EXPERIMENTAL NEUROLOGY 115, 2-6 (1992)

Hair Cell Regeneration in the Avian Vestibular Epithelium

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Research conducted in the past 4 years has shown that the avian vestibular system retains the capacity to generate hair cells postnatally. In the present paper we review information on postnatal proliferation and differentiation of hair cells in the avian vestibular system. In addition, we present preliminary accounts of recent experiments regarding regeneration of vestibular hair cells following aminoglycoside toxicity. The overall consensus is that the avian vestibular system is able to regenerate hair cells, both on an ongoing basis and after damage. © 1992 Academic Press, Inc.

INTRODUCTION

Until recently, it has been believed that proliferation and differentiation of hair cells in the inner ear of mammals and birds occurred only during prenatal and early postnatal development (12). Recent studies, however, have proved that production of new sensory hair cells does occur postnatally in the avian inner ear in both the auditory and the vestibular epithelia.

The functions of the vestibular system are to detect head movements and to determine the orientation of the head in space (3). These tasks are achieved by processing information that originates in the two vestibular labyrinths. Damage to the sensory epithelium of the vestibular labyrinth results in characteristic signs and symptoms that include: unsteadiness, inability to walk, head oscillations, and inability to track fast moving objects. In addition, animals lose their ability to perform difficult motor tasks such as acrobatic maneuvers in primates and flight in birds.

Each avian vestibular labyrinth houses six sensory structures: the lateral, superior, and posterior cristae ampullaris (ampullary organs) and the otolithic organs: the utricle, saccule, and the lagena. Supporting cells and two types of hair cells can be identified in the vestibular parenchyma. Hair cells are differentiated on the basis of their morphology and their innervation (16, 6). Type I hair cells are pear-shaped and nerve-enclosed. Type II hair cells have the form of an elongated cylinder, and their surface is in contact with several bouton-type nerve endings. Supporting cells form the basal layer of the epithelium. These cells are cuboidal in morphology, rest on the basement membrane, and extend to the free surface of the epithelium. On the lumenal surface of the supporting cells, several microvilli and a modified short kinocilium can be seen.

ONGOING PROLIFERATION IN THE VESTIBULAR EPITHELIUM

Jørgensen and Mathiesen (5) reported that a low, ongoing level of cell proliferation could be detected in the vestibular system of adult budgerigars (Australian parrots). Analysis of tissue from tritiated thymidine-injected animals revealed labeled cells in the sensory epithelium of both ampullary and otolithic organs. Labeled nuclei of Type II hair cells and supporting cells were seen throughout the parenchyma, indicating that newly formed sensory and supporting cells were added to the vestibular epithelium. Jørgensen and Mathiesen's study thus established that the production of vestibular sensory elements in the avian inner ear is not limited to the prenatal period.

Recently, we investigated whether the ongoing level of proliferation reported in the vestibular parenchyma of budgerigars could also be seen in chickens (11). Six-, thirteen-, and twenty-one-day-old chicks received an injection of 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg) every 12 h during a $2\frac{1}{2}$-day period. BrdU is a thymidine analogue incorporated into the DNA of dividing cells during the "S" phase of the cell cycle (14). Unlike tritiated thymidine, BrdU is antigenically distinct from normal thymidine and can be recognized by a monoclonal antibody and labeled with immunocytochemical techniques. Animals were sacrificed either 1 or 13 days after the last BrdU injection. Following intracardiac perfusion with a mixture of aldehydes, the vestibular organs were dissected, embedded in plastic, and 3-μm tissue sections were processed for BrdU-immunocytochemistry. A separate group of animals, whose tissue served as a positive control, were given five injections of tritiated thymidine (1.0 mCi/kg) and their vestibular organs were processed for tissue autoradiography.

Both BrdU-immunostaining and tissue autoradiography revealed mitotic activity in all ampullary and oto-

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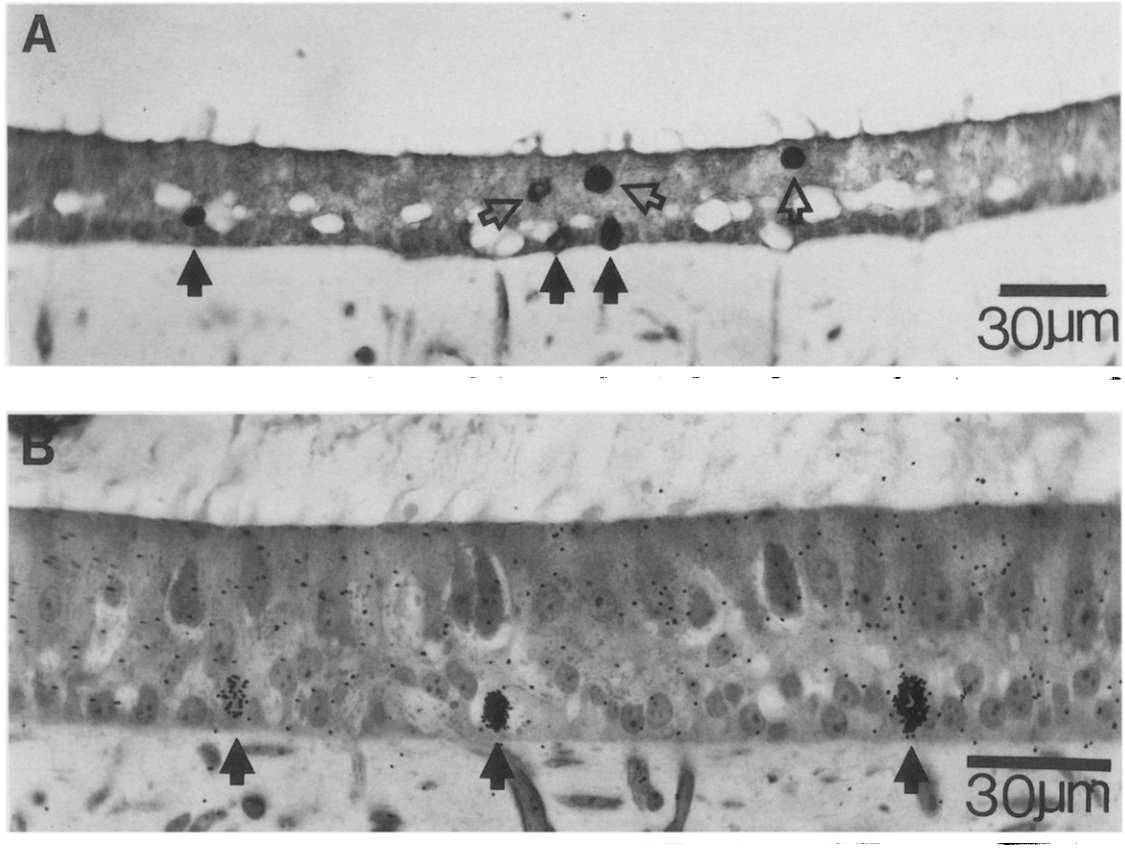

FIG. 1. Normal ongoing level of proliferation in the avian utricle. (A) This tissue, processed for BrdU-immunostaining, was obtained from an animal sacrificed 20 days after 5 days of BrdU injections. Filled arrows point to the nuclei of supporting cells that stained positively. Open arrows point to labeled Type II hair cells. (B) Ongoing proliferation as detected using tissue autoradiography. This tissue was obtained from an animal sacrificed 1 day after a 3-day course of tritiated thymidine. Three supporting cells are labeled (filled arrows).

lithic organs (Fig. 1). In the tissue of animals sacrificed 1 day after the last proliferation marker injection, positive nuclei were seen in the supporting cell layer only, next to the basement membrane. In tissue from animals allowed to survive 13 days, positive nuclei were seen in some Type II hair cells, in addition to label seen in the supporting cell layer. As previously described (5), the proliferative activity was not restricted to any specific area of the epithelium. Instead, a pattern of generalized proliferation was seen in all structures. The most significant conclusion of this study was that proliferation and hair cell differentiation also occurs in the vestibular organs of postnatal chickens. This finding provided support to Jørgensen and Mathiesen's (5) discoveries and added to the growing body of evidence indicating that cell proliferation occurs in the mature avian inner ear (1, 2, 13). Further analysis of the tissue from our experiment revealed a statistically reliable association between a labeled Type II hair cell and a labeled supporting cell, with the former located directly above the latter. In most tissue sections more than half of the labeled Type II hair cells are aligned with labeled supporting cells (Figs. 1 and 2). This finding suggests that supporting cells are progenitors of the new Type II hair cells. In addition, we were successful in labeling single supporting cells with two different proliferation markers, BrdU and tritiated thymidine. This discovery indicates that supporting cells of the mature avian vestibular system not only have preserved the capacity to proliferate, but can cycle through S phase repeatedly.

HAIR CELL REGENERATION FOLLOWING AMINOGLYCOSIDE TOXICITY

In a recent study, we sought to investigate whether the avian vestibular system possessed the ability to regenerate hair cells following drug damage and whether both Type I and Type II hair cells, normally present in the vestibular sensory epithelium, were regenerated (15). Ten-day-old chicks received daily intramuscular injections of streptomycin sulfate (600 mg/kg) for 7 days. Starting on the fifth day, these animals and age-matched control animals received twice-daily injections of tritiated thymidine (1.0 mCi/kg) for 3 days. Treatment and control animals were sacrificed 1, 20, or 60 days after the last proliferation marker injection. At the

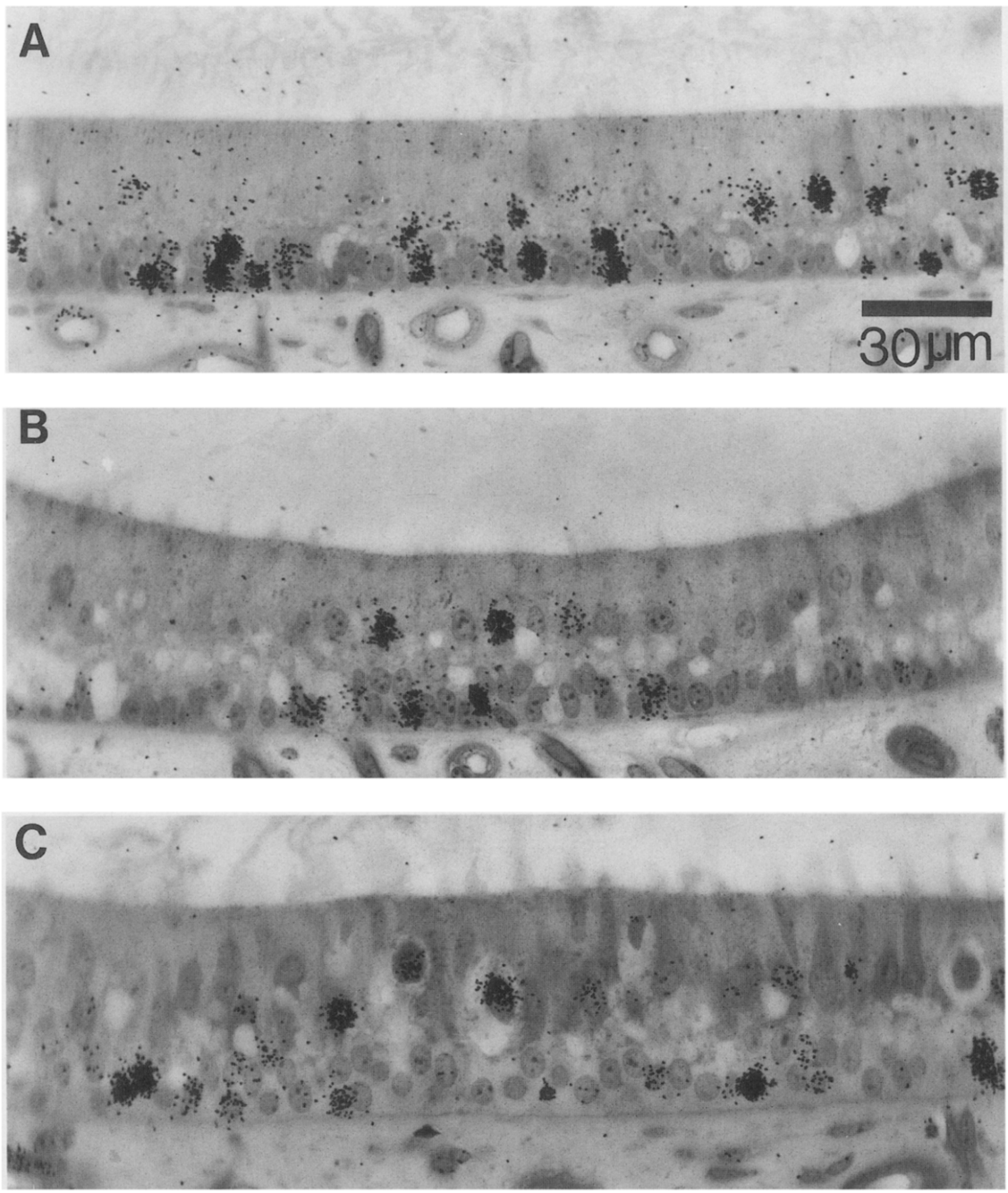

FIG. 2. Vestibular hair cell regeneration following streptomycin toxicity. (A) Histological signs of streptomycin lesion include loss of Type I hair cells and an important but less severe damage to Type II hair cells. In this utricle processed for tissue autoradiography, numerous labeled cells can be identified. (B) Twenty-day survival. Signs of regeneration include the reappearance of several Type II hair cells and a few Type I hair cells. In this micrograph, several supporting cells, and Type II hair cells have labeled nuclei. (C) Sixty-day survival. The anatomy of the utricles from treated animals is approximating that of untreated birds (see Fig. 1B). All three cell types normally present in the vestibular sensory epithelium can be easily identified. Labeled nuclei can be found on the three cell types.

appropriate time, animals were deeply anesthetized and perfused transcardially with fixative, and the vestibular organs were embedded in plastic. Following semi-thin serial sectioning the tissue was processed for autoradiography.

One day after the last aminoglycoside injection, the tissue of the streptomycin-treated animals shows clear signs of generalized toxicity (Fig. 2A). Type I hair cells cannot be found in the vestibular organs, leaving behind empty nerve chalices, and the number of Type II hair

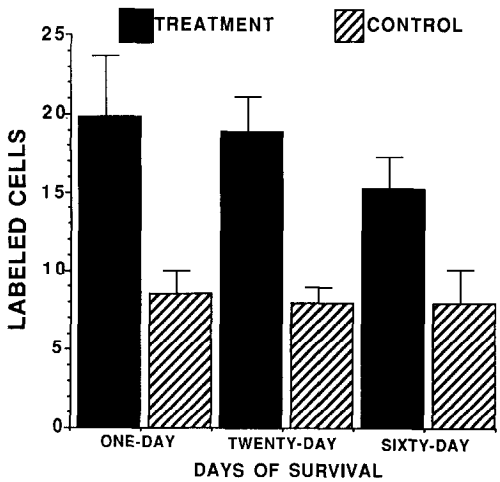

FIG. 3. Mean number of labeled cells per section per organ. The ordinate is a ratio, derived by dividing the number of labeled cells counted by the number of sections analyzed. Forty to sixty sections from throughout one superior crista ampullaris and one utricle of each animal were viewed under a microscope. The average of four animals for each group is displayed (Error bars = standard error of the mean).

cells is drastically reduced as compared to those of the untreated animals. Generalized thinning of the sensory epithelia of the treated subjects was seen, probably as a consequence of hair cell depletion. The row of supporting cells shows no conspicuous sign of damage. In many areas, however, the single layer of supporting cells has been substituted by two or three layers of cells. As previously reported (4, 7), the damage to the epithelium of the otolithic organs is not as severe as that seen in the ampullary organs, yet it is also generalized. Tissue autoradiography revealed mitotic activity in the supporting cell layer of all organs in both treated and untreated animals. The treatment group, however, had a much larger number of labeled nuclei than the untreated animals. The increase in the rate of proliferation in the treated versus untreated animals was quantified in the following manner: (i) 40 to 60 sections from one superior crista ampullaris and from one utricle of each animal were examined by light microscopy; (ii) the number of labeled supporting cells and labeled hair cells in each tissue section was counted and added to a running total; (iii) finally, in order to obtain a ratio, the total number of labeled cells was divided by the number of tissue sections examined. The average of such counts from four animals for each group is presented in Fig. 3. In the 1-day survival group, there are almost three times as many labeled cells in the tissue from streptomycin-treated animals than that of controls.

Twenty days after the last streptomycin injection, there are clear indications that regeneration of the epithelium is taking place (Fig. 2B). In the ampullary organs, numerous Type II hair cells can be seen in the sensory epithelium. Of interest, Type II hair cells can be found at the summit of the cristae as well as on the edges. In normal, untreated animals, the summit of the cristae is exclusively populated by Type I hair cells, while Type II hair cells occupy the lower $\frac{1}{4}$ of the organs. In addition to being able to identify Type II hair cells, an occasional Type I hair cell can also be seen. Some Type I hair cells are labeled while others are unlabeled. A salient feature is that the nerve calyces that can now be identified contain a single hair cell. Comparisons with tissue from untreated animals reveals that nerve calyces usually contain multiple hair cells. At this survival time, labeled nuclei were again found in supporting cells. The frequent association between a labeled hair cell and a labeled supporting cell previously reported (11) was again seen. Just as for the 1-day survival group, the number of labeled cells is reliably larger in the tissue of streptomycin-treated animals than in tissue from controls (Fig. 3).

Sixty days after the streptomycin schedule, the aminoglycoside-treated tissue looks more like the tissue from control animals (Fig. 2C). Type I hair cells can be identified inside nerve calyces. The cells are found at their usual location, and the number of hair cells per calyx has increased. Up to three hair cells can be counted inside a nerve calyx in the 3-μm sections. Although Type II hair cells can still be seen among nerve calyces in some areas, most are occupying their usual location on the skirts of the cristae. Analysis of the tritiated thymidine-labeled cells again reveals that streptomycin-treated animals have a larger number of labeled cells than untreated animals (Fig. 3).

GENERAL CONCLUSIONS

A considerable amount of information on proliferation and hair cell regeneration in the avian vestibular system has been compiled in the past 4 years. The avian vestibular system is able to generate hair cells postnatally on an ongoing basis and is able to regenerate hair cells following aminoglycoside toxicity. Moreover, all cell types normally present in the vestibular sensory epithelium can be replaced.

With the information currently available, it is reasonable to speculate that a population of supporting cells are progenitors of the regenerated vestibular hair cells. Supporting cells are the first cell-type to incorporate proliferation markers both in intact and damaged animals. In addition, this cell-type is the only one that remains in the tissue of streptomycin-treated birds. To date, supporting cells in the avian vestibular system have been grouped as one population. Detailed microscopic investigations of these cells might be able to describe different populations and establish the true precursors as has been recently done in the avian auditory system (8) and the vestibular system of fish (9).

We have shown that the level of proliferation in the avian vestibular system can be up-regulated in response to a lesion. The number of mitotic events seen after vestibulotoxicity is greater than that seen in untreated birds. This outcome indicates that proliferation in the avian inner ear is a dynamically regulated process. Additional experiments will elucidate whether more cells are coursing through the cell cycle or whether cells are cycling faster.

The vestibular system of birds and mammals share many structural and functional similarities (10). For this reason we believe that understanding the process of regeneration in the avian vestibular system may provide important clues toward the development of strategies to elicit hair cell regeneration in the mammalian vestibular and auditory epithelia.

ACKNOWLEDGMENTS

Supported by DC00395 and the Cora M. Poncin Foundation. The authors thank Dr. Elizabeth Oesterle for helpful comments on earlier versions of this manuscript and Janet Clardy for expert photographic assistance.

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