Chapter 2
Early Development of the Spiral Ganglion

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2.1 Introduction: Morphogenesis of the Spiral Ganglion

The spiral ganglion develops in parallel with the patterning and morphogenesis of the inner ear (Fig. 2.1). The inner ear arises from the otic placode, an ectodermal thickening that forms adjacent to the 5th and 6th rhombomeres of the hindbrain in vertebrates other than the lamprey (Kuratani et al., 1998). This occurs at the 8–10 somite stage of development, which corresponds to E8.5 in mouse (*Mus musculus*) (Anniko & Wikstrom, 1984) and stage 10 in chicken (*Gallus gallus*) (Hemond & Morest, 1991a). In birds and rodents, the placode subsequently invaginates and deepens to become an otic cup (Knowlton, 1967; Marovitz et al., 1977; Anniko & Schacht, 1984). The otic cup then detaches from the ectoderm and seals to form an ovoid otic vesicle that is closely apposed to the hindbrain and surrounded by mesenchyme.

Inner ear neurons develop from precursors in the anteroventral quadrant of the otic vesicle, which leave the epithelium and proliferate to form the cochlear-vestibular ganglion (CVG) just rostral to the developing inner ear. At this stage, the cochlear and vestibular ganglia are distinct yet closely associated with each other, as well as with the geniculate ganglion (Fig. 2.1), forming a three-ganglion complex. Morphologically, neuroblasts can be recognized during otic cup stages as otic epithelial cells that lose their columnar morphology and delaminate from the epithelium (Carney & Silver, 1983; Hemond & Morest, 1991b), forming a distinct CVG by the 22–24 somite stage in mice (Wikstrom &

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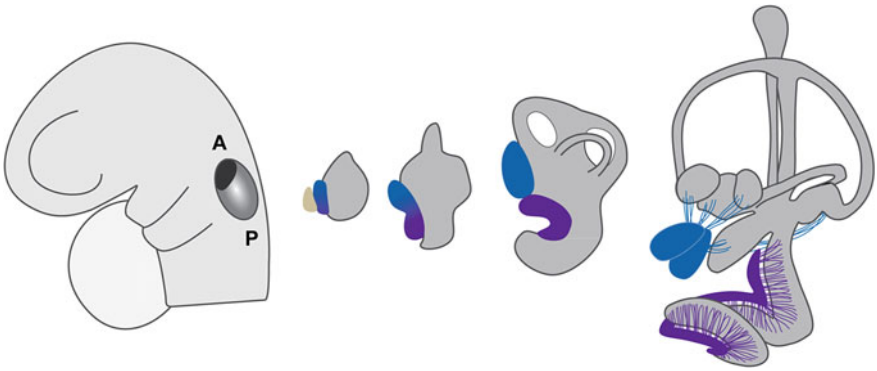


Fig. 2.1 Morphogenesis of the spiral ganglion. The spiral ganglion develops from a proneurosensory region (*dark gray*) in the anteroventral quadrant of the otic cup, beginning around E9 in mouse. Over the next week, the otic cup invaginates and acquires its mature three-dimensional structure. In parallel, the neurons delaminate to form a cochlear-vestibular ganglion (CVG). The vestibular (*blue*) and spiral (*purple*) ganglia gradually separate and eventually innervate the vestibular and auditory sensory epithelia respectively. The geniculate ganglion (tan) is initially attached to the CVG laterally (shown only for E10.5). This ganglion is separated from the CVG by the facial nerve and originates from a distinct placode

Anniko, 1987), with the auditory division positioned medial and ventral to the vestibular division and the entire neural anlage still attached to the geniculate ganglion (Sher, 1972). The spiral ganglion grows and extends together with the cochlear duct over the next several days (Carney & Silver, 1983), gradually separating from the vestibular and geniculate ganglia.

The basic sequence of events during spiral ganglion development is conserved across species, with a few notable exceptions. In every species examined, neurons seem to be the first differentiated cell type to appear in the inner ear. In both mice and birds, neurogenesis begins during otic cup stages and continues after the otic vesicle has closed and become free from the overlying ectoderm (Carney & Silver, 1983; Hemond & Morest, 1991a). However, the structures for hearing exhibit distinct forms. In mammals, the auditory sensory epithelium (the organ of Corti) spirals within a coiled cochlear duct. Birds (and reptiles) instead detect sound via the basilar papilla, which is located in the tube-like lagenar recess. To underscore its similar function, the avian hearing organ is often referred to as the cochlear duct, but it is important to note that these structures are not strictly analogous, as the lagenar recess also houses an additional sensory epithelium, the lagena macula, that is not present in the cochlea.

The lagena macula is a sensory organ found in birds, fish, reptiles, and amphibians but not in mammals (Harada et al., 2001). Most often considered vestibular in nature, its function remains unclear and may vary among species (Fritzsch & Straka, 2014). In pigeons, there is intriguing evidence for a role in magnetoreception, which is the ability to orient in response to the Earth's magnetic field (Wu & Dickman, 2011). From an evolutionary point of view, the lagena macula seems to

have been lost from mammals, possibly having been incorporated into the apex of the organ of Corti, which itself evolved from the basilar papilla (reviewed in Fritzsch et al., 2013). Because of these differences, auditory and vestibular neurons form distinct ganglia in mammals but remain as a CVG in birds. Whether this difference in organization affects the development of the auditory neurons is not clear, but the presence of the lagena macula should be borne in mind when making comparisons between species, especially because this sensory epithelium is innervated by a subset of neurons that project to distinct targets centrally (Mahmoud et al., 2013).

The fish inner ear also exhibits a number of salient differences from both the avian and mammalian ears. Most strikingly, fish such as the common model organism zebrafish (*Danio rerio*) do not develop a cochlea-like structure and have no basilar papilla, as fish rely instead on hair cells in the saccule and lagena for auditory function (Bigelow, 1904). This raises the question of whether the neurons that innervate the saccule and lagena in fish are more analogous to vestibular or auditory neurons in mammals. Circuit tracing studies have confirmed that the primary sensory neurons that innervate each of these structures convey information to different regions of the brain, suggesting that fish use the information from sound-induced vibrations in the saccule and lagena differently from the movement-induced vibrations of the utricle and semicircular canals (McCormick & Wallace, 2012). However, in many brainstem nuclei, there is also considerable overlap with projections that are vestibular in nature. In addition to the murky understanding of auditory versus vestibular identity, the timing of neurogenesis is also slightly different in fish. Whereas in birds and mammals, hair cells typically develop after neurons, both populations are produced at the same time in fish (Haddon & Lewis, 1996) and continue to be added long after hatching (Popper & Hoxter, 1984). Fish also differ in the size of the ganglion, which contains only a few hundred neurons (Popper & Hoxter, 1984) compared to approximately 8000 in mice (Johnson et al., 2011) and approximately 9000 in chickens (Ard & Morest, 1984).

The human spiral ganglion is even larger, with approximately 30,000 neurons (Rasmussen, 1940; Nadol, 1988; Spoendlin & Schrott, 1989), although it appears to pass through developmental stages similar to what has been described in mice and chickens (Streeter, 1906; Bibas et al., 2006; Locher et al., 2013). In addition, there is accumulating evidence that the same basic pathways operate in fish, mice, and chickens, though different specific players may be involved in each species (see Groves & Fekete, 2012 for review). Much less is known about the molecular basis of human spiral ganglion neuron development, but to date, no obvious differences have been described. Nevertheless, it is important to keep in mind that differences between species may exist, both among model organisms and between model organisms and humans. Because of the closer parallels to the human system, findings from chicken and rodents will be emphasized here. The chick has served as an excellent system for working out the earliest stages of neurogenesis because of its accessibility for acute embryological manipulations. Mice offer the advantage of genetics plus closer parallels to the human.

Morphogenesis of the spiral ganglion depends on a coordinated series of extrinsic and intrinsic patterning events that begin in the early otic vesicle with the

production of multipotent neurosensory progenitors. Neurosensory progenitors are progressively directed toward the spiral ganglion neuron (SGN) fate through a series of fate decisions. In parallel, the number and location of SGNs is controlled by selective expansion and culling of progenitor populations as well as directed migration away from the otic vesicle into the surrounding mesenchyme. As a result of these developmental events, the mature cochlea houses a population of SGNs that have both the intrinsic properties and the precise connections necessary for accurate transmission of sound information from hair cells to the central nervous system. Building on decades of careful anatomical and embryological studies, we now have a broad understanding of how these events unfold at the cellular level, and have begun to identify many of the signaling pathways and transcriptional networks that initiate and regulate early SGN development.

2.2 Origin of Inner Ear Neurons

2.2.1 The Otic Vesicle

In all species, neurogenesis is confined to spatially restricted regions of the otic cup, partially overlapping with zones that produce the sensory cells but excluded from those fated for non-neurosensory tissues in the mature inner ear. In rodents and birds, the neurons of the inner ear arise in the anteroventral quadrant of the otic vesicle (Figs. 2.1 and 2.2). Although intuited from histological studies, which revealed an obvious region of delamination (Knowlton, 1967; Carney & Silver, 1983), in vitro fate mapping studies ultimately confirmed that this portion of the otocyst produces neurons when dissected and cultured in isolation (Li et al., 1978; Adam et al., 1998), but that more dorsal and more posterior regions do not (Li et al., 1978). Similarly, dye labeling of the chick otic cup (Hamburger Hamilton [HH] stage 12) showed that CVG neurons are produced from the anterior compartment (Abello et al., 2007). Moreover, cells in this region rarely mixed with cells in the neighboring "non-neuronal" compartment and respected gene expression boundaries, indicating that the region of neurogenesis is patterned at an early stage.

The neurogenic zone itself appears to be further patterned, as vestibular neurons are generated before auditory neurons and from spatially distinct populations, as evidenced by dye labeling experiments in chicks (Bell et al., 2008) and genetic tracing experiments in mice (Koundakjian et al., 2007). Indeed, auditory and vestibular neurons appear to delaminate from different regions of the otic vesicle, with vestibular neurons developing close to the vestibular sensory epithelia and most auditory neurons instead delaminating from the boundary between the cochlea and saccule and then from the middle and apical turns of the cochlea itself (reviewed in Yang et al., 2011). Such spatial segregation is particularly extreme in fish, in which there are two separate neurogenic zones, with the anterior region producing neurons

that innervate the utricle and the posterior region producing neurons that innervate the saccule, with anterior neurogenesis occurring slightly earlier (Haddon & Lewis, 1996; Haddon et al., 1998; Sapede & Pujades, 2010).

Importantly, in each species, the neurogenic zone is closely associated with regions of the epithelium that produce sensory cells, namely the hair cells and supporting cells of the utricle, saccule, cristae, and cochlea. Hence, the neurogenic zone is actually contained within a larger "proneurosensory domain" (PNSD) that contains both neural and sensory progenitors, with the neurogenic region overlapping with the nascent sensory epithelia for the utricle and saccule (Cole et al., 2000; Raft et al., 2007). Other sensory areas, such as those for the cristae, can arise either from apparently non-neurogenic regions in the PNSD or outside of the PNSD altogether, as suggested by expression of sensory markers and fate mapping (reviewed in Fekete & Wu, 2002).

2.2.2 Other Potential Sources for SGNs

Although the otic vesicle is the primary source for inner ear neurons, there is a long history of studies considering the possibility of a contribution from the neural crest and/or neuroepithelium. In fact, Bartelmez argued strongly that auditory neurons derive from the neural tube based on his histological analysis of early human embryos (Bartelmez, 1922). In contrast, early embryological experiments using the larval salamander indicated that most if not all neurons derive from the otic placode, whereas the neural crest produces the Schwann cells that myelinate the inner ear neurons (Yntema, 1937). Similarly, when neural crest cells were transplanted from quails to chicks, many quail-derived glia populated the mature ganglia and eighth nerve, confirming that auditory Schwann cells share the same neural crest origin as those in the rest of the peripheral nervous system (D'Amico-Martel & Noden, 1983). More surprisingly, some quail-derived cells also appeared to develop as neurons, but these seemed more likely to be vestibular based on their location. However, it was not possible to rule out a contaminating non-neural crest cell population, nor were markers used to verify the neuronal identity of the quail-derived cells.

More recently, an argument for a neural crest contribution was made based on genetic fate mapping studies in mice (Freyer et al., 2011). In these experiments, neural crest cells were permanently marked by Cre-mediated recombination of fluorescent reporters. In mice that produce Cre under the control of the *Pax3* promoter, which is active throughout the dorsal neural tube, fluorescent cells could be seen moving from the neural tube into the otic vesicle, eventually contributing to the ganglion, maculae, and cochlea. However, these types of experiments can be difficult to interpret, as even undetectably low and likely physiologically irrelevant levels of Cre protein might be sufficient to induce recombination. In addition, *Pax3*

expression is not restricted to the neural crest, so many of these cells may be occasional neuroepithelial precursors that accidentally found themselves in the otic cup, which is pressed up against the hindbrain, but were nonetheless able to proliferate and differentiate within this new environment. In fact, dye labeling of cells in the early embryonic chick hindbrain revealed a similar contribution to various tissues of the inner ear, including the CVG (Ali et al., 2003). Hemond & Morest, (1991b) further noted a possible contribution from migratory cells formed at the boundary of the otic cup in chicks. These so-called "otic crest" cells seemed to stream toward multiple ganglia, but a specific contribution to the CVG was not defined.

In fact, many experiments argue that any non-otic contribution to the neurons of the inner ear is minimal or even nonexistent. For instance, complementary fate mapping studies using multiple independent *Cre* lines with expression in the otic vesicle, namely *Foxg1–Cre* (Hebert & McConnell, 2000), *Pax2–Cre* (Ohyama & Groves, 2004), and *Pax8–Cre* (Bouchard et al. 2004), suggest that the vast majority if not all inner ear neurons do in fact derive from the otic epithelium. In addition, fate mapping with *Wnt1–Cre* as well as a more restricted *Pax3–Cre* driver did not reveal any substantial contribution to the inner ear besides Schwann cells (Sandell et al., 2014). The conflicting results for *Wnt1–Cre* could reflect variation in the level of Cre activity in combination with different reporters and on different genetic backgrounds. Because the currently available "otic" *Cre* lines also mediate recombination in the neuroepithelium, albeit within highly restricted regions (Hebert & McConnell, 2000; Bouchard et al., 2004; Ohyama & Groves, 2004), generation of a truly otic-specific *Cre* line may be necessary for final resolution of this issue. Nonetheless, most studies indicate that the otic vesicle does indeed serve as the primary source for inner ear neurons.

2.3 Overview of SGN Development

SGNs pass through a number of developmental stages, from their origin in the proneurosensory domain to their final differentiation and maturation within the cochlea. Following on the careful descriptive studies performed by embryologists and anatomists at the beginning of the 20th century, our understanding of how SGN development progresses has been greatly aided by the more recent identification of genes that are expressed in a subset of cells and that have been shown to affect specific features of inner ear neuronal development. The first step is the production of proneurosensory progenitors, which are recognized by expression of Lunatic fringe (Lfng) (Morsli et al., 1998; Cole et al., 2000), Sox2 (Kiernan et al., 2005; Neves et al., 2007), and fibroblast growth factor 10 (FGF10; Pirvola et al., 2000; Alsina et al., 2004). Subsequently, a subset of proneurosensory cells upregulate expression of Delta-like 1 (Dll1) and Neurogenin1 (Neurog1) (Adam et al., 1998; Ma et al., 1998; Alsina et al., 2004; Brooker et al., 2006). Neurog1-positive precursors begin to express *Neurod1* as they delaminate from the otic vesicle (Liu et al.,

2000; Kim et al., 2001; Bell et al., 2008) and quickly down-regulate *Neurog1* (Evsen et al., 2013).

Delaminated neuroblasts continue to divide within the nascent ganglion, exiting the cell cycle along the basal-apical axis, starting in the mid-base region around E9.5 in mouse, with only a few neurons still dividing in the apical turn at E13.5 and none at E14.5 (Ruben, 1967; Matei et al., 2005). This general progression fits with *Neurog1*-based birthdating studies (Koundakjian et al., 2007), though it is important to bear in mind that onset of *Neurog1* precedes cell-cycle exit, so different aspects of timing are measured by these two methods. Early neuroblasts express Islet-1 (Adam et al., 1998; Li et al., 2004), which is sustained beyond the transient expression of Neurod1 (Radde-Gallwitz et al., 2004; Deng et al., 2014). The delaminated neurons begin expressing first Pou4f1 and then, after becoming postmitotic, Pou4f2 (Deng et al., 2014). Maturation is also marked by production of βIII-tubulin (i.e., TuJ1) (Radde-Gallwitz et al., 2004; Bell et al., 2008), which is maintained as the neurons differentiate and coalesce in distinct vestibular and spiral ganglia.

As they mature, neurons extend processes back toward the otic epithelium (Carney & Silver, 1983). In mice, neurons mature first in the base, with neurites present along the path by E11.5 (Carney & Silver, 1983) and peripheral processes extending into the cochlea by E12.5 (Farinas et al., 2001; Koundakjian et al., 2007; Appler et al., 2013). In parallel, SGNs extend central axons out of the ear and toward the auditory brainstem to form the auditory division of the eighth cranial nerve. The axons reach the hindbrain by E11.5 and quickly bifurcate, extending an ascending process rostrally toward the developing anterior ventral cochlear nucleus and a descending process caudally toward the posterior ventral cochlear nucleus and dorsal cochlear nucleus (Lu et al., 2011). By E15.5, SGN peripheral processes have penetrated the cochlear duct along its entire extent, and the central processes are topographically organized within each division of the developing cochlear nuclei (Koundakjian et al., 2007). The arrival of peripheral and central axons at their targets coincides with a peak in cell death first in the vestibular system and then in the cochlea (Nishizaki et al., 1998; Farinas et al., 2001). A similar sequence of events occurs in chicks (Ard & Morest, 1984; Whitehead & Morest, 1985; Molea & Rubel, 2003).

In mammals, there are two clear subtypes of SGNs that can be distinguished based on morphology by late embryogenesis (Bruce et al., 1997; Koundakjian et al., 2007). Type I SGNs extend radial projections directly toward the inner hair cells, whereas a minority population of Type II SGNs instead grow toward the outer hair cells and spiral toward the base. Initially, all SGNs produce Peripherin, but this expression is eventually restricted to Type II SGNs at postnatal stages (Hafidi, 1998). SGNs are further classified based on their spontaneous firing rates (Liberman, 1980) and variation in firing properties and gene expression (reviewed in Davis & Liu, 2011), but to date there are no molecular markers for early Type II SGNs or any other well-defined SGN subtype.

Efforts to understand the molecular pathways that govern early SGN development have uncovered important roles for several extrinsic pathways that pattern the otic

vesicle; promote neurogenesis; and control the specification, proliferation, and survival of committed SGN precursors. Many familiar signaling pathways are involved, often exerting distinct effects at different stages. Of particular importance are Notch signaling, as well as pathways activated by fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Sonic hedgehog (Shh), insulin growth factor (IGF), and the neurotrophins. In Sect. 2.4, the cellular and molecular events that govern SGN development are described, including the specific impact of relevant signaling pathways at each stage. Excellent reviews outlining the details of these pathways and their broader contributions to inner ear development are available for additional information (Wright & Mansour, 2003a; Varela-Nieto et al., 2004; Yang et al., 2011; Groves & Fekete, 2012; Kiernan, 2013; Neves et al., 2013).

2.4 Patterning the Proneurosensory Domain

Inner ear neurogenesis begins with the formation of the PNSD in the anterior otic cup during the earliest stages of development. As such, development of the PNSD is essentially a matter of establishing the anterior–posterior axis of the otic cup. The appearance of *L-fng* and other PNSD markers in the anterior otocyst is accompanied by a gradual restriction in the expression of nonsensory markers, such as Lmx1a/1b (Abello et al., 2007; Nichols et al., 2008; Vazquez-Echeverria et al., 2008) and Tbx1 (Raft et al., 2004), to the posterolateral domain as early as the 10–12 somite stage in mice (Fig. 2.2). Genetic fate mapping studies have confirmed that *Tbx1*-positive cells are excluded from the neurogenic zone (Xu et al., 2007). However, similar genetic fate mapping data for *L-fng* and *FGF10* are not available, leaving open the question of the degree of fate restriction within the PNSD at this early stage.

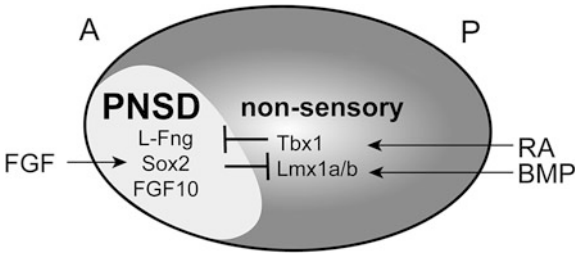


Fig. 2.2 Patterning the proneurosensory domain (PNSD). Extrinsic signals pattern the anterior–posterior (A-P) axis of the otic cup, with FGF inducing neurosensory development in the anterior and retinoic acid (RA) and BMPs inducing development of nonsensory structures in the posterior. As a result, PNSD markers such as L-fng, Sox2, and FGF10 are restricted to the anteroventral quadrant. These fate decisions are reinforced intrinsically by mutually antagonistic transcriptional networks, with Sox2 promoting the neurosensory fate and Tbx1 promoting the nonsensory fate

2.4.1 Patterning Signals from Outside of the Inner Ear

Initial efforts to understand how the PNSD is patterned showed that the anterior–posterior axis is fixed remarkably early. For instance, anterior pieces of the chick otic epithelium are able to generate neurons in vitro, even in the absence of any surrounding tissues (Adam et al., 1998). Similarly, when otic cups were transplanted with a reversed orientation in HH stage 16 chick embryos, the PNSD remained in its original position (Wu et al., 1998). However, a similar manipulation between HH stages 10–12 caused the *L-fng* domain to form in what was originally the posterior half of the otic cup (Bok et al., 2005). Thus, signals from the surrounding tissue initially influence where the PNSD will form, but the axis is fixed just after the 16 somite stage (HH stage 12). In contrast, the dorsal–ventral axis remains sensitive to changes in orientation for longer, with important cues provided by the notochord, floorplate, and dorsal neural tube (Wu et al., 1998; Bok et al., 2005). Changes to the dorsal–ventral axis can also affect the position of the neurogenic domain (Bok et al., 2005; Riccomagno et al., 2005), highlighting the complexity of the interactions that ultimately shape the PNSD. However, the timing of events suggests that signals along the anterior–posterior axis provide the primary cues for PNSD formation.

Embryological experiments have helped narrow down the possible source of the cues that pattern the otic cup. The hindbrain does not appear to provide essential anterior-posterior information, as rotations prior to the 16 somite stage did not affect the location of the PNSD (Bok et al., 2005). Instead, signals seem to come from the nearby periotic ectoderm and somatic mesoderm (Bok et al., 2011). A major component of this signal is retinoic acid (RA), which prevents neurogenesis when ectopically expressed in chicks or mice. Conversely, blockade of RA signaling causes expanded neurogenesis. Further, RA and other components of the pathway are present and actively maintained in tissues surrounding the early otic cup in chicks. A similar role for RA has been described in zebrafish (Radosevic et al., 2011), suggesting this is an evolutionarily conserved mechanism, though differences among species likely exist in terms of the source of RA, how the gradient is established, and how the signal is interpreted. Indeed, the complex expression patterns and wide range of teratogenic effects across species indicate that RA can affect multiple aspects of inner ear development (reviewed in Romand et al., 2006).

Additional critical patterning information appears to be provided by FGFs. In chicks, FGF8 is expressed close to the otic territory prior to the onset of neurogenesis and can promote neurogenesis when expressed ectopically (Abello et al., 2010). Conversely, broad pharmacological inhibition of FGF signaling caused a loss of PNSD markers, accompanied by an expansion of the nonsensory marker *Lmx1b*. Similar alterations in BMP signaling had no effect on neurogenesis, but did influence *Lmx1b* expression, confirming a role in anterior–posterior patterning. The expansion of a nonsensory marker in the absence of an effect on neurogenesis

indicates that FGF and BMPs may act independently to pattern the axis, with additional signals such as RA influencing the final outcome. For instance, as in chicks, an FGF ligand is required for anterior-posterior patterning in fish (Hammond & Whitfield, 2011), but some of these effects may be due to changes in expression of genes required for RA metabolism (Radosevic et al., 2011).

Whether FGFs regulate PNSD formation in mice remains unclear, due in part to differences in how and when neuronal development has been assessed. The PNSD appears to be present in *FGF3* mutants, though the CVG is noticeably smaller once it forms (Hatch et al., 2007; Vazquez-Echeverria et al., 2008). However, loss of both *FGF3* and *FGF10* can cause expanded neurogenesis (Vazquez-Echeverria et al., 2008). Similarly, *Neurod1*-positive cells form in ectopic locations in *Kreisler* mutant mice, which suffer from hindbrain patterning defects including reduced expression of *FGF3* and *FGF10*, and this phenotype can be partially rescued by restoration of either FGF. On the other hand, loss of the FGF2R (IIIb) receptor, which binds both FGF3 and FGF10, does not impair early CVG development (Pirvola et al., 2000; Pauley et al., 2003). However, because early PNSD development was not assessed, it is possible that a similar phenotype was missed in these animals (Pirvola et al., 2000). Altogether, the exact contribution of FGF3/FGF10 signaling is difficult to pinpoint due to the variability of reported double mutant phenotypes (Wright & Mansour, 2003b; Vazquez-Echeverria et al., 2008) and the fact that the phenotypes do not recapitulate what is seen in *FGF2R* mutants (Pirvola et al., 2000; Pauley et al., 2003). Why FGFs may antagonize neurogenesis in some contexts yet induce neurogenesis in others, as well as the specific contributions of various family members, will be important to work out in the future.

Although anterior–posterior signaling pathways function first, the final shape and size of the PNSD is also influenced by cues that pattern the dorsal–ventral axis. Shh from the notochord and floor plate provides a potent ventralizing signal that is necessary for normal expression of sensory and nonsensory markers (Brown & Epstein, 2011). However, loss of the PNSD from *Shh* mutant mice does not appear to be a direct effect on otic vesicle patterning, as otic-specific ablation of the Shh receptor Smoothened did not reproduce this effect. Instead, the change in PNSD gene expression appears to be secondary to expanded Wnt signaling from the neural tube, which is dorsalized in *Shh* mutants (Chiang et al., 1996; Riccomagno et al., 2005). In support of this idea, activation of Wnt signaling also inhibits neurogenesis (Ohyama et al., 2006; Brown & Epstein, 2011). However, importantly, Wnt signaling is not necessary for neurogenesis per se (Ohyama et al., 2006). Hedgehog proteins may play a more direct role during anterior–posterior patterning of the zebrafish otic vesicle, where they appear to work in parallel with—yet independently of—FGFs (Hammond et al., 2003, 2010; Hammond & Whitfield, 2011). The basis of these apparent differences among species is unclear but could reflect unique features of different types of vertebrate ears.

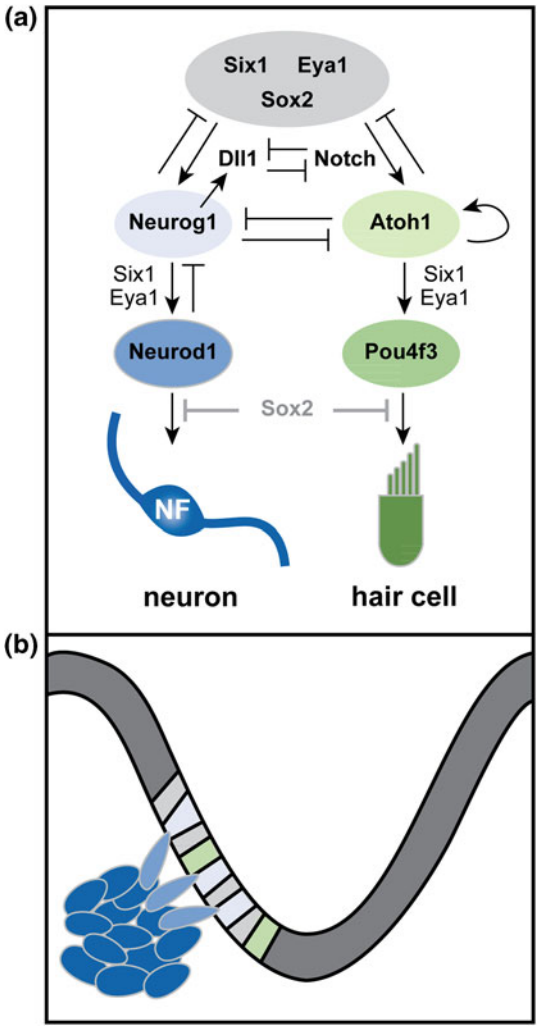
2.4.2 Intrinsic Patterning Mechanisms

Although signals extrinsic to the ear likely initiate the events that place the PNSD in the anterior otic cup, transcriptional networks and local cell–cell interactions play an important role in reinforcing these decisions and ensuring the neurosensory competence of cells within this domain. The transcriptional networks serve two complementary functions: to ensure expression of genes necessary for that cell's needs at that point in time and to control the activity of the network itself. The Cell–cell interactions coordinate these intrinsic events with the surrounding tissue.

One important player in the PNSD is the transcription factor Tbx1, whose expression is restricted to the posterolateral (i.e. nonsensory) region of the otocyst as early as the 10 somite stage in mice (Raft et al., 2004). *Neurog1* and *Neurod1*, which mark neural progenitors in the PNSD, are expressed in a complementary pattern to *Tbx1* in the otocyst (Fig. 2.2). In mice with excess *Tbx1*, neurogenesis is reduced, particularly in the posterior of the PNSD (Raft et al., 2004; Freyer et al., 2013). Conversely, in *Tbx1* mutant mice, *Neurog1* and *Neurod1* expression is increased (Raft et al., 2004; Xu et al., 2007), likely due to an expansion of the PNSD (Raft et al., 2004). Moreover, in *Tbx1* mutants, cells from the *Tbx1* domain populate the ganglion, something that never occurs in wild-type animals (Xu et al., 2007). Thus, Tbx1 actively represses the PNSD fate in nonsensory cells, thereby confining neurogenesis to a restricted domain. Extra *Neurog1*- and *Neurod1*-positive cells also develop in *Tbx1* mutant fish (Radosevic et al., 2011), and spiral ganglion defects have been reported in human patients with velocardiofacial/DiGeorge syndrome (Schmidt, 1985), which is linked to *TBX1* mutations (Yagi et al., 2003; Zweier et al., 2007). Hence, Tbx1 appears to play a basic, evolutionarily conserved role in determining where neurogenesis will occur.

If Tbx1 acts to prevent the neurosensory fate within the nonsensory region of the otocyst, then what factors promote this fate in the PNSD? The positively acting pathways are more complex, involving multiple transcriptional networks that interact with each other to drive production not only of neurons, but also of hair cells and supporting cells that will form the organ of Corti. One factor that appears to participate in many of these fate decisions is the SoxB1 family member Sox2. In mice, Sox2 is expressed in the ventral rim of the otic cup by E8.5 (Wood & Episkopou, 1999; Zou et al., 2008) and then in the ventrolateral otocyst at E9.5 (Mak et al., 2009). Subsequently, Sox2 can be detected both in delaminating neuroblasts and in the developing sensory epithelia (Kiernan et al., 2005; Mak et al., 2009). *Sox* genes show a similar expression in the PNSD in chicks, with expression complementing *Tbx1* and *Lmx1b* by the 10 somite stage (Abello et al., 2007, 2010).

Just as Tbx1 promotes the nonsensory fate, so does Sox2 drive cells down the neurosensory path. In mice harboring mutations in *Sox2*, neurosensory development is severely disrupted, with an early loss of both the prosensory domain (Kiernan et al., 2005) and of neurons (Puligilla et al., 2010). The loss of both cell types



suggests that Sox2 may be required for the initial specification of precursors in the PNSD. Moreover, in chicks, ectopic expression of Sox3 inhibits *Lmx1b*, hinting that mutually antagonistic interactions may solidify the nonsensory versus PNSD fate decision (Abello et al., 2010): wherever Tbx1 is on, Sox2 will be off and vice versa. Interestingly, *Tbx1* expression was not affected. Similarly, *Lmx1a* but not *Tbx1* shows reduced expression in *Kreisler* mutant mice, indicating there may be multiple parallel pathways that influence the nonsensory fate (Vazquez-Echeverria et al., 2008). Indeed, in *Lmx1a* mutant mice, *Sox2* expression expands, followed by the formation of fused sensory epithelia and a larger CVG (Nichols et al., 2008; Koo et al., 2009).

◀ **Fig. 2.3** Transcriptional control of neurogenesis. **a** Six1, Eya1, and Sox2 cooperate in PNSD progenitors (*gray*) to promote the neurosensory fate. Lateral inhibition mediated by Delta-like 1 (Dll1) and Notch steers neurosensory precursors toward either the neuronal (*blue*) or sensory (*green*) fate. In neural precursors, Neurog1 works with Six1 and Eya1 to promote differentiation, resulting in expression of Neurod1 and production of mature, neurofilament (NF)-positive neurons. Similarly, in prosensory progenitors (*green*), Atoh1 promotes its own expression and cooperates with Six1 and Eya1 to induce hair-cell differentiation pathways (i.e. Pou4f3) and production of hair cells. In some contexts, Neurog1 and Atoh1 show mutually antagonistic interactions. Downregulation of Sox2 occurs in differentiating neurons and hair cells; maintained expression of Sox2 interferes with maturation, suggesting that a decrease in Sox2 levels may be necessary for normal differentiation. **b** Diagram of neurogenesis in the context of the developing otocyst. Neuronal precursors are specified in the otic epithelium and then delaminate into the mesenchyme, where they continue to proliferate and then differentiate. Sensory precursors remain in the otic epithelium, where they ultimately produce both the hair cells and supporting cells of the organ of Corti (not shown)

As early acting factors such as Sox2 direct cells toward the neurosensory fate, additional transcriptional networks cooperate to determine whether individual progenitors will produce neurons, sensory cells, or both (Fig. 2.3). Unraveling the logic of this progressive fate restriction has been complicated by the fact that there seem to be multiple types of neurosensory progenitors within the PNSD. In favor of this idea, fate mapping in zebrafish has revealed three different types of precursors: neurosensory, neural, and sensory (Sapede et al., 2012). Similarly, in chicks, there is solid evidence that a common neurosensory progenitor produces both hair cells and neurons in the utricular maculae, but not in the other sensory organs, consistent with the presence of a heterogeneous precursor population (Satoh & Fekete, 2005).

The basis of the observed precursor heterogeneity remains unclear. One possibility is that hair cells and neurons derive from a similar early neurosensory progenitor that gradually shifts its potential over time, with the latest remnants of this population showing an extended ability to produce both hair cells and neurons in the maculae. Such a progenitor would be difficult to detect using standard fate mapping techniques. Alternatively, there may be a spatial segregation within the PNSD, with common progenitors limited to one subregion. Indeed, across species, proven bipotent progenitors exist in regions where the sensory domain, presaged, for example, by expression of *BMP4*, is found within the PNSD itself (Cole et al., 2000; Raft et al., 2007; Sapede et al., 2012).

Within the otocyst, local cell–cell interactions reinforce the intrinsic pathways that ultimately determine which cells will contribute to the neuronal lineage, influencing both the initial formation of the PNSD and the subsequent segregation of neuronal and sensory precursors. Both of these binary fate decisions appear to be under the control of the Notch pathway. As initially established in *Drosophila*, the Notch receptor interacts with a transmembrane ligand called Delta to mediate lateral inhibition and thereby promote the acquisition of distinct cell fates within a field of multipotent progenitors (reviewed in Schwanbeck et al., 2011). A second related ligand, Jagged, plays a similar role. In mammals, the basic pathway is conserved, with four Notch receptors, three Delta-like ligands, and two Jagged ligands. Ligand

binding induces cleavage of the intracellular domain (ICD) of the Notch receptor protein in the neighboring cell. The Notch-ICD then enters the nucleus to directly regulate expression of target genes. Among the target genes are additional transcription factors that feedback to increase expression of Notch itself while simultaneously decreasing Delta production in that cell. The overall consequence is that neighboring cells ultimately express either Delta or Notch and therefore adopt one of two possible fates, for instance, whether to become a neuron. Because of the lateral inhibition mechanism, early uniform expression of the ligand and receptor is often followed by a more salt-and-pepper–like appearance within that field of cells, reflecting the gradual emergence of two distinct cell fates. Thus, activation of the Notch receptor by a Delta-family ligand offers a direct way to convert an extrinsic signal into an intrinsic change in gene expression.

Understanding the precise effects of Notch signaling in otic neurogenesis has been challenging because of the presence of multiple ligands and receptors, as well as differences in the effects of these molecules throughout development (reviewed in Kiernan, 2013). However, several lines of evidence suggest that Notch signaling promotes neurogenesis during inner ear development. For instance, like Lfng (Morsli et al., 1998), which is a known regulator of the Notch pathway, the *Delta* homolog *Delta-like 1* (*Dll1*) is expressed in the PNSD at early stages (Abello et al., 2007; Daudet et al., 2007). In addition, pharmacological inhibition of Notch signaling in chicks can increase the number of Dll1+ cells (Daudet et al., 2007). Conversely, when *Dll1* is absent in mice, too many neurons develop, consistent with a loss of lateral inhibition between Dll1+ neurons and the surrounding cells (Brooker et al., 2006). Interestingly, the production of extra neurons in *Dll1* mutant mice apparently comes at the expense of the saccular and utricular maculae, whereas there is an increase in hair cell number in the cochlea, providing further evidence that there is a defined common neurosensory progenitor for only a subset of hair cells and neurons. Jagged ligands, on the other hand, may not be involved in inner ear neurogenesis (Zhang et al., 2000; Brooker et al., 2006; Neves et al., 2011).

One of the key consequences of Notch signaling is to induce expression of potent basic helix loop helix (bHLH) transcription factors, which act both to regulate Notch–Delta production and to induce cell-type–specific programs of gene expression. Similarly, whereas many genes are uniformly expressed in the PNSD, *Dll1* shows a more irregular pattern, apparently reflecting upregulation in early neuronal precursors (Adam et al., 1998; Abello et al., 2007; Daudet et al., 2007) which express the proneural bHLH transcription factors Neurog1 and Neurod1. In *Neurog1* mutant mice, no inner ear neurons form and *Dll1* expression is lost, consistent with the classic model of lateral inhibition, with Neurog1 acting both to enhance production of Dll1 and promote the neuronal fate (Ma et al., 1998; Raft et al., 2007). The Dll1- cells, on the other hand, likely adopt a prosensory fate, which is promoted by a different bHLH transcription factor, Atoh1 (formerly Math1), which is required for hair cell development in mice (Bermingham et al., 1999). When signaling downstream of Notch is prevented, *Neurog1* expression increases, as would be expected if Dll1 is no longer able to inhibit expression of *Neurog1* in neighboring cells (Raft et al., 2007). In addition, pharmacological inhibition of

Notch in chicks enhances the local upregulation of Dll1, indicating that lateral inhibition normally segregates Notch+ and Dll1+ populations within the PNSD (Daudet et al., 2007). Indeed, ectopic expression of the Notch-ICD is sufficient to create ectopic sensory regions and can divert neuroblasts to the hair cell fate in vivo (Daudet & Lewis, 2005; Hartman et al. 2010; Pan et al., 2010; Liu et al., 2012).

Because Neurog1 and Atoh1 are essential for neuronal and sensory development respectively, one attractive idea is that these two transcription factors participate in mutually antagonistic interactions within PNSD precursors that ultimately produce dedicated neuronal (i.e., Neurog1+) or sensory (i.e., Atoh1+) progenitors. If this model is true, then Neurog1 and Atoh1 must be coexpressed, at least at low levels, in any common neurosensory progenitor. This clearly appears to be the case in the utricle and saccule, where stripes of *Atoh1* expression appear within the neurogenic domain (Raft et al., 2007). Moreover, descendants of Neurog1+ cells, as marked by genetic fate mapping, do indeed populate the maculae, exactly as predicted both by gene expression studies and viral fate mapping (Morsli et al., 1998; Cole et al., 2000; Satoh & Fekete, 2005). Additional evidence has come from analysis of *Neurog1* and *Atoh1* mutant mice: loss of *Neurog1* is accompanied by an increased number of *Atoh1*-positive hair cell precursors in the developing utricular macula, whereas there are more neural precursors in the maculae of *Atoh1* mutant mice (Raft et al., 2007). Ultimately, however, fewer hair cells develop in *Neurog1* mutants, particularly in the saccule, indicating that the extra *Atoh1*-positive progenitors may not differentiate properly (Ma et al., 2000; Raft et al., 2007).

Together, these findings provide strong support for a common neurosensory progenitor in the maculae. Notably, the three major drivers of neuronal and hair cell fates in vertebrates—Neurog1, Neurod1, and Atoh1—are all bHLH factors that are closely related to each other and to bHLH factors with similar functions in sensory development in invertebrates. Indeed, it has been proposed that transient coexpression and cross-regulation of Neurog1, Neurod1, and Atoh1 may reflect an evolutionarily ancient series of interactions, with an expansion within the family eventually leading to a situation where individual bHLH factors segregate to neural versus sensory precursors (Pan et al., 2012).

The situation in the cochlea is less clear. First, *Atoh1* expression does not appear to overlap with the neurogenic region of the cochlea (Raft et al., 2007). Accordingly, genetic fate mapping revealed no contribution of *Atoh1*-positive progenitors to the CVG (Yang et al., 2010). Conversely, the *Neurog1* population does not produce any cochlear hair cells (Raft et al., 2007), despite massive labeling of the spiral ganglion neurons (Koundakjian et al., 2007; Raft et al., 2007). Nevertheless, there are some tantalizing phenotypes in the cochlea that suggest that Atoh1–Neurog1 interactions may occur, though perhaps only transiently. During normal development, neurons exit the cell cycle before hair cells (Matei et al., 2005). In contrast, in *Neurog1* mutants, hair cell precursors exit the cell cycle prematurely (Matei et al., 2005), and extra rows of hair cells develop in some regions (Ma et al., 2000). This phenotype can be explained in part by the absence of SGNs, which normally produce a Shh cue that prevents hair cells from exiting the cell cycle (Bok et al., 2013). However, intrinsic effects might also contribute: when Neurog1 is lost, a progenitor that

would normally become a neuron may instead select the alternate fate and differentiate as a hair cell (Matei et al., 2005).

According to this model, such a progenitor may in fact express extremely low levels of Atoh1, just not at high enough levels to drive Cre-mediated recombination for genetic fate mapping. In fact, Atoh1 positively regulates its own expression (Raft et al., 2007), so it is possible that the loss of Neurog1 relieves a block on this autoregulation, thereby allowing Atoh1 expression to accumulate and therefore divert a neural precursor to the sensory fate. This would explain the failure to observe any major contribution of Atoh1-derived cells to the ganglion in wild type mice: Neurog1 may be so efficient at promoting the neural fate that common progenitors per se do not exist in the wild-type cochlea, and any binary potential is revealed only once the Neurog1–Atoh1 feedback loop is disrupted.

Additional evidence that the Atoh1–Neurog1 feedback loop is evident only in restricted contexts has come from zebrafish. As in mice, neural (*neurod1*) and hair cell (*atoh1a*) markers are largely segregated, overlapping only in the posterior macula and not in the anterior (Sapede et al., 2012). Moreover, *Neurog1* mutant fish exhibit not only a loss of ganglion neurons, but also an increase in the number of hair cells, which differentiate prematurely and appear to derive from a *Neurog1*-positive progenitor. However, the phenotype is restricted to the site of overlapping expression, namely the posterior macula. Hence, as in mice, atoh1+/neurog1+ progenitors are restricted, in this case to the posterior maculae, with cells in the anterior maculae already committed to either the neural or sensory fate. Indeed, additional fate mapping studies in zebrafish suggest that precursors can become committed to the neuronal fate as early as the otic placode stage (Hans et al., 2013). Whether orthologous factors define restricted neural and sensory precursors in the early mouse otic vesicle remains to be determined.

2.5 Otic Neurogenesis

Within the PNSD population, neuronal precursors are ultimately specified through complex networks of transcription factors that involve both the pro-neurosensory factor Sox2 and the pro-neural factor Neurog1. Unravelling these interactions has been difficult, as many of the earliest acting factors have broad effects on inner ear morphogenesis in addition to their specific effects on auditory neurogenesis. For instance, loss of the transcription factor Six1 causes increased cell death and decreased cell proliferation throughout the otic vesicle by E9.5, which remains cystic with no CVG at E12.5 (Zheng et al., 2003). Hence, the failure in neurogenesis could in principle be secondary to gross patterning or growth defects. However, a series of studies indicate instead that Six1 and its partner Eya1 cooperate with Sox2 to control early neurogenesis directly.

2.5.1 *Six1 and Eya1*

Six1 and Eya1 cooperate in a network that reinforces the initial neurosensory versus nonsensory patterning of the otic vesicle and then plays an ongoing role in the maintenance of Neurog1. Six1 is a homeodomain protein that interacts with the Eya1 transactivator to control gene expression (Wong et al., 2013). Both Six1 and Eya1 are expressed in the ventral otic cup and are then maintained in the developing ganglion (Kalatzis et al., 1998; Zheng et al., 2003; Ahmed et al., 2012a). Six1 and Eya1 appear to act synergistically, with a range of phenotypes emerging in mice with differing degrees of Six1/Eya1 activity. When the network is completely blocked, PNSD development arrests early (Ahmed et al., 2012a). On the other hand, in single mutant mice, the PNSD appears to form (Zou et al., 2004). However, neurogenesis is clearly impaired from the earliest stages (Zou et al., 2004; Friedman et al., 2005). These results are consistent with the idea that Six1 and Eya1 act first during PNSD formation and subsequently during neurogenesis.

Analysis of an allelic series for *Eya1* revealed dose dependent effects that further confirm multiple roles for these genes (Zou et al., 2008). In *Eya1* null mutants, PNSD development is severely disrupted, with a loss of *L-fng* and expansion of *Tbx1* (Friedman et al., 2005; Zou et al., 2008). By contrast, in *Eya1* hypomorphs, *L-fng* expression is only reduced and *Tbx1* expression remains largely normal (Friedman et al., 2005). Nevertheless, *Neurog1* expression is still diminished, consistent with a role in neurogenesis that is independent of PNSD patterning. Although Six1 and Eya1 bind to each other and likely function within a common transcriptional complex (reviewed in Wong et al., 2013), it is important to remember that Eya1 is also a phosphatase and could therefore mediate some of its effects through signaling independent of Six1-mediated gene transcription.

2.5.2 *Sox2*

The effects of Six1 and Eya1 are influenced in part by the presence of Sox2. Indeed, in addition to its role in the early PNSD, Sox2 plays an ongoing role in neurosensory development, as reflected by its dynamic expression pattern and changing functions over time. At each stage, Sox2 is present in cells that exhibit some developmental plasticity. For instance, Sox2 is expressed initially throughout the PNSD, which has the potential to produce hair cells, supporting cells, or neurons (Wood & Episkopou, 1999; Mak et al., 2009). Subsequently, Sox2 continues to be produced in the prosensory domain (Dabdoub et al., 2008) and in developing neuroblasts (Puligilla et al., 2010). As the prosensory domain differentiates to form the organ of Corti, Sox2 is downregulated in hair cells, but maintained in supporting cells, which can behave like inner ear progenitors at neonatal stages (White et al., 2006). Sox2 is also expressed in Kölliker's organ (also called the greater epithelial ridge), a transient structure adjacent to the organ of Corti that eventually

becomes the inner sulcus. With the introduction of transcription factors such as Atoh1 (Woods et al., 2004) or Neurog1 (Puligilla et al., 2010), cells here can express markers for hair cells or neurons respectively, indicating that cells in Kölliker's organ are not yet locked into one defined fate.

Consistent with its expression in cells that have not yet committed to a final fate, Sox2 seems to inhibit differentiation. For instance, introduction of Sox2 can actually prevent cells from developing as hair cells, even if Atoh1 is present (Dabdoub et al., 2008). Atoh1, in turn, can inhibit *Sox2* expression. This leads to a model where Sox2 + precursors begin to produce Atoh1, which in turn increases its own expression and inhibits expression of both *Neurog1* and *Sox2*, thereby ushering the precursor from proliferation to differentiation. In support of this idea, hair cells differentiate prematurely in *Sox2* hypomorphs (Dabdoub et al., 2008). However, although Sox2 can antagonize hair cell differentiation when overexpressed, the presence of Sox2 is not fundamentally incompatible with hair cell differentiation, as Sox2 protein is in fact present in differentiating hair cells (Mak et al., 2009).

Sox2 may have similar effects on the behavior of neural precursors in the inner ear. Although the introduction of Sox2 was sufficient to cause cells in Kölliker's organ to express the neuronal marker βIII-tubulin (Puligilla et al., 2010), neither *Neurog1* nor *Neurod1* was induced, and the neurons failed to mature. In addition, only 39 % of electroporated cells took on a neuronal-like phenotype and this number decreased with developmental time. Sox2's potency is likely influenced by both Neurog1 and Neurod1 (Evsen et al., 2013). In fact, expression of *Sox2* is lower in delaminated neuroblasts, which instead express high levels of *Neurod1*. When Sox2 levels were forced to stay high by electroporation into the chick otic cup, *Neurog1* was induced, but the cells failed to progress to the next stage and neurogenesis failed. In contrast, electroporation of either *Neurog1* or *Neurod1* inhibited expression of *Sox2* and therefore increased the number of neurons. Together, these studies suggest that downregulation of Sox2 is necessary for neuronal differentiation, paralleling the situation for hair cell differentiation.

2.5.3 *Regulation of Transcriptional Activity in Neurosensory Progenitors*

Given the broad expression and function of transcription factors such as Six1, Eya1, and Sox2, how might such a network be poised to have specific effects on auditory neurogenesis? Like other transcriptional networks, the answer lies both in the presence of other transcription factors as well as the overall epigenetic status of the cell. For instance, the presence of the Notch-ICD appears to have important consequences for the outcome of Sox2 activity: when both are present, there is an expansion of sensory regions at the expense of neuroblasts (Hartman et al., 2010; Pan et al., 2010), and ectopic expression of Notch-ICD is sufficient to redirect neuroblasts to the hair cell fate in vivo (Pan et al., 2013). Similarly, Six1 and Eya1 can have dual effects on neuronal versus hair cell fate depending on the context

(Bricaud & Collazo, 2011; Ahmed et al., 2012b). Moreover, coexpression of Neurog1 and Neurod1 is only able to initiate neurogenesis, as the neurons that form fail to advance to a more mature state, as signaled by expression of neurofilament (NF). Hence, additional factors must influence how each of these regulators affects neuronal differentiation.

One important variable appears to be chromatin structure, which can affect which specific binding sites are available for active transcription (reviewed in Ronan et al., 2013). Although neither Eya1 nor Six1 is able to induce formation of ßIII-tubulin-positive neurons in the embryonic cochlea, co-electroporation of both induces production of Neurog1 and Neurod1, albeit with low efficiency, as most transfected cells instead adopt a hair cell fate (Ahmed et al., 2012a). However, when Six1 and Eya1 are introduced together with components of the SWI/SNF chromatin remodeling complex, approximately 85 % of the transfected neurons now express both Neurod1 and NF, with many fewer cells expressing hair cell markers. This effect requires Sox2, which apparently antagonizes the differentiation of Six1/Eya1+ cells into hair cells (Dabdoub et al., 2008; Ahmed et al., 2012a). Indeed, addition of Sox2 further augments the effectiveness of this treatment, such that 99 % of the transfected cells in Kölliker's organ now express NF. This might be influenced in part by the fact that cells in Kölliker's organ are derived from cells that once expressed *Neurog1* and may therefore already be biased toward the neuronal fate (Raft et al., 2007). The same transcriptional network (i.e., Six1, Eya1, SWI/SNF, and Sox2) can convert 3T3 fibroblasts into NF+ neurons with high efficiency, likely acting in part through synergistic effects on the activity of Neurog1 and Neurod1, which are induced in parallel (Ahmed et al., 2012a).

To add further complexity, the nature of the transcriptional complexes governing neurogenesis also evolves over time. For instance, in the developing chick inner ear, *Sox2* and *Neurog1* levels decrease in neuroblasts that have begun to express enhanced levels of *Neurod1* (Evsen et al., 2013). Moreover, Neurog1 and Neurod1 are able to promote neurogenesis when introduced into the chick otic cup, and these effects are accompanied by a loss of *Sox2*, likely through direct binding of these transcription factors to an enhancer in the *Sox2* locus. Presumably, the failure of Neurog1 and Neurod1 to induce mature neurons in cultured cochlear explants (Puligilla et al., 2010) reflects a difference in the availability of Sox2 in these two systems: Sox2 is naturally downregulated in the chick otic cup, but not in Kölliker's organ.

Taken together, a model emerges in which an Eya1/Six1/Sox2/SWI/SNF complex activates Neurog1 and Neurod1, which subsequently cooperate with a Sox2-negative complex that drives neuronal differentiation (Ahmed et al., 2012a; Wong et al., 2013). The importance of epigenetic regulation for SGN development is further underscored by analysis of CHD7, another chromatin remodeling enzyme that is responsible for CHARGE syndrome in humans, a disorder marked by hearing loss and many other developmental anomalies (Layman et al., 2010; Zentner et al., 2010). In mice, loss of *CHD7* impairs *Neurog1* expression and inner ear neurogenesis (Hurd et al., 2010). Hence, there are likely multiple pathways coordinating which regions of the genome are accessible to pro-neurogenic transcriptional complexes.

2.6 Delamination and Differentiation

Although the onset of *Neurog1* marks an important milestone in SGN development, this initial specification must be paired with tightly controlled delamination and differentiation to create a spiral ganglion with the correct number of cells in the proper location. Once specified, Neurog1+ neuroblasts pass through additional stages as they mature, with intrinsic factors endowing neuroblasts both with the ability to leave the otic epithelium and to respond appropriately to mitogenic and trophic cues in the environment.

2.6.1 Delamination

One of the earliest signs of maturation in Neurog1+ neuroblasts is expression of the closely related bHLH factor Neurod1 (Fig. 2.3). Neurod1 seems to take over from Neurog1 after the neuroblasts are specified, as hinted at by the upregulation of *Neurod1* and downregulation of *Neurog1* in post-delaminated neuroblasts (Evsen et al., 2013). Neurod1 is present at modest levels in the PNSD of the otic epithelium, but is more strongly expressed in the nascent ganglion (Liu et al., 2000; Kim et al., 2001). Consistent with this observation, Neurod1 is lost from *Neurog1* mutant mice (Ma et al., 1998), whereas Neurog1 expression is sustained and even increased in the absence of *Neurod1*, placing Neurod1 downstream in this hierarchy (Jahan et al., 2010a).

Neurod1 is clearly required for normal CVG development, likely affecting multiple stages of neuronal differentiation. In *Neurod1* mutant mice, the CVG is strongly reduced (Liu et al., 2000; Kim et al., 2001). This phenotype may have two origins. First, the neurons do not seem to delaminate normally. Many cells meant to produce Neurod1, as indicated by a β-galactosidase reporter, remain in the otic epithelium (Liu et al., 2000; Kim et al., 2001). Second, there is an increase in cell death, accompanied by reduced expression of two neurotrophin receptors: TrkB (Liu et al., 2000) and TrkC (Kim et al., 2001).

Although loss of cells is an early and drastic consequence, Neurod1 also appears to influence SGN differentiation beyond survival. For instance, activation of Neurod1 can have potent and long term effects for neuronal production in chicks (Evsen et al., 2013). In addition, analysis of *Neurod1* conditional knockouts has revealed changes in cochlear innervation (Jahan et al., 2010a). Whether these effects reflect intrinsic changes in SGN differentiation or instead are secondary to defects in the organ of Corti remains unclear as Neurod1 also affects the onset of hair cell differentiation (Jahan et al., 2010b).

Little is known about what controls the initial delamination of neurons out of the PNSD or the subsequent separation of the CVG into a distinct SG and VG. On delaminating, SGN neuroblasts come into contact with a new environment consisting of mesenchyme and neural crest cells, which will produce the glia of the

inner ear (D'Amico-Martel & Noden, 1983; Freyer et al., 2011; Sandell et al., 2014). The cells in this region are histologically distinct because they form a funnel-shaped structure, through which the differentiating SGNs appear to extend their earliest processes (Carney & Silver, 1983). Indeed, the earliest CVG neuroblasts appear to be enveloped by a "sleeve" of neural crest cells emanating from the fourth rhombomere of the hindbrain (Sandell et al., 2014). Similarly, neural crest cells form passages for migration of other placodally derived neurons (Freter et al., 2013). Ablation of neural crest in this region impairs extension of axons from differentiating CVG neurons (Sandell et al., 2014). In addition, when the cochlea is depleted of Schwann cells genetically, SGNs coalesce too close to the modiolus and can even migrate outside of the otic capsule (Morris et al., 2006; Mao et al., 2014).

The nature of the molecules that mediate the cell-cell interactions that guide SGN movements is not well understood. As in mice lacking Schwann cells, SGNs are displaced toward the modiolus in neurons lacking the transcription factor Gata3. This may mean that Gata3 determines how SGNs respond to glial-derived signals, though other interpretations are also possible (Appler et al., 2013). FGF2 has been put forward as one possible migratory signal, due to its ability to affect the migration of neuroblasts in vitro (Hossain et al., 2002). However, clear delamination defects have not been reported in any FGF mutant mice, though the CVG is often affected in poorly understood ways (Wright & Mansour, 2003a). One intriguing possibility is that canonical axon guidance molecules play a role. For instance, SGN cell bodies are somewhat mispositioned in the absence of Slit/Robo signaling (Wang et al., 2013). However, this phenotype arises after E13, suggesting that other signals direct the initial positioning of the SGN with Slit/Robo acting instead to keep SGNs in the proper location.

2.6.2 *Control of Proliferation and Differentiation*

After delaminating from the PNSD, Neurod1+ neuroblasts continue to proliferate before exiting the cell cycle and differentiating. FGF signals may influence how much proliferation occurs, though exactly how this works remains unclear because of the pleiotropic and redundant effects of various family members in vivo (Wright & Mansour, 2003a). This issue has been partly overcome by manipulating the FGF pathway acutely in cultured chick inner ear neurons. These studies revealed that in addition to its earlier role in neuronal specification, FGF also inhibits cell proliferation and drives neuroblasts to differentiate (Alsina et al., 2004). Conversely, pharmacological inhibition of FGF signaling has the opposite effect. A slightly different role has been proposed in zebrafish, in which exposure to high levels of FGF5 prevents slowly dividing neuroblasts in the nascent ganglion from differentiating (Vemaraju et al., 2012). These studies also uncovered a dose dependence that may influence the ultimate outcome of each FGF signal.

How FGFs affect the delamination and expansion of neuroblasts in mouse is not known. In mice lacking the shared FGF3/FGF10 receptor FGFR2, CVG neurons

seem to delaminate as they should, but the ganglion is obviously abnormal by E11 and exhibits excess cell death by E13 (Pirvola et al., 2000), with few neurons remaining by E18.5 (Pauley et al., 2003). Similarly, the CVG is smaller in *FGF3* mutants (Hatch et al., 2007), though the fact that FGF3 is restricted to the vestibular neurogenic zone (Koo et al., 2009) suggests this could reflect a selective loss of vestibular neurons. No obvious change in the CVG was noted in *FGF10* mutants (Pauley et al., 2003), and the phenotypes in *FGF3/10* double mutants are complicated to interpret because of the possible actions of each ligand at multiple stages of CVG development, as well as phenotypic variability (Wright & Mansour, 2003b; Vazquez-Echeverria et al., 2008).

As in other regions of the nervous system, the final number of neurons in the SG reflects a balance of proliferation and apoptosis. In chicks, IGF activation of the PI3 K/Akt pathway may contribute to the expansion of neuroblasts before they start to differentiate, influencing both proliferation and survival (Camarero et al., 2003; Varela-Nieto et al., 2004; Aburto et al., 2012). In contrast, IGF1 does not appear to be required for early SGN development in mice (Camarero et al., 2001). However, otic neuroblasts do show evidence of Akt pathway activation in mice, and there is a severe loss of Neurod1+ cells by E10.5 and increased cell death by E11.5 in mice with abnormal Akt signaling (Kim et al., 2013). Thus, early neuroblasts require signaling through the Akt pathway for their survival.

Apoptosis also affects subsequent stages of SGN development, after the neurons have exited the cell cycle and differentiated. Classic in vitro assays originally showed that SGN survival depends on target-derived cues present both in the sensory epithelia and in the hindbrain (Zhou & Van De Water, 1987). In vivo, apoptosis peaks in the SGN between E15.5 and E16.5 in mouse (Nishizaki et al., 1998), which coincides with the arrival of peripheral neurites in the organ of Corti (Farinas et al., 2001). Consistent with this observation, the total number of neurons in the spiral ganglion decreases from E15.5 to E17.5 (Farinas et al., 2001). A similar wave of cell death occurs after connections have been made in the chick basilar papilla (Ard & Morest, 1984). This naturally occurring period of cell death likely reflects a culling of SGNs that were unable to establish proper connections in the sensory epithelia.

There is abundant evidence that the brain-derived neurotrophic factor (BDNF) and neurotrophin (NT-3) neurotrophins are crucial for the survival of SGNs that have reached their target (reviewed in Yang et al., 2011). Both BDNF and NT-3 are produced in the developing sensory epithelia of the inner ear, whereas their respective receptors TrkB and TrkC are present in the developing neurons (Farinas et al., 2001). In the cochlea, NT-3 and BDNF are produced at slightly different times and in opposing apical-basal gradients. In addition, whereas NT-3 is broadly present in supporting cells, including those in Kölliker's organ, BDNF seems to be more restricted to hair cells. Consistent with these differing expression patterns, SGNs are strongly dependent on NT-3, particularly in the base where there is no BDNF during the initial stages of neurite outgrowth (Fritzsch et al., 1998; Farinas et al., 2001). This is exactly what would be predicted if NT-3 normally keeps SGNs alive as they extend processes into the cochlear duct. Although NT-3 appears to be the dominant player, BDNF is also involved, as inner ear neurons are completely

lost in *BDNF/NT-3* double mutants (Ernfors et al., 1995) and deletion of *TrkB* enhances the *TrkC* phenotype (Fritzsch et al., 1998). Importantly, in addition to these classic roles, BDNF and NT-3 also influence many other features of SGN differentiation, including axon guidance, synaptogenesis, and maturation of firing properties (reviewed in Yang et al., 2011 and Green et al., 2012. See also Chaps. 3 by Fritzsch et al., 4 by Davis and Crozier, and 7 by Green et al.).

2.7 SGN Specification

The astonishing diversity of neurons in the central nervous system has long captured the attention of developmental neurobiologists. Efforts to understand how different neuronal subtypes are generated have established a model where early acting transcription factors induce more generic fates that are progressively restricted over time, as shown both for spinal cord motor neurons and in the cortex (Leone et al., 2008; Philippidou & Dasen, 2013). Although the SG might at first glance appear to be comparatively homogeneous, similar subtype specification must still occur. First and foremost is the specification of the auditory and vestibular fates. In addition, within the auditory population in mammals, SGNs are further divided into Type I and Type II neurons. Type I SGNs, which comprise approximately 95 % of the population, innervate inner hair cells, whereas the minority Type II SGNs instead innervate outer hair cells (Spoendlin, 1972; Perkins & Morest, 1975). Type I SGNs can be further classified based on their firing properties, varying both along the tonotopic axis (Davis & Liu, 2011) and with regard to threshold sensitivity (Taberner & Liberman, 2005). Unfortunately, our knowledge of how these different types of SGNs arise during development remains rudimentary (Fig. 2.4).

2.7.1 Auditory Versus Vestibular

The decision whether to populate the auditory or vestibular division of the inner ear appears to be made quite early in development, with neuroblasts assigned to an auditory or vestibular fate prior to delamination. Histologically, the auditory neurons have more densely packed nuclei than the vestibular neurons, which develop more laterally (Sher, 1972) and are noticeably larger (Ard & Morest, 1984). As early as E12.5 in mouse, the two ganglia are already clearly distinct, though still attached (Carney & Silver, 1983). More recent studies further suggested that auditory and vestibular ganglion neurons can be distinguished even earlier, with vestibular ganglion neurons extending peripheral neurites along the future paths of

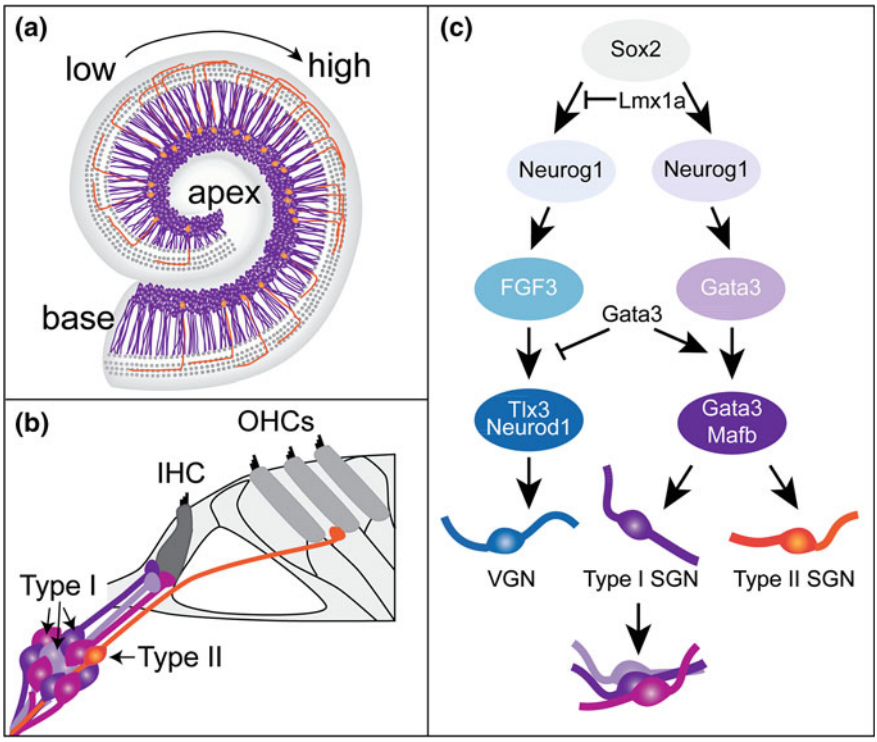


Fig. 2.4 Specification of SGNs. **a**, **b** SGNs are organized tonotopically from the apex to the base of the cochlea and show different firing properties depending on their location. Two basic types of SGNs can be recognized morphologically, with Type I SGNs (*purple*) projecting to inner hair cells (IHC) and Type II SGNs (*orange*) projecting to outer hair cells (OHCs). At any one point along the tonotopic axis, SGNs show additional heterogeneity, as evidenced by differences in spontaneous firing rates and gene expression. **c** Precursors for spiral (*purple*) and vestibular (*blue*) ganglion neurons are specified early in development. Lmx1a sets a medial–lateral boundary in the PNSD. Subsequently, early SGN progenitors maintain expression of Gata3, which both promotes auditory-specific programs of development and inhibits the vestibular fate. Mafb acts downstream of Gata3 to promote terminal differentiation of SGNs. The mechanisms that further diversify the SGN population remain unknown

the inferior and superior vestibular nerves at E10.5, followed 1 day later by the emergence of a fan of cochlear processes into the growing cochlear duct (Sandell et al., 2014).

Neurons in the murine vestibular ganglion also exit the cell cycle (Ruben, 1967) and express *Neurog1* (Koundakjian et al., 2007) earlier than those in the spiral ganglion. Similarly, in chicks, vestibular ganglion neurons are produced earlier (Bell et al., 2008). This temporal patterning is mirrored by a spatial patterning of the PNSD, with expression of the transcription factor Gata3 restricted to the medial PNSD (Lawoko-Kerali et al., 2004) and FGF3 marking the lateral division (Koo et al., 2009). Similarly, the earliest delaminated neuroblasts adjacent to these

domains express either auditory (i.e. Gata3) (Karis et al., 2001) or vestibular (i.e., Tlx3) markers (Lu et al., 2011).

Additional evidence for a spatial segregation of precursors within the PNSD has come from fate mapping studies in chicks, where neurons destined to innervate auditory or vestibular epithelia arise from physically distinct populations of precursors (Bell et al., 2008). Intriguingly, the same pattern was observed for the corresponding sensory epithelia, leading to a model where the PNSD is patterned along the dorsal-ventral axis, with neurogenesis preceding sensory cell production within defined regions for each sensory organ. In support of this idea, virus-based fate mapping in chicks has revealed that auditory and vestibular neurons rarely develop from a common neural precursor (Satoh & Fekete, 2005).

Which extrinsic cues may establish this pattern in the PNSD is not known. SGNs can still develop in the absence of Shh activity (Brown & Epstein, 2011). However, ectopic Wnt signaling can impose a "dorsal" identity and lead to the development of vestibular-like hair cells within the chicken cochlea (Stevens et al., 2003). As the lagena macula is a vestibular sensory epithelium that develops close to the basilar papilla, it is also possible that Wnt signaling acts more locally to affect the auditory-vestibular fate decision.

Although nothing is known about the extrinsic cues that might control the auditory versus vestibular neurogenic fate, the intrinsic factor Lmx1a may be involved in setting and reinforcing the boundaries that have been revealed by fate mapping. In the early otic vesicle, *Lmx1a* is present everywhere except for a small wedge where the neurosensory precursors are located (Nichols et al., 2008). However, *Lmx1a* is not fully excluded from the PNSD, but is in fact expressed within the auditory division (Koo et al., 2009). Here, *Lmx1a* appears to set the boundary between auditory and vestibular regions of the PNSD, as evidenced by ectopic production of vestibular neurons within the auditory domain in *Lmx1a* mutant mice. Interestingly, vestibular-like hair cells also appear in the cochlea of *Lmx1a* mutant mice, as predicted by the boundaries established by the fate mapping studies in chick (Bell et al., 2008). However, it remains unclear whether this phenotype reflects a change in the identity of a common "auditory" neurosensory progenitor or whether "vestibular" neurosensory progenitors are aberrantly migrating into the auditory zone.

The eventual differentiation of neurons with either auditory or vestibular-appropriate properties is controlled by intrinsic factors acting downstream of Neurod1. In fact, there are hints that Neurod1 may participate in this early segregation. After its initial role in generic neurogenesis, Neurod1 is selectively maintained in vestibular but not in more mature auditory neurons (Lawoko-Kerali et al., 2004; Jones & Warchol, 2009). Projections from auditory and vestibular ganglion neurons intermingle inappropriately in ear-specific *Neurod1* knockout mice, underscoring a possible role in subtype-specific properties of differentiation (Jahan et al., 2010a).

Another key player is Gata3, which is enriched in the medial PNSD where SGNs develop (Karis et al., 2001; Lawoko-Kerali et al., 2002, 2004). Moreover, Gata3 is maintained at high levels in SGNs throughout embryonic development and after

birth (Karis et al., 2001; Lawoko-Kerali et al., 2002, 2004; Appler et al., 2013). Although clearly auditory-enriched, *Gata3* is also transcribed transiently in the vestibular ganglion (VG), with a smattering of Gata3-positive vestibular ganglion neurons (VGNs) present by E11 (Lu et al., 2011). This observation suggests that Gata3 may not be the sole mediator of SGN identity. In support of this idea, in chicks, Gata3 is not expressed in dividing or migrating neuroblasts and is only upregulated in auditory neurons as they differentiate (Jones & Warchol, 2009). Together, these results indicate that Gata3 may play an important role beyond SGN specification.

As predicted by its auditory-enriched expression even within the PNSD, SGNs are lost from *Gata3* null embryos by E15, leaving only an apparent VG (Karis et al., 2001; Duncan et al., 2011). Similarly, conditional deletion of *Gata3* from the early otic vesicle prevents SGN development, while VGNs develop with no gross abnormalities (Duncan & Fritzsch, 2013). Thus, Gata3 clearly plays a primary role in SGNs, though more minor defects in a subpopulation of VGNs cannot be ruled out. Notably, the neurons that remain in *Gata3* null mutants still try to produce Gata3, as indicated by a lacZ reporter (Karis et al., 2001). This further suggests that Gata3's main function may be to drive execution of the auditory fate, but that other intrinsic factors are involved in the initial specification.

Analysis of other mouse mutants revealed an ongoing need for Gata3 after specification. When Gata3 is removed slightly later in development (Duncan & Fritzsch, 2013), the initial production of neurons appears normal, but the SGNs subsequently undergo cell death beginning at E12.5, apparently independent of any change in neurotrophin availability or responsiveness (Luo et al., 2013). In addition, the few remaining SGNs make highly abnormal connections both peripherally and centrally (Duncan & Fritzsch, 2013; Luo et al., 2013), indicating that Gata3 affects not only SGN specification and survival, but also differentiation.

Consistent with this interpretation, when *Gata3* is deleted after the neuroblasts have delaminated, SGNs differentiate prematurely (Appler et al., 2013). In these mice, Gata3 protein is preserved in the developing cochlea, and the organ of Corti shows no major defects, confirming that the changes in cochlear wiring reflect a direct role for Gata3 in SGNs. Although many features of the auditory identity are maintained, the mutant SGNs aberrantly express several vestibular markers and fail to transcribe some key auditory markers, including the transcription factor Mafb, which begins to be expressed in the base of the SG at E14.5 and is then maintained in postmitotic neurons (Yu et al., 2013). Mafb, in turn, acts downstream of Gata3 to direct later features of SGN differentiation. Taken together with the fact that many Gata3 target genes are not SGN-specific but are in fact expressed in both spiral and vestibular neurons in the wild-type scenario (Appler et al., 2013), these studies suggest that Gata3 guides SGNs through a prolonged period of differentiation by coordinating activation of generic and auditory-specific neuronal differentiation programs.

2.7.2 SGN Diversification

Even less is known about the further diversification of SGNs once they have been directed down the auditory path. SGNs are typically divided into two basic classes based on their morphology and pattern of innervation (Spoendlin, 1972). The vast majority are Type I SGNs, which are myelinated and extend unbranched radial processes to contact the inner hair cells. The remaining Type II SGNs, which comprise 5 % of the population, are unmyelinated, have smaller cell bodies, and extend thin processes that spiral along the cochlea to innervate multiple outer hair cells. A much stronger acoustic signal is required to evoke responses from Type II SGNs than from Type I SGNs, although both receive glutamatergic input (Weisz et al., 2009). These two populations are best recognized by their innervation patterns and by enriched expression of Peripherin in postnatal Type II SGNs (Hafidi, 1998). Although there is also heterogeneity among the auditory neurons in chickens (Whitehead & Morest, 1981; Rebillard & Pujol, 1983), Type I and Type II neurons analogous to those in humans have not been described, so only mammals are discussed here.

In the absence of early markers, it has been difficult to pinpoint exactly when Type II SGNs first appear in development. Electron microscopic studies confirmed the presence of Type II SGNs at birth in cats and mice, but not earlier (Romand & Romand, 1990). Similarly, the final pattern of Type I versus Type II innervation is not set until postnatal stages, with apparent rearrangement of neurites (Echteler, 1992; Huang et al., 2007), synaptic pruning (Huang et al., 2012), and differential cell death (Echteler et al., 2005; Barclay et al., 2011). With no reliable independent markers, however, it remains unclear which SGN populations are affected by each of these events. Nevertheless, Type II SGNs can be recognized during embryonic stages, as evidenced by the presence of a large growth cone that turns toward the base and grows among the region where the outer hair cells will eventually differentiate (Bruce et al., 1997; Koundakjian et al., 2007). In addition, Type I SGNs appear to be intrinsically programmed to avoid the outer hair cell region during synaptogenesis, as only Type I SGNs express EphA4 and signaling through this receptor is necessary to confine Type I neurites to the inner hair cell region (Defourny et al., 2013). Identifying the intrinsic programs that activate and maintain these differences is an important challenge for the future.

The Type I SGN population is itself further diversified at a functional level, though the extent and nature of this heterogeneity remain poorly defined. For instance, Type I SGNs exhibit different firing properties depending on their location along the tonotopic axis of the cochlea (see Chap. 4 by Davis and Crozier). These differences correlate with changes in protein expression, indicating that at least some of this variation may be genetically programmed (Flores-Otero et al., 2007). SGNs also express varying levels of calretinin and calbindin, but these differences have not yet been correlated with any clear electrophysiological differences (Liu & Davis, 2014).

Aside from these molecular differences, Type I SGNs also vary in their baseline firing profiles and respond to sound stimulation at different thresholds (Taberner & Liberman, 2005). SGNs with low spontaneous firing rates (low-SR) have higher dynamic range and thus contribute to hearing at higher sound intensities compared to those with high spontaneous firing rates (high-SR). These physiological differences come with morphological differences, as well (Liberman, 1980, 1982; Kawase & Liberman, 1992; Taberner & Liberman, 2005). Low-SR fibers preferentially innervate the modiolar side of the inner hair cell and develop smaller postsynaptic densities than the high-SR fibers, which instead cluster on the pillar side of the hair cell (Liberman et al., 2011). However, to date, there are no markers for either population, leaving open the question of when or how these differences are established.

2.8 Summary

The past decade has witnessed a remarkable flurry of discoveries into mechanisms of SGN development, made possible largely by the availability of improved molecular tools that overcome the technical hurdles for studying the cochlea. These studies have uncovered multiple extrinsic signaling pathways that determine when, where, and how many SGNs will develop. In parallel, complex transcriptional networks endow developing neurons with their unique properties, while simultaneously inhibiting alternative fates. This involves activation of cell-type–specific programs, as well as feedback loops within the pathway that turn off "early" genes and drive progenitors toward a progressively more differentiated state. Many transcription factors act at multiple stages and in multiple progenitor populations, with specificity achieved through the action of a few potent regulators.

Looking forward, this body of knowledge provides an important foundation for the next generation of scientific inquiry. Now that we have begun to gain traction on the question of how auditory fate is imposed on SGNs, it will be important to identify the pathways that further diversify this population, starting with the identification of reliable cell-type–specific markers. In addition, we still have very little grasp on the extrinsic pathways that divide the auditory and vestibular populations, let alone the cues that may promote any additional heterogeneity within the SGN population.

A deeper understanding both of the intrinsic and extrinsic pathways that act at each step of SGN development is crucial for current efforts to design effective stem cell-based therapies for deafness. For instance, with improved knowledge of the relevant signaling pathways that act at each step, scientists will be able to design protocols to steer naïve stem cells toward a specific fate, similar to approaches that have proven so successful for motor neurons (reviewed in Davis-Dusenbery et al., 2014). It may also be possible to reawaken developmental potential within cells in the mature cochlea by introducing cocktails of transcription factors such as Eya1/Six1 and Gata3, together with drugs that influence the chromatin state in these cells (see Chap. 9 by Nayagam and Edge).

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