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Appearance and Distribution of the 275 kD Hair-Cell Antigen During Development of the Avian Inner Ear

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ABSTRACT

The 275 kD hair-cell antigen (HCA) is a protein that was originally identified using immunological techniques in the inner ears of early hatchling and adult chickens. The HCA is specifically associated with the apical surface of sensory hair cells; in the vestibular system the antigen is distributed over the entire stereocilia bundle, but in the auditory system it only extends a short distance up the shafts of the stereocilia. The objectives of this study were to ascertain when the HCA is first expressed during inner ear development, to compare the temporal and spatial patterns of HCA expression with those of neurite ingrowth, and to determine how the distribution of the antigen observed in the auditory system arises during development. Serial sections of otocysts from embryonic day (ED) 4 to ED7.5 (stages 24 to 32) were stained with a monoclonal antibody to the HCA and polyclonal antibodies to the neuron–glial cell adhesion molecule in order to analyse patterns of HCA expression and neurite ingrowth. Nerve fibres are first observed in the anterior pole of the otocyst at ED4.5 (stage 24), and in the evaginating basilar papilla by ED5 (stage 26). The HCA first appears within the vestibular system in the anterior pole of the otocyst at ED5 (stage 26), and within the auditory system in the distal end of the basilar papilla at ED6.5 (stage 29). Serial section analysis indicates that expression of the HCA is always limited to areas of the epithelium where nerve fibres are found, although the delay between the onset of innervation and the onset of HCA expression varies from one region of the otocyst to another. The growth of stereocilia bundles in the auditory system was studied from ED10 to 2 days after hatching in sections from the medial to distal regions of the basilar papilla double labelled with rhodamine phalloidin and monoclonal anti-HCA. At ED12 the stereocilia bundles are 1.7 μm high and the staining observed with both phalloidin and the antibody extend to the same maximum height above the apical surface of the hair cell. The maximum height that anti-HCA staining extends up the stereocilia bundle remains almost constant between ED12 and postnatal day 2, but between ED15 and ED18 the stereocilia bundle grows rapidly in height, with a membrane domain lacking the HCA forming at the distal ends of the stereocilia. The restricted distribution of the HCA observed on the apical surface of mature auditory hair cells in the basilar papilla is therefore generated during the final growth phase of the stereocilia bundle by the accumulation of HCA-free membrane at the distal ends of the stereocilia.

Key words: neuron–glia adhesion molecule, cochlea, developmental biology

The inner ear is a highly complex sensory organ that develops from a thickening of the head ectoderm, the otic placode, via inductive interactions with the underlying mesenchymal tissue and the adjacent rhombencephalon (Yntema, '33, '50; Waddington, '37; Harrison, '45). The otic placode, after forming the otocyst, gives rise to at least seven distinct cell types within the inner ear, including the neurons of the VIIIth ganglion and the mechanosensory hair cells that they innervate. There are seven discrete

patches of sensory hair cells in the avian inner ear, six vestibular organs (three maculae and three ampullae), and one auditory organ known as the basilar papilla. The formation of the VIIIth ganglion, the differentiation of sensory hair cells, synaptogenesis, and the development of the stereocilia bundles in the chicken inner ear have been the subject of a considerable number of previous studies.

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These processes can be briefly described as follows. The neurons are the first cells to differentiate. They are generated within the otocyst around its inner luminal margin and then migrate out from the otocyst and condense around its ventromedial surface to form the VIIIth ganglion (Knowlton, '67; Meier, '78a,b; D'Amico-Martel and Noden, '83). These neurons then project one process up to the rhombencephalon and another process back into the otocystic epithelium. The hair cells then differentiate and synapses are subsequently elaborated (Vasquez-Nin and Sotelo, '68; Cohen and Fermin, '78; Hirokawa, '78; Ginzberg and Gilula, '79, '80; Rebillard and Pujol, '83; Fermin and Cohen, '84; Whitehead and Morest, '85a,b). Although both the Golgi (Whitehead and Morest, '85a) and the electron microscope studies (Vasquez-Nin and Sotelo, '68; Ginzberg and Gilula, '79, '80; Whitehead and Morest, '85b) of chick inner ear development have shown that neurites from the VIIIth ganglion penetrate the epithelium prior to hair cell differentiation, experimental evidence obtained with denervated avian otocysts developing ectopically *in vivo* (Swanson et al., '90) indicates that the presence of nerve fibres is not required for the initial differentiation of hair cells. The processes involved in the growth and development of stereocilia bundles in the basilar papilla have been described in considerable detail (Tilney et al., '86, '88; Tilney and DeRosier, '86), and it has also been demonstrated that the differentiation of location-specific stereocilia bundle morphologies occurs in denervated preparations (Corwin and Cotanche, '89).

Recent immunological studies have led to the identification of a 275 kD protein associated with the apical surface of sensory hair cells within the avian inner ear (Richardson et al., '90). The protein is referred to as the hair-cell antigen (HCA) and a monoclonal antibody directed against this protein is a highly specific immunohistochemical marker for sensory hair cells within the inner ears of early hatching and adult chickens. With vestibular hair cells, the HCA is found both on the apical, nonstereociliar surface of the cell and evenly distributed over the entire hair bundle. With auditory hair cells, the HCA is also found on the apical surface of the hair cell, but it is not distributed evenly over the hair bundle and only extends a short distance up the shafts of the stereocilia (Fig. 1). This restricted distribution of the HCA observed on the apical surface of auditory hair cells is most readily detected in the distal regions of the basilar papilla where the stereocilia are relatively long, and, in view of recent findings indicating that stereocilia grow by the addition of actin to their distal tips (Tilney and DeRosier, '86), prompted the suggestion that the HCA may only be expressed on auditory hair-cell stereocilia during the initial stages of their growth rather than at later stages. However, other explanations for such a distribution are possible. For example, the stereocilia bundles may reach their final height prior to expression of the HCA, and the HCA may then be selectively inserted into discrete domains on the cell surface. Alternatively, the HCA may be expressed over the entire apical surface as the stereocilia bundle grows and then be selectively removed from regions near the distal tips of the stereocilia.

The primary objective of the present study was therefore to determine the stage at which the HCA first appears during inner ear development. In particular we were interested to know whether the HCA is expressed either before or after the formation of the stereocilia bundle, and, if the antigen appeared early in development, how the expression

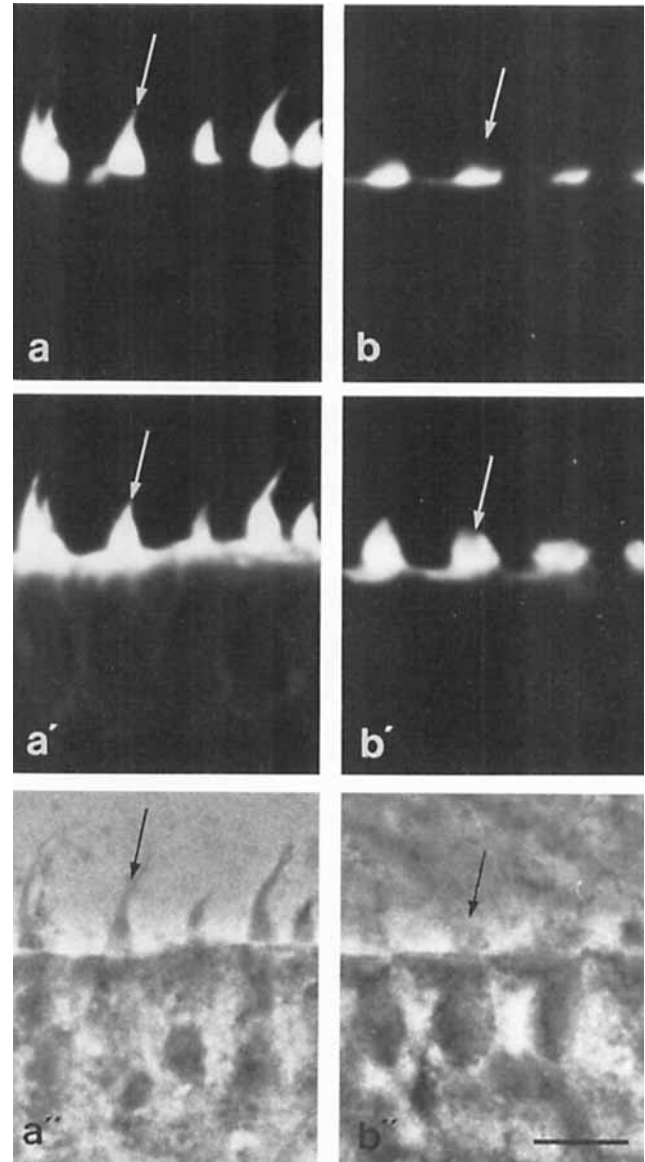


Fig. 1. Hair cells from the lagena macula (**a**, **a'**, **a''**) and the distal region of the basilar papilla (**b**, **b'**, **b''**) of a 2 day old chick double labelled for the hair-cell antigen (HCA) (**a**, **b**) and F-actin (**a'**, **b'**). Corresponding phase contrast images are shown in **a''** and **b''**, respectively. Arrows in **a**, **a'**, and **a''** and **b**, **b'**, and **b''** point to the same stereocilia bundles. Note how the HCA extends up the entire bundle in the lagena macula, but is only seen around the base of the bundle in the basilar papilla. Scale bar = 10 μ m.

pattern was related to the process of innervation. In a previous study of inner ear development (Richardson et al., '87), it was shown that the neuron-glial cell adhesion molecule (Ng-CAM, Grumet and Edelman, '84) is a good marker for early ingrowing nerve fibres in the chicken otocyst. The first part of this present study chronicles the appearance of the HCA during the early development of the otocyst, and compares the temporal and spatial patterns of Ng-CAM staining with those of HCA expression. In the second half of this study the distribution of the HCA in the distal, low-frequency region of the basilar papilla is examined during stereocilia bundle growth in order to determine

how the restricted distribution of the HCA observed on the apical surface of mature auditory hair cells arises during development.

MATERIALS AND METHODS

Chicken (*Gallus domesticus*) eggs of the Isa Brown variety were obtained from ISA Poultry Services (Peterborough, UK) and incubated at 38°C in a humid, forced-draught incubator. Eggs hatch between 20 and 21 days of incubation under these conditions. Early embryos were staged according to the criteria of Hamburger and Hamilton ('51). Later embryos (≥ 8 days of incubation) were staged according to the number of days of incubation. Tissues were fixed for 1 hour at room temperature by immersion in 3.7% (v/v) formaldehyde, 0.025% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2, washed 3 \times with phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM sodium phosphate, pH 7.2), equilibrated overnight with 30% (w/v) sucrose in PBS, embedded in 1% (w/v) low gelling point agarose (Sigma, Type VII) in PBS containing 18% (w/v) sucrose, and finally sectioned at either 7 or 10 μ m on a Reichert Jung Cryocut 1800. Cryostat sections were mounted on gelatin-coated glass coverslips and dried overnight at 37°C before use.

Sections were preblocked with Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 10% heat inactivated horse serum (HS) for 1 hour, and then incubated overnight in primary antibodies diluted in TBS/HS. Monoclonal mouse anti-HCA hybridoma supernatant and polyclonal rabbit Ig to Ng-CAM (R700) were both used at a dilution of 1:100. Antibodies to Ng-CAM were a gift from Prof. G.M. Edelman, The Rockefeller University, New York. After washing in TBS, bound monoclonal antibodies were labelled with FITC-conjugated rabbit anti-mouse Ig, followed by FITC-conjugated swine anti-rabbit Ig. Polyclonal rabbit antibodies were detected with a single layer of FITC-conjugated swine anti-rabbit Ig. To double label for the HCA and F-actin, rhodamine-conjugated phalloidin was added to the second layer of FITC-conjugated antibody to a final concentration of 0.2 μ g/ml. After labelling, the sections were washed with TBS and mounted in Tris-buffered glycerol (1 part 100 mM Tris-HCl, pH 8.4, 9 parts glycerol) containing 0.1% (w/v) p-phenylenediamine to retard bleaching of the fluorescent signal. Stained sections were observed with a Zeiss IM35 microscope equipped for epifluorescent illumination and photographed on Kodak TMY-400 film rated at 1600 ASA.

For tissues from the early stages of development, virtually complete serial 10 μ m section series through otocysts were collected from a total of nine embryos, and alternate sections from these series were then stained for the HCA and Ng-CAM. The otocysts were drawn with a camera lucida and darkfield microscopy and the positions of the Ng-CAM-positive fibres and the HCA then added to the tracings during subsequent observation of the sections under UV illumination. Results for the early stages of development were also derived from a considerable number of other specimens for which complete serial section series were not obtained.

Cochleas from two sets of animals were used to examine HCA distribution during growth of the stereocilia bundles. With one set, cochleas from embryonic day (ED) 13, ED15, ED17, and ED19 were sectioned transversely to the longitudinal axis of the duct until reaching a point 1 mm from the

distal end of the lagena, and then a small sample of 7 μ m sections was taken at this point in the basilar papilla. With the second set, cochleas from ED10, 12, 14, 16, 18, and 20 and 2 days posthatching were sectioned through the lagena macula until the distal tip of the basilar papilla was reached. Then, on the basis of information derived from the study of Tilney et al. ('86), the cochleas were sectioned until reaching a point calculated to be 75% from the proximal end of the basilar papilla. Sections of 7 μ m thickness were then taken at this point for double labelling. Measurements of the heights to which phalloidin staining and the HCA extended above the apical surface of the hair cells were made from micrographs printed at a magnification of $\times 650$ with the aid of a hand-held measuring magnifier. Only cells in which the hair bundles had been clearly sectioned parallel to the vertical axis of the cell were used for these measurements. All obliquely and partially sectioned hair bundle profiles were excluded from these measurements.

RESULTS

Innervation of the otocyst and appearance of the HCA

Previous studies have shown that the VIIIth ganglion becomes Ng-CAM positive by stage 24 (Richardson et al., '87). Serial section analysis reveals that Ng-CAM-stained fibres are present at this stage of development in the anteriormost end of the otocyst within the region that will eventually form the ampulla of the superior semicircular canal (Fig. 2a). Fibres are also observed at this stage more caudally within the ventromedial wall of the otocyst at the site where the otocyst is beginning to evaginate toward the midline (not shown). Staining cannot be detected using the anti-HCA antibody in either the anterior end of the otocyst (Fig. 2b), the evaginating ventromedial wall, or at any other site within the otocyst at stage 24. Staining with the anti-HCA antibody is first observed on the luminal surface of the epithelium in the presumptive anterior ampulla at stage 26 (Fig. 2d). The staining is punctate and is only found in the region of the epithelium where Ng-CAM-positive fibres can be seen (Fig. 2c). By stage 27, staining is observed with the anti-HCA antibody at several different sites on the luminal surface of the otocyst (Fig. 3a). These sites correspond to the sacculus (on the upper end of the dorsomedial wall), the posterior ampulla (located ventrally at the posterior end of the otocyst), the utriculus and the lateral ampulla (on the ventrolateral wall of the lateral expansion of the otocyst), and the ampulla of the superior semicircular canal (at the rostral end of the otocyst). With the exception of the patches corresponding to the future lateral and anterior ampullae which appear almost continuous, anti-HCA staining first appears in discrete, spatially separate locations on the luminal surface of the otocyst. Although the size of the patch within which anti-HCA staining is observed is initially smaller than the area innervated by Ng-CAM-stained fibres (see Figs. 2c,d), by stage 30 there is a close correspondence between the distribution of Ng-CAM-stained fibres within the sacculus, utriculus, and lateral ampulla and the sites at which the HCA can be observed on the luminal surface of the otocyst (Fig. 4).

Ingrowing nerve fibres stained with antibodies to Ng-CAM are first observed in the proximal end of the basilar papilla by stage 26, and can be seen throughout the entire length of the papilla by stage 28 (Fig. 5a). The HCA cannot be detected in the elongating basilar papilla at stage 28 (Fig.

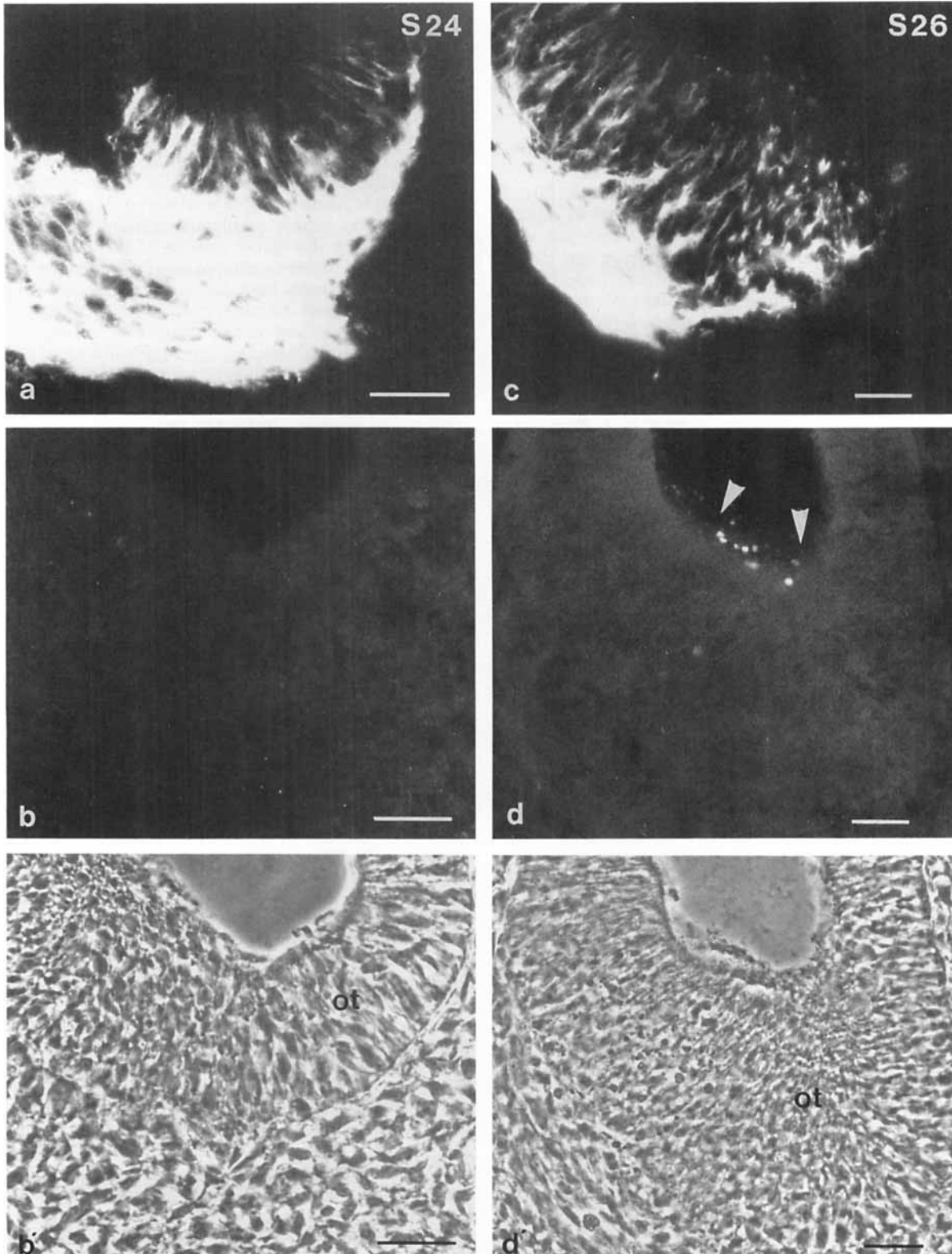


Fig. 2. Adjacent sections through the anteriormost ends of otocysts from stage 24 (**a**, **b**, **b'**) and stage 26 (**c**, **d**, **d'**) embryos stained with antibodies to the neuron–glial cell adhesion molecule (Ng-CAM) (**a**, **c**) and the HCA (**b**, **d**). Phase contrast images of **b** and **d** are shown in **b'**

and **d'**, respectively. Arrowheads in **d** demarcate the region of the lumenal surface where hair cells can be detected with the antibody to the HCA. *ot*, otocyst. Scale bars = 25 μ m.

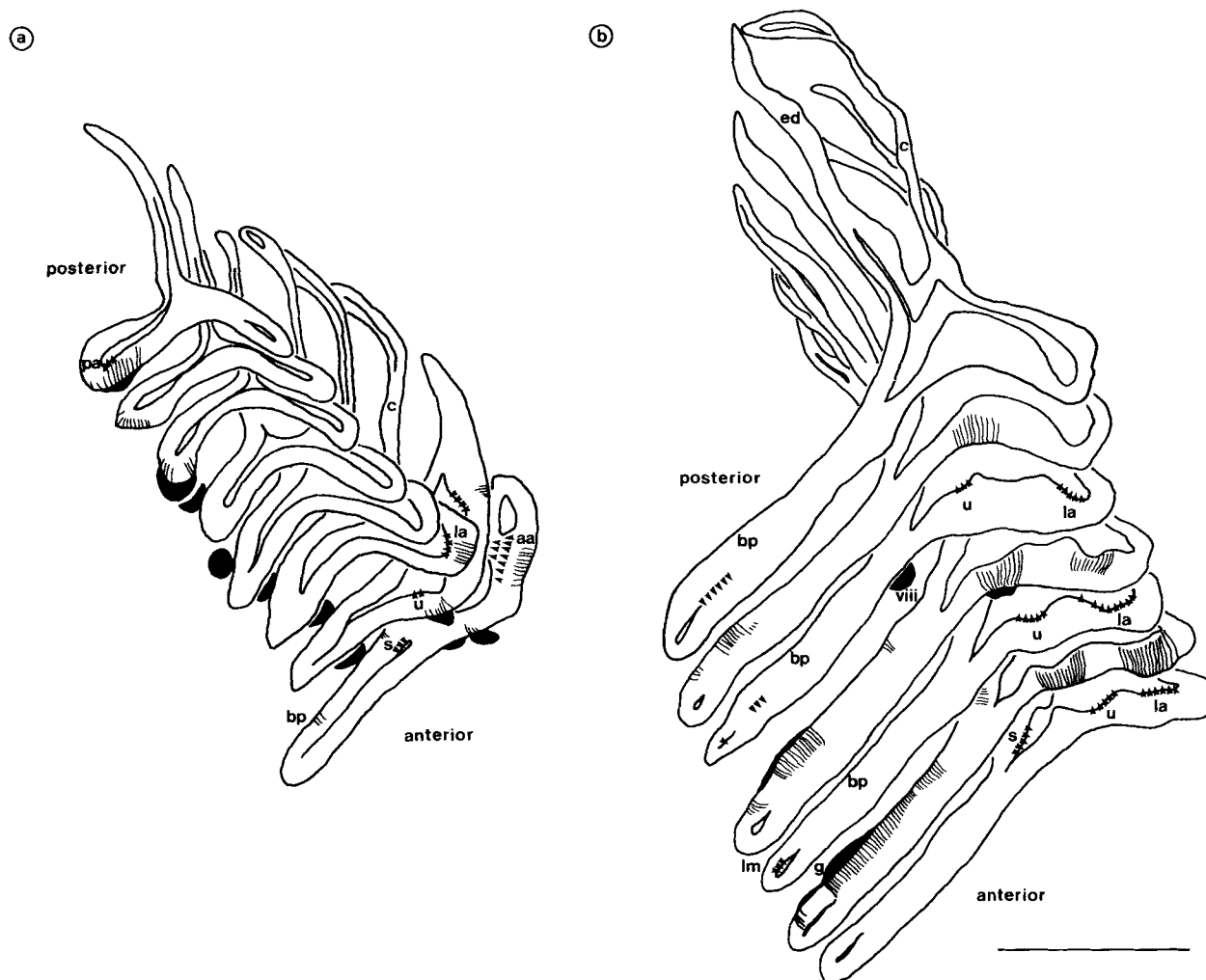


Fig. 3. **a:** Partial reconstruction of an otocyst from a stage 27 embryo. Only every fourth section is illustrated and the data from adjacent sections stained for either Ng-CAM or the HCA have been combined onto the one drawing. Slices are therefore at 50 μm intervals. **b:** Partial reconstruction of the otocyst from a stage 29 embryo in which alternate sections had been stained for the HCA or Ng-CAM. Seven adjacent 10 μm sections are shown. In a and b arrowheads indicate the positions of HCA staining, fine lines radiating into the epithelium in-

dicate the positions of Ng-CAM-positive fibres, and solid areas indicate the VIIIth nerve and portions of the acousticovestibular ganglion stained for Ng-CAM. aa, anterior ampulla of the superior semicircular canal; u, utricle; la, ampulla of the lateral semicircular canal; pa, ampulla of the posterior semicircular canal; s, sacculus; lm, lagena macula; bp, basilar papilla; g, cochlear ganglion, viii, eighth nerve; c, superior semicircular canal; ed, endolymphatic duct. Scale bar = 500 μm .

5b), and first appears at stage 29, in the distal end of the basilar papilla, at a site adjacent to a region within which a number of Ng-CAM-stained fibres can be seen (Figs. 5c,d). A partial reconstruction of a stage 29 otocyst illustrating the relative distributions of Ng-CAM staining and the HCA is presented in Figure 3b. By stage 29, staining with the monoclonal anti-HCA is also first seen in the lagena macula, which is located at the distal end of the cochlear duct (Fig. 3b). By stage 31, the HCA can be seen throughout the entire length of the basilar papilla, but the number of punctate stained spots observed along the length of the papilla is quite sparse. At stage 32 (Fig. 6), the HCA is readily detected throughout the entire length of the basilar papilla, which is approximately 700 μm long at this stage.

The results from the nine serial section series in which the distributions of the HCA and Ng-CAM were mapped throughout the otocysts are summarized in Table 1. Serial section analysis confirms that the HCA antigen only appears in areas within which Ng-CAM-stained fibres are

found and also indicates that the regions where the HCA appears are initially very small. For example, in the basilar papilla at stage 29, the HCA was only observed in two sections and extended for a distance of 50 μm along the papilla (see Figs. 3b, 5d). This would be, at maximum, a patch of HCA-positive cells about 25 μm in diameter, yet Ng-CAM-stained fibres are present throughout the length (~400 μm) of the basilar papilla at this stage. The differences between the stages at which Ng-CAM-stained fibres and the HCA appear in the various areas (see Table 1) indicate that the HCA appears with variable delays after the innervation of the different regions of the epithelium, with this delay being longer in the basilar papilla (36–48 hours) than it is in the vestibular system (12–24 hours).

Growth of stereocilia bundles in the basilar papilla

Examples of auditory hair cells in the medial to distal region of the basilar papilla at ED10, 12, 15, 17, and 20 that

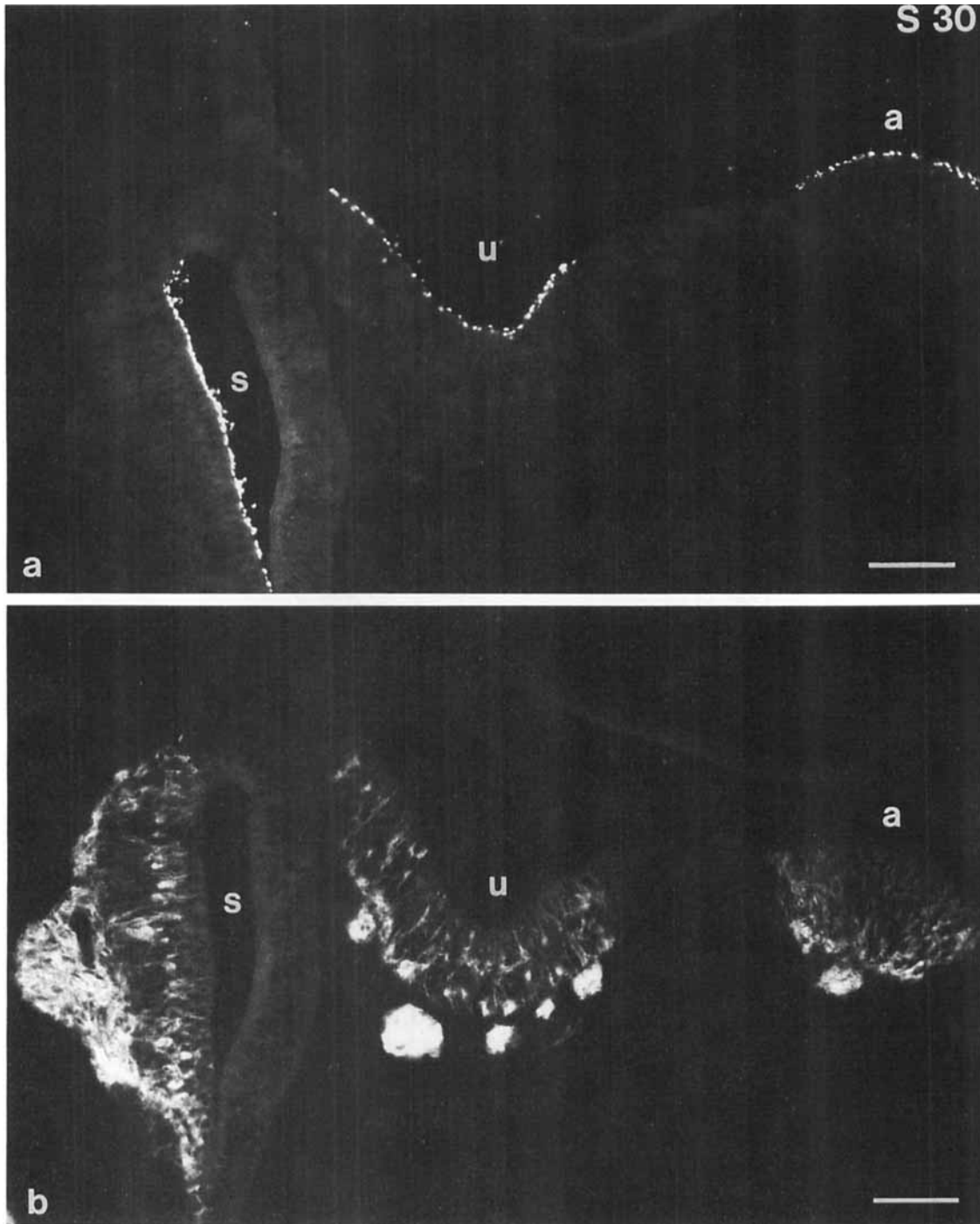


Fig. 4. Adjacent sections through the otocyst of a stage 30 embryo stained with antibodies to the HCA (a) and Ng-CAM (b). s, sacculus; u, utriculus; a, lateral ampulla. Scale bars = 50 μ m.

have been double labelled for F-actin and the HCA are presented in Fig. 7. Stereocilia bundles begin to stain intensely with phalloidin by ED12. Prior to this stage, phalloidin is a poor marker for hair cells relative to the anti-HCA antibody. Cuticular plates can be first seen in the hair cell cytoplasm below the stereocilia bundles using phalloidin staining at ED15 (Fig. 7c'), and an increment in the height of stereocilia bundles, observed using this tech-

nique, is most apparent between ED15 and ED18 (Fig. 7c',d'). Expansion of the apical, nonstereociliar surface of the hair cell is apparent between ED12 and ED20 using anti-HCA staining (Figs. 7b–e). Data obtained by measuring the maximum height to which phalloidin staining extends above the apical surface (considered to represent stereocilia bundle height), and the height to which the HCA extends above the surface, for the two different sets of

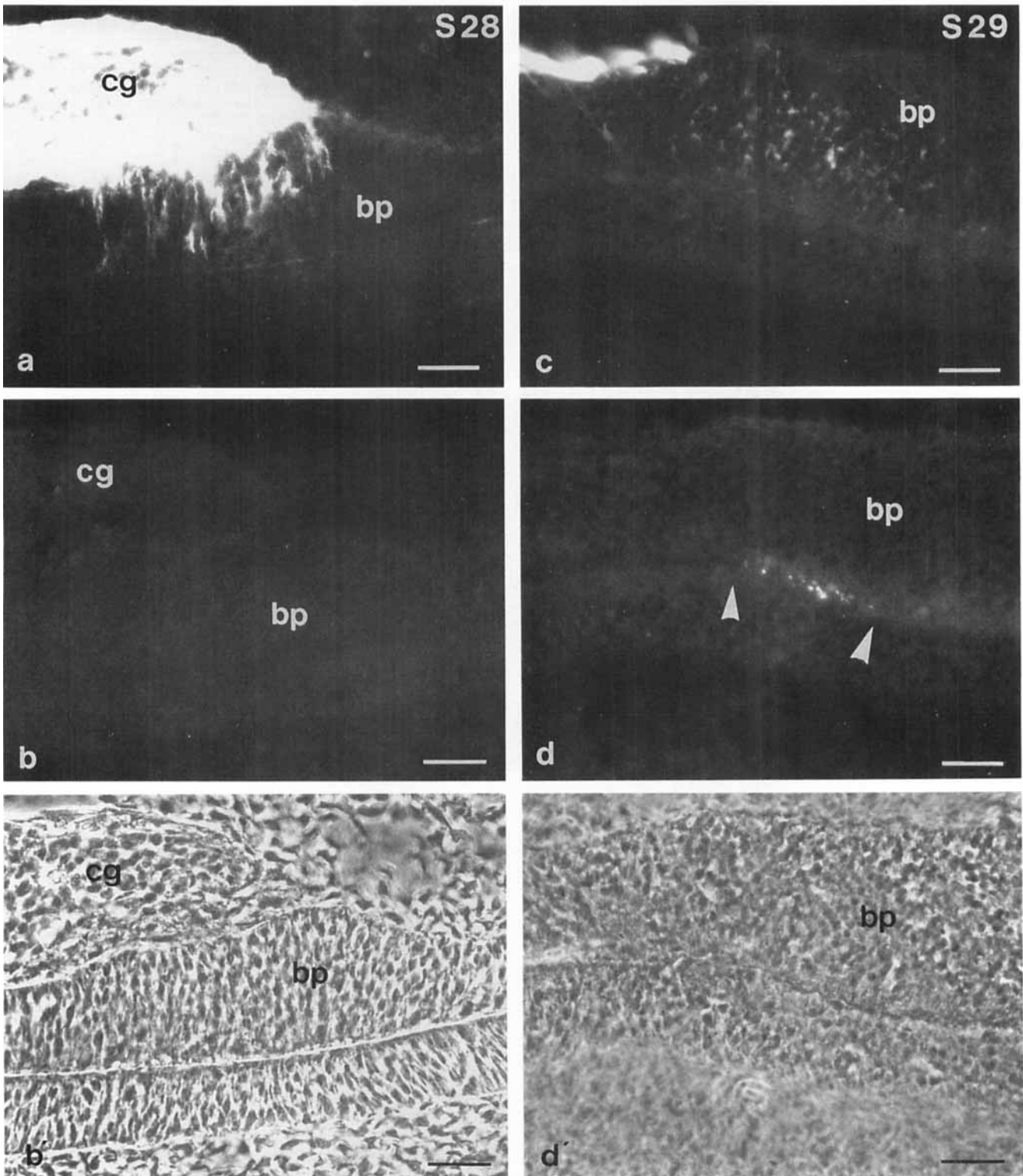


Fig. 5. Adjacent longitudinal sections through the basilar papillae of stage 28 (a, b, b') and stage 29 (c, d, d') embryos stained with antibodies to Ng-CAM (a, c) and to the HCA (b, d). Corresponding phase contrast images of b and d are shown in b' and d', respectively.

Arrowheads in d demarcate the region of the basilar papilla where hair cells are first detected with antibodies to the HCA. The distal end of the cochlear duct lies to the right of the figure in all sections. cg, cochlear ganglion, bp, basilar papilla. Scale bars = 10 μ m.

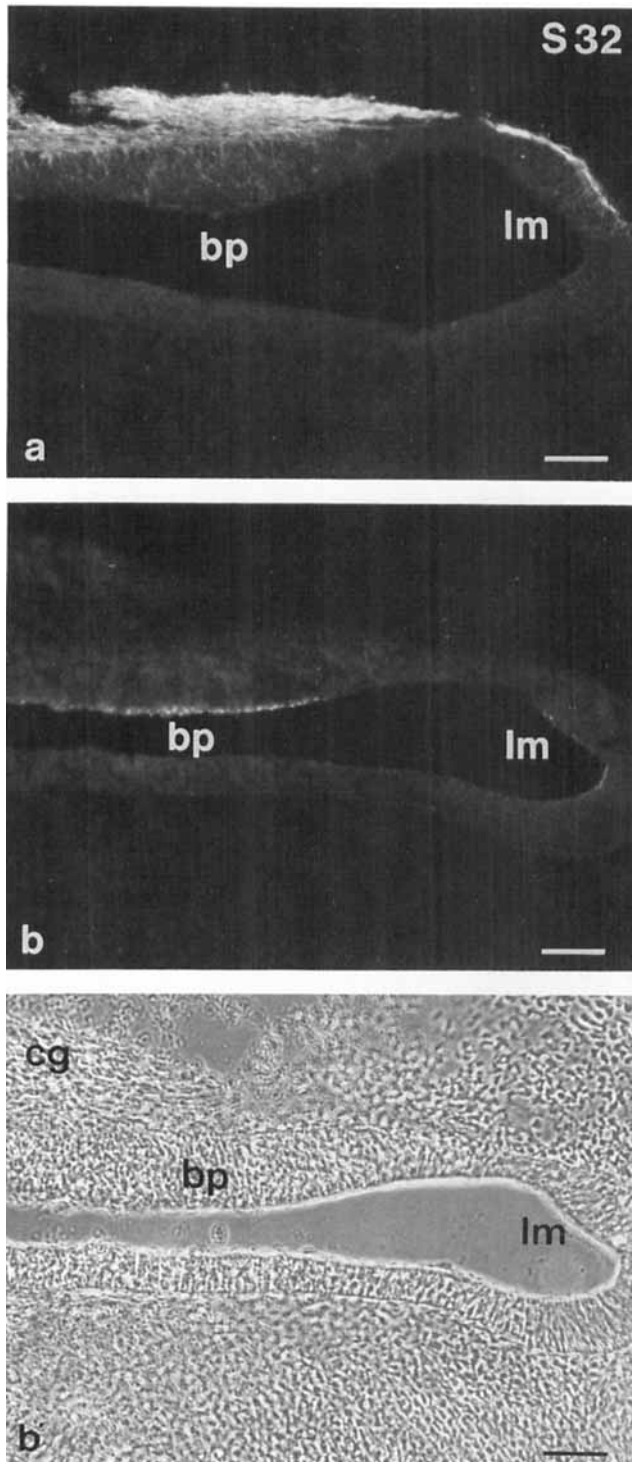


Fig. 6. Adjacent longitudinal sections through the basilar papilla and the lagena macula of a stage 32 embryo stained with antibodies to Ng-CAM (a) and the HCA (b). Corresponding phase contrast image of b is provided in b'. cg, cochlear ganglion; bp, basilar papilla; lm, lagena macula. Scale bars = 50 μ m.

cochleas used in this study are presented in Figure 8. With one of the sets of cochleas used in this study (Fig. 8a), the ducts were all sectioned to a fixed distance from the distal

TABLE 1. Summary of the Embryos Used for the Serial Section Analysis of HCA and Ng-CAM Distribution and the Results Obtained

Specimen	Stage	Approximate incubation time (days)	Ng-CAM		HCA	
			Vestibular	Auditory	Vestibular	Auditory
1	24	4.5	+ ¹	—	—	—
2	26	5.0	+ ²	+	+ ¹	—
3	26	5.0	+ ²	+	+ ¹	—
4	27	5.5	+	+	+ ³	—
5	28	6.0	+	+	+	—
6	29	6.5	+	+	+	+ ⁴
7	29	6.5	+	+	+	+ ⁴
8	31	7.0	+	+	+	+ ⁵
9	32	7.5	+	+	+	+

¹Staining only observed in the anterior end of the otocyst in the presumptive ampulla of the superior semicircular canal.

²Staining for neuron-glial cell adhesion molecule (Ng-CAM) observed in five presumptive vestibular sensory areas—the three ampullae of the semicircular canals, the utriculus, and the sacculus.

³Staining for the hair-cell antigen (HCA) observed in the three ampullae of the semicircular canals, the utriculus, and the sacculus.

⁴Staining for HCA only observed in the distal region of the basilar papilla and in the lagena macula.

⁵Staining first observed throughout the length of the basilar papilla.

extreme of the lagena, and, because of the increase in basilar papilla length that occurs with development, the cells used for the measurements may therefore come from slightly different points along the cochlea, with a tendency for the cells measured at the later stages to be located more distally than those measured at the early stages. With the second set of cochleas (Fig. 8b), growth of the duct was taken into consideration, and all cells measured were estimated to come from the same region, at a point 75% from the proximal end of the basilar papilla. Despite these methodological differences, both sets of data indicate that the height to which the HCA extends above the apical surface of the hair cell remains fairly constant during these later developmental stages, whilst the height of the stereocilia bundle as measured from the phalloidin staining increases most rapidly between ED15 and ED18 (Figs. 8a,b).

DISCUSSION

Differentiation of hair cells and appearance of the HCA

Although the transmission electron microscope studies of basilar papilla development (Cohen and Fermin, '78; Fermin and Cohen, '84; Whitehead and Morest, '85b) indicated that stereocilia bundles could be first recognised between stages 33 and 35 (ED7.5–ED9), Cotanche and Sulik ('84), using a combination of transmission and scanning electron microscopy, demonstrated that stereocilia bundles could be first identified in the distal region of the basilar papilla as early as stage 29 (ED6.5). Hair cells could not be readily distinguished from surrounding supporting cells at this early stage with the transmission electron microscope, and stereocilia bundles could only be identified in the scanning electron microscope as the presumptive stereocilia were slightly taller, thicker, and grouped together more closely than the microvilli on the surrounding cells. The data from this present study and that of Cotanche and Sulik ('84) imply that the appearance of the HCA in the basilar papilla correlates closely with the earliest morphological signs of stereocilia bundle differentiation.

Less information is available concerning the appearance of stereocilia bundles in the vestibular system. With the transmission electron microscope, Ginzberg and Gilula

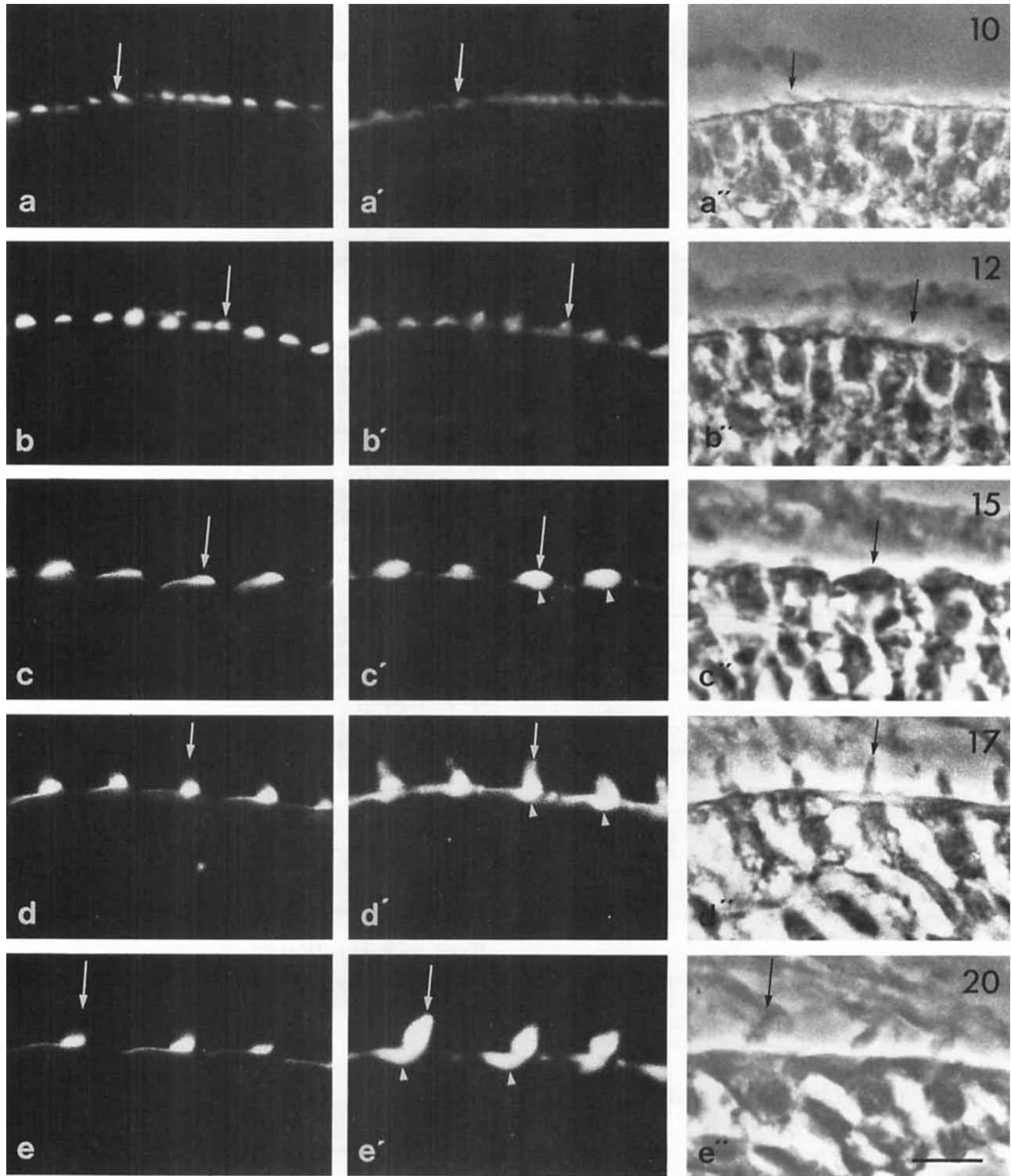


Fig. 7. Hair cells from the distal regions of the basilar papillae of embryos at ED10 (**a**, **a'**, **a''**), ED12 (**b**, **b'**, **b''**), ED15 (**c**, **c'**, **c''**), ED17 (**d**, **d'**, **d''**), and ED20 (**e**, **e'**, **e''**) double stained with anti-HCA antibodies (**a-e**) and phalloidin (**a'-e'**). Corresponding phase contrast images are given in **a''** to **e''**. For each set of micrographs the arrows

point to the same cell. Cuticular plates are indicated by arrowheads in **c'**, **d'**, and **e'**. Note how phalloidin is not a good marker for hair cells at ED10 and how the cuticular plate becomes apparent by ED15. Scale bar = 10 μ m.

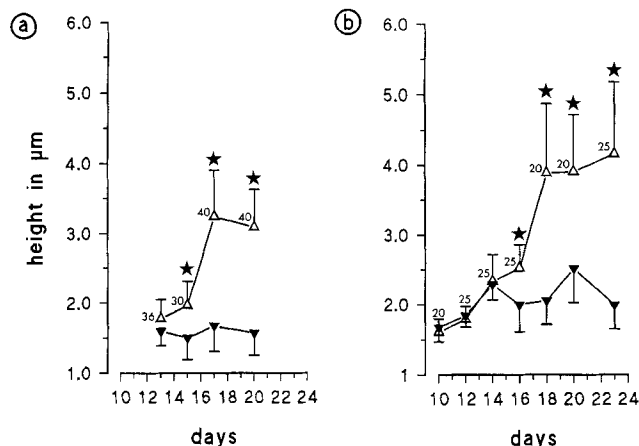


Fig. 8. Graphs show the length of stereocilia bundles as measured by phalloidin staining (Δ) and the height HCA staining extends above the apical surface of the hair cell (\blacktriangledown) as a function of developmental age in days. The day 23 point is data from a 2 day old chick. **a:** Data come from a region in the papilla 1 mm from the distal extreme of the duct. **b:** Data come from a point calculated to be 75% from the proximal end of the basilar papilla. Error bars, standard deviation of the mean; *n*, numbers are given beside each data point. Values for phalloidin staining that are significantly different from the values for HCA staining in a Student's *t*-test with $P < 0.001$ are indicated by stars.

(1979) reported the presence of well-organised stereocilia bundles by stage 32 (ED7.5). Definitive hair bundles were not reported prior to this stage, but presumptive hair cells could be identified on the basis of synaptic contacts between ingrowing neurites and epithelial cells by stage 28 (ED6). As the HCA is first seen in the distal basilar papilla at the time when stereocilia bundles can be first detected by scanning electron microscopy, the staining observed in the vestibular system with the HCA at stage 26 (ED5) probably reflects the appearance of stereocilia bundles in this region. If this is the case then stereocilia bundles first appear in the anterior end of the otocyst as early as stage 26 (ED5), and in all sensory regions of the vestibular system excepting the lagena macula (which is located at the distal end of the cochlear duct) by stage 27 (ED5.5). The onset of hair cell differentiation, as judged by the reorganisation of gap junctions among the pseudostratified epithelial cells of the otocyst (Ginzberg and Gilula, '79), occurs at stage 25 (ED4.5–ED5). The HCA is therefore expressed almost as soon as the hair cells differentiate, and would appear to be a suitable marker for distinguishing immature hair cells from supporting cells with the light microscope at a very early stage of development.

Innervation of the otocyst and expression of the HCA

Previous studies of chick otocyst innervation using Golgi staining and electron microscopy have reported that neurite ingrowth occurs at stage 25 (ED4.5–ED5) in the vestibular regions (Ginzberg and Gilula, '80) and between stages 26 and 31 (ED5 and ED7) in the basilar papilla (Whitehead and Morest, '85a,b). These results are both confirmed and extended by the present findings. Ng-CAM-positive fibres are first observed in the anterior end of the otocyst and in the evaginating dorsomedial wall at stage 24 (ED4.5), in the elongating basilar papilla at stage 26 (ED5),

and throughout the entire length of the basilar papilla by stage 29 (ED6.5).

Several conclusions can be drawn from comparing the spatial and temporal patterns of innervation with those of HCA expression. Firstly, the HCA is always expressed after nerve fibres have invaded the epithelium, but the delay between the arrival of fibres and the appearance of the HCA is variable. In the vestibular system the delay may be as little as 12 hours, in the distal regions of the basilar papilla it is around 36 hours, and in the proximal regions at least 48 hours. Secondly, although the HCA is only expressed within regions of the epithelium in which nerve fibres are found, these areas of HCA expression are usually smaller than the regions occupied by the fibres. Thirdly, the HCA is never expressed outside of the regions that contain fibres, and the final match obtained between nerve fibre distribution and the sites of HCA expression is very precise.

The variable delays observed between the time of nerve fibre invasion and the onset of HCA expression, and the initial mismatch between the sizes of the regions within which HCA-positive cells occur and nerve fibres are found, make it unlikely that the nerve fibres alone control either the timing or the site of HCA expression. Although some experimental studies (Gil-Loyzaga and Pujol, '87; Gil-Loyzaga, '90) have suggested that hair cell differentiation may be under neural control, experiments with denervated avian otocysts developing ectopically *in vivo* (Swanson et al., '90), and aganglionic mammalian otocysts developing *in vitro* (Van de Water, '76), have demonstrated that nerve fibres are not required for hair cell differentiation. Whilst hair cell differentiation and HCA expression may or may not be under neural control, the final match between nerve fibre distribution and sites of HCA expression, and therefore presumably the distribution of hair cells, is precise. If the nerve fibres are neither necessary for hair cell differentiation nor dictate where the hair cells will differentiate, and, in addition, arrive in the right locations before the hair cells have differentiated, then fibre guidance must be controlled by either the undifferentiated hair cell precursors or some other cell type. A "go-between" in the epithelium expressing an Ng-CAM ligand and capable of inducing hair cell differentiation would be one possibility, although one would then have to account for the spatial distribution of this element.

Birth of hair cells and appearance of the HCA

A recent study (Katayama and Corwin, '89) of cell production in the chicken cochlea has shown that the first hair cells born in the basilar papilla leave the mitotic cycle between stages 26 and 28 (ED5–E6). The appearance of the HCA in the basilar papilla at stage 29 (ED6.5) therefore occurs very soon (12–36 hours) after the first hair cells withdraw from mitosis, providing further evidence that the HCA is a good marker for the onset of hair cell differentiation. Katayama and Corwin ('89) also examined the spatio-temporal patterns of hair cell production and concluded that the first hair cells are generated in a thin band running longitudinally along most of the length of the basilar papilla. Expansion of the basilar papilla occurs with the addition of hair cells to the lateral and distal edges of this longitudinal band, with hair cells being added to both the superior and inferior edges at the distal end of the basilar papilla and only along the inferior edge at the proximal end of the cochlea. No obvious longitudinal gradients of hair cell

production, similar to those reported in the mouse cochlea (Ruben, '67), were detected, although hair cell differentiation (based on the appearance of stereocilia bundles) occurs in a distal to proximal wave (Cotanche and Sulik, '84). Although the results of this present study show that the HCA first appears in the distal end of the basilar papilla, it can be detected throughout the length of the papilla by stage 31 (ED7). Examination of the data of Katayama and Corwin ('89) indicates that the number of unlabelled (and therefore postmitotic) hair cells observed in an embryo injected with tritiated thymidine at 120 hours of incubation (approximately stage 26, ED5) is slightly greater in the distal end than at the proximal end (17% vs. 2%). However, there was no such evidence for any longitudinal, distal to proximal gradient in embryos injected at 148 hours of incubation (approximately stage 29, ED6.5) and greater, and the results are dominated by a very prominent lateral gradient of hair cell production across the papilla. Evidently, although hair cell production and HCA expression may initially occur in the distal end of the papilla at very early developmental stages, any such longitudinal bias toward the distal end would appear to be rapidly lost.

Distribution of HCA during stereocilia bundle growth

The development and differentiation of stereocilia bundles in the chick basilar papilla has been recently studied in considerable detail (Tilney et al., '86, '88; Tilney and DeRosier, '86). Essentially, once the stereocilia have first sprouted from the apical surface of the cell, growth of the stereocilia bundle occurs in three temporally distinct phases. During the first phase, between ED10 and ED12, the staircase pattern, with the stereocilia ranked in rows of different height within each bundle, is generated by the sequential onset of stereocilia elongation in the different rows. The second phase occurs between ED12 and ED17. During this phase there is a small increment in the overall height of the bundles in the distal half of the papilla and very little at all for those at the proximal end, but during this phase the stereocilia become considerably thicker as actin filaments are added around the actin bundle within each stereocilium, and the stereocilia rootlets grow down into the cuticular plate. Between ED17 and hatching the third phase occurs when the bundles in the distal half of the papilla again grow in height to reach their final form, with the sequential cessation of stereocilia growth in the different rows providing further height ranking within the bundle. Because the actin filaments within a stereocilia bundle are all polarized in the same direction (Flock and Cheung, '77; Tilney et al., '80), with the preferred end for actin monomer addition being located at the tip of each stereocilium, it has been argued (Tilney and DeRosier, '86) that stereocilia growth during the first and third phases of development must occur via the addition of actin monomers to the top of the filament bundle at the tip of the stereocilium, although rootlet elongation is considered to occur via actin monomer addition at the basal, nonpreferred end during the second phase of bundle development (Tilney and DeRosier, '86). The results of this study demonstrate that the differential distribution of the HCA and F-actin previously observed with hair cells in the distal regions of the basilar papilla in early hatchling and adult chickens (Richardson et al., '90) is generated during the final growth phase of the stereocilia bundle. The HCA is a detergent-

soluble, membrane associated protein (Richardson et al., '90), and although the accumulation of HCA-free membrane at the distal ends of the stereocilia during the final phase of stereocilia bundle growth may suggest that new membrane is added towards the distal end of the stereocilium, it is equally possible that newly synthesized membrane components are added around the stereociliar bases and then migrate through the plane of the membrane to their final location. How then is the HCA restricted to the apical, nonstereociliar surface of the hair cell and the region around the base of the stereocilia bundle? A simple hypothesis, similar to that recently suggested for generation of the three different actin assemblies present within the hair cell (Drenckhahn et al., '91), would be to restrict HCA mobility and limit its synthesis to the earlier stages of bundle development prior to the final elongation of the stereocilia. Whilst this is an attractively simple hypothesis, the apical, nonstereociliar surface of the hair cell over which the HCA is distributed also expands during these later, final stages of development when the stereocilia are elongating, suggesting that HCA expression is still occurring and implying that the local distribution of various apical membrane components may be regulated by other, as yet unidentified, mechanisms.

In conclusion, the results of this study indicate that the HCA is expressed very early during the development of the inner ear, shortly after the hair cells have withdrawn from the mitotic cycle and soon after nerve fibres from the VIIIth ganglion have invaded the otocyst. The distribution of the HCA observed in the basilar papilla is generated during the final stages of development by the accumulation of HCA-free membrane at the distal tips of the stereocilia. The function of the HCA remains to be elucidated, but the correlation of its appearance with the first and earliest signs of stereocilia bundle differentiation raises the possibility that the HCA may play a role in the morphogenesis of the stereocilia bundle.

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