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Review

Making connections in the inner ear: Recent insights into the development of spiral ganglion neurons and their connectivity with sensory hair cells

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ABSTRACT

In mammals, auditory information is processed by the hair cells (HCs) located in the cochlea and then rapidly transmitted to the CNS via a specialized cluster of bipolar afferent connections known as the spiral ganglion neurons (SGNs). Although many anatomical aspects of SGNs are well described, the molecular and cellular mechanisms underlying their genesis, how they are precisely arranged along the cochlear duct, and the guidance mechanisms that promote the innervation of their hair cell targets are only now being understood. Building upon foundational studies of neurogenesis and neurotrophins, we review here new concepts and technologies that are helping to enrich our understanding of the development of the nervous system within the inner ear.

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Contents

1. Introduction

The mammalian cochlea is a complex coiled organ composed of an array of cell types precisely arranged so that sound stimuli can be accurately transmitted from the outside world into the brain (Fig. 1). The two major sensory cell types of the peripheral auditory system are the mechanically sensitive hair cells (HCs),

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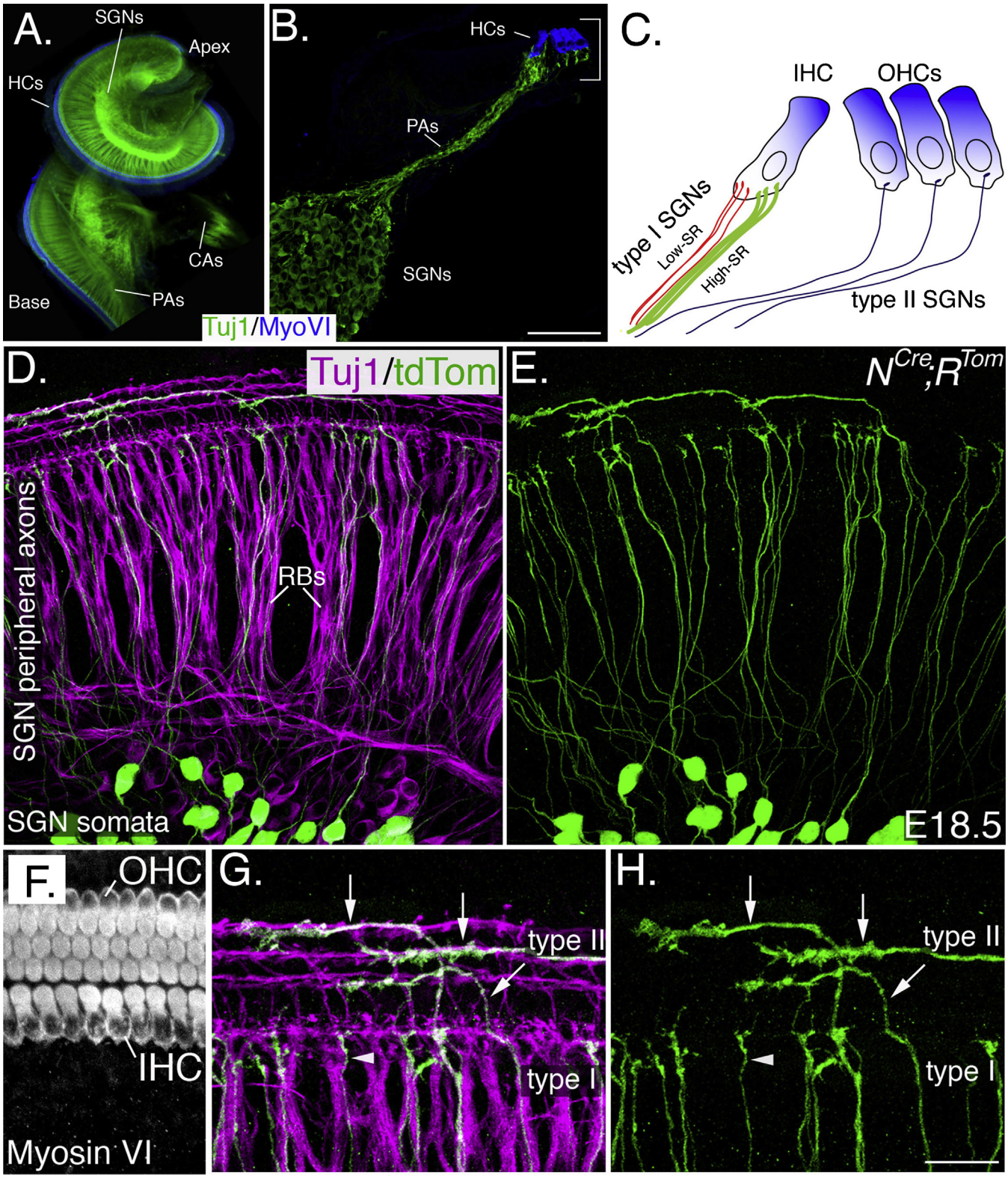

Fig. 1. Spiral ganglion neurons and hair cells in the developing cochlea. (A) The mouse cochlea in whole mount at embryonic day 16.5. (B) A cross section of the cochlea at postnatal day 2. For both A and B, the SGNs are marked with an antibody against Tuj1 (class III β-tubulin; green) and hair cells are marked with antibodies against Myosin 6 (blue). (C) Diagram illustrating the positions of inner and outer hair cells (IHCs and OHCs), type I and II SGNs, and low- and high-spontaneous rate (SR) SGNs relative to one another. We note that correlations between afferent morphology/position and spontaneous activity have been described in the cat [17], but are less conspicuous in the mouse [107]. (D and E) A whole-mount preparation of an E18.5 cochlea from an *Ngn1-Cre*^{ERT2}; *R26R-tdTomato* mouse, illustrating type I and type II innervation patterns. RBs, radial bundles. (F–H) A high magnification view from D; the arrowheads point to type I SGNs; the arrows point to type II SGNs.

and their afferent partners, the spiral ganglion neurons (SGNs). The HCs are located within the sensory domain of the cochlear epithelium, known as the organ of Corti (OC), and reside with a variety of supporting cell and non-sensory epithelial cell types. The SGNs are specialized bipolar neurons that extend peripheral axons (sometimes termed "dendrites") that couple to HCs at ribbon-type synapses, and centrally projecting axons that bifurcate, sending individual processes into the dorsal and ventral cochlear nuclei (DCN and VCN). An amazing and relatively unexplored aspect of this auditory relay system is the set of developmental mechanisms used to encode a frequency gradient along the longitudinal (or "tonotopic") axis of the spiraling cochlea: HCs and SGNs at the base of the cochlea respond to high-frequency sounds, whereas those located progressively toward the apex transmit lower-frequency sounds.

How is this remarkable sensory system established during development? The molecular mechanisms of hair cell and supporting cell development within the OC have been reviewed extensively [1–5] and will not be discussed here. Rather, our objective is to examine several recent advances in the development of the SGNs and how they traverse the complex anatomy of the developing cochlea before making functional connections with HCs. Several other excellent reviews have focused on these matters [6–8], but additional findings have emerged that have started to close the knowledge gap on several issues. First, we will start at the headwaters of SGN development where new light has been shed on the neuroblasts that give rise to the SGNs (see Section 3). Next, we will discuss the complex morphogenetic movements made by the SGNs along the lengthening cochlear duct and their maturation into bipolar neurons (Section 4). Subsequently, we describe several new findings on the accurate extension of SGN peripheral axons into the developing cochlear epithelium (Section 5). Finally we will discuss new insights on the tonotopic arrangement of the SGNs (Section 6), which may provide important insights into how physiological diversity along the frequency axis is generated. The scope of this review does not include important advances made in models of sensorineural deafness where the survival and maintenance of postnatal SGNs is of central focus (please see [9,10]), and does not include extensive discussion of the roles of neurotrophins (please see [11,12]).

2. Classification of SGNs

For decades, the SGNs have been subdivided into two main classes based on their innervation of inner or outer hair cells. Type I SGNs, which constitute 90–95% of the SGN population, terminate at the inner hair cells (IHCs) with each type I fiber forming a synapse on a single IHC (Fig. 1B–H). By contrast, type II SGNs (the remaining 5–10%) project past the IHCs and pillar cells, and into the Deiters' cell region before turning basally and forming synapses with multiple outer hair cells (OHCs). Considering the significantly greater number of type I fibers, it is assumed that IHCs and type I SGNs transduce the majority of all auditory input into the brain [13], whereas the activity of OHCs is thought to amplify auditory input [14]. In general, type I SGNs have a classic bipolar morphology and type II SGNs have more variability in their shape, appearing as either bipolar or psuedounipolar [15]. Both types of SGNs send a single peripheral process from their cell body toward the cochlear epithelium and a central process toward the brain; these central projections converge at the center of the cochlear modiolus, forming the auditory component of the eighth cranial nerve. For more details and historical references on type I and type II SGNs, including aspects of myelination and organelle content, please see Nayagam et al. [8]. As described toward the end of this review, the SGNs can be further distinguished based on their relative position along the tonotopic axis. Glutamate receptor (GluR) subunits and other factors relevant to hearing physiology show graded distributions between SGNs located at the cochlear apex (low frequency region) and SGNs located at the cochlear base (high frequency region) [16]. Finally, several mammalian systems indicate two classes of type I SGNs based on their spontaneous firing rates, caliber, and position of their afferent termini on the IHC. Thin SGN projections with slow spontaneous discharge rates (low-SR) project their afferent termini to the medial side of the IHC, whereas thicker SGN projections with fast spontaneous discharge rates (high-SR) project their afferent termini to the lateral side of the IHCs (Fig. 1C) [17,18].

In summary, SGNs are broadly classified as type I and type II, based on their innervation of IHCs and OHCs, respectively. Type I SGNs are further classified as low- or high-SR, and SGNs positioned at low- or high-frequency regions of the cochlea are distinguishable by their relative expression levels of factors related to synaptic transmission (such as GluRs). However, compared to other sensory systems like the retina [19], we have a relatively limited understanding of SGN diversity, and we are only now beginning to understand how different SGN subtypes arise during development.

3. Development of the cochleovestibular ganglion (CVG)

3.1. New insights into the origins of the SGNs: the re-emergence of the neural crest

What are the origins of the SGNs? Beginning around E9.0 in mouse, a subset of cells within in the otic vesicle initiates a neurogenic program (mediated by Neurogenin1 (Ngn1) and other genes; see Section 3.2), undergoes an epithelial-to-mesenchymal transition (EMT), and delaminates from the otic epithelium. These neuroblasts migrate a short distance away from the otocyst before coalescing to form the "cochleovestibular ganglion" (CVG; Fig. 2). Fate-mapping studies using inducible *Ngn1-Cre* mice have shown that the dorsal–lateral pool of CVG neurons goes on to form the vestibular ganglion, whereas the more ventral-medial pool of neurons goes on to form the spiral ganglion [20,21]. Delamination of neuroblasts continues through E12.5 with the last neurons arising mainly from epithelial cells of the posterior sacculus (a.k.a. "Ductus Reunions" [21,22]) (Fig. 2). In addition, it has been proposed that some SGNs (independent of the Ngn1-lineage) may arise from delaminating neurotrophin-expressing epithelial cells at the apex of the cochlea [7,23], but conclusive evidence supporting or ruling out this model has not yet emerged. Nevertheless, the conventional wisdom in the inner ear field for quite some time has been that the CVG neurons are derived primarily from epithelial cells originating in the otic placode [6,24]. A recent study by Freyer et al. [25] has altered this view.

Neural crest cells (NCCs) constitute a subset of midline neuroepithelial cells (NECs) that are known to contribute to various compartments of the inner ear including the stria vascularis (melanocytes [26]), the periotic mesenchyme [27], and inner ear glia [28]. In 1983, D'Amico and Noden performed chick-quail chimera experiments and observed that some NCCs contributed to various compartments of the inner ear, including neurons of the eighth cranial ganglion [28]. Concerns about aberrant mixing of donor and host cells in tissue grafting experiments have likely prevented these studies from being fully considered over the last two decades, however the results presented in Freyer et al., show exciting evidence that these earlier findings merit strong appreciation.

In the Freyer et al. study, NEC/NCCs were genetically labeled with GFP using *Wnt1*, *Pax3*, or *Hoxb1* Cre drivers and traced to different compartments in the inner ear over developmental time. Interestingly, NEC/NCCs were localized within the otocyst, with strong distribution in the "proneurosensory" domain (Fig. 2). Further, with

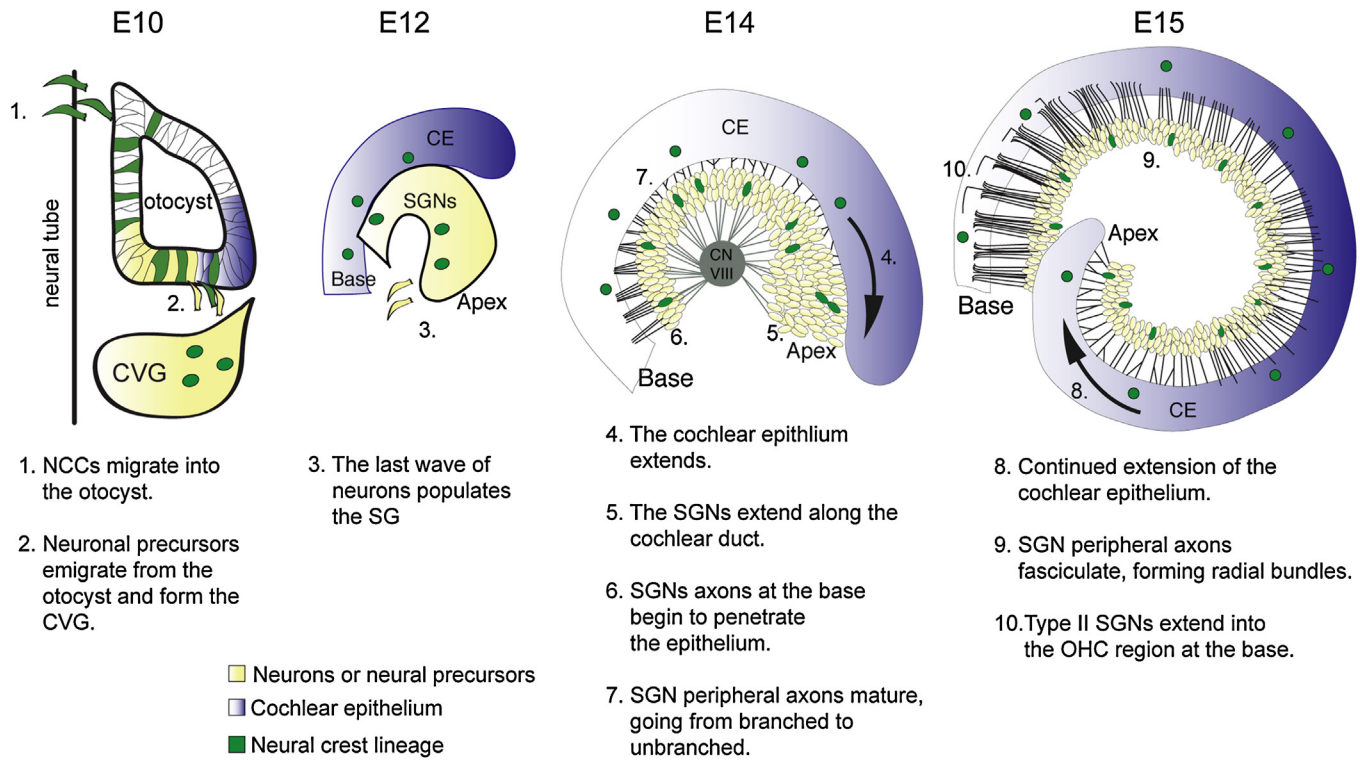

Fig. 2. Summary of SGN morphogenesis events prior to synapse formation with hair cells. Schematic representation of the developing spiral ganglion. Yellow cells indicate otocyst-derived cells within the CVG and SGN. Green cells represent cells originating in the neural tube that contribute to both the SGN and cochlear epithelium. Each numbered event corresponded to the region marked by the identical number below the schematics.

live imaging the authors were able to observe several NECs migrating into the medial otocyst. In the NEC/NCC reporter mice, GFP-positive cells were clearly present in the CVG and shown to be both NeuroD1 and Islet1-postive, suggesting they may have adopted the same developmental program as other neurons fate-mapped from the Ngn1 lineage [21]. These data suggest that NEC/NCC-derived cells contribute to the CVG after first being specified within the otocyst and the authors estimate that 20% of CVG neurons originate from the neural tube. At later developmental stages, there was clear representation of NEC/NCC-derived neurons in the mature spiral ganglion, as many of the NEC-labeled cells were also neurofilament-positive (Fig. 2). While these data renew our appreciation of the dual origin of the SGNs, they elicit several additional questions. In particular, it will be important to know whether the NEC-derived cells within the spiral ganglion give rise to any particular cell type, such as type I- or type II-neurons or high- or low-SR fibers, or have a preferential distribution along the tonotopic axis of the cochlea.

3.2. Specification of CVG neuroblasts

As discussed, the neuroblasts that will give rise to the CVG originate primarily from the anterior-ventral region of the otocyst. While the factors that specify this region of the otocyst to be neurogenic are not completely understood, significant progress has been made in understanding the initial patterning of the otocyst. Early in development, several classes of morphogens and transcription factors act in concert to specify the anterior–posterior and dorso-lateral axes of the otocyst [29,30]. These initial events are necessary to delineate the domains that eventually give rise to both the neural/sensory region (anterior otocyst) and the non-sensory region (posterior otocyst). As a result of these patterning events, the transcription factors Ngn1 [31] and, subsequently, NeuroD1, are upregulated in cells within the anterior region of the otocyst [32], leading to the specification of a neuronal fate in the majority of these cells. The expression of these proneural factors is maintained by the chromatin remodeling enzyme CHD7 [33] and further specification of CVG neurons is likely promoted by the transcription factor Sox2 [34].

It is well recognized that the Ngn1/NeuroD1 signaling cascade is required for the full formation of the CVG, but how these factors are upregulated in a subset of otocyst-derived cells has remained a mystery. Recent evidence has identified a new set of factors, Eya1 and Six1, which may act upstream of the Ngn1/NeuroD1 neurogenesis cascade in the developing inner ear [35]. *Eya1* and *Six1* double knock-out mice fail to generate *Ngn1* or *NeuroD1*-positive otic neuroblasts, suggesting a role in the specification of these cells. However, it is important to consider that these mutants also show defects in dorso-ventral otocyst patterning [35], suggesting that the loss of neuroblasts could be a secondary effect of improper patterning. Simultaneous ectopic expression of *Eya1* and *Six1* in either otocysts of developing mouse embryos or Kölliker's Organ of cochlear explants (non-sensory cells adjacent to the OC) *in vitro* drives the production of Ngn1/NeuroD1-positive neurons, supporting a direct role for these factors in neuronal specification. In Kölliker's Organ cells, the ectopic neurons were also shown to be neurofilament-positive, implicating Eya1 and Six1 not only in the specification of neurons, but also in their subsequent differentiation. In this study, the authors also demonstrate that Eya1 and Six1 interact directly with Sox2 and chromatin remodeling factors that may mediate *Ngn1*/*NeuroD1* transcription, but the precise mechanisms by which these factors cooperate to generate maturing (neurofilament-positive) neurons remain to be determined. In addition to the culture method used in this study, *Eya1*/*Six1* conditional knock-outs are also needed to confirm and extend these findings to in vivo.

3.3. Unresolved issues related to CVG formation

3.3.1. Mechanisms of migration of CVG neurons

In 1912, Streeter [36] first proposed the idea that the CVG arises from a population of cells that emigrates from the otic epithelium, and then Batten later helped champion this idea by showing, in sheep embryos, "stream[s] of cells" migrating from the otocyst into the anlage of the eighth cranial ganglion [37]. In 1983, Carney and Silver used transmission electron microscopy to demonstrate migratory neurons delaminating from the otic epithelium in regions where there was a conspicuous thinning of extracellular matrix around the wall of the otocyst. Here, it was first pointed out how the neurons of the CVG form a funnel that appears to orient "pioneer" axons to extend back into the epithelial cells of the otocyst [38], presumably initiating the process of otic innervation. Remarkably, we know very little about the mechanisms that help instruct the migration of neurons from the otocyst to the CVG. Ngn1 is required for the specification of neurons that form the CVG [31], and ectopically expressed Ngn1 in either chicken or mouse inner ears leads to more neurons [34,39]. But, Ngn1 expression is clearly not sufficient to drive cells out of the otocyst and into the CVG, because many Ngn1-lineage cells remain in the otocyst and eventually become other epithelial cell types [21]. Thus, it remains to be determined whether Ngn1 expression must be maintained for neuroblasts to delaminate, whether Ngn1 acts in concert with other factors to promote the emigration, or whether an undetermined alternative program acts independently of Ngn1. NeuroD1, on the other hand, appears to be more instructive for CVG neurons: in the absence of differentiation signals in *NeuroD1* null mice, not only is the delamination of CVG neurons delayed [40] but also neuronal survival is significantly reduced [32,40]. Given that NeuroD1 and other bHLH factors potentiate migration [41] in addition to their roles in neurogenesis, it is possible that NeuroD1 is involved (directly or indirectly) in the migratory program of neurons destined for the CVG. A more refined analysis of the roles of both Ngn1 and NeuroD1 during the delamination stages of CVG development is needed.

Central to the process of CVG formation is a classic phenomenon in developmental biology: the epithelial-to-mesenchymal transition (or "EMT"). In EMTs, epithelial cells down-regulate cell adhesion molecules such as E-cadherins, and upregulate a variety of factors known to promote cell motility such as Snail, Rac/Rho family GTPases and integrins [42]. Also, extensive work from studies of the EMT that occurs during neural crest migration has revealed the involvement of a remarkable number of factors and pathways [43]. Among these are the Notch and planar cell polarity (PCP) signaling pathways, raising the question of whether these same pathways control the migration of CVG neurons out of the otocyst. Both pathways are involved in various aspects of inner ear development [44,45], but the mechanisms by which these pathways or others control the delamination and migration of CVG neurons remains to be determined.

3.3.2. Division of vestibular and auditory ganglia within the CVG

At present, the signaling mechanisms that lead to the morphological partitioning and developmental specification of vestibular and auditory neurons within the CVG are unknown. Recent studies have now shown convincingly that vestibular neurons derived from a Ngn1 lineage are born earlier than SGNs [20], as may have been predicted given earlier work examining the morphological development of these structures [22] and the classic birth-dating studies by Ruben [46]. We also know that vestibular and auditory neurons are anatomically and physiologically distinct, and are programmed to project to different nuclei in the brainstem [47]. But, still unresolved are the developmental signaling mechanisms that underlie the specification and physical separation of these two neural subtypes. A major technological advance in this area has been the establishment of a database that allows for comparisons of the transcriptomes of vestibular and spiral ganglion neurons [48]. In this study, the authors performed a series of dissection and flow-cytometry-based sorting procedures to achieve distinguishable pools of vestibular and spiral ganglion cDNAs, which were then analyzed by gene chip microarray. A variety of candidates for distinguishing the two populations emerged from the vestibular and spiral ganglion pools and were subsequently validated by *in situ* hybridization. For example, this analysis revealed that members of the transforming growth factor-β (TGF-β) family, and surprisingly, major histocompatibility complex (MHC) family, are preferentially enriched in SGNs compared to vestibular neurons. It is now possible to use these data to investigate candidate factors for their ability to drive CVG neurons toward vestibular versus auditory fates, or to investigate factors that participate in ganglion segregation, neuronal differentiation and/or axonal path finding programs, for example.

4. Growth and alignment of SGNs

4.1. SGNs extend along the medial side of the cochlear duct

In terms of developmental signaling mechanisms underlying cochlear innervation, perhaps the most under investigated and complicated period of SGN development occurs between E12.5 and E15.5 in the mouse. During this period, SGN terminal mitosis occurs with the last cluster of SGNs eventually ending up in the cochlear apex [20,46] (Fig. 2). As the cochlear duct elongates [49] and coils, the SGN somata extend away from the vestibule, aligning themselves with supporting cell and hair cell progenitors located on the floor of the duct. How these movements by the SGNs occur remains a mystery, but at least four possibilities come to mind. First, as suggested by Yang et al. [7], SGNs may move passively with the extending cochlear duct, simply flowing along with or possibly being tethered to the adjacent epithelia and mesenchyme. To investigate this, one would need appropriate Cre drivers to conditionally eliminate factors in the cochlear epithelium that are thought to drive outgrowth, such as non-muscle myosins [49] or Fgf10 [50], without impacting expression in the SGNs. A second possibility is that SGNs undergo directed migration toward the apex, either being attracted toward the apex and/or repelled from the base by any of several families of guidance factors known to be expressed in the inner ear [51]. Although this process has not been described or examined specifically, on occasion we have observed SGNs at this developmental stage with fibroblast-like morphology and migratory behavior during live imaging (Coate et al., unpublished observations). Third, the SGNs may undergo a process well known in the developing cortex referred to as "somal translocation" [52]; in this case, an SGN could first extend its central and peripheral processes toward their targets, and subsequently shuttle its soma into alignment with the developing hair cells. A fourth possibility is that the SGNs use the Schwann cells as scaffolds for migration, similar to what has been shown for pyramidal neurons that use components of the Par complex and F-actin dynamics to drive migration along radial glia [53]. Consistent with this possibility is the observation that in both *Sox10* [54] and *ErbB2* mutants [55], which lack auditory glia, much of the ganglia appears to be grossly disorganized despite relatively normal differentiation. As noted [55], the loss of normal innervation may reflect the loss of trophic support in these mutants, but there is also the possibility that glia play a physical role in shaping the spiral ganglion lengthwise along the duct.

4.2. SGN maturation

To further illustrate how dynamic this phase of development is, while the SGN somata align along the cochlear duct, they also undergo a maturation process whereby they transition from having relatively complex branching patterns to a classic bipolar configuration [20]. Recent experiments using alkaline phosphatase expression under the control of *Ngn1-Cre*^{ERT2} (to achieve sparse numbers of labeled SGNs) have provided insight into the morphogenetic events underlying this process [20]. Starting at the base of the cochlea at E12.5, many of the peripheral axons of the SGNs are highly branched, whereas their central axons are unbranched. As development proceeds in a base-to-apex manner, the position of each SGN soma extends away from the cochlear epithelium while the peripheral axon branches are refined into a single process that projects into the sensory domain. This phase of development coincides with several other interesting events: dispersion of Schwann cells throughout the ganglia [55], differentiation and compartmentalization of adjacent otic mesenchyme cells [56,57], and specialization of hair and supporting cells within the prosensory domain [58–61]. It remains to be determined whether any of these latter events play a role in SGN maturation, or whether SGN maturation is primarily an intrinsic process.

Regardless of the external factors that might mediate SGN maturation, these changes must be carefully orchestrated through a series of events that produce a bipolar neuron at the correct location and stage of development. Recent studies [62] have shown that the transcription factor Gata3, which becomes preferentially expressed in SGNs compared to vestibular neurons over time [48], may normally regulate the timing of SGN peripheral axon extension. In mice, conditional deletion of *Gata3* in SGNs leads to precocious extension of their peripheral axons, and to somata that do not appropriately laminate along the length of the cochlear duct. In addition, fixed-specimen and time-lapse imaging studies demonstrate that Gata3-deficient SGN peripheral processes show aberrant path finding along their route to the cochlear epithelium. The loss of *Gata3* in SGNs confers an intrinsic transformation, as cultured SGNs display premature and augmented neurite outgrowth compared to controls. From transcriptome analyses, it appears that Gata3 controls the expression of factors related to SGN differentiation and maturation, which is consistent with the phenotypes observed. Interestingly, in later stages of SGN development, Gata3 acts as a survival factor for SGNs, as has been shown previously in other parts of the nervous system [63,64]. Overall, these studies represent the first insight into the control of SGN maturation during this period of development and emphasize the importance of timing of specific events (e.g. axon outgrowth) for appropriate innervation patterns of the cochlea.

5. Peripheral axon outgrowth

5.1. Development of peripheral axons

Before synapsing with auditory hair cells, the peripheral axons of the SGNs must navigate through a variety of cell types, all of which probably express a well-coordinated ensemble of guidance cues that act to generate stereotyped cochlear innervation patterns. As discussed, with some exceptions, most developing SGNs begin to extend peripheral processes between E12.5 and E15.5. As these processes extend they will encounter a number of different cell types including auditory glia and a mixture of cell types broadly referred to as "otic mesenchyme." Otic mesenchyme cells are grouped together based on their compartmentalization within the otic capsule, but actually consist of cells that originated as both paraxial mesoderm and neural crest cells [27]. Their derivatives will go on to form structures such as the auditory vasculature, spiral laminae and spiral limbus [56]. By E14.5, peripheral processes at the base of the cochlea can be observed within the prosensory domain of the cochlear epithelium (Fig. 2). During these early stages in mouse the SGNs freely explore the entirety of the prosensory domain, but then most (type Is) become confined to the basal side of the inner hair cell as hair and supporting cells begin to develop. Interestingly, the path finding behaviors of the SGNs in mouse at these early stages (E14.5 and E15.5) are very similar to the SGN segregation events that were elegantly described in the gerbil cochlea by Echteler [65], but at later time points (P0 and on). Type I and II SGNs become spatially distinct around E16 with type I afferent processes remaining along the row of inner hair cells, and type II afferents extending into the OHC layer and turning basally. Interestingly, type I and type II afferents are rarely observed to terminate in the nearby GER (type I) and LER (type II), suggesting that inhibitory mechanisms serve to restrict them to the prosensory region. Analysis of several mutant lines, including deletion lines for the transcription factor *Prox1*/Prox1 [66] or the transmembrane receptor *Fgfr3*/FGFR3 [67], have shown disruptions in type II SGN innervation patterns, but the specific guidance mechanisms that may be altered in either line remain unknown. As discussed below, a huge number of players that may regulate both attractive and repulsive signals within developing SGNs have been identified, and several specific guidance mechanisms have been demonstrated.

5.2. Axon guidance factors expressed in the developing cochlea

Over the last 20 years, expression studies have demonstrated that essentially all of the families of classic axon guidance factors and their receptors are present in the developing ear, suggesting that a complex array of signaling mechanisms is likely to control hair cell innervation patterns. Table 1 summarizes the known patterns of expression for the ephrins and their Eph receptors, netrin-1 and its DCC and UNC5 receptors, the semaphorins and their Neuropilin and Plexin receptors, and the Slits and their Robo receptors. Overall, more work has been focused on the ephrin/Eph receptor signaling system (reviewed by [68,69]), as it appears that nearly all of the members of both the ligand and receptor families are expressed in the cochlea at some point during development. Given that the Ephs and ephrins can signal uni-directionally or bi-directionally [70], and that their expression patterns in the cochlea are often overlapping, it seems that we are only at the starting point of understanding how these factors may precisely guide SGNs to their hair cell targets. In addition, there are a variety of cell adhesion molecules (CAMs) expressed in the cochlea [71–73], which are well known in axon guidance, as well as a variety of extracellular matrix factors that may provide preferential substrates for growing SGNs [74,75]. Also, morphogens such as members of the Wnt, FGF, Hedgehog, and TGFβ families, which are also known to exert guidance effects on growing axons (for reviews, see [76–78]), are also expressed and function within the developing cochlea [3]. The dissection of the role of these different factors in SGN guidance will probably require the careful generation of tissue-specific conditional mutants, since many of these factors are known to, or can be expected to, play roles in other aspects of cochlear development, such as patterning the OC.

5.3. SGN radial bundle fasciculation and otic mesenchyme

As the SGN peripheral axons extend through the otic mesenchyme on their way to the cochlear epithelium, they fasciculate to form arrays of "radial bundles" that are distributed orthogonally to the longitudinal axis of the cochlea. Each bundle contains

Table 1
Axon guidance molecules expressed in the developing cochlea.

Axon guidance family	Reported expression domain
Ephrins/Ephs	
Ephrin-A1	SC [93]
Ephrin-A2	SC [93]; SG [79,94]
Ephrin-A3, A4	SG [79,94]
Ephrin-B1, B2, B3	SG [48,79,94]
EphA3	SG [48];
EphA4	SG, HC [93]; OM [79,95]
EphA5, EphA7	SC [93]; SG [94]
EphB1, B2, B3	SG [94,48]
Not yet reported or possibly absent	Ephrin-A5, -A6; EphA1, A2, A8, A9, B4, B5
Netrins/DCC	
Netrin1	OV [96]; CE [97,98]
DCC	SG [98]
Neogenin	OM [96]
UNC5B	OV, OM, HC, SC [96]; SG [96,48]
UNC5C	OM, SG, C [96,48]
UNC5D	SG [96,48]
Not yet reported or possibly absent	UNC5A
Semaphorins/Nrps and Plexins	
Sema3A	OV [99]; SG [48]
Sema3B, 3D, 3F	OV [99]
Sema3C, 3E, 3G	SG [48]
Sema4A, 4B, 4C, 4F, 4G	SG [48]
Sema5A, 5B	SG [48]
PlexinA1, A3, A4	SG [100,101]
PlexinB1, B2	SG [48]
PlexinC1	SG [48]
PlexinD1	SG [48]
Neuropilin 1	OV [99]; SG [48]
Neuropilin 2	SG [48]
Not yet reported or possibly absent:	Sema4D, 4E, 5C, 6A-D, 7A; PlexinA3, B3
Slits/Robos	
Slit1	OV [102]
Slit2	OV [102]; C [103]; SAG [104]; SG [48]
Slit3	OV [105]; C [103]
Robo1, 4	SG [48,106]
Robo2	SG [106]; SAG [104]
Not yet reported or possibly absent	Robo3

Abbreviations: SC, supporting cell; HC, hair cell; SG, spiral ganglion; OV, otic vesicle; OM, otic mesenchyme; CE, cochlear epithelium (nothing otherwise specified); C, cochlea (nothing otherwise specified). We apologize to any of our colleagues if pertinent references were omitted because of space limitations.

between fifty and two hundred processes, with those bundles at the apex tending to be smaller than those at the base. As each bundle approaches the OC, it defasciculates into smaller fascicles containing 10–20 fibers that project toward individual hair cells. Until recently, the signaling factor(s) that controls this process was unknown. However, a study from Coate et al., demonstrated that the guidance receptor EphA4 is expressed on the surface of otic mesenchyme cells and influences the formation of radial fiber bundles [79]. EphA4 had previously been shown to be a chemorepellent to cultured SGNs [80] in addition to regulating critical aspects of auditory brainstem organization [81–83]. But in this study, the authors first determined that the transcription factor Pou3f4, which is expressed only by otic mesenchyme cells in the cochlea, is required for normal SGN peripheral axon fasciculation. Subsequent microarray transcriptome screening and mutant analysis demonstrated that Pou3f4 controls the expression of *Epha4*/EphA4 in otic mesenchyme, and that EphA4 normally activates ephrin-B2 expressed on the surface of SGNs to promote their fasciculation [79]. However, the specific molecular effects of ephrin-B2 reverse signaling in SGNs is still unclear. Ephrin-B2 may directly promote SGN axon-axon adhesion via factors such as integrins, or ephrin-B2 may mediate filopodial collapse leading to preferential adhesion to neighboring axons. Future studies focused on ephrin-B2 and its possible downstream targets within SGNs will be necessary to delineate these possibilities.

6. Tonotopy in the SGNs

A fundamental organizing principle of the auditory system is the spatial separation of complex sounds based on frequency, also known as tonotopy. In mammals, the cochlear apex is tuned to low frequency sounds, whereas more basal regions respond to progressively higher frequency sounds. Work over several decades has determined that frequency tuning within the mammalian cochlea is mediated through a combination of changes in passive properties of the basilar membrane, and possible active properties of the tectorial membrane and outer hair cells (reviewed by [84,85]). However, while SGNs might be expected to show morphological variation along the tonotopic axis, so far only a decrease in cell size at more apical positions has been reported (Fig. 3A). Importantly,

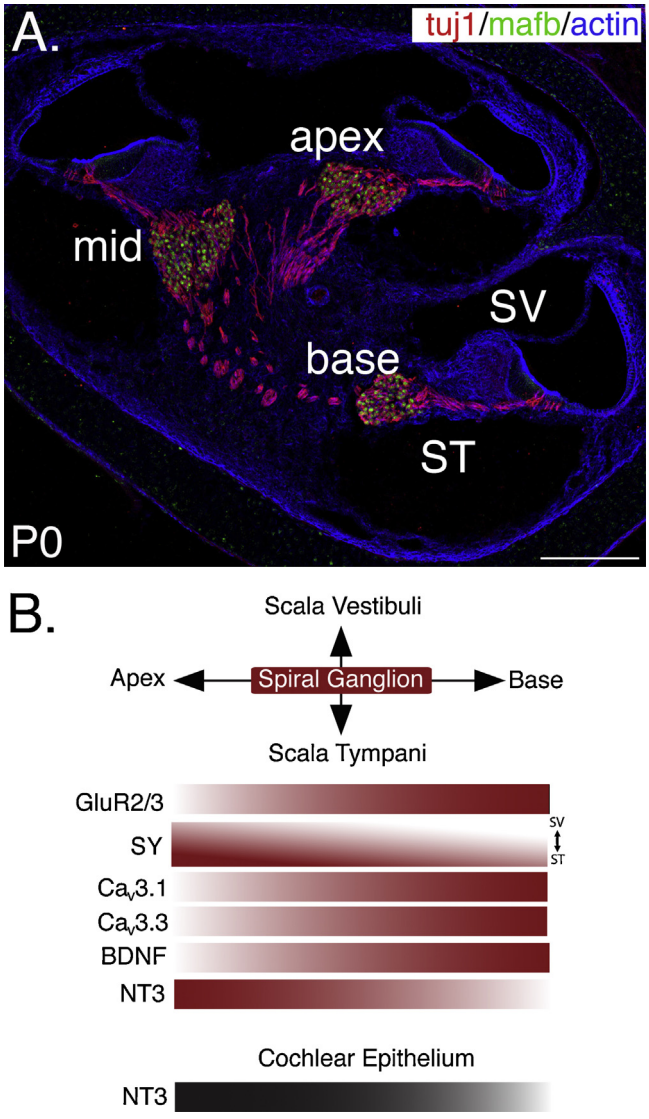

Fig. 3. Summary of factors expressed in tonotopic gradients in the neonatal cochlea. (A) A cross-section of the cochlea at P0. The SGNs are labeled with Tuj1 antibodies (red) and Mafb antibodies (green; specific for SGN nuclei) [48]. Actin (blue) is labeled with phalloidin. sv, scala vestibuli; st, scala tympani. (B) A schematic summary of the expression patterns of glutamate receptors 2 and 3 (Glur2/3), synaptophysin (SY), voltage-gated calcium channels (Ca_v) 3.1 and 3.1, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) along the tonotopic axis. Darker shading represents regions of stronger expression.

physiological and molecular studies have now identified marked changes in SGNs that correlate with longitudinal position and these specializations may account for their known differences in firing properties [86].

6.1. Tonotopic distribution of synaptic proteins and voltage-gated ion channels in SGNs

A recent report by Flores-Otero and Davis demonstrates how AMPA-type glutamate receptors and synaptophysin (SY), which are known to be involved in transmitting synaptic input from hair cells to the CNS, are expressed in graded distributions along different axes of the spiral ganglion [45]. As illustrated in Fig. 3, the expression levels of glutamate receptors 2 and 3 (GluR2 and GluR3) in the SGNs are enhanced at the base of the cochlea suggesting that these GluRs may facilitate high-frequency transmission. Conversely, the expression of SY is enriched at the apex suggesting that this factor may facilitate low-frequency transmission. It is important to note that the distribution of these factors appears in a smooth gradient along the frequency axis, rather than showing stark differences from one region to the next, consistent with how the cochlea conveys a gradient of sound frequencies. Also interesting was the keen observation that SY was expressed in a gradient along an additional axis between the scala tympani (ST) and the scala vestibuli (SV), which corresponds to the depth of the ganglion. Stronger levels of SY appear in SGNs that are positioned closer to the ST, compared to SGNs positioned near the SV (Fig. 3). What physiological or functional differences (e.g. spontaneous firing rate [17]) exists between SGNs along this additional axis of the ganglion remain to be determined.

As noted above, there are clear differences in the kinetic properties between apical and basal SGNs. These differences were previously explained by the differential distribution of potassium (K^+) channels in SGNs: the voltage gated K^+ channels, $K_v1.1$ and $K_v3.1$, and the large conductance calcium-activated potassium channel (BK) channel are more enriched in the base, whereas $K_v4.2$ is more enriched in the apex [86] (for illustration, see [11]). More recently, SGN action potentials were shown to depend on voltage gated calcium channels (VGCCs) [87], and antibody staining indicated $Ca_v3.1$ and $Ca_v3.3$ are distributed in a clear tonotopic gradient with enrichment in basal SGNs [87] (Fig. 3). Overall, the differential distribution of both synaptic proteins and channels suggests that the SGNs along the tonotopic axis are not generic, but physiologically specialized.

6.2. Regulation of tonotopic specializations by postnatal gradients of neurotrophins

Given the clear tonotopic differences in protein expression among the SGNs, it is intriguing to consider how these specializations arise. During development, BDNF and NT3 are important for the survival of SGNs in the cochlear apex and base, respectively [23]. Following the period of initial innervation, the expression of these factors continues and becomes progressively graded into adulthood: NT3 is expressed preferentially in the apex by cochlear satellite cells, hair cells [88] and SGNs [89], whereas BDNF is expressed predominantly in SGNs of the cochlear base [89] (Fig. 3). Recent studies have provided very intriguing evidence to suggest that the distribution of synaptic proteins and voltage-gated channels in the SGNs may be dictated by these regionalized concentrations of NT3 and BDNF [16]. Treating apical and basal SGNs with BDNF *in vitro* enhances their expression of GluR2/3, whereas NT3 treatment reduces GluR2/3 expression. Conversely, SY expression is enhanced by NT3 and reduced by BDNF [16]. Interestingly, this phenomenon holds true also for several voltage-gated potassium channels: for $K_v3.1$ and BK, their levels are reduced in basal neurons after NT3 treatment, but increased in apical neurons after BDNF treatment [86]. In addition, BDNF appears to augment the expression of $K_v1.1$, whereas NT3 appears to augment the expression of $K_v4.2$ [86]. Although the effects of BDNF and NT3 on synaptic proteins and ion channels has not yet been assessed in genetic models, these findings help establish an elegant model of how gradients of neurotrophins ultimately dictate how SGNs become specialized prior to hearing onset.

6.3. Future directions in the field of SGN development

In the past few years, several new mechanisms underlying SGN-HC connectivity have emerged and we now have a greater appreciation for what cells ultimately contribute to the SGN population, what factors regulate their neurogenic program, and what factors are important for their growth and stereotyped innervation patterns of the OC. It has also become apparent that SGNs are not all identical physiologically, and have developed specializations (e.g. along the tonotopic axis) that may have contributed to the relative sophistication of mammalian hearing.

Whereas, it is clear that the processes required for SGN development mirror many other events in the developing nervous system, it remains to be determined whether the factors that control those processes are similarly conserved. For example, neurons contributing to the CVG clearly go through an EMT – is this process similar to the one that occurs within the delaminating neural crest? In addition, a plethora of axon guidance factors are expressed in the cochlea during periods of hair cell innervation (Table 1), but only a handful of known guidance mechanisms have emerged. Which cues act as chemo-attractants? Which are chemo-repellents? As in other systems, one of the challenges will also be to understand how the SGNs integrate multiple guidance cues to generate a coherent and stereotyped response that ultimately targets their processes through the otic mesenchyme and into different hair cell regions of the OC.

In addition, there are several other cell types that have received little attention over the years, which should be considered when investigating SGN development. We know that efferent projections from the olivocochlear nucleus reside alongside SGN peripheral processes, but very little is known about whether they provide any guidance information to SGNs (or vice versa) to shape normal cochlear innervation patterns. In addition, there is very little known about how the vasculature develops within the inner ear and whether it impacts innervation patterns, as in the sympathetic nervous system, for example, where GDNF from blood vessel smooth muscle cells attracts sympathetic axons [90,91]. Fortunately, new technologies [48] and genetic resources such as Cre lines [92] are becoming increasingly available, which should accelerate our understanding of how SGNs develop and make functional connections with HCs in the cochlea.

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