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Postnatal production of supporting cells in the chick cochlea

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The auditory receptor organ in birds, the basilar papilla, is mitotically active after acoustic overstimulation or pharmological insult and is capable of self-repair. The damaged epithelium is repopulated with new hair cells and supporting cells. The cell production that underlies this regenerative self-repair is believed to be a response evoked by damage in populations of cells that normally become mitotically quiescent even before hatching. In contrast, regeneration in the vertebrate nervous system is often correlated with continued or recent neurogenesis in the tissue concerned. The hypothesis that there may be ongoing postnatal production of cells in the normal avian basilar papilla was investigated. Autoradiographic analysis of tritiated-thymidine-injected animals was used to look for the existence of newly formed cells in the basilar papilla of normal posthatch chickens. Several types of supporting cells, namely, organ supporting cells, border cells and hyaline cells, are produced postnatally in the normal chicken. Typically, they are added interstitially to the apical (distal) half of the basilar papilla.

Bird; Auditory; Cochlea; Basilar papilla; Supporting cells; Hair-cell regeneration

Introduction

Hair cells, the sensory receptors of auditory, vestibular, and lateral-line end organs, transduce mechanical stimuli into electrical signals and are involved in the detection of sound, linear and angular accelerations, water motion and substrate vibration. Hair cells are produced throughout life in the ears and the lateral-line organs of amphibians and fish (Tester and Kendall, 1969; Corwin, 1981, 1983, 1985; Jørgensen, 1981; Popper and Hoxter, 1984; Barber et al., 1985; Popper and Hoxter, 1990; Presson and Popper, 1990) and in avian vestibular end organs (Jørgensen and Mathiesen, 1988; Roberson et al., 1992). In many of these animals, inner-ear epithelia grow mainly through the addition of cells to the outer edges of the receptor epithelium (Lewis and Li, 1973, 1975). In contrast, in the vestibular end organs of some fish and in the avian inner ear, new cells are added throughout the receptor epithelium (Popper and Hoxter, 1990; Roberson et al., 1992).

Inner-ear epithelia that normally produce new hair cells and make new neural connections are suspected to be able to replace lost or damaged hair cells. Regeneration of this kind occurs in the lateral-line system of salamanders, where new hair-cell epithelia are produced when preexisting epithelia have been amputated or damaged (Stone 1933, 1937; Balak et al., 1990) and in avian and fish vestibular epithelia where new hair cells are produced after ototoxic antibiotic treatment (Yan et al., 1992; Weisleder and Rubel, 1992). Thus, in many systems, the cell production that underlies regenerative self-repair may be a postembryonic extension of hair-cell and supporting-cell production that occurs in embryos.

The idea of a link between regeneration and continuous growth or continued or recent neurogenesis, in general, is not a new one (for example, see Easter et al., 1981; Lyon and Stelzner, 1987; Birse et al., 1980). As reviewed by Holder and Clarke (1988), a correlation frequently exists between continuous neurogenesis and axon and neurosensory-cell regeneration in the vertebrate nervous system.

In contrast, hair-cell production in mammals, and in the auditory receptor epithelium in the bird, the basilar papilla, is thought to cease before birth. Counts of standing populations of hair cells in different aged cochleas suggest that hair-cell production ceases early in development (by embryonic day 10 in birds: Tilney et al., 1986). Investigations using radioactive tracing of DNA replication to investigate the timing of hair-cell production in the embryonic cochlea (Ruben, 1967; Katayama and Corwin, 1989) support this contention.

Regeneration occurs in avian basilar papillas that have lost hair cells after acoustic overstimulation (Cotanche, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988) or as a result of ototoxic antibiotic treatment (Cruz et al., 1987; Girod et al., 1991; Lippe et al., 1991). Thus, hair-cell regeneration does not

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appear to depend on epithelia with ongoing hair-cell production. Autoradiographic tracing of DNA replication has shown that the basis for this repair is the production of new hair cells and supporting cells at the sites of hair-cell loss (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Girod et al., 1989). The cell production that underlies this regenerative self-repair is believed to be a response evoked by damage in populations of cells that normally become mitotically quiescent even before hatching. In the avian cochlea the initiation of the regenerative repair is thought to depend on a preceding loss or injury of cells.

Evidence confirming that hair cells and supporting cells of the avian cochlea do not normally mitose during postembryonic life comes from the failure to observe tritiated-thymidine labeled cells in control ears and in the undamaged portions of overstimulated ears (Corwin and Cotanche, 1988; Jørgensen and Mathiesen, 1988; Ryals and Rubel, 1988; Girod et al., 1989). Ryals and Westbrook (1990), however, recently noted the production of a few new supporting cells and juxtaposed hair cells in several control animals. They suggest that there may be a very low level of hair-cell turnover and production in the normal adult quail ear. The purpose of this study is to determine whether ongoing production of basilar papilla cells occurs postnatally in the young domestic chicken. Tritiated thymidine and autoradiographic techniques were utilized to identify proliferating cells and their progeny.

Methods

Subjects

Fertilized chicken eggs of the White Leghorn variety were obtained from a local supplier and incubated at 37°C in a humid environment with turning. Hatching occurred at day 21. Hatchlings were raised in a quiet environment in our animal care facility prior to the beginning of tritiated-thymidine injections. No excessive noise or ototoxic exposures were known to have occurred. Nine normal posthatch chicks ranging from 9 to 17 days in age were used.

A cell proliferation marker, tritiated [^{3}H]-thymidine, was administered to the chicks through two routes: by means of a series of intramuscular injections or by a series of intramuscular injections combined with a subcutaneously placed osmotic pump. Specifically, eight of the nine experimental animals were divided into four groups of two animals based on the length of a series of tritiated-thymidine injections and the subsequent survival times: a 1-h group, a 21-h group, a 6-day group and a 30-day group. In each of these animals, tritiated thymidine (60–90 Ci/mol; ICN; IM) was injected at a dose of 10 μCi/gram body weight in a paradigm similar to that described previously by Girod et al. (1989). Subjects in the 1-h group received two tritiated-thymidine injections, 30 min apart, and were sacrificed 20–30 min after the last injection. Animals in the 21-h group were injected five times with tritiated thymidine during an 18-h period and were sacrificed 3 h after the last injection. Subjects in the 6-day group were injected 2 times per day for 3 days and underwent termination of the labeling period by injection of non-radioactive thymidine at 100 times the radioactive thymidine dose, twice a day for a total of 3 days. The animals were then sacrificed. The 30-day group animals were injected 2 times per day for 3 days and underwent termination of the labeling period by injection of non-radioactive thymidine at 100 times the radioactive thymidine dose, once or twice a day for a total of 6 days. The animals were then allowed to survive 21 days.

In the ninth chick, the 4-day animal, we attempted to insure that tritiated thymidine was continuously available. A seven-day osmotic pump (Model 2ML1; Alzet, Palo Alto CA) filled with 2 mls of tritiated thymidine (79 Ci/mmol; ICN) was implanted subcutaneously on the chicken's back. The pump released tritiated thymidine continuously at a rate of 10 μl per hour. After the implantation of the pump, tritiated thymidine (60–90 Ci/mol; ICN) was also injected into the thigh muscles of the chick 2 to 4 times a day at a dose of 2.5 μCi/gram body weight over a four-day period. The animal was sacrificed one hour after the last tritiated-thymidine injection.

Histology

At the end of the survival period, all animals were given an overdose of pentobarbital sodium, a small opening was made in the round window of each ear, and the ear was fixed by intralabyrinthine perfusion with 3.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) or a modified Karnovsky's Fixative (a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Cacodylate buffer (pH 7.4) with 0.001% $CaCl_2$). Perfused inner ears were held in fixative at 4°C for 4–24 h. The heads were washed in buffer (0.1 M Na/K phosphate buffer or 0.1 M cacodylate), and the cochlear ducts were dissected free from the temporal bone. Inner ears were post-fixed in 1% osmium tetroxide (in the appropriate buffer; pH 7.4) for one or two hours at room temperature. The ears were washed with buffer, dehydrated in a graded alcohol series, and embedded in Polybed 812 or Spurr epoxy resins.

One cochlea from each animal was cut transversely on a Sorvall MT2 microtome into 2 or 3 μm serial sections and processed for autoradiography (AR). For six of the animals, the 21-h group, the 6-day group and the 30-day group, three-to-nine serial sections were collected at 100 μm intervals through most of the basilar papilla, but at 50 μm intervals through the

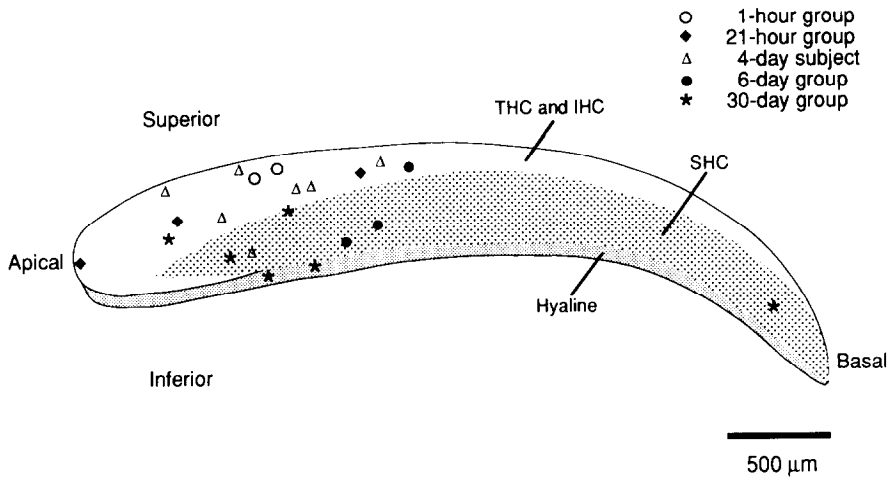

Fig. 1. Schematic of the basilar papilla of the young chicken illustrating the location of the tritiated-thymidine labeled cells. Pairs and triplets of labeled cells are represented by only one symbol. The criteria for a labeled cell was 5 or more silver grains overlying the cell nucleus. THC, tall hair cells; IHC, intermediate hair cells; SHC, short hair cells.

1.0–2.0 kHz region *. For two subjects, the 1-h group of animals, serial sections (3 μm) were collected from the apical half of the papilla ** and mounted. For the ninth animal, the 4-day animal, the entire basilar papilla was serially sectioned (2 μm), and all sections were collected and mounted. Sections were mounted on acid-washed, chrome-alum subbed slides and processed for autoradiography. The slides were coated with liquid photographic emulsion (Kodak NTB-2) diluted 1:1 with distilled water and stored at 4°C in light-tight boxes for 4–30 weeks. Slides were developed in cold Kodak D19 developer, rinsed in cold distilled water, and fixed in cold Kodak Rapid Fixer. After water washes, the slides were dried and stained lightly with 0.01% Toluidine blue. The slides were coverslipped, and the sections were examined under brightfield illumination for the presence of labeled cells. The criteria for a labeled cell was 5 or more silver grains overlying the cell nucleus. Customarily, silver grains are not present in unlabeled cells. The distinction between labeled cells and unlabeled cells was unambiguous.

* These animals also served as control animals for another experiment. In this other study, the experimental animals were exposed to an intense pure tone at 1.5 kHz. The region of the sound damage, 1.0 to 2.0 kHz, was studied more thoroughly than the adjoining nondamaged regions, and sections were collected at 50 μm intervals throughout this region. Sections were collected at 50 μm intervals throughout the 1.0 to 2.0 kHz region in the control animals as well.

** The 1-h group of animals were the last animals studied. They were prepared and sectioned after the other groups of animals had been examined, and after the general pattern of labeled cells had been determined. In view of the brevity of the thymidine pulse and the general absence of label in the basal half of the papilla in the other experimental animals, an absence of labeled cells was anticipated in the basal regions of the 1-h group animals. Consequently, the basal halves of these papillas were not sectioned. While unlikely, it is possible that some additional labeled cells may have been missed.

Results

Overview

As illustrated in Fig. 1, the auditory receptor epithelium, the basilar papilla, of the postnatal chicken is sickle shaped. It tapers to a point in the basal or proximal end and widens at the apical (distal) end. A schematic of a cross section of the basilar papilla is shown in Fig. 2. The superior edge of the basilar papilla sits above the superior fibrocartilagenous plate, and nerve fibers course to sensory epithelium from this edge. The basilar papilla is composed of hair cells, supporting cells, and unmyelinated terminal portions of cochlear nerve fibers. Several types of hair cells, namely tall, short and intermediate hair cells (Takasaka and Smith, 1971), and at least two types of supporting cells, organ supporting cells and border cells (Oesterle et al., 1992), have been identified in the basilar papilla. Hair-cell nuclei are located near the lumenal surface, above the nuclei of the organ supporting cells. Border cells are located at the inferior (abneural) edge of the basilar papilla and separate the sensory epithelium from the hyaline cells. Hair cells are easily differentiated from supporting cells at the light-microscopic level by their dark staining with toluidine blue, their location in the organ, and the presence of a cuticular plate and stereocilia at their lumenal surfaces.

^{3}H-thymidine labeling

Nine cochleas from the 9 subjects (one cochlea from each subject) were carefully examined for the presence of cells labeled by tritiated thymidine. Altogether this

amounted to 4550 sections *. Attention was focused on the basilar papilla and the hyaline-cell region **, on the presence and extent of the label, on the type of cell labeled, and on the location of the labeled cell. Within this restricted region, a total of twenty-six nonsensory cells are labeled by tritiated thymidine in the 9 ears. Their distribution is shown in Fig. 1. The nuclei of 21 organ supporting cells, 2 border cells and 3 hyaline cells were labeled, indicating the replication of DNA in these cells during the exposure to tritiated thymidine. Labeled cells were seen in 7 of the 9 cochleas; labeled cells were not detected in one 21-h animal and one 6-day animal.

Examples of labeled organ supporting cells in the superior and inferior regions of the basilar papilla are shown in Figs. 3a and 3b, respectively. In both instances, the nuclei of the labeled cell lies under the basal region of an intermediate hair cell, in very close proximity to the hair cell. A single organ supporting cell is labeled in each example, and label is undetectable in the nearby hair cells or supporting cells. Interestingly, in the animals with the shortest survival times, the 1-h group of animals (e.g., Fig. 4), the nuclei of all the labeled supporting cells ($N = 2$) lie immediately above the habenula in the superior region of the papilla. In view of the brevity of the thymidine pulse and the immediate fixation, it is not unreasonable to speculate that these cells are situated at the site where they replicated their DNA. In the 21-h group of animals the nuclei of some of the labeled supporting cells (2 of the three labeled supporting cells) lie near the lumenal surface of the epithelium, just under the hair cells (e.g., Fig. 3b). Label is undetectable in the nearby cells.

Labeled hyaline cells and border cells are pictured in Fig. 5. Two heavily labeled hyaline cells lying close to the inferior edge of the basilar papilla are shown in Fig. 5a. Nearby, short hair cells are unlabeled. Single labeled hyaline cells or two juxtaposed labeled hyaline cells have been observed. Labeled border cells are discernible in Fig. 5b. In this section one border cell is lightly labeled in contrast to a heavily labeled neighboring hyaline cell. Again, label is undetectable in nearby hair cells.

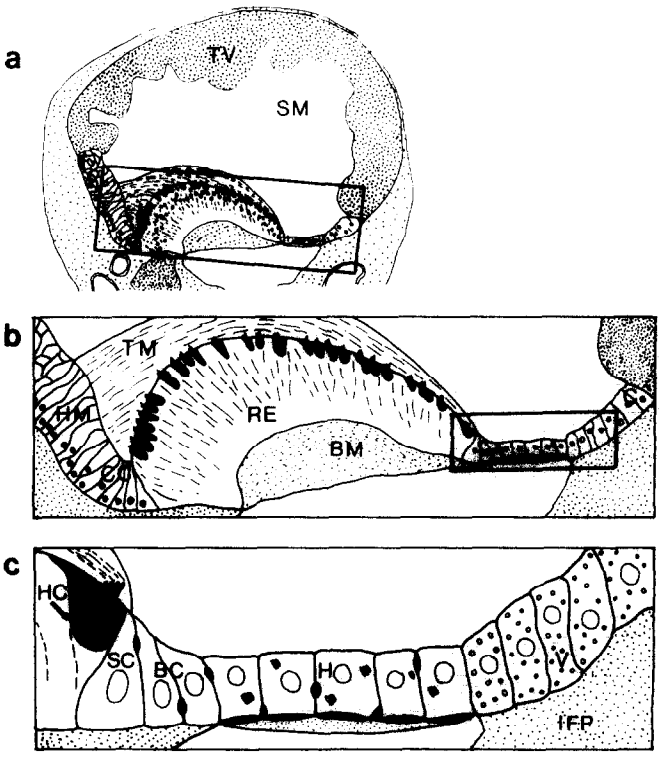

Fig. 2. Schematic reconstructions of chicken inner-ear tissue. (a) Schematic reconstruction of a cross section of the cochlear duct from the high-frequency region of the chicken cochlea. SM, scala media; TV, tegmentum vasculosum: (b) Schematic of the sensory epithelium and adjacent tissue. This is an enlargement of the boxed region in part (a). HM, homogene cell; CC, clear cell; TM, tectorial membrane; RE, receptor epithelium (basilar papilla); BM basilar membrane; C, cuboidal cells: (c) Schematic of the inferior region of the chicken basilar papilla and the neighboring supporting cells. This is an enlargement of the boxed region in part (b). HC, short hair cell; SC, organ supporting cell; BC, border cell; H, hyaline cells; V, vacuole cell; IFP, inferior fibrocartilaginous plate. (From Oesterle et al., 1992)

Labeled organ supporting cells are located in the inferior ($N = 7$), middle ($N = 4$) and superior ($N = 10$) parts of the organ. Heavily labeled (50 grains or greater), as well as lightly labeled (5–10 grains), supporting cells are seen (e.g., Fig. 5b). In contrast, heavily labeled basilar-papilla hair cells were never encountered. However, on two occasions 5 to 7 grains were observed over the nucleus of a hair cell (Fig. 6). As illustrated in Fig. 1, all but one of the labeled cells are scattered throughout the distal (apical) half of the papilla. These labeled cells appeared in all regions across the organ from superior to inferior (Fig. 1). A single labeled organ supporting cell was observed in the basal half of the papilla.

Labeled cells were observed in all groups of animals; labeled organ supporting cells were present in the 1-h group, the 21-h group, the four-day animal, the 6-day group and the 30-day group of animals. Labeled

* Of the 4550 sections examined, 1344 sections were from the 1-h animals, 954 were from the 21-h animals, 1226 were from the 4-day animal, 216 were from the 6-day animals, and 810 were from the 30-day animals.

** Findings regarding the cells in the basilar papilla proper, namely, the hair cells, organ supporting cells, and border cells, and the adjacent hyaline-cell region, are reported herein. Label is also seen in a variety of cell types outside the defined region (e.g., red blood cells, tympanic border cells, fibroblasts in the basilar membrane, cells in the spiral ganglion area, cells lining scala tympani, cells in inferior and superior fibrocartilaginous plates, capillary endothelial cells), but information regarding these cells will not be presented in this paper.

border cells and hyaline cells were also present in the 30-day subjects (Table I). Interestingly, in the 1-h and 21-h groups of animals all labeled cells occurred singly. That is, a single supporting cell was labeled, and label was undetectable in the neighboring cells (e.g., Fig. 3b). In the 4- and 6-day groups of animals, the majority

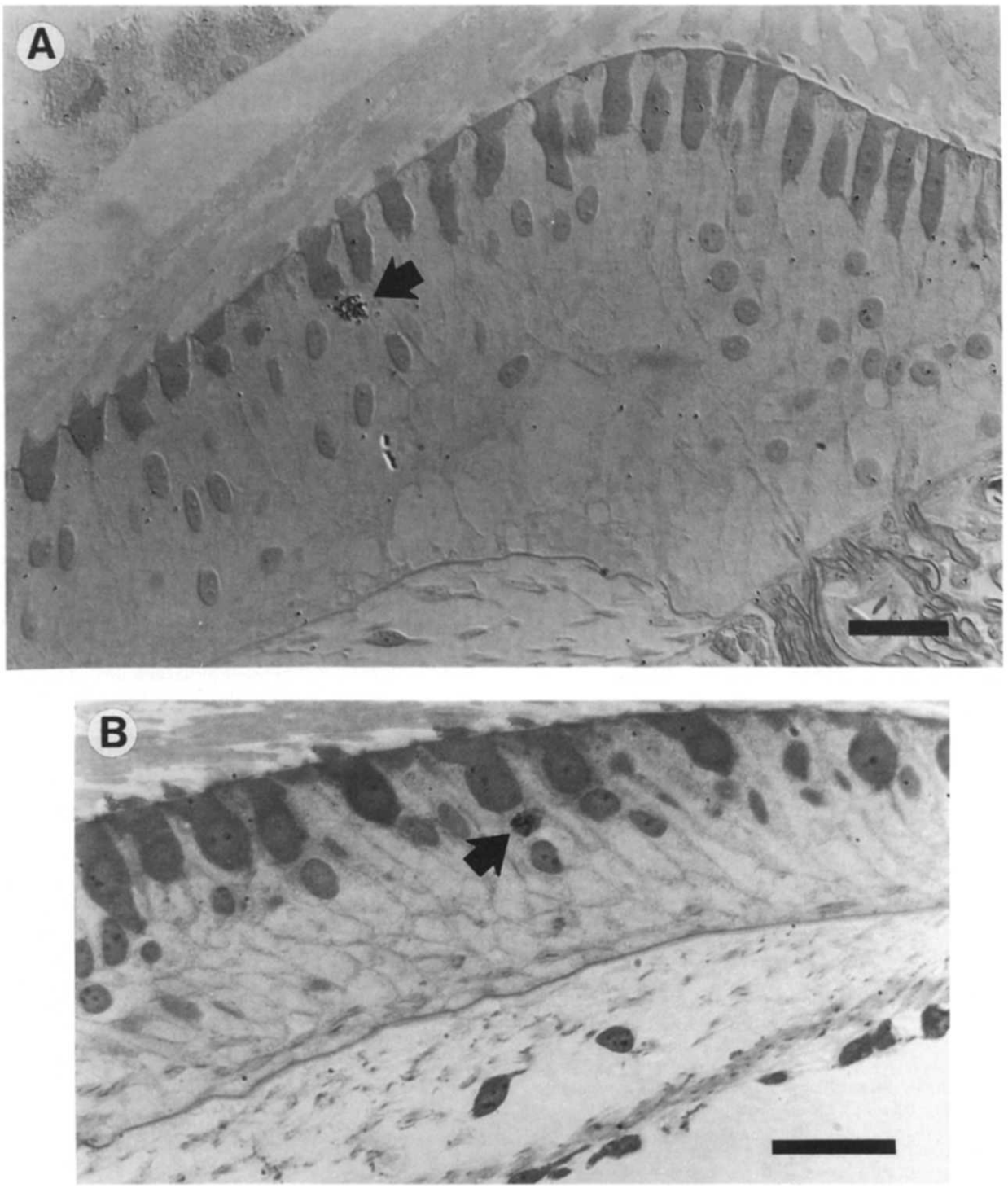

Fig. 3. Light micrographs of cross sections of the basilar papilla from normal chickens exposed to a cell proliferation marker, tritiated thymidine. Part (a) is viewed with Nomarski differential-interference contrast optics. Plane of focus is on the cells putting the overlying silver grains partially out of focus. (a) Exposed silver grains over the nucleus of an organ supporting cell (arrow) indicate the incorporation of ^{3}H thymidine into the DNA of this nucleus during the 'S' phase of the cell cycle. The labeled cell indicates the replication of DNA during the period of exposure to tritiated thymidine. The section is from a 4-day experimental group animal, approximately 1140 μm from the apex to the base. Scale bar = 20 μm. (b) Labeled nucleus of an organ supporting cell (arrow) in the basilar papilla from a 21-h experimental group animal. The section is approximately 515 μm from the apex to the base. Scale bar = 20 μm.

of labeled cells occurred singly (8 of 12 labeled supporting cells), but two pairs of labeled organ supporting cells were also present. In each instance, the nuclei of the labeled supporting cells were directly adjacent, and they are located at an equivalent height from the lumenal surface of the organ. In animals with a longer maturation period, the 30-day group of animals, the majority of labeled cells (5 of 9 labeled cells) were located in pairs or triplets (e.g., Fig. 5).

In one animal, the 4-day animal, tritiated thymidine was continuously available for a four day period. Presumably, all cells born during this time were labeled. This animal provides insights into the incidence and pattern of cell labeling. In total, 8 nonsensory cells, all organ supporting cells, were labeled in this animal, giving a specific labeling rate * of 0.05%, or 1 supporting cell in 1996. Supporting cells are produced at a very low rate at this stage in the chicken's life.

All eight organ supporting cells lay in the apical third of the papilla, at distances 450 to 1500 μm from the apex to the base of the papilla As illustrated in Fig. 1, they are located in the superior, middle, and inferior regions of the papilla. Lightly labeled (6–10 grains) and heavily labeled supporting cells (50 grains or more; e.g., Fig. 3a) are present. One supporting cell/supporting-cell pair was observed, all other cells were labeled singly.

Interestingly, in this animal, two very lightly labeled hair cells have 5 to 7 silver grains over their nuclei (e.g., Fig. 6). Three additional hair cells have 4 grains over their nuclei and thus fall below our 5 grain minimum. In each instance, label was undetectable in the neighboring cells. The lightly labeled hair cells were located at a distance approximately 1900 μm from the apex to the base. They lay in the inferior and middle part of the papilla. A well labeled hair cell was not detected.

Discussion

The primary objective of these experiments was to determine if there is postnatal production of cells in the auditory receptor organ of normal posthatch chickens. The principal finding is that some interstitial addition of new supporting cells to the chicken basilar papilla occurs during postembryonic life. A small number of new supporting cells are produced for at least several weeks postnatally.

To date, the majority of studies of DNA incorporation in the auditory receptor organ of the normal postnatal avian inner ear (Corwin and Cotanche, 1988; Jørgensen and Mathiesen, 1988; Ryals and Rubel, 1988; Girod et al., 1989) report an absence of label in hair cells and supporting cells, suggesting that cells of the avian basilar papilla do not normally divide during postembryonic life. Reports of an absence of label in nondamaged regions of sound-damaged animals (Corwin and Cotanche, 1988; Girod et al., 1989) support this contention. In contrast, a few newly formed supporting cells and juxtaposed hair cells were seen in normal young adult (3-month old) quail, leading Ryals and Westbrook (1990) to suggest that there may be a very low level of hair-cell production in the normal adult quail ear which is activated in the absence of massive trauma. Our results indicate that new cells (organ supporting cells, border cells and hyaline cells) are born in the inner ear of young, normal postnatal chickens. We have also detected the presence of tritiated-thymidine labeled supporting cells in nondamaged regions of sound-damaged chickens which range from 9 to 16 days in age during their exposure to ^{3}H thymidine (Oesterle, unpublished observations).

* To determine the specific labeling rate, the total number of supporting-cell nuclei present in the sections examined was estimated in the following manner. An unbiased estimate for the total number of supporting cells in our defined region of the cochlea (the basilar papilla and hyaline-cell region) was obtained using the disector principle (Sterio, 1984; Pakkenberg and Gundersen, 1988). A pair of sections was systematically selected every 100 sections through the papilla, using a random start in the first interval. Each pair served as a physical disector. The number of oil immersion fields that covered the length of the organ was noted for each disector, and a random number generator chose the field to be counted. The numerical density, NV, of supporting cells was obtained by counting nuclear profiles within an 80 μm by 80 μm counting frame, using a 100× oil immersion lens, NA 1.25. The NV was estimated by the equation, $\text{nuclei}/\mu^3 = \Sigma Q_i^- / t \cdot \Sigma a(\text{fra})$, where ΣQ_i^- = the total nuclear profiles observed in the reference section but not present in the look-up section, t = section thickness (and height of the physical disector), $\Sigma a(\text{fra})$ = cumulative area of the counting frames from each disector. The total volume, V(ref), of the region (basilar papilla and hyaline-cell region) was estimated by the method of Cavalieri (Cavalieri, 1966). The cochlea was observed under a 25× lens. A test grid of 20 μm by 20 μm squares was randomly placed over images selected from each disector pair. The number of points (P_i) corresponding to the upper left corner of each grid square lying over the defined region (cells above the basilar membrane between the most superior hair cell and the most inferior hyaline cell, i.e., the basilar papilla and the hyaline-cell region) was recorded. The equation $V(\text{ref}) = t \cdot k \cdot a(p) \cdot \Sigma P_i$, where t = section thickness, k = the number of sections in the interval between disectors, a(p) = the area associated with each point, ΣPi = the total number test grid points falling within the defined region. The total number of supporting cells is the product of NV·V(ref). Images for quantitative purposes were recorded by a Dage 68 video camera attached to a Zeiss Universal photomicroscope and displayed with a MacIntosh IIcx computer. NIH Image (NIH, Bethesda, MD) was used to create the counting frames and test grids directly on the video image of the tissue. Section thickness was verified with a length gauge (Heidenhain) attached to a Zeiss Universal microscope. Supporting-cell nuclei had been determined to be 4–7 μm in diameter, within the range for a 2 μm disector. The total number of supporting-cell nuclei present in all the sections collected was calculated, and the specific labeling rate was computed according to the following formula: Specific labeling rate = (number of labeled supporting-cell nuclei / total number of supporting-cell nuclei) × 100.

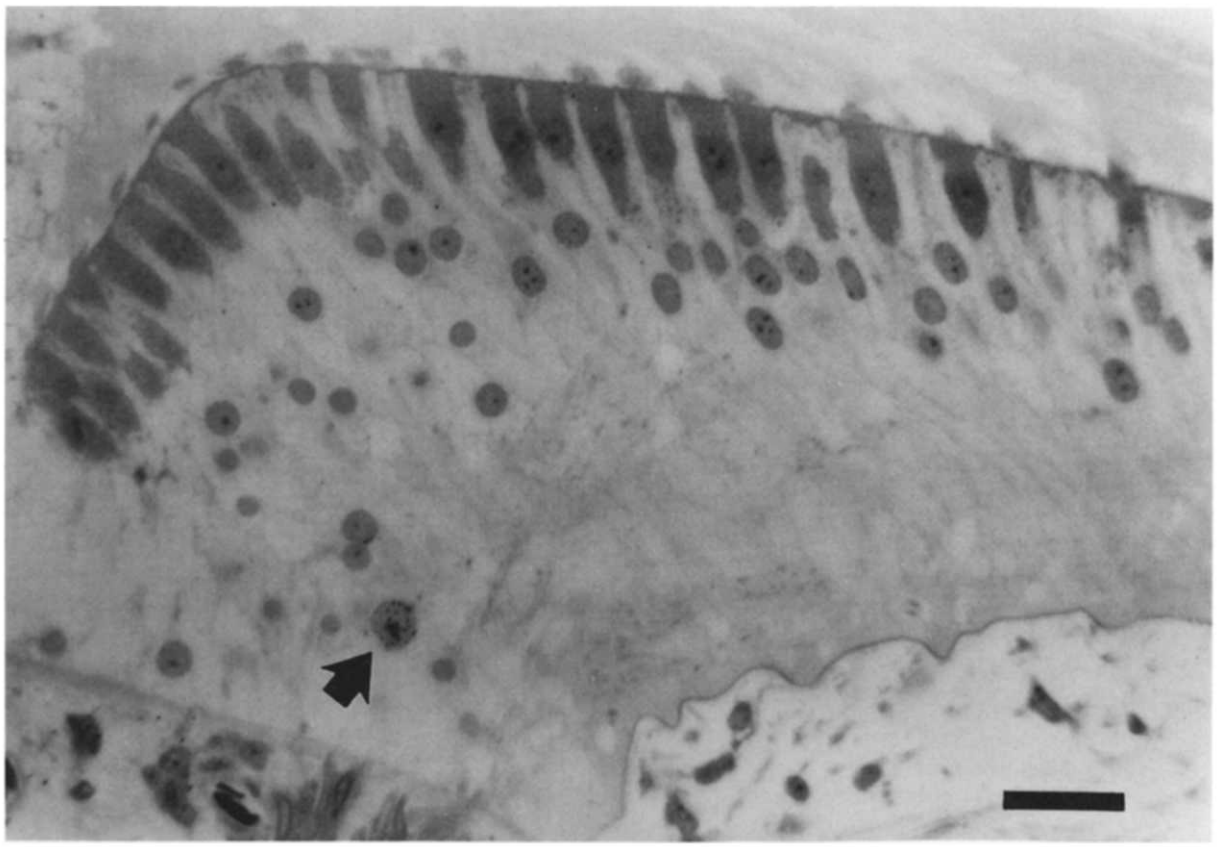

Fig. 4. Light micrograph of a cross section of the superior region of the basilar papilla in a normal juvenile chicken (13 days old). A labeled organ supporting cell (arrow) lies immediately above the habenula in this 1-h experimental animal. The section is located approximately 804 μm from the apex of the papilla. Scale bar = 20 μm.

The discrepancies between our findings and those of Corwin and Cotanche (1988) and Girod et al. (1989) in similarly aged chicks * may be due to the fact that the level of ongoing production is extremely low, and the mitotically active cells were missed. In the present study, all collected sections were examined for the presence of labeled cells within the defined region – the basilar papilla and hyaline-cell region. Labeled cells are very rare. For example, in one animal receiving the ^{3}H thymidine continuously for 4 days, only 8 labeled nonsensory cells were observed in the 1226 sections examined, and a specific labeling rate of 0.05%, or 1 supporting cell in 1996 supporting cells was calculated. Clearly, supporting cells are produced at a very low rate at this stage in the chicken's life.

Jørgensen and Mathiesen (1988) reported the absence of label in the basilar papilla of normal adult budgerigars. Again, it is conceivable that in the adult budgerigar, like the young chicken, the numbers of mitotically active cells are extremely low and, consequently, were missed. Alternatively, the postnatal production could cease with increased postnatal age. The report of labeled supporting cells and hair cells in adult quail (Ryals and Westbrook, 1990) argues against the latter possibility. Ryals and Westbrook (1990) noted the presence of several labeled supporting cells and hair cells in one section (approximately 100 sections were sampled throughout the papilla in each bird) in each of their two control birds, two normal 3-month old quail. A third alternative is that the difference between our findings and those of Jørgensen and Mathiesen (1988) are due to true species differences.

The presence of label in the nucleus of a cell indicates the replication of that cell's DNA during the period of exposure to tritiated thymidine Tritiated thymidine is incorporated into nuclear DNA during the 'S' phase of the cell cycle. The S phase of the cell cycle lasts roughly 7 h in the chicken (McFarlane and Callan, 1973; Grosset and Odartchenko, 1975), and cell division occurs during the M phase of the cell cycle, a number of hours after the completion of the S phase. Thus, a labeled cell can exist for a number of hours before it divides. In view of the time required for a labeled cell to progress through the cell cycle and divide, one would not expect to see divided cells (pairs of cells) in the 1-h or possibly the 21-h groups of

* Their subjects were 9 to 23 days old during the ^{3}H-thymidine injection period.

animals. In fact, all labeled cells occurred singly in the 1-h and 21-h groups of animals (Table I). Pairs and triplets of labeled cells were observed in the animals with longer incubation and recovery times, the 4-day group, the 6-day group, and the 30-day group of animals. In these animals the presence of pairs of labeled cells indicates that new cells have been born in the sensory epithelium; that some of the tritiated-thymidine labeled cells completed the cell cycle, divided, and produced daughter cells.

The percentage of the labeled cells that occurred in pairs or triplets increased with longer incubation and recovery times. For example, 25% of the labeled cells in the 4-d group of animals occured in pairs. Fifty and fifty-six percent of the labeled cells in the 6-day group and the 30-day group of animals, respectively, occurred in pairs or triplets. Single labeled cells were also present in these animals. The presence of single cells in animals with longer incubation and recovery times may have resulted from (i) a paired cell being located in a

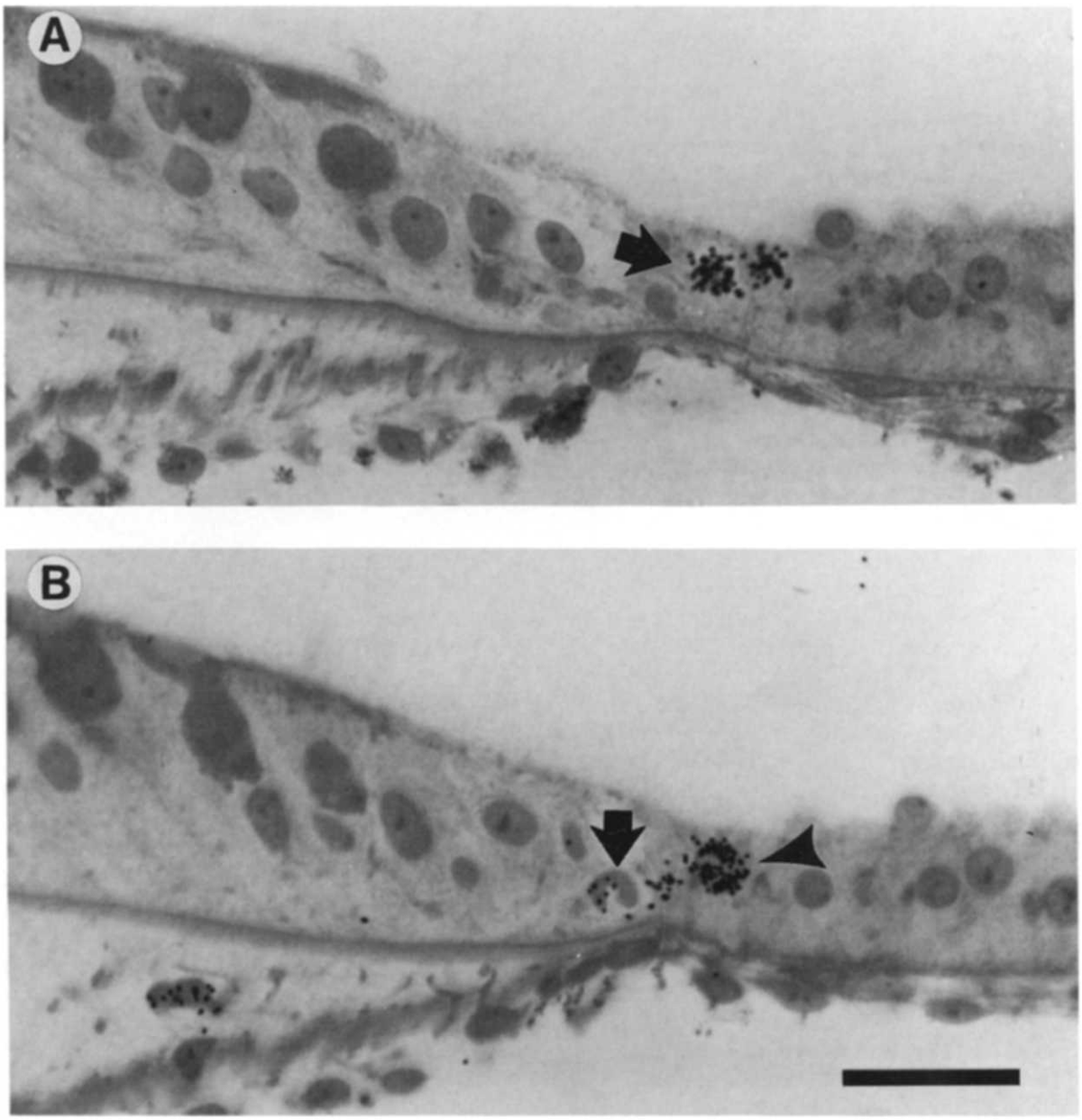

Fig. 5. Light micrographs of cross sections of the inferior region of the basilar papilla and the adjacent nonsensory cells in normal juvenile chickens (a) Well-labeled hyaline cells (arrow) in a 30-day experimental animal approximately 1215 μm from the apex to the base. Plane of focus is on the cells putting the overlying silver grains partially out of focus. (b) Labeled hyaline cells (arrowhead) next to a neighboring labeled border cell (arrow) in a 30-day experimental animal roughly 915 μm from the apex to the base. The damage apparent in these sections was done during the dissection, after the fixation. Magnification is the same for both parts of the figure. Scale bar = 20 μm.

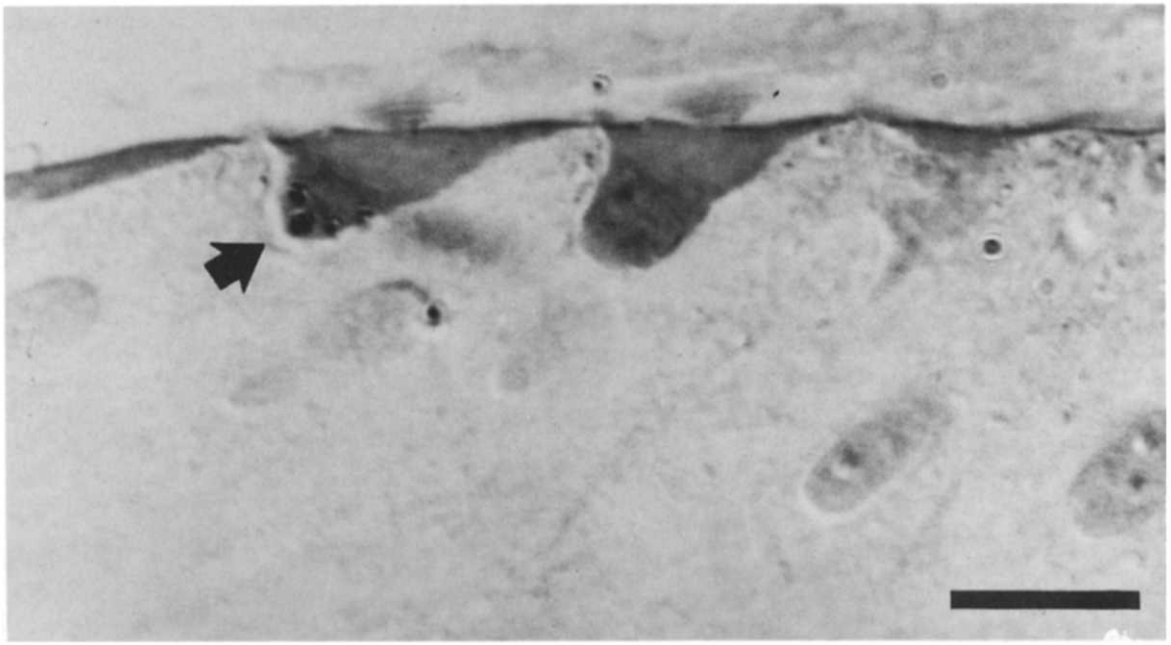

Fig. 6. Lightly labeled short hair cell (arrow) in the 4-day animal roughly 1900 μm from the apex to the base which is viewed with Nomarski differential-interference contrast optics. The hair cell is significantly labeled above background with 5 grains clustered over the nucleus, two of the five grains are clumped together. Unfortunately, the adjacent plastic sections were folded, and consequently, it could not be determined whether the grains were incorporated over the nucleus of the hair cell in several adjacent sections. Label is undetectable in nearby cells. Scale bar = 10 μm.

nearby section which was not saved; (ii) the daughter cell's migrating a distance away from the mother cell; (iii) the death of some of the newly formed cells, or (iv) some cells not progressing to the completion of the cell cycle. In the 4-day animal, the animal with an implanted osmotic pump, tritiated thymidine was still available at the time of sacrifice. Some of the single labeled cells in this animal may have replicated their DNA shortly before the animal was sacrificed.

What is the purpose of the persistent generation of supporting cells in the postnatal chicken basilar papilla?

The mitotic activity observed in the present study may simply be a reflection of new cells being added to a still growing receptor organ. In chickens the length and width of the basilar papilla continues to increase postnatally (Cotanche and Sulik, 1985; Tilney et al., 1986). A 16% increase in cochlear-duct length (containing the basilar papilla and lagena: Ryals et al., 1984) and a 19% increase in basilar-papilla length (Tilney et al., 1986) occurs from hatching to postnatal day 35 and 38, respectively. Significant increases in total duct length are not seen beyond postnatal day 35, but increases in basilar papilla length continue at least to postnatal day 50 (Ryals and Rubel, 1984). Papilla width increases roughly 10% * from hatching until postnatal day 38 (Tilney et al., 1986). The postnatal growth has been attributed to an expansion of the surface areas of a proliferatively static population of hair cells (Cotanche and Sulik, 1985; Tilney et al., 1986) or to changes in overall dimensions of the sensory cells and/or increases in the size or number of supporting cells along the basilar papilla (Ryals et al., 1984).

Results of this study establish definitively that a small number of new supporting cells are born in the postnatal chicken basilar papilla. The observed intussusceptive addition of new supporting cells throughout the apical half of the existing sensory epithelium may play a role in the postembryonic growth of the basilar papilla.

An alternative explanation for the continued supporting-cell production is that there may be a low rate of ongoing supporting-cell proliferation coupled with a low basal turnover rate. The population of supporting cells in the postnatal basilar papilla may be slowly renewing itself. The idea of a supporting cell renewal in inner-ear end organs is not without precedence. Supporting cells in the saccular epithelium of toads are thought to have an average lifespan of roughly 30 days (Corwin, 1986).

Meanwhile, the findings reported herein regarding the prolonged production of supporting cells in the basilar papilla of juvenile chickens may be of importance for interpreting hair-cell regeneration findings. The self-repair of the sensory epithelia after damage could be the result of the following mechanisms: (i) an

* This value was extrapolated from graphs in Fig. 3 of Tilney et al. (1986) and, consequently, is a rough estimate of the actual increase in width.

TABLE I

Number of labeled cells in the experimental animals

experimental group	organ supporting cells (SC)	border cells (BC)	hyaline cells (H)	hair cells (HC)	labeled pairs and triplets [1]
1 hr (n = 2)	2	–	–	–	–
21 hr (n = 2)	3	–	–	–	–
4 day (n = 1)	8	–	–	2	1 (SC/SC)
6 day (n = 2)	4	–	–	–	1 (SC/SC)
30 day (n = 2)	4	2	3	–	2 (BC/BC/H; H/H)

[1] The types of supporting cells comprising the labeled pairs and triplets are indicated in the parenthesis with the following abbreviations: SC, organ supporting cell; BC, border cell; H, hyaline cell; HC, hair cell.

up-regulation of the normally low levels of mitosis. That is, the rate of mitosis is rapidly increased after the insult to the organ, many new cells are rapidly produced, and it is these new cells or their progeny which serve to repopulate the damaged sensory epithelium; (ii) mitotically quiescent cells in the epithelia are stimulated to reenter the cell cycle; or (iii) a combination of (i) and (ii). The small numbers of mitotically active cells seen in this study and the general absence of mitotically active cells in the basal region of the papilla, a region known to regenerate rapidly after ototoxic drug insult, suggest that mechanism (i) is unlikely.

Is there a low level of hair-cell turnover in the avian basilar papilla, or are hair cells produced normally at a very low rate?

Ryals and Westbrook (1990) recently reported the presence of several well labeled hair cells in two of their four control animals (3-month old quail). They suggested that there may be some very low level of hair-cell turnover and production in the normal adult quail ear which is activated in the absence of massive trauma. In view of a decrease (6%) in the number of hair cells present in older quail (3–6 years) relative to that in younger (three month) quail, Ryals and Westbrook (1988, 1990) suggest that the labeled hair cells in their normal (non-traumatized) quail are probably replaced cells rather than cells added to the end organ. Interestingly, two lightly labeled hair cells (5–7 silver grains) were detected in the present study (e.g., Fig. 6). However, a well-labeled hair cell was never observed. The absence of a well-labeled hair cell and the unfortunate loss of information from adjacent sections (due to section folding) in each instance makes these data difficult to interpret. The possibility that the label was due to background can not be ruled out entirely when the label is so light.

These data, however, still raise the possibility that there may be a very low rate of new hair-cell production normally in the postnatal avian basilar papilla. In the inner ear of the shark, where more than 80% of the 200 000 hair cells in the adult macula neglecta are produced postembryonically, a very low incidence of hair-cell labeling was observed by Corwin (1981). Specifically, Corwin reports that one hair cell in 1250 hair cells (a specific labeling rate of 0.08%) and one supporting cell in 77 supporting cells (a specific labeling rate of 1.3%) are labeled in the shark *. Hair-cell labeling in the normal shark inner ear was extremely infrequent and significantly decreased relative to supporting-cell labeling. Extrapolating this to the chicken inner ear, an extremely low incidence of hair-cell labeling would be predicted in the chicken basilar papilla since the incidence of labeled supporting cells is in itself already quite low, and the postembryonic growth in the chicken inner ear is substantially decreased relative to that in the shark. To illustrate, a supporting-cell specific labeling rate of 0.05% was obtained in the present study, a rate substantially decreased relative to the 1.3% incidence of supporting-cell label reported for the shark. Assuming that the chick has an equivalent ratio of hair-cell to supporting-cell label as the shark, it is anticipated that the ratio of hair-cell specific labeling in the chick would be roughly 0.003% or 1 labeled hair cell in 33 333 hair cells. If there are 10 405 hair cells in the postnatal chicken papilla (Tilney et al., 1986), it is predicted that 1 hair cell would be labeled in 3.2 postnatal chick papillas with the labeling protocols employed in this study. Two lightly labeled hair cells were observed in the present study.

Interestingly, in the vestibular sensory epithelia in the avian inner ear, new hair cells and supporting cells are produced normally postnatally (Jørgensen and Mathiesen, 1988; Roberson et al., 1992). In view of the close developmental relationship between vestibular and auditory organs and the postnatal supporting-cell production observed in this study, it is not unreasonable to speculate that hair-cell production in the young chicken cochlea may also continue postnatally at an extremely low rate. Future detailed studies need to be undertaken to address this question.

Where do the new supporting cells originate?

The identity of the hair-cell precursors in the regenerating basilar papilla still remains to be determined.

* In determining these values of specific labeling rates, Corwin pooled data from five sharks where slightly different tritiated-thymidine administration protocols were used. Specifically, three sharks received single tritiated-thymidine injections and were sacrificed at 6, 12, and 48 h. One animal received two injections, at 95 and 48 h before sacrifice, and another animal received three injections at 123, 98, and 48 h before sacrifice.

Indirect evidence from sound-damaged basilar papillas suggest that hyaline cells and organ supporting cells are potential hair-cell precursor cells (Girod et al., 1989; Raphael, 1992). As shown here, organ supporting cells, border cells and hyaline cells are produced in the normal end organ. Two organ supporting cells located immediately above the habenular area were labeled in the 1-h animals, animals which were sacrificed immediately after receiving a brief pulse of tritiated thymidine (for 50–60 min). In view of the brevity of the tritiated-thymidine pulse and the immediate fixation, it is not unreasonable to speculate that we may have labeled a rare precursor cell. Conceivably, their very low level of production could be increased and modified in response to damage to the organ. By 30 days, the labeled cells are found in the inferior half or close to the inferior edge of the basilar papilla (Fig. 1). In between, there is a gradual progression toward the inferior edge with longer survival times (longer survival times after the cessation of the tritiated-thymidine injections). One possible explanation for this pattern of label is that the precursor cells are located above the habenular region, and that they migrate to the lumenal surface where they divide (Raphael, 1992; Tsue et al., 1993), and they and/or their progeny then migrate toward the inferior edge with longer survival times. An alternative explanation is that supporting cells in other parts of the organ, for example supporting cells in or near the inferior region of the organ, are also capable of mitosis. The low numbers of labeled cells detected in the present study make it difficult to evaluate between these possibilities with any certainty. In general, these data are supportive of the idea that organ supporting cells, border cells, hyaline cells, or an undifferentiated stem cell that exists in the epithelia, but may not be recognized as distinct from the differentiate supporting cells, are all still candidates for potential hair-cell precursor cells.

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