

Hair cell replacement in the avian inner ear following two exposures to intense sound

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Summary

The present study is concerned with the degree to which the avian cochlea retains the capacity to regenerate hair cells following repeated exposures to intense sound. Two groups of chicks were exposed once to an intense pure tone for 48 h at either 2 or 16 days of age. In a third group, they were exposed to the same stimulus at both ages. Structural alterations of the auditory epithelium were assessed both qualitatively and quantitatively at 0, 12 or 26 days following the single exposure at 2 days of age, and at 0 or 12 days following the single or second exposure at 16 days of age. The numbers of hair cells lost in the twice-exposed birds and those exposed once at 2 days of age were approximately 24 and 36%, respectively, and were not significantly different. Interestingly, the single exposure at 16 days of age caused greater hair cell loss (61%). Twelve days after overstimulation, the hair cell population in all experimental groups returned to near normal levels due to the emergence of new hair cells. This observation of hair cell replacement extends the early findings that birds are able to repair their acoustically damaged ears after either a single or repeated overexposure.

Introduction

Exposure to intense sound produces two localized lesions in the avian cochlear neuroepithelium: the basilar papilla (Cotanche, 1987a; Cotanche *et al.*, 1987; Henry *et al.*, 1988; Marsh *et al.*, 1990). One of these damaged areas, the stripe lesion, which appears as a narrow band of missing hair cells, is located in the middle of the epithelium of the basal region of the papilla (Cotanche, 1987a; Cotanche *et al.*, 1987; Henry *et al.*, 1988; Marsh *et al.*, 1990). The second site of injury, the patch lesion, is found on the abneural edge of the papilla in the region most sensitive to the exposure frequency, and is situated apical to the stripe lesion (Cotanche, 1987a; Cotanche *et al.*, 1987; Henry *et al.*, 1988; Marsh *et al.*, 1990). The patch lesion displays several levels of structural alterations, i.e., distorted aspects of the hair cell stereociliary bundles, altered apical surfaces of both hair and supporting cells, 30–35% hair cell loss, and damage to the tectorial membrane (Cotanche, 1987a,b; Cotanche *et al.*, 1987, 1991; Corwin & Cotanche, 1988; Henry *et al.*, 1988; Ryals & Rubel, 1988; Marsh *et al.*, 1990; Cotanche & Dopyera, 1990; Raphael, 1993; for review see Saunders *et al.*, 1992; Rubel, 1992; Cotanche *et al.*, 1994). Hair cell replacement, partial tectorial membrane regeneration, and restoration of cell surface regions have been

reported within 1 week of acoustic trauma in the bird cochlea (Cotanche, 1987a,b; Cotanche *et al.*, 1987, 1991; Corwin & Cotanche, 1988; Henry *et al.*, 1988; Ryals & Rubel, 1988; Marsh *et al.*, 1990; Cotanche & Dopyera, 1990; Raphael, 1993; for review see Saunders *et al.*, 1992; Rubel, 1992; Cotanche *et al.*, 1994).

The present study is a continuation of an earlier investigation on the effects of repeated acoustic overstimulation on the avian auditory periphery. Previously, we reported that birds recovered most of their lost hearing and partially regenerated their tectorial membrane following the second of two intense sound exposures (Adler *et al.*, 1993). In addition, overstimulation variables such as exposure age and number of exposures affected the degree of both hearing restoration and tectorial membrane injury (Adler *et al.*, 1993). Since the previous investigation had yet to determine the extent of hair cell damage and repair, the main observations of the early research helped develop two aims of the present study, that is, (1) to indicate if hair cells are replaced after multiple overstimulation and (2) to determine the degree of hair cell changes following a single or repeated exposure to intense sound. Specifically, the present investigation focuses on the extent of hair cell

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loss and replacement in the single and twice-exposed ears at various recovery periods.

Materials and methods

Experimental design

Newly hatched chicks (*Gallus domesticus*) were obtained from Truslow Farms (Cumberland, MD). The experimental and control groups have been described elsewhere (Adler *et al.*, 1993). Briefly, 'early' or 'late'-exposed birds were subjected once to a 0.9 kHz tone at 120 dB SPL (relative to 20 μ Pa) for 48 h at 2 or 16 days of age. 'Twice'-exposed animals received the same stimulus two times, the first at 2 days and the second at 16 days of age.

At the end of the exposure, the chicks were further grouped by recovery duration. The late and twice-exposed cochleae were examined at 0 or 12 days of recovery, while the early-exposed basilar papillae were considered at 0, 12, or 26 days post-exposure. Papillae were also obtained from age-matched, non-exposed control groups for comparison against the exposed birds. Each experimental or control group contained between four and 11 samples of the papilla, each from a different animal. Either *t*-tests or analyses of variance (ANOVA) for independent samples were used to determine differences among groups, and $p < 0.05$ was accepted as an indication of reliable differences.

Preparation for scanning electron microscopy

The basilar papillae were prepared for examination by scanning electron microscopy (SEM) as described in detail elsewhere (Adler *et al.*, 1993). There was, however, one modification. A partial digestion of the tectorial membrane by 0.005% protease type XXVII (SIGMA) in phosphate buffered saline (pH 7.0) for 2–5 min prior to osmication was performed in the present study. This digestion facilitated the removal of the overlying tectorial membrane from the surface of the sensory epithelium. The protease use was justified further, because it reduced the risk of damage by tweezers to the sensory cells. The protease digestion technique was first described elsewhere (Stone & Cotanche, 1992; Cotanche, personal communication).

After acetone dehydration, final dissection, critical point drying, and gold/palladium sputter-coating, the specimen was examined with an AMRAY 1400 scanning electron microscope. Photomicrographs were taken of the whole basilar papilla at 200 \times magnification and then assembled into a montage. In addition, pictures were obtained at 2000 \times magnification to provide a greater detail of individual hair cells.

Hair cell analysis

While the patch lesion was obvious on all papillae immediately following overstimulation (see for example, the bottom cochlea of Fig. 1 in Marsh *et al.*, 1990), it became less apparent at 12 or 26 days post-exposure because of the emergence of new hair cells. The 12 or 26-day recovered papillae looked very similar to those of the age-matched control animals. In order to identify the patch area in the post-exposure groups, we used as indicators some signs of structural repair such as new hair cells and/or surface

alterations of hair and supporting cells. After the patch location and borders were determined, a template was traced around the approximate region. For each recovery duration (0, 12 or 26 days), the sound-damaged ears were matched with those of control non-exposed birds on the basis of the width, length and shape of the papilla. The approximate patch site was then situated in the control papilla, and the template was used to draw a patch outline at that site. This matching method was modified from elsewhere (Marsh *et al.*, 1990).

The presence of a stereociliary bundle was used as a criterion for identifying a hair cell. All hair cells were counted within the patch outline of all exposed and control papillae. A montage exhibiting a papilla with the patch outline was placed on a digitizing tablet and the patch perimeter was traced with a stylus in order to compute the patch area. This area assessment, along with hair cell counts, allowed us to calculate hair cell density (hair cells per 1000 μm^2).

Results

The effects of a single intense sound exposure or multiple exposures on the chick inner ear at various recovery intervals were described in two ways. First, the structural aspects of hair and supporting cells were detailed in normal and acoustically damaged birds. Second, the hair cells were counted within the patch lesion in a quantitative attempt to determine the effects that the age and/or number of exposures had on hair cell density.

Structure

The normal chick basilar papilla had a crescent shape, and displayed an orderly distribution of over 10 000 hair cells (Tanaka & Smith, 1978; Tilney *et al.*, 1986; Marsh *et al.*, 1990). Figure 1A shows the hexagonal shape of the apical surfaces of control hair cells, each of which displays a strict staircase organization of the stereociliary bundle. In addition, each hair cell was surrounded by five or six supporting cells. The luminal surfaces of the supporting cells appeared mossy and slender (Fig. 1A).

The acoustically damaged inner ears exhibited both the stripe and patch lesions, as described above. The present investigation, however, considered the patch lesion only, because it was located in a cochlear region most affected by the sound exposure. Figures 1B and C illustrate the damage to the sensory epithelium immediately following the acoustic overstimulation. The most obvious consequence of the damaging stimulus was the disruption of the orderly cellular organization on the papilla surface. Many hair cells were missing, and the apical surfaces of surviving hair cells were shrunken and oval (Fig. 1B, C). In addition, hair cell stereocilia were altered such that they appeared elongated, floppy and/or fused (Figs. 1B, C). Supporting cells were affected by overstimulation,

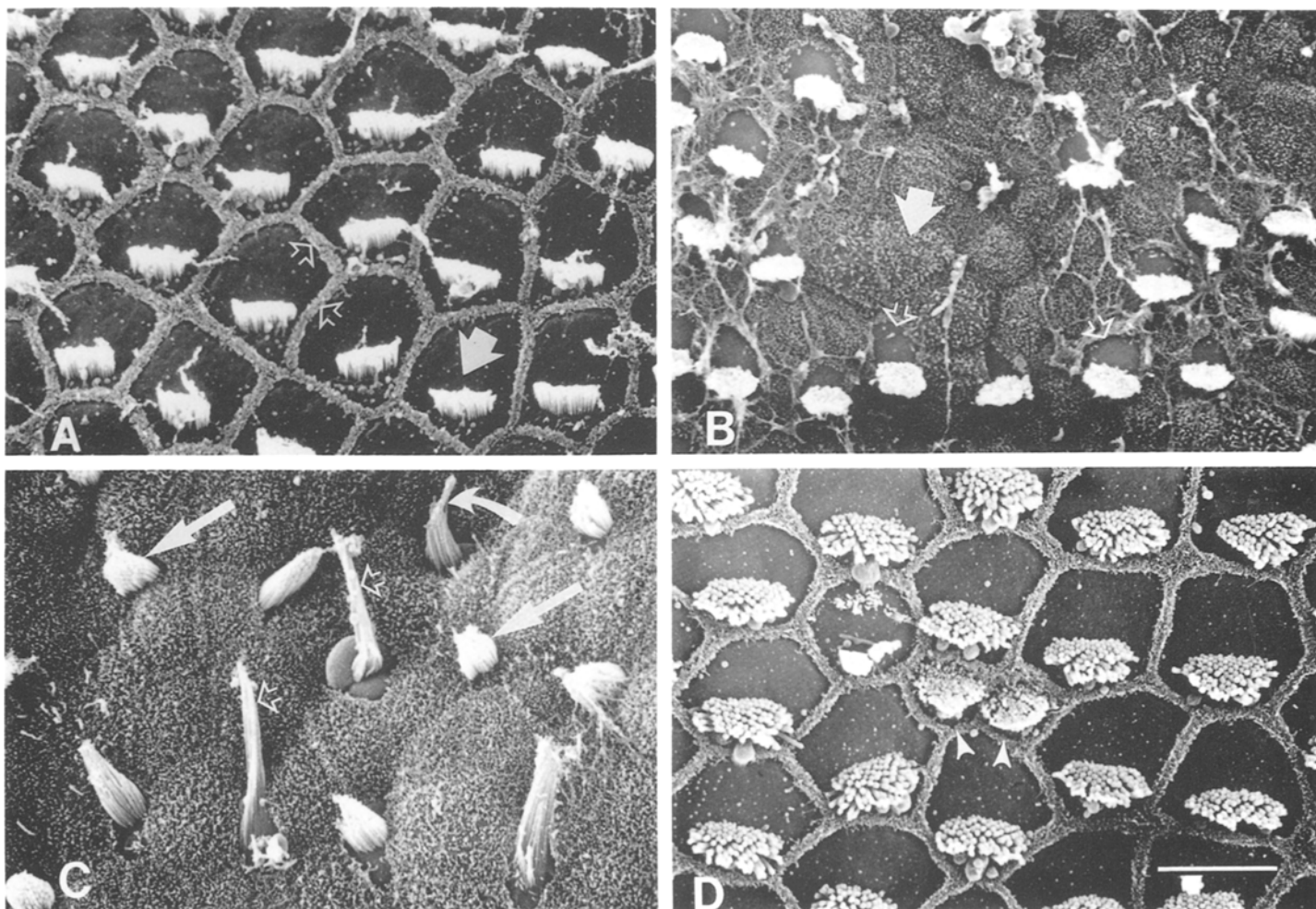


Fig. 1. Photomicrographs exhibit papilla regions at 2000 \times . Each region contains both sensory hair cells and supporting cells. (A) Control at 18 days of age. The hair cell surface area is hexagonal and smooth except for the stereocilia (solid arrow). Supporting cells have a slender, mossy luminal surface (open arrows). (B) Immediately after the early exposure to intense sound. Several hair cells are missing, and the surface appearances of both surviving hair cells and supporting cells are distorted. Note that the apical surface of hair cells is shrunken and oval (open arrows), while the supporting cells appear to expand their luminal surface, setting their microvilli apart (solid arrow). (C) Immediately after the late exposure. Note the altered organization of stereocilia. They are elongated (open arrows), fused (solid arrows), or floppy (curved arrow). (D) Twelve days following the second of two exposures. The surfaces of both hair cells and supporting cells have regained their normal appearance. A pair of new hair cells can be observed (arrowheads). Scale bar = 10 μ m.

as shown in Fig. 1B and C. Their luminal surfaces were swollen, and, as a result, displayed numerous microvilli.

New hair cells were identifiable 12 days post-exposure (on the basis of their small surface area; Fig. 1D). Moreover, the apical surfaces of both surviving hair and supporting cells, as well as the structural organization of the stereocilia, returned to near normal (Fig. 1D). These observations of hair cell damage and repair were similar to the early findings of many investigators (Saunders & Tilney, 1982; Cousillas & Rebillard, 1985, 1988; Saunders & Coppa, 1986; Cotanche, 1987a; Cotanche *et al.*, 1987, 1991; Marsh *et al.*, 1990; Cotanche & Dopyera, 1990; Raphael, 1992, 1993).

Hair cell analysis

The extent of papilla injury produced by different sound exposure variables was quantitatively assessed; Fig. 2 shows hair cell densities in early, late, and twice-exposed animals after various recovery intervals. The experimental data were compared with those from age-matched control groups.

It is important to note that patch size varied as a result of the differing exposure conditions (Adler *et al.*, 1993). Consequently, the control patch size matched to that produced by the late exposure was larger than the patch lesions induced by the early exposure or by the second of two exposures. We compared hair cell densities in the control patch sites at all ages, and determined that these densities were essentially the

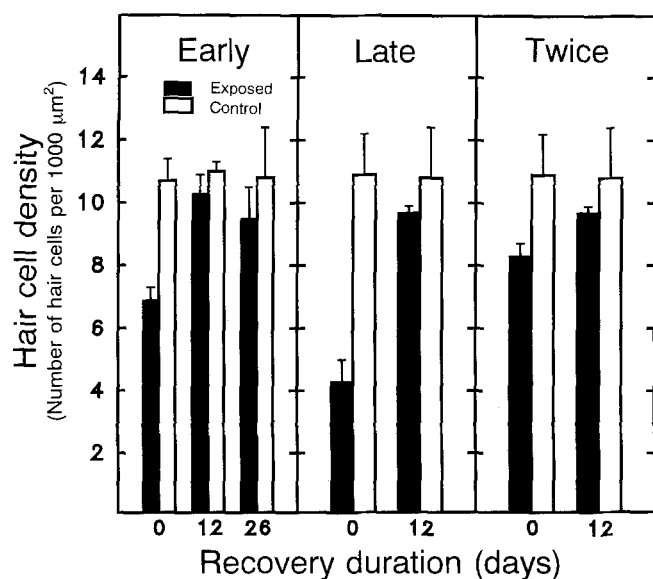


Fig. 2. Hair cell density is plotted as a function of recovery duration. At 0 days post-exposure, all experimental groups lost hair cells, but 12 days later the hair cell density returned to near normal. For details on the effects of intense sound exposure age and number of exposures on hair cell density, see text. The vertical bars indicate the standard error above the mean value.

same (Fig. 2). For statistical purposes, control data were gathered into a single group at each age.

Acoustic overstimulation caused hair cell loss in all experimental groups at 0 days post-exposure, and this is clearly seen in Fig. 2. Independent *t*-tests yielded significant differences in hair cell density between experimental and control animals at 0 days recovery (Table 1).

Exposure age and number of intense sound exposures, however, appeared to affect the degree of hair cell loss (Fig. 2; Table 2). A one-way ANOVA revealed reliable variances in hair cell loss among the three experimental groups at 0 days post-exposure ($F = 19.1$, $df = 2.19$, $p < 0.0001$). Scheffé *post-hoc* tests yielded significant differences in hair cell density between the early and late-exposed groups and between the twice and late-exposed groups (Table 2). However, a third Scheffé test revealed that the difference in hair cell density between the early and twice-exposed subjects was due to chance sampling (Table 2). Two conclusions emerged from the hair cell density analyses at 0 days post-exposure: (A) the late

Table 2. Scheffé paired comparisons tests in hair cell density among experimental groups at 0 days post-exposure.

Group comparison	Mean difference*	<i>p</i>
Early vs late	2.6	< 0.01
Early vs twice	1.4	n.s.
Late vs twice	4.0	< 0.0001

* measured in number of hair cells per 1000 μm².

Table 1. Independent *t*-tests between experimental and control hair cell densities.

Recovery duration*		Group comparison		
		Early vs control	Late vs control	Twice vs control
0	<i>t</i>	-4.95	-8.80	-4.18
	<i>df</i>	11	13	18
	<i>p</i>	< 0.001	< 0.001	< 0.001
12	<i>t</i>	-1.05	-1.37	-1.58
	<i>df</i>	7	18	20
	<i>p</i>	n.s.†	n.s.	n.s.
26	<i>t</i>	-1.42	-	-
	<i>df</i>	18		
	<i>p</i>	n.s.		

* measured in days

† n.s. = not significant

exposure produced greater hair cell loss (61%) than the early exposure (36%) at 0 days of recovery; and (B) the second of two exposures yielded less hair cell loss (24%) than seen in the late-exposed group, even though hair cell loss was measured at the same age (18 days). It is of interest to note that regions lacking hair cells (the so-called 'wedges' described by Cotanche and colleagues (1987) and Cotanche (1987a)) were found in the patch lesion after the early exposure or the second of two exposures, whereas in most of the late-exposed cochleae, a major portion of the patch exhibited a total 'wipe-out' of hair cells (Adler *et al.*, 1993).

Almost every lost hair cell was replaced after 12 days in all the exposed groups (Fig. 2), as indicated by independent *t*-tests that revealed chance sampling between control and experimental birds (Table 1). In addition, the observation that the late exposure produced greater hair cell loss than either the early exposure or the second of two exposures at 0 days post-exposure, followed by nearly complete hair cell replacement in all 12-day recovered exposed groups (Fig. 2; Table 1) indicated that more hair cells were regenerated when the lesion was larger.

The results of an independent *t*-test indicated random differences in hair cell density between control and early-exposed groups at 26 days of recovery (Table 1; Fig. 2). This chance sampling suggested that no further hair cell regeneration occurred between the 12th and 26th day post-exposure.

Discussion

The present study demonstrates directly that birds retain the capacity to replace hair cells following two traumatic acoustic overstimulations, confirming previous suggestions based on electrophysiological (Adler *et al.*, 1993) and behavioural findings (Niemi-

et al., 1994). This conclusion has received further support from recent immunocytochemical techniques that have identified new hair cells one week after the second of two octave-band noise exposures (Adler & Raphael, unpublished data). However, it would be interesting to determine whether the birds can repair their ears after many intense sound exposures.

Several investigations have shown almost complete restoration of hearing in birds after two exposures to intense sound (Adler *et al.*, 1993; Niemiec *et al.*, 1994). In addition, Niemiec and colleagues (1994) reported a significant recovery of hearing function in adult quail following a third exposure to an intense octave band. However, the question remains as to how cochlear repair contributes to auditory restoration in the injured animals. Birds that were exposed once to intense sound regained approximately 70–75% of the lost hearing by 3 days post-exposure, and this occurred before any new hair cells appeared on the sensory surface of the papilla (McFadden & Saunders, 1989; Raphael, 1992; Saunders *et al.*, 1992; Pugliano *et al.*, 1993; Niemiec *et al.*, 1994). This suggested that the repair of cochlear structures (for example, tectorial membrane regeneration and supporting cell reorganization) may play a prominent role in hearing restoration (McFadden & Saunders, 1989; Saunders *et al.*, 1992; Pugliano *et al.*, 1993; Raphael, 1993). However, the tectorial membrane may remain incompletely repaired (Cotanche, 1987b; Adler *et al.*, 1993), and as a consequence, the micromechanics of the affected ears may be abnormal prior to a subsequent exposure. Thus, it is possible that the role of the tectorial membrane and other papilla structures may vary in returning the lost auditory function to birds after multiple episodes of overstimulation. It is important to determine the roles of hair cells and other cochlear structures in the process of hearing restoration following a single or repeated acoustic overexposure.

The effects of the two exposure variables (age and number of exposures) on hair cell density confirmed and extended the findings of our previous study (Adler *et al.*, 1993). In both the previous and present investigations, it was shown that increasing exposure age resulted in a larger extent of papilla damage, and this included a higher percentage of hair cell loss and a larger lesion area. It was suggested that the greater degree of injury might be due to developmental processes of the middle and inner ears (Adler *et al.*, 1993; for structural and functional maturation in the chick middle ear, see Saunders, 1985; Saunders *et al.*, 1986, 1993; Cohen *et al.*, 1992a,b; for chick inner ear development, see Ryals *et al.*, 1984; Tilney *et al.*, 1986).

In addition, we reported a lesser degree of injury (i.e., a smaller patch lesion area and a lower percentage of hair cell loss) in the twice-exposed basilar papillae

than in the late-exposed cochleae (Adler *et al.*, 1993). This difference in inner ear damage may be due to altered micromechanics of the basilar papilla with an incompletely repaired tectorial membrane (Adler *et al.*, 1993) and/or 'protective' mechanisms of the inner ear (Raphael, 1991). However, the 'protective' processes of the basilar papilla may be in doubt, because the time interval used in our study was too long. Also, there is some disagreement between this investigation and the study of Niemiec and colleagues (1994) on hearing recovery following repeated exposures to intense sound. Adler and colleagues (1993) showed more complete hearing recovery in the twice-exposed chicks than in the late-exposed chicks, and provided support for the hypothesis that the first intense sound exposure 'protected' the ear against future exposures. On the other hand, Niemiec and colleagues (1994) indicated that an increasing number of exposures was followed by a slower rate of hearing recovery, suggesting that the 'protective' mechanisms of the cochlea may decline with multiple traumatic experiences with sound. Further study on these 'protective' processes is called for.

It is of interest to note that the late intense-sound exposure produced a greater level of hair cell regeneration in the present study than either the early exposure or the second of two exposures. This result is remarkably similar to that described in a previous investigation demonstrating a larger extent of papilla damage, (i.e., a higher percentage of hair cell loss and subsequent replacement) following a single sound exposure of greater intensity (Adler *et al.*, 1992). This similarity suggested that the processes contributing to the larger degree of hair cell replacement are the same following a more damaging stimulus, whether it occurs at an older age or from a greater sound pressure level. Possible contributing factors (for example, a larger room for inner ear repair and an increasing number of intracellular agents leading to mitotic divisions) have been described elsewhere (Adler *et al.*, 1992) and, with further research, may provide a useful tool for unravelling the processes of hair cell regeneration.

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