Clonal analysis of the relationships between mechanosensory cells and the neurons that innervate them in the chicken ear

Takunori Satoh and Donna M. Fekete*

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA
*Author for correspondence (e-mail: dfekete@purdue.edu)

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Summary

In vertebrates, hair-cell-bearing mechanosensory organs and the neurons that innervate them share a common placodal origin. In the inner ear, the peripheral neurons for both auditory and vestibular systems emigrate from the otic placode as neuroblasts, and divide, differentiate and innervate only one of six to eight distinct sensory organs. How these neurons find their correct target is unknown, although one suggestion is that they synapse with clonally related cells. To test this idea for both the middle and inner ears of chicken embryos, lineage analysis was initiated at the time of neuroblast delamination by labeling progenitors with replication-defective retroviruses. The vast majority (89%) of clones were restricted to a single anatomical subdivision of the sensory periphery or its associated ganglia, indicating limited clonal dispersion. Among the remaining clones, we found evidence of a shared neurosensory lineage in the middle ear. Likewise, in the inner ear, neurons could be related to cells of the otic epithelium, although the latter cells were not widely distributed. Rather, they were restricted to a region in or near the utricular macula. None of the other seven sensory organs was related to the ganglion neurons, suggesting that a common lineage between neurons and their targets is not a general mechanism of establishing synaptic connections in the inner ear. This conclusion is further strengthened by finding a shared lineage between the vestibular and acoustic ganglia, revealing the presence of a common progenitor for the two functional classes of neurons.

Key words: Labyrinth, Placode, Otocyst, Cell lineage, Neuroblast, Chick

Introduction

Mechanosensory systems in vertebrates all share some basic features. They all consist of epithelial sensory organs containing supporting cells and mechanosensory hair cells, which are innervated by peripheral ganglion neurons. These peripheral components have similar embryonic origins. The ganglion neurons, the sensory organs and the associated non-sensory structures are derived primarily from a single source – the placodal ectoderm. The otic placode gives rise to the inner ear epithelium, including several types of sensory organ, and the auditory and vestibular ganglia that innervate them (Fritzsch et al., 1998). A few neural-crest derived neurons are located in the vestibular ganglion (D'Amico-Martel and Noden, 1983); however, those neurons are now considered to belong to the proximal facial ganglion (von Bartheld, 1990). There is a report that cells emigrating from the ventral neural tube may also supply cells to the chicken inner ear (Ali et al., 2003), although this source of migratory neuroepithelial cells remains controversial (Erickson and Weston, 1999; Yaneza et al., 2002). In birds, the epibranchial placode generates a hair-cell-bearing sensory organ in the middle ear called the paratympanic organ and the neurons of the geniculate ganglion that supply its afferent innervation (D'Amico-Martel and Noden, 1983; von Bartheld, 1990). In fish and amphibians, cranial placodes give rise to neuromast mechanosensory organs and their afferent neurons. An additional contribution from the neural crest to the neuromasts has been observed (Collazo et al., 1994).

It has been proposed that a delaminating neuroblast may carry positional information to direct its neurite back to the sensory region from which it delaminated (Fritzsch et al., 2002). This suggests that neurons might recognize their synaptic partners because they are clonally related. Although dye labeling of the zebrafish lateral line failed to confirm this hypothesis (Gompel et al., 2001), definitive studies for other mechanosensory organs are lacking. We sought to address this issue in two classes of mechanosensory organs located in the middle and inner ears of the bird. The inner ear poses a particular challenge for neuronal targeting, as, here, a single placode gives rise to two general classes of neurons (vestibular and auditory) and multiple different sensory organs. In the bird ear, this includes four maculae, three cristae and one auditory organ (the basilar papilla). The logistics of how neurons sort to their correct targets in the periphery could, in theory, be simplified if the neuroblasts leave the otic ectoderm with information that allows them to project back to the progeny of their own sister cells. A lineage relationship between auditory neurons and the basilar papilla could not be confirmed in a prior study where progenitors were targeted at otocyst stages in chick (Lang and Fekete, 2001). This study seeks evidence that bipotent neurosensory progenitors might be present at earlier stages of otic development.

The molecular basis of cell-fate specification between the two classes of inner ear neurons, auditory and vestibular, is unresolved. Expression of the transcription factor, Gata3, distinguishes neuroblasts apparently destined for auditory (Gata-3+) versus vestibular (Gata3–) ganglion fates even before they emigrate from the otocyst in mouse (Lawoko-Kerali et al., 2004). This early segregation could provide a mechanism by which the neuroblasts acquire different identities before delaminating (Fekete and Wu, 2002).

Inner ear lineages are also of interest in the context of evolutionary homologies. For inner ear mechanosensory organs, the issue has been raised of whether they share an ancient evolutionary relationship to insect mechanosensory organs (Eddison et al., 2000; Fritzsch et al., 2000; Lewis, 1991). Both systems use Notch-Delta signaling during key stages of neuroblast delamination (Eddison et al., 2000) and Atonal homologs for cell fate specification (Bermingham et al., 1999; Chen et al., 2002; Zheng and Gao, 2000). In the invertebrate organs, mechanosensory cells and their supporting cells share a common lineage with the neurons that supply the innervation (Gho et al., 1999; Hartenstein and Posakony, 1989). It could be argued, then, that finding a shared lineage between neurons and sensory cells in vertebrate mechanosensory organs further strengthens the developmental parallels between vertebrate and invertebrate mechanosensory organs.

Gene expression patterns indicate extensive regionalization of the ear by the otocyst stages in several vertebrate classes (reviewed by Fekete, 1999; Riley and Phillips, 2003; Torres and Giraldez, 1998). This pre-patterning may serve to specify the major parts of the ear, segregating them into separate lineages. By contrast, fate mapping in *Xenopus* showed that cells originating from restricted regions of the otic vesicle can colonize widely separated sensory organs (Kil and Collazo, 2001). It is therefore essential to conduct clonal analysis on a higher vertebrate, to ask whether the cell dispersion seen in the frog is a general feature of developing inner ears.

This lineage study was initiated to address three questions. Does each neuron originate from the same focal part of the sensory primordium that it will subsequently innervate? Is there a separate origin of auditory versus vestibular neuroblasts? And can clones disperse across the different subdivisions of the inner ear? We find that in the middle ear, a clonal relationship between the ganglion and the sensory organ was common. In the inner ear, a shared lineage between neurons and sensory organs of the inner ear is also possible, although it is less common than separate lineages. Furthermore, an individual neuron is not necessarily related to the sensory organ it innervates in the ear. The auditory and vestibular neurons can originate from a shared lineage, although the majority derive from separate lineages. Finally, we find that individual clones do not colonize more than one anatomical subdivision of the inner ear, with the exception of clones that include ganglion neurons.

Materials and methods

Production of virus stocks

LAP(A) is a mixed *Avian Leukemia Virus* (ALV) virus stock of the A-envelope subgroup carrying both the RDlac1 genome (encoding *lacZ*) and a library of CHAPOL genomes [encoding AP (Golden et al., 1995)]; it was made and titered at 1×10^6 infectious units/ml (i.u./ml) as described (Lang and Fekete, 2001). LAP(G) carries similar viral genomes in a capsid pseudotyped with VSV-G envelope. CHAPOL(G) is a pseudotyped stock carrying only the CHAPOL genomes. Pseudotyped virus stocks were generated by co-transfection of plasmids separately encoding *gag-pol* (pCMV-*gagpol*2), VSV-G [pMD-G; a gift from Connie Cepko (Chen et al., 1999)] and viral genomes. We created a plasmid, pCMV-*gagpol*2, in which the ALV *gag-pol* gene was driven by the CMV promoter. *gag-pol* was excised from pRIA [a gift from Connie Cepko (Chen et al., 1999)] by *Sac*I and *Cla*I, and cloned between the *Sac*I and *Acc*I sites of pDsRed1N1 (Clontech) after first deleting the DsRed1 gene by *Bam*HI/*Not*I digestion following blunting by Klenow polymerase. Plates (20×10 cm) with 40% confluent DF1 chicken embryo fibroblasts (gift of Doug Foster; ATCC #CRL012203) were washed with D-MEM, transfected with 2 μg pCHAPOL, 1 μg pMD-G and 1 μg pCMV-*gagpol*2 using 25 μl Polyfect (Qiagen) per plate. To make LAP(G), 1 μg each of pCHAPOL and pRDlac1 were transfected. Cells were incubated for 3 hours with 25 μM chloroquine and washed with PBS. HEPES (pH 7.4, 10 mM) was added to maintain a neutral pH. Supernatants containing viruses were harvested every 6-8 hours at 36-84 hours post-transfection, and centrifuged at 900 ***g*** for 20 minutes to remove cell debris. A high-speed spin at 77,000 ***g*** for 2.5 hours was used to pellet the virus. The pellets were resuspended in PBS, dispensed into 20 μl aliquots and stored in liquid nitrogen. Virus stocks were titered by serial dilution on DF-1 cells, and confirmed to be helper-free in duplicate assays as described (Morgan and Fekete, 1996).

Injection and detection of viruses

All embryos were assigned stages (s) according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Virus was delivered to the otic cup (stages 9.5-15) or otic vesicle (stages 16-18) of SPAFAS standard White Leghorn chicken embryos as described (Homburger and Fekete, 1996; Lang and Fekete, 2001). Embryos were fixed at E9-13 in 4% paraformaldehyde in PBS at 4°C for 30-60 minutes, washed with PBS and frozen-sectioned at 25-35 μm. Slides were processed for β-galactosidase or alkaline phosphatase histochemistry (Fekete et al., 1994). Selected sections were stained with Hoechst 33342 and 1:500 mouse anti-NF160 (Sigma) followed by anti-mouse IgG_1-Alexa-fluor-488 (Molecular Probes).

After removing coverslips, AP+ cells and a minimal amount of surrounding tissue were collected with 30 gauge needles under a dissection microscope. Similar sized fragments were picked from AP-negative regions as controls. PCR amplification and sequencing of the variable region of CHAPOL proviral DNAs were carried out as described (Golden et al., 1995).

Electroporation of plasmid DNA into chicken otic cup

Episomal marker plasmid, pRep4-CMV-GVP-H2B-EYFP, was constructed from pREP4 (Invitrogen) as the backbone by inserting DNA fragments of cytomegalovirus immediately early promoter from pDsRed1N1 (Clontech), a fragment containing *Gal4-VP16* gene, 14 repeats of UAS and E1b promoter from EFGVPUlynUH2B (Koster and Fraser, 2001) and human *Histone2B* (Kanda et al., 1998) fused with *EYFP* (Clontech). Plasmid pRep4-CMV-GVP-H2B-EYFP was introduced to the right otic cup of 26 embryos (stages 11-14) using electroporation. An epoxy-insulated tungsten microelectrode with 20 μm exposed tip was placed on the right otic epithelium as the cathode, and a 0.5 mm tungsten wire anode was placed to the left of the head. The otic cup was filled with 1 μg/μl plasmid using a beveled glass micropipette attached to the Picospritzer. Electroporation consisted of five square pulses of 10-20V, 1 mseconds each, applied using a BTX T820 pulse generator. On E9, heads from 15 surviving embryos were fixed with 4% paraformaldehyde in PBS for 45 minutes on ice, frozen, sectioned at 50 μm and stained with 1:100 Alexa-fluor-564-phalloidin (Molecular Probes) and 1:500 mouse anti-NF160 (Sigma) followed by anti-mouse IgG_1-Alexa-647 and TOTO3 (Molecular Probe).

EYFP-positive nuclei in the statoacoustic ganglia were counted using a Nikon E800 microscope with 10× objectives, and selected nuclei were analyzed three-dimensionally to judge anatomical identity using MRC1024 confocal microscope with 60× objective in *z*-series optical sections.

Results

VSV-G pseudotyping markedly improves the efficiency of retroviral infection in chicken otic epithelium

One of the major goals of the present study was to look for possible lineage relationships between neurons and mechanosensory organs of the inner ear. Of necessity, then, we wished to target progenitors before the neuroblasts delaminate from the otic epithelium. In the chicken, neuroblast migration begins at the otic cup stage, with most cells emigrating between stage 14 and stage 21 (Adam et al., 1998). We were unable to successfully infect chicken otocysts at the earliest stages of neuroblast delamination using replication-defective avian retroviral vectors as lineage tracers (T. Zenner and D.M.F., unpublished). By comparison, retroviruses pseudotyped with the G-protein coat of *vesicular stomatitis virus* (VSV-G) are reported to infect neural progenitors earlier, and at much higher efficiency, in both mice and chickens (Chen et al., 1999; Gaiano et al., 1999). We sought evidence that this might also be true for placodes.

We generated a pseudotyped virus stock carrying a VSV-G envelope around the *Rous Sarcoma Virus* (RSV)-based protein core. Packaged within this were two different replication-defective viral genomes, CHAPOL and RDlac encoding human placental alkaline phosphatase (AP) and β-galactosidase, respectively. We compared results using this pseudotyped mixed virus stock to a stock carrying virions of similar genotypes coated instead with the RSV A-subgroup envelope protein. However, the two virus stocks differed nearly sevenfold in concentration, with the A-subgroup stock having the higher titer (6.7×10^6 versus 1×10^6 infectious units/ml).

Viruses were delivered to the otic cup or vesicle in 147 embryos. For each virus subgroup, we examined 3-41 embryos per stage (mean=15 embryos per stage). Ears were scored as positive if they showed histochemical staining within the otic epithelium or its ganglia for either AP or β-galactosidase on embryonic days 7-10 (E7-E10). Injections with G-subgroup viruses at stages 14, 15, 16, 17 and 18 yield positive ears in 17%, 57%, 40%, 85% and 100% of cases, respectively. By comparison, A-envelope viruses of higher titer delivered at the same stages yield clones in 0%, 0%, 11%, 33% and 70% of injected ears. These results indicate that VSV-G pseudotyped viruses are considerably more effective as reagents for lineage analysis in the chick inner ear.

Distribution of AP+ cells

For lineage analysis, we targeted the ear as early as possible to try to generate large and potentially more varied clones. We used CHAPOL(G) virus at titers of 1×10^6 i.u./ml (131 embryos) or 4.5×10^6 i.u./ml (147 embryos). Virus was delivered near the otic cup at stages 9.5-14.5, and the ears were processed on E10-13. Out of 278 injected ears, 80% have at least one AP-positive (AP+) cell in the paratympanic organ, inner ear epithelium or the associated ganglia.

AP+ cells are unevenly distributed throughout the various subdivisions of the inner ear, as summarized in Fig. 1. They are found most frequently in the non-sensory part of utricle and the endolymphatic apparatus, including both the duct and the sac. We separately scored AP+ cells located in sensory versus non-sensory regions of the major anatomical subdivisions of the ear. In total, 18% of injected embryos have sensory clones. The frequency of infecting any single sensory organ varies as follows: paratympanic organ>basilar papilla>utricular macula>superior crista>saccular macula>lagenar macula> posterior crista>lateral crista. There is no obvious variation in the sensory organ labeling with respect to the stage of injection, with the possible exception of the lagenar macula, which is labeled only by early injections (stage 10.5-11). The statoacoustic ganglia are labeled with moderate efficiency at all injection stages, as are non-sensory cells in the remaining parts of the labyrinth. Owing to the method of injection, infected cells are not restricted to the inner ear, but can also include other ectodermal derivatives. For example, AP+ cells are found in the epidermis, the trigeminal ganglion, the geniculate ganglia and the paratympanic organ.

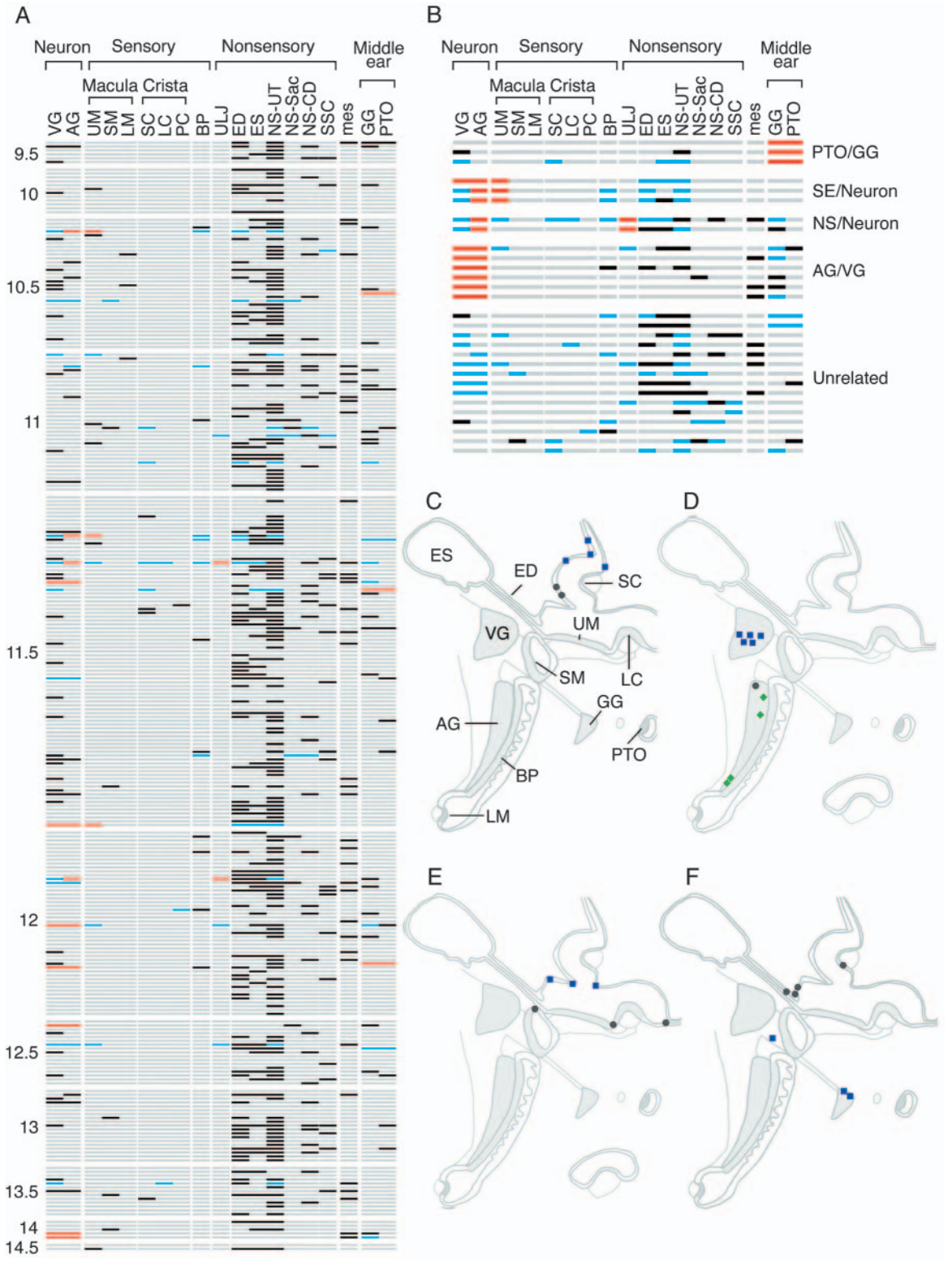
In previous lineage studies in chicken embryos, it has been estimated that the average delay between the stage of virus injection and the time of viral integration is ~15 hours (Fekete et al., 1994; Fekete et al., 1998). Using the same estimate, we expect that injections at stages 9.5-14.5 will infect otic progenitors at stages 12-17. Lineage data were primarily obtained from injections at stages 10.5-12 that infect progenitors approximately at stages 13-16. This is well within the time when otic neuroblasts are actively delaminating. We thus expect that many of the labeled neurons are derived from progenitors that were infected while they were still resident within the otic cup. Occasional evidence of mesenchymal infection in 30% of the ears indicates the virus may be delivered deeper than the ectoderm, possibly labeling some neuroblasts after they delaminate. However, as most (46/64, 72%) of the ears with neuronal clones do not also show evidence of mesenchymal infection, the majority of the neuronal lineages marked in this study probably originate from progenitors infected before delamination.

Clones are usually restricted to distinct anatomical parts of the inner ear

The virus stock, CHAPOL(G), is a library of replication-incompetent vectors that each carry a 24 bp tag in addition to the AP gene. Clonal analysis of infected ears was performed by amplifying and sequencing the 24 bp variable fragment independently from one or a small cluster of AP+ cells. We selected 29 ears of greatest interest, primarily because they contained AP+ cells in both sensory organs and ganglia. From these, 629 small pieces of tissue (picks) containing one or more AP+ cells were removed for analysis. Slightly less than half (306 picks) were successfully amplified by PCR and sequenced. By comparison, PCR product was amplified from only three out of 52 AP-negative picks chosen based on proximity to AP+ tissue. Among the sequenced PCR products, 40 contain more than one library sequence and were not considered further. Multiple sequences are thought to originate from progenitor cells that accept more than one virion. In total, sequencing of AP+ picks yielded 139 unique sequences or clones. None of the picks has an identical sequence with picks

from other ears, suggesting that viruses carrying any single tag are not over-represented within the library.

The distribution of successfully amplified picks and the resulting clonal relationships are shown schematically in Fig. 1B, with specific examples in Fig. 1C-F. Of the confirmed clones, the vast majority (124/139) do not extend beyond a single anatomically defined structure in the inner or middle ears. The 15 clones identified as having members colonizing more than one structure will be discussed in detail later. In summary, three out of the 15 are associated with the middle ear and geniculate ganglion. Five clones show members both in the inner ear and one or both of its ganglia and seven clones are dispersed across the two otic ganglia. Among clones restricted to the inner ear epithelium, only a single clone



contains cells located in two anatomically distinct structures. In this case, clonally related cells are present in the superior ampulla and the adjacent part of the superior semicircular canal (Fig. 1C). In all other ears, AP+ cells in different anatomical parts of the inner ear are clonally independent, even if they are located in close proximity to each other.

Despite the early injection paradigm, only 14% (39 of 278) of ears have AP+ cells located in inner ear sensory organs. Only a fraction of those, a scant four ears, has AP+ cells in multiple sensory organs. Successful sequencing of three of these cases failed to indicate a clonal relationship between different sensory organs (basilar papilla and utricular macula, n=2; basilar papilla, utricular macula, superior crista and lateral crista, n=1; see Fig. 1B). The probability of finding more than two independent viral infections in sensory epithelia can be estimated using Poisson regression analysis with the assumption of independence of each sensory organ (not shown). The estimated frequency of 1.35% matches the observed frequency of 1.40%. The distribution of inner ear sensory clones supports the hypothesis that progenitors colonizing each sensory organ may already be separated by the time the proviral DNA integrates into the host cell genome.

Neurons and sensory organs can share a common progenitor in the middle ear

Sixteen ears have AP+ cells in the paratympanic organ. Nine out of these (56%) also have AP+ cells in the geniculate ganglion that resides immediately adjacent to the organ and is the source of its innervation (Fig. 1). We successfully tested the clonal relationships in five of them, and found three cases where AP+ cells in the paratympanic organ and geniculate ganglion are clonally related (Fig. 2). If we extrapolate the sequencing results to include the unsuccessful cases, we estimate that approximately one-third (5.4/16) of ears with infection of the paratympanic organ will show evidence of a shared neurosensory lineage. No clonal relationship was found between the geniculate ganglion and either the inner ear or the statoacoustic ganglion in nine tested cases. In one case (Fig. 1F), neuronal cells in the facial nerve (between the geniculate and vestibular ganglia) are clonally related to the cells in geniculate ganglion. We have no samples where AP+ cells spanned both the sensory and non-sensory epithelium of the paratympanic organ (not shown). We conclude that on or shortly after E2, there are multipotent epibranchial placode progenitors that give rise to both sensory cells and neurons.

Fig. 1. Distribution of AP+ cells in all 278 ears (A), all sequenced picks from 29 ears (B) and representative infected ears (C-F). (A,B) Each row indicates an individual ear, sorted (A) by stage of virus delivery, shown along the left edge, and (B) by clonal relationships of interest. Vertical columns depict the parts of the ear. Within each row, gray regions are AP negative and black regions have one or more AP+ cells that did not yield clonal information (either because of lack of PCR product or because the samples were not picked). Red regions have one or more sequenced picks that are related to sequenced cells located in another region. In other words, the red bars show the distribution of clones with dispersed members. Blue regions have at least one sequenced pick that is not related to picks from other regions of the same ear. (C-F) Schematics showing the distribution of clones for four individual ears. Similar schematics are shown in subsequent figures. Within each ear, different symbols depict separate clones. Grey circles indicate sequenced picks unrelated to any other picks; i.e. each grey circle is a separate clone. Ears are oriented with medial to the left and dorsal to the top. Left ears are flipped for uniform orientation. Unsequenced AP+ cells are not indicated. Abbreviations: AG, acoustic ganglion and nerve; VG, vestibular ganglion and nerve; UM, utricular macula; SM, saccular macula; LM, lagenar macula; SC, superior crista; LC, lateral crista; PC, posterior crista; BP, basilar papilla; ULJ, utricle-lateral ampulla junction; ED, endolymphatic duct; ES, endolymphatic sac; NS-UT, non-sensory part of the utricle; NS-Sac, non-sensory part of the saccule; NS-CD, non-sensory part of the cochlear duct; SSC, semicircular canal and ampulla; mes, periotic mesenchyme; GG, geniculate ganglion; PTO, paratympanic organ; SE, sensory epithelium; NS, non-sensory epithelium. Cells in the proximal facial ganglion are included in VG due to difficulty in distinguishing the two structures.

Neurons and sensory organs can share a common progenitor in the inner ear

Nearly 90% of the clones appear to be restricted to a single anatomical compartment. One major exception is a class of clones including cells in the utricular macula. In three out of eight tested cases, cells in this vestibular sensory organ are related to cells in the statoacoustic ganglia (Fig. 3). In total, 23% (3/13) of all ears containing utricular sensory clones show evidence of a shared neurosensory lineage.

The clone shown in Fig. 3A, red triangles, has a particularly large number of cells. It includes two picks from the vestibular ganglia, six picks from the acoustic ganglia and seven picks from the utricular macula. The AP+ cells in both ganglia are judged to be neurons because they have plump nuclei, generous cytoplasm and/or a labeled process (Fig. 3E,F), and their cell bodies are neurofilament positive (not shown). Our criterion for judging AP+ epithelial cells as sensory is based on their location relative to neurofilament-labeled processes (Fig. 3H′). The AP+ sensory cells of this clone are distributed as two major clusters across the utricular macula (Fig. 3D,G). One cluster is arrayed very close to the medial edge of the macula. Here, the cells disperse along the anteroposterior axis of the organ, but do not cross over into the non-sensory territory. The other cluster of AP+ cells spans about 200 μm in diameter and is located more laterally at ~85% of the width of the macula. We were interested in whether this cluster might span the striola, where hair cell polarity reverses. To address this, we compared this specimen with uninfected ears of the same age that were sectioned in the same orientation and stained for GATA3 immunoreactivity to locate the striola (data not shown). We conclude that the two separate clusters of clone A were both restricted to the medial side of the striola (shown as a black curve in Fig. 3D). Another 10 ears had smaller numbers of AP+ cells in the utricular macula that were all located medial to the striola. These results are consistent with the presence of two possible lineage boundaries, one that bisects the utricular macula along the center of the striola, and a second located at the most medial extreme edge of the sensory patch.

Neurons and non-sensory epithelium can share a common progenitor in the inner ear

Although some otic neurons can be related to the utricular macula sensory organ, we also found that they can share a lineage with non-sensory cells adjacent to the utricular macula. In two out of three tested ears, cells in the acoustic ganglion

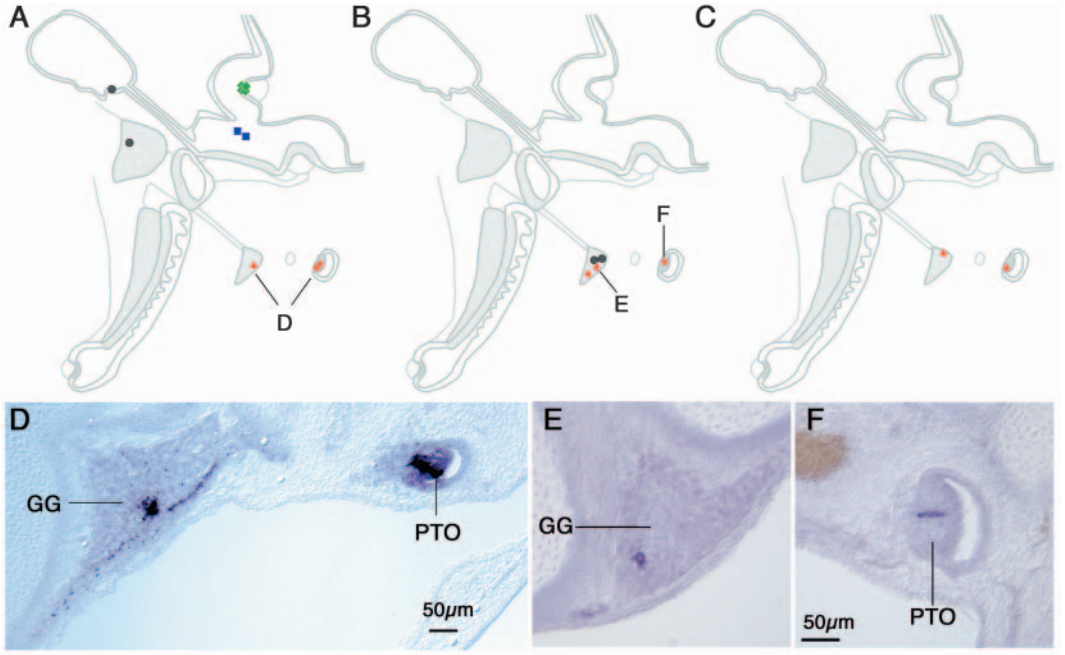


Fig. 2. Neurons and sensory cells are related in the middle ear. (A-C) Three examples containing a clone (red triangles) with members in both the geniculate ganglion and the paratympanic organ. See legend to Fig. 1 for details. Grey circles indicate sequenced picks unrelated to any other picks; i.e. each grey circle is a separate clone. (D-F) Histological images of two clones. Transverse sections with medial leftwards and dorsal upwards. GG, geniculate ganglion; PTO, paratympanic organ.

are clonally related to non-sensory epithelial cells residing between the utricular macula and the lateral crista (Fig. 4). We refer to this region as the utricle-lateral ampulla junction (ULJ). Its identity as non-sensory is indicated by the absence of neurofilament-positive nerve fibers penetrating into the epithelium (not shown). Except for this region, we found no other clonal relationships between neurons and non-sensory epithelium (n=10, Figs 1 and 2). Furthermore, in no case are non-sensory regions related by lineage to sensory organs.

Independent lineages of neurons and glia in the inner ear ganglia

Using retroviral vectors, we have shown that sensory cells in the chicken inner ear can be clonally related to cells in the otic ganglia. We assume the ganglion cells are neurons, and in many cases this is obvious from their morphology. However, there is another cell type in the ganglion, the satellite cell, that sends a thin rim of cytoplasm around the soma of a ganglion neuron (Fig. 5A). Because of the dispersed nature of the AP reaction product, in certain cases it can be difficult to differentiate between labeling of a ganglion neuron and labeling of its satellite cell. Although chick-quail transplant data showed that these glial cells arise from the neural crest rather than the otic placode (D'Amico-Martel and Noden, 1983), we wished to confirm this using another method.

We used electroporation of plasmid encoding EYFP to label only ectodermally derived cells of the otic cup. To observe EYFP expression after the neurons and satellite glial cells were differentiated, we designed a plasmid vector that was retained in transfected cells for at least a week. This vector has the following features: a *Gal-4*/UAS cassette to enhance expression, *EYFP* fused with *Histone2B* for nuclear localization to facilitate double-labeling with antibodies and OriP/*EBNA-1* viral replicon for episomal replication and segregation to each daughter cell (Fig. 5B).

On E9, electroporated ears have EYFP+ cells that are widely distributed in the ear epithelium, ganglia and nearby epidermis on the right side. The *Gal4*-UAS cassette made it considerably easier to distinguish EYFP+ cells from negative cells in comparison with cells labeled with plasmids lacking the *Gal4*-UAS cassette (data not shown). No EYFP+ cells are found in the periotic mesenchyme, confirming that plasmid does not penetrate beyond the surface ectoderm under these electroporation conditions.

Nine out of 15 specimens contain EYFP+ cells in the statoacoustic ganglia. These ears were processed for fluorescent labels to distinguish neurons from satellite cells. The cytoplasm of satellite cells (and Schwann cells) is rich in F-actin and can be readily stained by phalloidin conjugated to a fluorochrome. By contrast, neuronal somas have a paucity of F-actin but instead are stained by anti-neurofilament-160 (Fig. 5C). We used confocal microscopy to observe 128 EYFP+ cells in these ganglia. The vast majority (n=122) clearly have a neurofilament-enriched cytosol surrounding their EYFP+ nucleus (an example in Fig. 5D), while none of the remainder are heavily stained by phalloidin. These data provide independent confirmation that only the neurons, and not their associated glial cells, arise from the otic placode. Therefore, from our retrovirus lineage study, we can conclude that the AP+ cells in the otic ganglia found to be clonally related to other otic placode derivatives, such as the inner ear epithelial cells, are indeed neurons and not glial cells.

Acoustic and vestibular ganglion neurons can share a common progenitor

In 28 ears with AP+ cells in the acoustic ganglion, 22 ears (79%) have AP+ cells in the vestibular ganglion. This strong correlation indicates the possibility that a shared progenitor pool populates these two ganglia. AP+ cells in both ganglia were picked from 15 ears and analyzed for clonal relationships. In seven ears, we could confirm that single clones were dispersed across the acoustic and vestibular ganglia. Three of these are shown schematically in Fig. 5E-G (red triangles in

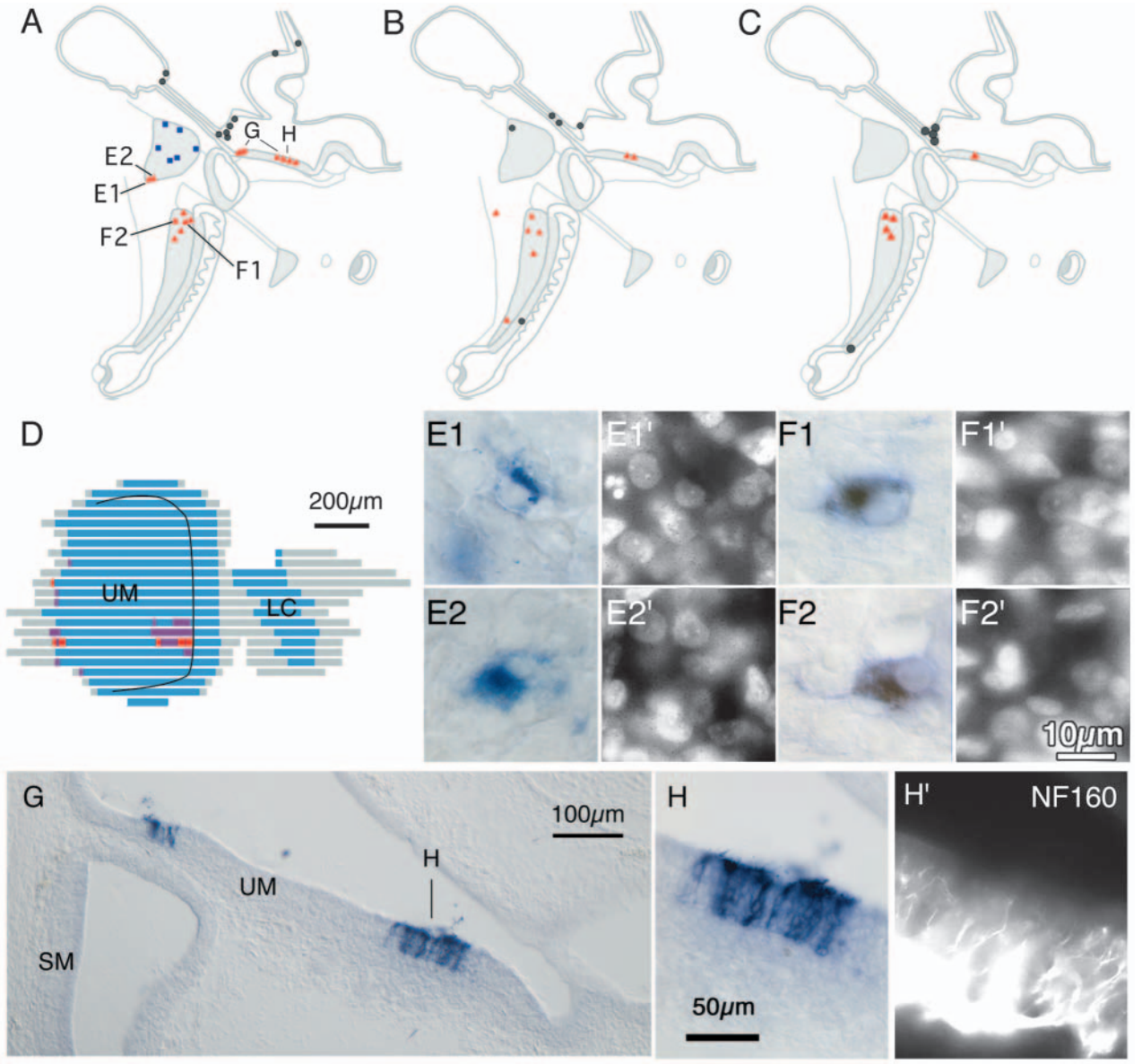


Fig. 3. Neurons and sensory organs are related in the inner ear. (A-C) Three cases with clones composed of neural and sensory progeny (red triangles). E1,F1, etc. indicate the subsequent histological panels showing the red clone. (D) Reconstruction from serial sections, with AP+ sequenced picks (red) and AP+ cells not sequenced (purple) overlaid on the sensory parts (blue) of the utricle and lateral ampulla. Medial is leftwards and anterior is upwards. The approximate location of the striola is indicated by a curved black line. (E1-F2′) AP+ cells in the vestibular (E1-E2′) and auditory (F1-F2′) ganglia, respectively. Adjacent panels show Hoechst nuclear staining. (G) Transverse section containing AP+ cells in both medial and lateral parts of utricular macula, oriented as in A, with higher magnifications in H (AP staining) and H′ (NF160 immunostaining). UM, utricular macula; LC, lateral crista; SM, saccular macula.

each panel). Another is the ear shown in Fig. 3A that also has utricular macula members.

There were 22 ears with AP+ cells in both ganglia. If we extrapolate from our successful sequencing data, ~10 of these will have clones that disperse across both ganglia. The other 12 should have clones that are restricted to each ganglion (e.g. Fig. 1D). Six ears have clones in the acoustic but not vestibular ganglion and 36 ears have clones in the vestibular but not acoustic ganglion. In total, among the ears displaying neuronal clones, we estimate that 84% (54/64) will have clones that remain confined to individual ganglia.

The avian acoustic ganglion is unusual in that it includes vestibular neurons projecting to the lagenar macula (Fischer et al., 1994; Kaiser and Manley, 1996). We therefore had to consider the possibility that some of the AP+ neurons located in the acoustic ganglia might be lagenar-projecting vestibular neurons. Electron microscopic analysis (Fischer et al., 1994) and tract tracing of lagenar afferents back to their cell bodies of origin (A. Campero, T.S. and D.M.F., unpublished) both indicate that lagenar and acoustic ganglion neurons do not spatially overlap. Rather, lagenar-projecting neurons are located just beyond the medial edge of the acoustic ganglion, within the large nerve bundle traveling between the lagenar macula and the vestibular ganglion. Auditory neurons are

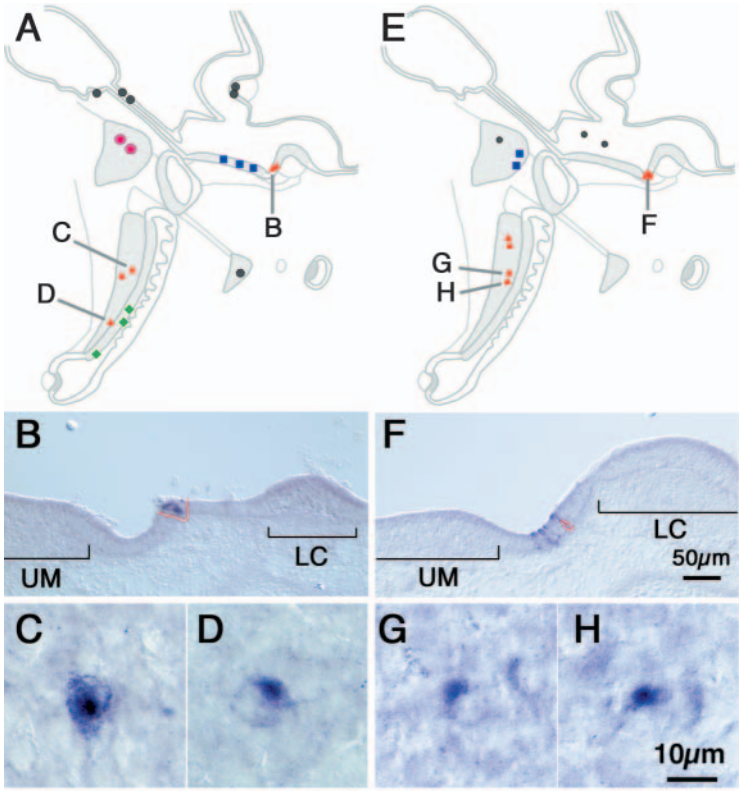


Fig. 4. Neurons and non-sensory cells are related in the inner ear. (A-D and E-H) Two separate examples. Each ear contains a clone (red triangles) containing AP+ cells in the acoustic ganglion and the non-sensory epithelium between the utricle and the lateral ampulla, the ULJ. Higher-power images depict the epithelial (B,F) and neuronal (C,D and G,H) members of the clones. Brackets indicate the positions of sensory epithelia. UM, utricular macula; LC, lateral crista.

distributed throughout the entire acoustic ganglion. We conclude that most of the AP+ cells in the acoustic ganglia that share a lineage with vestibular cells are, in fact, auditory neurons, based on their spatial distribution within the ganglion. This indicates that vestibular neurons and auditory neurons can be clonally related, and that auditory neurons can be related to cells in a vestibular sensory organ.

Discussion

Clonal relationships between neurons and the sensory organs that they innervate

A schematic summarizing the lineage relationships observed in the present study is shown in Fig. 6A. Lineage studies can address the issue of whether neurons tend to make synaptic connections with their clonal relatives in mechanosensory systems in vertebrates. The question arises, in part, because in these systems the peripheral sensory components and the peripheral neuronal components both originate from a single source – an ectodermal placode. The paratympanic organ of the bird middle ear is representative of this class of organs. We confirmed that the paratympanic organ could share a lineage with neurons in the geniculate ganglion that supplies its innervation. However, we were unable to trace the peripheral dendrites of the labeled neurons sufficiently far to determine whether or not they terminate beneath the clonally related hair cells. Thus, it remains formally possible that geniculate neurons and the precise hair cells they innervate in the paratympanic organ may not be direct descendants of a common progenitor.

In the inner ear, a clonal relationship between functionally connected cells could not be confirmed for the majority of sensory clones. We find that the overwhelming majority of neurogenic progenitors generate only neurons (59/64 clones, 92%). Only a few of them generate both epithelial and neuronal progeny (5/64 clones, 8%). The epithelial cells in these neurosensory lineages are remarkably restricted in location either to the sensory epithelium of the utricle or to the non-sensory epithelium between the utricle and lateral crista. Even when utricular macular cells are related to the neurons, in two out of three cases their clonal relatives are auditory neurons rather than vestibular. Furthermore, auditory and vestibular neurons are themselves related by lineage in a subset of clones. Obviously, neurons of these two classes will innervate separate sensory patches. Therefore, the hypothesis that axonal targeting in the inner ear is mediated by lineage relationships between neurons and sensory cells appears unlikely.

Distribution of neurogenic and prosensory domains

One of the outstanding lineage questions for inner ear development has been whether neurons and sensory organs share a common progenitor. A shared lineage was proposed based on homology to mechanosensory organs in flies, where neurons, mechanoreceptors and their support cells all arise from a single sensory organ progenitor (SOP) cell (Adam et al., 1998). Early during ear development, both lineages express some of the same neuroblast-associated proteins, including the LIM-homeodomain protein, Islet1, in the mouse (Radde-Gallwitz et al., 2004) and the homoebox transcription factor, Prox1, in the chicken (Stone et al., 2003). Further circumstantial evidence of a shared lineage can be deduced from gene expression studies showing spatial and temporal overlap between neurogenic and prosensory markers (Fig. 6B). The neurogenic genes, *neurogenin1* and *NeuroD*, identify a broad neurogenic domain in the ventral otocyst, although they are excluded from the most posterolateral region, which is occupied instead by cells expressing an inhibitor of neural fate determination, *Tbx1*, in the E9.5 mouse (Raft et al., 2004). By E10, neurogenic gene expression is largely co-extensive with expression of *Lunatic fringe* (*Lfng*), a gene that may identify a sensory-competent zone (Cole et al., 2000). Only in the most posterior part of the vesicle does *Lfng* expression extend beyond the neurogenic domain (Fekete and Wu, 2002; Raft et al., 2004). In the chicken, the combined expression domains of *Lfng* and *Serrate-1* define a putative sensory-competent region (Cole et al., 2000). This region, and later the prosensory patches, are also marked by the cell-adhesion molecule BEN (Goodyear et al., 2001). *Lfng* is co-expressed with *Fgf10*, a gene that acts upstream of *neurogenin1* to regulate the size of the neurogenic domain in the chicken (Alsina et al., 2004). Thus, in both the mouse and the chicken, there is evidence that neurogenic and the presumed sensory-competent domains are mostly co-extensive.

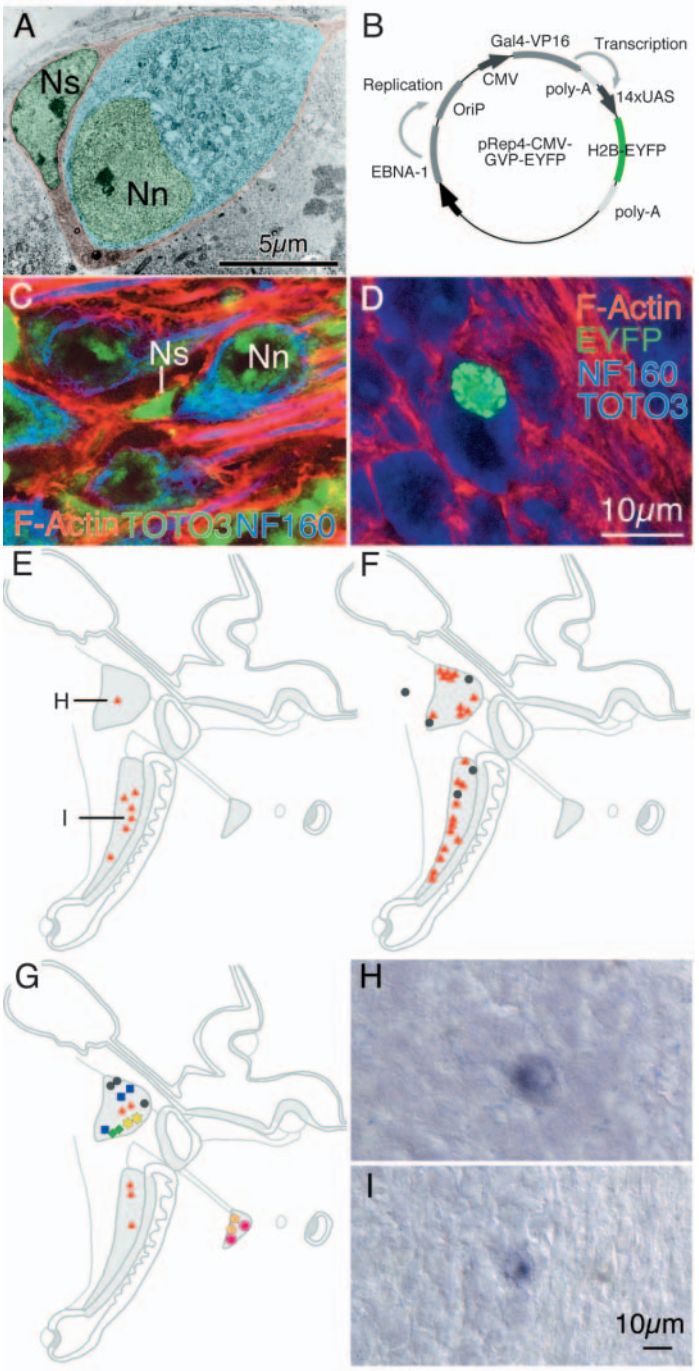


Fig. 5. Neurons in the auditory and vestibular ganglia are related, but satellite cells are not. (A-D) Satellite cells in the statoacoustic ganglion do not originate from the otic cup. (A) Electron micrograph of the acoustic ganglion at E10. False-coloring shows nuclei of a neuron (Nn) and a satellite cell (Ns) in green, the cytosol of the satellite cell in red, and the cytosol of the neuron in blue for ease of comparison to the fluorescent images in C and D. (B) Schematic diagram of the plasmid used for electroporation. (C) Confocal micrograph of E10 vestibular ganglion triple-labeled and false-colored for neurofilament 160 immunoreactivity (blue), phalloidin (red) and TOTO3+ DNA (green). (D) Confocal micrograph of E9 vestibular ganglion neurons labeled by electroporation. EYFP-positive nuclei (green), phalloidin (red), TOTO3 (blue) and neurofilaments (blue) are labeled. (E-G) Three ears with clones (red triangles) that span the two ganglia. (H-I) Histological detail of the picks indicated in E.

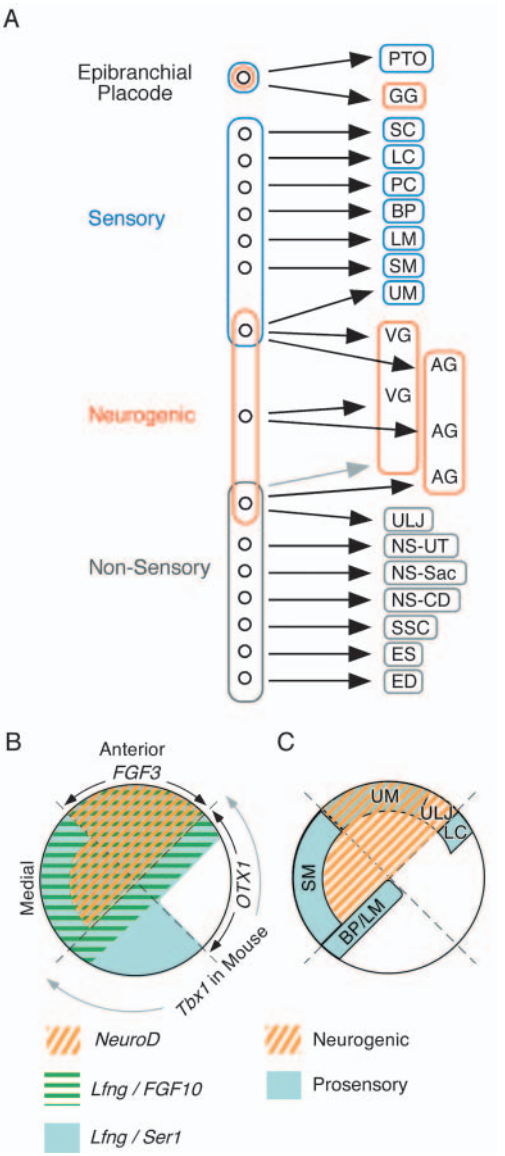


Fig. 6. Summaries of: (A) observed lineages from otic cup injections; (B) gene expression patterns used to define neurogenic and sensory-competent regions on the ventral floor of the otic cup/otocyst of chicken and mouse; and (C) predicted fate map looking down onto the ventral floor of the otic cup/vesicle of the chicken, showing prosensory, neurogenic and non-sensory (white) domains. The primordia of the anterior and posterior crista are not shown in C because they would be expected to be located slightly more dorsally than the other primordia (Cole et al., 2000). Abbreviations: AG, acoustic ganglion and nerve; VG, vestibular ganglion and nerve; UM, utricular macula; SM, saccular macula; LM, lagenar macula; SC, superior crista; LC, lateral crista; PC, posterior crista; BP, basilar papilla; ULJ, utricle-lateral ampulla junction; ED, endolymphatic duct; ES, endolymphatic sac; NS-UT, non-sensory part of the utricle; NS-Sac, non-sensory part of the saccule; NS-CD, non-sensory part of the cochlear duct; SSC, semicircular canal and ampulla; mes, periotic mesenchyme; GG, geniculate ganglion; PTO, paratympanic organ.

How much of the sensory-competent zone actually generates sensory organs rather than neurons remains unresolved in the absence of a high-resolution fate map of the region. In general, gene expression data suggest that only a subset of sensory-competent cells will ultimately generate sensory organs in the chicken ear. A comparison of *Lfng* and/or *Serrate-1* as sensory-competent markers with *Bmp4* (Cole et al., 2000) and *Prox1* (Stone et al., 2003) as prosensory markers suggests that the first sensory primordia are specified near the anterior and posterior poles of the sensory-competent zone. The sensory primordia then arise sequentially in discrete regions of the sensory-competent domain.

It is in the context of these prior studies that we consider our lineage data, and offer a predicted fate map for the neurogenic, sensory-competent and prosensory primordia (Fig. 6C). The last we define as the regions within the sensory-competent zone that will ultimately form individual sensory organs. Within the sensory-competent domain, the prosensory regions map mostly along the edges. Neurogenic progenitors fill in the anterior and central part of the sensory-competent region, overlapping with prosensory cells only in the region of the primordial utricular macula, as suggested by our lineage data. This location is interesting in view of a previous report that ongoing delamination of neurons from the utricular primordium was observed as late as stage 27 in the chicken (Stone et al., 2003). There is also overlap of the neurogenic zone with non-sensory cells in the ULJ; we presume these cells arise from within the sensory-competent zone, but fail to acquire a sensory fate.

It is still unclear whether the primordium of the basilar papilla and lagenar macula (BP/LM) is also neurogenic. Previous studies indicate that most neuroblasts delaminate from ventral pole of otic vesicle (Adam et al., 1998; Alsina et al., 2004; Stone et al., 2003), which is close to or overlapping with the primordial BP/LM (Cole et al., 2000). However, we failed to find evidence of clonal relationships between acoustic sensory epithelium and the statoacoustic ganglia in four successfully tested ears. We can add to these data another five ears derived from a previous lineage study performed at otic vesicle stages (Lang and Fekete, 2001). Because of the small number of samples, we cannot exclude the possibility of a shared lineage between these two auditory tissues. It may be that neurogenic progenitors are lineally separated from the primordial BP at an early stage but remain in the epithelium for several days. They would then be intermingled with sensory precursors when they begin to delaminate. Without clear evidence that this is the case, in Fig. 6B we place the BP/LM primordium beyond the neurogenic domain in the posterior part of the otic cup.

Both shared and separate lineages give rise acoustic and vestibular ganglion neurons

It has been suggested that auditory and vestibular neuroblasts might arise from separate developmental compartments in the medial versus lateral halves of the ventral otocyst (Fekete and Wu, 2002). Gata3 expression in the medial half of the neurogenic region (Lawoko-Kerali et al., 2004), combined with loss of the spiral ganglion but not the vestibular ganglion in the Gata3-null (Karis et al., 2001), suggests that the two ganglia may be derived from separate pools of progenitors in the mouse otocyst. The majority of our neural lineage data supports this idea for the chick ear: we estimate that 84% of ears with infected neurons contain clones that colonize either acoustic or vestibular ganglia, but not both. On the other hand, the segregation of the two pools must be incomplete, as we estimate that the remaining 16% of ears with neural clones will contain a clone that spans both ganglia. Such clones are generated from progenitors uncommitted to auditory or vestibular ganglion fate at the time of infection. We do not know how long these progenitors remain in the otic ectoderm after infection, whether their progeny might be dispersing within the plane of the epithelium, or when their progeny become committed relative to when they delaminate. Nonetheless, the presence of clones that colonize both ganglia suggests that the neurogenic region is not bisected by a strict lineage (or compartment) boundary according to auditory versus vestibular fates, at least up to stage 16, or ~15 hours after the latest time (stage 14) at which virus injection yielded bipotential clones.

Dispersion of clonally related cells in the otic epithelium

In the medial wall of utricle and in the endolymphatic apparatus, the sizes of AP+ clusters are relatively small, typically fewer than five cells (data not shown). In other regions of the inner ear, clusters typically contain a larger number of cells that can be somewhat dispersed within a single anatomical subdivision (Fig. 1C,E; data not shown). For example, some clones in the utricular macula and the BP contain more than 50 scattered cells, although their dispersion appears to be restricted by the borders formed with surrounding non-sensory epithelium (e.g. Fig. 3D, Fig. 4A). In the context of these regional differences in clone size, we note that the dorsomedial wall of the otic vesicle, which generates the endolymphatic apparatus, has substantially lower mitotic activity than the medial and ventral walls at stage 16-28 (Lang et al., 2000). Thus, regional differences in the proliferation potential of otic progenitors may explain much of the variation in size and dispersion of individual clones

Conclusion

In summary, we report the first systematic lineage studies initiated at the time of neuroblast emigration from the cranial placodes of the middle and inner ears. Even at these early stages, there was little dispersion of clonally related cells across anatomical subdivisions of the inner ear. Neurogenic progenitors were the exception, as they could disperse across both auditory and vestibular ganglia, and they could have clonal members that remained behind in the sensory epithelium in both middle and inner ears. These data demonstrate that bipotential neurosensory progenitor cells are present in cranial placodes.

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