THE JOURNAL OF COMPARATIVE NEUROLOGY 379:603–616 (1997)

Neurofilament Proteins in Avian Auditory Hair Cells

ELIZABETH C. OESTERLE,* DIANA I. LURIE, AND EDWIN W RUBEL
Department of Otolaryngology and Head and Neck Surgery, Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seattle, Washington 98195

ABSTRACT

The distribution of middle-weight neurofilament protein (NF-M), an intermediate filament of neurons, was examined in the developing and mature avian inner ear by using immunocytochemical techniques. NF-M was detected in auditory hair cells and VIIIth cranial nerve neurons. NF-M-positive hair cells are first detected at embryonic day 11 (E11) in superior hair cells in the midproximal (midfrequency) region of the chicken basilar papilla. With time, increasing numbers of hair cells express NF-M. Two developmental gradients occur: 1) a radial gradient, in which superior hair cells are labeled first, and progressively more inferiorly located hair cells are labeled during ontogeny, and 2) a longitudinal gradient, in which hair cells in the midproximal region are labeled first, and then progressively more distal (low-frequency) hair cells are labeled. There is also a small proximally directed progression of NF-M expression. By E19, NF-M-positive hair cells are found throughout the distal and midproximal regions, and this expression is maintained through 3 weeks posthatching. By 22 weeks posthatching, NF-M staining in hair cells is markedly diminished; staining is seen in only a few tall hair cells in the distal one-fourth of the papilla and in short hair cells in the distal one-half of the papilla. NF-M is never expressed by hair cells at the proximal (high-frequency) end of the papilla at any time examined. These findings suggest that some cell types that have traditionally been classified as nonneural may express neurofilament and that the basilar papilla of the neonatal chicken is not morphologically mature. J. Comp. Neurol. 379:603–616, 1997.

Indexing terms: inner ear; cochlea; intermediate filaments

Intermediate filament proteins (IFs) are one of the three major fibrous protein systems making up the eukaryotic cell cytoskeleton (see Schliwa, 1986, for a review). IFs are 8–11 nm in diameter, and five major subclasses of IFs have been defined from biochemical and immunological data: the neurofilaments, cytokeratins, vimentin, desmin, and glial fibrillary acidic protein. Cell types in mammalian tissue differ in the content and type of IFs expressed, and many studies have shown that the cell-specific expression of the various IFs in normal and in transformed cells may be used to identify their embryonic origin, developmental stage, and possibly cell function (Franke et al., 1979, 1982; Osborn and Weber, 1983; Virtanen et al., 1985; Erickson et al., 1987).

Neurofilaments (NFs), the intermediate filaments specific to neurons, are the predominant cytoskeletal elements in many nerve cells. Their functions remain unclear, although it is hypothesized that they confer mechanical stability and play a role in determining axonal caliber (see Hoffman et al., 1984). NFs are heteropolymers composed of three protein subunits with molecular weights of about 68 kilodaltons (kD), 150 kD, and 200 kD in mammals (Hoffman and Lasek, 1975; Liem et al., 1978). These proteins are often referred to as the low (NF-L), middle (NF-M), and high (NF-H) molecular-weight NF subunits. NF-L, NF-M, and NF-H are found in phosphorylated or dephosphorylated forms: Axonal NFs are heavily phosphorylated, whereas the NFs in perikarya and dendrites are dephosphorylated or phosphorylated to only a small degree (Sternberger and Sternberger, 1983; Robinson and Anderton, 1988).

Neurofilament expression in the inner ears of a variety of species has been described for both developing and adult animals. Many studies report the expression of NFs in

Contract grant sponsor: National Institute on Deafness and Other Communication Disorders, National Institutes of Health; Contract grant numbers: DC00395, DC02388.

Diana I. Lurie's current address is Department of Pharmaceutical Sciences, University of Montana, Missoula, MT 59812.

*Correspondence to: Elizabeth C. Oesterle, Ph.D., Virginia Merrill Bloedel Hearing Research Center, Box 357923, University of Washington, Seattle, WA 98195-7923. E-mail: Oesterle@otomail.u.washington.edu

Received 21 May 1996; Revised 4 October 1996; Accepted 17 October 1996

cranial nerve VIII fibers (auditory and vestibular) and their cell bodies (see, e.g., Dau and Wenthold, 1989; Raphael et al., 1987; Kuijpers et al., 1988, 1991; Anniko et al., 1989; Anniko and Arnold, 1990; Hafidi et al., 1990; Berglund and Ryugo 1991; Pirvola et al., 1991; Bauwens et al., 1992). In contrast, only a few studies have described the presence of neurofilaments in the mechanosensitive hair cells, the sensory receptors, of the inner ear.

NF-M has been detected in auditory hair cells during the development of the mouse cochlea (Hasko et al., 1990). Both inner and outer hair cells transiently express this neurofilament protein during the period from embryonic day 17 to postpartum day 10. Neurofilaments have also been detected in some vestibular hair cells in adult mammals; proteins detected with anti-NF-L, anti-NF-M, and dephosphorylated anti-NF-H/M antibodies form a small ring structure in type I vestibular hair cells in the adult guinea pig. No such immunostaining was found in guinea pig type II vestibular hair cells or in rat vestibular hair cells of either type (Deschesne et al., 1994). Ring-like neurofilament structures have also recently been detected in some auditory hair cells in posthatch chickens (Ofsie and Cotanche, 1996).

In this study, we examined the immunocytochemical distribution of neurofilament in the avian inner ear in the hope of identifying cell-type-specific markers for the avian inner ear cells. The identification of hair-cell-specific markers would serve as a useful tool for future studies of hair cell genesis, differentiation, and regeneration. We examined how antibodies that are specific for phosphorylated and dephosphorylated NF-M and NF-H labeled auditory and vestibular sensory epithelia in the mature chicken inner ear. Two phosphorylation-independent NF-M antibodies were found to label hair cells in the mature chicken basilar papilla, the auditory sensory receptor epithelium. A developmental study of NF-M expression was undertaken to determine when auditory hair cells express NF-M. Basilar papilla hair cells first express NF-M early in development (E11). Neurofilament expression is then increased such that, by E19, neurofilament-positive hair cells are found throughout the distal (low-frequency) and midproximal (midfrequency) regions of the basilar papilla. This expression is maintained through 3 weeks posthatch. By adulthood, NF-M staining in auditory hair cells is markedly diminished, and only a few hair cells in the very distal end of the papilla and short hair cells in the distal half of the papilla still express NF-M. NF expression was never detected in type I or type II vestibular hair cells.

MATERIALS AND METHODS

Subjects

Neonatal chickens, adult chickens, and chicken embryos (white leghorn; H&N International, Redmond, WA) were used for investigation. Fertilized chicken eggs were incubated at 37–38°C in a humidified environment and hatched in the University of Washington vivarium in AAALAC-approved facilities. The chickens were raised in communal brooders with unrestricted access to food and water. Embryos were killed at stages 35–45 (embryonic days (E) 8, 9, 10, 11, 12, 13, 15, 17, and 19) using Hamburger and Hamilton's (1951) staging scheme. Two to six embryos were used for each developmental stage. Four hatchlings, 1–21 days old, were used, and two adult chickens were obtained directly from the supplier and used at 22 weeks of age. All experiments were approved by the University of Washington Animal Care Committee under PHS grants DC00395 and DC02388.

In vivo tissue preparation

Neonatal and adult chickens were deeply anesthetized with an overdose of sodium pentobarbital prior to surgery. Subjects equal to or younger than E19 were not anesthetized prior to rapid decapitation. For E13 and older subjects, the bony capsule at the distal end near the lagena was opened widely, and a small opening was made in the round window. Individual ears were then fixed by intralabyrinthine perfusion with either methanol Carnoy's or Bouin's fixative prior to immersing the head in the same fixative. An intralabyrinthine perfusion was not performed on subjects younger than E13; the heads were immersion fixed. Heads were immersed in fixative for 4–6 hours at room temperature. They were then washed with 100% methanol, washed again with 100% ethanol, and hydrated with a graded ethanol series. They were then stored at 4°C for 1–3 days in 70% EtOH. The cochlear tissue was prepared in one of two ways for immunocytochemical processing: 1) paraffin sections or 2) whole papilla preparations. Specifically, to obtain paraffin sections of the basilar papilla, the cochlear ducts were dissected free from the head (for E10 and older subjects; for E8 and E9 embryos the whole head was processed). Following additional hydration with a graded ethanol series, the isolated cochlear ducts were stained with eosin diluted in phosphate-buffered saline (PBS; pH 7.4) and washed with PBS. Cochlear ducts were then dehydrated with a graded ethanol series, cleared in methyl salicylate, and infiltrated with Paraplast Plus at 60°C. The entire duct was sectioned transversely at 10 µm, and a one-in-four series was mounted onto bleached-washed, chrom-alum-subbed slides. Immunocytochemistry was performed, as discussed below, by using the avidin-biotin-peroxidase complex (ABC) staining procedure (Hsu et al., 1981). In later experiments, sections were counterstained with thionin. Sections were dehydrated and cleared prior to coverslipping with DPX mountant (BDH Limited, Poole, England). To obtain whole papilla surface preparations (Ofsie and Cotanche, 1995), the stria vascularis and tectorial membrane were carefully removed with fine forceps, and immunocytochemical procedures were run on the entire basilar papilla. The papillae were mounted in glycergel (Dako Corp., Carpinteria, CA) and coverslipped.

Culturing procedures

Culturing techniques were similar to those described previously (Oesterle et al., 1993). Cochlear ducts were dissected free from 7–18-day-old posthatch chickens using sterile technique and placed freely floating into wells of 24-well culture plates filled with culture medium. The culture medium consisted of basal medium Eagle (BME) supplemented with Earle's balanced salt solution (EBSS; 2 parts BME to 1 part EBSS), 0.5% D-glucose, and 5% fetal bovine serum (all these medium components were from Gibco, Grand Island, NY). Each organ was cultured individually, one organ per well. The culture well plates were placed on a slowly moving nutator (Clay Adams, Parsippany, NJ) and incubated at 37°C under an atmosphere of 5% CO_2 in air for 2 or 7 days. Culture medium was not exchanged during the culture period. Three end organs were grown in culture for 2 days, and six end organs were

TABLE 1. Properties of the Monoclonal Antibodies Used[1]

Antibody name	Subunit specificity	Phosphorylation state of epitope	Epitope location
RMO 80	NF-M only	P[ind]	
RMO 100	NF-M and -H	P[+]	
RMO 270	NF-M only	P[ind]	Tail (C-terminal)
RMO 286	NF-M only	P[ind]	
RMO 308	NF-M only	P[ind]	
RMdO 21	NF-M only	P[−]	

[1]The phosphorylation states of the epitopes recognized by the antibodies are: P[+], diminished immunoreactivity after dephosphorylation; P[−], increased immunoreactivity after dephosphorylation; P[ind], no change after dephosphorylation. NF-M, middle-weight neurofilament protein; NF-H, high-weight neurofilament protein.

cultured for 7 days. After the completion of the culture period, the explants were immersion fixed for 4–6 hours with methanol Carnoy's fixative and processed histologically as described above.

Antibodies

The panel of monoclonal antibodies used in this study and their subunit and domain specificities are listed in Table 1. They were obtained from Virginia Lee (University of Pennsylvania) and have been previously described (Lee et al., 1987; Balin and Lee, 1991; Landmesser and Swain, 1992). RMO 270 recognizes a phosphorylation-independent epitope of the medium-weight neurofilament subunit that is highly conserved across species; it recognizes the extreme carboxy-terminal end of the molecule (Lee et al., 1987).

Immunohistochemical procedures

Paraffin sections were deparaffinized, rinsed for 5 minutes in Tris buffer, rinsed for 5 minutes in Tris buffer with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), and blocked with 4% normal goat serum for 20 minutes. This and all other immunocytochemical reagents (except for the ABC reagent) were prepared in 1% BSA/0.1% sodium azide in Tris buffer. The sections were then incubated overnight in RMO antisera (1:1, 1:50, 1:100) at 4°C in a humidified chamber. The next day, the sections were rinsed for 5 minutes in each of the Tris buffers, incubated in 1:400 biotinylated goat anti-rabbit serum (Vector Laboratories, Burlingame, CA) for 1 hour, rinsed in the Tris buffers, and then incubated with the avidin-biotin complex (Vectastain ABC Elite kit; Vector). The sections were then rinsed for 10 minutes in Tris buffer alone and developed with the chromagen. The chromagen used was diaminobenzidine (0.25; Sigma) and 1 mM imidizole and 0.1% hydrogen peroxide in Tris buffer. The colored end product was viewed using light microscopy with conventional optics or Nomarski differential interference contrast optics.

Controls

Method and antibody specificity were checked by substituting nonimmune sera for the primary antibody and by using a series of dilutions of the primary antibody. Staining of eighth-nerve fibers served as a within-tissue positive control.

Western blot analysis of cochlear duct tissue

Chicks (n = 35; 4–7 days posthatch) were decapitated, and the cochlear ducts (360 mg) were dissected out, minced on ice, and homogenized in 10 ml of buffer A [100 mM MES, pH 6.8, 1 mM $MgSO_4$, 1 mM EDTA, 1% Triton X-100, 1 mM benzamidine, 0.002% PMSF (w/v), 2 µg/ml leupeptin, 0.5% pepstatin A and 20 KIU/ml aprotinin]. The homogenate was clarified by centrifugation at 40,000g for 60 minutes at 4°C. The supernatant was saved, and the pellet containing the neurofilaments was rehomogenized in 10 ml of buffer A without detergent and centrifuged as described above. The washed pellet was resuspended in 75 µl of Laemmli sample buffer (Laemmli, 1970), and protein concentration was determined according to Bradford (1976). The samples were run on 8.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were blotted on nitrocellulose, and the monoclonal antibody RMO 270 diluted 100-fold in TBST-B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, 0.1% BSA) was used for Western analysis. Immunoreactive proteins were visualized with a secondary antibody coupled to alkaline phosphatase.

Electron microscopy

Protocols identical to that published previously (Oesterle et al., 1992) were used. Briefly, ears from three normal chickens (white leghorns) ranging from 10 to 14 days in age were perfused with a modified Karnovsky's fixative (mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 0.001% $CaCl_2$. They were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at room temperature, washed with buffer, dehydrated in a graded alcohol series, and embedded with Spurr epoxy resin. A series of ultrathin sections (90 nm) was collected at a distance 55% from the basal end of the papilla. Sections were stained with uranyl acetate and lead citrate before examination with a JEOL 1200 EX transmission electron microscope.

RESULTS

Overview of basilar papilla and cell types

A cross section through the cochlear duct of the chicken is shown in Figure 1. Detailed descriptions of the structural organization of the cochlear duct in the developing and mature chicken ear have been published elsewhere (Retzius, 1884; Jahnke et al., 1969; Cohen and Fermin, 1978; Hirokawa, 1978; Tanaka and Smith, 1978; Smith, 1981, 1985; Tilney and Saunders, 1983). Both auditory and vestibular sensory receptor epithelia lie within the duct. The auditory sensory receptor epithelium, the basilar papilla, lies on the basilar membrane and is composed of auditory hair cells (tall, short, and intermediate hair cells), supporting cells, and unmyelinated terminal portions of cochlear nerve fibers. Clear cells and hyaline cells abut the superior and inferior edges of the basilar papilla, respectively. The lagenar macula (not shown), a vestibular sensory receptor epithelium, lies at the distal end of the cochlear duct, distal to the basilar papilla. It is composed of type I and II vestibular hair cells, supporting cells, and unmyelinated terminal portions of vestibular nerve fibers.

Antibody screen on P10 chickens

All monoclonal anti-NF antibodies (RMO 80, 100, 270, 286, 308, and RMdO 21) were screened on inner ear tissue of P10 chickens. Auditory and vestibular nerve fibers (afferent and efferent) were strongly immunolabeled by all NF antibodies, whereas supporting cells within the auditory and vestibular sensory receptor epithelia and nonsensory cell types immediately adjacent to the sensory epithelia (e.g., hyaline cells, clear cells, transition epithelium)

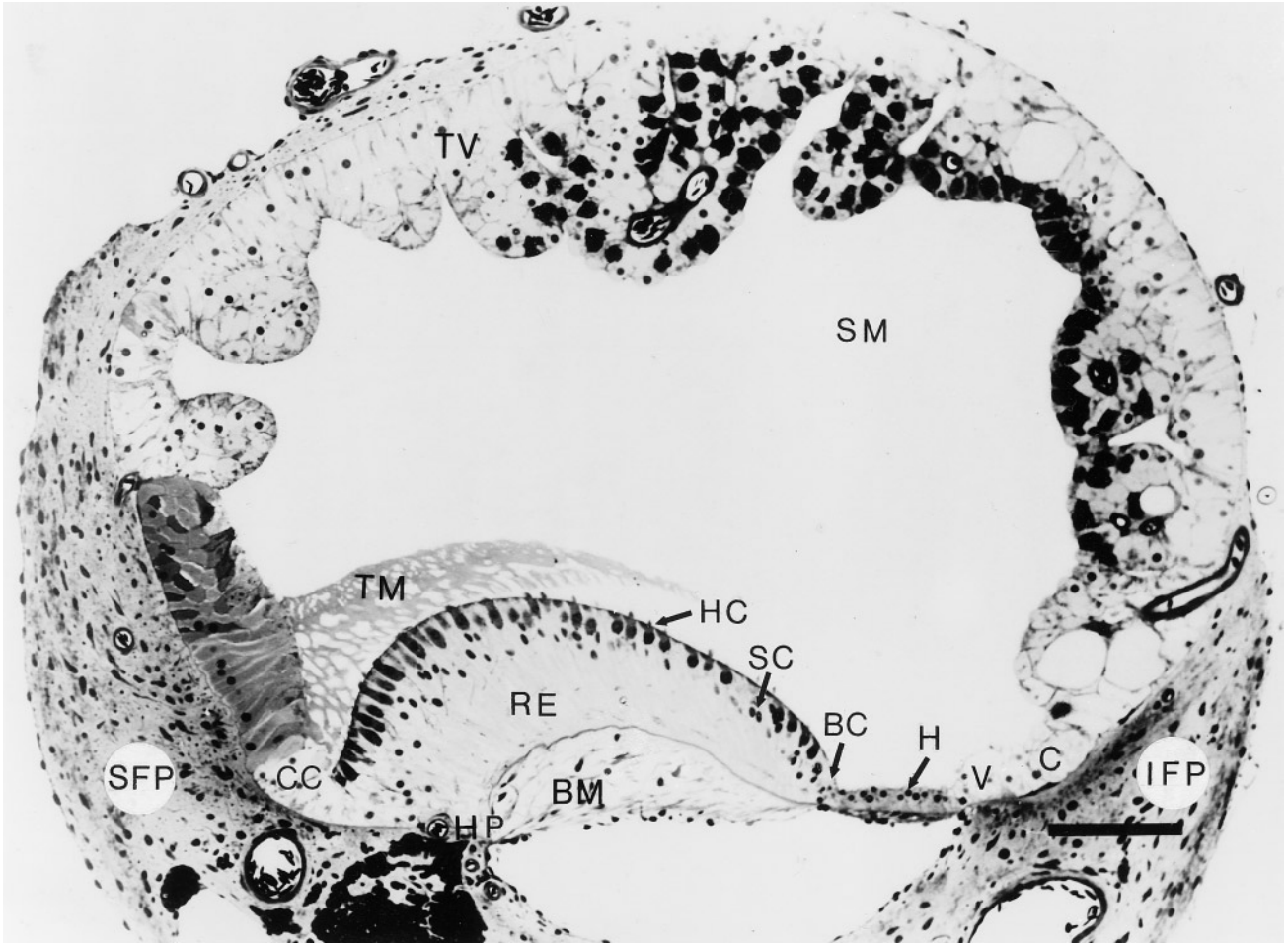

Fig. 1. Light micrograph of a transverse section through the cochlear duct of the posthatch chicken. The receptor epithelium (RE) is composed of hair cells (HC), organ supporting cells (SC), border cells (BC), and unmyelinated terminal portions of cochlear nerve fibers. Hyaline cells (H) lie inferior to the receptor epithelium and extend inferiorly towards the inferior fibrocartilaginous plate (IFP). Vacuole cells (V) and cuboidal cells (C) rest on the inferior fibrocartilaginous plate. BM, basilar membrane; SM, scala media; CC, clear cells; TM, tectorial membrane; TV, tegmentum vasculosum; HP, habenula perforata; SFP, superior fibrocartilaginous plate. Modified from Rubel and Ryals (1982) and Oesterle et al. (1992). Scale bar = 100 μm.

were unlabeled. The RMO 270 and RMO 286 antibodies labeled auditory, but not vestibular, hair cells. RMO 270 and RMO 286 are directed against NF-M. The primary objective of these experiments was to characterize the expression and distribution of NF-M in auditory hair cells. To this end, patterns of NF expression were examined in the basilar papillae of adult and developing chickens by RMO 270 staining. Findings regarding NF-M expression in the adult ear will be presented first, followed by a discussion of the developing inner ear.

Western blot results

The RMO 270 antibody labels neurofilament protein in the chicken basilar papilla. Figure 2 is a Western blot of cochlear duct tissue taken from posthatch chickens that was immunolabeled with RMO 270. Pure neurofilament protein was run in lane 1, and 7 μg of the chicken cochlear duct preparation (Triton X-100 insoluble material) was run in lane 2. The RMO 270 antibody labeled a single band located at approximately 180 kD in both lanes, confirming the presence of neurofilament protein in the chicken cochlear duct.

Neurofilament staining in adult ears

Nerve. Strong anti-NF-M binding is present within fibers of the VIII cranial nerve that innervate the auditory and vestibular sensory epithelia of the adult ear (Fig. 3A,C,E). Portions of the fibers within and below the sensory receptor epithelium are very strongly immunostained. NF-positive efferent fibers are also present in the hyaline cell region, nonsensory epithelia next to the basilar papilla (not shown). The perikarya of the ganglion neurons are heavily stained with neurofilament, whereas the nuclei are NF negative (not shown).

Adult sensory receptor epithelia. Only a few hair cells in the adult ear are stained by anti-NF-M. Staining with RMO 270 is limited to a few tall hair cells in the distal one-fourth of the basilar papilla located on the superior (neural) side of the papilla (Fig. 3A,B, Table 2) and to short hair cells in the distal one-half of the papilla (Fig. 3D).

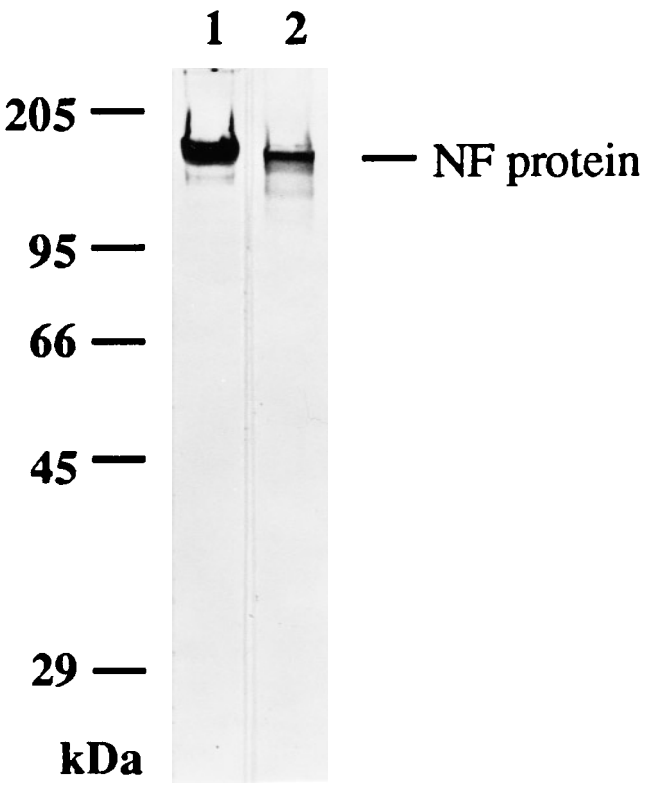

Fig. 2. Western blot analysis of cochlear duct tissue. **Lane 1** was loaded with 1.6 µg of pure neurofilament protein. **Lane 2** corresponds to 7 µg of cochlear duct preparation (Triton X-100-insoluble material). RM0-270 MAb was used to detect neurofilament proteins as described in Material and Methods.

Inferiorly (abneural) located tall hair cells are all NF negative (Fig. 3A,C). NF staining in superior tall hair cells is cytoplasmic in nature and occurs throughout the cell body (Fig. 3B). Staining is not observed in the hair cell nuclei, stereocilia, or cuticular plate.

Short hair cells in the distal half of the papilla show prominent NF staining under their lumenal surface (Fig. 3C) and in the region under the cuticular plate. Again, staining is not observed in the cuticular plate or stereocilia. Supporting cells throughout the papilla are unlabeled by anti-NF-M (Fig. 3A–F), and NF expression could not be detected in vestibular hair cells (in the lagenar macula) at the light-microscopic level (not shown).

Neurofilament staining in the developing ear

Nerve. Strong anti-NF-M binding is present within fibers of the VIII cranial nerve that innervate both the auditory and the vestibular sensory epithelia at all developmental ages studied. Neurofilament-positive nerve fibers are seen in inner ear sensory epithelia at E8, the first developmental age studied. At E8–10, stained fibers extend well into the sensory epithelium, with some fibers almost reaching the lumenal surface (Fig. 4A,B). Numerous NF-positive fibers are also present within the inferior fibrocartilaginous plate, and positive NF label is seen in the tegmentum vasculosum in distal (low frequency) and midproximal (midfrequency) regions of the basilar papilla (Fig. 4A). Anti-NF staining is especially prominent in the region of tegmentum located immediately above the homogene cells. Cochlear and vestibular ganglion cells are strongly labeled by NF; the cell bodies stain intensely, whereas the nuclei remain unstained. By E11–12, NF-positive fibers can be detected in the hyaline cell region. This overall pattern of NF staining in nerve fibers and ganglion cells is observed at all developmental stages studied through E17.

At E17, NF staining in the tegmentum begins to decrease in intensity, and it is undetectable in neonatal and adult chickens. NF label in the inferior fibrocartilaginous plate is also decreased at E19 relative to that seen at earlier developmental stages, and it is absent in adult chickens. In contrast, nerve fibers within the auditory and vestibular sensory epithelia, fibers in the hyaline region, and cell bodies of all cochlear and vestibular ganglion cells remain positive for NF through adulthood.

Embryonic sensory receptor epithelia. Hair cells and supporting cells are present at E8–10 in both auditory and vestibular sensory epithelia, and they are NF (RMO 270) negative (Fig. 4A,B, Table 2). NF continues to be undetectable in vestibular hair cells at all later developmental stages studied (through 22 weeks posthatch). NF is expressed in auditory hair cells during later developmental stages. At E11, NF-positive hair cells are found in midproximal regions of the basilar papilla (Fig. 4C,D). A few superior (neural) hair cells label in these regions (Fig. 4C). The stain is located in nonnuclear cytoplasmic areas of the hair cells (Fig. 4D). Inferior (abneural) hair cells in this region, and hair cells in the distal and proximal regions of the papilla, are NF negative at this time. At E12, the numbers of NF-positive hair cells have increased, and the pattern of staining has extended along the papilla; many more superior hair cells are stained in the midproximal region of the papilla, and a few NF-positive hair cells are now present in the distal region as well. With time, the NF-staining pattern continues to extend along two dimensions: 1) and more inferiorly located hair cells become labeled with time (radial gradient) and 2) progressively more distal hair cells become labeled with time (a mid- to distal longitudinal gradient). There is also a small proximally directed progression of neurofilament expression. By E13, all classes of hair cells (tall, intermediate, and short) are NF positive in the midproximal region of the papilla, and many more distal hair cells are labeled. By E19, all hair cells are NF positive (Fig. 4E,F), except hair cells at the extreme ends of the papilla. Numerous hair cells at the proximal tip, and a few hair cells at the very distal end of the papilla, are NF negative.

Neonatal sensory receptor epithelia. In the neonatal ear, in marked contrast to the adult ear, many hair cells are stained by anti-NF-M. NF-M staining patterns in the basilar papilla of 1–3-week-old posthatch chickens strongly resemble those seen at E19 (Table 2). All types of auditory hair cells, tall, intermediate and short, are labeled by anti-NF-M (Fig. 5A–F). The hair-cell cytoplasm is strongly NF positive, whereas the stereocilia, nucleus, and cuticular plate are all NF negative (see, e.g., Fig. 5B,D,F). The cytoplasmic NF staining is filamentous in nature.

Hair-cell expression of NF-M in neonatal chickens varies as a function of basilar papilla location. NF-positive hair cells are located in the distal and midproximal regions of the papilla (Fig. 5A–D), but hair cells located in the proximal 400 µm of the papilla (Fig 5E–H) are NF negative. NF-positive hair cells are first detected at distances approximately 13% from the proximal end (n = 4, SD =

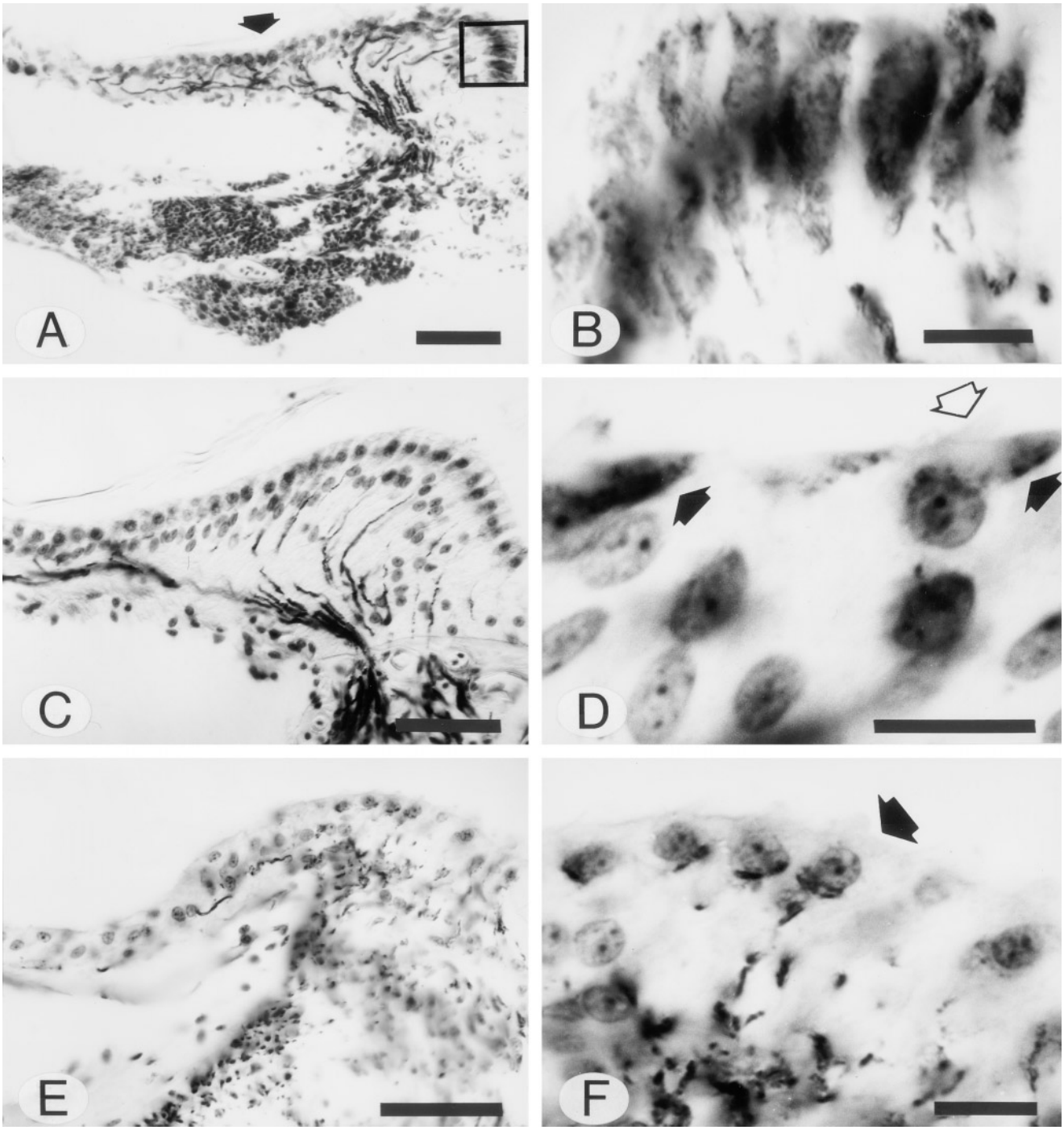

Fig. 3. Immunocytochemical localization of middle-weight neurofilament protein (NF-M; with RMO 270) in 22-week-old chickens. All sections were counterstained with thionin to label cell nuclei. **A:** Very distal region of the basilar papilla (a distance 86% from the proximal end). Note the cytoplasmic stain in superior tall hair cells (boxed region) and the absence of stain in inferior tall hair cells (arrow). **B:** Higher magnification of the boxed region in A. **C:** Distal region of the basilar papilla (79% from the proximal end). Note the absence of stain in the hair cells and the presence of stained nerve fibers in and below the sensory epithelium (SE). **D:** Neurofilament labeling in short hair cells (solid arrows) in the distal half of the papilla (63% from proximal end). The stereocilia (open arrow) and cuticular plate of the short hair cell are unlabeled. **E:** Proximal tip of the cochlear duct (20% from the proximal end). Note the absence of stain in the hair cells. **F:** Higher magnification of the papilla shown in E. Arrow points to an unlabeled hair cell. Scale bars = 50 μm in A, 10 μm in B,D,F, 40 μm in C,E.

2.2, range 11–16%). Initially, a few superior hair cells are labeled, with progressively more hair cells being labelled across the superior to inferior axis as one advances towards the low-frequency (distal) end (Fig. 6).

Further evidence for the presence of neurofilaments within the cytoplasm of hair cells in the neonatal chicken comes from an electron microscopic examination of basilar-papillar hair cells. Intracellular filaments with a diameter

TABLE 2. Summary of NF Expression for RMO 270[1]

	Proximal		Midproximal		Distal		Lagena	
Age	HC	N	HC	N	HC	N	HC	N
E8–10	−	+	−	+	−	+	−	+
E11	−	+	+	+	−	+	−	+
E12–19	−	+	+	+	+	+	−	+
P1–21 days	−	+	+	+	+	+	−	+
22 weeks	−	+	−	+	+	+	−	+

[1]HC, hair cell; N, neuron; E, embryonic day; P, posthatch day.

of approximately 10 nm can be observed within the hair cell cytoplasm (Fig. 7).

Neurofilament staining in vitro

Cochlear ducts from 1–2-week-old posthatch chickens were isolated and grown in culture for 2 or 7 days. During this time, NF staining of cochlear nerve fibers decreased markedly. This was due to the degeneration of the axons that occurs in culture within 24 hours (Oesterle et al., 1993). Cultured auditory hair cells remain strongly NF positive even after 7 days in vitro. This is further evidence that the staining observed is due to NF within the hair cell cytoplasm and not to the presence of axons surrounding the hair cell body. Furthermore, it shows that innervation is not necessary for the maintenance of the NF staining.

DISCUSSION

This study demonstrates the presence of NF-M in a nonneural cell type, in hair cells in the avian auditory sensory epithelium. Hair cells, by classical definition, are nonneural cells because they do not have an axon or dendrites. Hair cells do, however, generate electrical potentials, have synaptic activities, release neurotransmitters, and have presynaptic structures, and they are neuron-specific enolase immunoreactive (Deschesne et al., 1985). Hair cells in the chicken basilar papilla express NF, the intermediate filament of neurons, as early as E11 (Table 2), around the time when the various types of hair cells (short, intermediate, or tall) first become distinguishable from one another (Cohen and Fermin, 1978; Hirokawa, 1978). By E19, neurofilament-positive hair cells are found throughout the distal and midproximal regions of the papilla, and this expression is maintained until 3 weeks posthatch. By adulthood, NF expression in hair cells decreases markedly such that expression appears limited to a few tall hair cells at the very distal end of the papilla and short hair cells in the distal half of the papilla only. Hair cells located at the very proximal end of the papilla never appear to express NF.

The expression of NF-M in hair cells, along with reports of NF expression in several other nonneural cell types (adult bovine adrenal chromaffin cells grown in culture: Grant et al., 1990; Ehrlich et al., 1994; human T lymphocytes: Murphy et al., 1993; immature Schwann cells: Kelly et al., 1992; Roberson et al., 1992; embryonic heart muscle: Bennett and DiLullo, 1985; Semba et al., 1990; chicken erythrocytes during development: Granger and Lazarides, 1983), suggests that NF is not specific only to neurons. Hence, NF can no longer be considered a definitive cell-type-specific marker for neural cells, because NF is expressed in several nonneural cell types as well, especially during ontogeny.

NF in auditory hair cells

The immunolabeling of eighth nerve fibers and spiral ganglion cells by NF seen in this study corresponds well to the classical localization of NF as previously reported by a number of investigators (e.g., Raphael et al., 1987; Kuijpers et al., 1988, 1991; Anniko et al., 1989; Dau and Wenthold, 1989; Anniko and Arnold, 1990; Hafidi et al., 1990; Berglund and Ryugo, 1991; Pirvola et al., 1991; Bauwens et al., 1992; Ofsie and Cotanche, 1995). However, most previous studies of the inner ear using antibodies to various intermediate filaments did not report the presence of neurofilaments in hair cells (Anniko et al., 1987; Raphael et al., 1987; Thornell et al., 1987; Schrott et al., 1988; Wikström et al., 1988; Dau and Wenthold, 1989; Anniko and Arnold, 1990; Berggren et al., 1990; Hafidi et al., 1990; Kuijpers et al., 1991; Pirvola et al., 1991; Bauwens et al., 1992).

Two groups of investigators (Hasko et al., 1990; Ofsie and Cotanche, 1996) have reported the presence of NF in auditory hair cells. Hasko and colleagues (1990) observed a transient expression of NF in auditory hair cells in the developing mouse (Hasko et al., 1990). They first detected the expression of NF-M in organ of Corti hair cells, in both inner and outer hair cells, in the basal (high-frequency) region of E19 mouse cochleas. By 2 days postpartum (P2), NF-positive hair cells were found throughout the entire length of the cochlea. By P10, staining of the hair cells began to diminish, and, by P21, NF-M could no longer be detected in either hair cell type. The loss of NF expression by mouse hair cells appeared to occur during the onset of auditory function, as judged from the onset of cochlear microphonics (P8–11), compound action potentials (P9–12), and behavioral responses to sound (P11; Rubel, 1978; Romand, 1983). Similar to findings reported here, a developmental gradient of NF expression was seen in the developing mouse cochlea. A basal-to-apical gradient of NF expression was described by Hasko and colleagues, but the existence of a radial gradient was not reported. One difference between our data and those reported for the mouse is that the very basalmost (proximal) hair cells in the chicken never appear to express NF.

Mice and chicken data also differ with regard to the timing of the NF expression in hair cells. NF expression in chicken hair cells begins near the onset of auditory function, whereas, as was mentioned previously, NF expression in mouse hair cells decreases during the onset of auditory function. In chick, onsets of the cochlear microphonic (E13; Vanzulli and Garcia-Austt, 1963), compound action potential (E12; Rebillard and Rubel, 1981; Saunders et al., 1973), brainstem evoked potential (E11–12; Saunders et al., 1973), and behavioral responses (E14; reviewed in Rubel, 1978) occur around the time when NF is first expressed in papillar hair cells. At E19, when NF staining patterns strongly resemble those seen in posthatch chickens, tuning characteristics of the primary afferents are remarkably mature and resemble those in posthatch chicks (Jones and Jones, 1995a), and the tonotopic map of the avian cochlea (for CFs between 100 and 1700 Hz) is stable and not significantly different from the maps of P2 and P21 chickens (Lippe and Rubel, 1985; Lippe, 1987; Manley et al., 1987; Jones and Jones, 1995b). NF expression is then maintained by most chicken hair cells until at least until 3 weeks posthatch. Reasons for the

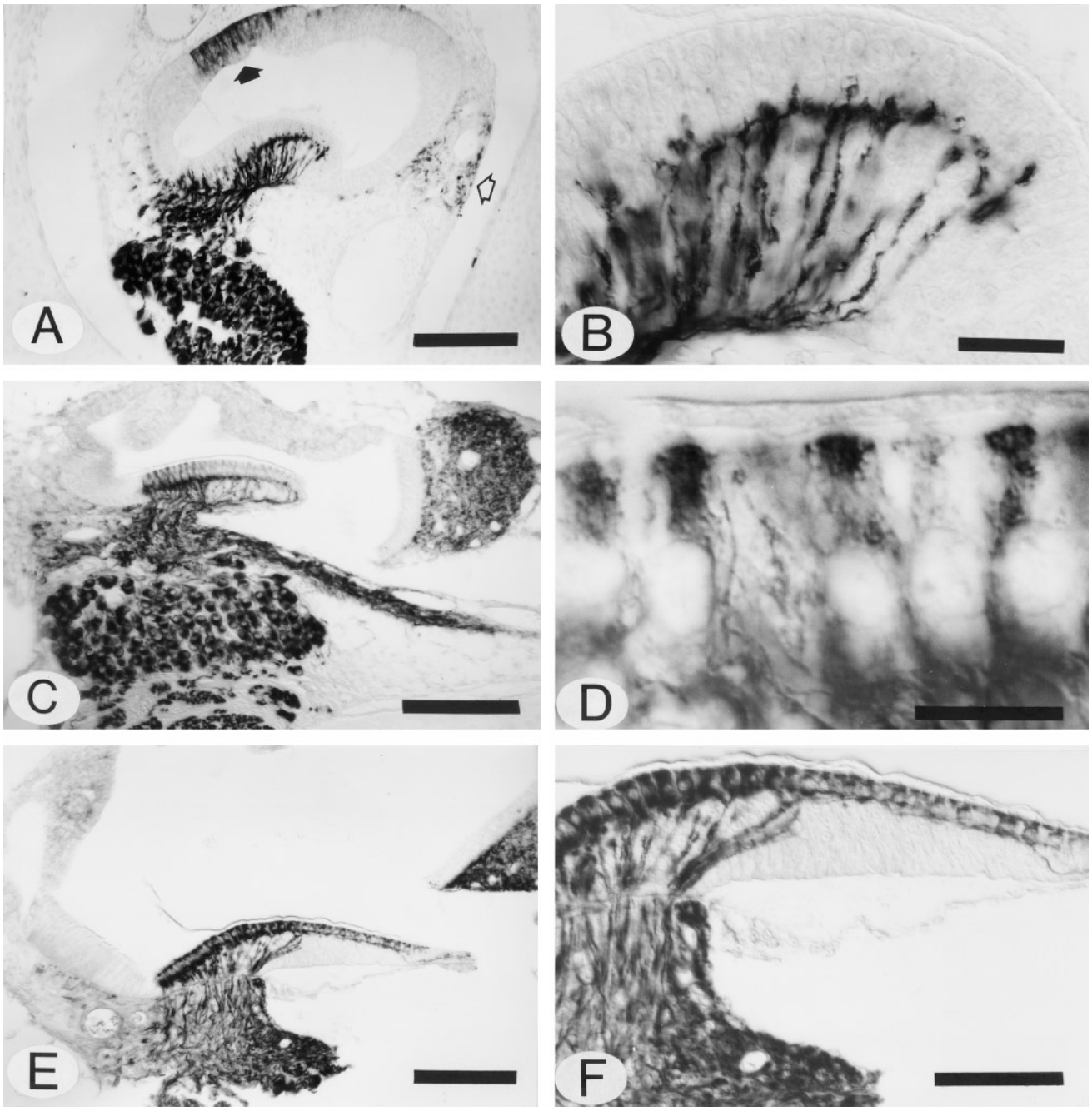

Fig. 4. Immunocytochemical localization of NF-M (RMO 270) in developing chicken cochlear ducts. The sections were not counterstained. **A:** Note the absence of NF stain in the hair cells of this E10 embryo and the presence of stain in the tegmentum (solid arrow), inferior cartilaginous plate (open arrow), and spiral ganglion. **B:** Higher magnification of the sensory receptor epithelium shown in A. Neurofilament-stained nerve fibers travel up to the unstained hair cells. **C:** Midproximal region of an E11 embryo. The disarticulation of the basilar papilla from the inferior cartilaginous plate (IFP) is the result of damage that occurred during the dissection. **D:** Higher magnification of the auditory sensory receptor epithelium shown in C. Some superior (abneural) hair cells are strongly immunostained by the anti-NF-M antibody. Note the filamentous nature of the cytoplasmic stain and the absence of NF stain in the nucleus. **E:** E17 embryo. All hair cells in this section are NF positive. **F:** E17. Higher magnification of the basilar papilla shown in E. Tall and short hair cells are immunostained. Scale bars = 100 μm in A,C, 20 μm in B, 10 μm in D, 50 μm in E,F.

differences in the temporal pattern between mice and chicken are unknown at present.

Recently, a second group of investigators also reported the presence of NF in auditory hair cells. Ofsie and Cotanche (1996), using a cocktail containing monoclonal antibodies to NF-L, NF-M, and NF-H, noted the presence of NF in basilar-papillar hair cells in normal and noise-damaged chickens that were 1–2 weeks in age. They did not examine NF expression in either the developing or the adult chicken. Ofsie and Cotanche found that tall and

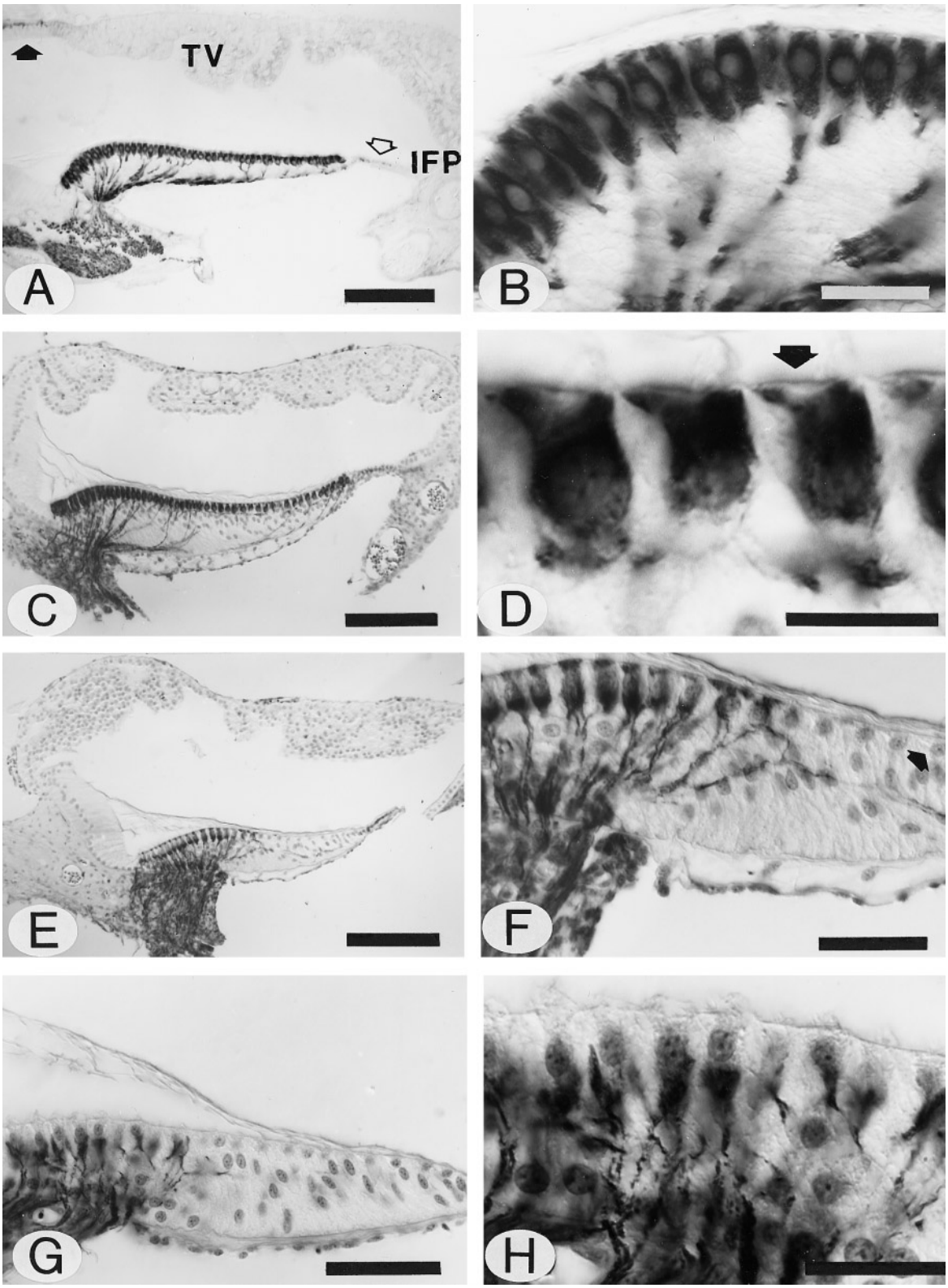

Fig. 5. Immunocytochemical localization of NF-M (with RMO 270) in E19 and P10 chickens. **A:** Distal region of the basilar papilla in a P10 chicken. Note the immunopositive nerve fibers in the lagena macula (solid arrow) and hyaline cell region (open arrow) and the absence of label in the inferior cartilaginous plate (IFP) and tegmentum vasculosum (TV). The section was not counterstained. **B:** Higher magnification of the basilar papilla shown in A. All hair cells are intensely labeled with RMO 270. **C:** Midproximal region of the basilar papilla in a P10 chicken. All hair cells are intensely stained. The section was counterstained with thionin. **D:** Higher magnification of the basilar papilla shown in C. Cuticular plates (arrow), stereocilia, and hair cell nuclei are not stained by RMO 270, whereas the rest of the hair cell cytoplasm is intensely labeled. **E:** Proximal region of the basilar papilla in an E19 embryo. Section was counterstained with thionin. **F:** Higher magnification of the basilar papilla shown in E. Note that the superior hair cells are stained, whereas inferior hair cells (arrow) are unstained. **G:** Proximal tip of the cochlear duct of an E19 embryo. Section was counterstained with thionin. **H:** Higher magnification of the basilar papilla shown in G. All hair cells are unlabeled by RMO 270. Scale bars = 100 µm in A,C,E, 20 µm in B,H, 10 µm in D, 30 µm in F, 50 µm in G.

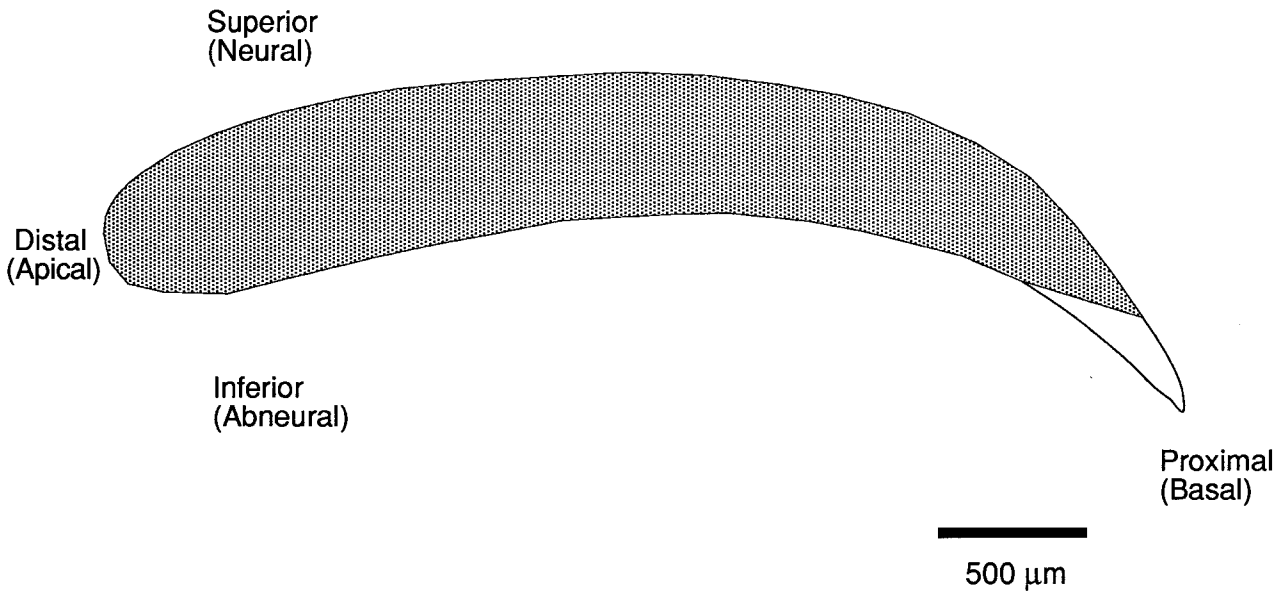

Fig. 6. Schematic diagram of the basilar papilla of 1–3-week-old posthatch chickens illustrating the location of NF-M-positive hair cells. The shaded area represents the region of NF-M-positive hair cells, and the white area represents the region of NF-M-negative hair cells. Scale bar = 500 µm.

short hair cells in the distal half of the neonatal papilla exhibit diffuse perinuclear NF labeling. In the proximal half, short hair cells overlying the basilar membrane are unlabeled by NF, but tall hair cells along the superior edge are labeled. The tall hair cells have an annular (ring) structure in the basal portion of their cytoplasm that is NF positive. Unlike Ofsie and Cotanche (1996), we observed extensive anti-NF-M labeling throughout the cytoplasm of hair cells in the distal half of the papilla. Ofsie and Cotanche's report of an absence of label in proximal short hair cells is in agreement with findings reported here regarding the neonatal ear. The similarity of labeling patterns seen in the Ofsie and Cotanche study and in our study suggests that much of the label seen by Ofsie and Cotanche might be due specifically to NF-M expression.

After screening six NF antibodies in the adult, we chose to study the development of the one that was immunopositive in adults (RMO 270). It is conceivable that staining with some of the other adult-negative antibodies would also show embryonic staining, and this might be a fruitful direction for future research.

NF in vestibular hair cells

Recently, Deschesne et al. (1994) reported that antibodies to NF-L, NF-M, and dephosphorylated NF-H immunostain an annular ring structure in type I vestibular hair cells in adult guinea pigs, but not in adult rats. NF immunoreactivity was not detected in type II vestibular hair cells. The intense cytoplasmic NF-M staining seen in the avian auditory hair cells was never seen in the avian vestibular hair cells. However, the possibility that a small annular ring structure similar to that reported by Deschesne and coworkers exists within avian vestibular hair cells cannot be eliminated. Intense staining of nerve fibers innervating the vestibular hair cells made identification of a small ring structure within the basal regions of the hair cells impossible with the classical light and differential optics (Nomarsky) used in this study.

Developmental gradients in the avian basilar papilla

The basilar papilla, like the mammalian cochlea, does not develop synchronously along its length (Bredberg, 1968; Rubel, 1978; Cotanche and Sulik, 1984; Fermin and Cohen, 1984; Chandler, 1984). Longitudinal and radial developmental gradients have been identified previously in the chicken basilar papilla. The terminal mitoses of hair cell precursor cells in the developing chicken basilar papilla are reported to proceed through appositional addition (Katayama and Corwin, 1989). The first hair cells are formed in a longitudinal band that extends along most of the length of the sensory epithelium (SE). The epithelium then grows further by adding new hair cells to the edges of the first band of cells. A radial gradient (superior to inferior) of hair cell genesis occurs in the proximal half of the SE.

With regard to the differentiation of hair cells, hair cells in chicken (Fermin and Cohen, 1984) and duck (Chandler, 1984) first begin to differentiate in the midbasal region of the basilar papilla and progress along what is largely a proximal-to-distal gradient, although there is also a small proximally directed progression. A radial gradient also exists: Tall hair cells develop earliest, followed by intermediate hair cells and finally by short hair cells.

Afferent innervation is also reported to proceed longitudinally and radially along the cochlea (Fermin and Cohen, 1984). Tall hair cells are innervated first and show the earliest synaptic specializations. Then, intermediate hair cells and, finally, short hair cells are innervated (Fermin and Cohen, 1984). Hair cells in the midbasal region are innervated before those situated in the more distant proximal and distal regions (Fermin and Cohen, 1984).

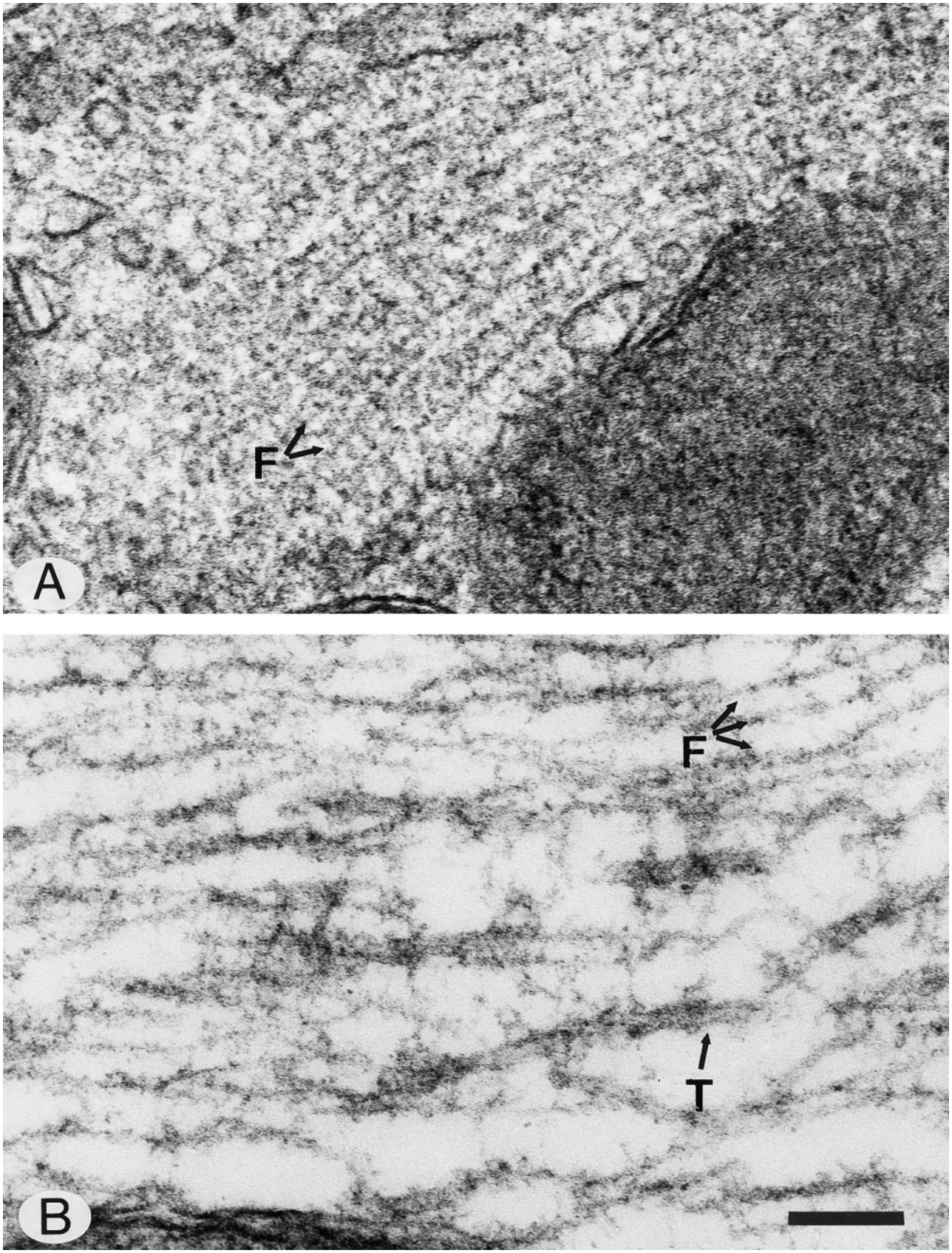

Fig. 7. Electron micrographs illustrating the presence of intracellular filaments in an 11-day-old posthatch chicken. **A:** Basal subnuclear region of a superior tall hair cell located at a distance 55% from the proximal end. **B:** Axoplasm of an afferent nerve fiber. T, microtubule; F, filament. A and B are identical in magnification. Scale bar = 100 nm.

Whitehead and Morest (1985), on the other hand, did not detect any developmental gradients for the development of afferent innervation in the chicken cochlea. The spatiotemporal pattern of NF expression demonstrated in this study parallels the longitudinal and radial gradients of hair cell differentiation, with the exception that the proximal hair cells never express NF.

Function of NF in auditory hair cells

Despite intensive study, the precise roles(s) of neurofilaments in cells remains largely unknown. The presence of NFs in hair cells is surprising in that NFs are widely believed to be neuron specific, and they are not usually found in nonneural cells. As discussed above, NFs may be expressed in adult bovine adrenal chromaffin cells grown in culture (Grant et al., 1990; Ehrlich et al., 1994), human T lymphocytes (Murphy et al., 1993), immature Schwann cells (Kelly et al., 1992; Roberson et al., 1992), embryonic heart muscle (Bennett and DiLullo, 1985; Semba et al., 1990), and chicken erythrocytes during development (Granger and Lazarides, 1983). The significance of this nonneuronal expression is unclear.

NF is unlikely to be involved directly in initially triggering the differentiation of hair cells, insofar as hair cells at E8–10 show no staining. Hair cells in the avian basilar papilla first begin to express NF at E11. At this time, the three types of hair cells become distinguishable (Cohen and Fermin, 1978), stereocilia of proximal hair cells and inferior hair cells in the distal portion of the papilla obtain their mature orientation (Shield and Cotanche, 1990), synaptic bars form in hair cells across from afferent endings (Cohen and Fermin, 1978, Fermin and Cohen, 1984), and brainstem evoked potentials to tones are seen (Saunders et al., 1973). Compound action potential (CAP) thresholds are seen at high levels at E12, and the cochlear microphonic is detected at E13 (Vanzulli and Garcia-Austt, 1963). The tuning characteristics of the primary afferents at E19 are remarkably mature and resemble those in posthatch chicks (Jones and Jones, 1995a). The antigen may be involved in the later stages of differentiation that result in specialized hair cell morphology or function.

Neonatal and adult chickens differ substantially with regard to hair cell expression of NF-M. This points to the existence of morphological differences between the neonatal and the adult basilar papilla, and calls into question the idea that the functioning of the neonatal papilla accurately reflects that of the adult bird. Several other lines of evidence also suggest that the auditory system of the chicken continues to develop for some time after hatching. Tucci and Rubel (1990) found that thresholds for brainstem evoked potentials continue to decline up to 20 weeks of age. Ryals and colleagues (1984) found that the cochlear duct grows 16% in length between hatching and P35. Behavioral thresholds in adult chickens (Saunders and Salvi, 1993) are lower than those reported for young chicks (Gray and Rubel, 1985).

The question still remains regarding why hair cells express NF. In spite of the fact that NFs were first visualized by silver staining in the late nineteenth century, their functions are not fully understood. They are thought to help neurons establish and maintain the structure of their axons (reviewed in Xu et al., 1994), and abnormal accumulation of NFs has been seen as a common early feature of many motor neuron disorders (Hirano, 1991). Conceivably, the production of NF by hair cells affects some function of the cytoskeleton in a manner that relates to the specific properties of these hair cells. Does neurofilament provide hair cells with additional structural stability that is required in particular regions of the cochlea at certain times? Clearly, additional work must be carried out to elucidate the roles of NF in developing auditory hair cells.

In summary, this study demonstrates the presence of NF-M in a nonneural cell type, in auditory hair cells in the avian inner ear, and suggests that NF cannot be considered a definitive cell-type-specific marker for neural cells. All types of hair cells, e.g., short, intermediate, and tall, can express NF, and NF expression changes as a function of age. Neonatal and adult chickens differ substantially with regard to hair cell expression of NF, suggesting that the neonatal papilla is not an accurate model for the adult bird.

ACKNOWLEDGMENTS

This research was supported by research grants DC00395 and DC02388 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health. The authors thank Paul Schwartz and Janet Schukar for assistance with photographic work, Dale Cunningham for assistance with TEM, Dr. Flavio Solca for help with the gels, Simone Han for assistance with antibody staining, and Dr. Bill Lippe for helpful comments regarding the manuscript.

LITERATURE CITED

Anniko, M., and W. Arnold (1990) Cytoskeletal network of intermediate filament proteins in the adult human vestibular labyrinth. Acta Otolaryngol. *470:*40–50.

Anniko, M., L.-E. Thornell, and I. Virtanen (1987) Cytoskeletal organization of the human inner ear. Acta Otolaryngol. *437:*29–54.

Anniko, M., B.-I. Wenngren, L.-E. Thornell, and I. Virtanen (1989) Expression of intermediate filament proteins in the inner ear of the dancer mouse mutant. Acta Otolaryngol. *108:*45–54.

Balin, B.J., and V.M.-Y Lee (1991) Individual neurofilament subunits reassembled in vitro exhibit unique biochemical, morphological and immunological properties. Brain Res. *556:*196–208.

Bauwens, L.J., J.C. DeGroot, J.E. Veldman, F.C. Ramaekers, and E.H. Huizing (1992) Expression of intermediate filament proteins in the adult human vestibular labyrinth. Acta Otol. Rhinol. Laryngol. *101:*479–486.

Bennett, G.S., and C. DiLullo (1985) Transient expression of a neurofilament protein by replicating neuroepithelial cells of the embryonic chick brain. Dev. Biol. *107:*107–127.

Berggren, D., M. Anniko, L.-E. Thornell, F.C.S. Ramaekers, and I. Virtanen (1990) Intermediate filament proteins in the embryonic inner ear of mice under normal conditions and after exposure to ototoxic drugs. Acta Otolaryngol. *109:*57–65.

Berglund, A.M., and D.K. Ryugo (1991) Neurofilament antibodies and spiral ganglion neurons of the mammalian cochlea. J. Comp. Neurol. *306:*393–408.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72:*248–254.

Bredberg, G. (1968) Cellular pattern and nerve supply of the human organ of Corti. Acta Otolaryngol. Suppl. (Stockholm) *236:*1–135.

Chandler, J.P. (1984) Light and electron microscopic studies of the basilar papilla in the duck, Anas platyrhynchos II. Embryonic development. J. Comp. Neurol. *222:*523–542.

Cohen, G.M., and C.D. Fermin (1978) Development of hair cells in the embryonic chick's basilar papilla. Acta Otolaryngol. *86:*342–358.

Cotanche, D.A., and K.K. Sulik (1984) The development of stereociliary bundles in the cochlear duct of chick embryos. Dev. Brain Res. *16:* 181–93.

Dau, J., and R.J. Wenthold (1989) Immunocytochemical localization of neurofilament subunits in the spiral ganglion of normal and neomycin-treated guinea pigs. Hearing Res. *42:*2532–63.

Dechesne, C.J., A. Sans, and A. Keller (1985) Onset and development of neuron-specific enolase immunoreactivity in the peripheral vestibular system of the mouse. Neurosci. Letters *61:*299–304.

Deschesne, C.J., E. Scarfone, P. Atger, and G. Desmadryl (1994) Neurofilament proteins form an annular superstructure in guinea-pig type I vestibular hair cells. J. Neurocytol. *23:*631–640.

Ehrlich, M.E., M. Evinger, S. Regunathan, and G. Teitelman (1994) Mammalian adrenal chromaffin cells coexpress the epinephrine-synthesizing enzyme and neuronal properties in vivo and in vitro. Dev. Biol. *162:*480–490.

Erickson, C.A., R.P. Tucker, and B.F. Edwards (1987) Changes in the distribution of intermediate-filament types in Japanese quail embryos during morphogenesis. Differentiation *34:*88–97.

Fermin, C.D., and G.M. Cohen (1984) Developmental gradients in the embryonic chick's basilar papilla. Acta Otolaryngol. *97:*39–51.

Franke, W.W., B. Appelhans, E. Schmid, C. Freudenstein, M. Osborn, and K. Weber (1979) Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibody to prekeratin. Differentiation *15:*7–25.

Franke, W.W., E. Schmid, D.L. Schiller, S. Winter, E.D. Jarasch, R. Moll, H. Denk, B.W. Jackson, and K. Illmensee (1982) Differentiation-related expression of proteins of intermediate-size filaments in tissues and cultured cells. Cold Spring Harbor Symp. Quant. Biol. *46:*431–453.

Granger, B.L., and E. Lazarides (1983) Expression of the major neurofilament subunit in chicken erythrocytes. Science *221:*553–556.

Grant, N.J., D. Aunis, and O.K. Langley (1990) Neurofilament expression in bovine chromaffin cells. Acta Histochem. Suppl. *38:*71–75.

Gray, L., and E.W Rubel (1985) Development of absolute thresholds in chickens. J. Acoust. Soc. Am. *77:*1162–1172.

Hafidi, A., G. Despres, and R. Romand (1990) Cochlear innervation in the developing rat: an immunocytochemical study of neurofilament and spectrin proteins. J. Comp. Neurol. *300:*153–161.

Hamburger, V., and H.L. Hamilton (1951) A series of normal stages in the development of the chick embryo. J. Morphol. *88:*49–92.

Hasko, J.A., G.P. Richardson, I.J. Russell, and G. Shaw (1990) Transient expression of neurofilament protein during hair cell development in the mouse cochlea. Hearing Res. *45:*63–74.

Hirano, A. (1991) Cytopathology of amyotrophic lateral sclerosis. Adv. Neurol. *56:*91–102.

Hirokawa, N. (1978) Synaptogenesis in the basilar papilla of the chick. J. Neurocytol. *7:*283–300.

Hoffman, P.N., and R.J. Lasek (1975) The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. J. Cell Biol. *66:*351–356.

Hoffman, P.N., J.W. Griffin, and D.L. Price (1984) Control of axonal caliber by neurofilament transport. J. Cell Biol. *99:*705–714.

Hsu, S.M., L. Raine, and H. Fanger (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J. Histochem. Cytochem. *29:*1349–1353.

Jahnke, V., P.-G. Lundquist, and J. Wersäll (1969) Some morphological aspects of sound perception in birds. Acta Otolaryngol. *67:*583–601.

Jones, S.M., and T.A. Jones (1995a) Neural tuning characteristics of auditory primary afferents in the chicken embryo. Hearing Res. *82:*139–148.

Jones, S.M., and T.A. Jones (1995b) The tonotopic map in the embryonic chicken cochlea. Hearing Res. *82:*149–157.

Katayama, A., and J.T. Corwin (1989) Cell production in the chicken cochlea. J. Comp. Neurol. *281:*129–135.

Kelly, B.M., C.S. Gillespie, D.L. Sherman, and P.J. Brophy (1992) Schwann cells of the myelin-forming phenotype express neurofilament protein NF-M. J. Cell Biol. *118:*397–410.

Kuijpers, W., E.L. Tonnaer, T. Peters, and F.C. Ramaekers (1988) Intermediate filaments in the developing and mature rat inner ear. Paper presented at the Inner Ear Biology Workshop, London.

Kuijpers, W., Tonnaer, E.L., Peters, T.A. and Ramaekers, F.C. (1991) Expression of intermediate filament proteins in the mature inner ear of the rat and guinea pig. Hearing Res. *52:*133–146.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227:*680–685.

Landmesser, L., and S. Swain (1992) Temporal and spatial modulation of a cytoskeletal antigen during peripheral axonal pathfinding. Neuron *8:*291–305.

Lee, V.M.-Y., M.J. Carden, W.W. Schlaepfer, and J.Q. Trojanowski (1987) Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. J. Neurosci. *7:*3474–3488.

Liem, R.K.H., S.-H. Yen, G.D. Solomon, and M.L. Shelanski (1978) Intermediate filaments in nervous tissues. J. Cell Biol. *79:*637–645.

Lippe, W.R. (1987) Shift of tonotopic organization in brainstem auditory nuclei of the chicken during late embryonic development. Hearing Res. *25:*205–208.

Lippe, W.R., and E.W Rubel (1985) Ontogeny of tonotopic organization of brainstem auditory nuclei in the chicken: Implications for development of the place principle. J. Comp. Neurol. *237:*273–289.

Manley, G.A., J. Brix, and A. Kaiser (1987) Developmental stability of the tonotopic organization of the chick's basilar papilla. Science *237:*655–656.

Murphy, A., K.C. Breen, A. Long, C. Feighery, E.B. Casey, and D. Kelleher (1993) Neurofilament expression in human T lymphocytes. Immunology *79:*167–170.

Oesterle, E.C., D.E. Cunningham, and E.W Rubel (1992) Ultrastructure of hyaline, border and vacuole cells in chick inner ear. J. Comp. Neurol. *318:*64–82.

Oesterle, E.C., T.T. Tsue, T.A. Reh, and E.W Rubel (1993) Hair-cell regeneration in organ cultures of the postnatal chicken inner ear. Hearing Res. *70:*85–108.

Ofsie, M.S., and D.A. Cotanche (1995) Innervation patterns of normal and noise-damaged chick cochleae. Assoc. Res. Otolaryngol. Abstr. *18:*85.

Ofsie, M.S., and D.A. Cotanche (1996) Distribution of nerve fibers in the basilar papilla of normal and sound-damaged chick cochleae. J. Comp. Neurol. *370:*281–294.

Osborn, M., and K. Weber (1983) Tumor diagnosis by intermediate filament typing: A novel tool for surgical pathology. Lab. Invest. *48:*372–394.

Pirvola, U., E. Lehtonen, and J. Ylikoski (1991) Spatiotemporal development of cochlear innervation and hair cell differentiation in the rat. Hearing Res. *52:*345–355.

Raphael, Y., G. Marshak, A. Barash, and B. Geiger (1987) Modulation of intermediate-filament expression in developing cochlear epithelium. Differentiation *35:*151–162.

Rebillard, G., and E.W Rubel (1981) Electrophysiological study of the maturation of auditory responses from the inner ear of the chick. Brain Res. *229:*15–23.

Retzius, G. (1884) Das Gehörorgan der Wirbelthiere, Vol. II. Das Gehörorgan der reptilien, der Vögel und der Säugethiere. Stockholm: Samson and Wallin.

Roberson, M.D., A.D. Toews, J.F. Goodrum,. and P. Morell (1992) Neurofilament and tubulin mRNA expression in schwann cells. J. Neurosci. Res. *33:*156–162.

Robinson, P.A., and B.H. Anderton (1988) Neurofilament probes—A review of neurofilament distribution and biology. Rev. Neurosci. *2:*1–40.

Romand, R. (1983) Development of the cochlea. In R. Romand (ed): Development of Auditory and Vestibular Systems. New York: Academic Press, pp. 47–89.

Rubel, E.W (1978) Ontogeny of structure and function in the vertebrate auditory system. In M. Jackson (ed): Handbook of Sensory Physiology, Vol. IX. Development of Sensory Systems. New York: Springer-Verlag, pp. 135–237.

Rubel, E.W, and B.M. Ryals (1982) Patterns of hair cell loss in chick basilar papilla after intense auditory stimulation. Acta Otolaryngol. *93:*31–41.

Ryals, B.M., H.B. Creech, and E.W Rubel (1984) Postnatal changes in the size of the avian cochlear duct. Acta Otolaryngol. *98:*93–97.

Saunders, J.C., R.B. Coles, and G.R. Gates (1973) The development of auditory evoked responses in the cochlea and cochlear nuclei of the chick. Brain Res. *63:*59–74.

Saunders, S.S., and R.J. Salvi (1993) Psychoacoustics of normal adult chickens: Thresholds and temporal integration. J. Acoust. Soc. Am. *94:*83–90.

Schliwa, M. (1986) The Cytoskeleton: An Introductory Survey. New York: Springer.

Schrott, A., G. Egg, and H. Spoendlin (1988) Intermediate filaments in the cochleas of normal and mutant (w/wv, sl/sld) mice. Arch. Otorhinolaryngol. *245:*250–254.

Semba, R., M.T. Asano, and K. Kato (1990) Physiological expression of neural marker proteins in the heart of young rats. Dev. Brain Res. *54:*217–220.

Shield, M.J., and D.A. Cotanche (1990) SEM analysis of the developing tectorial membrane in the chick cochlea. Hearing Res. *47:*147–158.

Smith, C.A. (1981) Recent advances in structural correlates of auditory receptors. In D. Ottoson (ed): Progress in Sensory Physiology 2. New York: Springer-Verlag.

Smith, C.A. (1985) Inner ear. In A.S. King and J. McLeland (eds): Form and Function in Birds, Vol. 3. London: Academic Press, pp. 273–310.

Sternberger, L.A., and N.H. Sternberger (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc. Natl. Acad. Sci. USA *80:*6126–6130.

Tanaka, K., and C.A. Smith (1978) Structure of the chicken's inner ear: SEM and TEM study. Am. J. Anat. *153:*251–272.

Thornell, L.-E., M. Anniko, and I. Virtanen (1987) Cytoskeletal organization of the human inner ear. Acta Otolaryngol. *437:*5–27.

Tilney, L.G., and J.C. Saunders (1983) Actin filaments, stereocilia, and hair cells of the bird cochlea I. Length, number, width, and distribution ofstereocilia of each hair cell are related to the position of the hair cell on the cochlea. J. Cell Biol. *96:*807–821.

Tucci, D.L., and E.W Rubel (1990) Physiologic status of regenerated hair cell in the avian inner ear following aminoglycoside ototoxicity. Otolaryngol. Head Neck Surg. *103:*443–450.

Vanzulli, A., and E. Garcia-Austt (1963) Development of cochlear microphonic potentials in the chick embryo. Acta Neurol. Latin Am. *9:*19–23.

Virtanen, I., M. Miettinen, V.P. Lehto, A.L. Kariniemi, and R. Passivuo (1985) Application of monoclonal antibodies to intermediate filaments. Ann. NY Acad. Sci. *455:*635–648.

Whitehead, M.C., and D.K. Morest (1985) The development of innervation patterns in the avian cochlea. Neuroscience. *14:*255–276.

Wikström, S.O., M. Anniko, L.-E. Thornell, and I. Virtanen (1988) Developmental stage-dependent pattern of inner ear expression of intermediate filaments. Acta Otolaryngol. *106:*71–80.

Xu, Z., D.L.-Y. Dong, and D.W. Cleveland (1994) Neuronal intermediate filaments: New progress on an old subject. Curr. Opin. Neurobiol. *4:*655–661.