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Research paper

Gentamicin ototoxicity in the saccule of the lizard *Podarcis Sicula* induces hair cell recovery and regeneration

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Abstract

There is little information available on the susceptibility of reptilian saccule hair cells to ototoxin-induced sensory damage. In this study, we report morphological evidence of hair cell recovery and regeneration after damage induced by gentamicin in the saccule of a lizard. We perform morphological analysis using scanning electron microscopy and confocal laser scanning microscopy with actin and calbindin as markers for hair cells and tubulin as a marker for supporting cells. The data were consistent: gentamicin induced damage in the hair cells, and the damage increased with increasing duration of treatment. Initially, the saccule appeared unhealthy. Subsequently, the sensory hair cells became compromised, with fused stereovilli, followed by widespread loss of hair cell bundles from the hair cells. Finally, numerous hair cells were lost. Morphologically, the saccule appeared normal 28 days after gentamicin treatment. Using a mitogenic marker, we tested whether or not there is hair cell regeneration following administration of gentamicin. We found evidence of bromodeoxyuridine incorporation first in supporting cell nuclei and subsequently in hair cell nuclei. This indicates that a process of sensory epithelium repair and hair cell regeneration occurred, in both extrastriolar and striolar regions, and that the recovery was due to both the proliferation of supporting cells and, as seems likely, self-repair of hair cell bundles.

Keywords: Aminoglycoside antibiotics; Calbindin; BrdU; Inner ear; Recovery

1. Introduction

The saccule is a sensory organ of the vestibular apparatus that responds to linear acceleration and gravity. Hair cells in the saccule are less susceptible to injury caused by exposure to aminoglycosides than are the hair cells in the utricle and cristae of guinea pigs (Li and Forge, 1995; Li et al., 1995) and in the utricle of chicks (Kil et al., 1997). Gale et al. (2002) reported that bullfrog saccular hair cells tolerate low doses of gentamicin and subsequently undergo repair. However, there is little information available on the susceptibility of reptilian saccule hair cells to ototoxin-induced sensory damage. Bagger-Sjoback and Wersäll (1976) studied the toxic effects of gentamicin on the basilar papilla in a lizard and we have previously demonstrated hair cell regeneration in the crista ampullaris (which is involved in dynamic balance) of the lizard *Podarcis sicula*. We have now extended our investigation to the saccule (involved in static balance) of gentamicin-treated *P. sicula*.

We examined the fate of the saccular hair cells after exposure to three doses of gentamicin (Gm) to determine whether there was any recovery and whether this was due to repair of damaged hair cells, to regeneration or, most likely, to both. We performed a morphological analysis using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) with actin as a marker

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CaB, calbindin-D-28-K; CaM, calmodulin; CBPs, calcium-binding proteins; CLSM, confocal laser scanning microscope; DABCO, diazabicyclo-octane; DW, distilled water; FITC, fluorescein isothiocyanate; Gm, gentamicin; PBS, buffer phosphate saline; SEM, scanning electron microscope; TBS, Tris buffer saline; TRITC, tetramethylrhodamine isothiocyanate

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for hair cells. Actin is the major cytoskeletal component of hair cells and is also present in the tight junctions that connect these cells at the reticular lamina level (Flock and Cheung, 1977; Macartney et al., 1980; Slepecky and Chamberlain, 1982, 1985; Daudet et al., 1998). Tubulin was used as a marker for supporting cells, since their cytoskeleton is rich in microtubules (Balsamo et al., 1995). We used calbindin, a calcium-binding protein present in hair cells of the sensory epithelium of *P. sicula* saccules (Piscopo et al., 2003, 2004) and in chick saccules (Balsamo et al., 2000) to determine whether the regenerated cells were hair cells. Calcium-binding proteins such as parvalbumin (Celio, 1990; Steyger et al., 1997; Daudet et al., 1998) and calmodulin (Stone et al., 1996; Steyger et al., 1997; Ogata and Slepecky, 1998; Ogata et al., 1999) have been used previously as markers for hair cells.

We looked for regeneration of cells following administration of Gm, using 5-bromo-2′-deoxyuridine (BrdU), an S-phase-associated marker that is incorporated into newly synthesized DNA strands (Gratzner, 1982), to label cells that were in the process of dividing.

2. Materials and methods

Lizards were captured with the authorization of 1/06/2000N. SCN/2D/2000/9213 del Ministero dell'Ambiente.

Adult specimens of *P. sicula* (average weight ~7.00 g) were collected from sites around Naples, Italy. The lizards were maintained in the laboratory under natural conditions of light (10–13 h/day) and temperature (14–24 °C). They were then decapitated under general anaesthesia with ether vapours according to the institutional animal care and use committee.

2.1. Gm treatment

Sixty-three lizards received 150 mg/kg of Gm (Schering-Plough, Italy) daily by subcutaneous injection. Seven groups, each of nine lizards, were euthanized at different times after injection: one group 4 h after one Gm injection (1Gm/4 h), one group 4 h after two Gm injections (2Gm/4 h), one group 4 h after three Gm injections (3Gm/4 h), one group 3 days after the third Gm injection (3Gm/3 d), one group 8 days after the third Gm injection (3Gm/8 d), one group 18 days after the third Gm injection (3Gm/18 d) and one group 28 days after the third Gm injection (3Gm/28 d) (Table 1). From each group, three lizards were processed for SEM, three for CLSM and three for immunofluorescence. At each time point, the saccule of two lizards (untreated), one for SEM and one for CLSM, were used as controls.

2.2. BrdU treatment

Twenty one lizards, (three lizards from each group: 1Gm/4 h, 2Gm/4 h, 3Gm/4 h, 3Gm/3 d, 3Gm/8 d, 3Gm/18 d, 3Gm/28 d), received a subcutaneous injection of BrdU (100 mg/kg in saline; Sigma, St Louis, MO, USA) daily from the first day of Gm injection until the day of sacrifice (1Gm + 1BrdU/4 h, 2Gm + 2BrdU/4 h, 3Gm + 3BrdU/4 h, 3Gm + 6BrdU/3 d, 3Gm + 11BrdU/8 d, 3Gm + 21BrdU/18 d, 3Gm + 31BrdU/28 d) (Table 1).

Seven groups, each containing one lizard, were treated with BrdU injections alone for the same amount of time as a control for BrdU uptake (Table 1). The lizards were then processed for immunofluorescence. As a positive control for BrdU incorporation and staining, the intestines of lizards treated with BrdU only or Gm plus BrdU were removed and processed for immunofluorescence in the same manner as tissue from the saccule.

2.3. Scanning electron microscopy

For SEM, the heads were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 3 h at 4 °C, according to Avallone et al. (2003). After microdissection of the vestibular end-organs, the saccules were isolated with fine microforceps, rinsed in PBS and post-fixed in 1% OsO_4 in the same buffer for 1 h at 4 °C. After several rinses in PBS, the specimens were subjected to serial dehydration followed by critical-point drying. The specimens were mounted on aluminium stubs, coated with gold and

Table 1
Animals used for each group and gentamicin and BrdU treatment

Samples	Treatment 1st group	Treatment 2nd group	Treatment 3rd group	Treatment 4th group	Treatment 5th group	Treatment 6th group	Treatment 7th group	Samples total
Three lizards for SEM	1Gm/4 h	2Gm/4 h	3Gm/4 h	3Gm/3 d	3Gm/8 d	3Gm/18 d	3Gm/28 d	21 lizards
Three lizards for CLSM	1Gm/4 h	2Gm/4 h	3Gm/4 h	3Gm/3 d	3Gm/8 d	3Gm/18 d	3Gm/28 d	21 lizards
Three lizards for IF	1Gm + 1BrdU/4 h	2Gm + 2BrdU/4 h	3Gm + 3BrdU/4 h	3Gm + 6BrdU/3 d	3Gm + 11BrdU/8 d	3Gm + 21BrdU/18 d	3Gm + 31BrdU/28 d	21 lizards
One lizard for IF	1BrdU/4 h	2BrdU/4 h	3BrdU/4 h	6BrdU/4 h	11BrdU/4 h	21BrdU/4 h	31BrdU/4 h	Seven lizards
One lizard for SEM	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated	Seven lizards
One lizard for CLSM	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated	Seven lizards

examined using a Cambridge Stereoscan 250 MKIII Microscope.

2.4. Indirect immunofluorescence for confocal laser scanning microscopy

For CLSM analysis, the heads were fixed in 100% methanol for 2 h at 4 °C, then embedded in paraffin by the standard procedure. Serial sections were deparaffinized and hydrated in a graded sequence of methanol to H_2O, then treated with 0.25% Triton-X100 and 0.1% Tween 20 in 0.1 M PBS (pH 7.4) for 1 h. After several rinses in 0.1 M PBS (pH 7.4), some sections were incubated overnight at 4 °C with FITC-conjugated primary antibodies against β-actin (clone AC-15; 1:500) (Sigma, St Louis, MO, USA) and against α-tubulin (clone B 5-1-2; 1:500) (Sigma). After rinses in the same buffer (6×10 min), the sections were incubated with TRITC-conjugated anti-mouse IgG (from goat; 1:400) in 0.1 M PBS (pH 7.4) for 2 h at room temperature. Other sections were incubated overnight at 4 °C using a primary antibody against calbindin-D-28K (mouse IgG1 isotype) (Sigma) and FITC-conjugated rabbit anti-mouse IgG as a secondary antibody, each one diluted 1:1000. The nuclei were visualized by DAPI (Sigma) staining. Both steps were performed in a moist, dark chamber. After several rinses in 0.1 M PBS (pH 7.4), the specimens were mounted using diazabicyclo-octane (Sigma). Pre-immune sera were used as negative controls. Fluorescence

Fig. 1. SEM (A) saccule sensory epithelium of untreated lizards: hair cells with an enlarged, bulb-like kinocilium (arrow) and stereovilli in an organ pipe configuration (arrowhead); (B) (1Gm/4 h): the hair cells appear compromised (arrows); (C) (2Gm/4 h): hair cells appear damaged and the stereovilli seem to be fused; (D) (3Gm/4 h): several hair cell bundles have disappeared from the saccule (arrow), and many hair cells are abnormal, with swellings or blebs on the apical surfaced (arrowheads); (E, F) (3Gm/3 d): widespread loss of hair bundles from the hair cells in both extrastriolar (E) and striolar (F) regions is evident (arrows), apical blebs are present (arrowheads).

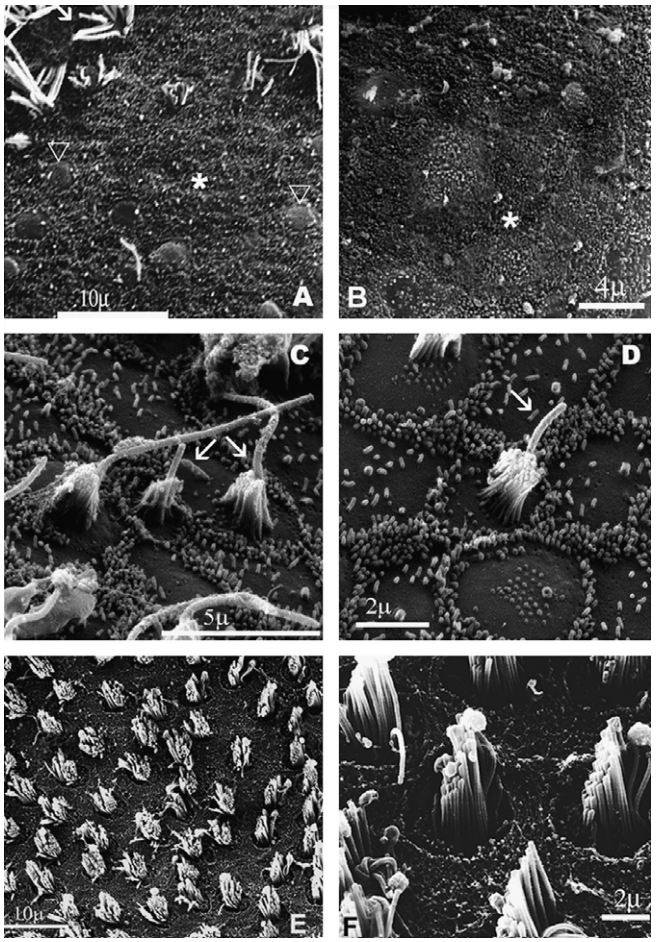

Fig. 2. SEM (A) (3Gm/8 d): in extrastriolar region numerous hair cells are missing; note the supporting cells invading the region of pre-existing hair cells to produce a "scar" (asterisk). A few remaining mature hair cell bundles and some mature hair cell bundles undergoing degeneration are evident (arrow); some hair cells that had lost their hair bundles retain cuticular plates (arrowheads); (B) (3Gm/8 d): in striolar region the epithelium appears like a mosaic of supporting cells forming a "scar"; (C) (3Gm/18 d): in extrastriolar region small hair cell bundles show features of immaturity with stereovilli of almost equal height surrounding a much longer kinocilium (arrows); (D) (3Gm/18 d): in striolar region hair cell showing small hair cell bundles and a longer kinocilium, which are therefore probably immature, are present (arrow); (E, F) (3Gm/28 d): sensory epithelium has regained an apparently normal morphology comprising hair cells with an enlarged, bulb-like kinocilium and stereovilli that are graduated in length.

observations were performed a confocal microscope (Leica TCSNT) with laser argon–krypton 7.5 mW multilines. Focal series of horizontal planes of sections were monitored simultaneously using the 488 nm laser line and band-pass 530/30 for FITC, and the 568 nm laser line and a long-pass filter 590 for TRITC. The Leica TCSNT confocal microscope was equipped with an AOTF filter. This filter minimizes cross-talk during simultaneous detection of FITC/TRITC labels. The nuclei, stained by DAPI, were monitored with a UV laser line.

2.5. Indirect immunofluorescence for BrdU detection

Lizard heads were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2.5 h at room temperature. After several rinses in the same buffer, the saccules were dissected out and treated with 0.2 M ammonium chloride in cacodylate buffer for 1 h at room temperature. The specimens were dehydrated with ethanol and propylene oxide and embedded in Epon. Serial sections of specimens were cut at 2 μm thickness and

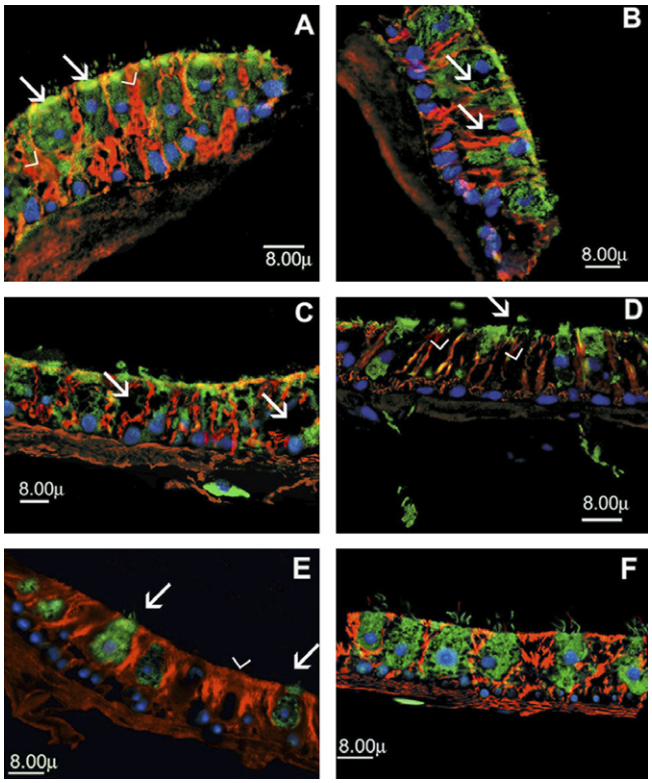

Fig. 3. CLSM: anti-actin (green) labels the cytoplasm of hair cells, anti-tubulin (red) labels the cytoplasm of supporting cells; DAPI (blue) stains the nuclei. (A) Saccule sensory epithelium of untreated lizards: anti-actin (green) labels the cytoplasm of hair cells (arrows); anti-tubulin (red) labels the cytoplasm of supporting cells (arrowheads); (B) (3Gm/4 h): hair cells appear damaged (arrows); (C) (3Gm/3 d): widespread loss of the sensory cells' hair bundles and damage is evident (arrows), just a few intact hair cells bodies are present; (D) (3Gm/8 d): many hair cells are lost (arrowheads), some hair cell debris are evident in the luminal space above the epithelium (arrows), but no intact hair cell bodies are evident; (E) (3Gm/18 d): large supporting cells invade the region previously occupied by hair cells (arrowheads); note a few newly formed hair cells with small tufts of stereovilli (arrows); (F) (3Gm/28 d): sensory epithelium shows a normal appearance.

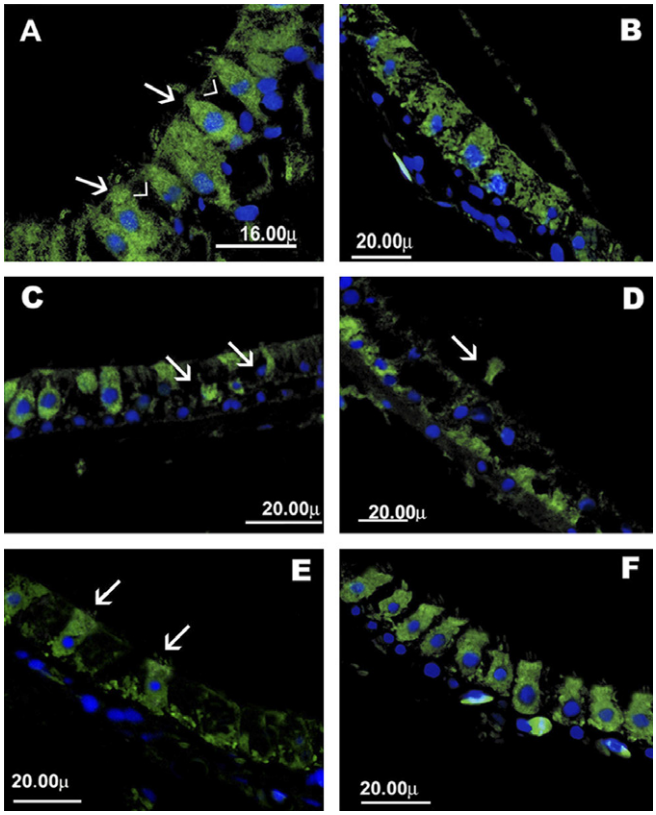

Fig. 4. CLSM: anti-calbindin (green) labels the cytoplasm of hair cells; DAPI (blue) stains the nuclei. (A) Saccule sensory epithelium of untreated lizards: anti-calbindin (green) strongly labels the cytoplasm of hair cells (arrows), but not the supporting cells' cytoplasm (arrowheads); (B) (3Gm/4 h): hair cells appear damaged; (C) (3Gm/3 d): widespread loss of hair bundles of the hair cells and damage is evident (arrows), just a few intact hair cells bodies are present; (D) (3Gm/8 d): many hair cells are lost and cellular contents are present in the lumen (arrow), but no intact hair cell bodies are evident; (E) (3Gm/18 d): note a few newly formed hair cells with small tufts of stereovilli (arrows); (F) (3Gm/28 d): sensory epithelium shows a normal appearance.

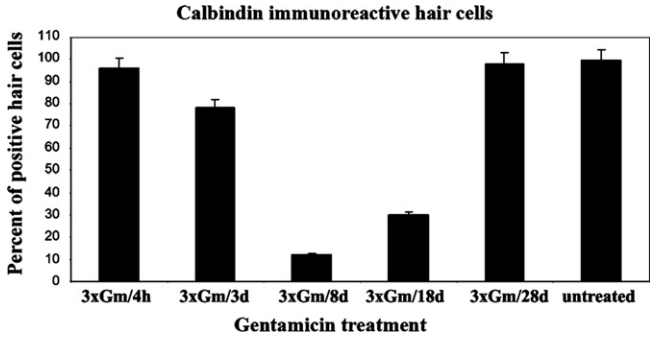

Fig. 5. Mean number of hair cells in the saccule, for each group of treated lizards (3Gm/4 h, 3Gm/3 d, 3Gm/8 d, 3Gm/18 d, 3Gm/28 d) using calbindin as marker, expressed as percentage of the mean number of hair cells in the respective control samples (untreated), ±SD. The significance of differences was $P < 0.01$ (ANOVA).

mounted on slides coated with poly-L-lysine (0.01%). The plastic was removed from the sections by immersing the slides in a solution of 2.4 M KOH in propylene oxide:-methyl alcohol (2:1) for 6 min. The slides were rinsed with methyl alcohol and washed thoroughly, first in water for 10 min and then in distilled water for 5 min. The slides were treated with 2 N HCl in PBS for 20 min at 37 °C to denature DNA and rinsed in distilled water. The sections were then incubated in: (1) protease K (4 μg/ml; Sigma) in 0.01 M Tris–HCl (pH 7.4) for 8 min at room temperature and rinsed in 0.1 M PBS and 0.5% bovine serum albumin (BSA; Sigma); (2) rabbit serum (Sigma) 1:20 in 0.1 M Tris-buffered saline (TBS), 0.25% Tween 20 and 0.5% BSA for 1 h at 37 °C; (3) anti-BrdU monoclonal antibody (Sigma) 1:1000 in 0.1 M TBS, 0.25% Tween 20 and 0.5% BSA overnight at room temperature and rinsed in the same buffer; and (4) rabbit anti-mouse IgG FITC conjugated (Sigma) 1:100 in the same buffer for 1 h at 37 °C, and finally rinsed several times in the same buffer.

Pre-immune sera, instead of specific antisera, were used in the control sections. Each step was performed in a moist chamber. The prepared sections were mounted in glycerol and observed under a Zeiss Axioskop microscope equipped with a UV lamp. Fluorescent images were captured with an Optronic camera and digitalized by PC software (Kontron KS300; Kontron Elektronik, Eching, Germany).

2.6. Statistical analysis

For each group of treated lizards, the mean number of saccule hair cells counted in 100 CLSM micrographs at a magnification of 1000×, using calbindin as marker, was expressed as a percentage of the mean number of hair cells in the respective control samples, ±SD. Statistical analysis was conducted with systematic sampling on the same regions for each group, and 75 micrographs of extrastriolar and 25 micrographs of striolar regions were analysed. Statistical analysis was performed by single factorial analysis of variance (ANOVA). *P* values less than 0.01 were considered significant.

For each group of lizards treated with Gm and BrdU, 100 sections were used for quantitative analysis of the mean numbers ±SD of labelled hair and supporting cell nuclei. Statistical analysis was performed by single factorial ANOVA. *P* values less than 0.01 were considered significant.

3. Results

The saccule sensory epithelium of untreated specimens contained hair cells with an enlarged, bulb-like kinocilium and stereovilli in an organ pipe configuration, surrounded by supporting cells with small microvilli projecting from the luminal surfaces (Fig. 1A).

The hair cells appeared compromised after one Gm injection (1Gm/4 h) (Fig. 1B). After the second Gm injection (2Gm/4 h), the hair cells appeared damaged and the stereovilli seemed to be fused (Fig. 1C); after the third Gm injection (3Gm/4 h), several hair cell bundles had disappeared from the saccule and many hair cells were abnormal, with swellings or blebs on the apical surface (Fig. 1D). Three days after the end of Gm treatment (3Gm/3 d), there was widespread loss of hair cell bundles from the hair cells in both extrastriolar and striolar regions (Fig 1E and F). The greatest damage appeared 8 days after treatment (3Gm/8 d); there was almost complete (Fig. 2A) or complete (Fig. 2B) loss of hair cell bundles. Some hair cells that had lost their hair bundles retained cuticular plates (Fig. 2A); in the region in which hair cell loss was observed, the epithelium appeared like a mosaic of supporting cells forming a 'scar'. Eighteen days after the end of Gm treatment (3Gm/18 d), there was still widespread loss of hair bundles from the hair cells in the extrastriolar and striolar regions, but some hair cells with small hair bundles, which were therefore probably immature, were present (Fig. 2C and D). Twenty-eight days after the end of Gm treatment (3Gm/28 d), the sensory epithelium had regained an apparently normal morphology comprising hair cells with an enlarged, bulb-like kinocilium and stereovilli that were graduated in length (Fig. 2E and F). This observation could indicate that a process of sensory epithelium repair and hair cell regeneration had occurred in both the extrastriolar and the striolar region.

CLSM showed actin (Fig. 3A) and calbindin (Fig. 4A) immunoreactivity in the cytoplasm of hair cells and tubulin immunoreactivity (Fig. 3A) in the cytoplasm of supporting cells. Calbindin immunolabelling was not detected in the supporting cells and there was no immunoreactive distinction between the striolar and extrastriolar regions (Fig. 4A). Incubation with pre-immune sera resulted in a complete lack of immunoreactivity (data not shown). The CLSM data for antibodies against tubulin and actin and for antibody against calbindin-D-28K were consistent with those obtained by SEM. After the third Gm injection (3Gm/4 h), the hair cells appeared damaged (Fig. 3B and Fig. 4B); 3 days after Gm treatment (3Gm/3d) there was widespread loss of the sensory cells' hair bundles and damage was evident, with just a few intact hair cell bodies present (Fig. 3C and Fig. 4C). The retention of immunoreactivity observed in the epithelium was probably due to debris from dead hair cells. The greatest damage was observed 8 days after Gm treatment (3Gm/8 d): many fragments were present throughout the body of the epithelium and hair cell debris was evident in the luminal space above the epithelium, but no intact hair cell bodies were evident (Fig. 3D and Fig. 4D). Eighteen days after Gm treatment (3Gm/18 d), with the two antibodies against tubulin and actin, the lack of hair cells persisted; expanding supporting cells had invaded the region previously occupied by hair cells, and a few newly formed hair cells with small tufts of stereovilli and properly placed nuclei were present (Fig. 3E). Even using anti-calbindin antibody as a marker for hair cells, a lack of hair cells was observed (Fig. 4E). Twenty-eight days after Gm treatment (3Gm/28 d), the

sensory epithelium appeared normal (Fig. 3F and Fig. 4F), though a reduction in tissue thickness was evident (Fig. 3F).

Fig. 5 shows the mean number of hair cells in the saccule for each group of treated lizards using calbindin as marker, expressed as percentage of the mean number of hair cells in the respective control samples, ±SD. The significance of differences was $P < 0.01$ (ANOVA).

Immunofluorescence showed evidence of BrdU incorporation in the epithelium of the saccule of lizards treated with BrdU daily from the first day of Gm injection until the day of sacrifice. A few BrdU-labeled nuclei of supporting cells (i.e. in the supporting cell layer of the epithelium) were already present in the 2Gm + 2BrdU/4 h group (Fig. 6A), and the number of labeled supporting cell nuclei increased progressively in the following order:

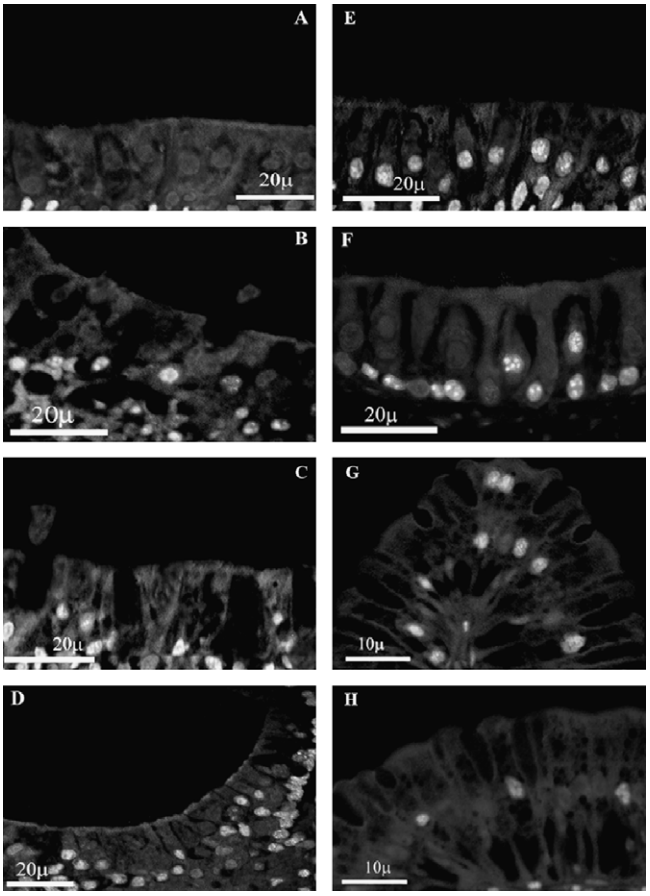

Fig. 6. Immunofluorescence of saccule treated with Gm + BrdU (A–E), and with BrdU only (F). (A) 2Gm + 2BrdU/4 h: a few BrdU-labeled nuclei of supporting cells (i.e. in the supporting cell layer of epithelium) are already present; (B) 3Gm + 6BrdU/3 d: incorporation of BrdU in hair cells nuclei (i.e. in the HC layer of epithelium) appears in a few nuclei; (C) 3Gm + 11BrdU/8 d: some BrdU-labeled nuclei of hair cell are detected; (D) 3Gm + 21BrdU/18 d: BrdU-labeled nuclei of hair cells increase; (E) 3Gm + 31BrdU/28 d: incorporation of BrdU in hair cell nuclei appear increased significantly; (F) 3BrdU/4 h: lizards treated with BrdU only show evidence of BrdU incorporation in some hair cell nuclei; (G) control intestinal sections in the Gm plus BrdU showed BrdU incorporation evidence; (H) control intestinal sections in the BrdU-only-treated lizards showed some epithelium cells with BrdU-labeled nuclei.

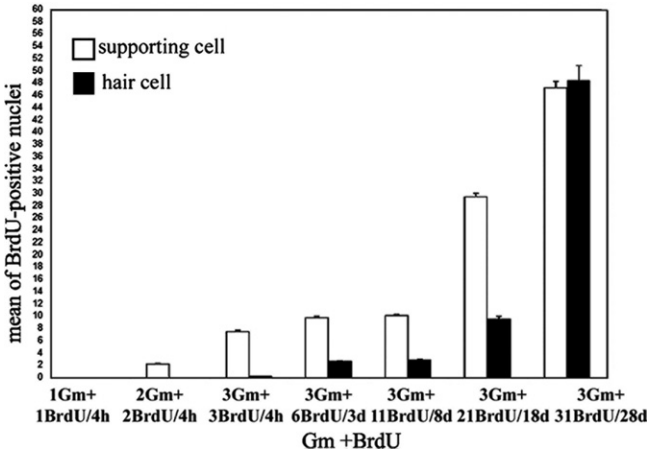

Fig. 7. Mean number of labelled hair and supporting cell nuclei, ±SD, for each group of the Gm + BrdU-treated lizards. The significance of difference was $P < 0.01$ (ANOVA).

3Gm + 3BrdU/4 h, 3Gm + 6BrdU/3 d, 3Gm + 11BrdU/8 d, 3Gm + 21BrdU/18 d and 3Gm + 31BrdU/28 d (Fig. 6B–E). BrdU was incorporated into very few hair cell nuclei (i.e. in the hair cell layer of the epithelium) in the 3Gm + 3BrdU/4 h group. Greater numbers of BrdU-labeled hair cell nuclei were detected in the 3Gm + 6BrdU/3 d, 3Gm + 11BrdU/8 d and 3Gm + 21BrdU/18 d groups (Fig. 6B–D), and the number increased significantly in the 3Gm + 31BrdU/28 d group (Fig. 6E), indicating a regenerative process. Lizards treated with BrdU only showed evidence of BrdU incorporation in some hair cell nuclei in the 3BrdU/4 h group (Fig. 6F), and the number increased progressively in the subsequent treatment groups. Control intestinal sections in both the Gm + BrdU and BrdU only-treated lizards showed evidence of BrdU incorporation (Fig. 6G and H). All negative controls showed no fluorescence.

Fig. 7 shows the mean number of labelled hair and supporting cell nuclei, ±SD, for each group of the Gm + BrdU-treated lizards. The significance of difference was $P < 0.01$ (ANOVA).

4. Discussion

In this study, on the grounds of SEM and CLSM analysis, we can conclude that Gm induces damage in hair cells and that the damage increases progressively with increasing duration of treatment. Initially, the saccule appeared unhealthy. The hair cells then became compromised, with fused stereovilli, followed by widespread loss of hair bundles from the hair cells. Eventually, numerous hair cells were lost. Subsequently, some hair cells with small hair bundles, which were therefore probably immature, were evident. Twenty-eight days after Gm treatment, the morphology of the saccule appeared normal, with no signs of ototoxic damage. This finding is consistent with Carranza et al. (1997), who demonstrated recovery of the sensory epithelium in the posterior crista of the bullfrog 4 weeks

post-Gm treatment. In contrast, Baird et al. (1993) found regenerating hair cells in the saccular and utricular maculae as early as 4–48 h after aminoglycoside treatment. Furthermore, the cellular morphology had returned to normal 7–9 days after treatment, earlier than in the present study. In a previous study (Avallone et al., 2003), we found normal morphology 10 days after Gm injection in the crista ampullaris of *P. sicula*. It is likely that the accelerated recovery was due to more limited damage caused by the use of either a smaller dose of Gm and/or a single high dose.

Calbindin immunolabelling was not detected in the supporting cells and there was no immunoreactive distinction between the striolar and extrastriolar regions. This uniformity in immunolabelling is consistent with Sans et al. (1987), Dechesne et al. (1988) and Usami et al. (1995) and with that observed with the calcium-binding proteins calretinin (Dechesne et al., 1991), parvalbumin (Dememes et al., 1993) and calmodulin (Stone et al., 1996; Ogata and Slepecky, 1998) in the vestibular end-organs of other vertebrates. Thus, in *P. sicula*, immunohistochemical labelling of calbindin provides a good marker for the detection of hair cells and enables their differentiation from supporting cells in the sensorial epithelium. Our calbindin labelling results demonstrate that the mosaic of the regenerating hair cells was nearly re-established by the 28th day after the cessation of Gm treatment in all examined regions of the saccule.

Evidence of BrdU incorporation, first in supporting cell nuclei and subsequently in hair cell nuclei, indicates that the recovery of hair cells following Gm damage in the saccule predominantly follows a pathway that includes mitotic events, and suggests that new hair cells arise from supporting cells. Studies on birds, amphibians and fish have led to the suggestion that new hair cells result from proliferation of supporting cells in these species (Corwin and Cotanche, 1988; Girod et al., 1989; Balak et al., 1990; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Tsue et al., 1994; Presson et al., 1996; Gleich et al., 1997).

On SEM, some hair cells had lost their hair bundles and retained cuticular plates. We believe that it is likely that a proportion of hair cell bundle renewal does not require mitosis but occurs due to a self-repair ability. BrdU incorporation into hair cell nuclei in BrdU only-treated lizards suggests that hair cell renewal also occurs in untreated lizards, but it is greatly amplified by Gm treatment. Our immunofluorescence and SEM studies indicate that regeneration processes are strongly implicated in the repair of damaged sensory epithelium.

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