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Neural Crest Contributions to the Ear: Implications for Congenital Hearing Disorders

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Abstract

Congenital hearing disorders affect millions of children worldwide and can significantly impact acquisition of speech and language. Efforts to identify the developmental genetic etiologies of conductive and sensorineural hearing losses have revealed critical roles for cranial neural crest cells (NCCs) in ear development. Cranial NCCs contribute to all portions of the ear, and defects in neural crest development can lead to neurocristopathies associated with profound hearing loss. The molecular mechanisms governing the development of neural crest derivatives within the ear are partially understood, but many questions remain. In this review, we describe recent advancements in determining neural crest contributions to the ear, how they inform our understanding of neurocristopathies, and highlight new avenues for further research using bioinformatic approaches.

Keywords

development; Schwann cells; cochleovestibular ganglion; melanocytes; stria vascularis; neurocristopathy

1. Introduction

Congenital hearing disorders affect millions of children worldwide. The ear is an extraordinarily complex and sensitive sensory system, such that even slight perturbations in normal development can lead to severe or profound hearing loss. Decades of research efforts have offered considerable insights into our understanding of how the ear develops and the genetic basis for congenital hearing loss, with the ultimate goal of devising better therapeutic strategies for hearing restoration. Using a variety of animal models, researchers have made progress toward mapping the embryonic origins of each component of the ear, including structures derived from the neural crest (NC).

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The neural crest is a transient cell population arising from the process of neurulation (formation of the brain and spinal cord) in early embryonic development. First described in 1868 by Wilhelm His Sr. as “Zwischenstrang”, (His, 1868) the neural crest is often referred to as “the fourth germ layer” for its substantial contributions to nearly every organ system in the vertebrate body. He initially proposed that the inner ear anlage is entirely derived from the neural crest due to its close proximity to the nascent neural tube. Despite later evidence of otic placodal origins of the inner ear (Von Kupffer, 1891), careful study of cranial neural crest pioneered by Nicole Le Douarin and colleagues (Le Douarin and Kalcheim, 1999) revealed that NC derivatives do indeed comprise several structures of the outer, middle, and inner ear. Many of these structures have been characterized using powerful genetic lineage tracing tools in animal models. Exactly how aberrant development of NC derivatives in the ear contribute to congenital hearing loss is an active field of inquiry, and many open research questions remain. In the following sections, we discuss current understanding of the development of specific NC-derived ear structures and cell populations. We also explore how perturbation of these structures and cells can lead to congenital hearing loss and highlight avenues for future research.

2. Neural Crest Contributions to the Outer Ear: cartilage of the auricle and external auditory canal

The outer ear serves important roles in sound localization and amplification, as sound waves from the environment are funneled towards the tympanic membrane. The auricle (or pinna) and external auditory canal (EAC), which collectively comprise the outer ear, develop from neural crest-derived cartilage and are covered in ectoderm-derived skin. Defects in neural crest development, particularly in cartilaginous structures, can lead to small malformed ears (microtia) or, in extreme cases, complete absence of the outer ear (anotia). While many outer ear defects can be surgically repaired, significant morphological abnormalities in outer ear development are often associated with middle ear developmental defects and hearing loss (Ishimoto et al., 2007; Kountakis et al., 1995; Mallo, 2003). Unfortunately, much less is known about the underlying genetic mechanisms of outer ear development compared to other ear structures.

In humans, the outer ear develops from the first and second pharyngeal arches, transient developmental structures that ultimately give rise to various components of the head and neck. NCCs migrate ventrally from the dorsal aspect of the nascent neural tube to populate the mesoderm-filled pharyngeal arches. Six auricular hillocks of His form from the first and second pharyngeal arches during the sixth week of gestation (Streeter, 1922). Over subsequent weeks, each hillock undergoes substantial morphogenesis to form well-defined regions of the auricle (Fig 1A). The cleft between the first and second pharyngeal arches ultimately becomes the external auditory canal. While this dual-arch origin of the outer ear is well established for human development, detailed lineage tracing has shown that the mouse auricle is entirely derived from only the second pharyngeal arch, and the EAC arises by invagination of mesenchymal NCCs within the first pharyngeal arch (Minoux et al., 2013). Despite these key differences between human and mouse outer ear development,

studies in mice have substantially improved our understanding of the genes underlying many human neurocristopathies affecting the ear (Table 1).

Mutations in *HOXA2* are associated with microtia, with and without hearing loss (Alasti et al., 2008; Brown et al., 2013; Cox et al., 2014). *HOXA2* is both necessary and sufficient for second pharyngeal arch identity and subsequent auricle development. Deletion of *HoxA2* in the mouse either globally or specifically in neural crest before E12.5 results in homeotic transformation of the second pharyngeal arch into first arch structures, an almost complete absence of the auricle, and duplicate EACs (Gendron-Maguire et al., 1993; Rijli et al., 1993). The period between E12.5 and E13.5 represents a critical window for the requirement of *Hoxa2* in auricle development, as a small outer ear still develops when *Hoxa2* is conditionally deleted after E13.5 (Santagati et al., 2005). Mice that ectopically express *Hoxa2* in neural crest cells within the first pharyngeal arch exhibit polyotia, or duplicate auricles, supporting the sufficiency of *Hoxa2* for outer ear development (Minoux et al., 2013).

Given the extensive defects in outer ear development observed in *Hoxa2* mutants, it is not surprising that mutations in other genes involved in patterning the first and second pharyngeal arches can also cause similar defects in auricle and ear canal development. Mutations in transcription factors *EYA1* and *SIX1* cause Branchio-otic and Branchio-oto-renal syndromes (BOS/BORS), which are characterized by defects in craniofacial structures derived from the first and second pharyngeal arches (Abdelhak et al., 1997; Ruf et al., 2004). Patients with BOS/BORS present with a wide variety of outer, middle and inner ear defects, including cup-shaped auricles with simplified structure and hypoplastic EACs. Work in mice showed that *Eya1* is activated by *Hoxa2* in a subset of NCCs at the base of the early auricle and is required for outer ear patterning (Minoux et al., 2013). Later in development, *Eya1* interacts with *Six1* to regulate differentiation of cartilaginous and skeletal tissues in multiple NC-derived structures, including the first and second pharyngeal arches (Xu et al., 1999; Zhang et al., 2017). Pathogenic variants in *SIX5*, another member of the *SIX* gene family, can also cause BOS/BORS (Hoskins et al., 2007), but potential interactions of *SIX5* with *EYA1* and/or *HOXA2* have not yet been explored.

Goosecoid (GSC) is another essential regulator of pharyngeal arch neural crest development, as mutations in this gene cause SAMS disorder (Short stature, Auditory canal atresia, Mandibular hypoplasia, Skeletal abnormalities) (Lemire et al., 1998; Parry et al., 2013). Studies in chick embryos demonstrated that while *Gsc* is expressed in both the first and second pharyngeal arches, *Hoxa2* specifically represses *Gsc* expression in the first arch but not the second (Grammatopoulos et al., 2000), thereby contributing to the regulation of pharyngeal arch identity. Additionally, Hox family members *Hoxa1* and *Hoxb1* also regulate outer ear development. Double knockout mice that lack expression of both *Hoxa1* and *Hoxb1* fail to produce migratory cranial NCCs and subsequently fail to develop any outer ear structures (Gavalas et al., 1998; Rossel and Capecchi, 1999).

Defects in outer ear development can also arise after the establishment of pharyngeal arch patterning by Hox proteins. The transcription factor *Hmx1* is expressed in the first and second pharyngeal arches in mice at E10.5, after cranial NCCs have already migrated to this

region (Yoshiura et al., 1998). Mutations affecting *Hmx1* expression, particularly in a conserved non-coding element downstream of *Hmx1*, can lead to Oculo-Auricular Syndrome (OAS) in both rodents and humans (Quina et al., 2012; Vaclavik et al., 2011). Similarly, insufficient *Tfap2a* function does not impede NCC induction, specification, or migration to the arches, but impairs the differentiation of NCCs to cartilaginous structures. Branchio-Oculo-Facial Syndrome (BOFS), characterized by hypoplastic outer ears, is caused by mutations in *TFAP2A* (Brewer et al., 2004; Milunsky et al., 2008; Milunsky et al., 2011).

The development of pharyngeal arch NCCs relies on signaling input from neighboring mesoderm-derived cells in the core of each arch. The requirement of this interaction is evidenced in patients with 22q11.2 Deletion Syndrome, who experience haploinsufficiency of *TBX1* and a variety of defects in NC-derived craniofacial structures, including the outer, middle, and inner ear. However, *TBX1* is restricted to the mesodermal cores of the second pharyngeal arch and is not expressed in NCCs, thereby acting in a non-cell autonomous manner to regulate the development of outer ear cartilage (Arnold et al., 2006).

While mutations in transcription factors are common in many of the neurocristopathies associated with outer ear malformations, erroneous function of other molecules can also lead to related phenotypes. Endothelin signaling is repeatedly used in the development of many neural crest derivatives, including NCCs in the pharyngeal arches. Endothelin 1 (EDN1), produced and secreted by the overlying ectoderm of the first pharyngeal arch, acts on Endothelin Receptor A (EDNRA) in NCCs (Tavares et al., 2012) and helps regulate proliferation and outgrowth of the pharyngeal arches (Sasaki et al., 2013). Mutations in either *EDN1* or *EDNRA* are associated with defects in outer ear development, including Auriculo-Condylar Syndrome (ACS) and Mandibulofacial dysostosis with alopecia (MFDA) (Clouthier et al., 2013; Gordon et al., 2013; Gordon et al., 2015). The chromatin remodeler CHD7 is expressed in a wide variety of tissues in the early embryo and is specifically required for neural crest induction at the neural plate border and maintenance of NCCs (Bajpai et al., 2010). Pathogenic variants in *CHD7* cause CHARGE Syndrome (Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth/development, Genitourinary abnormalities, and Ear defects) (Visser et al., 2004). The diverse hallmark features of CHARGE highlight the impact of CHD7 function in neural crest derivatives throughout the body, including all compartments of the ear. Patients with CHARGE often present with simplified, square-shaped auricles, although outer ear appearance may vary among individuals (Bergman et al., 2011; Hsu et al., 2014). Treacher-Collins Syndrome, caused by mutations in *TCOF1*, is characterized by a host of craniofacial malformations including variable severity of microtia and EAC atresia (Dixon et al., 2006; Dixon, 1995). TCOF1 is a nucleolar protein that cooperates with UBF to regulate ribosomal gene transcription (Valdez et al., 2004) and may also participate in DNA damage repair in neuroepithelial cells fated to become neural crest (Sakai and Trainor, 2016). How TCOF1 exerts its neural crest-specific functions remains unknown.

3. Neural Crest Contributions to the Middle Ear

Within the air-filled mammalian ear cavity, NCCs contribute to three structures: the ossicles (malleus, incus, and stapes), the auditory bulla and tympanic ring, and a portion of the epithelial lining of the middle ear cavity (Fig 1B). The precise anatomic positioning of each of the three ossicles, along with their ability to freely move within the middle ear cavity, are essential for hearing. Failure of normal development of middle ear components leads to conductive hearing loss. Many defects of middle ear development can be surgically repaired; however, such developmental defects are sometimes associated with other craniofacial abnormalities that can make reparative surgeries extraordinarily difficult. Thus, understanding the mechanisms of middle ear development is important for improving treatment strategies for conductive hearing loss.

3.1 Ossicles

The malleus mechanically transmits vibrations of the tympanic membrane (eardrum) through the incus and to the stapes, which contacts the cochlea through a small opening in the otic capsule called the oval window. The piston-like action of the stapes footplate on the oval window causes movement of the cochlear fluid, endolymph, which is then interpreted by the neurosensory structures of the inner ear (see section 4). Thus, the ossicles work in an elegantly simple manner to directly translate sound waves from a relatively large surface area (the eardrum) onto a much smaller membrane (the oval window) to substantially amplify the sound that can be detected by the inner ear. The critical nature of ossicular function is demonstrated in animals and patients who experience conductive hearing loss as a consequence of stiffened or fixed ossicles (otosclerosis). Congenital otosclerosis is caused by defects in the development of NCCs that form the ossicles.

The malleus and incus develop from the proximal end of Meckel's cartilage, a rod of cartilaginous tissue that forms from the first pharyngeal arch. Both of these ossicles transform from cartilage to bone by endochondral ossification during weeks 19-24 of human gestation (Richard et al., 2017) and approximately E14.5 in the mouse (Anthwal et al., 2013). Most of the stapes, except for a mesoderm-derived portion of the footplate that contacts the oval window, develops from Reichert's cartilage comprised of NCCs of the second pharyngeal arch. The stapes is the last of the ossicles to fully ossify in both humans and mice (Anthwal et al., 2013).

Given the shared pharyngeal arch origins of the ossicles and the cartilage of the outer ear, many of the key genes required for outer ear development described in the previous section are also critical for patterning the ossicles. *Hoxa1*^{-/-}; *Hoxb1*^{-/-} double knockout animals fail to form ossicles (Gavalas et al., 1998; Rossel and Capecchi, 1999), a particularly interesting finding given that expression of these genes is confined to the second pharyngeal arch, yet first-arch derivatives are severely affected. This indicates that crosstalk between NCCs of the first and second arches is required for the patterning and formation of all three ossicles, regardless of their arch of origin. Further support for this crosstalk comes from studies of *Tbx1*, an essential transcription factor expressed in the mesoderm-derived core of the second pharyngeal arch that works in a non-cell autonomous manner to guide the trajectory of migratory NCCs to the second pharyngeal arch. In *Tbx1*^{-/-} mice, the second

arch-derived stapes is severely hypomorphic or completely absent in some animals, and the malleus and incus may also be malformed (Moraes et al., 2005). Patients with 22q11.2 Deletion Syndrome, in which expression of *TBX1* is reduced, also present with stapedial defects as well as simplified square-shaped auricles as mentioned in section 2 (Loos et al., 2016; Weinberg and Wright, 1978). Interestingly, a very similar middle ear phenotype is observed in mice with loss of function of *Chd7*, where the neural crest-derived portions of the stapes are most severely affected with milder defects observed in the malleus and incus (Hurd et al., 2011; Ogier et al., 2014). Noggin signaling has also been implicated in middle ear ossicle development, as patients with mutations in *NOG* present with stapes ankylosis (fixation of the stapes footplate in the oval window) and occasionally other skeletal features, such as fused joints in the hands and feet (Quesnel et al., 2015; Takano et al., 2016; Usami et al., 2012). Patients with *NOG* mutations and *Nog*^{+/-} mutant mice develop an accessory cartilaginous appendage near the head of the stapes that attaches to the otic capsule and results in stapedial fixation (Hwang and Wu, 2008; Quesnel et al., 2015). Noggin is a secreted protein that antagonizes BMP signaling early in embryonic development, including during the early phases of neural crest induction, delamination from the neural tube, and migration (Mayor et al., 1995; Sela-Donenfeld and Kalcheim, 1999, 2000). Interestingly, mutations in *NOG* are not associated with other neural crest-related phenotypes, which suggests that some specific development processes may be more sensitive to the effects of *NOG* gene dosage.

3.2 Middle ear cavity

The process of middle ear cavitation starts with invagination of the first pharyngeal pouch towards the otocyst, which then further extends and becomes filled with mesenchymal NCCs (Park and Lim, 1992; Thompson and Tucker, 2013). At approximately E17.5 in the mouse, the endodermal pouch opens medially, allowing NCCs to migrate into the newly formed middle ear space, while the ossicles undergo chondrogenesis. At approximately P6, NCCs undergo a mesenchymal-to-epithelial transition to form the epithelial lining of the anterior attic of the middle ear cavity. These NC-derived epithelial cells can be distinguished from the rest of the endoderm-derived middle ear by their lack of cilia as revealed by lineage tracing using *Wnt1-Cre* in conjunction with epithelial cell markers (Thompson and Tucker, 2013).

Defects in cavitation cause conductive hearing loss, since the middle ear cavity must be filled exclusively with air to allow ossicle movement. Conductive hearing loss caused by incomplete middle ear cavitation occurs in many patients with Treacher Collins Syndrome (TCS) (Dixon et al., 2007; Pron et al., 1993; Richter et al., 2010). *TCOF1*, one of 3 genes altered in Treacher-Collins syndrome, is required in mice for disintegration of neural crest-derived mesenchyme and subsequent creation of the lining of the anterior middle ear cavity (Richter et al., 2010). Precise construction of the middle ear cavity is also required for the regular removal of fluid or other contaminants from the middle ear down to the Eustachian tube. Residual mesenchyme in the middle ear cavity poses an increased risk for middle ear infections (Jaisinghani et al., 1999), which can impair the treatment of conductive hearing loss in patients with neurocristopathies associated with middle ear cavity defects, such as TCS and CHARGE Syndrome (Richter et al., 2010; Shah et al., 1998).

Recent work has demonstrated a role for Cadherin-11 (*Cdh11*) in middle ear cavitation. *Cdh11* is expressed in migratory NCCs at the onset of delamination from the neural tube and more broadly in the surrounding mesodermal migratory environment, and helps mediate cell-substrate adhesion during migration (Langhe et al., 2016). *Cdh11*^{-/-} mice experience conductive hearing loss due to failure of mesenchymal NCCs to undergo a mesenchymal-to-epithelial transition (MET) to form the anterior epithelial lining of the middle ear cavity (Kiyama et al., 2018). This finding is particularly interesting because despite its early, widespread expression early in mouse development and known functions in NCC migration, Cadherin-11 does not appear to be required for successful infiltration of cranial NCCs into the pharyngeal arches (Hoffmann and Balling, 1995; Kimura et al., 1995). Intriguingly, this study found that ossicle development was unaffected by Cadherin-11 loss, although a prior study observed defects in osteoblast differentiation in the mesoderm-derived tibia bones of *Cdh11*^{-/-} mice (Di Benedetto et al., 2010). Taken together, these results suggest different roles for *Cdh11* in the development of NC-derived versus mesoderm-derived bone structures and *Cdh11* has previously unappreciated roles in middle ear development. Other members of the cadherin family, including *Cdh7*, *Cdh19*, and *Cdh20* also play important roles in pre- and post-migratory NCCs by mediating their interactions with the migratory environment (reviewed by (Taneyhill and Schiffmacher, 2013)). Further research is needed to determine if these other cadherins are involved in middle ear development, or if cadherins are regulated by neurocristopathy causative genes.

3.3 Auditory bulla and tympanic ring

The auditory bulla is the bony structure that encapsulates the entire middle ear. While it has been shown to be neural crest derived, not much is known regarding the mechanisms of its development. *Tcof1*^{-/-} knockout mice exhibit defective middle ear cavitation and the size of the auditory bulla is significantly reduced (Richter et al., 2010). It remains unknown if this phenotype is unique to *Tcof1* mutations, or if other neurocristopathy mouse models have similar phenotypes but the auditory bulla has yet to be specifically assessed. The auditory bulla is challenging to study because this structure develops postnatally (P6-P9) and many neurocristopathy mouse models with severe defects in craniofacial bone do not survive long beyond birth.

The tympanic ring is a transient bony structure in the middle ear that eventually becomes part of the auditory bulla in the postnatal period. The tympanic ring serves as a scaffold or anchor for the tympanic membrane as it develops. Additionally, the tympanic ring plays an important role in inhibiting the medial growth of the external auditory canal (EAC) beyond the middle ear (Aimi, 1983). These functions of the tympanic ring have clinical significance, as abnormal formation of the tympanic ring is associated with EAC atresia in SAMS disorder (Lambert and Dodson, 1996). Patients with SAMS Disorder harbor mutations in *GSC*, which is required for mesenchymal NCCs from the first pharyngeal arch to coalesce and differentiate into osteoblasts (Rivera-Perez et al., 1999). A recent study has also revealed a novel role for COUP-TFII (also known as *Nr2f2*) in tympanic ring development (Hsu et al., 2017). In neural crest-specific *Nr2f2* knockout mice, growth of the tympanic ring is stunted and invagination of the EAC is misguided. A downstream target of COUP-TFII, *Sox9*, is an important regulator of condensation of pre-osteoblast mesenchymal NCCs.

Precisely how COUP-TFII may be involved in neurocristopathies associated with craniofacial defects remains to be determined.

4. Neural Crest Contributions to the Inner Ear

The majority of inner ear structures are derived from the otic placode, an ectodermal thickening adjacent to rhombomere 5 in the early mammalian embryo. At approximately E9.5 in the mouse, the otic placode invaginates and pinches off from the overlying ectoderm to form the otic vesicle or otocyst, which, over the next week of embryonic development, undergoes substantial morphogenesis to become the vestibular system and cochlea. Concurrently, cranial neural crest cells migrate from rhombomere 4 toward the nascent otocyst and ultimately differentiate into two critical cell types in the inner ear: glial cells of the cochleovestibular ganglion and melanocytic intermediate cells of the cochlear stria vascularis (Fig 1C). Additionally, there is some disputed evidence for neural crest origins of inner ear neurosensory components (Freyer et al., 2011). Recent studies on each of these cell types are described below.

4.1 Glia of the cochleovestibular ganglion

In the earliest stages of inner ear formation, neural crest cells serve instructive, supporting roles prior to the onset of glial cell differentiation. At approximately E9.5 in the mouse, migratory NCCs arrive at the anteroventral region of the nascent otocyst, where neuroblasts delaminate to form the cochleovestibular ganglion. The physical interactions between NCCs and placode-derived neuroblasts have been carefully mapped in the mouse using a unique 6.5Pax3-Cre, in which Cre recombinase is under the control of a neural crest-specific enhancer of Pax3 (Sandell et al., 2014). NCCs derived from rhombomere 4 form a “corridor” alongside the neural tube and, upon arriving at the otocyst, form a sheath or sleeve around newly delaminated neuroblasts. Some of these NCCs continue their migratory path beyond the otocyst and infiltrate the first and second pharyngeal arches to give rise to outer and middle ear structures, as described in sections 2 and 3.

NCCs intermingle with placode-derived neuroblasts as they proliferate, exit the cell cycle, and begin neuronal differentiation. As early neurites extend towards the hindbrain, NCCs construct a lattice-like structure to guide these central projections. Interestingly, otic placode neurogenesis is required for cranial NCC targeting to the appropriate region. Disruption of placodal neurogenesis by electroporation of diphtheria toxin into the placodal ectoderm in chick prevents the migration of *Sox10*⁺ NCCs to the otocyst but not the initial formation of NCC corridors alongside the neural tube (Freter et al., 2013). Reciprocally, NCCs are required for development of the cochleovestibular ganglion. Removal of rhombomere 4 NCCs at the 6-9 somite stage in chick results in isolated cochleovestibular ganglia which lack central projections towards the hindbrain (Sandell et al., 2014). However, *Wnt1*-Cre;*Sox10*^{flox/flox} embryonic mice that have significant loss of NCCs do not exhibit defects in central projections of cochleovestibular ganglion neurons toward the hindbrain (Mao et al., 2014). These conflicting results may be due to species differences in the requirement of NCCs in development, or differences in the methods of NCC ablation. Despite the importance of placodal neuron/cranial NCC interactions, the signaling molecules exchanged

by these two cell populations to coordinate cochleovestibular ganglion development remain unknown. Canonical axon guidance molecules such as Slit/Robo may play a role, but these cues are not active until several days after the initial formation of the CVG in mouse development (Wang et al., 2013). Exactly how NCCs instruct the initial formation and patterning of the CVG and how NCCs may be involved in the division between the spiral ganglia and vestibular ganglia remain open questions.

Cells that comprise the NCC corridors subsequently differentiate to become myelinating Schwann cells. These Schwann cells encase both the peripheral axons of Type I spiral ganglion neurons that innervate inner hair cells in the organ of Corti and non-myelinating satellite glia that envelope neuronal cell bodies within the spiral ganglia. NC-derived Schwann cells serve an instructive role in guiding the trajectory of spiral ganglion peripheral axons, reminiscent of their function earlier in development as a scaffold for the initial formation of the cochleovestibular ganglion. The requirement of Schwann cells for development of inner ear neural architecture has been demonstrated in mouse models (Mao et al., 2014; Morris et al., 2006). *ErbB2*^{-/-} mice, which fail to develop Schwann cells, exhibit spiral ganglion neuronal projections that extend beyond the organ of Corti to the lateral wall of the cochlea and appear highly disorganized (Morris et al., 2006). Interestingly, a very similar phenotype occurs in *Wnt1*-Cre;*Sox10*^{flx/flx} embryonic mice that lack *Sox10* specifically in neural crest lineages (Mao et al., 2014). Together, these results suggest that NC-derived Schwann cells inhibit the wayward neuritic expansion of spiral ganglion neurons. Additional studies are needed to identify these potential Schwann cell-derived inhibitory cues. Importantly, these mouse models do not survive beyond birth; hence, the impact of *Sox10* or *ErbB2* loss on mature hair cell innervation and hearing has not been determined.

Efforts to determine the requirement of NC-derived glia for survival and maintenance of developing spiral ganglion neurons have led to some confusing results. Loss of Schwann cells leads to increased spiral ganglion neuron apoptosis in *ErbB2*^{-/-} (Morris et al., 2006), but not *Wnt1*-Cre;*Sox10*^{flx/flx} (Mao et al., 2014) embryos. In contrast, mouse embryos harboring a *Sox10*-LacZ knock-in allele that disrupts normal *Sox10* expression and impairs Schwann cell development exhibit slightly reduced spiral ganglion neuronal apoptosis compared to wild type littermates (Breuskin et al., 2010). These differences in experimental findings may be due to the genetic means by which NCCs were eliminated in the various mice. Schwann cells and supporting cells in the organ of Corti express a plethora of shared transcription factors, including *ErbB2* and *Sox10*. Global ablation of either of these genes would therefore affect both Schwann cells and supporting cells, making it difficult to distinguish specific functions of either cell type. Interestingly, reduced neurotrophin production was noted in *ErbB2*^{-/-} mutants, whereas NT-3 and BDNF production in *Wnt1*-Cre;*Sox10*^{flx/flx} cochleae was unchanged (Mao et al., 2014), suggesting phenotypic differences in these mice may be attributed to differential neurotrophin production. Given that supporting cells should be spared in *Wnt1*-Cre;*Sox10*^{flx/flx} mice, it is likely that supporting cells provide neurotrophic support and work alongside NC-derived Schwann cells to promote the survival and maintenance of spiral ganglion neurons. However, this hypothesis has not been directly tested.

The exclusive neural crest origin of glial cells in the inner ear has been a long-held dogma in developmental biology. However, a recent study identified progenitor cells in the embryonic mouse cochlea that are not of neural crest origin (that is, they are not marked by *Wnt1*-Cre lineage tracing) yet are capable of producing glial cells in the spiral ganglia (Xu et al., 2017). These cochlear progenitor cells derive from an *Eya1*⁺ cell lineage, likely originating from the otic placode, and are also capable of producing hair cells and supporting cells in the organ of Corti. This was the first study to suggest that glial cells anywhere in the peripheral nervous system, including the inner ear, derive from a non-neural crest source, and has implications for the impact of disrupted glial cell development in the neural function of the inner ear. For example, NC and non-NC derived glia may exhibit vastly different functions, responses to therapy, and sensitivities to injury.

4.2 Melanocytes of the stria vascularis

All melanocytes in vertebrates, including melanocytic intermediate cells of the stria vascularis, are derived from the neural crest. These intermediate cells are perhaps the best studied of all the NC derived structures of the ear, because normal functioning of these cells is absolutely essential for hearing. Intermediate cells contribute to the ionic composition of endolymph, a potassium-rich fluid that fills the scala media within the cochlear duct. Without endolymph, hair cells are unable to generate endocochlear potentials (EP) in response to movement of their stereociliary bundles and thus unable to relay sound (Kim et al., 2013). Patients and animal models lacking intermediate cells, or the potassium pumps they express, exhibit profound sensorineural hearing loss. Importantly, mutations in the genes encoding Connexin gap junction proteins Cx26 and Cx30 are among the most common causes of congenital sensorineural hearing loss, and affect the ability of intermediate cells to recirculate potassium ions ((Liu and Zhao, 2008; Wingard and Zhao, 2015)

The specification, differentiation and maturation of intermediate cells is controlled by many of the same genes that are required for the development of melanocytes in skin. Indeed, hypopigmentation of skin, hair, and eyes is often associated with defects in the development of intermediate cells and profound sensorineural hearing loss, as is the case in Waardenburg syndrome (WS). WS Types I and III are usually caused by heterozygous pathogenic variants in *PAX3* (Pingault et al., 2010). While sensorineural hearing loss is the most common feature in WS, its severity is highly variable and can either be congenital or progressive and of later onset (Pingault et al., 2010). While a variety of missense, truncating, and deletion mutations in *PAX3* have all been identified in WS patients, the type of mutation does not seem to predict severity of hearing loss (Baldwin et al., 1995; Tassabehji et al., 1995), suggesting that gene dosage of *PAX3* is critical for intermediate cell development. Determining how *PAX3* regulates intermediate cell development has been challenging because *Pax3* mutant mouse models do not recapitulate the inner ear phenotypes observed in WS patients. *Pax3*^{spotch} and *Pax3*^{spd} mutant mice exhibit a variety of other neural crest phenotypes, including splotches of hypopigmentation and neural tube closure defects, but do not have hearing loss (Steel and Smith, 1992; Tassabehji et al., 1992). However, *Pax3*-*Cre/Cre* homozygous knock-in mice, which have reduced *Pax3* expression, have complete loss of stria vascularis melanocytes (Kim et al., 2014). Interestingly, a subset of *Pax3*-

Cre/Cre embryos exhibit exencephaly as a result of failed neural tube closure; in these embryos, the entire inner ear is hypoplastic. This finding suggests that *Pax3* may participate in hindbrain signaling mechanisms that regulate inner ear development, but this hypothesis has not been experimentally tested. Further research is needed to determine how *Pax3* regulates not only melanocyte development, but also other components of the inner ear.

Patients with WS Type IV, characterized by WS Type I features alongside Hirschsprung disease, harbor mutations in *SOX10*, another critical gene for the melanocyte differentiation program. *Sox10* function in the developing mouse stria vascularis is challenging to study for the same reasons described in section 4.1 – multiple other cell types, including cochlear progenitor cells, supporting cells, and NC-derived glial cells robustly express *Sox10*, making it difficult to isolate the function of *SOX10* specifically in cochlear melanocyte precursors. As such, most knowledge of *Sox10* in melanocyte development comes from work conducted in NC-derived skin melanocytes. Interestingly, *PAX3* works synergistically with *SOX10* to activate *Mitf*, a gene considered to be the “master regulator” of the melanocyte differentiation program. Mutations in *MITF* cause WS Type II, which has the same hallmark features of WS Type I without lateral displacement of the inner canthus of both eyes (Pingault et al., 2010). Upon activation of *MITF*, *SOX10* works in turn with *MITF* to promote expression of genes required for melanocyte identity, including *Dct* and *Tyr* (Murisier et al., 2007). *BRG1* (encoded by *Smarca4*) was recently shown to be recruited by *SOX10* and *MITF* to the distal enhancers of melanocyte-specific genes, including *Tyrl* (Marathe et al., 2017). It remains to be tested whether these interactions also occur in cochlear melanocytes.

In addition to these melanocyte-specific defects, non-cell autonomous mechanisms also contribute to the development of stria vascularis melanocytes. The transcription factor *Pou3f4* is required in otic fibrocytes for signaling to intermediate cells and for stimulating expression of the Kir4.1 potassium channel in intermediate cells. *Pou3f4*^{-/-} knockout mice lack Kir4.1 expression and the endolymph produced lacks appropriate amounts of potassium for hair cell mechanotransduction to occur (Song et al., 2011). Interestingly, *Pou3f4* is not expressed in any NCCs in the ear or later on in differentiated intermediate cells, suggesting a critical role for signaling from otic fibrocytes to NCCs in intermediate cell development. The contribution of other non-NCC lineages to promoting or inhibiting intermediate cell development remains to be explored.

4.3 Other lineages (neurosensory components)

Several studies have examined the precise contributions of neural crest lineages to neurosensory components of the inner ear. Using *Wnt1*-Cre and *Pax3*-Cre transgenic mouse lines, Freyer et al. observed spiral ganglion neurons and cochlear hair cells derived from neuroepithelial lineages, presumably including neural crest cells (Freyer et al., 2011). However, similar lineage tracing experiments conducted by other research groups failed to identify neural crest-derived neurosensory cochlear components (Karpinski et al., 2016; Mao et al., 2014; Sandell et al., 2014). There are two important caveats one must consider in the interpretation of these studies. First, the *Wnt1*-Cre transgenic mouse line employed by each of the aforementioned studies does not capture all cranial neural crest derivatives. In fact, a

substantial proportion of the neural crest cells that comprise the sensory cranial ganglia robustly express the canonical neural crest markers Sox10 and FoxD3 but do not exhibit *Wnt1*-Cre recombinase activity (Karpinski et al., 2016). As such, *Wnt1*-Cre mice have been valuable for initial characterization of neural crest in ear development, but no exhaustive fate mapping has been done to identify neural crest contributions to the inner ear. Second, the *Pax3*-Cre mouse line used by Freyer et al. labels a much broader neuroepithelial cell population derived from the neural tube, which presumably also includes neural crest cells. Freyer et al. found significant contributions of *Pax3*-Cre derived cells to cochlear hair cells and neurons of the spiral ganglia. However, the *Pax3*-Cre transgene is broadly expressed throughout the developing neural tube, so the precise developmental origin of these cells may extend beyond the NC. Taken together, these studies provide an important first step in interrogating potential NC origins of inner ear neurosensory components.

Neural crest contributions to the inner ear have also been studied using the *6.5Pax3*-Cre transgenic mouse line in which Cre recombinase is expressed under the control of a neural crest-specific enhancer of *Pax3* (also described in section 4.1) (Sandell et al., 2014). In this study, no NC-derived cochlear hair cells or spiral ganglion neurons were identified. However, *6.5Pax3*-Cre is not expressed in all neural crest cells, and thus comes with some of the same caveats as the widely used *Wnt1*-Cre mice. Other genetic tools have also been used to label cells of the organ of Corti and neural crest. Caution must be taken in the choice of lineage markers used to trace NCCs versus other cell types. For example, *Gata3* is highly expressed in the organ of Corti (Duncan and Fritzsche, 2013; Fritzsche et al., 2015) and in some cranial neural crest derivatives, and has known critical roles in neural crest cell development and neuronal differentiation (George et al., 1994; Lakschmanan et al., 1999; Lieu et al., 1997). Clearly, further research is needed to determine the extent to which the neural crest contributes, if at all, to neurosensory components of the organ of Corti.

5. Avenues for Future Research

As many questions remain about how NCCs contribute to the developing ear, burgeoning technical advancements in bioinformatics and epigenomics have the potential to fill in the knowledge gaps and pave the way for therapeutic applications in patients with neurocristopathies affecting ear development. The past decade of research has revealed critical roles for epigenetic factors, including chromatin remodelers and DNA methyltransferases, in neural crest development. Indeed, multiple neurocristopathies affecting ear development and hearing are caused by loss of function of epigenetic factors, including CHARGE syndrome (*CHD7*, (Choo et al., 2017; Vissers et al., 2004)), Kabuki syndrome (*KMT2D*, (Bogershausen and Wollnik, 2013)), Williams syndrome (*WSTF*, (Barnett et al., 2012)), and Brachydactyly Mental Retardation syndrome (*HDAC4*, (Williams et al., 2010)). However, the specific roles of epigenetic factors in NC derivatives in the ear are largely unknown. As CRISPR/Cas9 gene editing has become more widespread, interrogating the functions of known disease-causing genes *in vivo* is more accessible and affordable than ever. Additionally, methods for characterizing and analyzing the epigenomic landscape in specific tissues or cell types at discrete stages of development, including whole-genome bisulfite sequencing and ATAC-Seq, could be applied to ascertain the chromatin and

DNA methylation landscape of not only cranial NCCs, but other cell types contributing to the development of the ear.

As described in section 4.3, the contributions of NCCs to inner ear neurosensory components have not yet been fully resolved, partly due to the considerable overlap of gene expression profiles of NCCs and otic placode-derived cells in the developing inner ear. The recent surge in bioinformatic techniques could be readily applied to parse out gene expression patterns in hair cells and supporting cells in the organ of Corti with great precision. For example, the power of single cell RNA-Seq to detect unique cell types in even very small cell populations (Saliba et al., 2014) has the potential to help researchers identify any genetic differences that may distinguish between NC- versus placode-derived neurosensory cells that could be further investigated.

A better understanding of neural crest contributions to the ear, particularly the inner ear, could be applied to translational applications. Partial restoration of hearing has been achieved with new cochlear gene therapy techniques (Lustig and Akil, 2012; Zhang et al., 2018). While these experiments have been targeted primarily at recovery of hair cell function, these techniques could be applied to replenishment of melanocytic intermediate cells in the stria vascularis or Schwann cells in spiral ganglia. Further research is needed to determine if a critical window of development is necessary for the successful restoration of intermediate cells or Schwann cells.

6. Conclusions

In this review, we have summarized how the neural crest contributes to specific structures and cell types in the outer, middle, and inner ear, including recent advancements in determining the molecular genetic underpinnings of NC-derived ear components. We have also discussed how aberrant NC development can lead to neurocristopathies that are associated with congenital hearing loss. An emerging theme in the literature is the requirement of crosstalk between NCCs and non-NCCs in constructing all parts of the ear. While much progress has been made in mapping these cellular interactions, the underlying genetic drivers have been difficult to identify. With the rapid rise of technical advancements in bioinformatics and characterization of gene expression landscapes with single-cell resolution, the coming years will be fruitful for research in development of the ear and the neural crest.

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Highlights

- The neural crest (NC) contributes to components of the outer, middle, and inner ear
- Disorders of NC development are frequently associated with hearing loss
- Understanding NC contributions to the ear will improve hearing restoration therapies

Figure 1. Neural crest contributions to the ear.

Cranial neural crest cells (NCCs) contribute to the outer, middle, and inner ear. NCC-derived components are highlighted in light blue. **(A)** The six hillocks of His are visible in the human embryo at the 6th gestational week. Hillocks 1-3 and 4-6 arise from the first and second pharyngeal arches, respectively. Cranial NCCs derived from rhombomere 4 migrate ventrally, infiltrate the pharyngeal arches, and ultimately give rise to the cartilage of the outer ear. The major features of the outer ear arising from each hillock are: 1) tragus, 2) crus of helix, 3) helix, 4) crura of antihelix, 5) antihelix, and 6) lobe. **(B)** The middle ear arises from the cavitation of mesenchyme derived from cranial neural crest. Mesenchymal NCCs from Meckel's cartilage undergo chondrogenesis to form the three ossicles: the malleus (m), incus (i) and arch of the stapes (s). The stapes footplate is derived from mesoderm. In the process of middle ear cavitation, a subset of mesenchymal NCCs undergoes a mesenchymal-to-epithelial transition (MET) to form the epithelial lining of the anterior middle ear cavity. **(C)** Cross section of the cochlea reveals the spiral ganglia that harbor afferent neurons innervating hair cells of the organ of Corti. Neural crest-derived myelinating Schwann cells and non-myelinating satellite glia are found within the spiral ganglia and around the

peripheral axons of Type I spiral ganglion neurons innervating inner hair cells. Additionally, melanocytic intermediate cells within the stria vascularis lining the lateral wall of the cochlear scala media are neural crest-derived.

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Table 1.

Neurocristopathies associated with congenital hearing loss or defects in ear development

Neurocristopathy Associated with Hearing Loss	Ear Phenotype	Causative Gene(s)	Affected Neural Crest Derivative(s)	References
Microtia with or without hearing loss	Small, dysmorphic outer ear	<i>HOXA2</i>	Cartilage of auricle derived from 2 nd pharyngeal arch NCCs	(Alasti et al., 2008; Brown et al., 2013; Piceci et al., 2017)
Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities (SAMS) Disorder	Auditory canal atresia, conductive hearing loss	<i>GSC</i>	First pharyngeal arch NCCs contributing to EAC formation	(Lemire et al., 1998; Parry et al., 2013)
Postaxial Acrofacial Dysostosis Syndrome (POADS), a.k.a. Miller Syndrome	Cup-shaped ears	<i>DHODH</i>	Cartilage of auricle	(Ng et al., 2010)
3MC Syndrome	Conductive or mixed hearing loss	<i>COLEC10, COLEC11, MASPI/3</i>	Middle ear ossicles, inner ear structures unknown	(Munye et al., 2017; Rooryck et al., 2011)
Auriculo Condylar Syndrome (ACS)	Dysmorphic outer ears, conductive hearing loss	<i>EDN1, EDNRA, PLCB4, GNAI3</i>	Cartilage of auricle, middle ear ossicles (fusion of malleus and incus)	(Clouthier et al., 2013; Kokitsu-Nakata et al., 2012; Propst et al., 2013)
Branchio-Oculo-Facial Syndrome (BOFS)	Conductive hearing loss	<i>TFAP2A</i>	Cartilage of auricle, Middle ear ossicles	(Brewer et al., 2004; Milunsky et al., 2008; Milunsky et al., 2011)
Branchio-Otic and Branchio-Oto-Renal Syndromes (BOS/BORS)	Dysmorphic outer, middle, and inner ear; preauricular pits, conductive and/or sensorineural hearing loss	<i>EYA1, SIX1, SIX5</i>	Cartilage of auricle, middle ear ossicles, cochlear hypoplasia in some cases	(Lindau et al., 2014; Smith and Schwartz, 1998)
Klippel-Feil Syndrome (KFS)	Conductive, mixed, or sensorineural hearing loss, microtia, narrow external ear canal	<i>MEOX1, GDF3, GDF6</i>	Cartilage of auricle and ear canal, middle ear ossicles	(McGaughran et al., 1998; Tassabehji et al., 2008)
Treacher-Collins Syndrome (TCS)	Dysmorphic outer ear and middle ear, conductive hearing loss	<i>TCOF1, POLR1C, POLR1D</i>	Cartilage of pinna, middle ear ossicles, auditory bulla	(Richter et al., 2010)
22q11.2 Deletion Syndrome	Conductive and sensorineural hearing loss	<i>TBX1</i>	Auricle cartilage, middle ear ossicles,	(Arnold et al., 2006; Van Eynde et al., 2016)
Craniosynostosis (CS)	Conductive hearing loss	<i>COL2A1, FGFR2, 21, 22, 23, TWIST, MSX2, EFNB1</i>	Middle ear cavitation and ossicles	(Church et al., 2007; Farlie et al., 2016; Goh et al., 2018)
Mandibulofacial dysostosis with alopecia (MFDA)	Dysmorphic outer ears, periauricular skin tags and/or pits, conductive hearing loss	<i>EDNRA</i>	Cartilage of auricle, ossicles, defective Meckel's cartilage development	(Gordon et al., 2015; Zechi-Ceide et al., 2010)

Neurocristopathy Associated with Hearing Loss	Ear Phenotype	Causative Gene(s)	Affected Neural Crest Derivative(s)	References
CHARGE Syndrome	Dysmorphic outer and middle ear, conductive and/or sensorineural hearing loss	<i>CHD7</i>	Cartilage of pinna, middle ear ossicles, cochlear hypoplasia in some cases	(Bosman et al., 2005; Hurd et al., 2011; Hurd et al., 2007; Hurd et al., 2010; Vissers et al., 2004)
Tietz Albinism-Deafness Syndrome (TADS)	Complete sensorineural hearing loss	<i>MITF</i>	Loss of stria vascularis melanocytes	(Izumi et al., 2008; Smith et al., 2000)
Craniofacial-deafness-hand Syndrome (CDHS)	Profound sensorineural hearing loss	<i>PAX3</i>	Likely melanocytes of stria vascularis	(Asher et al., 1996; Drozniewska and Haus, 2014; Sommer and Bartholomew, 2003)
Waardenburg Syndrome Type I, III	Profound sensorineural hearing loss	<i>PAX3</i>	Loss of stria vascularis melanocytes	(Pingault et al., 2010)
Waardenburg Syndrome Type II	Profound sensorineural hearing loss	<i>MITF</i>	Loss of stria vascularis melanocytes	(Pingault et al., 2010)
Waardenburg Syndrome Type IV	Profound sensorineural hearing loss	<i>EDNRB, EDN3, SOX10</i>	Likely melanocytes and glia, perhaps also supporting cells	(Chaoui et al., 2011; Ederly et al., 1996; Tuysuz et al., 2009)
Charcot-Marie-Tooth and Deafness Syndrome	Profound sensorineural hearing loss	<i>PMP22, MPZ, GJB1,</i>	Schwann cells	(Rance et al., 2012)