

Class III β -Tubulin Expression in Sensory and Nonsensory Regions of the Developing Avian Inner Ear

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ABSTRACT

A previous study showed that class III β -tubulin, a widely used neuron-specific marker, is expressed in mature and regenerating hair cells but not the support cells of the avian inner ear. We investigated the expression of this marker in the developing avian inner ear. We found that class III β -tubulin is not neuron-specific in the avian embryo, but appears to accumulate in neuronal cell types, including hair cells, about the time of their differentiation. In the developing inner ear, some degree of class III β -tubulin immunoreactivity is found in all regions of the otic epithelium from its formation as the otic placode (stage 10 [embryonic day, E1.5]) until about stage 21 (E3.5), when the prospective tegmentum vasculosum begins to lose its staining. By stage 35 (E8-9), most of the nonsensory epithelia have lost their class III β -tubulin staining, leaving distinct regions of staining between the morphological compartments of the inner ear. Concurrent with the loss of staining from nonsensory regions, the hair cells of the sensory epithelia accumulate class III β -tubulin, whereas the supporting cells decrease their staining. We also observed a similar pattern of development in another hair cell organ, the paratympanic organ. Double labeling with the 275 kD hair cell antigen (HCA) indicated that the majority of hair cells identifiable with class III β -tubulin are HCA-positive. Additionally, presumptive hair cells were identified which were not within defined sensory epithelia. Our findings show that class III β -tubulin can be used as an early marker for hair cell differentiation in all hair cell sensory epithelia in the chicken. *J. Comp. Neurol.* 406:183-198, 1999. © 1999 Wiley-Liss, Inc.

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The vertebrate inner ear develops from a neurogenic ectodermal thickening, the otic placode, which invaginates and separates from the ectoderm to form the otocyst. The otocyst subsequently develops into the morphologically complex and multifunctional membranous labyrinth consisting of approximately eight sensory epithelia and at least two specialized nonsensory epithelia (Rubel, 1978; Fritzsche et al., 1998). Several sensory structures, e.g., the cochlea, utricle, and semicircular canals, segregate into distinct spatial domains during development. At least two neuronal cell types are generated by the otic epithelium: the neurons of the acoustic-vestibular ganglion (AVG) which migrate from the otic epithelium to the ganglion (D'Amico-Martel and Noden, 1983), and sensory hair cells. The sensory hair cells appear to be generated within the otic epithelium after the ganglion cell precursors migrate (D'Amico-Martel, 1982; Katayama and Corwin, 1989). We do not know, however, whether the cells migrating to the

AVG originate from the same regions in which sensory hair cells subsequently develop.

The molecules and mechanisms involved in these developmental processes are just beginning to be elucidated (e.g., Oh et al., 1996; Wu and Oh, 1996; Kiernan et al., 1997; Whitfield et al., 1997; Wu et al., 1998; reviewed in Fritzsche et al., 1998). Many molecules are being investigated and have shown distinct expression patterns within the otic epithelium. Bone morphogenetic protein-4 (BMP4), the mouse homolog of the *Drosophila* muscle segment

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homeobox (*msh*) gene, *Msx-1*, and the p75 nerve growth factor receptor (p75NGFR) have been used to elucidate the generation of the individual sensory epithelia (Wu and Oh, 1996), and appear to be biochemically segregated shortly after the otocyst separates from the ectoderm (stages 19–24 [embryonic day E2.9–E4]). Additionally, two homeobox genes, the sensory organ homeobox-1 (SOHo-1) and *Gallus gallus* homeobox protein-6 (GH6), have been found to be restricted to regions that develop into the semicircular canals (Kiernan et al., 1997).

Until recently, the principle distinguishing feature of hair cell differentiation has been the formation of stereocilia. Cotanche and Sulik (1984) showed that chicken hair cells begin to morphologically differentiate at about stage 29 (E6) in the distal basilar papilla (i.e., the chicken cochlea), and that this morphological differentiation proceeds in a distal-to-proximal gradient. More recently, a hair cell marker, the hair cell antigen (HCA), has been found to be expressed contemporaneously with stereocilia differentiation, and used to investigate the differentiation of hair cells and formation of the hair cell mosaic in the developing chick basilar papilla (Bartolami et al., 1991; Goodyear et al., 1995; Goodyear and Richardson, 1997).

Stone et al. (1996a) recently demonstrated that class III β -tubulin is expressed in normal and regenerating sensory hair cells of the posthatch chicken inner ear, and can be used as a marker for the differentiation of hair cells during drug-induced hair cell regeneration. The TuJ1 antibody, which binds to the isotype-defining domain of class III β -tubulin (Lee et al., 1990), has been widely used to study developing neurons and their precursors. This antibody has been used to study the differentiation, distribution, and migration of many neuronal cell types in various regions of the nervous system including the telencephalon (Menezes and Luskin, 1994; Gates et al., 1995), olfactory bulb (Gonzalez and Silver, 1994), optic tectum (Snow and Robson, 1995), and the peripheral trigeminal system (Moody et al., 1989). In particular, Moody et al. (1989) showed that class III β -tubulin is expressed in cells migrating from the trigeminal placodes prior to and during their migration to the trigeminal ganglion.

Given its usefulness as a hair cell differentiation marker in the sensory epithelium of the posthatch chicken, we decided to investigate the expression of class III β -tubulin during the early development of the chicken inner ear. We found that class III β -tubulin is not neuron-specific in the developing avian embryo, but appears to accumulate in neuronal cell types about the time of their differentiation. In the developing inner ear, class III β -tubulin is found in all regions of the otic epithelium from its formation as the otic placode until about stage 21 (E3.5), when the prospective tegmentum vasculosum begins to lose its staining. By stage 35 (E8–9), most of the nonsensory epithelia have lost their class III β -tubulin staining, leaving distinct regions of staining between the utricle and saccule, saccule and cochlear duct, and in the outer circumference of the semicircular canals. Concurrent with the loss of class III β -tubulin staining from nonsensory regions, the hair cells of the sensory epithelia accumulate class III β -tubulin, whereas the supporting cells decrease their staining. We also observed a similar pattern of development in another hair cell organ, the paratympanic organ. Double-labeling with antibodies to HCA and class III β -tubulin indicated that the majority of hair cells identifiable with class III β -tubulin are HCA-positive. Additionally, presumptive hair

cells were identified that were not located within defined sensory epithelia. A preliminary report of a portion of this study has been presented in abstract form (Stone et al., 1996b).

MATERIALS AND METHODS

Fertilized White Leghorn chicken eggs (*Gallus domesticus*) were purchased from H & N International (Redmond, WA) and incubated at 38°C in a humidified, forced-draft incubator. Embryos were staged according to Hamburger and Hamilton (1951). In this paper, the developmental stage of the embryo will be referred to by both the stage and the corresponding age appropriate to the Hamburger and Hamilton staging, e.g., stage 10 (E1.5). A total of 108 embryos (stages 10 [E1.5] to 35 [E8–9]) were used in this investigation. In general, 2–4 embryos at each age were used for observations reported from tissue sections, and 5–11 embryos at each age were used for wholemount preparations. All the procedures were approved by the Animal Care Committee of the University of Washington.

Antibodies

Two antibodies against class III β -tubulin, TuJ1 and β -III, were used in this study. The TuJ1 antibody is a monoclonal antibody that binds the N-terminal, isotype-defining domain of class III β -tubulin (Lee et al., 1990). The β -III antibody is a polyclonal antibody raised specifically against the N-terminal, isotype-defining domain of class III β -tubulin (Moody et al., 1996; A. Frankfurter, personal communication). Labeling patterns and intensities were identical with the two antibodies. Additionally, the HCA antibody was used to label developing hair cells in wholemount preparations. TuJ1 and β -III were obtained from Dr. Anthony Frankfurter (University of Virginia, Charlottesville, VA), and the HCA antibody was obtained from Dr. Guy Richardson (University of Sussex, Falmer, Brighton, UK).

Fixation and immunohistochemistry

In the procedures described below, all washes in phosphate-buffered saline (PBS) were done at pH 7.4, and all washes in Tris were done at pH 7.6.

Sectioned embryos. Embryos used for paraffin sections were immersion-fixed according to one of four fixation protocols: (1) ice-cold 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4 for 5–7 hours; (2) ice-cold 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4/3% sucrose for 21–23 hours; (3) ice-cold methacarn (1 part glacial acetic acid:3 parts chloroform:6 parts absolute methanol) for 4–6 hours; and (4) ice-cold buffered methacarn (1 part glacial acetic acid:2 parts chloroform:6 parts absolute methanol: 1 part 10 \times modified chick Ringers; 10 \times modified chick Ringers consists of 1.54 M NaCl, 60 mM KCl, 50 mM HEPES, 10 mM EDTA at pH 7.4 after Lurie and Rubel, 1994) for 4–5 hours. Whole embryos were removed from eggs and placed directly in fixative where the extra-embryonic membranes were removed. The entire embryo was then transferred to fresh fixative. When the fixation period was complete, paraformaldehyde-fixed tissue was washed with PBS, and methacarn-fixed tissue was washed with 70% ethanol. The embryos were then staged and embedded in paraffin. For embryos older than stage 21 (E3.5), only the head region of the embryo was embedded. Whole-head, transverse sections, 6- μ m- or 10- μ m-thick,

were cut through the hindbrain of the embryos (except for three embryos which were sagittally sectioned). A one-in-five series of sections was mounted onto ethanol/HCl-washed, chrome-alum gelatin-subbed slides, and stained with hematoxylin and eosin in order to identify the otocyst. Sections encompassing the region of the otocyst were mounted onto ethanol/HCl washed, chrome-alum gelatin-subbed slides for immunohistochemistry. For methacarn-fixed embryos which were stage 25 (E4.5) or younger, all remaining sections encompassing the otocyst were used. Additionally, all remaining sections encompassing the otocyst were used for two stage 29 (E6) embryos and one stage 35 (E8–9) embryo. All other tissue was mounted in a one-in-five series.

Sections were deparaffinized, rinsed in 70% ethanol, then incubated for 15 minutes in absolute methanol containing 0.5% H_2O_2 to inhibit endogenous peroxidases. After rinsing and rehydration, nonspecific binding of secondary antibody was blocked with a blocking solution of 4% normal horse serum, 1% bovine serum albumin, 0.1% Triton X-100 in PBS. The sections were then incubated overnight at 4°C in primary antibody, TuJ1 or β -III, diluted to 1:500, 1:1,000, or 1:3,000 in blocking solution. The next day, the sections were washed in PBS, incubated 60 minutes in a 1:400 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), washed in PBS, and treated for 60 minutes with the avidin-biotin-horseradish peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories). The tissue was then transferred to 50 mM Tris, preincubated with 0.375 mg/ml DAB (3,3'-diaminobenzidine; Sigma, St. Louis, MO), and stained by using 0.375 mg/ml DAB/0.01% H_2O_2 /50 mM Tris for 3–10 minutes. The sections were coverslipped with DPX, and examined by using light microscopy with conventional or differential interference contrast optics.

Wholemouted embryos. Embryos used for whole-mount analysis were fixed according to two protocols: (1) direct immersion in ice-cold-buffered methacarn for 1 hour, and (2) direct immersion in 4% paraformaldehyde made with a microtubule-stabilizing buffer, PHEM (50 mM PIPES, 25 mM HEPES, 8 mM EGTA, 2 mM Mg^{++} , 28 mM Cl^- , and 136 mM Na^+ ; Trout et al., 1994) with a final pH of 7.3. These embryos were fixed for 1 hour at room temperature or 37°C to prevent cold destabilization of the microtubules. The embryos were then washed in PBS and staged.

After staging, whole embryos were immersed in ice-cold, 70% ethanol for at least 10 minutes, then immersed in -20°C acetone for 7–10 minutes to improve permeabilization (D. Frost, personal communication). After the acetone, the embryos were reimmersed in ice-cold, 70% ethanol and allowed to warm to room temperature before being rehydrated. (All incubations listed below that are greater than 10 minutes were performed on a nutator at 4°C.) The tissue was then incubated for 2 hours in blocking solution (4% normal horse serum, 1% bovine serum albumin, 0.1% saponin, PBS) with additional saponin added to raise the concentration to 1%, and incubated for 36–48 hours in primary antibody (TuJ1 or β -III) diluted to 1:1,000 in blocking solution. The secondary antibody, a 1:400 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories) in blocking solution, was applied for 36–48 hours. Next, an avidin-conjugated fluorophore (1:750 avidin-Bodipy (Molecular Probes, Eugene, OR) or 1:1,000 avidin-Cy5 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA))

diluted in blocking solution was applied for 24–36 hours. The tissue was then washed, cleared in a graded series of buffered glycerols, and mounted in an anti-fade solution of 1% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma)/90% buffered glycerol/0.2% NaN_3 . The cleared embryos were mounted between two #1 coverslips, and imaged with a BioRad MRC-1024 confocal laser scanning microscope running BioRad LaserSharp software, version 2.1A. The images were imported into and analyzed by using public domain NIH image software (version 1.61; developed at the U. S. National Institutes of Health and available on the Internet at <http://128.231.98.16/nih-image/>), transferred to Adobe Photoshop, and printed with a Tektronix Phaser IISDX dye-sublimation printer.

In order to counterstain only the cell nuclei, some embryos were also treated for 1 hour with a 10- μ g/ml solution of RNase A at 37°C to eliminate binding of propidium iodide to RNA, then incubated with a 10 μ g/ml solution of propidium iodide (Molecular Probes) for 1 hour.

Wholemouted otocysts. Cochlear ducts or otocysts were dissected from embryos fixed with room temperature, 4% paraformaldehyde/PHEM or ice-cold, buffered methacarn as described above for whole embryos, and washed in PBS. The sensory epithelia were treated with 0.5% H_2O_2 in PBS for 15 minutes to block endogenous peroxidase activity. After rinsing with PBS, 10% normal horse serum in 0.05% Triton X-100/PBS was applied for 20 minutes to block nonspecific immunoglobulin binding. For single-labeling experiments, wholemounts were reacted with the TuJ1 monoclonal antibody (1:1,000) for 2 hours at room temperature or overnight at 4°C. Tissue was treated with Bodipy/fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:300; Molecular Probes) for 30 minutes, or biotinylated horse anti-mouse IgG (1:200; Vector Laboratories) for 30 minutes followed by the avidin-biotin-horseradish peroxidase reagent (ABC kit BA-2000; Vector Laboratories). Up to this point, all rinse steps were performed in PBS. The tissue incubated with the horseradish peroxidase (HRP) reagent was transferred to 50 mM Tris and treated with 0.04% DAB/0.05% H_2O_2 /Tris for 3–10 minutes. The tissue was then rinsed once in Tris and stored in Tris until cleared and mounted in 1% DABCO/90% buffered glycerol/0.2% NaN_3 . After treatment with the fluorescent secondary antibody, wholemounts were washed in PBS, then cleared and mounted in 1% DABCO/90% buffered glycerol/0.2% NaN_3 .

For β -III/HCA colabeling, the β -III antibody was detected using Bodipy/FITC-conjugated anti-rabbit IgG (1:300; Molecular Probes), whereas the HCA antibody was detected in the same tissue with Cy5-conjugated anti-mouse IgG (1:300; Jackson ImmunoResearch Laboratories, Inc.).

Controls. The specificity of the antibody and immunohistochemical protocol was determined by substituting nonimmune, mouse sera in blocking solution, or just blocking solution, without addition of primary antibody. Staining of the brainstem (Moody et al., 1989; Lee et al., 1990) and retina (Lee et al., 1990; Watanabe et al., 1991; Snow and Robson, 1995) was used as within-tissue positive controls.

RESULTS

Although very few studies have used the TuJ1 antibody in paraffin-embedded tissue (Katsetos et al., 1993), we

chose to use paraffin sections in order to optimize morphology. Both of the antibodies to class III β -tubulin used in this study, hereafter referred to as TuJ1 for the monoclonal antibody and β -III for the polyclonal antibody, provided specific staining in paraffin-embedded tissue using either paraformaldehyde or methacarn fixation. The staining was judged to be specific because of: (1) the very low background staining observed within experimental sections; (2) the juxtaposition of heavily labeled regions adjacent to regions with an absence of label in either the same tissue (e.g., the otocyst), or between two different tissues (e.g., otic epithelium and otic capsule); and (3) the absence of staining in sections used as negative controls (i.e., incubated with nonimmune mouse sera or blocking solution with no primary antibody added). Labeling was always observed in internal positive control tissues such as retinal ganglion cells (Lee et al., 1990; Watanabe et al., 1991; Snow and Robson, 1995), peripheral nerve fibers (Moody et al., 1989), and the marginal and intermediate zones of the neural tube (Moody et al., 1989; Lee et al., 1990) whenever primary antibody was applied. Both of these antibodies consistently showed the same tissue-specific staining pattern and intensity, and were used interchangeably.

A comparison of paraffin sections from methacarn-fixed tissue against sections from paraformaldehyde-fixed tissue revealed that some regions of the otocyst that are clearly stained with TuJ1 or β -III in methacarn-fixed tissue, are often unstained or just barely stained when the tissue is fixed with paraformaldehyde (Fig. 1). The tissues that label with paraformaldehyde fixation, however, are always a subset of the tissues that label with methacarn fixation. Neuronal elements in both the central and peripheral nervous systems label regardless of fixation protocols. The degree of staining in paraformaldehyde-fixed tissue also appears to be a function of fixation time. Embryos that are fixed for short periods of time (< 7 hours) consistently showed more staining than embryos fixed for longer times (> 20 hours) as shown in Figure 1B and C. These observations suggest that paraformaldehyde fixation, when combined with paraffin embedding, reduces the binding of class III β -tubulin antibodies, resulting in the labeling of only those tissues containing the largest amounts of class III β -tubulin. We did not observe any differences between wholemounted tissue fixed with methacarn or 1 hour fixation with 4% paraformaldehyde.

The following results are based primarily on observations from methacarn-fixed paraffin sections and paraformaldehyde-fixed wholemount preparations. Wholemount preparations were used to examine expression in the youngest embryos (stage 14 and younger) and to look at hair cell differentiation in the inner ear sensory epithelia with and without double labeling with HCA. First, we will present an overview of the pattern of class III β -tubulin expression in the developing membranous labyrinth from stages 10 (E1.5) through 35 (E8–9), and then discuss the development of expression in sensory hair cells and the developing sensory epithelia. We will then address the expression of class III β -tubulin in another hair cell sensory organ, the paratympanic organ, and finally, the neuronal specificity of class III β -tubulin in the developing chick embryo. A later report will deal specifically with AVG cell differentiation and migration.

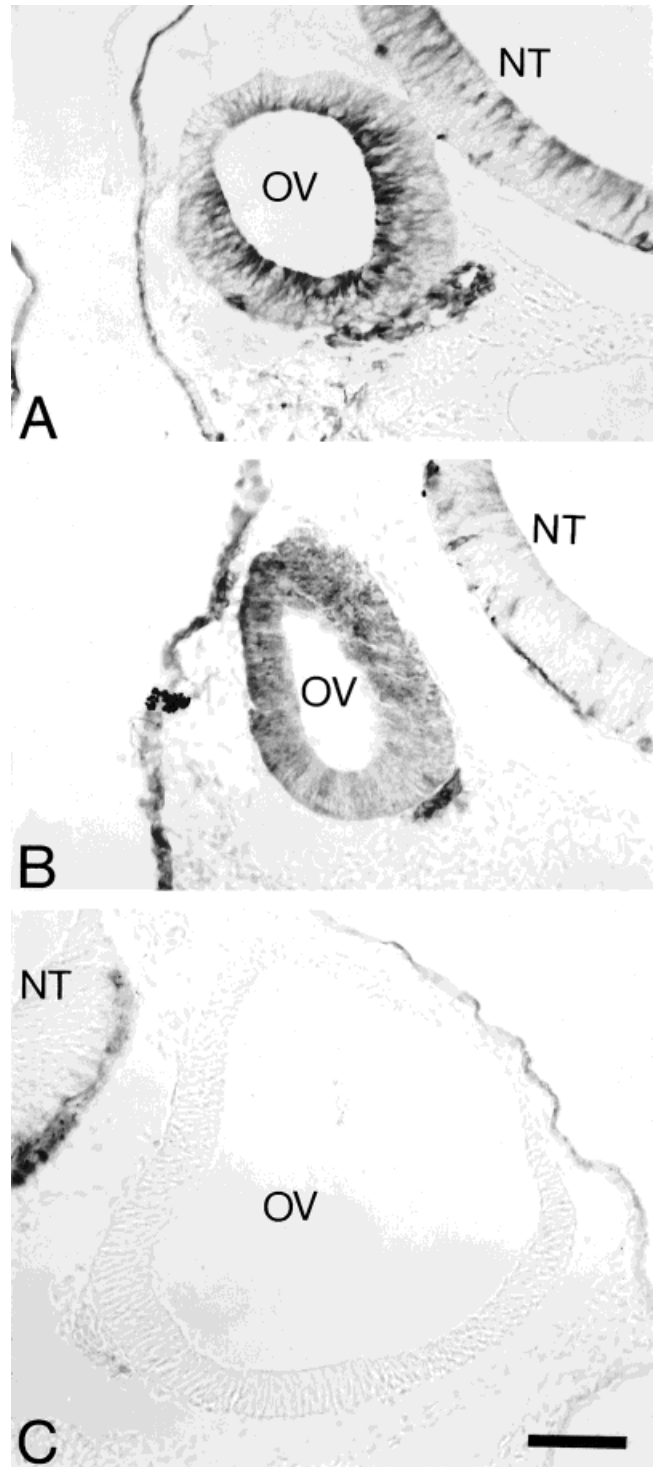


Fig. 1. Effects of fixation on the detection of class III β -tubulin. The overall staining with class III β -tubulin antibodies in the otic vesicle (OV) and neural tube (NT) is less distinct and less intense when the tissue is fixed in paraformaldehyde rather than methacarn, especially for long periods of time. Some neuronal elements stain well regardless of fixation. **A:** Stage 16 (embryonic day, E2.2) embryo fixed for 4 hours in methacarn and reacted with the β -III antibody. **B:** Stage 17 (E2.4) embryo fixed for 6 hours in 4% paraformaldehyde and reacted with the TuJ1 antibody. **C:** Stage 19 (E2.9) embryo fixed for 22.5 hours in 4% paraformaldehyde and reacted with TuJ1 antibody. Dorsal is up in A–C. Medial is to the right in A and B, and to the left in C. Scale bar = 50 μ m for A–C.

Class III β -tubulin expression in the developing inner ear

Figure 2 illustrates the overall staining pattern for class III β -tubulin at stages 10 (E1.5), 12 (E2.0), 16 (E2.2), 21 (E3.5), 25 (E4.5), and 27 (E5) in the otocyst. In the thickened, pseudostratified regions of the otocyst, class III β -tubulin is heavily localized to the apical regions of the cells, resulting in dense staining in the luminal region of the epithelium. Streaks of staining project into the more lightly labeled basal regions of the epithelium, where the cell nuclei are packed tightly together. This dense, luminal staining may be due to the displacement of cytoplasm lumenally by the closely packed, basally located nuclei. A similar pattern was reported by Troutt et al. (1994) using a different anti- β -tubulin antibody not specific for a particular isotype. In many areas that are immunoreactive for class III β -tubulin, the density of the cells and the density of the staining prevents the resolution of individual cells. Thus, we can not determine whether or not all cells are labeled in these regions, but clearly the majority are labeled. In order to avoid repeating a description of this staining pattern, we will use the term "luminal-streaky" to refer to blocks of epithelium which show dense apical staining and streaks of staining into the basal regions of the epithelium.

Frequently, luminal-streaky staining is interrupted by labeled mitotic spindles, especially during the early development of the otocyst (e.g., Fig. 2B and C). In some regions of the otic epithelium, class III β -tubulin-labeled cells are seen next to or crossing the basal lamina. We believe that these cells are prospective ganglion cells migrating from the epithelium and will examine them more closely in another report. At later stages, heavily labeled nerve fibers can be seen penetrating and coursing through the epithelium, but are often indistinguishable from the streaks extending from the lumen when they are oriented radially.

As shown in Figure 2, class III β -tubulin is expressed in the otic epithelium as early as the initial formation of the otic placode (stage 10 [E1.5]; Fig. 2A). From stage 10 (E1.5) to stage 25 (E4.5; Fig. 2B–E), expression of class III β -tubulin is detected in all regions of the otic epithelium. Figure 2D illustrates that, by stage 21 (E3.5), an abrupt reduction in the density of staining can be seen at the ventral tip and along the lateral wall of the nascent cochleosaccular duct, where the tegmentum vasculosum is expected to develop (Knowlton, 1969; Cotanche and Sulik, 1982). By stage 25 (E4.5; Fig. 2E) and stage 27 (E5; Fig. 2F), staining in the lateral wall of the cochleosaccular duct is no longer detectable in the distal region of the duct, but becomes increasingly more dense as one approaches the proximal region of the cochleosaccular duct. At later stages, the abrupt transition in staining becomes more pronounced as the unstained lateral wall of the cochleosaccular duct expands toward the utricle (Fig. 3; stage 29 [E6]).

A second distinct region of reduced class III β -tubulin staining, also marked by an abrupt transition in staining (bracket in Fig. 3 inset), is detected at about stage 29 (E6) in the nonsensory epithelia lateral and adjacent to the utricular macula. The endolymphatic sac and distal endolymphatic duct also show reduced class III β -tubulin staining by stage 27 (E5; data not shown).

Between stage 27 (E5) and stage 35 (E8–9), many nonsensory regions of the epithelia lose their class III

β -tubulin staining. Nevertheless, dense luminal-streaky class III β -tubulin staining persists in certain regions of the nonsensory epithelia: (1) a wedge of cells, giving the impression of a keystone in an arch, continues to label heavily along the outer circumference of the semicircular canals (Fig. 4A); (2) the pseudostratified epithelium at the confluence of the endolymphatic duct, utricle, and sacculle continues to label (arrows in Fig. 4B); and (3) the cells adjacent to the basal tip of the basilar papilla (arrowhead in Fig. 4B), near to where the superior fibrocartilaginous plate will form, also continue to label. The nonsensory character of this last region was confirmed in three ways: (1) examination of sequential sections at stage 35 (E8–9) when the proximal tip of the basilar papilla is well defined and contains hair cells; (2) observation that the tectorial membrane arises from a subset of these labeled cells; and (3) examination of wholemounted otocyst containing both the basilar papilla and saccular macula at stages 34–35 (E8–9) in which the proximal tip of the basilar papilla is easily identified by identification of class III β -tubulin-positive hair cells and morphology. In all cases, the nonsensory area between the saccular macula and basilar papilla was class III β -tubulin-positive and continuous with both sensory epithelia (not shown).

Hair cell differentiation and sensory epithelia development

In the developing sensory epithelia of the inner ear, both antibodies differentially label a subset of lumenally placed cells. This subset of cells can be identified by their flask-shaped or gourd-shaped appearance, with the heavily labeled neck of the gourd extending to the lumen. Figure 5A shows that, by stage 29 (E6), the cell's neck stands out against the less intense luminal-streaky pattern that is still present as this phenotype emerges. The unlabeled nuclei are located close to the lumen and often partially surrounded by label or have a labeled tail extending towards the basal lamina. By stage 35 (E8–9), cells in the vestibular sensory epithelia with this phenotype also have stereocilia bundles (Fig. 5B). This morphological phenotype is similar to the presumptive hair cells described by Ginzberg and Gilula (1979) and Whitehead and Morest (1985a,b) in developing sensory epithelia in the chicken, and to differentiating hair cells described by Stone et al. (1996a) in regenerating chicken basilar papilla. In addition, TuJ1 has been shown to label mature hair cells, but not support cells, in the posthatch chicken basilar papilla (Stone et al., 1996a). We consider these gourd-shaped cells to be, and will refer to them as, immature hair cells.

Prior to stage 29 (E6), we were not able to uniquely identify hair cells in paraffin-sectioned tissue due to the presence of luminal-streaky staining throughout the sensory epithelia. In wholemounted material reacted with the class III β -tubulin antibodies, however, immature hair cells can be identified by stage 26 (E4.5–5) because of the intense labeling of their apical cytoplasm. Identification of these cells as hair cells was confirmed by labeling with anti-HCA. Figure 6 shows a projection of a confocal z-series through the apical surface of a stage 26 (E4.5–5) saccular macula. The intense peaks of class III β -tubulin labeling in the apical cytoplasm of the immature hair cells are evident. The majority of class III β -tubulin-positive hair cells also label for HCA. HCA labeling was not observed in class III β -tubulin-negative cells.

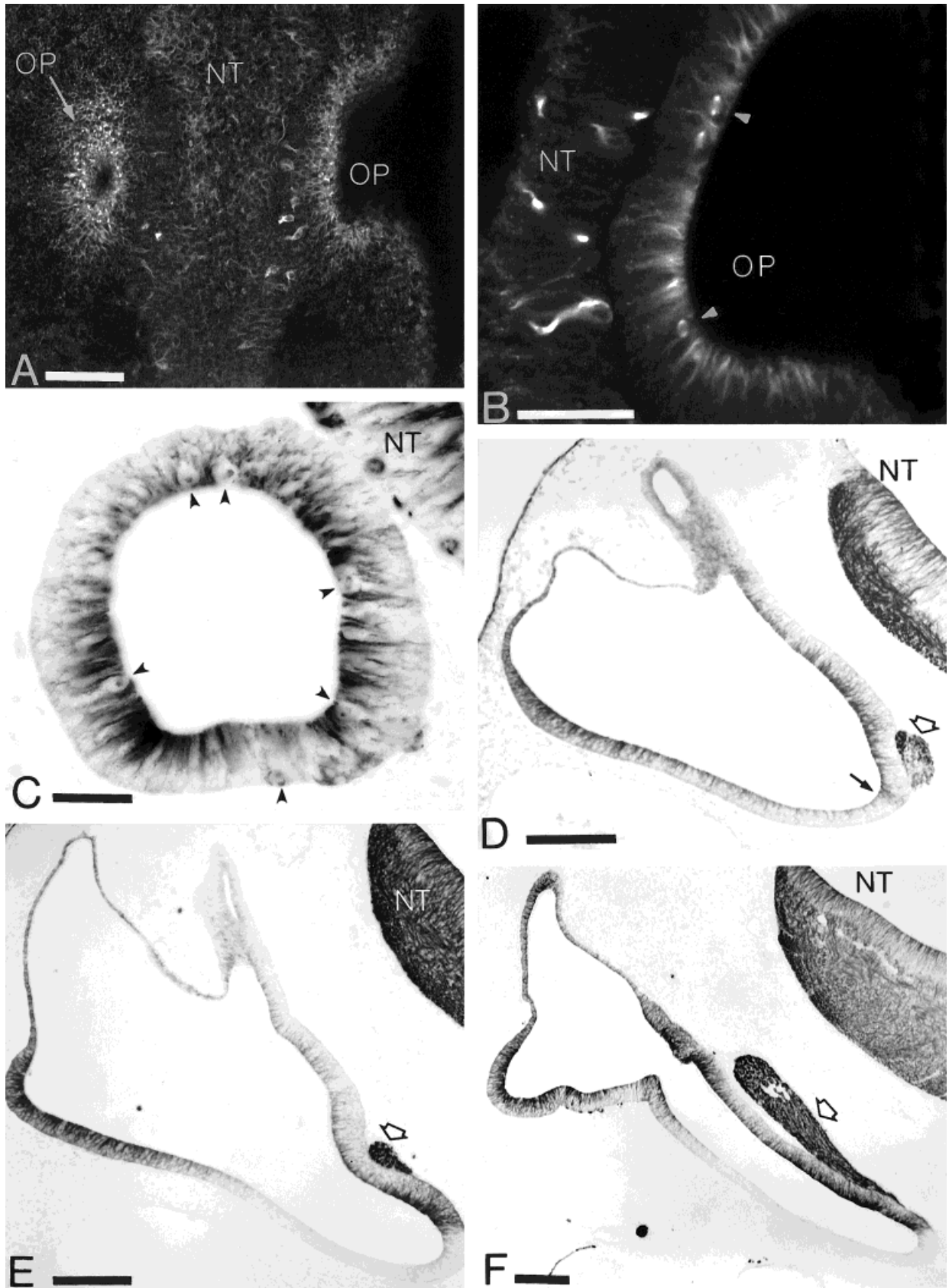


Figure 2

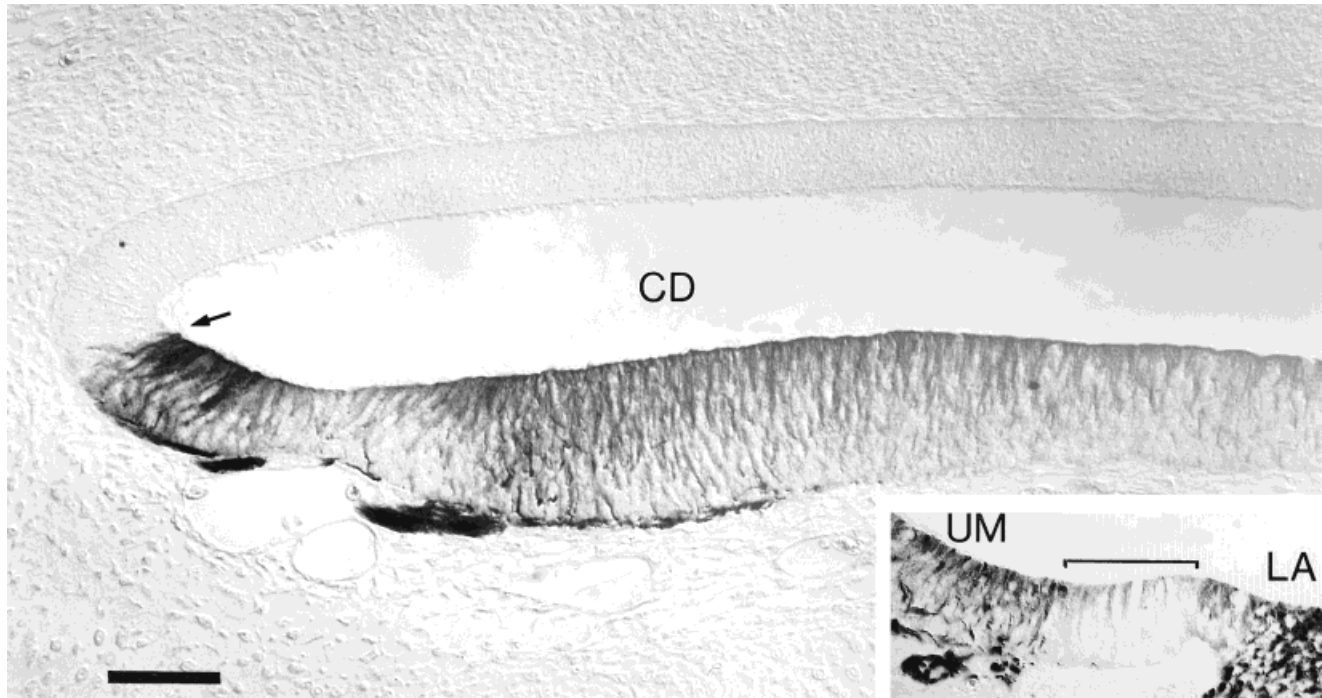


Fig. 3. Stage 29 (embryonic day, E6) cochlear duct (CD) showing that the lateral wall of the cochlear duct is unlabeled, and that staining ends abruptly at the distal tip (arrow). Note the lumenal-streaky staining in the sensory epithelium. **Inset:** Stage 29 (E6)

utricular macula (UM) and lateral ampulla (LA) showing the unlabeled perimacular region (bracket). Scale bar = 40 μ m for main image; 50 μ m for inset.

By stage 29 (E6), class III β -tubulin expression can identify immature hair cells in all of the vestibular sensory epithelia, except the macula lagena and macula neglecta, in both paraffin sections and wholmount preparations. As shown in Figures 5 and 6, immature hair cells are often packed together such that their cell bodies appear to be either in contact or overlapping. In the utricular macula, the immature hair cells are not evenly distributed. There is an increased density of immature hair cells laterally in the region where the utricular striola will form, whereas in the medial expanse of the macula, which will form the

nonstriolar region, immature hair cells appear more widely distributed. The lateral grouping of immature hair cells suggests that patterning of the utricular macula into striolar and nonstriolar regions may already be occurring. However, without a way of determining the bundle orientation, or Type I versus Type II hair cells, we cannot establish this relationship with certainty. By stage 35 (E8–9), the lumenal-streaky staining in the vestibular sensory epithelia has nearly disappeared, so that almost all the staining is confined to hair cells and nerve fibers.

Class III β -tubulin immunoreactivity can first identify immature hair cells in the distal basilar papilla at stage 29 (E6; Fig. 7), when hair cells are first beginning to develop stereocilia (Cotanche and Sulik, 1984), and first express HCA (Bartolami et al., 1991; Goodyear et al., 1995; Goodyear and Richardson, 1997). Again, the immature hair cells are often packed tightly together (Fig. 7). By stage 34/35 (E8–9), class III β -tubulin labeling shows immature hair cells along the entire length of the basilar papilla, indicating that the differentiation of hair cells in the basilar papilla with respect to class III β -tubulin occurs in a distal-to-proximal gradient as described for stereocilia formation (Cotanche and Sulik, 1984) and HCA labeling (Bartolami et al., 1991; Goodyear et al., 1995; Goodyear and Richardson, 1997).

Occasionally, isolated class III β -tubulin-positive cells that look like immature hair cells were observed in regions of the otic epithelium not associated with a sensory epithelium in both sectioned and wholmounted material. An example of one of these cells is shown in Figure 8.

Furthermore, in wholmount preparations, we detected numerous HCA-positive cells which are also class III

Fig. 2. Class III β -tubulin staining in the otic epithelium at stages 10 (embryonic day, E1.5, A), 12 (E2.0, B), 16 (E2.2, C), 21 (E3.5, D), 25 (E4.5, E), and 27 (E5, F). A and B are horizontal confocal sections from wholmounted embryos fluorescently labeled using TuJ1. Anterior is up in both A and B. C–F are transverse paraffin sections stained with β -III. Dorsal is up and medial is to the right in C–F. **A:** Stage 10 (E1.5) embryo showing the class III β -tubulin staining in the neural tube (NT) and both otic placodes (OP). **B:** Stage 12 (E2.0) otic pit (OP) showing the lumenal-streaky pattern of staining and labeled mitotic spindles (arrowheads). **C:** Stage 16 (E2.2) otic vesicle showing lumenal-streaky staining in all regions of the otic epithelium. Labeled mitotic spindles (arrowheads) are also located in all regions of the otic epithelium. **D:** Stage 21 (E3.5) otic vesicle showing lumenal-streaky staining in all regions of the otic epithelium. Note the abrupt change in staining at the ventromedial tip of the otic vesicle (arrow). The large open arrows in D–F indicate the acoustic-vestibular ganglion (AVG). **E:** Stage 25 (E4.5) inner ear showing staining in all regions of the epithelium except the ventrolateral wall of the cochlear duct where the tegmentum vasculosum will develop. **F:** Stage 27 (E5) inner ear showing that most regions of the otocyst are still labeled, except for the lateral wall of the cochlear duct. Scale bars = 100 μ m in A, D–F; 50 μ m in B; 30 μ m in C.

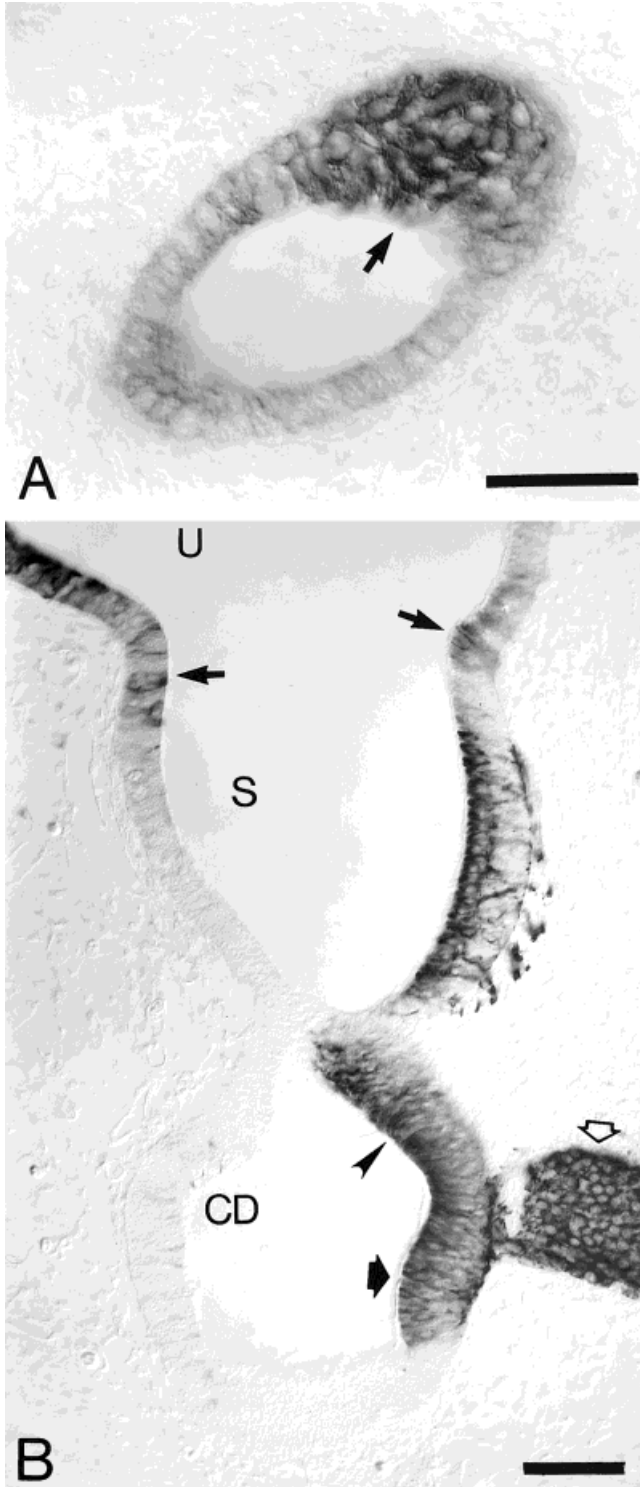


Fig. 4. Persistent nonsensory labeling in the inner ear at stage 35 (embryonic day, E8–9). **A:** The superior semicircular canal showing the "keystone in an arch" labeling in its outer circumference (arrow). **B:** Persistent labeling (arrows) around the junction of the utricle (U), saccule (S), and endolymphatic duct (continuous with epithelium at upper right), and between the saccule and the proximal region (arrowhead) of the cochlear duct (CD; see text). Large open arrow indicates the cochlear ganglion; large filled arrow indicates the basilar papilla. Dorsal is up and medial is to the right in both panels. Scale bars = 30 μ m in A; 50 μ m in B.

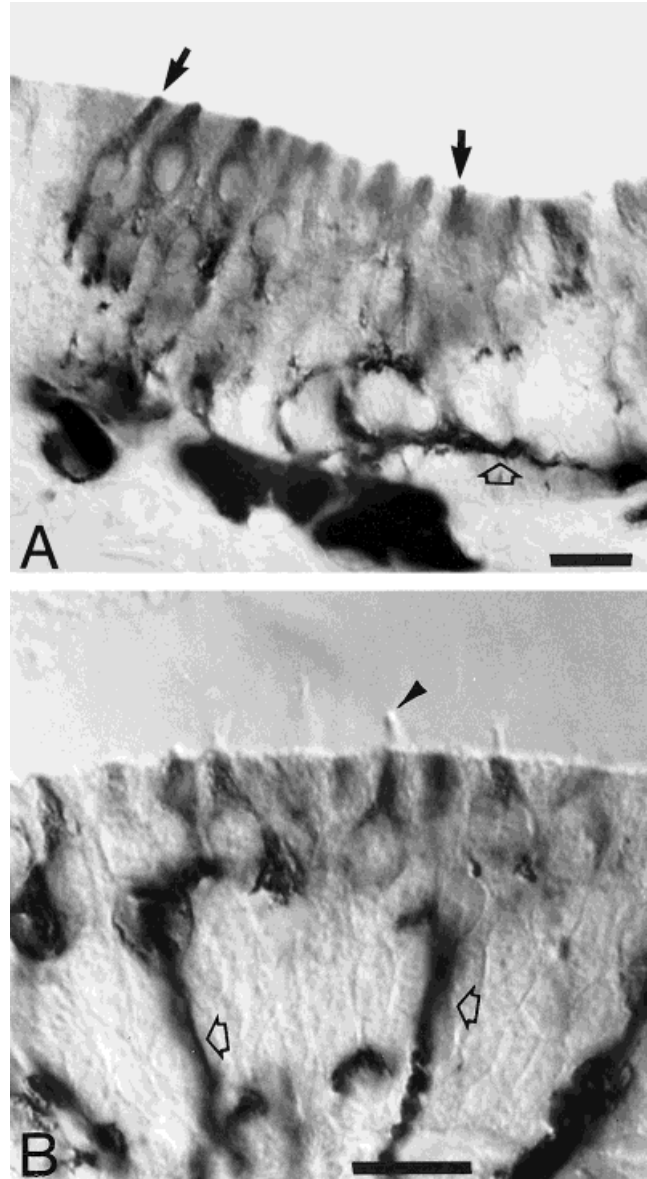


Fig. 5. Class III β -tubulin labeling in hair cells. **A:** Gourd-shaped, class III β -tubulin-labeled immature hair cells (arrows) in the stage 29 (embryonic day, E6) utricular macula. **B:** Gourd-shaped, class III β -tubulin-labeled immature hair cells with stereocilia (arrowhead) in the stage 35 (E8–9) lateral crista. Open arrows indicate nerve fibers. Scale bars = 10 μ m.

β -tubulin-positive in regions outside of the identifiable vestibular sensory epithelia (data not shown). These cells, however, are typically located in regions near the established saccular and utricular maculae.

Class III β -tubulin expression in the developing paratympanic organ

Class III β -tubulin is also expressed in another hair cell sensory organ, the paratympanic organ, in a similar manner as in the otocyst. The paratympanic organ is located in the middle ear of birds, develops from the first eibranchial placode and first pharyngeal pouch, and is innervated by the geniculate ganglion, which is also

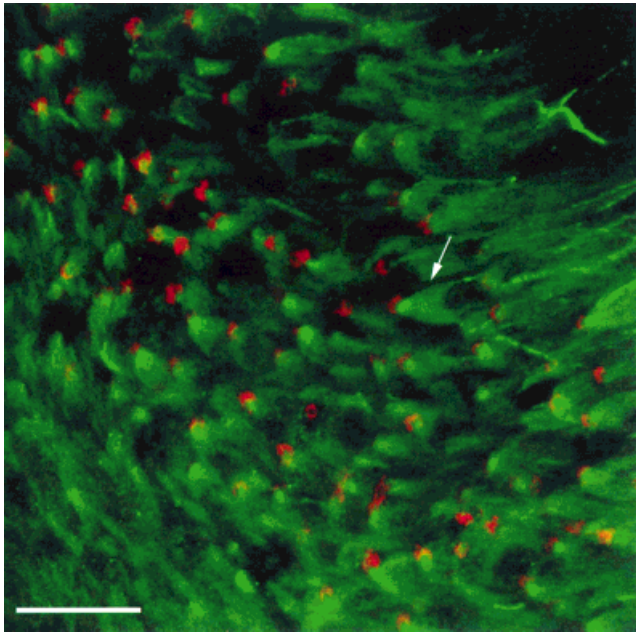


Fig. 6. Confocal image showing class III β -tubulin-labeled immature hair cells in the stage 26 (embryonic day, E4.5–5) saccular macula. Immature hair cells form peaks of class III β -tubulin labeling (green), the majority of which colabel with hair cell antigen (HCA) labeling (red). A colabeled hair cell is indicated by the arrow. This image is a projection of a 4- μ m confocal z-series acquired by looking down on the saccular macula in a wholemounted otocyst. Scale bar = 20 μ m.

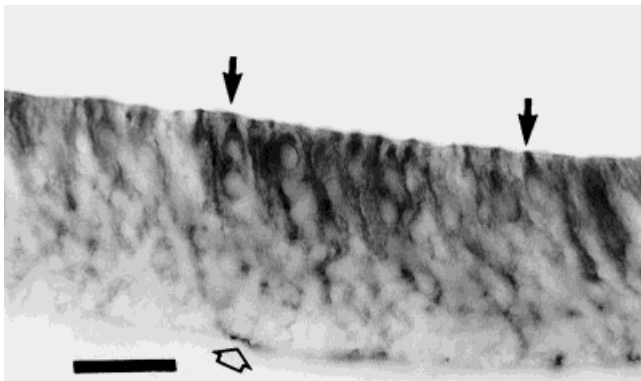


Fig. 7. Immature hair cells (arrows) emerging from the luminal-streaky staining of the distal basilar papilla at stage 29 (embryonic day, E6). Note that cochlear ganglion fibers (open arrow) are beginning to penetrate the basilar papilla. Scale bar = 20 μ m.

derived from the first epibranchial placode (D'Amico-Martel and Noden, 1983; von Bartheld, 1990). As shown in Figure 9A, class III β -tubulin-expressing cells from the first epibranchial placode fuse with the epithelium of the first pharyngeal pouch as early as stage 16 (E2.2). At stages 21 (E3.5) and 25 (E4.5), the epithelium of the pharyngeal pouch, which is still in contact with the first epibranchial placode, is labeled in the same luminal-streaky pattern as found in the otocyst (Fig. 9B). By stage 27 (E5), the paratympanic organ can be identified in the lateral pocket of the first pharyngeal pouch. Figure 9C

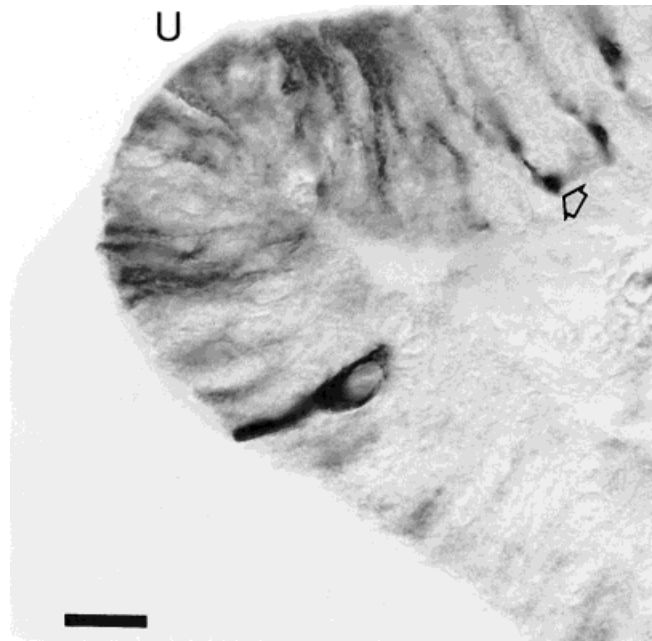


Fig. 8. Labeled gourid-shaped immature hair cell lying in the lateral wall of the saccule near its merger with the utricle (U) at stage 29 (embryonic day, E6). Large open arrow indicates nerve fiber penetrating the utricular macula. Dorsal is up and medial is to the left. Scale bar = 10 μ m.

shows that, at stage 27 (E5), this organ contains distinct immature hair cells.

Class III β -tubulin is not neuron-specific in the developing chick embryo

Previous studies have indicated that class III β -tubulin is not restricted to neuronal structures in the developing chick embryo. Class III β -tubulin expression has been reported in the ectodermal layers of the extra-embryonic membranes (Lee et al., 1990), trigeminal placodes (Moody et al., 1989; Lee et al., 1990), lens (Lee et al., 1990), glomus cells of the carotid body (Kameda et al., 1994), C cells in the ultimobranchial organ (Kameda et al., 1993; Kameda, 1995), caudal-ventral somites (Lee et al., 1990), and caudal mesonephric duct (Lee et al., 1990). Our observations in the developing head of the chick embryo corroborate these findings. Additionally, we observed that the non-neuronal labeling is much more widespread in methacarn-fixed tissue than in paraformaldehyde-fixed tissue. In addition to the structures indicated above, we found that TuJ1 and β -III consistently label a number of other structures: ectoderm, Rathke's pouch, first and second epibranchial placodes, olfactory placode, lens placode, mesenchyme of the branchial arches, tissue surrounding developing membranous bone, isolated cells in the mesenchyme immediately underneath the ectoderm, the ventral wall of the embryonic pharynx, the dorsal and ventral walls of the first pharyngeal pouch, and the developing thyroid anlage. Some of these structures are illustrated in Figure 10 and listed in Table 1.

We also found that class III β -tubulin expression can be detected in the ectoderm and neural tube as early as stage 9 (E1.3), which is earlier than previously reported (stage 12 [E2.0]; Lee et al., 1990). Additionally, we observed that

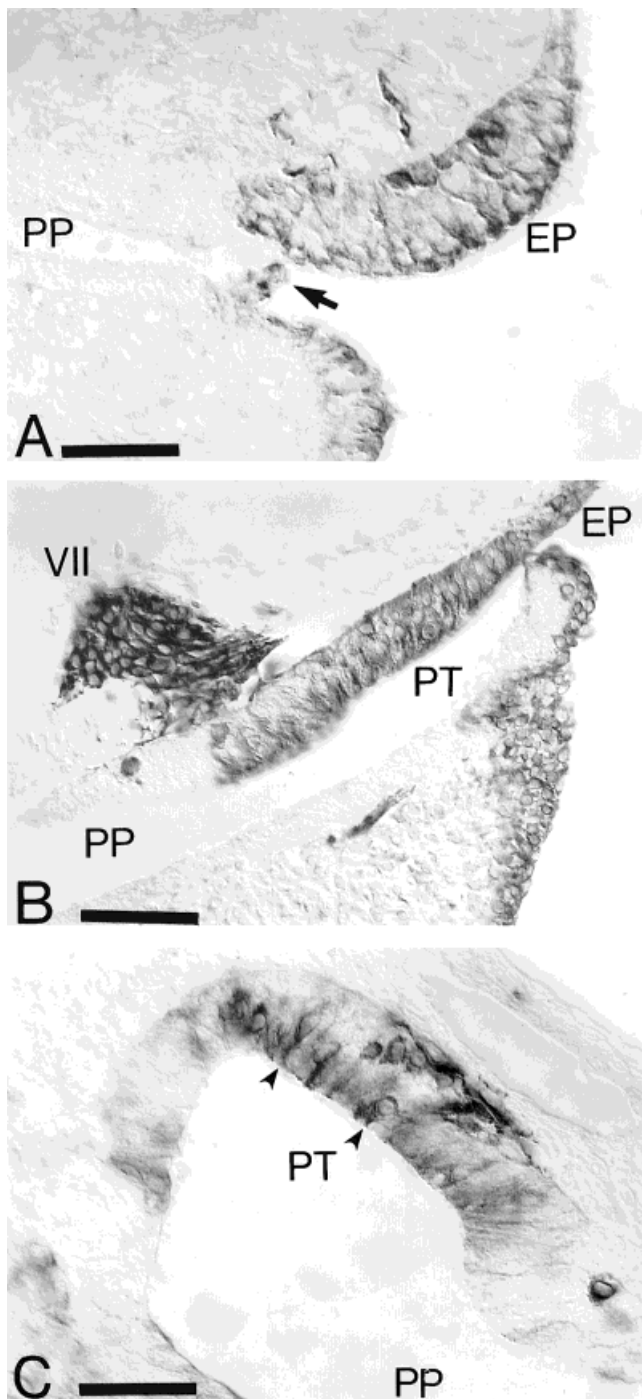


Fig. 9. Class III β -tubulin labeling in the developing paratympanic organ (PT). **A:** Fusion (arrow) between the first epibranchial placode (EP) and the first pharyngeal pouch (PP) at stage 16 (embryonic day, E2.2). **B:** Lumenal-streaky class III β -tubulin labeling in the epithelium of the presumptive paratympanic organ at stage 21 (E3.5) in the first pharyngeal pouch. Note the heavily labeled facial nerve ganglion cells (VII) next to the paratympanic organ. **C:** Stage 27 (E5) paratympanic organ with class III β -tubulin-positive immature hair cells (arrowheads) emerging from the lumenal-streaky staining of the epithelium. Dorsal is up in A–C. Medial is to the left in A and B, and to the right in C. Scale bars = 50 μ m.

TuJ1 and β -III typically produce the same lumenal-streaky pattern as we observed in the otic epithelium in other thickened epithelia expressing class III β -tubulin, including the proliferative zone of the neural tube, (see Figs. 1, 2, and 10E). In the proliferative zone of the neural tube, the staining is not limited to the trailing processes of densely stained cells migrating from the proliferative zone. Before stage 25 (E4.5), large regions of the proliferative zone exhibit lighter, lumenal-streaky staining and the staining appears to label all the cells in these regions. After stage 25 (E4.5), all the cells of the proliferative zone appear to be labeled. Labeled mitotic spindles were also observed in the proliferative zone of the neural tube as well as the otic epithelium and ectoderm (data not shown).

DISCUSSION

A previous study showed that class III β -tubulin, a widely used neuron-specific marker, is expressed in mature and regenerating hair cells but not the support cells of the avian inner ear (Stone et al., 1996a). In this study, we investigated the expression of this marker in the developing avian inner ear. We found that class III β -tubulin is not neuron-specific in the developing avian embryo, but appears to accumulate in neuronal cell types, including hair cells, about the time of their differentiation. In the developing inner ear, class III β -tubulin immunoreactivity was found in all regions of the otic epithelium from its formation as the otic placode until about stage 21 (E3.5), when the prospective tegmentum vasculosum begins to lose its staining. By stage 35 (E8–9), most of the nonsensory epithelia have lost their class III β -tubulin staining, leaving distinct regions of staining in nonsensory regions between the morphological compartments of the inner ear, and in the outer circumference of the semicircular canals. Concurrent with the loss of class III β -tubulin staining from nonsensory regions, the hair cells of the sensory epithelia accumulate class III β -tubulin, whereas the supporting cells decrease their staining for class III β -tubulin. We also observed a similar pattern of development in a hair cell organ, the paratympanic organ, that is not derived from the otocyst.

In the following sections, we will first discuss the neuronal specificity of class III β -tubulin, its accumulation in neuronal cell types, including hair cells. We will then discuss our observations with respect to several aspects of inner ear development, in particular: the determination of hair cells and support cells, the commonality of developmental programs in hair cell structures of different embryonic origins, the determination and differentiation of nonsensory areas of the membranous labyrinth, and hair cell dependence on innervation throughout development.

Neuronal specificity of class III β -tubulin and neurogenesis

Class III β -tubulin is widely used as a marker to detect neurons shortly after the onset of differentiation (e.g., Moody et al., 1989, 1996; Lee et al., 1990; Watanabe et al., 1991; Easter et al., 1993; Katsetos et al., 1993; Gonzalez and Silver, 1994; Menezes and Luskin, 1994; Gates et al., 1995; Memberg and Hall, 1995; Snow and Robson, 1995). Earlier studies indicated that embryonic expression of class III β -tubulin is not solely restricted to neuronal cell types (see Table 1). Our results also indicate that class III β -tubulin is expressed more widely in non-neuronal tis-

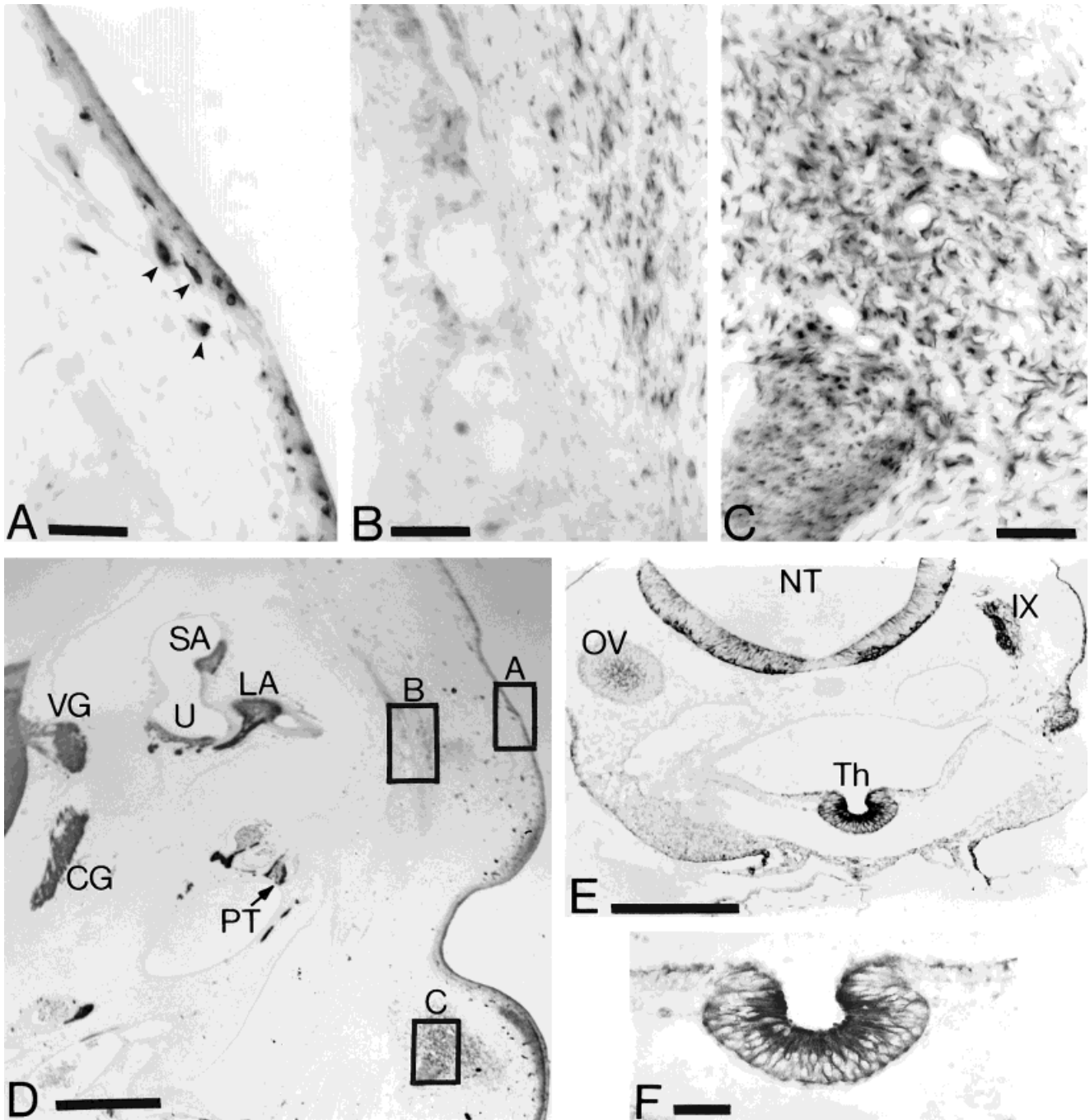


Fig. 10. Non-neuronal class III β -tubulin staining at stage 16 (embryonic day, E2.2) and stage 35 (E8–9). A–C are high magnification images of the regions indicated in D. **A:** Class III β -tubulin-positive cells underlying the ectoderm (arrowheads). **B:** Class III β -tubulin-tissue associated with bone formation in the head. **C:** Class III β -tubulin positive mesenchyme near developing bone in the jaw. **D:** Low power image of a section from the head of a stage 35 (E8–9) embryo showing staining in various non-neuronal tissues. Dorsal is up

and medial is to the left. **E:** Non-neuronal staining in the branchial arch mesenchyme and thyroid anlage (Th) at stage 16 (E2.2). Dorsal is up. **F:** High power image of the thyroid anlage indicated in E. CG, cochlear ganglion; IX, glossopharyngeal nerve; LA, lateral ampulla; NT, neural tube; OV, otic vesicle; PT, paratympic organ; SA, superior ampulla; U, utricle; VG, vestibular ganglion. Scale bars = 50 μ m in A, B, C and F; 500 μ m in D; 200 μ m in E.

sues of the developing embryo than previously reported. In addition to expression in the nonsensory parts of the developing labyrinth, we found specific staining with the class III β -tubulin antibodies in several other clearly

identifiable, non-neuronal tissues that were not previously reported, including the ventral wall of the embryonic pharynx (endodermal origin), mesenchymal tissue surrounding developing bone in the head (possibly of neural

TABLE 1. Tissues Expressing Class III β -Tubulin in the Developing Chicken Embryo

| | |
|---|---|
| Previously reported: | |
| C-cells of the ultimobranchial organ | Kameda et al. (1993); Kameda (1995) |
| Glomus cells in the carotid body | Kameda et al. (1994) |
| Extra-embryonic membranes (ectodermal layers) | } Lee et al. (1990) |
| Lens | |
| Caudal mesonephric duct | |
| Caudal-ventral somite | |
| Trigeminal placodes | } Lee et al. (1990); Moody et al. (1989) |
| Neural tube-intermediate zone | |
| Neural tube-marginal zone | |
| Neural crest-derived neurons | |
| Placodally-derived neurons | |
| Reported in this paper: | |
| Ectoderm | |
| Embryonic pharynx- ventral wall | |
| first pharyngeal pouch | |
| thyroid anlage | |
| Epibranchial placodes (first and second) | |
| Lens placode | |
| Neural tube-proliferative zone | |
| Mesenchyme- | |
| in the branchial arches | |
| around developing bone | |
| isolated cells underlying the ectoderm | |
| Olfactory placode | |
| Otic placode, otic pit, and otocyst | |
| Paratympenic organ | |
| Rathke's pouch | |

crest origin), the thyroid anlage (endodermal origin), and the ectoderm (including regions giving rise to epidermis). The non-neuronal staining in the tissue around forming bone is easily distinguishable from nerve fibers running through the mesenchyme because the nerve fibers are very heavily stained and the labeled mesenchymal cell bodies are small, fusiform, and too numerous to be neuronal precursors in these regions which normally do not have large populations of neurons. Previous studies also correlated the onset of class III β -tubulin expression with terminal mitosis and the onset of neuronal differentiation, because class III β -tubulin immunoreactivity was first detected in cells migrating from the proliferative zone of the neural tube, cells with neuronal morphologies, and neuronal populations undergoing terminal mitosis (Moody et al., 1989; Lee et al., 1990). This correlation relied on the absence of class III β -tubulin immunoreactivity in mitotic figures and proliferating neurogenic tissues, particularly the proliferative zone of the neural tube and neurogenic placodes. Our observations, however, show that class III β -tubulin immunoreactivity can be detected in the proliferative zone of the neural tube, neurogenic placodes, and otic epithelium. Immunoreactive mitotic spindles were also observed in these same structures. Hence, the expression of class III β -tubulin is not, by itself, a reliable indicator of neuronal differentiation during embryonic development.

The widespread immunoreactivity observed with both of these antibodies is clearly due to binding of the primary antibody and not a result of endogenous peroxidases, or adsorption of the secondary antibody or ABC reagents. Our use of methacarn, which fixes tissue by denaturation and coagulation, resulted in the observation of staining in more tissues than previously reported. Because the TuJ1 antibody has been shown to be specific for class III β -tubulin and not to cross-react with other isotypes of β -tubulin or other proteins in chick embryo extracts under denaturing conditions (Lee et al., 1990), we believe that the observed staining in methacarn-fixed tissue is the

result of specific binding of the antibodies to class III β -tubulin. Additionally, the carboxyl terminus of chicken class III β -tubulin ends in a lysine residue (Sullivan and Cleveland, 1986). Because lysine is one of the targets of cross-linking fixatives like paraformaldehyde (Bancroft and Stevens, 1990), it is likely that less of the epitope would be preserved with cross-linking fixatives than with coagulative fixation. Earlier reports of class III β -tubulin as a neuronal marker used paraformaldehyde fixation, with fixation times ranging from 1 to 24 hours (Moody et al., 1989; Lee et al., 1990). As a result, low levels of class III β -tubulin expression may have been missed in previous studies due to the destruction of the epitope during fixation. In situ hybridization with specific probes to class III β -tubulin mRNA may be needed in the future to confirm that cells staining moderately with antibodies to class III β -tubulin actually express the molecule.

Our experience with the fixation sensitivity of these antibodies suggests that, as fixation time increases, only those cells with the largest amount of the class III β -tubulin epitope are stained. Because neuronal cell types were the only cells identified with TuJ1 in earlier studies, neuronal cells probably have significantly more class III β -tubulin than other cell types. Lee et al. (1990) were the first to suggest the accumulation of class III β -tubulin in neurons. They noted that labeled cells in the marginal zone of the neural tube were more intensely stained than cells migrating out of the proliferative zone, and observed weakly labeled cells in the dorsal root ganglion as neurons began to differentiate. Additionally, Lee et al. (1990) ran immunoblots of protein from whole embryonic chick heads at stages 12 (E2.0) through 23 (E3.5-4), and showed that the relative abundance of class III β -tubulin increases during this period when the number of differentiating neurons is increasing. These observations led these authors to suggest that class III β -tubulin accumulates in neuronal cell types as they begin to differentiate.

Our observations support this hypothesis, but in a different context than suggested by Lee et al. (1990). Fixation with methacarn, as compared to paraformaldehyde, appears to be much less detrimental to the detection of class III β -tubulin by TuJ1 and β -III, thereby allowing us to detect lower levels of class III β -tubulin expression. As a result, we see staining in more cell types and structures. Nevertheless, we always see dense staining in neuronal cells in the neural tube, cranial ganglia, neurogenic placodes, and otic epithelium. Instead of seeing these cells appear on a "background" of no class III β -tubulin expression, we see these cells emerge from cell populations expressing lower levels of class III β -tubulin, and are able to recognize them because of their accumulation of class III β -tubulin and distinctive morphology. Hair cells provide an excellent example of this phenomenon. The otic epithelium clearly expresses class III β -tubulin prior to and during the period of hair cell differentiation. Beginning at stage 29 (E6.0), we are able to distinguish hair cells from the other labeled epithelial cells because of their morphology, luminal location, and because they stain more heavily than the surrounding epithelium, i.e., both the surrounding epithelium and the hair cells express class III β -tubulin, but hair cells express much more and stain more densely. These observations suggest that, early in development, class III β -tubulin is expressed at low levels in most neurogenic epithelia, such as the otic epithelia and neural tube. As neuronal cells begin to differentiate, their expres-

sion of class III β -tubulin is significantly upregulated. The expression of class III β -tubulin in other cell phenotypes, however, is eventually downregulated.

At this time one can only speculate on the molecular events underlying the bidirectional regulation of class III β -tubulin expression in neurogenic versus non-neurogenic cell types during differentiation. Anderson and colleagues have described a 21-base pair sequence of genomic DNA designated as the neuron-restrictive silencer element (NRSE; also called RE-1; Mori et al., 1990; Wuenschell et al., 1990; Mori et al., 1992; Kraner et al., 1992) and an associated protein binding factor, the neuron-restrictive silencer factor (NRSF; also called REST or XBR; Chong et al., 1995; Schoenherr and Anderson, 1995a,b; Palm et al., 1998). This complex has been shown to negatively regulate the expression of a large number of neuronal genes in neuronal precursors and non-neuronal cells (Mori et al., 1990; Wuenschell et al., 1990; Kraner et al., 1992; Mori et al., 1992; Chong et al., 1995; Schoenherr and Anderson, 1995a; Schoenherr et al., 1996; Bessis et al., 1997; Edelman and Jones, 1998; Kallunki et al., 1998). The chicken class III β -tubulin gene has at least one NRSE and has been shown to be regulated by NRSF (Schoenherr et al., 1996). Therefore, one is tempted to speculate that a constitutive level of class III β -tubulin is expressed in all inner ear precursor cell types and that changes in class III β -tubulin levels are mediated by the NRSE/NRSF system as neuronal and non-neuronal cells undergo differentiation.

Class III β -tubulin expression during hair cell and sensory epithelia differentiation

We can first identify immature hair cells by class III β -tubulin immunoreactivity in wholemount preparations of the saccular macula at about stage 26 (E4.5–5). By stage 29 (E6.0), immature hair cells are detected in all the sensory epithelia. The appearance of hair cells with class III β -tubulin in the saccular macula at stage 26 (E4.5–5) correlates with the appearance of hair cells as indicated by HCA labeling (this paper; Goodyear et al., 1995), and the elimination of gap junctions from hair cells reported by Ginsberg and Gilula (1979). Similarly, in the basilar papilla, class III β -tubulin-positive hair cells can be identified at the same time that hair cells can be identified by HCA expression (Bartolami et al., 1991; Goodyear et al., 1995; Goodyear and Richardson, 1997) or stereocilia using scanning electronic microscopy (SEM; Cotanche and Sulik, 1984). Furthermore, class III β -tubulin staining follows the same distal-to-proximal gradient of hair cell maturation (Cotanche and Sulik, 1984; Bartolami et al., 1991; Goodyear et al., 1995; Goodyear and Richardson, 1997). This evidence shows that accumulation of class III β -tubulin occurs early during hair cell differentiation, and is contemporaneous with other available indicators of hair cell differentiation.

Lateral inhibition has been suggested as a possible mechanism for the developmental determination of hair cells versus support cells, and the subsequent organization of hair cells and support cells into a mosaic where the hair cells are isolated from each other by the supporting cells (Goodyear et al., 1995; Goodyear and Richardson, 1997). This model proposes that, at some point in hair cell and support cell differentiation, a hair cell begins to inhibit its neighbors from also differentiating into hair cells through contact-mediated signals. Thus, all the cells contacting a

hair cell become supporting cells. Variants of this idea have been suggested as mechanisms that could produce the hair cell-support cell mosaic seen in the epithelia of the inner ear (Goodyear et al., 1995). All of these models suggest that hair cell-hair cell contacts should not be observed. One advantage of class III β -tubulin over other hair cell markers is that it allows us to see the morphology of the cell body of the developing hair cell at about the time that it begins to form stereocilia. Early hair cells have a gourd-shaped appearance with a narrow neck extending to the lumen. The lumenal area of these hair cells is much smaller than the amount of space occupied by the cell body located below the lumen. Although we recognize that hair cell-hair cell contact cannot be resolved using the techniques in this study, this gourd-shaped morphology, which is present as the hair cells and supporting cells are differentiating, and the close packing of immature hair cells, suggests that there is ample opportunity for hair cell-to-hair cell contacts below the lumen of the epithelium, but does not preclude the possibility of thin supporting cell processes separating the cells. The morphology of early differentiating hair cells suggests that there could be significant contact occurring between hair cells below the lumen where the mosaic is observed. Additionally, sublumenal contact between hair cells has been noted as late as hatching in chicken (Fermin and Cohen, 1984) and in adult quail (Ryals et al., 1992). These data suggest that, if lateral inhibition is the mechanism by which hair cells and support cells acquire their distinctive phenotypes, the timing and degree of contact between cells may be more important than just whether contact is occurring.

The paratympanic organ shows a similar sequence of development as the otic epithelium. It is derived from an ectodermal placode, the first epibranchial placode, which appears to invade the distal first pharyngeal pouch as suggested by class III β -tubulin staining. Class III β -tubulin is expressed in a lumenal-streaky pattern throughout the early development of the paratympanic organ, and accumulates in immature hair cells emerging from the lumenal-streaky epithelium as they begin to differentiate. BMP4, a marker used to identify developing sensory epithelia in the inner ear, also localizes to the distal end of the first pharyngeal pouch where the paratympanic organ develops, as early as stage 27 (E5), a stage when immature hair cells are already present, and possibly as early as stage 16 (E2.2; Figs. 1 and 2 in Wu and Oh, 1996). These data suggest that similar processes of development are occurring in these two hair cell-producing structures, even though they are derived from different anlagen, and that a common program of development may be occurring during the early differentiation of these sensory organs.

Class III β -tubulin expression in the nonsensory regions of the inner ear

Beginning at about stage 21 (E3.5), the expression of class III β -tubulin in all regions of the otic epithelium begins to give way to a distinct region of reduced class III β -tubulin expression in the lateral wall of the cochlear duct. This region is where the tegmentum vasculosum will develop (Knowlton, 1969; Cotanche and Sulik, 1982), and is indicated by an abrupt change in the density of staining at the distal tip of the cochleosaccular duct evagination. The staining becomes increasingly more dense as one approaches the utricle. This abrupt change in staining is contemporaneous with the specification of the sensory

epithelia in the cochlear duct as indicated by BMP4, Msx-1, and p75NGFR (Wu and Oh, 1996), suggesting that the determination of the tegmentum vasculosum may be occurring as a distinct functional unit at the same time as the sensory epithelia. This result indicates that the sensory and nonsensory regions of the inner ear are being determined during the same period of development.

As development proceeds, more nonsensory regions begin to lose their class III β -tubulin staining, in particular the endolymphatic sac, the perimacular region lateral but adjacent to the utricular macula, and regions involved in the formation of the semicircular canals. By stage 35 (E8–9), most of the nonsensory regions of the inner ear have lost their class III β -tubulin staining, leaving distinct regions of persistent staining between the medial walls of the cochlear duct and saccule, the confluence of the saccule, utricle and endolymphatic duct, and in the outer circumference of the semicircular canals. The first two of these regions occurs at the morphological transitions between regions of the inner ear, and the staining in the semicircular canals is located at the farthest extremes of the epithelia. One can imagine that these regions lie remote from possible signals directing morphogenesis and have not yet been directed to differentiate.

With respect to the formation of semicircular canals, the idea that the persistent staining in the semicircular canal lies remote from morphogenetic signals suggests that there is a signal directing the differentiation of the fusion plates of the semicircular canals which decreases with distance from the center of the fusion plate. This construct would place the signaling and differentiation, i.e., the morphogenetic action, of cells forming the semicircular canals in the fusion plates, which subsequently disappear, and not in the remaining semicircular canals whose epithelia may have a predominately passive role. In *Xenopus*, Haddon and Lewis (1991) demonstrated that the epithelia of the fusion plates are a source of glucosaminoglycans, which drive the formation of the semicircular canals, thus showing that these cells are differentiating from the other cells in the epithelium of the semicircular canal. Additionally, Fekete et al. (1997) showed that, in chicken, cell death first occurs at the center of the fusion plates and appears to expand outward. Recently, Kiernan et al. (1997) proposed a model of semicircular canal determination based on the boundary model developed by Fekete (1996) and staining pattern exhibited by two homeobox genes, SOH-1 and GH6. These two genes display patterns of expression remarkably similar to those we see with class III β -tubulin in the thin-walled nonsensory parts of the otocyst once the semicircular canals begin to form. The model proposed, however, focuses on the specification of structures seen in the mature labyrinth to explain the parceling of the labyrinth into different structures during development and does not account for the complex morphogenetic movements required to form the semicircular canals. The model suggested above would imply that the cells undergoing specification and specialization in the formation of the semicircular canals lie in the fusion plates and subsequently are not represented in the mature labyrinth.

Isolated hair cells and hair cell dependence on innervation

Around stage 29 (E6.0), we observed gourd-shaped, class III β -tubulin-positive cells, and HCA-positive/class III

β -tubulin-positive cells in putative nonsensory regions of the utricular and saccular spaces. Although some of these cells may be part of a distinct population of nonsensory cells, because some of these cells also express HCA, we suspect that some are isolated hair cells. These cells lie in regions devoid of innervation, and away from the identifiable utricular and saccular maculae. Hair cells without innervation are not observed in mature sensory epithelia. Several studies suggest that the presence of the AVG or contact between hair cells and nerve fibers is not required for hair cell differentiation (Fell, 1928; Orr, 1968; van de Water, 1976; Hirokawa, 1977, 1978; Corwin and Cotanche, 1989; Swanson et al., 1990; Sokolowski et al., 1993). There is evidence, however, that hair cells and other receptor cells derived from the octavolateralis system do require some interaction with innervating fibers for their long-term maintenance (> 2 weeks). Whereas short term denervation of these sensory epithelia does not produce any substantial degeneration (Fell, 1928; Orr, 1968; van de Water, 1976; Corwin and Cotanche, 1989; Swanson et al., 1990; Sokolowski et al., 1993), long-term denervation results in a loss or dedifferentiation of hair cells within these sensory epithelia (Favre and Sans, 1991; Weisleder et al., 1994, 1996). The extended length of time required for hair cells to disappear suggests that the process may be secondary to other changes in the epithelium, or gradual changes of a yet to be determined regulatory pathway normally set by neuronal interactions. We conclude that the initiation of hair cell or sensory epithelia differentiation occurs independent of neuronal influences, but that at some point in development, hair cells or sensory epithelia become dependent on some neuronal influence for long-term maintenance and hence, these isolated hair cells deteriorate. The best test of this hypothesis will be studies of the developing AVG and inner ear sensory epithelia in knockout mutants, such as the neurogenin1 knockouts (Ma et al., 1998), that do not seem to develop an AVG.

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