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J Neurocytol. Author manuscript; available in PMC 2007 September 27.

Published in final edited form as:
J Neurocytol. 2003 February ; 32(2): 185–197.

Cell type-specific reduction of β tubulin isotypes synthesized in the developing gerbil organ of Corti

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Abstract

There are seven isotypic forms of the microtubule protein β tubulin in mammals, but not all isotypes are synthesized in every cell type. In the adult organ of Corti, each of the five major cell types synthesizes a different subset of isotypes. Inner hair cells synthesize only β_I and β_{II} tubulin, while outer hair cells make β_I and β_{IV} tubulin. Only β_{II} and β_{IV} tubulin are found in inner and outer pillar cells, while β_I, β_{II}, and β_{IV} tubulin are present in Deiters cells, and β_I, β_{II} and β_{III} tubulin are found in organ of Corti dendrites. During post-natal organ of Corti development in the gerbil, microtubules are elaborated in an orderly temporal sequence beginning with hair cells, followed by pillar cells and Deiters cells. Using β tubulin isotype-specific antibodies, we show that, in the gerbil cochlea, the same three isotypes are present in each cell type at birth, and that a cell type-specific reduction in the isotypes synthesized occurs in hair cells and pillar cells at an unusually late stage in development. No β tubulin isotypes were detected in mature afferent dendrites, but we show that this is because few microtubules are present in mature dendrites. In addition, we show that primary cilia in inner hair cells, a feature of early development, persist much later than previously reported. The findings represent the first description of developmental cell type-specific reductions in tubulin isotypes in any system.

Introduction

The ubiquitous structural protein tubulin is found in cells as microtubules consisting of α and β tubulin monomers. Mammalian β tubulin exists as seven isotypes, termed β_I, β_{II}, β_{III}, β_{IVa}, β_{IVb}, β_V, and β_{VI}, each a separate gene product synthesized without alternative splicing (Ludueña, 1998). The amino acid sequences of the seven isotypes are 75–96% identical, but several of them are also among the most highly conserved in evolution. For example, the chicken and mouse β_I isotypes differ by only two residues (Ludueña, 1998). The conservation of isotype sequence in mammals and in other vertebrates has led to the multi-tubulin hypothesis, the proposition that the multiple functions of microtubules may require different forms of tubulin (Fulton & Simpson, 1976). This hypothesis predicts that isotypes will be selectively synthesized in different cell types according to function.

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In post-mitotic organ of Corti development, microtubules are elaborated in a specific temporal pattern, beginning with hair cells at post-natal day 0 (P0), then in pillar cells by P3 and Deiters cells by P6 (Hallworth *et al.*, 2000). A recent study using four β tubulin isotype-specific antibodies has shown that, in gerbil organ of Corti, the isotypes are differentially synthesized in several cell types (Hallworth & Ludueña, 2000). To be specific, inner hair cells (IHCs) were found to have only β_I and β_{II}, while outer hair cells (OHCs) had only β_I and β_{IV}. Both inner and outer pillar cells (IPs and OPs) showed only β_{II} and β_{IV}, while Deiters cells showed β_I, β_{II}, and β_{IV}. Selective synthesis of β tubulin isotypes has also been described in the vestibular system and in nasal epithelia (Perry *et al.*, 2003;Woo *et al.*, 2002).

We here ask, how is the adult configuration of β tubulin isotypes achieved during development? Are microtubules equipped with the mature isotype composition during *de novo* synthesis, or are isotypes added in a cell-specific temporal sequence? Or, a further possibility, are all isotypes present initially and the number of isotypes in each cell type selectively pruned in development? To answer this question, we have taken advantage of the progressive post-natal elaboration of microtubules in gerbil organ of Corti. We examined the distribution of β tubulin isotypes in the developing organ of Corti during the first thirty days of post-natal development using isotype-specific antibodies and have compared the results to the previously described adult pattern.

Materials and methods

The distribution of β tubulin isotypes was examined in developing (P0 to P30) and adult gerbil cochlea using indirect immunofluorescence in whole mounts and frozen sections. Gerbils were anesthetized with Nembutal and cardiac perfused with heparinized saline solution followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Cochleae were dissected out, post-fixed for one hour, and decalcified if older than P6 with EDTA. The apical, middle and basal turns were dissected out for processing as whole mounts. For sections, cochleas were equilibrated, after decalcification if necessary, in 30% sucrose in PBS as a cryoprotectant, and were then quickly frozen in O.C.T. compound (Miles Labs, Elkart, IN). Frozen sections, 8–10 μm thick, were cut on a cryostat (Leica Microsystems, Bannockburn, IL). Specimens were blocked and permeabilized in PBS containing 1% bovine albumin serum, 0.25% Triton-X and 5% normal goat serum. The presence of β tubulin isotypes was detected using monoclonal antibodies obtained from hybridomas created by fusion of Sp2/0 or NS1 cells with spleen cells harvested from mice immunized with rat C-terminal β tubulin isotype peptides (Banerjee *et al.*, 1988,1990,1992;Roach *et al.*, 1998). The primary antibodies were made visible by counterstaining with goat anti-mouse IgG coupled to fluorescein isothiocyanate (Sigma, St. Louis, MO) or Alexa 488 (Molecular Probes, Eugene, OR). Whole mounts and sections were sealed under coverslips in 50% PBS: 50% glycerol containing 1% n-propylgallate. Negative controls were performed by omitting the primary antibody. Pre-incubation control experiments were performed using the rat c-terminal β tubulin isotype peptides described earlier. An anti-β tubulin antibody (not isotype specific) was used as a positive control (Sigma, clone TUB 2.1; Gozes & Barnstable, 1982).

Specimens were observed using an Axioskop II epifluorescence microscope (Carl Zeiss Jena, Jena, Germany) equipped with 40× and 100× objectives and digital images were acquired using a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Specimens were also imaged with a Radiance 2000 confocal microscope (Bio-Rad Corporation, Hercules, CA) on an Eclipse 800 epifluorescence microscope (Nikon Instruments, Melville, NY). Images were prepared for presentation using Photoshop (Adobe Systems, San Jose, CA).

For electron microscopy, gerbils were anesthetized as above and perfused with a solution of 3% paraformaldehyde and 1% glutaraldehyde in P.B.S. After post-fixation for 1 hour, and

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decalcification if necessary, cochleas were dissected into individual cochlear turns, prior to further fixation in 1% osmium tetroxide in P.B.S. Subsequently, cochlear turns were dehydrated in an ascending alcohol series, passaged through propylene oxide, and infiltrated with 3:1, 1:1 and 1:3 mixtures of propylene oxide:Eponate-12 (Ted Pella Inc, Redding, CA). Specimens were further infiltrated with 100% Eponate-12 overnight, prior to polymerization with fresh resin mixed with DMP-30 (catalyst) in flat embedding molds at 50°C for 2 days. Blocks were thin-sectioned (gold-silver reflectance) and collected using single-slot nickel grids coated with 2% nitrocellulose in amyl acetate. Grids were stained with 2% aqueous uranyl acetate for 30 minutes, then with 2% lead citrate for 15 minutes at room temperature, and subsequently observed in a Phillips CM 100 transmission electron microscope.

Animal care and handling was performed in compliance with protocols approved by the Creighton University and University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committees.

Results

In the figures below, all whole mount images are presented with the modiolus above, and all sections are presented with the modiolus to the left.

THE ORGAN OF CORTI DURING POST-NATAL DEVELOPMENT IN THE GERBIL

The anatomical configuration of the organ of Corti in the developing and adult gerbil is illustrated in Figure 1, as seen in both whole mounts and sections. At birth, the gerbil organ of Corti consists of a single layer of modified epithelial cells that are post-mitotic but incompletely developed (Fig. 1A). The innermost layer of organ of Corti cells, that is, those closest to the modiolus (the central core of the cochlea), are inner hair cells (IHCs). More peripherally are two supporting cell types, the inner and outer pillar cells (IPs and OPs). More peripheral still are three rows of outer hair cells (OHCs). The OPs are also intercalated between the first (innermost) row of OHCs. Another class of supporting cell, the Deiters cell, is intercalated between the OHCs in the second and third rows and also forms a continuous row peripheral to the OHCs. Both IHCs and OHCs at birth possess on their apical (most superficial) surfaces actin-based stereocilia that will eventually function as mechano-electric transducers of sound. As is normal with epithelial cells, tight junctions form a diffusion barrier close to their apical surfaces of organ of Corti cells.

As the organ of Corti matures (Fig. 1B), the IHCs expand towards the modiolus and the pillar cells become separated to form an open space called the tunnel of Corti. The OHCs also elongate, more so in the apical (low frequency) cochlea than the basal (high frequency) cochlea. Most microtubules in the organ of Corti are assembled after birth (Hallworth *et al.*, 2000). Mature anatomical appearance and physiological function are apparent by 20 days after birth.

DEVELOPMENT CHANGES IN THE β TUBULIN ISOTYPES IN IHCs

At birth (P0), three of the four β tubulin isotypes studied were present in IHCs, namely β_I, β_{II} and β_{IV} tubulin. Filamentous labeling, characteristic of microtubules, was observed in IHCs using the antibodies for these three isotypes but not the antibody to β_{III} tubulin. The label was observed in the form of an aster at the cell apex, as has been described previously (Hallworth *et al.*, 2000). An example of the labeling pattern in IHCs is shown in Figure 2A (β_I tubulin in a whole mount of organ of Corti at P0). The IHC labeling pattern for β_{II} tubulin at P0 (Fig. 2B) was identical to that for β_I tubulin. β_{IV} tubulin in IHCs was also found in the same aster pattern at P0 (Fig. 2C, whole mount). In radial section (Fig. 2D), label for β_{IV} tubulin in IHCs can be seen to extend from the cell apex to the base. Thus, three β tubulin isotypes were present in

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inner hair cells early in postnatal development. No labeling for β_{III} tubulin was found in IHCs at P0 or at any later stage in development.

As previously described (Steyger *et al.*, 1989;Hallworth *et al.*, 2000), the organization of labeling for β tubulin isotypes in adult IHCs was very different from that in the neonate. Labeling in the mature IHC consisted of filaments originating from a circumferential apical ring, approximately at the level of the tight junctions, and was seen to ramify diffusely throughout the cell. Figure 2E shows labeling for β_I tubulin in IHCs in an adult whole mount. Both the circumferential ring (*r*) at the apex and the somatic filamentous tubulin are evident. A similar labeling pattern was seen in the adult for β_{II} tubulin (Fig. 2F). The adult pattern of β_{II} tubulin labeling is established by P16. These observations suggest that a major reorganization of IHC microtubules takes place during development.

A remarkable feature of β tubulin isotype development in IHCs is the decline in β_{IV} tubulin synthesis after P20. As shown in Figure 2G, filamentous labeling for β_{IV} tubulin was observed in IHCs late in postnatal development (confocal image in a whole mount at P21). However, by P27 β_{IV} tubulin was no longer synthesized in IHCs. As shown in Figure 2H, a confocal microscope image of a whole mount at P27, labeling for β_{IV} tubulin was present only in IPs and OPs. No labeling was present at the position corresponding to IHCs. Between P20 and P27, therefore, levels of β_{IV} tubulin in IHCs apparently decline to zero while β_I and β_{II} tubulin levels remain constant during adult life.

DEVELOPMENT CHANGES IN THE β TUBULIN ISOTYPES IN OHCs

In the first few days of post-natal life, the same three isotypes were present in OHCs as in IHCs (β_I, β_{II} and βIV tubulin). For all three antibodies, the labeling in OHCs was observed in the form of asters, as in IHCs (Fig. 3A, P3). Note, in the inset to Figure 3A, the presence of a single kinocilium on the OHC, indicated by (*k*). Similar OHC labeling patterns were seen for β_I tubulin, as can be seen by inspection of Figure 2A. Figure 3B shows the label pattern for β_{II} tubulin in OHCs in a section of organ of Corti at P0. Therefore, the same three isotypes were observed in OHCs as in IHCs in identical patterns in early post-natal development. In the adult, contrary to our observation in IHCs, OHC labeling did not reorganize (Hallworth *et al.*, 2000). Instead, label in OHCs continued to originate from a point close to the apex, just as in the neonate. Figure 3C and D show examples of filamentous label for β_I tubulin and β_{IV} tubulin (a confocal image) in adult whole mounts. Note in Figure 3C the discrete aster-like apical origin of adult OHC labeling (*a*). No labeling for β_{III} tubulin was found in OHCs at any stage in development.

Just as β_{IV} tubulin levels declined in IHCs after P20, β_{II} tubulin levels in OHCs declined over the same period. Labeling for β_{II} tubulin was present in OHCs at P9 (Fig. 3E), but by P27 levels of β_{II} labeling in OHCs had declined to undetectable levels (Fig. 3F). The decline began at P20 and was essentially complete by P27.

DEVELOPMENT CHANGES IN THE β TUBULIN ISOTYPES IN PILLAR CELLS

β tubulin isotype labeling first appeared in pillar cells as early as P0 in the basal turn (Hallworth *et al.*, 2000), and somewhat later in middle and apical turns. Three isotypes were present, β_I, β_{II}, and β_{IV} tubulin, in identical patterns. An example (Fig. 4A) shows β_{IV} tubulin at P0. Initially, label in IPs was seen as a tooth crown pattern at the cell apex, as previously reported (Hallworth *et al.*, 2000). As development progressed, the characteristic filamentous labeling in pillar cells was seen to form in a progressive fashion from cell apex to base. The phalangeal process of the OP cell developed at the same time, as shown in Figure 4B, a whole mount at P9. In the adult, both IPs and OPs showed prominent labeling for β_{II} tubulin. Figure 4C, a whole mount, shows labeling for β_{II} tubulin in the long processes of OPs. Figure 4D shows a

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view of IPs and OPs in a whole mount labeled for β_{IV} tubulin in the adult whole mount. No labeling for β_{III} tubulin was found in pillar cells at any stage.

Labeling for β_I tubulin in pillar cells was observed to decline after P20. Figure 4E shows a confocal image of β_I tubulin in IPs and OPs at P20. Levels of β_I tubulin labeling declined first in IPs, while levels in OPs declined more slowly. By P27, β_I tubulin was virtually absent in pillar cells (Fig. 4F).

NO CHANGE IN THE β TUBULIN ISOTYPES IN DEITERS CELLS

Unlike hair cells and pillar cells, the levels of β tubulin isotypes in Deiters cells did not undergo a selective reduction during development. Labeling for β tubulin isotypes first appeared in Deiters cells by P6. Labeling for the three isotypes, β_I, β_{II} and β_{IV} tubulin, was observed in identical patterns. No labeling for β_{III} tubulin was found in Deiters cells during development. At P6 through P9, label appeared as a loosely gathered bundle of filaments extending from the apex of the cell to the base. Figure 5A, a whole mount, shows β_I in Deiters cells (*D*) at P6. In cross-sections, β_{IV} tubulin was also evident in Deiters cells at that stage (Fig. 5B). The label can be seen to extend through most of the length of the cell. By P9, β_{IV} tubulin labeling in Deiters cells (Fig. 5C) was in the form of more organized but still splayed bundles throughout the cell. In the adult (Fig. 5D), labeling for β_I tubulin bundle in Deiters cells was condensed in a single bright strand, as previously reported (Hallworth *et al.*, 2000).

DEVELOPMENT CHANGES IN THE β TUBULIN ISOTYPES IN ORGAN OF CORTI INNERVATION

The somas of auditory nerve primary afferent neurons innervating the organ of Corti are found in the spiral ganglion of the modiolus. Their dendrites extend to the IHC and OHC regions of the organ of Corti, while their axons exit the cochlea via the modiolus. A population of small diameter efferent fibers, originating from cell bodies in the auditory brainstem, traverse the spiral ganglion and form the intraganglionic spiral bundle. Branches from the IGSB then innervate the organ of Corti, where they form two bundles, the inner spiral bundle under the IHCs, and the outer spiral fibers under the OHCs.

At P0, three β tubulin isotypes (β_I, β_{II}, and β_{III}) were found in developing organ of Corti neural processes. Figure 6A shows labeling for β_{II} tubulin at P3 in radial afferent dendrites and in the intraganglionic spiral bundle. Figure 6B shows β_{III} tubulin in the inner spiral bundle under the IHC at P3. Figure 6C shows a con-focal image of tunnel crossing fibers and outer spiral fibers labeled for β_I tubulin at P16. The fibers may be characterized by their elevated position in the tunnel of Corti as efferent fibers going to outer hair cells. Label of fibers in the habenula perforata (the channels in the modiolus through which the afferent dendrites reach the organ of Corti) was sparse, as shown in Figure 6D (same specimen as Fig. 6C).

However, after P9, label of fibers in the habenula diminished, and in the adult, none of the four isotypes was present in afferent dendrites, which are characterized by their large diameters and are the preponderant nerve process in the habenula. By P16, no large diameter nerve fibers in the organ of Corti or habenula were labeled by any of the antibodies (Fig. 6E). In the adult, three antibodies (those to β_I, β_{II}, and β_{III} tubulin) labeled only a few fine caliber fibers in the habenula. Figure 6E shows the fine fibers labeled for β_{III} tubulin in a whole mount of an adult. Similar observations were made for β_I and β_{II} tubulin. The lack of label in large diameter fibers in the habenula does not seem to be an effect of insufficient antibody penetration, because the antibody labels the small diameter fibers adequately, as seen in Figure 6E. Rather, levels of β tubulin isotype label in afferent dendrites declined during maturation, because very few microtubules were present. Studies using the pan-β tubulin antibody failed to reveal any labeling in adult afferent dendrites (data not shown; see also Hallworth *et al.*, 2000).

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The near-absence of microtubules in spiral ganglion dendrites in the adult was further confirmed using electron microscopy. Longitudinal filaments in basal turn dendrites from a P2 animal had an average diameter of 28.6 nm (standard deviation 3.5 nm, $n = 24$), typical of microtubules (Figure 7A, indicated by arrows). These microtubules were observed in processes that were not surrounded by Schwann cells, which indicates that at this stage myelination had not yet begun. Microtubules were seen in dendrites in both apical and basal turns. At P9, large filaments, consistent in diameter with microtubules, were only sparsely seen in spiral ganglion dendrites (Fig. 7B). The diameter of the large filaments was 27.2 nm (standard deviation 5.3, $n = 8$). These were usually single microtubules that were extensively cross-linked to intermediate filaments. In the adult, longitudinal fibers in spiral ganglion dendrites were extensively cross-linked and had an average diameter of 10.0 nm(standard deviation 1.4, $n = 23$), which is characteristic of intermediate filaments (Fig. 7C). Very few microtubules were seen in dendrites.

KINOCILIA ON ORGAN OF CORTI CELLS DURING DEVELOPMENT

Solitary kinocilia were observed at birth on many cell types in the developing organ of Corti, including hair cells, supporting cells, and the superficial cells of the greater epithelial ridge. Figure 8A shows β_I tubulin in kinocilia in a whole mount at P0. Kinocilia are seen on IHCs, OHCs and at least one Deiters cell (*arrows*). Similar views of kinocilia on hair cells may be seen in Figure 2A(labeled for β_I tubulin) and Figure 3A(labeled for β_{II} tubulin). Figure 8B shows kinocilia (*arrow*) on OHCs in a cross section of organ of Corti at P3, labeled for β_{II} tubulin. β_{III} tubulin was not detected in kinocilia, but β_{IV} tubulin was present. This is shown in Figure 8C, which shows a whole mount of organ of Corti at P0. Note also the extensive labeling of kinocilia on cells of the greater epithelial ridge and on Deiters cells in this specimen. Figure 8D shows an electron micrograph of an IHC kinocilium at P9, to illustrate the microtubules present.

Kinocilia were not usually observed on most cell types after P6. However, using these antibodies, we have observed kinocilia as late as P20 on IHCs. Figure 8E shows kinocilia on IHCs at P18 in a whole mount, labeled for β_{II} tubulin.

Discussion

Our findings are summarized in diagrammatic form in Figure 9 and are summarized in Table 1.

In previous studies, we have demonstrated the β tubulin isotype compositions of several different cell types in the adult cochlea, vestibular system and nasal epithelia (Hallworth & Ludueña, 2000;Woo *et al.*, 2002;Perry *et al.*, 2003). We here show that the β tubulin isotype composition of microtubules in organ of Corti hair cells, pillar cells and dendrites is initially the same at birth, but, at a late stage in post-natal development, hair cell and pillar cell microtubules undergo a selective reduction in the number of β tubulin isotypes. Deiters cells, whose microtubules are the last to be elaborated, did not show a β tubulin isotype change.

The reduction in the number of β tubulin isotype in hair cells and pillar cells occurs after P20, well after the developmental period in which the most extensive post-mitotic changes in cellular morphology take place, and well after physiological responses are generally considered mature. In contrast, we noted that all β tubulin isotypes in cochlear dendrites disappear during development, but we showed that this is because the microtubules themselves disappear. We also described a finding, incidental to the main purpose of this study, that cilia are present on IHCs much later in organ of Corti development than previously reported.

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These data represent the first description of the developmental course of tubulin isotype synthesis at the cellular level in any system. Lewis *et al.* (1985) demonstrated a decline of mRNA transcripts of four α and β tubulin isotypes in mouse brain from birth to maturity. However, it is unclear whether those results represent cell-type specific changes in isotypes or changes in the relative abundance of one cell type over another. Havercroft and Cleveland (1984) showed tissue-specific changes in the levels of certain chicken β tubulin isotypes in brain, but, again, the information obtained was not cell-type specific. With the results presented here, we show a novel pattern, that three isotypes are synthesized early in development and that subsequently the number of isotypes is pruned, in a different way in each cell type.

Our findings do not permit any direct assignment of isotype-specific functions to the β tubulin isotypes. Microtubules in hair cells appear to have the usual functions of providing structural integrity and providing pathways for transport of macromolecules and vesicles. The unusually dense bundles of microtubules in the pillar cells and Deiters cells (Slepecky, 1996), which are complexed with actin filaments, appear to confer substantial axial rigidity to those cells. However, the β tubulin isotype compositions of each cell type are different, and do not suggest simple assignments of function to isotypes. Further, there are no obvious microtubule-related functions that change between P20 and P27 in either hair cells or supporting cells. However, our findings do, if indirectly, support the position that the isotypes may have specific roles, either individually or in combination.

It remains possible that the levels of β tubulin isotypes were not reduced but rather that the antibody epitopes became masked by specific microtubule binding proteins in each cell type or by modification of the epitope itself. Microtubules are known to undergo a variety of post-translational modifications, including acetylation, detyrosination, and polyglutamylation. Such post-translational modifications are associated with resistance to depolymerization by colchicine and by cold (Bulinksi & Gundersen, 1991). It is possible that post-translational tubulin modifications may be responsible for epitope masking that gives the appearance of reduced isotype levels. Slepecky *et al.* (1995) showed that microtubules in supporting cells are extensively modified, while those in hair cells are not. However, our antibodies are effective in both hair cells and supporting cells in the adult, which argues against any selective modification of epitopes. Further, while changes in post-translational modification of microtubules do occur in cochlear development (Tannenbaum & Slepecky, 1997), the changes are complete by P14 and therefore could not account for the changes in labeling that we have observed.

We did not observe label for β_{III} tubulin in hair cells at any stage in development. This is in agreement with the results of Hallworth and Luduena (2000) in the adult. The absence of β_{III} tubulin in non-neuronal cell types in the developing and adult cochlea is in agreement with previous observations that β_{III} tubulin is rarely synthesized in cells other than neurons (Burgoyne *et al.*, 1988;Joshi & Cleveland, 1989). β_{III} tubulin is commonly referred to as the "neuron-specific" tubulin, although it has also been reported in colon (Roach *et al.*, 1998) and in Sertoli cells (Lewis & Cowan, 1988). In contrast, Stone and Rubel (2000) reported β_{III} tubulin in both mature and regenerating chick cochlea hair cells. The antibody used in that study, TUJ1 (Lee *et al.*, 1990) was raised to chick brain β_{III} tubulin. We cannot be certain that our antibody recognizes exactly the same epitope that TUJ1 recognizes. Therefore it is possible that there is β_{III} tubulin present that our antibody does not recognize.

We observed kinocilia on all organ of Corti cells in the early stages of post-natal development. These are primary cilia, a common feature of epithelial cells in development and in many sites in the mature animal (Pazour & Whitman, 2003). Cilia on organ of Corti cells have been reported to atrophy during early post-natal development. We now report that cilia on gerbil

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IHCs persist up to P20. This is much later than the latest previously reported, P10 in the mouse (Lim & Anniko, 1985).

We found that cilia were labeled with the same isotypes as microtubules in the rest of the cell, including, but not limited to, β_{IV} tubulin. This is significant because β_{IV} tubulin is thought to be a specific requirement of axonemes (Renthal *et al.*, 1993;Raff *et al.*, 1997). In other ciliated tissues, the results suggest that all isotypes present are used but that β_{IV} tubulin and possibly β_I tubulin are required (Renthal *et al.*, 1993;Jensen-Smith *et al.*, 2003). In this context, it is interesting that all four isotypes are found in the cilia, dendrites, somas and axons of olfactory neurons, but β_{IV} tubulin is not found in the immature stem cells of olfactory neurons (Woo *et al.*, 2002), which suggests that β_{IV} tubulin specifically begins to be synthesized in olfactory neurons during maturation.

In summary, our results imply that there are systematic changes in β tubulin isotype composition in individual cell types of the developing mammalian organ of Corti and that these changes are not clearly linked to known morphological or physiological changes. Further study may reveal if the developmental changes in tubulin isotype composition correspond to subtle or even major changes in the functional properties of these cells. The strong evolutionary conservation of the differences among tubulin isotypes obliges us to consider the possibility that these functional changes may be important.

Acknowledgments

Supported by N.I.H. grant CA26376, U.S. Army grant DAMD17-98-1-8246, and Welch Foundation grant AQ-0726 to R.F.L., and N.I.H. grant DC02053 to R.H. H.C.J.-S. is a Clare Booth Luce fellow. We thank Juany Rehling for excellent technical assistance in the electron microscopy portion of this study and Bernd Fritzsch for useful comments on the manuscript. We also thank the Taub Foundation and the Nebraska Health Future Foundation for assistance toward the purchase of the confocal microscope, and our anonymous reviewers for perceptive and helpful comments.

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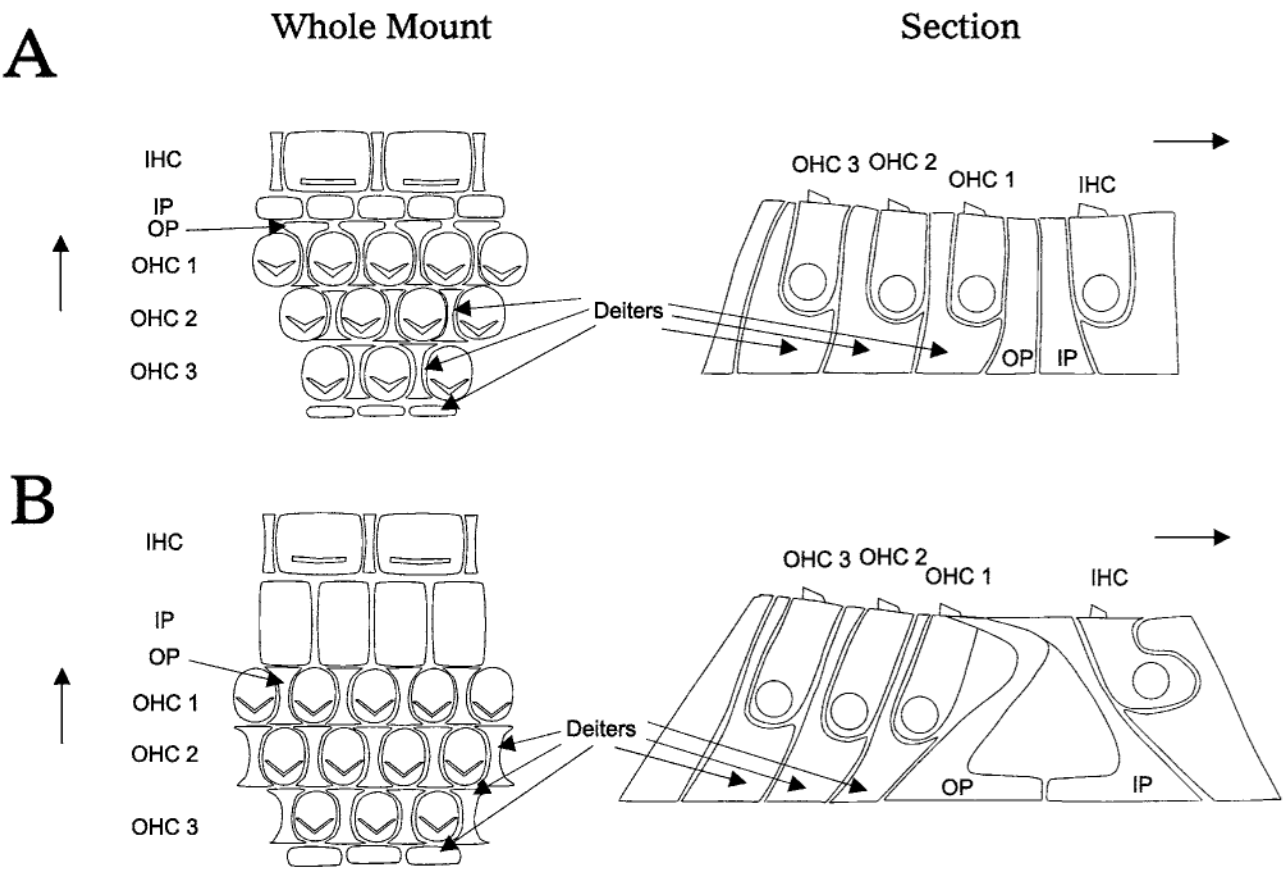

Fig 1.

Diagram of the organ of Corti as seen in the whole mount (left) and in radial section (right). (A) Representative diagrams of organ of Corti in the early stages of post-natal development. The development stage depicted approximately represents P3 in the basal turn, a stage at which microtubules have not year appeared in outer pillar cells and Deiters cells. (B) Representative diagrams of mature organ of Corti. The location and approximate configurations of the apical stereocilia bundles are indicated on the hair cells. *Labels*: IHC = inner hair cell, OHC1 −3 = first, second, and third rows of outer hair cells, IP = inner pillar cell, OP = outer pillar cell. Arrows next to each diagram indicate the direction of the modiolus.

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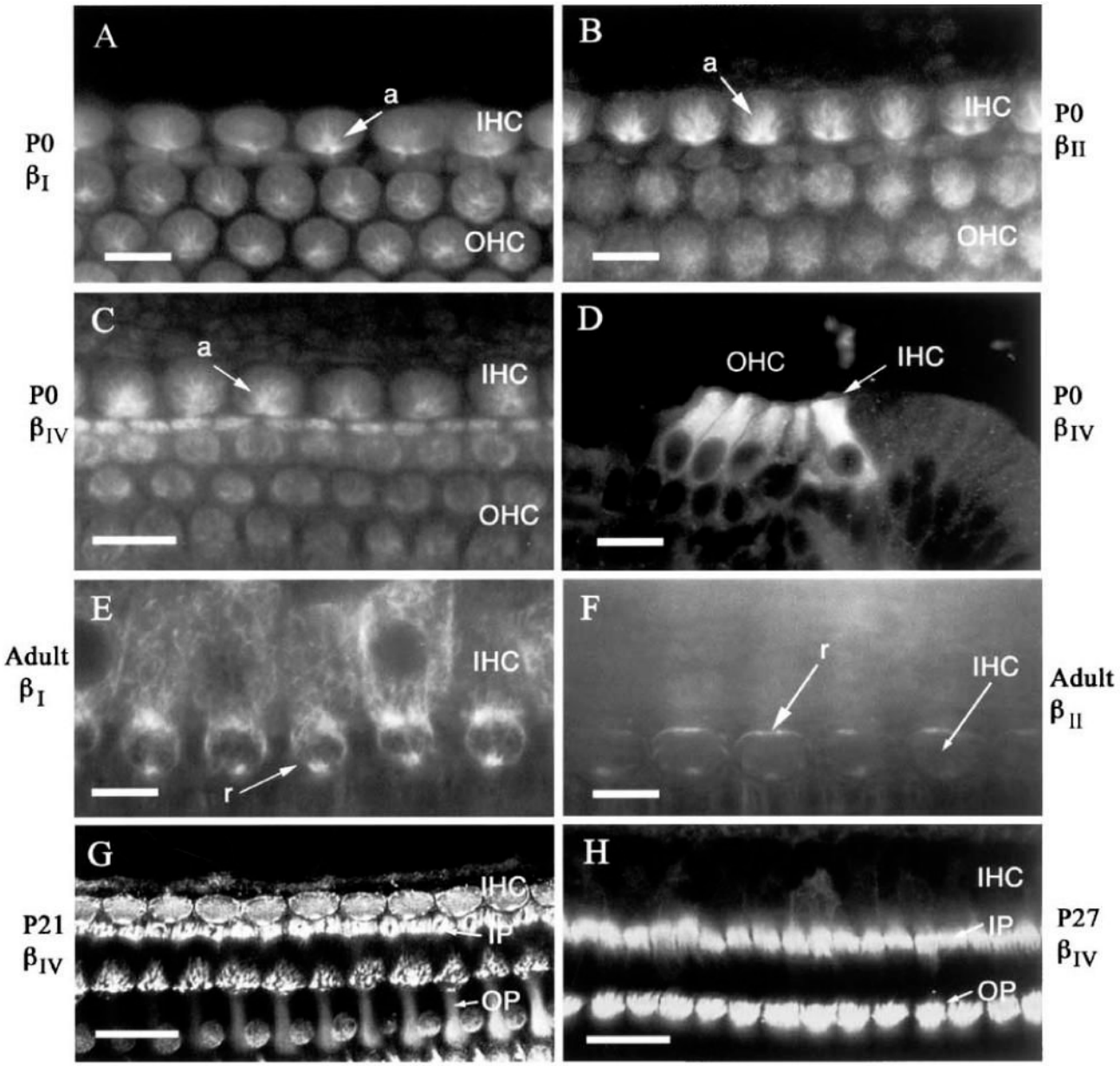

Fig 2.

β tubulin isotypes during post-natal development of IHCs. (A) Label for β_I tubulin in a whole mount of organ of Corti at P0, basal turn. Label in IHCs consists of an aster (*a*) at the apex. (B) Label for β_{II} tubulin in a whole mount of organ of Corti at P0, showing a similar aster pattern (*a*). (C) Label for β_{IV} tubulin in a P0 whole mount of organ of Corti, showing the aster-like label pattern (*a*) characteristic of IHCs. (D) β_{IV} tubulin label in IHCs in a section of organ of Corti at P0. (E) Label for β_I tubulin in a whole mount of adult organ of Corti. In contrast to the earlier stages in development, label is organized in a ring (*r*) at the cell apex. (F) β_{II} tubulin label in a whole mount of adult organ of Corti, showing a similar arrangement. (G) Confocal image of label for β_{IV} tubulin label in a whole mount of organ of Corti at P21, showing presence of β_{IV} tubulin in IHCs. (H) Confocal image of β_{IV} tubulin label in a whole mount of organ of Corti at P27, showing absence of β_{IV} tubulin in IHCs. Scale bars: (A, B, C, D, E, F) 10 μm,

(G, H) 20 μm. *Labels*: IHC = inner hair cell, OHC = outer hair cell, IP = inner pillar cell, OP = outer pillar cell, a = aster, r = apical ring.

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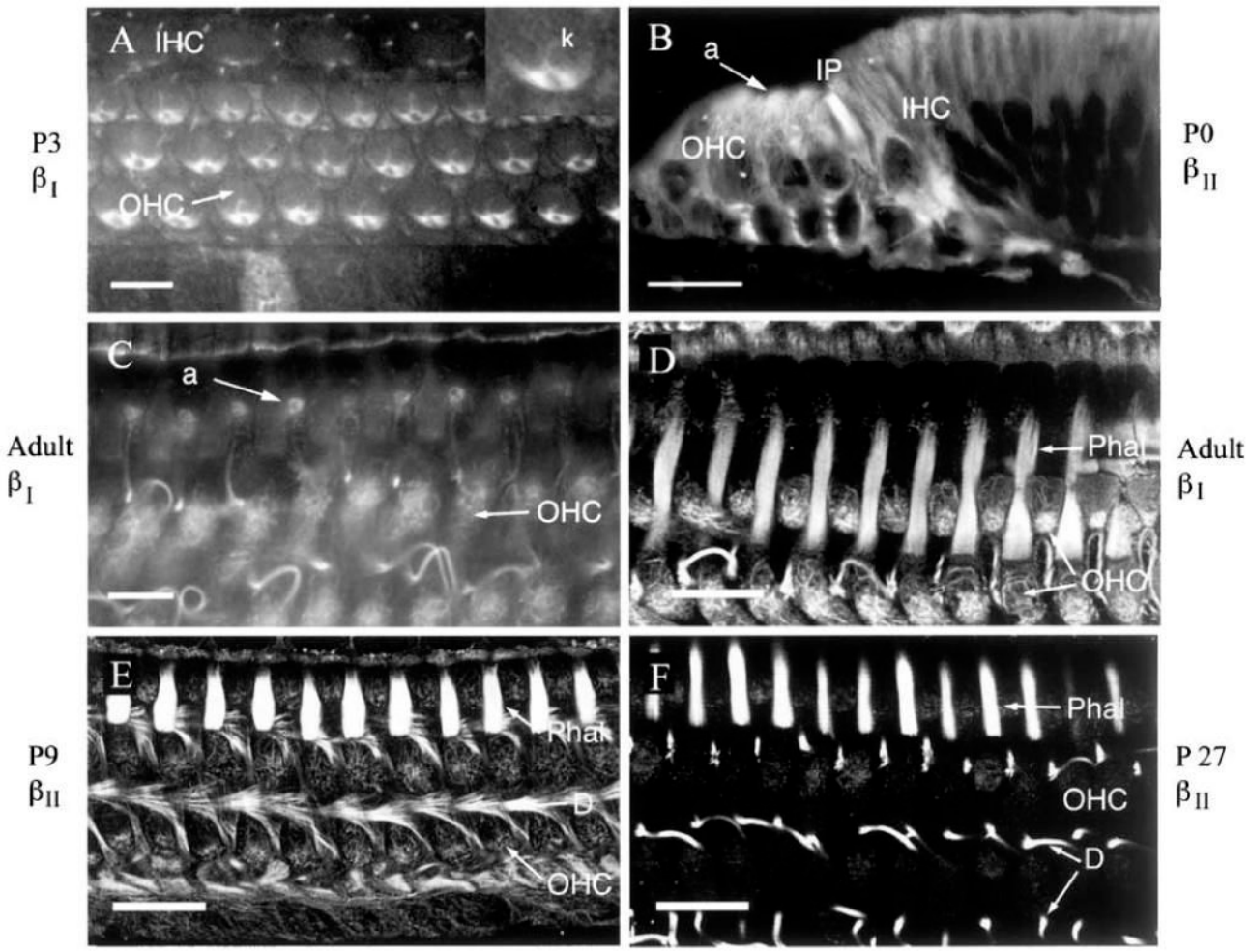

Fig 3.

β tubulin isotypes during post-natal development of OHCs. (A) β_{II} tubulin in a whole mount of organ of Corti at P3. The inset shows a kinocilium on a third row OHC indicated by (*k*). (B) β_{II} tubulin label in a section of organ of Corti at P0. (C) Label for β_I tubulin in a whole mount of adult organ of Corti. Label is visible in the OHC in the form of an apical aster (*a*). (D) Similar view, confocal, of label for β_{IV} tubulin in OHCs in an adult whole mount. (E) β_{II} tubulin in OHCs in a whole mount of organ of Corti at P9. (F) β_{II} tubulin in a whole mount of organ of Corti at P27. Label for β_{II} tubulin is absent from OHCs. Scale bars: (A, C) 10 μm, (B, D, E, F) 20 μm. *Labels*: IHC = inner hair cell, OHC = outer hair cell, IP = inner pillar cell, OP = outer pillar cell, Phal = phalangeal processes of Deiters cells, D = Deiters cell, a = aster.

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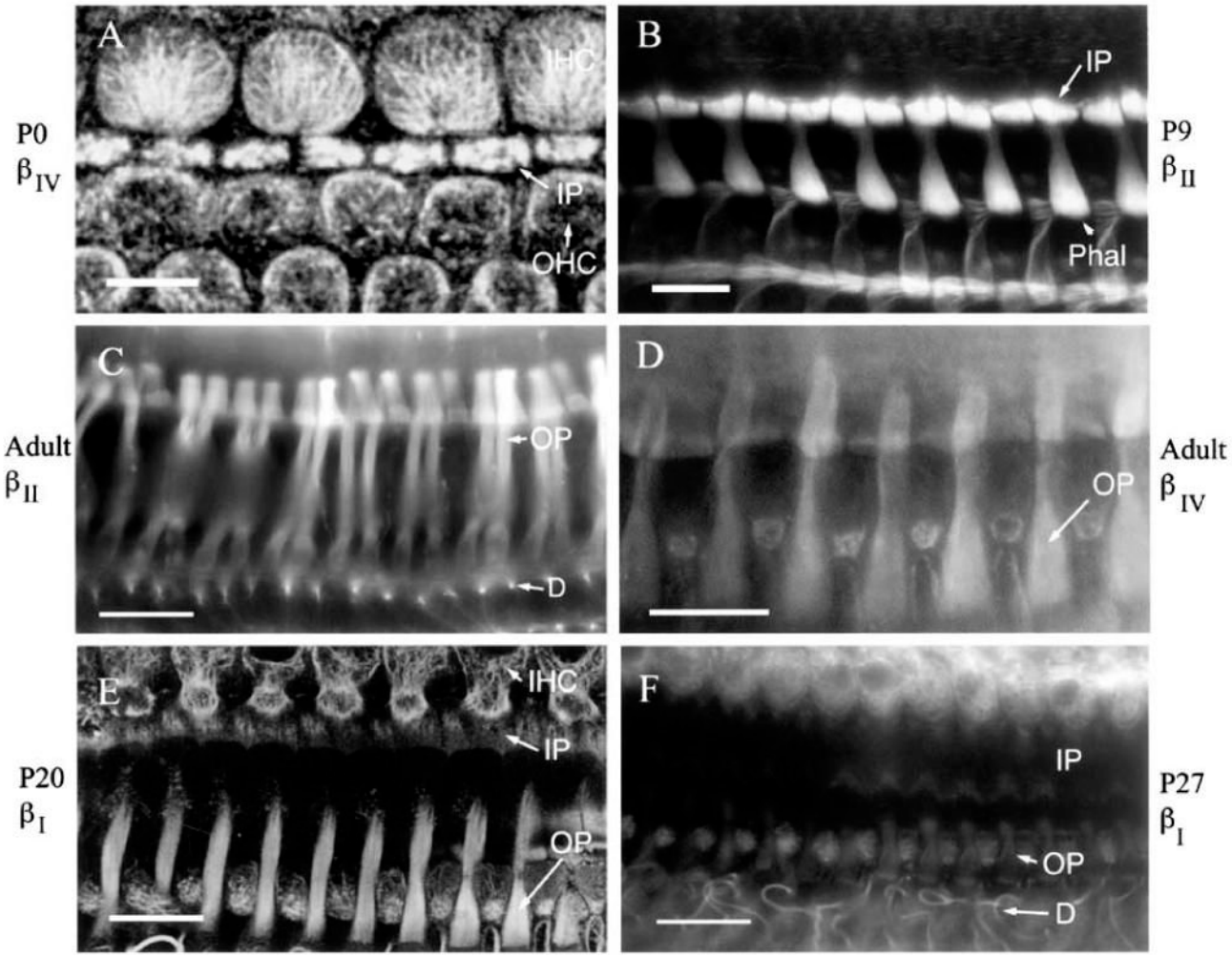

Fig 4.

β tubulin isotypes during post-natal development of pillar cells. (A) Label for β_{IV} tubulin in IPs at P0 in a whole mount, observed with the confocal microscope. (B) Label for β_{II} tubulin in a whole mount at P9, in the phalangeal process *(Phal)*. (C) Prominent labeling for β_{II} tubulin in the long processes of OPs in an adult whole mount. (D) Similar labeling pattern for β_{IV} tubulin in an adult whole mount. (E) Confocal view of β_{I} tubulin in a whole mount at P20, showing label in IPs and OPs. (F) β_{I} tubulin in a whole mount, at P27, showing absence of label in IPs and faint labeling in OPs. Scale bars: (A, B, D, E) 10 μm, (D) 25 μm, (F) 20 μm, *Labels*: IP = inner pillar cells, OP = outer pillar cells, Phal = phalangeal processes of OP, IHC = inner hair cells, D = Deiters cells.

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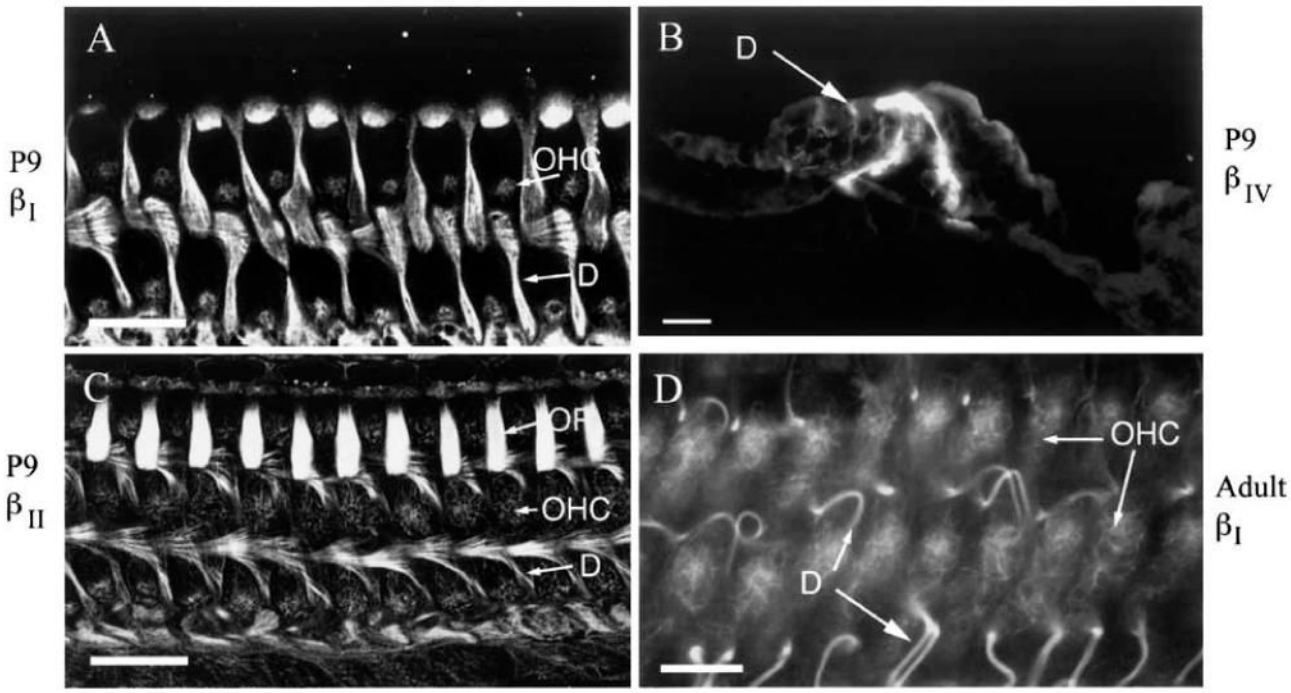

Fig 5.

β tubulin isotypes during post-natal development of Deiters cells. (A) Label for β_I tubulin in a whole mount of organ of Corti at P9. (B) Label for β_{II} tubulin in a section of organ of Corti at P9. Label in Deiters cells is indicated by (*D*). The other bright label is in pillar cells. (C) Confocal view of β_{II} tubulin label in Deiters cells at P9, whole mount (same figure as Fig. 2D). (D) Label for β_I tubulin in a whole mount of adult organ of Corti. Scale bars: 10 μm, all panels. *Labels*: D = Deiters cells, OHC = outer hair cells, OP = outer pillar cells.

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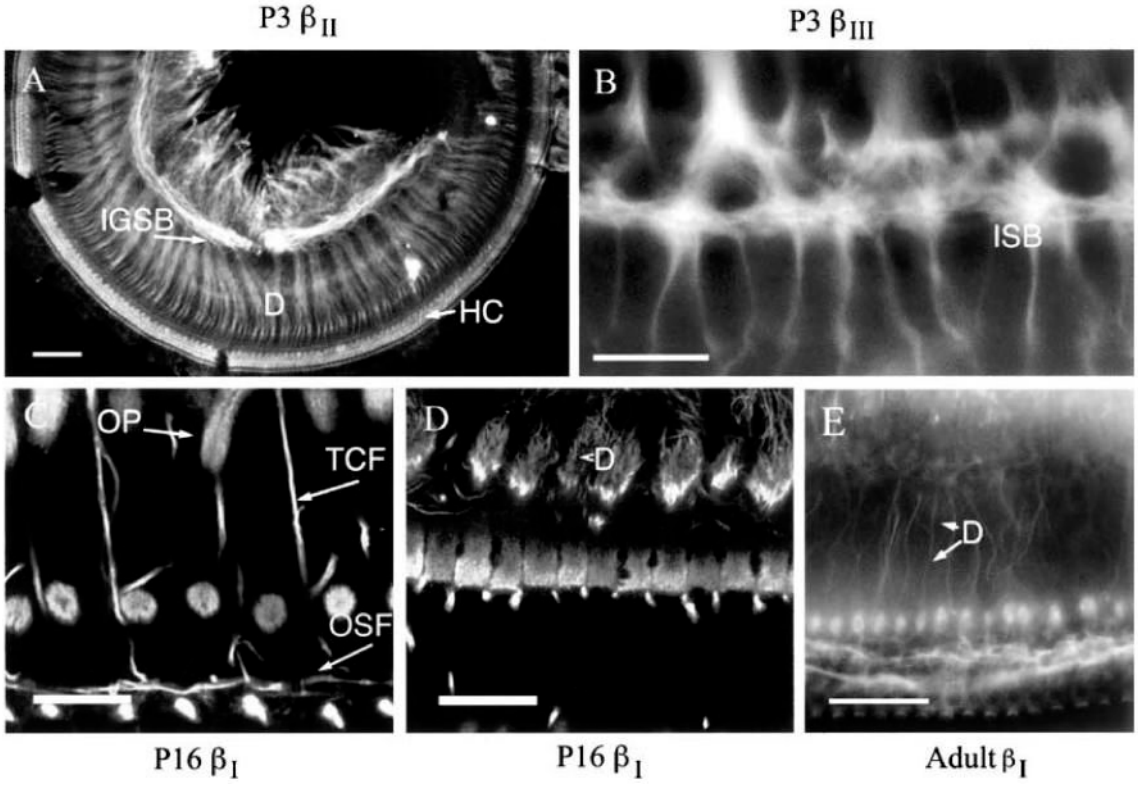

Fig 6.

β tubulin isotypes during post-natal development of cochlear innervation. (A) Afferent dendrites, hair cells and fibers of the intraganglionic spiral bundle in the apical turn at P3, labeled for β_{II} tubulin. (B) Detail of label in organ of Corti fibers under IHCs at P3, labeled for β_{III} tubulin. (C) Labeling for β_I tubulin in tunnel crossing fibers in the organ of Corti in a P16 whole mount, viewed in the confocal microscope. (D) Fibers labeled for β_1 tubulin in the habenula perforata labeled for β_I, at P16, also viewed in the confocal microscope (same specimen as D). (E) Fine caliber fibers labeled for β_I tubulin in the modiolus of an adult. Scale bars: (A) 100 μm, (B, C, D) 10 μm, (E) 50 μm. *Labels*: D = Deiters cells, HC = hair cells, IGSB = intraganglionic spiral bundle, ISB = inner spiral bundle, TCF = tunnel crossing fibers, OSF = outer spiral fibers, OP = outer pillar cells.

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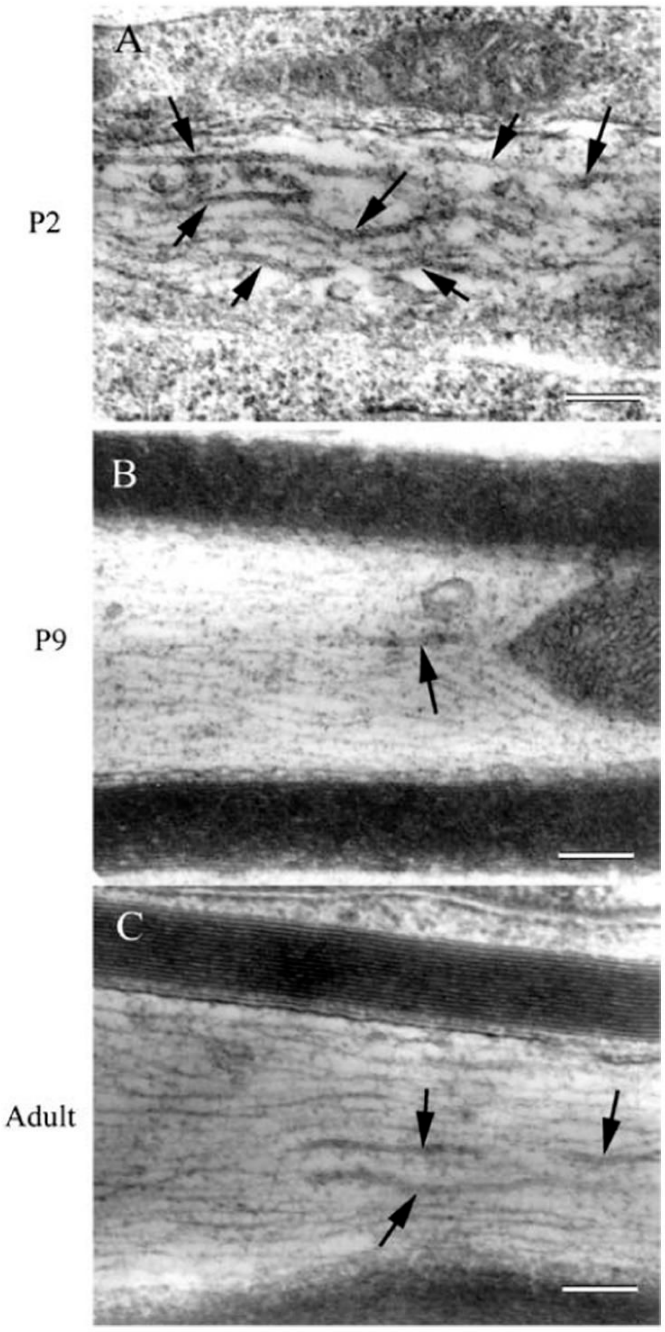

Fig 7.

Loss of microtubules in afferent dendrites during development. (A) Electron micrograph of afferent dendrites in the habenula perforata at P2, showing extensive microtubule networks, individual microtubules, indicated by arrows. (B) Electron micrograph of afferent dendrites at P9, showing sparse microtubules (arrow). (C) Electron micrograph of afferent dendrites in the adult. Microtubules are few in number (arrow). Scale bar: 200 nm in all panels.

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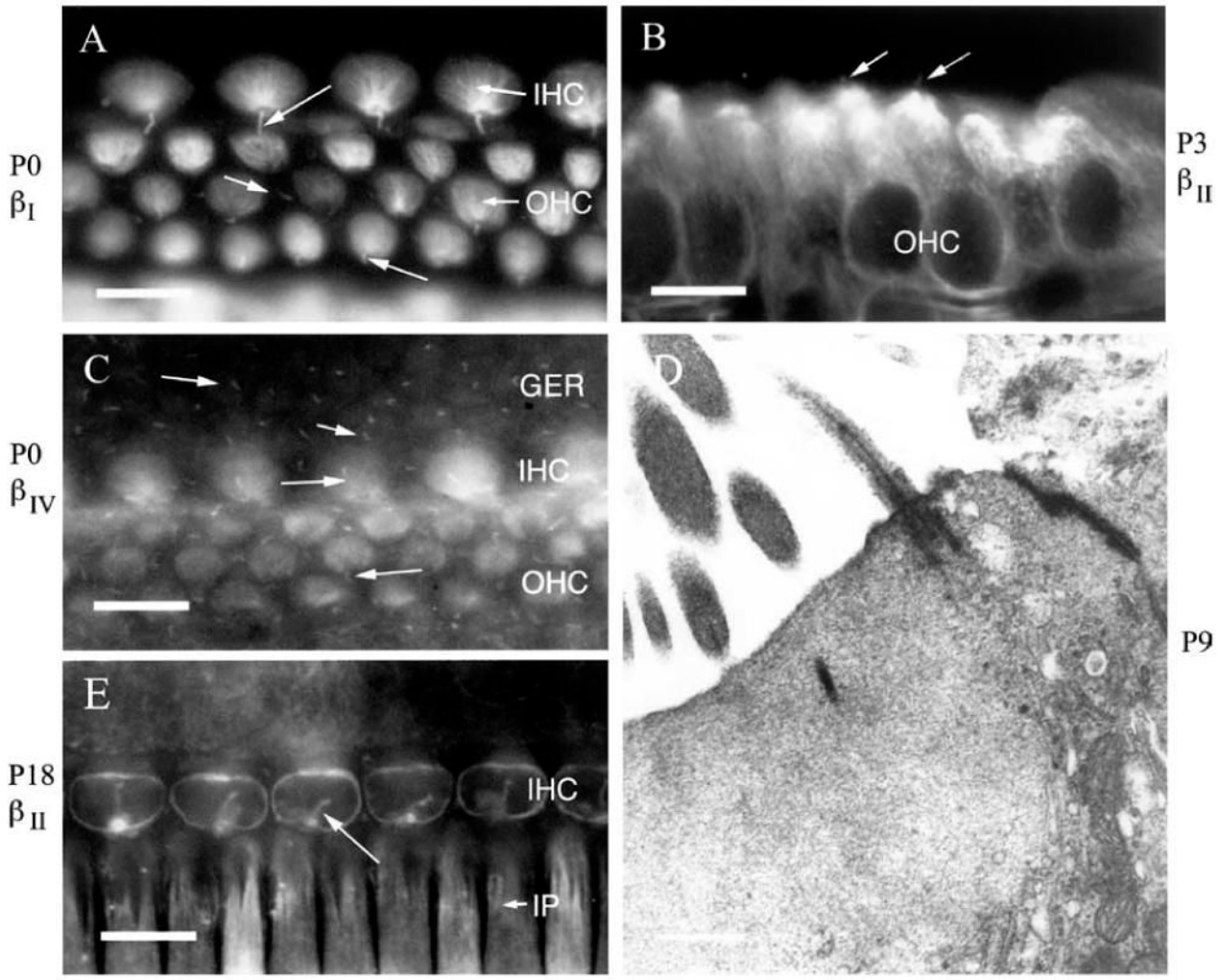

Fig 8.

Kinocilia on organ of Corti cells during development revealed using the antibodies to β tubulin isotypes. (A) β_I tubulin labeling in kinocilia at P0 in a whole mount. Note kinocilia on IHCs, OHCs, and Deiters cells (*arrows*). (B) β_{II} tubulin labeling in an oblique cross section of organ of Corti, at P3, showing kinocilia on OHCs (*arrow*). (C) Kinocilia labeled for β_{IV} tubulin in kinocilia in a P0 whole mount. Kinocilia are seen on OHCs, IHCs and the cells of the greater epithelial ridge (*GER*). Note kinocilia on IHCs, and OHCs (*arrows*). (D) Electron micrograph of cilium on IHC at P9. (E) Kinocilia on IHCs at P18 (*arrow*). This example shows β_{II} tubulin labeling. Scale bars: (A, B, C, D) 10 μm, (E) 500 nm. Labels: GER=greater epithelial ridge, IHC = inner hair cells, OHC = outer hair cells, IP = inner pillar cells.

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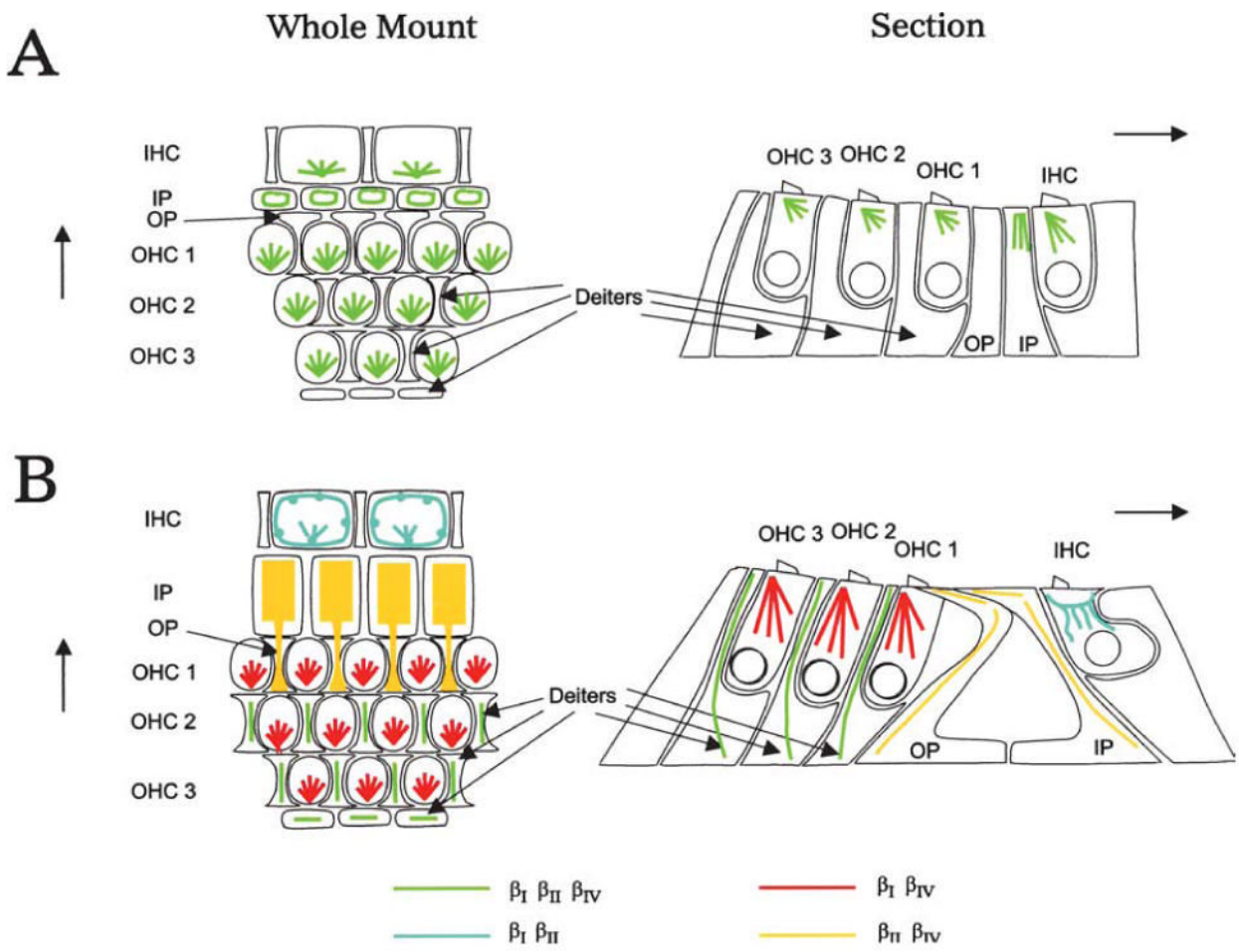

Fig 9.

The findings of this study, superimposed on the same outline diagram as Figure 1. (A) Representative diagrams of organ of Corti in the early stages of post-natal development (P3). (B) Representative diagrams of mature organ of Corti. *Labels*: IHC=inner hair cell, OHC1-3 = first, second, and third rows of outer hair cells, IP = inner pillar cell, OP = outer pillar cell. Arrows next to each diagram indicate the direction of the modiolus. The color key to the isotype composition of the microtubules in each cell type is given below the figures. Labels: IHC—inner hair cell, IP = inner pillar cell, OP = outer hair cell, OHC1-3 = first, second and third row outer hair cells.

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Table 1

Summary of β tubulin isotypes present in developing and adult organ of Corti cells.

	Isotypes at first observation of microtubules	***Isotypes in mature microtubules***	***Isotypes lost***	***Developmental stage of loss***
IHC	β_I β_{II} β_{IV}	β_I β_{II}	β_{IV}	P20 – P27
OHC	β_I β_{II} β_{IV}	β_I β_{IV}	β_{II}	P20 – P27
IP	β_I β_{II} β_{IV}	β_{II} β_{IV}	β_I	P20 – P27
OP	β_I β_{II} β_{IV}	β_{II} β_{IV}	β_I	P20 – P27
Deiters	β_I β_{II} β_{IV}	β_I β_{II} β_{IV}	none	n/a
Afferent dendrites	β_I β_{II} β_{III}	none	all	P9 – P16