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The Molecular and Developmental Basis of the Evolution of the Vertebrate Auditory System

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We review the molecular basis of the auditory system development and evolution. The auditory periphery evolved by building on the capacity of vestibular hair cells to respond to higher frequency mechanical stimulation. Evolution altered accessory structures to transform vestibular to auditory receptors. Auditory neurons are derived from vestibular neurons, possibly through the expression of the zinc finger protein GATA3. The bHLH gene *Neurogenin1* is expressed in the area of the developing vestibular nuclei whereas the bHLH gene *Atoh1* is expressed in the developing auditory nuclei. *Atoh1* null mice show an almost complete loss of cochlear nuclei. Overall, the ear, sensory neurons and brainstem auditory nuclei show molecular conservation embedded in an organ-specific molecular context. This results in the modification of the developmental pathways governed by these conserved molecules. These data are consistent with the emerging insight that morphological evolution is primarily driven by the modification of gene expression regulation.

The word "homology" is classically used to characterize evolutionarily related organs, regardless of variation in form and function exhibited in different species. During evolution, novel functions can be acquired by morphologically transforming ancestral structures or by developing new structures, seemingly from scratch through novel molecular developmental programs. A clear definition of what new organs are will ultimately dependent on our ability to dissect, at the molecular level, the developmental pathways that give rise to these structures in order to uncover whether preexisting molecular modules were combined or whether those molecular modules evolved de novo to generate a seemingly new structure. Given the modular nature of most developmental programs and the dependency of transcription factor signaling on the context within which they are expressed, separating those two basic issues of evolutionary biology will remain contentious for the near future. This is nowhere more obvious than in the formation of major germinal layers during embryonic development—ectoderm, endoderm, and mesoderm—for which recent molecular evidence suggests that cellular specification akin to mesoderm formation occurs in coelenterates, species that have traditionally been claimed to lack formation of mesoderm (Spring et al., 2002). Thus, molecular and cellular specification events can predate the morphological appearance of novelties, making it difficult to establish exactly when a novelty first appears in evolution. Ultimately, then, the distinction between novelties in a true sense as opposed to apparent novelties that rely on transformed ancestral genes and developmental

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modules, will depend on the extent to which new or conserved molecular features are expressed in these developmental programs.

Mechanosensory cells have recently become a prime example for a deep, molecular based homology, a term used to identify homologous molecular developmental pathways serving equivalent developmental functions in both protostomes and deuterostomes (Shubin, Tabin, & Carroll, 1997). Similar to the vertebrate and insect limbs for which this term was originally coined, the formation of mechanosensory cells critically depends on developmental factors (such as the bHLH gene *atonal/Atoh1)* that are highly conserved across phyla (Barald & Kelley, 2004; Ben-Arie et al., 1996; Montcouquiol & Kelley, 2003; Wang et al., 2002) and that can even be found in neurosensory cells of coelenterates such as jelly fish (Seipel, Yanze, & Schmid, 2004). Based on these findings, it appears likely that mechanosensory cells that use these closely related bHLH genes for their development in the context of other, equally conserved genes are homologous regardless of how different they may appear at the morphological level (Fritzsch & Beisel, 2004; Fritzsch et al., 2005). This molecular conservation and cellular homology does not, however, extend to the level of macroscopic mechanosensory organs such as insect and vertebrate auditory organs, simply because pathways can undergo inheritance with modifications. Despite these morphological differences in sound detection organs, it is likely that these molecularly and developmentally conserved cells had the potential to be used for sound detection, and that organs specialized for sound detection, in both insects and vertebrates, may represent a case of parallel morphological evolution whereby mechanosensory transducer cells are brought into a position that allows them to extract sound related mechanical energy. Consistent with this idea is the fact that all sound pressure receiving auditory organs, in vertebrates and invertebrates alike, appear to come about through the evolutionary transformation of already existing mechanosensory structures into these more specialized organs (Caldwell & Eberl, 2002; Fritzsch, 1999; Fritzsch & Beisel, 2004; Yager, 1999). Transformations that gave rise to a vertebrate auditory system require minimally the formation of an appropriate endorgan, the assignment of primary sensory cells and the formation of dedicated processing nuclei in the central nervous system (Figure 1).

In this overview we will address all three issues, with a special emphasis on the molecular developmental processes underlying their evolution. Specifically, we will provide an overview of new developmental data on various mechanosensory systems within the theoretical framework of their evolution. In particular, we will provide evidence supporting the following hypotheses:

(1) That auditory hair cells are derived, both developmentally and evolutionarily, from vestibular hair cells
(2) That primary auditory neurons (spiral ganglia) are derived both developmentally and evolutionarily, from primary vestibular neurons
(3) That hindbrain auditory nuclei are either totally new or are derived (both developmentally and evolutionarily) from hindbrain electrosensory nuclei.

In principle, all three aspects of auditory system evolution require, in analogy to the well known multiplication and diversification of genes, the multiplication of existing cell population(s), the diversification of these populations and, finally, the specialization of these populations to function appropriately in their new

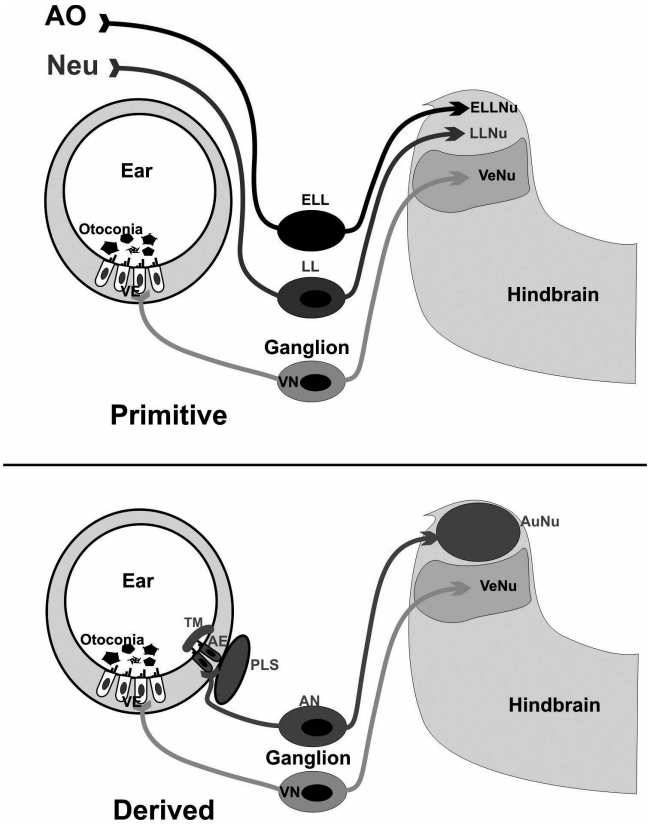

Figure 1. This scheme shows the transformation of the ear, sensory neurons and brainstem from a nonauditory, primitive condition, into the derived condition, enabling a tetrapod vertebrate to hear. The primitive ear has vestibular sensory epithelia (VE) that are connected with vestibular sensory neurons (VN) to the vestibular nuclei of the brainstem (VeNu). Additional sensory systems in primary aquatic vertebrates are the ellectroreceptive ampullary organs (AO) and the mechanosensory neuromasts of the lateral line (NEU). These organs are connected via specific sets of sensory neurons (ELL, LL) to specific brainstem nuclei (ELLNu, LLNu). Derived landvertebrates have lost these senses and have a sound pressure receiving sense, called hearing. This sense is characterized by the auditory epithelium (AE) that sits at or near a sound conducting perilymphatic system (PLS) and is covered with a tectorial membrane (TM). Auditory neurons (AN) conduct the information from the auditory epithelium to the auditory nuclei (AuNu) of the brainstem. This basic organization may have evolved in the aquatic ancestor of landvertebrates, but was modified in amphibians through the addition of the amphibian papilla and in amniotes through the formation of the cochlea.

context. The alternative scenario of a de novo formation of the mechanosensory cellular populations dedicated to sound reception in the ear is unlikely, as all auditory organs of vertebrates clearly evolve from existing vestibular organs (Fritzsch, 1999; Manley, Popper, & Fay, 2004). Likewise, it is reasonable to assume that vertebrate auditory sensory neurons are evolutionary transformations of ancestral vestibular neurons that acquired novel functions through molecular alterations of their development, since both neuronal populations originate from the ear. In contrast to the auditory periphery, the evolution of the central auditory nuclei seems to follow a different pathway. We will present data showing that the formation of auditory nuclei of mammals depends critically on the very same gene that allows identification of mechanosensory cells across phyla, *Atoh1*. This gene is not only essential to establish the homology of mechanosensory cell development across phyla but, in

vertebrates, is also involved in the formation of dorsal cell populations in the spinal cord and hindbrain which, together, form highly derived and novel parts of the hindbrain such as the cerebellum and auditory nuclei. While this may seem to be confusing at first glance, it is important to realize that genes and their effects depend on the context in which they are expressed (Wang et al., 2002). Consequently it is insufficient to establish homology across evolution considering only single gene expression without taking their molecular context into account, Some of the genes that establish the context for ear development, such as *Eya1*, *Six1, Pou* and *Pax* genes (Zou, Silvius, Fritzsch, & Xu, 2004), are not expressed in other areas of *Atoh1* expression such as the intestine, but are also essential for eye development across phyla, indicating a more basal homology of sensory organs across phyla (Fritzsch & Beisel, 2004; Kozmik et al., 2003).

Auditory Hair Cells are Derived, both Developmentally and Evolutionarily, from Vestibular Hair Cells

For hearing, an endorgan must be generated within the ear that is not required for other mechanical reception such as vestibular sensation. That is to say, this endorgan has to be redundant to vestibular function and can therefore be modified to extract sound related energy from the stimuli impinging on an animal without interfering with other mechanosensory functions of the ear. Making the sensory epithelium of an auditory organ requires therefore the differential regulation of proliferation and specification of hair cell precursors. Assuming that all auditory sensory cells across phyla are derived from ancestral mechanosensory cells, we have to understand how these cells multiplied and subsequently diversified to achieve their novel function. To understand this aspect of auditory evolution we must consider the auditory mechanosensory cells within the context of an altered periphery that allows these cells to access sound energy.

Hair cells in the vertebrate ear and their mechanosensory cellular equivalents in non-vertebrates appear to be highly conserved with respect to their cellular differentiation genes. Some of these genes, like the fly bHLH gene *atonal* and its vertebrate homologue *Atoh1* (Figure 2) are involved in mechanosensory cell specification across phyla and can be mutually exchanged (Ben-Arie et al., 1996, 2000; Wang et al., 2002). Other genes that are essential for both insect and vertebrate mechanosensory differentiation are also highly conserved in their sequence and appear to function across phyla in a comparable context. Such genes are the Pou domain factor *Pou4f3* and the zinc finger factor *senseless/Gfi1* (Clough, et al., 2004; Nolo, Abbott, & Bellen, 2000; Wallis et al., 2003; Xiang et al., 2003). In addition, several mutations in conserved genes affect both insect and mammals (Caldwell & Eberl, 2002; Fritzsch & Beisel, 2004). Although details are still unclear, it appears that putative mechanotransducer channels (Corey et al., 2004) may possibly also be conserved and common to several phyla (Duggan, Garcia-Anoveros, & Corey, 2000). This high degree of conservation of cell fate specifying transcription factors across mechanosensors implies a high degree of molecular developmental conservation across phyla that is likely related to the great antiquity of mechanosensors and the regulation of expression of the mechanotransducer channel in these cells (Fritzsch, Beisel, & Bermingham, 2000). Such conservation renders any idea of an independent and parallel evolution of auditory mechanosen-

sory transducer cells across phyla difficult to defend and leaves open essentially only the alternative that mechanosensory cells involved in sound reception represent independent transformations of ancestral mechanosensory cells that have led to the acquisition of a novel function (Figure 3). Below we will provide two examples of conversion of general mechanosensors to specialized hearing organs.

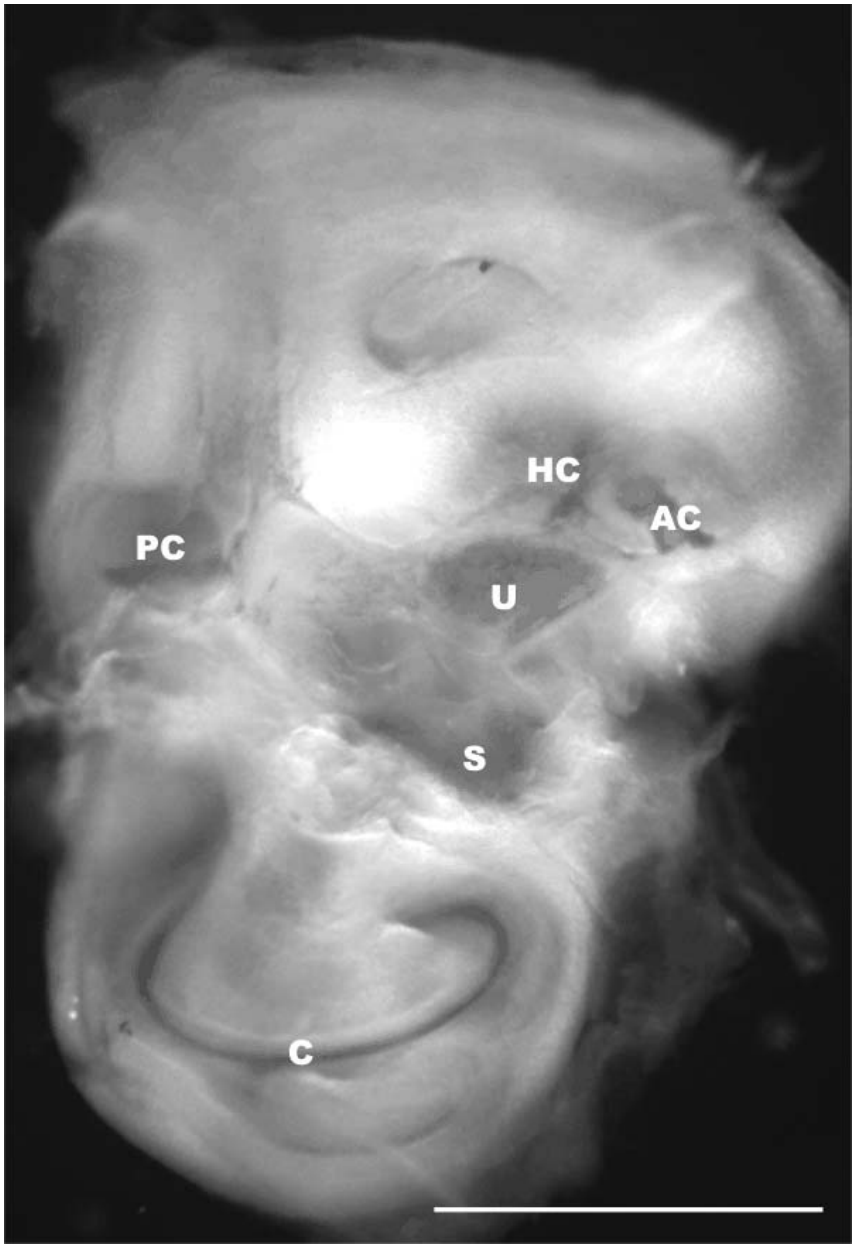

Figure 2. This image shows the distribution of *Atoh1* in inner ear hair cells of an embryonic day 18.5 mouse ear using the *LacZ* reporter construct that transcribes β-galactosidase in places of *Atoh1* gene expression. Note that all hair cells of both the vestibular and cochlear sensory epithelia are *Atoh1* positive. AC, anterior crista; C, cochlea; HC, horizontal crista; S, saccule; PC, posterior crista; U, utricle. Bar indicates 1 mm.

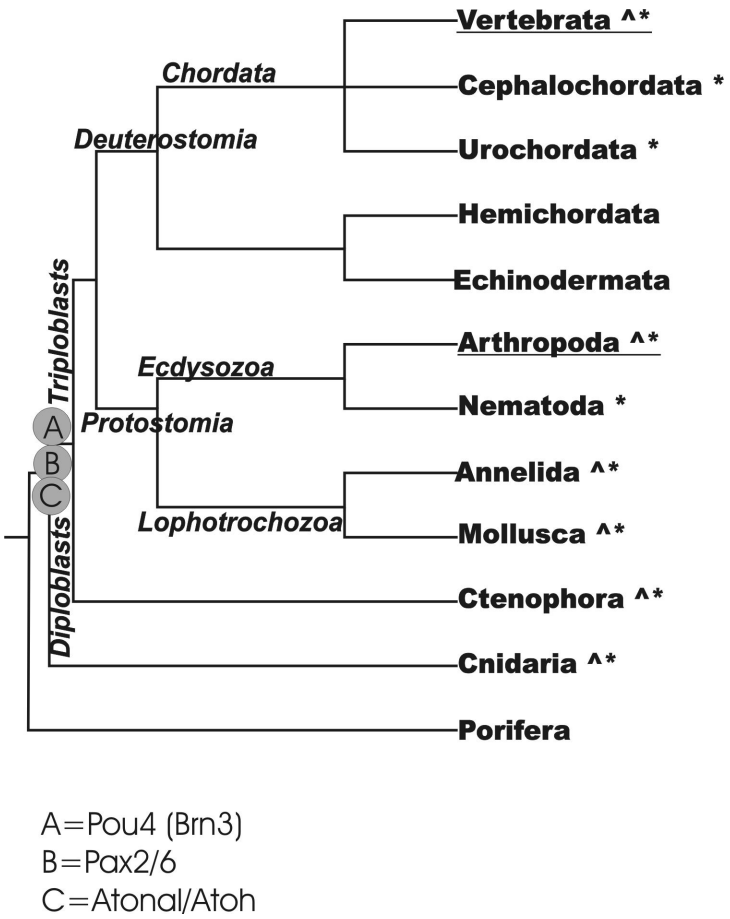

Figure 3. Critical steps in mechanosensory and auditory evolution are depicted as nodes on a cladogram of metazoan taxa. Mechanosensory cells (indicated by *) and mechanosensory multicellular organs (indicated by ^) are known for all multicellular taxa, excluding sponges (Porifera). The simplest assumption therefore is that mechanosensory cells and organs are ancestral to diploblasts and the divergent appearance of those cells and organs in crown group taxa is related to transformational similarities. Genetic analysis has shown that the *Pou* domain factors and *Pax* factors can already be identified in sponges and, in diploblasts, are associated, among other relations, with mechanosensory cell and organ formation. Triploblasts such as insects (a taxon of the arthropoda) and vertebrates require the bHLH *atonal/Atoh1* for mechanosensory cell development. They also have a unique molecular basis for epithelial polarity formation conserved between arthropods (insects) and chordates (vertebrates). The conservation of the *Atoh1* gene is so great that replacing of the insect with the vertebrate gene, and vice versa, leads to rescue of mechanosensory cell formation in both taxa. Such structural and functional conservation across deuterostome and protostome taxa is referred to as 'deep homology'. Hearing, here used to describe sound pressure reception, apparently evolved multiple times independently in arthropods and vertebrates (indicated by underlining). It appears that different mechanosensory organs in each case were modified to become hearing organs.

Scolopidial Organs in Invertebrates

Scolopidial organs are proprioreceptors that measure the relative movement of proximal to distal parts of insect legs. Insects have transformed some of their legs into mouth parts and antennae, rendering those proprioreceptors useless in some of their original function and allowing them to adopt a novel function. This novel function will maintain the basic modality of such an organ (i.e., to

measure movement between proximal and distal parts of the leg) but become driven by sound. To achieve this, the distal part of the antenna has to increase its surface area in order to allow near field or direct acoustic stimulation, as employed in some insect hearing (Yager, 1999). Such increases of antennal surface may also be related to the surface increase needed by a second sensory system present on the antenna, the olfactory receptors. Once a sufficient surface to mass ratio is reached, this distal process can be moved by direct or near field sound (Tauber & Eberl, 2003). These movements will result in stimulation of the scolopidial organs in the proximal antennal segment, thereby eliciting proprioreceptive responses that encode sound stimuli. In flies, this organ, known as the Johnston's organ, consists of a sensory array that appears to be uniquely dedicated to the reception of sound that is greatly enlarged during development with little overt alteration in the overall scolopidial structure (Yager, 1999). How the dramatic increase in size of this organ relates to sensitivity and spectral analysis carried out by these modified scolopidial organs remains to be determined.

Formation of the Cochlea in Vertebrates

In vertebrates, the most obvious transformation of the vestibular sensory array is the formation of the mammalian cochlea (Figure 4). Both in size and number of hair cells, the cochlea can, in certain mammals, outclass all other inner ear sensory organs combined. Nevertheless, even small sets of hair cells are sufficient for some limited hearing capability, as in turtles or amphibians (Smotherman & Narins, 2004), despite an obvious correlation between increased numbers of hair cells, added length of the cochlea and broader range of frequencies (Lewis, Leverenz, & Bialek, 1985). The cochlea of mammals or basilar papilla of other sarcopterygian vertebrates appears to be an ontogenetically transformed part of the saccule (Fritzsch, 1992; Fritzsch, 2003b). This conclusion is derived from several descriptive and molecular ontogenetic studies using markers such as lunatic fringe and brain-derived neurotrophic factor, *Bdnf* (Farinas et al., 2001; Morsli et al., 1998) that indicate the progressive segregation of the mouse cochlea from the saccule during development (Fritzsch, Barald, & Lomax, 1998). Similar progressive developmental segregations of various sensory epithelia have long been noticed in amphibians (de Burlet, 1934; Fritzsch et al., 1998) and have been proposed as a general mechanism of forming new sets of hair cells that are functionally uncommitted and thus allow functional alterations in the course of evolution (Fritzsch et al., 2002). After a new cochlea epithelium segregated during development, splitting the saccular and cochlear sensory patch into discrete areas situated each in its own recess (Fritzsch, 1992; Fritzsch, 2003b), the evolutionary transformation of the cochlea from a vestibular to an auditory receptor required the co-option of molecules that could govern the emergence of novel properties.

Recent evidence suggests that transformation of hair cells from a cochlear to a vestibular type is under the control of *Wnt* signaling pathway (Stevens et al., 2003), supporting their basic homology and indicating a possible molecular switch. Thus, as *Wnt* signaling is apparently necessary for the development of the vestibular hair cell phenotype (Stevens et al., 2003), the auditory sensory epithelium of tetrapods may have evolved from a gravistatic sensory epithelium by downregulating the *Wnt* pathway mediated activation of vestibular hair cell differentiation, thus

allowing the emergence of a new phenotype, the auditory hair cell. *Wnt* signaling may also be involved in the transformation of otoconia to a tectorial membrane as numerous proteins are common to otoconia and tectorial membrane matrix (Goodyear & Richardson, 2002). It seems possible that minute alterations, perhaps mediated by the sensory epithelium as a consequence of absence of *Wnt* induced differentiation (Stevens et al., 2003), turned an otoconia bearing saccular epithelium into an epithelium, covered instead with a tectorial membrane (the basilar papilla) thus rendering it functionless for the transduction of gravity and other linear acceleration stimuli, and allowing their phenotypic switch to become an auditory receptor (Figures 1 and 4). Similar transformations are well known for some teleost fish where part of the utricle, a gravistatic organ, becomes transformed into a sound pressure receiver (Fritzsch, 1999). In contrast to the fly model presented above, tetrapod vertebrate ears are predominantly sensitive to sound pressure and require appropriate association with sound conducting pathways to the basilar papilla. At which point and through which additional morphological alteration this basilar papilla, redundant and irrelevant for vestibular function, acquired access to sound pressure is an issue of ongoing controversy (Clack, 2002; Fritzsch, 1992).

Primary Auditory Neurons (Spiral Ganglia) are Derived Both Developmentally and Evolutionarily, from Primary Vestibular Neurons

For hearing, sensory neurons have to be specified and/or generated that connect a novel inner ear auditory endorgan to an area of the central nervous system dedicated to sound processing. We will use the term "auditory sensory neurons" in vertebrates for a population of specialized sensory neurons that connect a specific peripheral "auditory" sensory epithelium to a specific central target, usually referred to as auditory or cochlear nuclei. Through development and evolution, auditory neurons must accomplish the following tasks:

(1) They must acquire the ability to reach auditory, rather than vestibular, epithelia in the periphery.
(2) They must acquire the ability to project to auditory, rather than vestibular, nuclei in the CNS.

In mice, auditory neurons are closely related developmentally to vestibular neurons. Spiral auditory neurons develop, much like vestibular neurons, through the signaling of the bHLH gene *Neurogenin 1* (*Neurog1*). Abrogation of *Neurog1* leads to complete absence of all vestibular and cochlear sensory neurons (Fritzsch et al., 1998; Ma, Anderson, & Fritzsch, 2000) thus showing that, as much as *Atoh1* is essential for all sensory hair cell differentiation of the ear, so is *Neurog1* essential for all sensory neuron formation of the ear. All sensory neurons express early on another bHLH gene, *Neurod1,* and require this gene for cell survival and for proper migration (Kim et al., 2001; Liu et al., 2000). Interestingly, there is a differential effect between cochlear and vestibular neurons, since loss of *Neurod1* causes more cochlear neurons to die, whereas many vestibular neurons survive (Kim et al., 2001). In later stages *Neurod1* has been reported to be differentially distributed in vestibular and cochlear sensory neurons (Lawoko-Kerali et al., 2004). More recently, a gene that is uniquely expressed in the spiral sensory neurons has been found, *Gata3* (Karis et al., 2001; Lawoko-Kerali, Rivolta, & Holley, 2002). Based on the function of this gene product in other systems, it is possible that this gene is

involved in the unique pathfinding properties of spiral auditory neurons. However, owing to the early lethality and apparent loss of spiral neurons in *Gata3* null mice, conditional mutants for *Gata3* in the spiral neurons will be needed to verify this idea. Nevertheless, examination of central auditory projection using *Gata3*-tau-LacZ construct reveals that Gata3 is uniquely associated with the central processes of auditory sensory neuron, which show extensive projections to the cochlear nuclei as early as E12.5 (Figure 5).

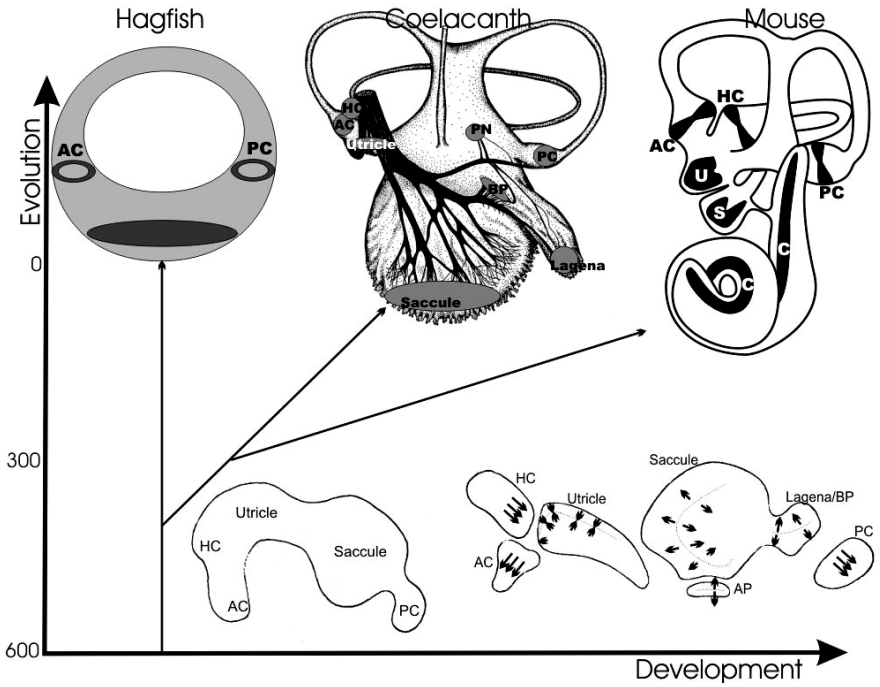

Figure 4. Critical steps in the ear evolution of vertebrates are shown and how these steps may relate to the ontogenetic reorganization. A simple ear, consisting of two canal cristae and a single otoconia bearing endorgan (as found in hagfish) has been diversified in the course of evolution (indicated by Millions of years) through formation of multiple sensory patches, each localized in a specific position and innervated by distinct bundles of nerve fibers (eight organs in the coelacanth, *Latimeria*). Further evolution has resulted in elaboration of the ancestral basilar papilla (BP) near the lagena/saccular recess into the coiled cochlea of mammals (mouse). Note that all mammals, except monotremes, have lost the lagena and have reduced the papilla neglecta. Development shows that anatomically or molecularly specified sensory epithelia progressively split into smaller components, each one differentiating into a discrete sensory organ. This split is not a function of size alone as very small epithelia consisting of few cells still form whereas giant single epithelia are also found that consist of several thousand hair cells. The last epithelia to segregate in development in amphibians are also the last to appear in evolution (lagena, basilar papilla, amphibian papilla). Available data suggest that ear epithelium formation is a simple case of terminal addition largely recapitulating evolution during development. The molecular basis for the segregation and formation of specific recesses during development remains largely unknown. AC, anterior crista; AP, amphibian papilla; BP, basilar papilla; C, cochlea; HC, horizontal crista; MC, macula communis; PC, posterior crista; S, saccule; U, utricle.

Two other similarities between vestibular and cochlear sensory neurons are the expression of the POU domain factor *Pou4f2* and the two neurotrophin factors *Ntrk2* and *Ntrk3*. *Pou4f2* appears to be involved in pathfinding, but no details

exist that would suggest that there is a differential effect in the two sensory neuron populations of the ear (Huang et al., 2001). *Pou4f2* is also involved in neurotrophin receptor upregulation in all ear sensory neurons (Huang et al., 2001) whereas *Pou4f3* is involved in upregulation of neurotrophins (*Bdnf* and *Ntf3*) in hair cells (Clough et al., 2004). Although all vestibular and cochlear sensory neurons coexpress the two neurotrophin receptors *Ntrk2* and *Ntrk3* (Farinas et al., 2001; Pirvola et al., 1992), single neurotrophin-null mice have a differential effect on vestibular and cochlear innervation. It appears that the vestibular innervation depends quantitatively more on *Bdnf* and its receptor *Ntrk2* whereas the cochlear innervation depends more on *Ntf3* and its receptor *Ntrk3* (Ernfors et al., 1995). However, examination of the pattern of innervation of vestibular and cochlear epithelia by sensory cells showed that this dominance is not receptor or ligand specific (Fritzsch et al., 1997). Rather, more recent data suggest that the neurotrophins are functionally equivalent and that the differential effects reflect the spatio-temporal expression pattern of a given neurotrophin (Farinas et al., 2001). Simply speaking, one neurotrophin can functionally replace the other only if expressed at the same time and place. This suggestions of the importance of spatio-temporal expression patterns was recently verified with the use of transgenic mice in which *Ntf3* replaced *Bdnf* (Agerman et al., 2003) or in which *Bdnf* replaced *Ntf3* (Farinas et al., 2001; Tessarollo, Coppola, & Fritzsch, 2004). These data also revealed that the altered pattern of expression of *Bdnf* under *Ntf3* promoter control in these transgenic mice resulted in rescue of the *Ntf3* null phenotype in the cochlea but also caused a massive misrouting of vestibular fibers into the cochlea. Apparently this misrouting came about through the premature upregulation of *Bdnf* under *Ntf3* promoter control in the basal turn of the cochlea, an area that normally expresses *Bdnf* only much later in development. Interestingly, no such misrouting has been reported for the cochlear fibers into the vestibular sensory epithelia of *Bdnf* transgenic mice that express *Ntf3* under *Bdnf* promoter control, suggesting that BDNF is indeed the major attraction for any fiber in the ear. This conclusion is consistent with the expression of *Bdnf* in all differentiated hair cells, which are known to attract fibers to them even when showing an abnormal distribution (Fritzsch, Signore, & Simeone, 2001). In addition, misexpression of BDNF in areas outside of sensory epithelia can attract fibers even in the absence of hair cells (Tessarollo et al., 2004). Thus, while initial data implied a differential effect of the neurotrophins expressed in the ear on vestibular and cochlear neuron survival and possibly pathfinding, more recent data have shown that both types of fibers are attracted by the same neurotrophin (BDNF) that is expressed in all hair cells. Differential effects on pathfinding and survival are related to the spatio-temporal patterns of expression of *Bdnf* and *Ntf3*, and do not reflect unique properties of these two neurotrophins (Fritzsch et al., 2004). Such ideas are consistent with the fact that both neurotrophin receptors are co-expressed in vestibular and cochlear neurons. They are also consistent with the emerging concept that cis-regulatory elements are major driving forces of evolution (Carroll, Grenier, & Weatherbee, 2005).

Recent evidence suggests that spiral and vestibular neurons may originate from distinct areas of the developing otocyst (Fritzsch, 2003a). It is possible that such unique origins bestow unique identities to these neuronal populations. It is also possible that peripheral target finding is less of a challenge since neurons may actually leave their trailing migratory processes near their site of delamination.

Thus the distal processes may need to navigate comparatively short distances to find their targets (Fritzsch, 2003a; Fritzsch & Beisel, 2003). While such a mechanism may provide some crude epithelial specificity, it is unlikely that it will be important for intraepithelial fiber segregation. In this context it has been proposed that sensory neurons and hair cells have a clonal relationship (Ma et al., 2000) that might also be important for targeting specific hair cell populations and for the size-matching of sensory neuron and hair cell populations (Fritzsch et al., 2002). Interestingly, the basic assumption of a clonal relationship has been established for chicken (Satoh & Fekete, 2005) but it remains unclear how this translates to numerical and pathfinding regulations of sensory neurons.

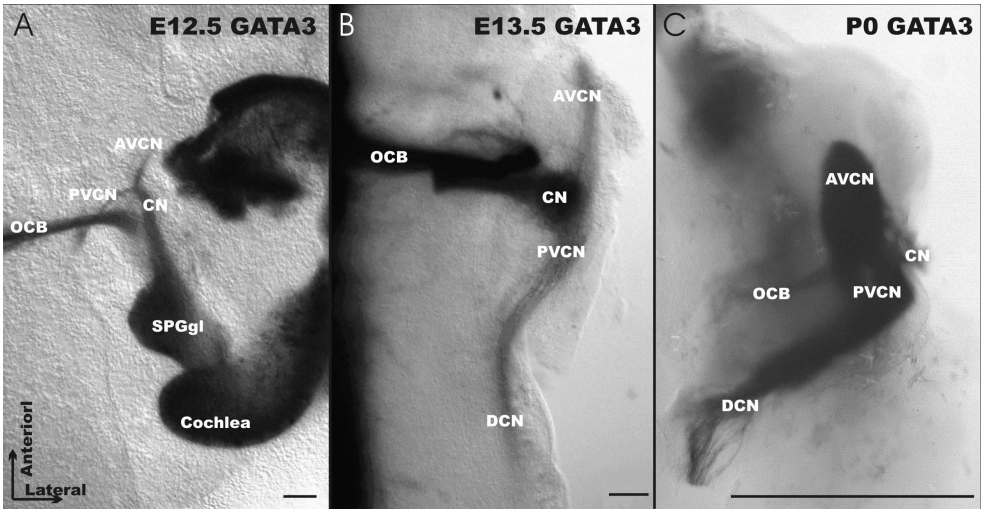

Figure 5. The expression of *Gata*3 is shown as revealed with the *tau-LacZ* construct in whole mounted brains. Anterior is up, midline is to the right. Note that both auditory afferents forming the cochlear nerve (CN) and inner ear efferents forming the olivo-cochlear bundle (OCB) are β-galactosidase positive. The spiral ganglion cells (SPGgl) delaminate from the growing cochlea which is also *Gata3* positive (A). The earliest afferent projection of the spiral ganglia (SPGgl) reaches both the anterior and posterior ventral cochlear nucleus (AVCN, PVCN) at E12.5 (A). The olivo-cochlear bundle of efferents (OCB) is entering at this stage into the vestibular ganglion. One day later (B), the auditory afferent projections have reached not only the ventral but also the dorsal cochlear nuclei (DCN). In newborns, the ventral cochlear projections have grown substantially and the dorsal cochlear nucleus projection reaches along the ventricle toward the caudal aspect of the cochlear nuclei. Bar indicates 100 µm in A,B, 1 mm in C.

Even less is known about the molecular basis of central pathfinding of either vestibular or cochlear neurons (Rubel & Fritzsch, 2002). Whether or not GATA3 plays a role in this process, as its apparent distribution in the centrally projecting 'auditory' sensory fibers suggests (Figure 5), remains to be demonstrated. Unfortunately, *Gata3* null mutants are early lethal and do not develop a cochlea (Karis et al., 2001). Targeted mutations that eliminate GATA3 only in sensory neurons are needed to investigate this issue further. Clearly, peripheral reorganization of afferents does not affect their central targeting and absence of functional hair cells is also of little consequence (Tessarollo et al., 2004; Xiang et al., 2003). Establishing the molecular basis of central projection patterns will be a crucial step towards understanding how these neurons develop appropriate central projections and how sensory neuron regeneration can be accomplished.

In summary, only two molecular changes can thus far be associated with the unique projection properties of auditory sensory neurons, the differential expression of neurotrophins that maintain a segregated projection of vestibular and auditory sensory neurons in the periphery (Tessarollo et al., 2004) and the expression of *Gata3* in auditory sensory neurons (Karis et al., 2001). When these alterations were implemented in evolution of auditory neurons and how they causally relate to the differential projection of auditory neurons is not yet fully established.

Overall, while the mechanosensory transduction system and its development appear to have evolved early and to be conserved across phyla, the vertebrate ear has evolved around this conserved neurosensory element a unique structure for sound acquisition. This was apparently achieved by co-opting existing genes from other developmental modules of the body into the ear to govern formation of the cochlea and formation of auditory sensory neurons (Fritzsch & Beisel, 2004; Xiang et al., 2003). Based on our current insights, the vertebrate ear can be viewed as the product of continuous alteration of an existing program to govern sensory hair cell and sensory neuron development by co-opting existing genes to generate a novel context that alters function of those genes thereby generating a novel outcome.

Hindbrain Auditory Nuclei are either Totally New or Derived (Both Developmentally and Evolutionarily) from Hindbrain Electrosensory Nuclei

For the evolution of hearing, second and higher order auditory signal processing areas in the central nervous system have to be generated that are specifically dedicated to process sound information. Two possible ways by which neurons can be made available to perform new functions will be considered below:

(1) Increased proliferation of an existing population (Fritzsch et al., 1998) can form redundant and therefore potentially uncommitted neurons (see the above outlined interpretation of the auditory sensory periphery).

(2) Loss of an old input and/or target frees neurons of their previous functional constraints and allows them to adopt new function. The mammalian middle ear ossicle evolution is an example for such a scenario.

Beyond these problems surrounding the formation of new central neurons, establishing a new function would require either (1) alteration of selector gene expression through, upstream changes in regulatory factors gradients which activate these genes, (2) mutation of selector genes which result in changes in the expression pattern and thereby changes in the pattern of activation of downstream genes, or (3) mutation of downstream genes to respond differently to selector genes. There is growing evidence that many selector genes may have a stable pattern of expression among chordates (Holland et al., 2000) and even hemichordates (Lowe et al., 2003). Differences in activation of downstream genes or mutations in downstream genes, combined with evolutionary changes in selector gene expression (Carroll et al., 2005) may be the major modes by which variation in the nervous system organization is generated while conserving the overall topography of certain transcription factor expression.

Increased Proliferation of an Existing Population

Increased proliferation and formation of more neurons clearly happened in the evolution of vertebrates, as exemplified by the absolute and relative (with respect to body size) enlargement of the brains of humans compared to those of bony fish. Moreover, specific areas may in some vertebrates become differentially enlarged, whereas they may be entirely absent in other species. For example, the cerebellum can become the largest part of the bony fish brain, much like the forebrain has become the largest part in human brains (Nieuwenhuys, Donkelaar, & Nicholson, 1998). These enlargements must certainly result from increased proliferation, which could result from a simple mutation in genes regulating the proliferation of precursor cells. For example, adding one round of mitosis could potentially double the total number of neurons being generated, some of which could develop an entirely different identity. How this new identity could be achieved is still unclear but one possibility would be that the enlarged population of postmitotic neurons could experience slightly different levels of selector gene products which may in turn determine different phenotypes. The differences in function would be obtained by (1) innervating a different target and/or by (2) receiving different inputs through differential migration.

Loss of an Old Input and/or Target

A second possible scenario for the formation of uncommitted neurons may be the loss of either the target or the input of a preexisting neuronal population. This might eliminate the constraints normally acting on these neurons and would thus allow them to acquire a new function. One well known example for this second scenario is the evolution of the middle ear ossicles of land vertebrates: There is good comparative and developmental evidence suggesting that these ossicles are derived from former jaw-supporting ossicles (Fritzsch, 1999; Knoll & Carroll, 1999). Once their original functional constraint—supporting the jaws—was lost, they underwent a radical change in function. While there is evidence for evolutionary loss of both inputs and targets, it is not yet proven that the neurons, freed by such a process from their previous functional constraints, during development are in fact modified to perform a novel, different function or whether they are simply lost (Restrepo, Manger, & Innocenti, 2002). However, it is clear that experimental changes of inputs can alter the function of existing neuronal networks (Pallas, 2001). Thus, while experimental evidence tends to support the notion of the functional lability of neurons, there is little evolutionary evidence to support it. The importance of such a process on brain evolution in general, and the auditory system in particular, has not yet been worked out.

Irrespective to how the new auditory neurons evolved in the first place, they have to achieve new input/output relationships to mediate any new function. New inputs can come about through differential migration path that allows them to achieve a new input or through alteration of their molecular properties so that they become permissive for new axons to make contacts. Likewise, delivering a new function requires the establishment of novel output connections. Comparative neuroanatomy offers very few well understood examples that highlight migration related input change and navigation related output changes. The best example is pro-

vided by the system of olivo-cochlear neurons (Fritzsch, 1999). Briefly, inner ear efferents are developmentaly and evolutionarily derived from facial branchial motoneurons. In mice, inner ear efferents migrate differently from facial branchial motoneurons into a new position to receive auditory input—rather than premotor facial input—and project to the ear (instead of facial muscle fibers). A single gene is thus far known that may mediate the differential projection, *Gata3* (Karis et al., 2001). It is also possible that expression of other genes (or their absence of expression) plays a role in differential migration of facial motoneurons and olivocochlear efferents (Muller et al., 2003). Overall, the segregation of the facial branchial motor system and the olivocochlear system highlight but one possibility of how connections within the brain can be changed. More detailed connectional data are needed to show how often such changes have happened in the brain.

In conclusion, there is evidence for both ongoing invasion of certain fibers into novel territories and for rather precise pathway selection. Certain areas, like the forebrain, may benefit from a less constrained developmental program that enables them to form a wider array of initial connections from which only certain connections will be later selected (Clarke & Innocenti, 1990). However, recent developmental data on auditory thalamo-cortical forebrain connection indicates that a high level of precision in thalamo-cortical projections is achieved initially, likely under the guidance of genetic, rather than auditory, influence (Gurung & Fritzsch, 2004). Follwing this theoretical assessment of brain changes, we will now present some data that relate to the development and evolution of the auditory nuclei in the brainstem.

Central Auditory Pathways Likely Evolved not by Capturing other Sensory Pathways but by Expansion of Cell Populations under the Control of *Atoh1*

Transformational identity, similar to the evolutionary transformation of the hyomandibular bone into the stapes of the terrestrial ear, was the leading idea for the evolution of auditory nuclei until some 20 years ago. Larsell (1967) and others proposed that auditory nuclei derive from mechanosensory lateral line nuclei. It was further suggested that auditory input from the ear replace, evolutionarily as well as during metamorphosis of frogs, the disappearing lateral line input (Larsell, 1967). However, it was established that not all frogs lose the lateral line system during metamorphosis, and that those that retain the lateral line system also have auditory nuclei (Fritzsch, 1990; Fritzsch et al., 1988). Specialized auditory nuclei are are also found in some bony fish that fully retain the mechanosensory lateral line system (McCormick, 1999). In addition, there is a direct spinal output in the lateral line nuclei, but not in the auditory nuclei (Coombs, Goerner, & Muenz, 1989). Thus, neither the apparent coincidence of loss of one nucleus and appearance of another nor the detailed connections support this idea at the moment. The only factors supporting it are its more generalized connections to the midbrain.

More recent data suggest yet another transformation, related to the discovery that an electroreceptive sense is primitive for all jawed vertebrates (Bullock & Heiligenberg, 1986), including amphibians (Fritzsch, 1981). The loss of this primitive sense and the formation of recognizable auditory nuclei coincide among bony fish, amphibians and amniotes (Fritzsch, 1990). Moreover, within a given class of vertebrates, such as amphibians, some either have electroreception with the spe-

cialized central nervous system nuclei but no specialized auditory system (salamanders, caecilians) whereas others have lost the sense of electroreception but have apparently gained central auditory nuclei with or without losing the mechanosensory lateral line (Fritzsch, 1990; McCormick, 1999). In addition, and in contrast to the mechanosensory lateral line nuclei, electroreceptive and auditory nuclei in mammals show topological similarities in the rostro-caudal extent (from trigeminal to glossopharyngeal roots or from rhombomere 2-6) whereas lateral line and vestibular nuclei extent from rhombomere 1 to rhombomere 7/8. Thus, at least a spatial correlation exists between loss of the ancient sense of passive electroreception and gain of auditory nuclei in the brainstem with the overall topology of the mammalian auditory nuclei, but not with birds. In addition, electroreceptive and auditory nuclei are the most dorsal structures in the hindbrain alar plate, suggesting the expansion of the hindbrain through the formation of a suprastructure not unlike the cerebellum. However, connectional details show that the auditory system has brainstem nuclei (superior olive, lemniscal nuclei) not recognized in the electroreceptive or mechanosensory lateral line system (McCormick, 1999). If the auditory nuclei of tetrapods represent a transformation of a dormant developmental program for electroreceptive central nuclei, refuting such scenarios on topological and connectional grounds will be almost impossible. Other information needs to be considered to establish what the likely evolutionary history of the auditory system might be.

Limitations of the Functional Replacement Hypothesis

Replacement suggestions assume at their very core an independent loss of peripheral sensory organs and their afferents while retaining the central nuclei. Most developmental data have clearly shown that a loss of innervation of those central auditory nuclei would likely cause them to disappear before they receive a different input (Rubel & Fritzsch, 2002). Nevertheless, such functional reconnections have been experimentally shown for forebrain connections (von Melchner, Pallas, & Sur, 2000) and certainly are a possibility worth exploring. However, attempts to experimentally reorganize the lateral line or auditory input by transplanting or extirpating the ear in frogs have seen little alterations in the remaining projections (Fritzsch, 1990; Fritzsch, 1999). It also needs to be stressed that this entire idea is not yet tested at the molecular level: no specific lateral line, electroreceptive or auditory markers are known that are expressed in all sensory nuclei of each of these systems and across taxa.

Molecular Developmental Evidence for the Origin of Auditory Nuclei

More recent molecular data show that functional systems may be developmentally connected by shared activation of transcription factors (Bermingham et al., 2001; Qian et al., 2001) and suggests that such molecules may indeed also exist for auditory nuclei. For example, auditory nuclei appear to express unique markers in some songbirds (Akutagawa & Konishi, 2001). It is therefore possible that identical transcription factors may govern the development of both the peripheral receptors (i.e., sensory neurons, sensory cells) and the central nuclei (i.e., lateral line, electroreceptive, and auditory nuclei in the brainstem). If this pleiotrophic effect

can be demonstrated for certain genes, it would appear more plausible that all parts of these systems are lost or gained concomitantly during evolution, presumably owing to a single or a few mutations. This would eliminate the core assumption of the replacement hypothesis and both auditory periphery and the functionally related auditory nuclei would then need to be considered as evolutionary novelties that nevertheless might represent transformations of a general column of cells extending throughout the hindbrain and spinal cord (Xiang et al., 2003) by co-opting singling molecules also relevant for ear development (Figure 6).

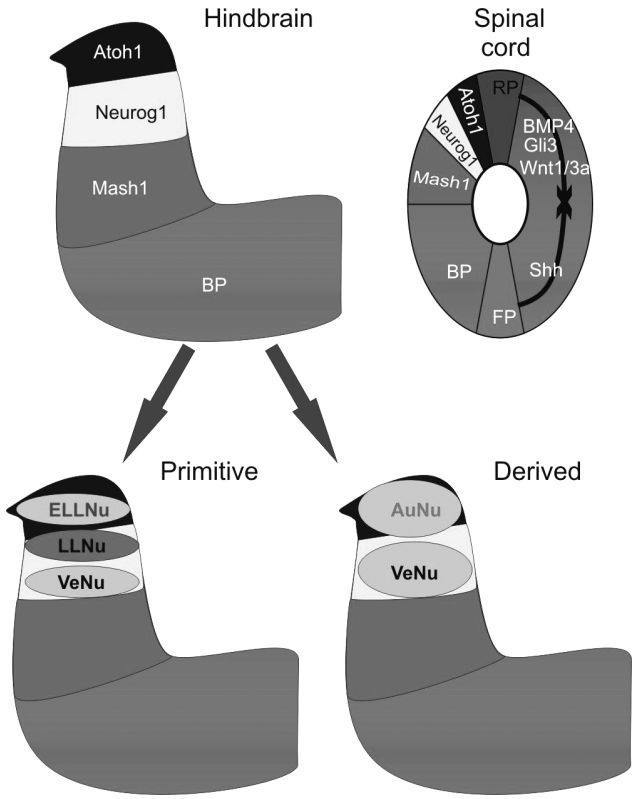

Figure 6. This scheme shows the molecular patterning as revealed by numerous studies for the spinal cord (Bermingham et al., 2001; Gowan et al., 2001) and its possible application for the hindbrain alar plate organization (top images). Note that a gradient composed of several diffusible molecules, that are ancestral to chordates and insects, is setting up the specific expression domains of the three bHLH genes shown here, *Atoh1*, *Neurog1*, and *Mash1*. The detailed contribution of these expression domains to the various hindbrain nuclei is only known for the *Mash1* domain (Qian et al., 2001). It is, however, clear that the vestibular nuclei likely derive from a *Neurog1* expressing domain with an unknown contribution by the *Atoh1* expressing domain. In primary aquatic vertebrates, the mechanosensory (LLNu) and electrosensory nucleus (ELLNu) may be derived entirely or in parts from the *Atoh1* expressing zone, but this is not yet known (bottom left). In landvertebrates, auditory nuclei (AuNu) may be derived in parts or entirely from the *Atoh1* expressing zone as we show below. *BMP4*, bone morphogenetic protein 4; BP, basal plate; FP, floor plate; *Gli3*, Gli-Kruppel Family Member 3; RP, roof plate; *Shh* sonic hedgehog; *Wnt1/3a*, wingless-type MMTV integration site family member 1 and 3a.

Beyond the temporal coincidences in appearance and loss of various sensory systems, recent experimental work suggests that specific genes, such as bHLH genes and homeotic selector genes, may be uniquely associated with the formation

of specific components of the auditory nuclei (Xiang et al., 2003). For example, like the cerebellar granule cells, cells of the cochlear nuclei of mice express the bHLH gene *Atoh1* (Bermingham et al., 2001) and the dorsal cochlear nuclei require the bHLH gene *Neurod1* for their development (Liu et al., 2000). This similarity in the molecular developmental network of the ear and the auditory nuclei connects evolutionary conservation of hair cells to the evolution of brainstem novelties such as the cerebellum, inferior olive and the pontine nuclei. Moreover, based on similarities in dorso-ventral patterning genes such as sonic hedgehog or BMP-4 on both ear and spinal cord (Gowan et al., 2001; Morsli et al., 1998; Riccomagno et al., 2002; Xiang et al., 2003) it is possible to speculate that similar transcription factors are regulated by these identical upstream patterning factors in both the hind brain and the ear.

We have therefore recently conducted an expression analysis of the hindbrain for two bHLH genes important for ear development, *Neurog1* and *Atoh1*. Specifically, we wanted to understand how the temporal and spatial expression pattern of these two genes in the hindbrain relates to their expression in the ear and spinal cord. We analyzed *Neurog1* expression using a probe obtained from Dr. Q. Ma (Fritzsch et al., 1998) and for *Atoh1* we used a probe obtained from Dr. H. Zoghbi (Bermingham et al., 1999).

For this study we used the following mouse lines: *Atoh1-LacZ* knock ins; *Atoh1* null mutants, *Gata3-Tau-LacZ* knock ins; *Neurod1-LacZ* knockins, *Neurog1-GFP* knock ins (Bermingham et al., 1999; Fritzsch et al., 1998; Karis et al., 2001; Kim et al., 2001). In all mice the gene was replaced either by a construct containing the LacZ (β-galactosidase) reporter (with or without a Tau translocator) or a Neocassette. Mice were bred as single, double or triple heterozygotes and single or double mutants with a heterozygotic reporter gene were obtained at the Mendelian frequency. Embryos were removed from the mothers at various times, fixed by perfusion and reacted for β-galactosidase reaction as previously described (Fritzsch et al., 1997). Brains and ears were postfixed in 4% paraformaldehyde, dissected and imaged using MetaMorph software. Images were grouped to plates using CorelDraw software. In situ hybridization for *Neurog1* and *Atoh1* was conducted using probes as previously described (Pauley et al., 2003). Visualizing the dorsal projection of *Neurog1* required extra long time of reaction of DMP-purple for detection of the alkaline phosphate enzyme attached to the antibody used to detect the dioxigenin labeled *in situ* probes so that is could be visualized.

In the hindbrain, *Neurog1* expression showed the well known ventral expression in the basal plate and a much fainter dorsal band of expression (Figure 7). The dorsal expression of *Neurog1* became first visible at E10.5 and showed a conspicuous longitudinal gradient: it was most pronounced caudally where it overlapped with the *Neurog1* expression domain in the spinal cord (Ben-Arie et al., 1997). One day later, expression had expanded more rostrally and showed a faint upregulation in the cerebellum. The more caudal expression in the hindbrain faded and by E12.5 we found this dorsal expression only in the more rostral part of the hindbrain. Around the lateral recess of the IVth ventricle the expression diminished but turned into a broad band of expression in the cerebellum. Overall, the topology of *Neurog1* expression in the hindbrain is consistent with the projection of the vestibular part of the VIIIth nerve. This suggests that parts of the vestibular nuclei may derive from a *Neurog1* expressing domain. This idea is supported by the

known evolution of deep cerebellar nuclei being derived from vestibular nuclei as anterior vestibular nuclei project in lamprey much like the deep cerebellar nuclei in mammals, through the superior cerebellar peduncle to the contralateral midbrain (Fritzsch et al., 1998). We are currently analyzing vestibular nuclei and cerebellar nuclei projections in *Neurog1* null mutants to verify this suggestion. *Neurog1* is also the transcription factor that regulate sensory neuron formation in the ear (Fritzsch et al., 1998; Gowan et al., 2001). Most recent data show that expression of *Neurog1* in various tissues is regulated by distinct parts of the promoter region (Nakada et al., 2004). It is noteworthy that this analysis has thus far not identified the promoter for *Neurog1* expression in the dorsal hindbrain or the ear. It is therefore possible that *Neurog1* expression in the ear and the vestibular nuclei is mediated by an identical promoter region that is driven by similar regulators. If proven, the regulation of *Neurog1* in the ear and the brainstem could be via identical upstream genes, thus linking ear and brainstem development at a molecular level.

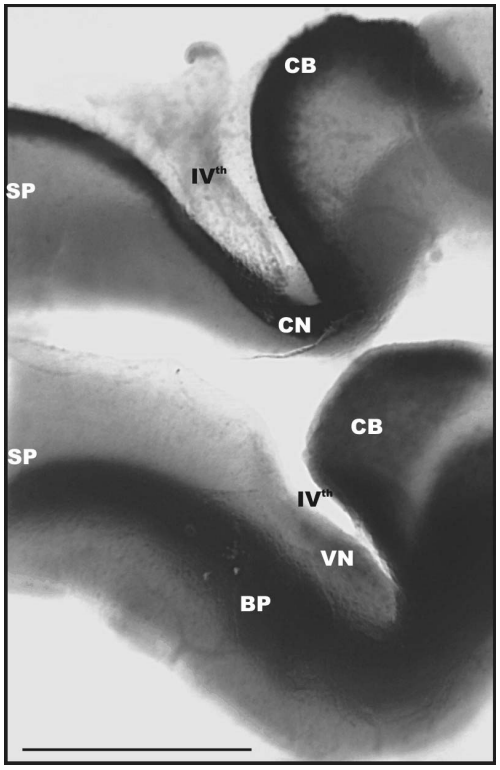

Figure 7. The expression of the bHLH genes *Atoh1* (top) and *Neurog1* (bottom) is revealed in this *in situ* hybridization of two E12.5 mouse hindbrains. Note the extensive expression of *Atoh1* along the entire dorsal margin of the spinal cord and the hindbrain including the future cochlear (auditory) nuclei (CN) as well as the cerebellum (CB, top). In contrast, the most prominent expression of *Neurog1* is in the basal plate (BP) of the hindbrain. However, at this stage there is also a clear dorsal expression (VN) that is immediately ventral to the *Atoh1* expression. This ventral expression continues into the cerebellum (CB) where it expands to form a large area at this stage. Most of the expression is transient and thus makes it difficult to establish directly which vestibular and auditory nucleus neurons are derived from either of these two areas of expression. IVth, IVth ventricle; SP, spinal cord. Bar indicates 1mm.

We have recently also investigated the expression of *Atoh1* in the hindbrain and analyzed the formation of cochlear nuclei in *Atoh1* null mutant mice. Those mice are known to lose embryologically a number of dorsal aspects of the hindbrain such as the external cuneate nucleus and the cerebellum (Bermingham et al., 2001). Our data support the notion that *Atoh1* expression is dorsal to the *Neurog1* expression in an apparently non-overlapping population (Figure 7). Starting at around E12.5, the areas of the future dorsal and ventral cochlear nuclei increase in size, and cochlear nuclei are, much like the cerebellum, positive for *Atoh1* expression (Figure 7).

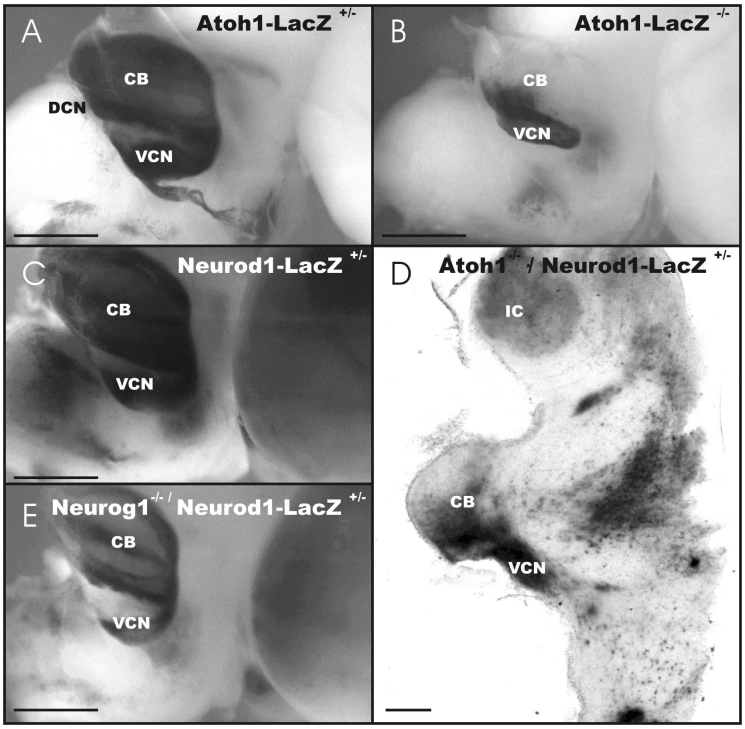

Figure 8. Laterally viewed hindbrains of 18.5 days old embryos show the expression of *Atoh1* as revealed with the *LacZ* reporter in a heterozygotic and null mutant littermate (A,B) as well as the effects of either *Atoh1* (D) null mutation or *Neurog1* (E) null mutation on *Neurod1* expression in cochlear nuclei. Note that *Neurod1* is, like *Atoh1*, strongly expressed in the cerebellum and cochlear nuclei (A,C), but is also in other brain stem nuclei like the inferior colliculus (IC in E). Loss of *Neurog1* reduces only slightly the cochlear nuclei (E), presumably because of the loss of auditory and vestibular sensory neurons and their afferent fibers which critically depend on *Neurog1*. In contrast, in *Atoh1* null mutant mice almost the entire cochlear nuclei have disappeared except for an aggregation of extensively β-galactosidase positive cells that appear immature (VCN in D). CB, cerebellum; DC, dorsal cochlear nucleus; IC, inferior colliculus; VCN, ventral cochlear nucleus. Bar indicates 1mm (A-C,E) and 100 µm (D).

We also investigated the appearance of cochlear nuclei in *Neurog1* and *Atoh1* null mutant mice, making use of other markers to highlight the distribution of structures outside the cochlear nuclei such as *Neurod1* (Figure 8). Our data suggest that auditory nuclei are almost completely absent in *Atoh1* null mice at E18.5 (Figure 8), a finding that was recently largely confirmed (Wang, Rose, & Zoghbi, 2005). We also find that the apparent size of both the cerebellum and the cochlear

nuclei is reduced in *Neurog1* null mice (Figure 8). *Atoh1* null mutants, like *Pou4f3* null mutants, retain some auditory fibers whereas all sensory neurons are lost in Neurog1 null mutants (Ma et al., 2000). We interpret the dramatic reduction in the cochlear nuclei in *Atoh1*-null mutants as a direct effect whereas the minor reduction in Neurog1 null mice could present an indirect effect related to the absence of sensory fiber projections from the ear. We are currently investigating the central projection and connections of the cochlear nuclei through retrograde filling with lipophilic dyes (Gurung & Fritzsch, 2004).

These data suggest that mammalian auditory nuclei derive in large parts from *Atoh1* expressing populations that extend from the cerebellum to the spinal cord. Regional specialization of this longitudinal zone is likely mediated through the various Hox genes expressed in the different rhombomeres of the hindbrain (Cambronero & Puelles, 2000; Cramer, Fraser, & Rubel, 2000). Future studies need to dissect the molecular basis for the apparent differences in the longitudinal origin of auditory nuclei in mammals and birds (Fritzsch, 2003).

Conclusions

We present here an overview of ideas and data concerning the neuronal aspect of auditory system evolution. Specifically we discuss the formation of auditory sensory epithelia to extract auditory signals rather than vestibular signals, the evolution and development of auditory sensory neurons to conduct information from the sensory epithelia to the brain, and of auditory nuclei in the brainstem. Current data on the molecular basis of hair cell and sensory neurons suggest that vestibular and cochlear hair cells and sensory neurons share many developmental transcription factors. We interpret this as evidence that the auditory periphery is derived from and homologous to certain vestibular parts of the ear. Multiplication, segregation and diversification, well known principles in molecular evolution, may have also played a major role at the molecular and cellular level for the evolution of the auditory periphery. However, combining those cells into morphologically distinct auditory receptors may have occurred several times and independently among aquatic vertebrates, as in each case the sensory epithelia were associated with a unique sound conduction system. In contrast to this interpretation of the ear evolution, evolution of tetrapod central auditory nuclei may have occurred through a combination of two events, loss of the ancient sense of electroreception and expansion of an *Atoh1* dependent precursor population either *de novo* or through changed use of the ancestral electroreceptor nucleus anlage. Our data on *Atoh1* null mice support that this gene is essential not only for the formation of all hair cells but also for the specific formation of auditory nuclei. Most important is the apparent use of similar cell type-specifying factors in the ear and the brain. In the ear, *Atoh1* and *Neurog1* are in their expression temporally segregated and generate two distinct cell populations, hair cells and sensory neurons, respectively. In the hindbrain, however, *Neurog1* and *Atoh1* show a broader temporal overlap but appear to be segregated into dorsal and ventral expression zones that apparently are important for the formation of cochlear nuclei and vestibular nuclei, respectively. More detailed investigations of the brains of *Neurog1* and *Atoh1* mutants are needed to verify these suggestions. The apparently primary absence of cochlear nuclei in *Atoh1* null mutants would render these mutations an excellent model in which to

test for the changes in the formation of thalamo-cortical connection in the absence of any brainstem input that has been recently predicted (Gurung & Fritzsch, 2004). More detailed understanding of upstream factors regulating transcription factor expressions simultaneously in the ear and hindbrain are needed to verify or refute ideas of co-evolution of the entire auditory system through common transcription factor regulation, an emerging theme in developmental evolutionary biology (Carroll et al., 2005).

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