered sound processing in the brain. The inferior colliculus (IC) is the first level of auditory space map projection that shows some amelioration of primary

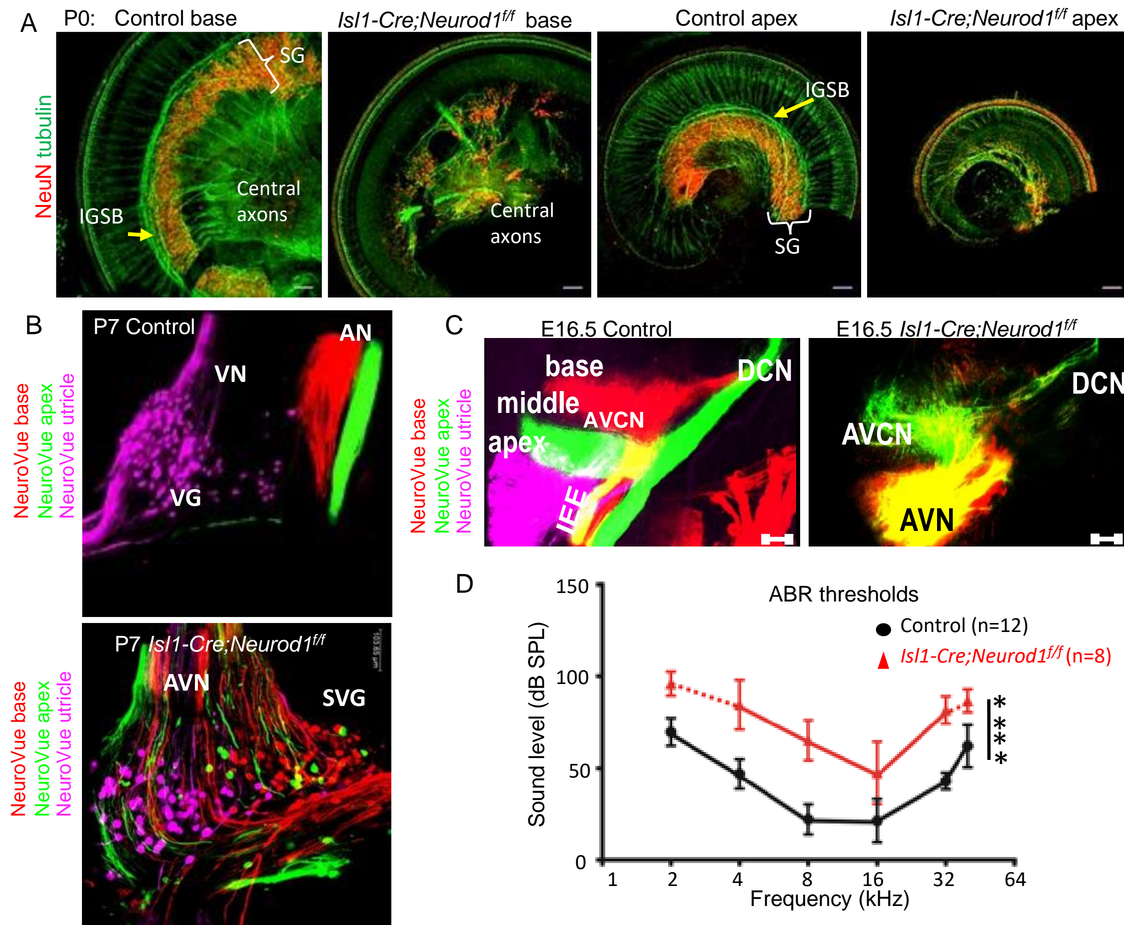

Fig. 8: Altered innervation and distribution of cochlear neurons is associated with hearing impairment of *Isl1-Cre;Neurod1$^{f/f}$* . (A) Representative whole-mount immunolabeling of the cochlea shows distribution of spiral ganglion (SG) neurons (anti-NeuN, a neuronal soma marker) and innervation (labelled by anti-acetylated α-tubulin). Note a missing intraganglionic spiral bundle (IGSB) in mutant compared to control (arrows indicate IGSB). Scale bars, 50 µm. (**B**) NeuroVue dye labeling from the utricle (magenta), the base (red) and apex (green) show a unique pattern of labeled projections forming the aberrant auditory-vestibular nerve (AVN) and "spiro-vestibular" ganglion ("SVG") in *Isl1-Cre;Neurod1$^{f/f}$* compared to segregated neurons and fibers in the auditory nerve (AN) and vestibular nerve (VN) of control animals. (**C**) Injections of different colored dyes label distinct bundles of neuronal fibers (auditory nerve, AN) projecting to the cochlear nucleus (CN) and vestibular nuclei in controls. The tonotopic organization of the CN subdivisions in controls is shown in the antero-ventral (AVCN) and the dorsal cochlear nucleus (DCN) with low frequency fibers labeled from the apex and high frequency from the base and no labeling between (the middle cochlea). The only mixed bundle (yellow) are inner ear efferents (IEE). In mutant, neuronal fibers completely overlap forming a mixed-labeled audio-vestibular nerve (AVN, yellow) and are restricted to the ventral part of the CN with just a few fibers occasionally expanding to the DCN. The overlapping fibers in the AVCN indicate a loss of the tonotopic organization of the central projections (yellow fibers). (**D**) The average ABR thresholds of control and mutant mice. 5 of the 8 *Isl1-Cre;Neurod1$^{f/f}$* 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 ± SD; two-way ANOVA (****$P < 0.0001$). Scale bars, 100 µm.

afferent dysfunction (Buran et al., 2010; Pelgrim et al., 2018). The tuning properties of IC neurons of *Isl1-Cre;Neurod1*$^{f/f}$ were significantly altered, including an enlarged frequency range, increased spontaneous activity, higher excitatory thresholds, and worsened tuning capabilities (Figure 9). The tuning characteristics of IC neurons

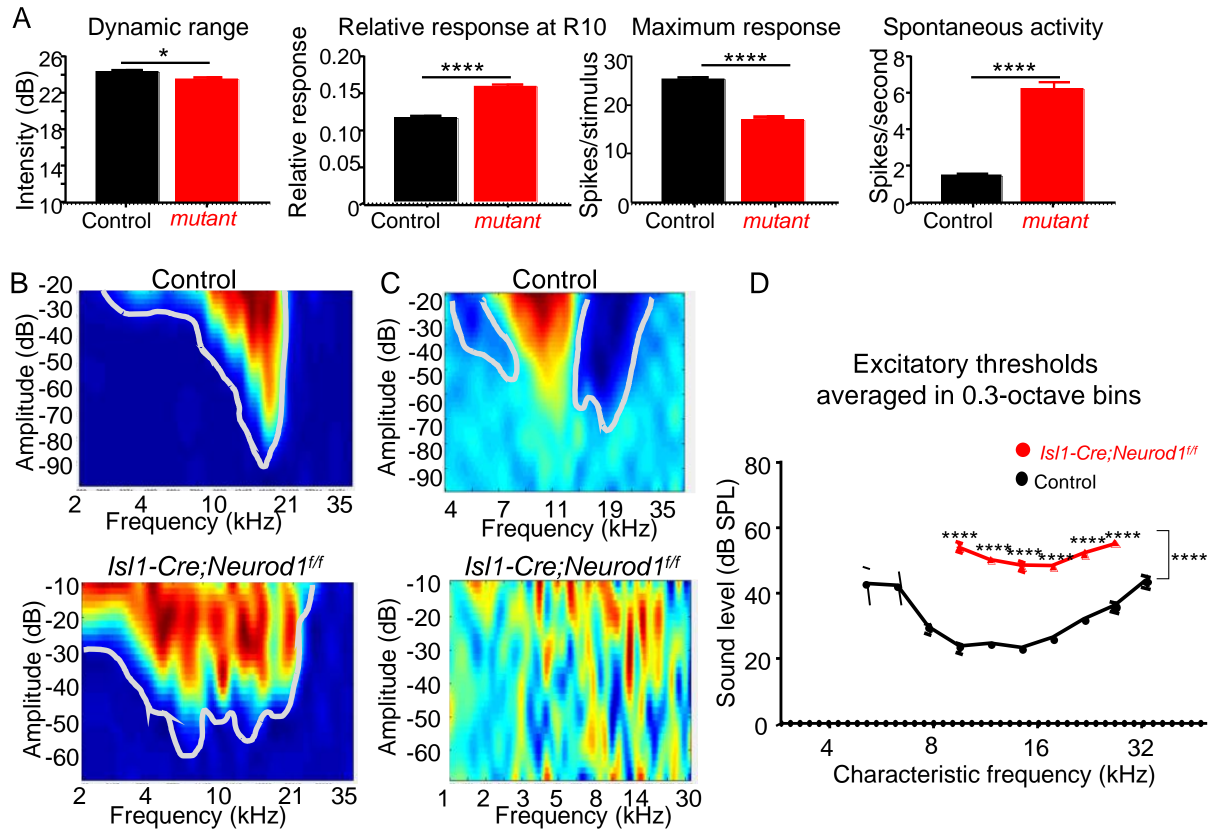

Fig. 9 Characteristics of inferior colliculus neurons are altered by the changes in the cochlea of *Isl1-Cre;Neurod1*$^{f/f}$ mice. (**A**) Comparison of the rate-intensity function (RIF) parameters in control and mutant mice: dynamic range, relative response at the RIFs point R10 (R10, 10% of the RIF response magnitude), maximum response magnitude and spontaneous activity. Data are the mean ± SD; unpaired t-test (*P < 0.05, ****P < 0.0001). For all extracellular recordings *Isl1-Cre;Neurod1*$^{f/f}$ (n = 9; 432 units from the IC) and control (n = 10; 480 units) were used. (**B**) Representative examples of tuning curves recorded in the inferior colliculus (IC). (**C**) 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 mutant. (**D**) Excitatory IC thresholds are shown as averages in 0.3-octave bins. Data are the mean ± SEM. Two-way ANOVA with Bonferroni post-hoc test (****P < 0.0001).

revealed striking differences in the shape of the excitatory receptive fields between mutant 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 *Isl1-Cre;Neurod1*$^{f/f}$ mice (Figure 9B), suggesting multiple inputs from the lower levels of the auditory system. Two-tone stimulation, employed 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 mutant (Figure 9C). Consistent with the ABR thresholds, the excitatory thresholds of IC units were higher in mutants than in control animals in all measured frequencies (Figure 9D). Modifications in intensity coding in the IC of *Isl1-Cre;Neurod1$^{f/f}$* 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 *Isl1-Cre;Neurod1$^{f/f}$* 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 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, Clause and Noh, 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. Such effects have never been investigated in other sensory systems due to the lack of models. It remains to be established how these plastic reorganization in the cochlea and in the central auditory pathway alter molecular properties of neurons.

2.2.2. *Isl1* deletion modifies molecular properties of auditory neurons and the tonotopic map.

Transgenic modulations of *Isl1* indicate important roles of ISL1 in the maintenance and function of neurons and hair cells and as a possible contributing factor in neurodegeneration (Huang et al., 2013; Chumak et al., 2016; Bohuslavova et al., 2017; Chumak et al., 2021). These studies suggest that ISL1 plays a role in developing neurons and sensory cells, but no direct evaluation of ISL1 function has been performed. To circumvent the pleiotropic effects of ISL1 in embryonic development we used *Neurod1^{Cre}* to delete *Isl1* specifically in the inner ear neurons (*Neurod1-Cre;Isl1$^{f/f}$*). Using RNA profiling, morphological, and physiological analyses, we showed that the LIM homeodomain transcription factor ISL1 coordinates genetic networks

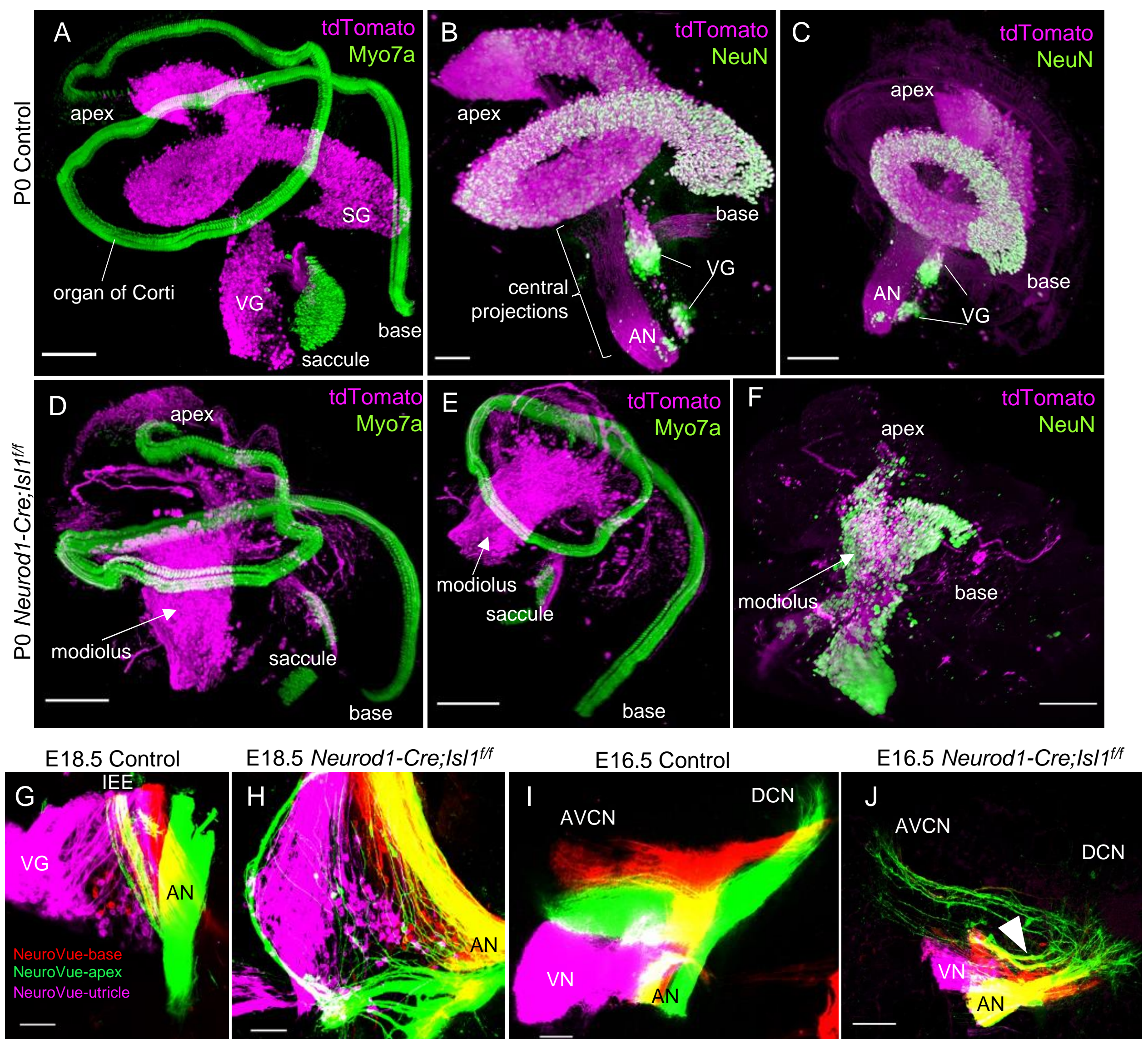

Fig. 10 Neurons in the *Neurod1-Cre;Isl1*$^{f/f}$ inner ear are abnormally distributed and project disorganized central projections to the cochlear nucleus. (**A-C**) In the control cochlea (reconstructed in 3D), tdTomato^{+} and NeuN labeled neurons form a coil of the spiral ganglion (SG) in the Rosenthal's canal and anti-Myo7a labeled hair cells show the spiral shape of the organ of Corti. Parts of the vestibular ganglion (VG), the auditory nerve (AN), and the saccule with Myo7a^{+} hair cells are shown. (**D-F**) In the mutant cochlea, tdTomato^{+} and NeuN labeled neurons are abnormally located in the conical central part of the cochlea, the modiolus (arrows), in contrast to the spiral of the organ of Corti with Myo7a^{+} hair cells. (**G**) Images of dye applications into the apex, base, and utricle show distinct and spatially restricted bundles of neuronal fibers of the auditory nerve (AN) projecting to the CN and the vestibular ganglion (VG) in controls. The only mixed bundle (yellow) is inner ear efferents (IEE). (**H**) In mutant, the segregation of central axons is lost, as fibers labelled from the apex and base are mainly overlapping in the AN (yellow fibers), and spiral ganglion neurons are mixed with VG neurons to form an aberrant ganglion, the "spiro-vestibular" ganglion. Note no IEE are recognizable in *Neurod1-Cre;Isl1*$^{f/f}$. (**I**) Axonal projections from cochlear neurons to the cochlear nucleus bifurcate with one branch synapsing in the dorsal (DCN) and the other innervating the anteroventral CN (AVCN). The tonotopic organization of the CN subdivisions is shown with low-frequency afferents labelled from the apex (green) and high frequency from the base (red) and organized as parallel fibers in isofrequency bands in controls. (**J**) In mutant, cochlear afferents overlap, the branch synapsing in the AVCN is reduced and disorganized, and the DCN branch is represented by just a few fibers. Arrowhead indicates overlapping fibers. Scale bars: 200 μm (A, C-F), 100 μm (B, G-J).

affecting the molecular characteristics of SGNs, pathfinding abilities, and auditory

information processing. The elimination of *Isl1* in neurons during inner ear development results in a migration defect of SGNs, disorganized innervation in the cochlea, unsegregated and reduced central axons, and reduced size of the cochlear nucleus (Figure 10).

Our RNA profiling of SGNs demonstrated transcriptome changes induced by a loss of *Isl1* affecting the molecular characteristics of neurons and pathfinding abilities, including neurotransmission, the structure of synapses, neuron migration, axonogenesis, and expression of crucial guidance molecules (Figure 11). Consistent with the central role of ISL1 in sensory neuron developmental programs (Sun et al., 2008), regulatory networks of signaling molecules and transcription factors were affected in *Neurod1-Cre;Isl1$^{f/f}$* neurons, such as proneural bHLH factors (members of NeuroD, Olig, and Nscl families), LIM-only (*Lmo2*, *Lmo3*) and LIM homeodomain transcription factors (*Lhx1, Lhx2, Isl2*), transcription activation complexes for coordination of particular differentiation programs Eyes absent (*Eya4* and *Eya2*) and Sine oculis (*Six2*), POU homeodomain trans-regulatory factors (*Pou3f2* and *Pou4f2*), and FGF signaling molecules (*Fgf10, Fgf11, Fgf13, Fgf14*) and their

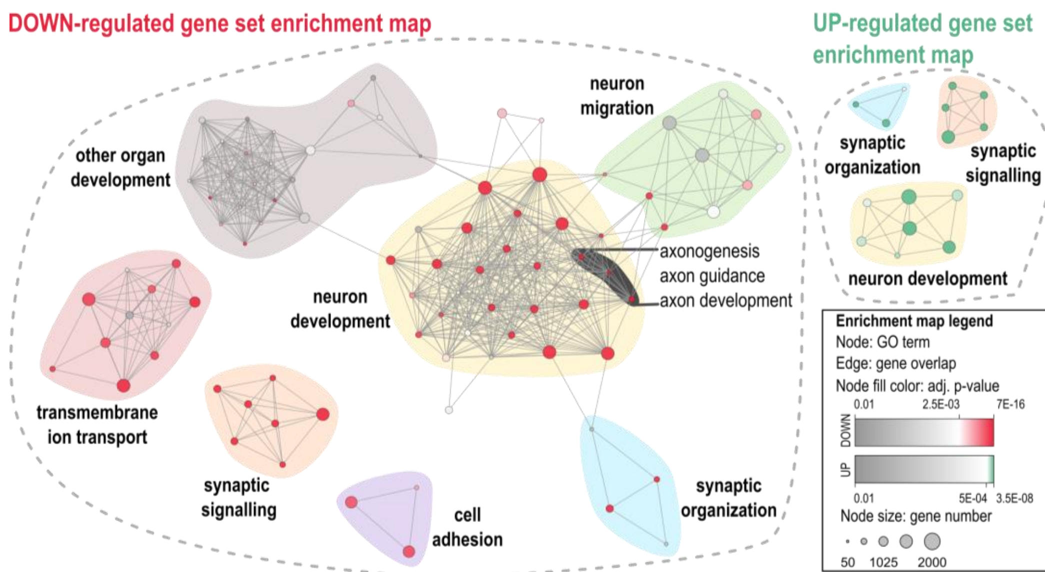

Fig. 11. ISL1-mediated transcription signature in cochlear neurons. Enrichment map of differentially expressed down- and upregulated genes identified by RNA sequencing in comparison between *Neurod1-Cre;Isl1$^{f/f}$* and control neurons . Each node represents a GO term; edges depict shared genes between nodes. Node size represents a number of genes of the mouse genome per the GO term, and node fill color represents a GO term significance. Each GO set cluster was assigned with representative keywords.

downstream targets (*Etv1, Etv4, Etv5*). Interestingly, the transcription factor *Gata3* was downregulated, suggesting that ISL1 is upstream of the Gata3 transcriptional network of neuronal differentiation programs (Appler et al., 2013). Thus, ISL1 orchestrates a complex gene regulatory network driving multiple aspects of neuronal differentiation in the cochlea and defining neuronal features.

This neuronal phenotype of *Neurod1-Cre;Isl1$^{f/f}$* was accompanied by hearing impairment, abnormalities in sound processing in the IC, and aberrant auditory behavior (Figure 12). As a result of disorganized primary auditory neurons with derailed

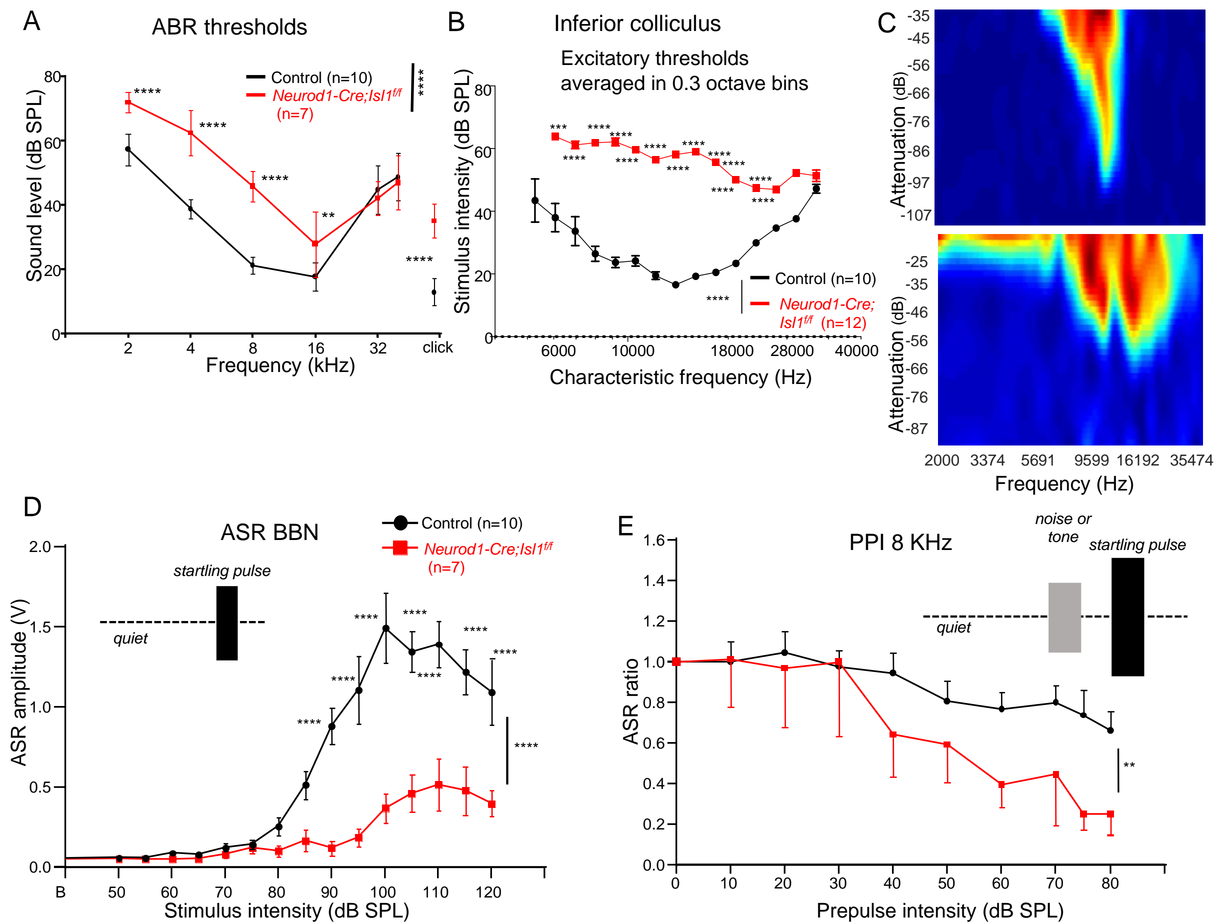

Fig. 12. Hearing dysfunction and aberrant auditory behavior of *Neurod1-Cre;Isl1$^{f/f}$*. (**A**) The average auditory brainstem response (ABR) thresholds of mutant and control mice are analyzed by click-evoked ABR. Data are expressed as mean ± SD. (**B**) Excitatory thresholds of the inferior colliculus neurons at different CFs are shown as averages in 0.3-octave bins. Data are expressed as mean ± SEM. (**C**) Representative examples of tuning curves recorded in the inferior colliculus display impairments in tuning properties with broad and irregular receptive fields in *Neurod1-Cre;Isl1$^{f/f}$* compared to control mice. (**D**) The acoustic startle reflex (ASR) thresholds for broadband noise (BBN) bursts are shown. Data are expressed as mean ± SEM. Holm-Sidak method multiple comparison t-test. (**E**) Prepulse inhibition (PPI) responses for tone pips of 8 kHz tone prepulse intensity on the relative ASR amplitudes are displayed; ASR ratio = 1 corresponds to the ASR amplitude without a prepulse (uninhibited ASR). Data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-hoc tests were used in A, B, E. *P < 0.05, **P < 0.01 ***P < 0.001, ****P < 0.0001.

central projections, the characteristics of persistent auditory function in the IC were altered with worsened tuning capabilities of IC units and their increased spontaneous activity and threshold elevations and decreased dynamic range. The peripheral deficit in sound encoding results in abnormal auditory behavior of *Neurod1-Cre;Isl1$^{f/f}$*. The startle reactions of *Neurod1-Cre;Isl1$^{f/f}$* were reduced. Plasticity of the startle response is also evident in the PPI responses of *Neurod1-Cre;Isl1$^{f/f}$* mice, in which a weak prestimulus suppresses the response to a subsequent startling stimulus. *Neurod1-Cre;Isl1$^{f/f}$* mice demonstrated PPI impairment for the pure tone of 8 kHz, reflecting abnormal sensorimotor gating due to hyperactivity of the central auditory system (Geyer, McIlwain and Paylor, 2002; Hickox and Liberman, 2014).

The unexpected close-to-normal hearing function and auditory signal processing at high frequencies suggest some preservation of tonotopic organization in the *Neurod1-Cre;Isl1$^{f/f}$* cochlea for the propagation of acoustic information. These results may reflect a timeline of sequential neuronal differentiation from the base to the apex, as the first differentiated neurons are in the base and the last SGNs undergo terminal mitosis in the apex (Matei et al., 2005). As the cochlea extends, differentiating neurons migrate along the cochlea to form the spiral ganglion. This process is completely disrupted in the *Neurod1-Cre;Isl1$^{f/f}$* cochlea, and many neurons are concentrated in a conical-shaped central structure, the modiolus, with only a portion of neurons in the Rosenthal's canal. Notably, the functional and spatial organization of SGNs may differ in transcription factor networks required for their differentiation programs. For example, some *Neurod1* lacking neurons survive, form a rudimental cochlear ganglion, and establish bipolar connections to their targets in *Neurod1* null mice (Kim et al., 2001) or the otocyst-deleted *Neurod1* conditional mutant (Filova et al., 2022a). Consistent with these findings, some neurons of *Neurod1-Cre;Isl1$^{f/f}$* may not require ISL1 for their differentiation, and the migration mode, and are in normal position in the Rosenthal’s canal, resulting in a partial preservation of auditory system function at high frequencies (Figure 12A, D).

Altogether, our study provides compelling evidence that ISL1 is a critical regulator of SGN development, affecting neuronal migration, pathfinding abilities to form cochlear wiring, and central axonal projections. As such, ISL1 represents an essential factor in the regulation of neuronal differentiation to produce functional

neurons in cell-based therapies and stem cell engineering (Pavlinkova, 2020; Zine, Messat and Fritzsch, 2021). Additionally, this unique model contributes to our understanding of how disorganization of the neuronal periphery affects information processing at higher centers of the central auditory pathway at the physiological and behavioral levels.

In this study we only assessed the functional role of *Isl1* in the inner ear neurons. Notably, the elimination of *Isl1* in developing neurons is only a part of the story. The expression pattern of ISL1 indicates that ISL1 may control the development of multiple inner ear progenitors (Radde-Gallwitz et al., 2004). The expression of ISL1 in sensory precursors is downregulated as hair cell differentiation is initiated and thus, ISL1 is not detected in the differentiated sensory hair and supporting cells in vestibular and cochlear epithelia (Radde-Gallwitz et al., 2004). It is conceivable that ISL1 may play a role in the specification of sensory fate and the regulation of the initial sequential events in sensory precursor development. Further studies will be needed to fully uncouple regulatory mechanisms in inner ear development by targeted eliminations of *Isl1* in sensory precursors.

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II. Selected publications as a basis of this thesis

(IF from the year 2022/23, [number of citations] from Web of Sci, 22-09-2023)

1. Dvorakova, M.; Jahan, I.; Macova, I.; Chumak, T.; Bohuslavova, R.; Syka, J.; Fritzsch, B.; **Pavlinkova, G*.** Incomplete and delayed Sox2 deletion defines residual ear neurosensory development and maintenance. *Scientific Reports*. 2016, 6:38253. IF 4.6 [27]
2. Chumak, T.; Bohuslavova, R.; Macova, I.; Dodd, N.; Buckiova, D.; Fritzsch, B.; Syka, J.; **Pavlinkova, G***. Deterioration of the Medial Olivocochlear Efferent System Accelerates Age-Related Hearing Loss in Pax2-Isl1 Transgenic Mice. *Molecular Neurobiology* 2016, 53 (4), 2368-83. IF 5.1 [15]
3. Bohuslavova, R.; Dodd, N.; Macova, I.; Chumak, T.; Horak, M.; Syka, J.; Fritzsch, B.; **Pavlinkova, G***. Pax2-Islet1 Transgenic Mice Are Hyperactive and Have Altered Cerebellar Foliation. *Molecular Neurobiology* 2017, 54 (2), 1352-1368. IF 5.1 [6]
4. Kersigo, J.; Pan, N.; Lederman, J. D.; Chatterjee, S.; Abel, T.; **Pavlinkova, G.**; Silos-Santiago, I.; Fritzsch, B. A RNAscope whole mount approach that can be combined with immunofluorescence to quantify differential distribution of mRNA. *Cell and Tissue Research* 2018, 374 (2), 251-262. IF 3.6 [25]
5. Macova, I.; Pysanenko, K.; Chumak, T.; Dvorakova, M.; Bohuslavova, R.; Syka, J.; Fritzsch, B.; **Pavlinkova, G***. Neurod1 Is Essential for the Primary Tonotopic Organization and Related Auditory Information Processing in the Midbrain. *Journal of Neuroscience* 2019, 39 (6), 984-1004. IF 5.3 [37]
6. Fritzsch, B.; Elliott, K. L.; **Pavlinkova, G.**; Duncan, J. S.; Hansen, M. R.; Kersigo, J. M. Neuronal Migration Generates New Populations of Neurons That Develop Unique Connections, Physiological Properties and Pathologies. *Frontiers in Cell and Developmental Biology* 2019, 7. IF 5.5 [27]
7. Dvorakova, M.; Macova, I.; Bohuslavova, R.; Anderova, M.; Fritzsch, B.; **Pavlinkova, G***. Early ear neuronal development, but not olfactory or lens development, can proceed without SOX2. *Developmental Biology* 2020, 457 (1), 43-56. IF 2.7 [24]
8. **Pavlinkova, G*.** Molecular Aspects of the Development and Function of Auditory Neurons. *International Journal of Molecular Sciences* 2020, 22 (1). IF 5.6 [7]
9. Filova, I.; Dvorakova, M.; Bohuslavova, R.; Pavlinek, A.; Elliott, K. L.; Vochyanova, S.; Fritzsch, B.; **Pavlinkova, G*.** Combined Atoh1 and Neurod1 Deletion Reveals Autonomous Growth of Auditory Nerve Fibers. *Molecular Neurobiology* 2020, 57 (12), 5307-5323. IF 5.1 [16]
10. Elliott, K. L.; Kersigo, J.; Lee, J. H.; Jahan, I.; **Pavlinkova, G.**; Fritzsch, B.; Yamoah, E. N. Developmental Changes in Peripherin-eGFP Expression in Spiral Ganglion Neurons. *Frontiers in Cell Neuroscience* 2021, 15, 678113. IF 5.3 [11]
11. Chumak, T.; Tothova, D.; Filova, I.; Bures, Z.; Popelar, J.; **Pavlinkova, G.***; Syka, J*. Overexpression of Isl1 under the Pax2 Promoter, Leads to Impaired Sound Processing and Increased Inhibition in the Inferior Colliculus. *International Journal of Molecular Sciences* 2021, 22 (9). IF 5.6 [1]

12. Elliott, K. L.; **Pavlinkova, G.**; Chizhikov, V. V.; Yamoah, E. N.; Fritzsch, B. Development in the Mammalian Auditory System Depends on Transcription Factors. *International Journal of Molecular Sciences* 2021, 22 (8). IF 5.6 [29]
13. Filova, I.; Bohuslavova, R.; Tavakoli, M.; Yamoah, E. N.; Fritzsch, B.; **Pavlinkova, G*.** Early Deletion of Neurod1 Alters Neuronal Lineage Potential and Diminishes Neurogenesis in the Inner Ear. *Frontiers in Cell and Developmental Biology* 2022, 17 February. IF 5.5 [7]
14. Petitpré, Ch.; Faure, L.; Uhl, Ph; Fontanet, P., Filova, I., **Pavlinkova, G.**; Adameyko, I.; Hadjab, S.; Lallemend, F. Single-cell RNA-sequencing analysis of the developing mouse inner ear identifies molecular logic of auditory neuron diversification. *Nature Communications* 2022, 13, 3878. IF 16.6 [16]
15. Filova, I.; Pysanenko, K.; Tavakoli, M.; Vochyanova, S.; Dvorakova, M.; Bohuslavova, R.; Smolik, O.; Fabriciova, V.; Hrabalova, P.; Benesova S, Valihrach L, Cerny J, Yamoah EN, Syka J, Fritzsch B, and **Pavlinkova, G*.** ISL1 is necessary for auditory neuron development and contributes towards tonotopic organization. *PNAS* 2022, Sept 8, 119 (37) e2207433119 IF 11.1 [8]

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