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Hair cell regeneration in the bullfrog vestibular otolith organs following aminoglycoside toxicity

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Adult bullfrogs were given single intraotic injections of the aminoglycoside antibiotic gentamicin sulfate and sacrificed at postinjection times ranging from 0.5 to 9 days. The saccular and utricular maculae of normal and injected animals were examined in wholemount and cross-section. Intraotic 200 μM gentamicin concentrations resulted in the uniform destruction of the hair bundles and, at later times, the cell bodies of saccular hair cells. In the utriculus, striolar hair cells were selectively damaged while extrastriolar hair cells were relatively unaffected. Regenerating hair cells, identified in sectioned material by their small cell bodies and short, well-formed hair bundles, were seen in the saccular and utricular maculae as early as 24–48 h postinjection. Immature versions of mature hair cell types in both otolith organs were recognized by the presence or absence of a bulbed kinocilia and the relative lengths of their kinocilia and longest stereocilia. Utricular hair cell types with kinocilia longer than their longest stereocilia were observed at earlier times than hair cell types with shorter kinocilia. In the sacculus, the hair bundles of gentamicin-treated animals, even at 9 days postinjection, were significantly smaller than those of normal animals. The hair bundles of utricular hair cells, on the other hand, reached full maturity within the same time period.

Ototoxicity; Hair cell; Regeneration; Otolith organs; Bullfrog

Introduction

Proliferation and differentiation of sensory hair cells occurs in mammals only during embryonic development. The auditory and vestibular systems of fish (Corwin, 1981, 1985; Popper and Hoxter, 1984), amphibians (Li and Lewis, 1979; Corwin, 1985), and birds (Jorgenson and Matthiesen, 1988; Katayama and Corwin, 1989; Robertson et al., 1992), on the other hand, produce hair cells at a low level throughout life. Newly produced hair cells in fish and amphibians are primarily localized to a distinct peripheral growth zone at the edge of the sensory epithelium. In birds, newly produced hair cells are found in all regions of the sensory epithelium. More importantly, birds and fish retain the capacity to rapidly increase the rate of hair cell regeneration following the elimination of hair cells due to ototoxic drugs (Cruz et al., 1987; Hashino et al., 1991; Lippe et al., 1991; Yan et al., 1991) or noise exposure (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Girod et al., 1989). In these lower vertebrates, regenerated hair cells develop morphologically normal hair bundles (Cotanche, 1987; Corwin and Cotanche, 1989; Duckert and Rubel, 1990) which, in both the auditory (Tucci et al., 1990) and vestibular (Jones and Nelson, 1992) organs, appear to be fully functional.

Vestibular and cochlear ototoxicity is a well-known side effect of aminoglycoside antibiotics (Lim, 1986; Schacht, 1986). These drugs reversibly block hair cell transduction channels (Kroese and van den Bercken, 1980; Kroese et al., 1988; Jamarillo and Hudspeth, 1991) and, after longer exposure, irreversibly disrupt hair bundle morphology (Takumida et al., 1989a–c) and cytoplasmic organelles (DeGroot et al., 1990; Hashino et al., 1991), resulting in the degeneration and extrusion of hair cells. Hair cells in the vestibular organs are differentially sensitive to ototoxic drugs, cells in central regions being more sensitive than cells in more peripheral locations (Lindeman, 1969; Yan et al., 1991).

Regeneration of hair cells following exposure to ototoxic drugs has not been previously studied in amphibians. In addition, previous studies of hair cell regeneration have not attempted to examine the development and differentiation of specific hair cell types within individual inner ear endorgans. This is of critical importance in the vestibular organs, where hair cells with differing cell body and hair bundle morphology are located in close proximity to one another (Lindeman, 1969; Wersall and Bagger-Sjoback, 1974). Varia-

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tions in hair bundle morphology are particularly striking in the vestibular organs of the bullfrog, where several hair cell types have previously been described (Lewis and Li, 1975; Baird and Lewis, 1986). These hair cell types have recently been shown to differ dramatically in their physiological response properties (Baird, 1992, 1993a,b).

In the present study, the aminoglycoside antibiotic gentamicin sulfate was used to induce the degeneration of hair cells in the vestibular otolith organs of the bullfrog. The primary aim of this study was to determine whether hair cells in the bullfrog otolith organs would regenerate following exposure to ototoxic drugs. Our results reveal that hair cells in both the saccular and utricular maculae regenerate following ototoxic insult and that immature versions of mature hair cell types in these endorgans are identifiable by their hair bundle morphology.

Preliminary accounts of portions of this data have recently been presented in abstract form (Baird et al., 1993).

Methods

Intraotic injection of gentamicin sulfate

Adult bullfrogs (*Rana catesbeiana*) of either sex weighing 100–200 g were anesthetized by immersion in 0.2% MS-222. Unlike similar experiments in mammals (Takamida et al., 1989a–c), birds (Cruz et al., 1987; Duckert and Rubel, 1990; Lippe et al., 1991) and fish (Yan et al., 1991), systemic injections of gentamicin did not result in the degeneration of vestibular hair cells in amphibians. We therefore used an intraotic approach to administer gentamicin to the inner ear. Using asceptic technique, the right otic capsule was ventrally exposed through a small hole in the roof of the mouth and perforated, using a deburred 25 gauge syringe, at two points immediately above the saccular macula. We then carefully introduced a 10 μl Hamilton syringe into one of these perforations and slowly injected 9.5 μl of a 500–2000 μM gentamicin sulfate solution dissolved in low-Ca^{2+} HEPES-buffered saline into the otic capsule. Mean otic capsule volume, measured in 5 animals, was estimated to be 47.7 ± 3.0 μl. This volume, which represents an upper limit on the total (endolymphatic and perilymphatic) volume of fluid space encapsulated by the otic capsule, suggests that our injections resulted in 100–400 μM intraotic gentamicin sulfate concentrations. We believe, although this was not verified histologically, that this injection procedure resulted in a localized rupture of the membranous labyrinth and a mixing of the endolymphatic and perilymphatic fluids. Following intraotic injections, the otic capsule was sealed with bone wax and bullfrogs were allowed to recover from anesthesia.

Removal of the vestibular otolith organs

Bullfrogs injected with gentamicin sulfate were reanesthetized with 0.2% MS-222 and decapitated 0.5–9 days following gentamicin injection. As in previous studies (Baird, 1992, 1993a,b), saccular and utricular maculae were dissected from the membranous labyrinth in cold, oxygenated HEPES-buffered saline and trimmed of excess nervous and connective tissue to improve the visibility of hair bundles. Otolith membranes were removed with gentle mechanical agitation following a 15–45 min proteolytic digestion in 50 μg/ml subtilopeptidase BPN' (Sigma). Excised saccular and utricular maculae were fixed by immersion for 2–4 h in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.25), dehydrated in an ascending series of ethanol solutions, and mounted in depression slides.

Hair cell counts

Measurements of hair cell density were made from the saccular and utricular maculae of 5 normal, 1 saline-injected, and 15 gentamicin-injected animals. These measurements were made by viewing wholemount maculae under Nomarsaki illumination with x40/0.9 and x63/1.25 oil immersion objectives and counting the number of hair cells located within a 75×100 μm rectangle. Hair cells were counted only if they had a clearly recognizable hair bundle and cuticular plate. These counts were made in the central portion of the sacculus and in both the striolar and medial extrastriolar regions of the utriculus. The results of three independent counts were made and averaged for each endorgan.

Hair cell morphology

To examine the cellular and hair bundle morphology of hair cells in more detail, wholemount maculae were embedded in glycol methacrylate (Polysciences, JB-4) and serially sectioned at 8 μm on a rotary microtome (LKB, Historange). For 10–15 individual sections, the boundaries of the sensory epithelium and, in the utriculus, the striolar region, were traced and stored to hard disk via a video processor board (ITI, FG-100). Hair cell types in normal material were identified, as in previous studies (Baird, 1992, 1993a,b), from their macular location and hair bundle morphology. The macular location of utricular hair cells was determined from their position relative to the striolar border and the reversal of hair bundle polarization. Hair bundle morphology was classified by the size of the hair bundle, the presence or absence of a bulbed kinocilium, and the relative lengths of the kinocilium and longest stereocilia.

Hair cells from gentamicin-treated animals were identified as damaged, undamaged, or regenerating from their cell body and hair bundle morphology. Damaged hair cells had swollen cell bodies, pyknotic

nuclei, and/or fused, splayed, or missing hair bundles. Regenerating hair cells were identified by their small cell bodies, small nuclei, and the presence of short, well-formed hair bundles (see Results). To our surprise, it was possible, based upon the presence or absence of a bulbed kinocilium and the relative lengths of the kinocilium and longest stereocilia, to identify immature versions of mature hair cell types in both the saccular and utricular maculae.

Results

The general appearance of the saccular and utricular maculae from one of 5 uninjected animals is shown in Figs. 1 and 2. From above, both maculae are kidney-shaped. The utriculus, unlike the sacculus, is divided into medial and lateral parts by the striola, a 50–100 μm ribbon-shaped zone, that runs for almost the entire length of the sensory epithelium near its lateral border (Fig. 2A). In cross section, this region is distinguished from flanking extrastriolar regions by the wider spacing of its hair cells and the elevated height of its apical surface (Fig. 2B). Hair cells within the striola reverse their hair bundle polarization near the lateral border of the striola, separating the striola into medial and lateral parts.

The cellular organization of both maculae is similar, consisting of a pseudostratified columnar epithelium of sensory hair cells interspersed with non-sensory supporting cells (Figs. 1A and 2B). Hair cells occupy the upper two-thirds of the sensory epithelium, while supporting cells span its entire distance. The nuclei of hair cells are positioned apical to those of supporting cells.

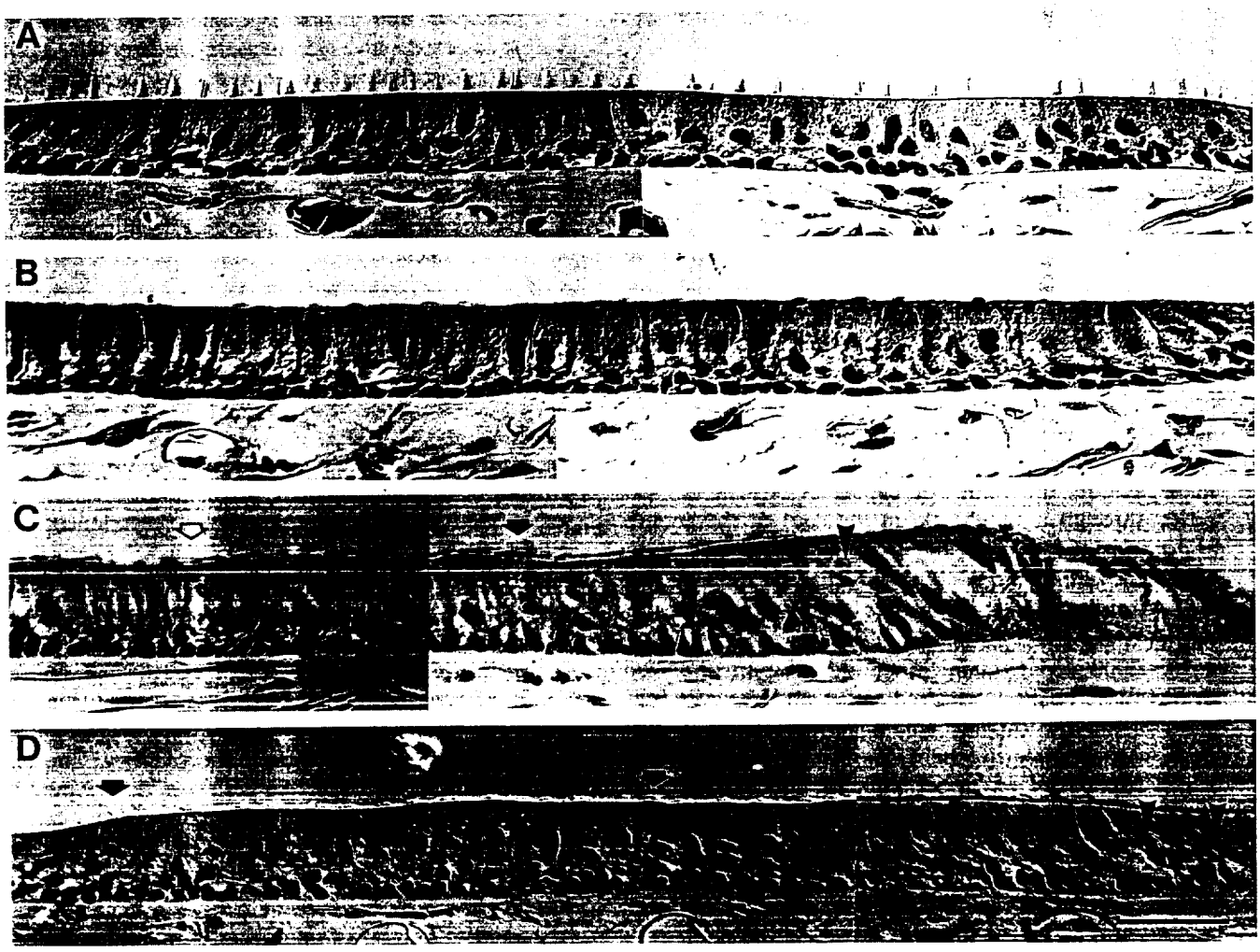

Fig. 1. Nomarski photomicrographs of toludine-blue stained cross-sections of the right saccular macula from an uninjected animal (A) and animals sacrificed 1 day (B), 2 days (C), and 7 days (D) after 200 μM gentamicin injection. In B, note the universal loss of hair bundles and the beginnings of cellular damage in the peripheral margin (far right). In C, a partially extruded hair cell (solid arrow), regenerating hair cell (solid pointer) and the opening left by an extruded hair cell (open arrow) are shown. Note also the large epithelial holes and apical migration of supporting cell nuclei in the peripheral margin (far right). In D, note regenerating hair cells with no hair bundles (solid pointers) and with long kinocilia (solid arrows). Numerous hair cells with short, bulbed kinocilia are also seen. Scale bar, 50 μm (A–D).

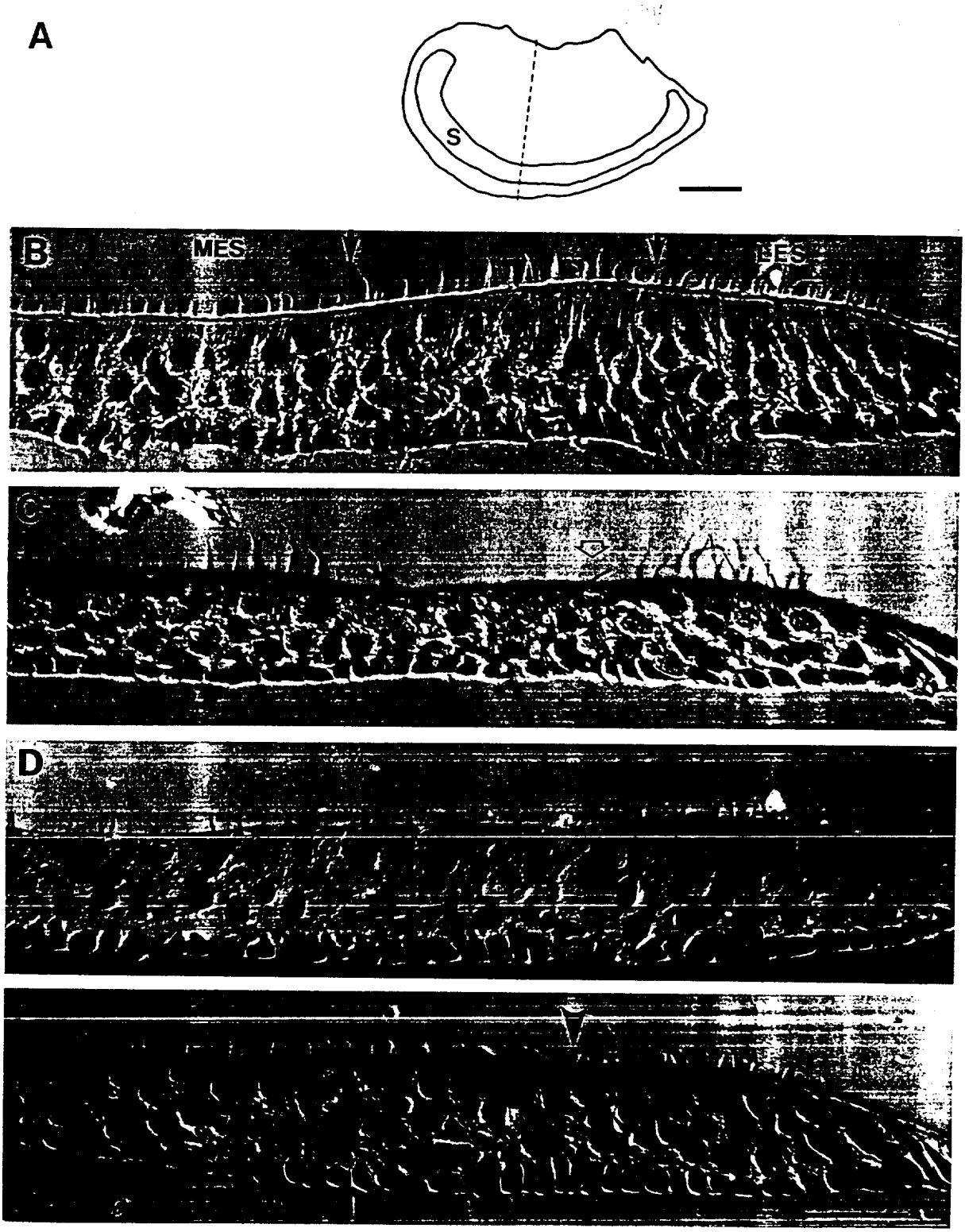

Fig. 2. Surface reconstruction (A) of the wholemount utricular macula. Dashed line x----x' indicates the plane of section seen in subsequent figures. The striola, a ribbon-shaped zone, separates the extrastriola into medial and lateral parts. Nomarski photomicrographs of toludine-blue stained cross-sections of the right utricular macula from an uninjected animal (B) and animals sacrificed 1 day (C), 3 days (D) and 9 days (E) after 200 μM gentamicin injection. In C, open arrow indicates an undamaged striolar hair cell with hair bundle morphology similar to that of extrastriolar hair cells. Pointers in D and E indicate regenerating hair cells. MES, medial extrastriola; S, striola; LES, lateral extrastriola. Scale bars, 250 μm (A); 25 μm (B–E).

The basal surfaces of supporting cells rest on a basement membrane which separates the sensory epithelium from its afferent and efferent innervation.

The great majority of saccular hair cells have bulbed kinocilia no longer than their longest stereocilia (Fig. 1A). These hair cells occupy the entire central region of the macula. A second type of hair cell, with unbulbed kinocilia longer than its longest stereocilia, is located around the macular perimeter. This hair cell is known from previous studies to be a growth precursor of mature saccular hair cells (Li and Lewis, 1979; Corwin, 1985). Hair cells in the utriculus, unlike the sacculus, differ significantly in hair bundle morphology in different membrane regions (Lewis and Li, 1975; Baird and Lewis, 1986). The predominant hair cell type in the utriculus has short stereocilia and kinocilia 2–6 times as long as its longest stereocilia. These hair cells are found throughout the medial and lateral extrastriola and, more rarely, in the striolar region. Three other hair cell types, with a variety of hair bundle morphologies, are confined to the striolar region.

Cell body and hair bundle morphology in an animal injected with HEPES-buffer was similar to that of uninjected animals. In gentamicin-treated animals, on the other hand, rapid changes in the cell body and hair bundle morphology of saccular and utricular hair cells were observed following gentamicin injection. The effects of gentamicin treatment were quantified by comparing hair cell density in the saccular and utricular maculae of normal animals with those of animals injected with HEPES-buffer or varying concentrations of gentamicin sulfate (Fig. 3). Hair cell density in the utricular striola and extrastriola of an animal 24 h after injection of HEPES-buffer was only slightly less than that in normal animals (0.012 vs. 0.014 and 0.028 vs. 0.030, respectively). This difference was not statistically significant ($P > 0.05$). Injections of gentamicin sulfate, on the other hand, caused large decreases in hair cell density. Hair cell density in the sacculus began to drop for gentamicin concentrations as low as 100 μM and continued to decrease with increasing gentamicin concentration. Injections of 200 μM gentamicin sulfate resulted, within 24 h, in the complete degeneration of all saccular hair cells. In the utriculus, on the other hand, gentamicin concentrations < 200 μM did not significantly decrease striolar or extrastriolar hair cell density below that of normal animals ($P > 0.4$). For 200 μM concentrations, there was a significant drop in striolar ($P < 0.0005$) but little or no effect on extrastriolar ($P > 0.4$) hair cell density. Animals did not survive 400 μM gentamicin concentrations. We therefore chose to use 200 μM intraotic injections for the remainder of these studies.

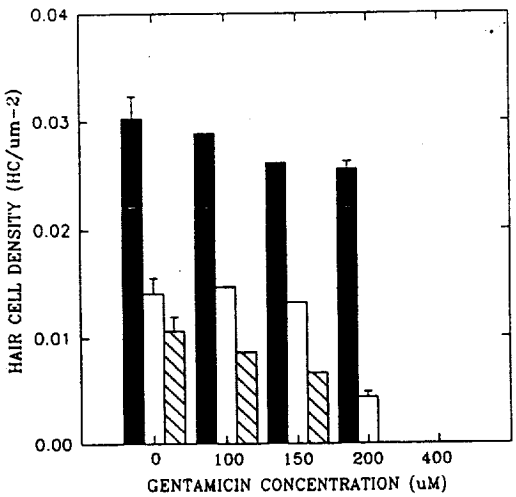

Fig. 3. Scale bar graphs of hair cell density in the sacculus and the striolar and medial extrastriolar regions of the utriculus for 5 uninjected animals and 5 animals sacrificed 1 day after receiving injections of varying concentrations of gentamicin sulfate. Values, means ± standard deviations. Sacculus, (▨); Utricular striola (□); Utricular extrastriola (■).

The first sign of gentamicin toxicity, seen within 12–24 hours following gentamicin injection, was a gradual degeneration of the hair bundle (Figs. 1B and 2C). This degeneration began with a splaying or fusion of individual stereocilia and culminated with the disappearance of the hair bundle. By 24–48 h, further changes were observed in the sensory epithelium of the saccular and utricular maculae. These included hair cells with swollen nuclei and cell bodies, hair cells with absent or pyknotic nuclei, and hair cells with breaks in their plasma membrane (Figs. 1C and 2D). Partially extruded hair cells, particularly in the saccular macula, were often observed (solid arrow, Fig. 1C). Large epithelial holes surrounded on either side by supporting cells were also seen, suggesting the extrusion of many additional hair cells from the sensory epithelium (open arrow, Fig. 1C). These holes were especially prominent in the peripheral margin of the saccular macula (far right, Fig. 1C) and in the striola region of the utricular macula, where they often resulted in a flattening of the apical surface of this region relative to surrounding extrastriolar regions (Fig. 2C). Supporting cells, on the other hand, had normal cellular morphology.

In the sacculus, hair cells were uniformly damaged by gentamicin injection (Figs. 1B and C), although cellular damage was often greater in the peripheral margin than in more central regions (Fig. 1C). Hair cells in the utricular macula, on the other hand, displayed a differential sensitivity to gentamicin (Fig. 2C). Hair cells in the extrastriolar regions of the utriculus displayed few, if any, signs of gentamicin toxicity. The great majority of striolar hair cells were severely damaged and degenerated within 12–24 h of gentamicin injection. The hair bundle morphology of undamaged striolar hair cells was similar to that of extrastriolar hair cells (Fig. 2C).

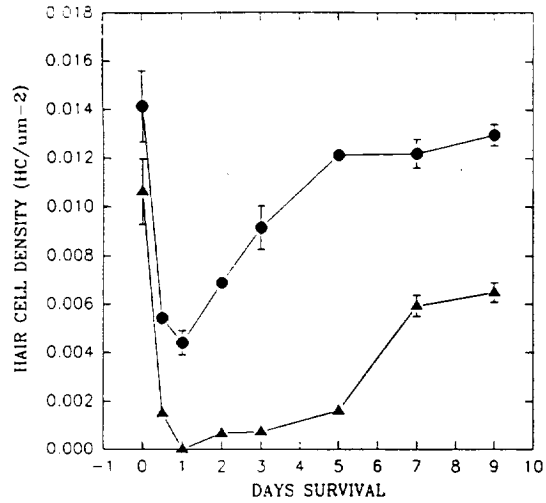

Fig. 4. Hair cell density in the sacculus (▲) and utricular striola (●) plotted against post-injection time for 12 animals receiving 200 μM gentamicin injections. Values for 5 uninjected animals plotted at 0 days post-injection. Values, means ± standard deviations.

To test for the possible recovery of hair cells at later postinjection times, we plotted hair cell density in the saccular and utricular maculae versus postinjection time for 12 animals injected with 200 μM gentamicin sulfate (Fig. 4). The sacculus recovered slowly from gentamicin toxicity, showing little or no recovery for 5 days. At this point, hair cell density increased significantly, restoring half of the normal hair cell density by 9 days. The utricular striola recovered more rapidly, displaying a rapid increase in hair cell density for the first 3 days post-injection and a slower increase from this point up to 9 days post-injection. Hair cell density in the utricular striola of animals sacrificed 9 days postinjection was not significantly different from that in normal animals ($P > 0.10$).

The recovery of hair cell density demonstrated in Fig. 4 suggested that new hair cells were being formed following the degeneration of hair cells damaged by gentamicin toxicity. This conclusion is based upon two assumptions: (1) damaged hair cells rapidly and completely lose their sensory hair bundles, and (2) recovering or regenerating hair cells can be recognized in wholemount preparations by the presence of their sensory hair bundles. To test the latter assumption, we studied sectioned material from animals sacrificed at later postinjection times to more closely examine the cell body and hair bundle morphology of individual hair cells.

By 2–3 days postinjection, obvious signs of repair were evident in the most damaged areas of the saccular and utricular maculae of gentamicin-injected animals. These included the rapid proliferation of supporting cells. In the sacculus, supporting cells at the peripheral margin were seen to migrate from the basement membrane to more apical positions (far right, Fig. 1D), suggesting that these cells might be redifferentiating into hair cells. Similar migrations were observed, although less consistently, in the central sacculus and utricular striola (Figs. 1C and 2D). In addition, newly formed cells, typified by small, narrow cell bodies and small nuclei, were seen in both the saccular and utricular maculae (pointers, Figs. 1C and D; 2D and E). Many of these cells also exhibited mitotic figures (Fig. 5), enabling us to unequivocally identify hair cells undergoing metaphase (Figs. 5A and B), anaphase (Fig. 5C), and telophase (Fig. 5D). Newly formed hair cells were also recognized by their weaker nuclear staining density in toluidine-blue stained material.

Newly formed hair cells, unlike mature hair cells or supporting cells, initially occupied intermediate positions within the sensory epithelium and did not contact the apical surface. Upon contacting this surface, these

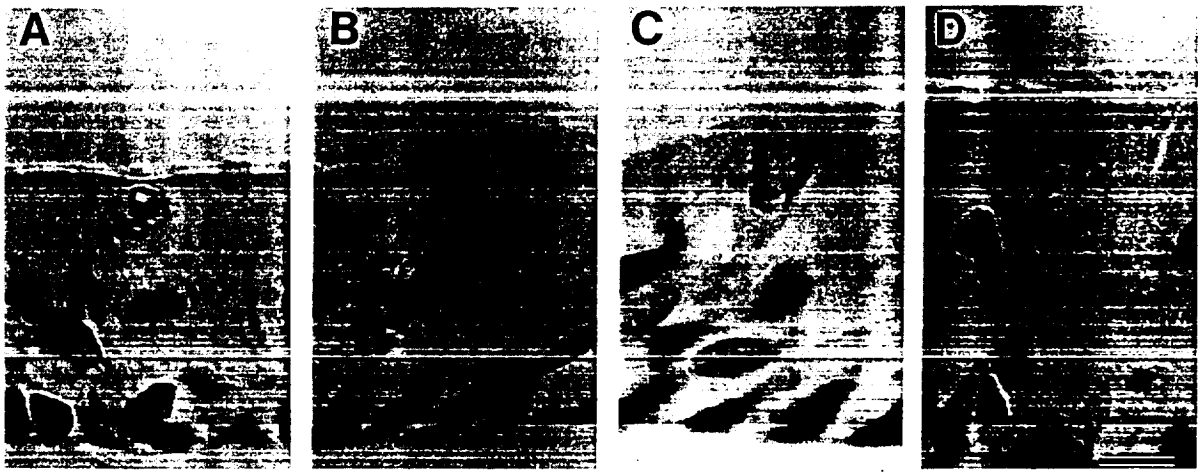

Fig. 5. Nomarski photomicrographs of newly formed hair cells in the saccular macula undergoing metaphase (A.B), anaphase (C) and telophase (D). Scale bar, 10 μm (A–D).

cells began to acquire the hair bundle characteristic of mature hair cells. Newly formed hair cells were observed as early as 2 days and as late as 9 days postinjection. By 7–9 days postinjection, the cellular morphology of the saccular and utricular maculae in gentamicin-injected animals more closely resembled that seen

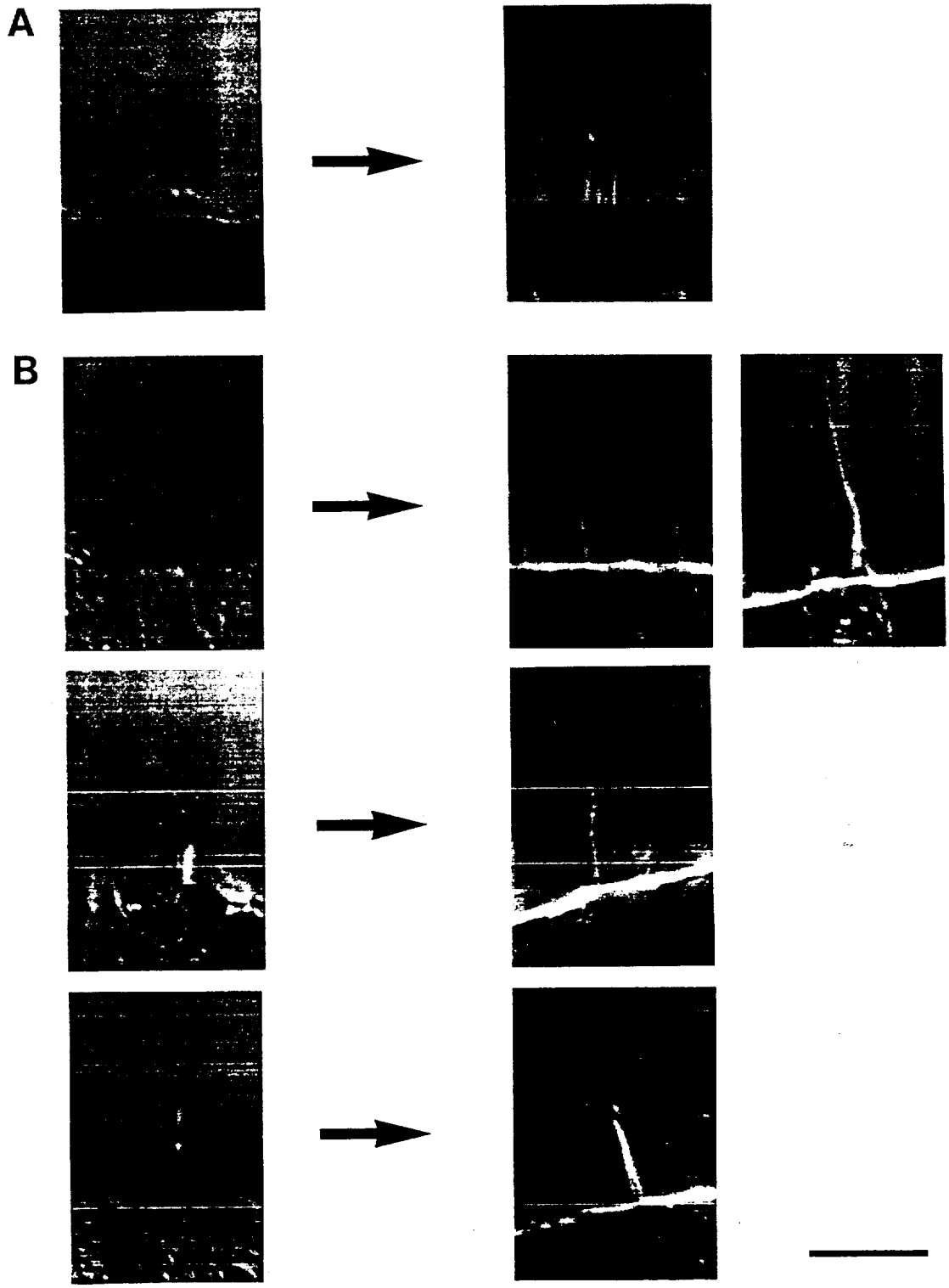

Fig. 6. Nomarksi photomicrographs of immature (left) and mature (right) hair cell types in the saccular (A) and utricular (B) maculae. Immature saccular hair cell taken from animal sacrificed 7 days postinjection; immature utricular hair cells taken from animals sacrificed 3–5 days postinjection. Scale bar, 10 μm.

in normal material (Figs. 1D and 2E). The cell bodies of hair cells and supporting cells were more ordered and normal in appearance. The nuclei of hair cells and supporting cells, as in normal material, were well separated. Epithelial holes in the sensory epithelium, although still apparent (Figs. 1D and 2E), were smaller and less frequent than at earlier postinjection times.

To our surprise, immature versions of most of the mature hair cell types in the bullfrog sacculus and utriculus could readily be identified by their distinctive hair bundle morphology (Figs. 6A and B). As in the normal sacculus (Fig. 1A), regenerating hair cells in the peripheral margin and, more rarely, in the central region had unbulbed kinocilia longer than their longest stereocilia (solid arrows, Fig. 1D). Most hair cells in the central region had short, bulbed kinocilia equal in length to their longest stereocilia (Figs. 2E and 6A). In both cases, the hair bundles of newly formed hair cells were significantly shorter than those of mature saccular hair cells, even in animals sacrificed 7–9 days postinjection.

In the utriculus, four mature hair cell types have been distinguished by the presence or absence of a bulbed kinocilium and the relative lengths of their kinocilium and longest stereocilia (Lewis and Li, 1975; Baird and Lewis, 1986). Two of these hair cell types have short stereocilia and kinocilia 2–6 times longer than their longest stereocilia, differing only in the absolute lengths of their kinocilia and longest stereocila. Short hair bundles with similar morphology were often seen in gentamicin-treated animals (Fig. 6B, top). The remaining two types possess bulbed or unbulbed kinocilia approximately equal in length to their longest stereocilia. Immature versions of both of these hair cell types were also observed in gentamicin-injected animals (Fig. 6B, middle and bottom). The hair bundles of newly formed utricular hair cells, unlike saccular hair cells, reached similar lengths as their mature counterparts by 7–9 days postinjection.

Hair cells appeared to repopulate the utricular striola in a fixed order. At 2–5 days postinjection, the great majority of newly formed hair cells had kinocilia longer than their longest stereocilia (Fig. 6B, top). These hair cells, as in normal material, were largely restricted to the outer striolar rows (Fig. 2E). Hair cells with shorter bulbed and unbulbed kinocilia (Fig. 6B, middle and bottom) appeared only at later postinjection times.

Discussion

The results of the present study clearly demonstrate that vestibular hair cells in the bullfrog, like those in birds (Cruz et al., 1987; Hashino et al., 1991; Lippe et al., 1991) and fish (Yan et al., 1991), are sensitive to aminoglycoside antibiotics. More importantly, our findings demonstrate that vestibular endorgans, like their auditory counterparts (Cruz et al., 1987; Hashino et al., 1991; Lippe et al., 1991), are capable of regenerating new hair cells following aminoglycoside toxicity. Using autoradiogaphy and immunocytochemical labeling, Weisleder and Rubel (1992) have recently shown that vestibular hair cells in the bird also exhibit regeneration following exposure to aminoglycoside antibiotics. Although hair cells in the amphibian vestibular endorgans are known to proliferate at a low level throughout adult life (Li and Lewis, 1979; Corwin, 1985), these findings represent, to our knowledge, the first direct evidence of hair cell regeneration in amphibians.

The degeneration and subsequent regeneration of hair cells seen in this study was more rapid than that seen in other studies. This may be due to the way in which gentamicin was administered to the inner ear. With the exception of one early study (Lindeman, 1969b), most investigators (Cruz et al., 1987; Hashino et al., 1991; Lippe et al., 1991), have administered aminoglycyoside antibiotics systemically, resulting in a slow, sustained delivery of these antibiotics to all parts of the body. Our intraotic injection procedure, by contrast, was designed to rapidly deliver a concentrated dose of antibiotic to the inner ear. We are not certain why vestibular hair cells in the bullfrog are not affected by systemic injections of aminoglycoside antibiotics. Our results do, however, emphasize the ability of the vestibular organs to rapidly reproduce new hair cells in response to ototoxic insult. Moreover, they are in good agreement with the results of Yan et al. (1991), who also did not observe hair cell damage in the sacculus after systemic injections of gentamicin in the fish.

It has long been known that aminoglycoside antibiotics can induce cochlear and vestibular ototoxicity. The biochemical mechanism(s) underlying ototoxicity, however, are only poorly understood (Lim, 1986; Schacht, 1986). Schacht and his colleagues (1986) have proposed that aminoglycosides bind electrostatically with negatively charged components of the hair cell plasma membrane. In addition, polycationic aminoglycosides, such as gentamicin and streptomycin, bind to and block negatively charged hair cell transduction channels (Kroese and van den Berken, 1980; Kroese et al., 1988; Jamarillo and Hudspeth, 1991), located at or near the tips of the stereocilia (Hudspeth, 1982; Jaramillo and Hudspeth, 1991). Both of these interactions are believed to be reversible and antagonized by divalent cations. A second, more crucial, interaction is the energy-dependent transport of aminoglycosides into hair cells by endocytosis (Takada et al., 1985; DeGroot et al., 1990) and its subsequent binding to phosphatidylinositol 4,5-biphosphate (PIP_2) (Schacht, 1986). This binding inhibits the hydrolysis of IP_2, preventing its physiological function (Berridge, 1984; Nishizuka,

1984), and disturbs membrane structure, resulting in non-specific permeabilities of the plasma membrane to external ions.

Hair cells in the vestibular organs were differentially sensitive to gentamicin, saccular hair cells being affected at lower gentamicin concentrations than their utricular counterparts. Within the utriculus, hair cells in the striola were affected more than hair cells in extrastriolar regions. Vestibular organs in mammals (Lindeman, 1969b) and fish (Yan et al., 1991) are also known to exhibit such regional sensitivity, hair cells in central regions being more sensitive than hair cells in more peripheral locations. In our study, selective sensitivity to gentamicin was correlated with hair cells with particular hair bundle morphologies. This is the first evidence that such sensitivity is correlated with hair cell type rather than epithelial location per se.

The cellular basis for selective gentamicin sensitivity is not well understood. One possibility is that some hair cells are able to slow or prevent the access of aminoglycoside to intracellular compartments, perhaps by varying the level of intracellular calcium at their apical surface. This would be expected to antagonize the electrostatic interaction of aminoglycosides with the transduction channel and plasma membrane, preventing or delaying its intracellular entry and subsequent deleterious effects. A second possibility is that the intracellular effects of aminoglycoside antibiotics are more deleterious in some hair cells than in others. The primary site for these antibiotics appears to be the endoplasmic reticulum and Golgi complex (DeGroot et al., 1990). Hair cells in central regions of the vestibular endorgans possess a more extensive endoplasmic reticulum and larger numbers of mitochondria than hair cells located in more peripheral regions (Yan et al., 1991). Whatever its cellular basis, however, this phenomenon provides investigators with a useful tool to study the degeneration and regeneration of specific hair cell populations in the vestibular organs.

The signals and cellular mechanism(s) which trigger the production of new vestibular hair cells are not known. Hair cell recovery could potentially result from a number of processes, including the migration of undamaged hair cells to damaged regions, the recovery of partially damaged hair cells, the redifferentiation of other cell types into hair cells, and the production of new hair cells by mitosis. Migration of undamaged hair cells cannot contribute to recovery in the sacculus, where a near-complete loss of the existing hair cell population is observed. While migration of undamaged hair cells from extrastriolar regions could contribute to hair cell recovery in the utricular striola, our results suggest that this is unlikely. First, such a migration would be expected to produce a reduction in extrastriolar hair cell density. This was not observed. Secondly, a restoration of the normal distribution of striolar hair cell types would imply that differentiated extrastriolar hair cells were able to redifferentiate into striolar hair cell types. The existence of distinctive immature versions of mature striolar hair cell types suggests that this does not occur. Our results do not reveal if partially damaged hair cells are able to recover after gentamicin exposure. Two lines of evidence, however, suggest that this is not the case. First, the loss of hair cell nuclei and the creation of large epithelial holes following gentamicin exposure suggests that damaged hair cells are destroyed and extruded from the sensory macula. Studies in the auditory system also suggest that only differentiating hair cells are capable of hair bundle assembly (Corwin and Cotanche, 1989; Tilney, 1986), seemingly ruling out the possibility that mature hair cells can regrow their hair bundles.

The large loss of hair cells in the sacculus and utricular striola argues strongly that recovery must involve the mitotic production of new hair cells. This is supported by the presence of regenerating cells with mitotic figures in gentamicin-treated but not normal animals. These regenerating hair cells were present only in regions of hair cell loss suggesting that, as in the auditory organs, such loss is necessary for mitosis to occur. Mitotic division of a precursor population is also responsible for the restoration of hair cell populations in other lower vertebrates following acoustic trauma (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Girod et al., 1989; Popper and Hoxter, 1990; Rubel, 1991) and aminoglycoside toxicity (Cruz et al., 1987; Hashino et al., 1991; Lippe et al., 1991). One obvious candidate for this population is some subset of supporting cells (Girod et al., 1989; Popper and Hoxter, 1990; Rubel, 1991). Our results further reveal that supporting cells, following the destruction of existing hair cells, migrated toward the apical surface, leaving no cell nuclei adjacent to the basement membrane. This migration was not necessarily associated with mitotic hair cells, suggesting that supporting cells may also redifferentiate directly into hair cells.

Surprisingly, regenerating hair cells strongly resembled miniature versions of mature hair cells at all stages of their development and could be classified into a number of hair cell types by the same morphological criteria used to identify their mature counterparts. Regenerating hair cells, for example, had kinocilia 2–6 times longer or approximately equal to their longest stereocilia. Similarly, regenerating hair cells with bulbed kinocilia, differing only in absolute kinociliary and stereociliary length, were consistently seen in gentamicin-treated animals. This suggests that the hair bundle morphology of vestibular hair cells, as in auditory hair cells (Corwin and Cotanche, 1989), is location-specific and predetermined early in the regeneration process. Our results do not reveal whether hair cell types transform their hair bundle morphology dur-

ing the regenerative process or if they differentiate from independent precursor cells. In the utricular striola, however, hair cells appeared to regenerate in a fixed order, suggesting that morphologically distinct hair cell types may represent intermediate stages in morphogenic development. In the bullfrog sacculus, moreover, mature hair cells with short, bulbed kinocilia are believed to develop from hair cells with long, unbulbed kinocilia (Li and Lewis, 1979; Corwin, 1985). Regenerating saccular hair cells with both types of hair bundle morphology were seen in our material. However, hair cells with long, unbulbed kinocilia were usually located in the peripheral margin. Moreover, most saccular hair cells, even at early postinjection times, had short, bulbed kinocilia, arguing against a compulsory involvement of hair cells with long, unbulbed kinocilia in the regenerative process. This would seem to contradict the results of Corwin (1985), who argues that developing saccular hair cells initially have long kinocilia which shrink during development to attain the bulbed kinocilium typical of mature saccular hair cells. It is, of course, possible that the processes underlying hair cell regeneration do not mirror those underlying normal development.

Our results emphasize the importance of the vestibular otolith organs as model systems for studies of hair cell regeneration. These organs, particularly the utricular macula, possess a number of hair cell types, which are distinguishable both by their hair bundle morphology (Lewis and Li, 1975; Baird and Lewis, 1986) and their physiological response properties (Baird, 1993a,b). As our results demonstrate, immature versions of these hair cell types can easily be recognized and studied at various stages of development. Studies of the morphological and physiological changes that occur during hair cell regeneration should reveal what signals trigger regeneration in lower vertebrates and hopefully lead to an understanding of the basic mechanisms underlying differentiation and repair in these important receptor cells.

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References

Baird, R.A. (1992) Morphological and electrophysiological properties of hair cells in the bullfrog utriculus. Ann. NY Acad. Sci. 656, 12–26.

Baird, R.A. (1993a) Comparative transduction and tuning mechanisms of hair cells in the bullfrog utriculus. I. Responses to intracellular current. J. Neurophysiol. (Submitted).

Baird, R.A. (1993b) Comparative transduction and tuning mechanisms of hair cells in the bullfrog utriculus. II. Sensitivity and response dynamics to mechanical stimulation. J. Neurophysiol. (Submitted).

Baird, R.A. and Lewis, E.R. (1986) Correspondences between afferent innervation patterns and response dynamics in the bullfrog utricle and lagena. Brain Res. 369, 48–64.

Baird, R.A., Torres, M.A. and Schuff, N.A. (1993) Hair cell regeneration in the amphibian vestibular otolith organs following gentamicin ototoxicity. Assoc. Res. Otolaryngol. 16, (in press).

Berridge, M.J. and Irvine, R.F. (1989) Inositol phosphates and cell signalling. Nature 341, 197–205.

Corwin, J.T. (1981) Postembryonic production and aging of inner ear hair cells in sharks. J. Comp. Neurol. 201, 541–553.

Corwin, J.T. (1985) Perpetual production of hair cells and maturational changes in hair cell ultrastructure accompany postembryonic growth in an amphibian ear. Proc. Natl. Acad. Sci. (USA) 82, 3911–3915.

Corwin, J.T. and Cotanche, D.A. (1988) Regeneration of sensory hair cells after acoustic trauma. Science 240, 1772–1774.

Corwin, J.T. and Cotanche, D.A. (1989) The development of location-specific hair cell stereocilia in denervated embyronic ears. J. Comp. Neurol. 288, 529–537.

Cotanche, D.A. (1987) Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. Hear. Res. 30, 181–196.

Cruz, R.M., Lambert, P.R. and Rubel, E.W. (1987) Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. Arch. Otolaryngol. Head Neck Surg. 113, 1058–1062.

DeGroot, J.C.M., Meeuwsen, F., Ruizendaal, W.E. and Veldman, J.E. (1990) Ultrastructural localization of gentamicin in the cochlea. Hear. Res. 50, 35–42.

Duckert, L.G. and Rubel, E.W. (1990) Ultrastructural observation on regenerating hair cells in the chick basilar papilla. Hear. Res. 48, 161–182.

Girod, D.A., Duckert, L.G. and Rubel, E.W. (1989) Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear. Res. 42, 175–194.

Hashino, E., Tanaka,, Y. and Sokabe, M. (1991) Hair cell damage and recovery following chronic application of kanamycin in the chick cochlea. Hear. Res. 52, 356–368.

Hudspeth, A.J. (1982) Extracellular current flow and the site of transduction by vertebrate hair cells. J. Neurosci. 2, 1–10.

Jamarillo, F. and Hudspeth, A.J. (1991) Localization of the hair cell's transduction channels at the hair bundle's top by iontophoretic application of a channel blocker. Neuron 7, 409–420.

Jones, T.A. and Nelson, R.C. (1992) Functional recovery of the peripheral vestibular system following severe streptomycin injury. Assoc. Res. Otolaryngol. 15, 161.

Jorgensen, J.M. and Mathiesen, C. (1988) The avian inner ear: continous production of hair cells in vestibular sensory organs, but not in the auditory papilla. Naturwissenschaften 75, 319–320.

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

Kroese, A.B.A. and Bercken, J. Van Den. (1980) Dual action of ototoxic antibiotics on sensory hair cells. Nature 283, 395–397.

Kroese, A.B.A., Das, A. and Hudspeth, A.J. (1988) Blockage of the transduction channels of hair cells in the bullfrog's sacculus by aminoglycoside antibiotics. Hear. Res. 37, 203–218.

Lewis, E.R. and Li, C.W. (1975) Hair cell types and distributions in the otolithic and auditory organs of the bullfrog. Brain Res. 83, 35–50.

Li, C.W. and Lewis, E.R. (1979) Structure and development of

vestibular hair cells in the larval bullfrog. Ann. Otol. Rhinol. and Laryngol. 88, 427–437.

Lim, D.J. (1986) Effects of noise and ototoxic drugs at the cellular level in the cochlea: a review. Am. J. Otolaryngol. 7, 73–99.

Lindeman, H.H. (1969a) Studies on the morphology of the sensory regions of the vestibular apparatus. Ergeb. Anat. Entwickl. 42, 1–113.

Lindeman, H.H. (1969b) Regional differences in sensitivity of the vestibular sensory epithelia to ototoxic antibiotics. Acta. Otolaryngol. 67, 177–189.

Lippe, W.R., Westbrook, E.N. and Ryals, B.M. (1991) Hair cell regeneration in the chicken cochlea following aminoglycoside toxicity. Hear. Res. 56, 203–210.

Nishizuka, Y. (1984) Turnover of inositol phospholipids and signal transduction. Science 225, 1365–1370.

Popper, A.N. and Hoxter, B.H. (1984) Growth of a fish ear. I. Quantitative analysis of sensory hair cell and ganglion cell proliferation. Hear. Res. 15, 133–142.

Popper, A.N. and Hoxter, B.H. (1990) Growth of a fish ear. II. Locations of newly proliferated hair cells in the saccular epithelium of Astronotus ocellatus. Hear. Res. 45, 33–40.

Roberson, D.F., Weisleder, P., Bohrer, P.S. and Rubel, E.W. (1992) Ongoing production of sensory cells in the vestibular epithelium of the chick. Hear. Res. 57, 166–174.

Rubel, E.W. (1991) Regeneration of hair cells in the avian inner ear. In: A. Dancer, D. Henderson, R. Salvi and R.P. Hamernik (Eds.), Noise-Induced Hearing Loss, St. Louis: Mosby Year Book, pp. 204–227.

Ryals, B.M. and Rubel, E.W. (1988) Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240, 1774–1776.

Schacht, J. (1986) Molecular mechanisms of drug-induced hearing loss. Hear. Res. 22, 297–304.

Takada, A., Bledsoe, S.C., Jr. and Schacht, J. (1985) An energy-dependent step in aminoglycoside ototoxicity: prevention of gentamicin ototoxicity during reduced endolymphatic potential. Hear. Res. 19, 245–251.

Takumida, M., Bagger-Sjoback, D., Wersall, J. and Harada, Y. (1989) The effect of gentamicin on the glycocalyx and the ciliary interconnections in vestibular sensory cells: a high-resolution scanning electron microscopic investigation. Hear. Res. 37, 163–170.

Takumida, M., Bagger-Sjoback, D., Harada, Y., Lim, D. and Wersall, J. (1989) Sensory hair fusion and glycocalyx changes following gentamicin exposure in the guinea pig vestibular organs. Acta Otolaryngol. 107, 39–47.

Takumida, M., Wersall, J. and Bagger-Sjoback, D. (1989) Initial changes in the sensory hair-cell membrane following aminoglycoside administration in a guinea pig model. Arch. Otorhinolaryngol. 246, 26–31.

Tilney, L.G., Tilney, M.S., Saunders, J.C. and DeRosier, D.J. (1986) Actin filaments, stereocilia and hair cells of the bird cochlea. III. The development and differentiation of hair cells and stereocilia. Dev. Biol. 116, 100–118.

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

Weisleder, P. and Rubel, E.W. (1992) Hair cell regeneration in the avain vestibular epithelium. Exp. Neurol. 115, 2–6.

Wersall, J. and Bagger-Sjoback, D. (1974) Morphology of the vestibular sense organs. In: H.H. Kornhuber (Ed.), Vestibular System: Basic Mechanisms, Handbook of Sensory Physiology, Springer-Verlag, New York, pp. 124–170.

Yan, H.Y., Saidel, W.M., Chang, J.S., Presson, J.C. and Popper, A.N. (1991) Sensory hair cells of a fish ear: evidence of multiple types based on ototoxicity sensitivity. Proc. R. Soc. Lond. B 245, 133–138.