Scanning Microscopy

Volume 10 | Number 4 Article 18

10-7-1996

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Scanning Microscopy Vol. 10, No. 4, 1996 (Pages 1127-1142) 0891-7035/96$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

HAIR BUNDLE MORPHOLOGY ON SURVIVING HAIR CELLS OF THE CHICK BASILAR PAPILLA EXPOSED TO INTENSE SOUND

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(Received for publication March 28, 1996 and in revised form October 7, 1996)

Abstract

Exposure to intense sound produces a well-defined "patch" lesion on the chick basilar papilla in which 30-35% of the short hair cells are lost. The present study compares various aspects of sensory hair bundle morphology on surviving hair cells in the patch lesion with hair bundles from matched locations on nonexposed control papilla immediately after removal from the exposure and 12-days post exposure. The height and thickness of the hairs, the total number of hairs in the bundle, the width of the bundle, and the area and perimeter of the apical surface of the hair cell were quantified from scanning electron microscope photomicrographs. An attempt was also made to determine if there was a consistent microstructure to the pattern of hair cell loss within the lesion area. Similar observations in 12-day recovered ears are also presented.

The results indicated that stereocilia height increased and width decreased on surviving hair cells in the exposed ear. The width of the hair bundle, the hair cell surface area, and perimeter also decreased. However, the number of hairs per cell remained unchanged, and there was no evidence of any consistent organization to the hair cell loss within the patch across a number of specimens. These observations indicated that the hair bundles on short hair cells underwent changes as a consequence of intense sound exposure. The results after 12 days of recovery were complicated by developmental changes on the papilla and incomplete maturation of the newly regenerated hair cells. It remains to be seen whether these changes were the result of cell sampling in the sound-damaged ear or were due to true structural alterations within the sensory hairs themselves.

Key Words: Chick, basilar papilla, acoustic injury, hair cells, stereocilia, scanning electron microscopy.

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Introduction

Acoustic injury to the chick cochlea has become an important model not only for studying the processes associated with hair cell regeneration, but also for exploring the underlying mechanisms related to the functional recovery of the auditory system. Exposure to intense sound produces two areas of damage on the surface of the avian sensory epithelium (the basilar papilla), referred to as the "patch" and "stripe" lesions. (Cotanche, 1987a; Cotanche *et al.*, 1987).

The patch lesion is located on the abneural side of the basilar papilla at the approximate tonotopic location for the exposure frequency. Within the patch lesion, there is a 30 to 35% loss of short hair cells (Cotanche, 1987a; Henry *et al.*, 1988; Marsh *et al.*, 1990) and retraction of the tectorial membrane (Cotanche, 1987b). There are also changes to the surface organization of the sensory epithelium characterized by a decrease in the apical surface area of surviving hair cells and by an increase in surface area of the surrounding supporting cells (Cotanche, 1987a; Marsh *et al.*, 1990; Raphael, 1993; Saunders *et al.*, 1992). In addition, there appears to be some organization to the hair cell loss within the patch lesion, with regions devoid of hair cells, referred to as "wedges," frequently observed (Cotanche *et al.*, 1987).

The avian model is of particular interest in the study of acoustic injury to the inner ear for several reasons. First, the peripheral auditory system demonstrates nearly complete functional recovery within two weeks of removal from the exposure (Cohen and Saunders, 1993; Saunders *et al.*, 1996a,b). Indeed, in as short a time as three days post-exposure, a significant degree of hearing has returned (Adler *et al.*, 1992, 1993; Cohen and Saunders, 1993; McFadden and Saunders, 1989; Pugliano *et al.*, 1993a,b; Saunders, *et al.*, 1992, 1996a,b). This functional recovery is also accompanied by a remarkable degree of structural recovery. Within 12-days post-exposure, new hair cells have repopulated the patch lesion, the "honeycomb" layer of the tectorial membrane has regenerated, and the cellular organization of the sensory surface has returned to a more normal appearance (Adler

and Saunders, 1995; Cotanche, 1987a,b; Corwin and Cotanche, 1988; Henry *et al.*, 1988; Raphael, 1993). The stereociliary bundles of the newly regenerated hair cells have also attained a proper orientation on the sensory surface (Cotanche and Corwin, 1991). Although the structural recovery is impressive, it remains incomplete. Not all the hair cells lost to the exposure are replaced by newly generated hair cells (Marsh *et al.*, 1990), nor is the hexagonal mosaic of the hair cell field completely restored (Cotanche, 1987a; Henry *et al.*, 1988). In addition, only the lower layer of the tectorial membrane appeared to regenerate (Adler *et al.*, 1993; Cotanche, 1987b).

Although many aspects of structural damage and repair have been studied on the chick basilar papilla, the appearance of sensory hair (stereocilia) bundles on surviving hair cells in the patch lesion has yet to be systematically compared with the appearance of corresponding hair bundles on hair cells in the non-exposed ear. The purpose of this study was to evaluate the morphology of surviving short hair cell stereocilia in the patch lesion of the exposed ear with stereocilia on short hair cells from corresponding papilla locations in the non-exposed ear.

Materials and Methods

Subjects and exposure

One day old white leghorn chicks (*Gallus domesticus*) were obtained from a commercial breeder (Truslow Farms, Cumberland, MD). One group of chicks was exposed to a 0.9 kHz pure tone at 120 dB sound pressure level (relative to 20 μPa) for 48 hours beginning one day after hatching. A second unexposed group of age-matched chicks served as the controls. The protocol for use of animals in this study was approved by the Animal Care and Use Committee at the University of Pennsylvania.

A 40 cm diameter circular cage (made from "chicken wire") was divided into six wedge-shaped sections. The floor of the cage was located approximately 20 cm below a 30 cm diameter speaker. The acoustic stimulus was measured at approximately 3 cm and 6 cm above the cage floor for three locations in each wedge compartment. The variability in SPL was never more than $\pm$ 1.0 dB over the entire cage, and the second and third harmonics of the exposure tone were at least 45 dB below the fundamental. The exposed chicks were individually held in the compartments, and food and water were available to the animals throughout the exposure.

Tissue preparation

Immediately, or 12 days after the exposure, experimental animals and age-matched controls were euthanized with a 0.5 ml intracardiac injection of a 50% urethane (ethyl carbamate) solution. These exposed and control groups are referred to here as 0-day or 12-day recovery animals. The temporal bones were harvested from the skull and placed in culture medium (Leibowitz, L-15; Gibco Inc., Buffalo, NY). Further dissection in culture medium exposed the surface of the basilar papilla. This was accomplished by removing the overlying bony capsule, the outer membrane over scala vestibuli, and the tegmentum vasculosum. The above sequence took between four and five minutes. The specimens were then placed in an 80 μg/ml solution of Sigma type VII protease (Substilisin Carlesburg; Sigma, St. Louis, MO) for 5 minutes. The protease treatment facilitated removal of the tectorial membrane.

The tissue was then fixed at 4°C in 4% glutaraldehyde for 24 hours and then in 1% OsO_4 for 1 hour. After fixation, the specimens were dehydrated in serial rinses of acetone up to a concentration of 90%. Further dissection in 90% acetone removed the tectorial membrane and subsequently revealed the hair cell field on the sensory surface. The tissue was then prepared for scanning electron microscopy (SEM) by dehydrating it in 100% acetone, critical point drying in CO_2, and sputter-coating with gold palladium to a depth of about 0.1 μm. Both the control and exposed groups were identically prepared.

Tissue evaluation

The coated specimens were examined with a Philips (Eindhoven, The Netherlands) XE-20 SEM at an accelerating voltage of 20 kV. Photomicrographs from the SEM (obtained as either Polaroid prints or electronic videoprints) were taken from various angles to most effectively display the hair bundles so that measurement error was minimized, for example, due to the tilt of the specimen. The parameters described below were all measured from micrographs taken from individual cells.

It was necessary to identify the corresponding location of the patch lesion on the control papillae. This was accomplished by making a videoprint montage of each papilla at 200 X magnification. The specimens were oriented so that the montages presented the sensory surface as viewed from above. The patch area on exposed specimens was then outlined and its distance from the apical (distal) end of the papilla was measured. The total length of the papilla was also determined. This length varied from one ear to the next, but control and exposed samples of approximately equal length were selected as matched pairs. The location of the patch lesion was then transposed to an equivalent location on the matched control ear. The perimeter of the patch was then outlined on these matched ears and only hair cells within the confines of this perimeter were sampled. The parameters evaluated here were derived from 9 control

and 14 exposed papillae. A videoprint of each patch lesion, or the equivalent location on the control ear, was taken for each specimen. A dot was then placed on these micrographs representing the position of each sampled hair cell. We then qualitatively combined these dots onto a representative cartoon of the lesion area to assure ourselves that the sampling of hair cells was homogeneously distributed throughout the lesion.

Measured variables

Eight features of the short hair cells within the lesion were measured. Multiple variables were easily examined on pictures of the same cell, and every effort was made to distribute our measures across as many cells and papilla specimens as possible. This strategy was adopted to avoid biasing hair cell samples toward any one specimen. The mean and standard deviation were calculated for each variable, and these were statistically compared between control and exposed ears.

Stereocilia height: The height of the tallest hairs in the bundle were measured from the point of contact on the apical surface of the cell to the tip of the stereocilia. Two examples of the tallest row of hairs in the bundle are presented in Figures 1A and 1B. The actual measurements were made from the photomicrographs using the calibration bar and calipers to express the height in μm. Each hair in this row was measured and the average height over all hairs constituted the value for that cell. Every effort was made to view the tallest row of hairs at approximately right angles as shown in these two examples. In many instances this was not possible, but we estimate that the tilt of the viewing angle above the horizontal surface of the hair cell never exceeded 12°-15°.

Stereocilia thickness: The thickness (width) of each hair in the tallest row was also measured, and the average across the row constituted the estimate of hair thickness for that cell. Width was always measured at the mid-point of the stereocilia (see Figs. 1A and 1B).

Number of tallest hairs: From micrographs, such as those in Figures 1A and 1B, the total number of hairs in the tallest row were counted.

Hair bundle width: Micrographs, such as those in Figures 1A and 1B, were again used to measure the width of the bundle. This width was measured at the base of the hairs and was defined by the distance in μm between the outermost hairs in the tallest row.

Total number of hairs: The total number of hairs in the bundle were counted from images such as those in Figures 1C or 1E. The latter figures viewed the hair bundles from above. Care was taken to ensure that all of the stereocilia were visible and cells were discarded whenever there was uncertainty about identifying all the hairs.

Hair cell area and perimeter: A digitizing tablet, stylus, and computer software (Sigma Scan; Jandel Inc., San Raphael, CA) were used with micrographs such as those in Figures 1E and 1F to measure the surface area and perimeter of the apical hair cell surface. The pictures were placed on the tablet and the stylus was swept about the perimeter of the cell. The software then calculated the perimeter and area. The calibration bar on the micrograph allowed the measurements to be made directly in μm or μm^2.

Microstructure within the lesion: Six specimens were identified from all of the 0-day recovered papilla in which the width and length of the patch lesion were nearly identical. A montage of the papilla surface, as viewed from above, was then assembled at a magnification of 500 X for each of these specimens. Each montage was placed on a light box and then overlaid by a transparency on which was printed a 48 x 12 grid. Each grid square represented 256 μm^2 on the montage surface. The grid was oriented so that the 0, 0 square was placed over apical/abneural corner of the lesion (lower left corner in Figure 1D). The lower axis of the grid was then adjusted to parallel the abneural edge of the lesion. The number of hair cells contained within each grid square was then counted, and this was only done within the confines of the lesion. The presence of a hair cell was defined by the identification of a hair bundle. When a bundle was bisected by either a horizontal or a vertical grid line, a 0.5 was added to the cell counts of the grid squares bisecting the bundle. If debris on the papilla surface blocked a particular grid square, an average of the cell counts in adjacent squares to the left and right was used as an assigned value.

The data from the grid squares were used with graphic software (Sigma Plot, Jandel Inc.) to create contour plots of the patch lesion. Since we were interested in identifying areas of missing hair cells, only those contour lines representing hair cell densities of 2.5 or less per square were plotted.

Twelve-days recovery: Finally, measures of hair height, hair thickness and bundle width in the patch lesion of exposed papillae, at 12 days of recovery, were obtained. Data were also obtained from age-matched control cells at the equivalent papilla location of the patch. The procedures used in collecting these 12-day recovery data were the same as those described above.

Results

The photomicrographs in Figures 1A, 1C and 1E were obtained from control ears, while those in Figures

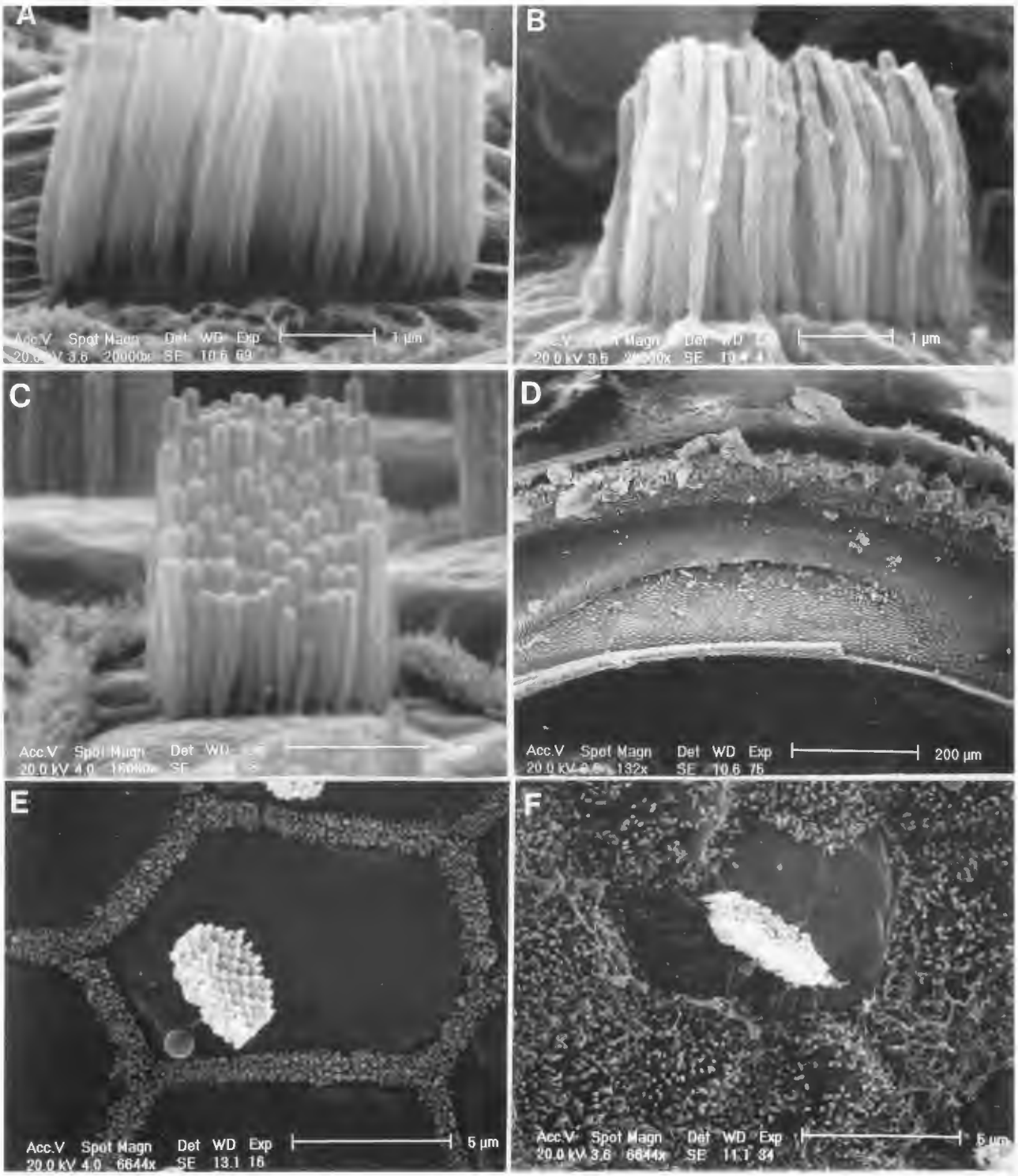

Figure 1. Figures **1A** and **1B** show the tallest row of stereocilia on a control and 0-day recovered hair cell. The reduction in bundle width and hair thickness, as well as the appearance of particles on the hairs is apparent in the 0-day recovered cell (Fig. **1B**). Figure **1C** views the hair bundle from the front and these micrographs were used to count the total number of hairs in the bundle. Figure **1D** shows the damaged tectorial membrane over the patch lesion. The superior edge of the papilla is toward the top. The apical (left side) and basal (right side) extent of the lesion were defined by the limits of tectorial membrane destruction. Figures **1E** and **1F** illustrate the apical surface of a control and 0-day recovered hair cell, respectively. The reduction in the area of the exposed cell is apparent.

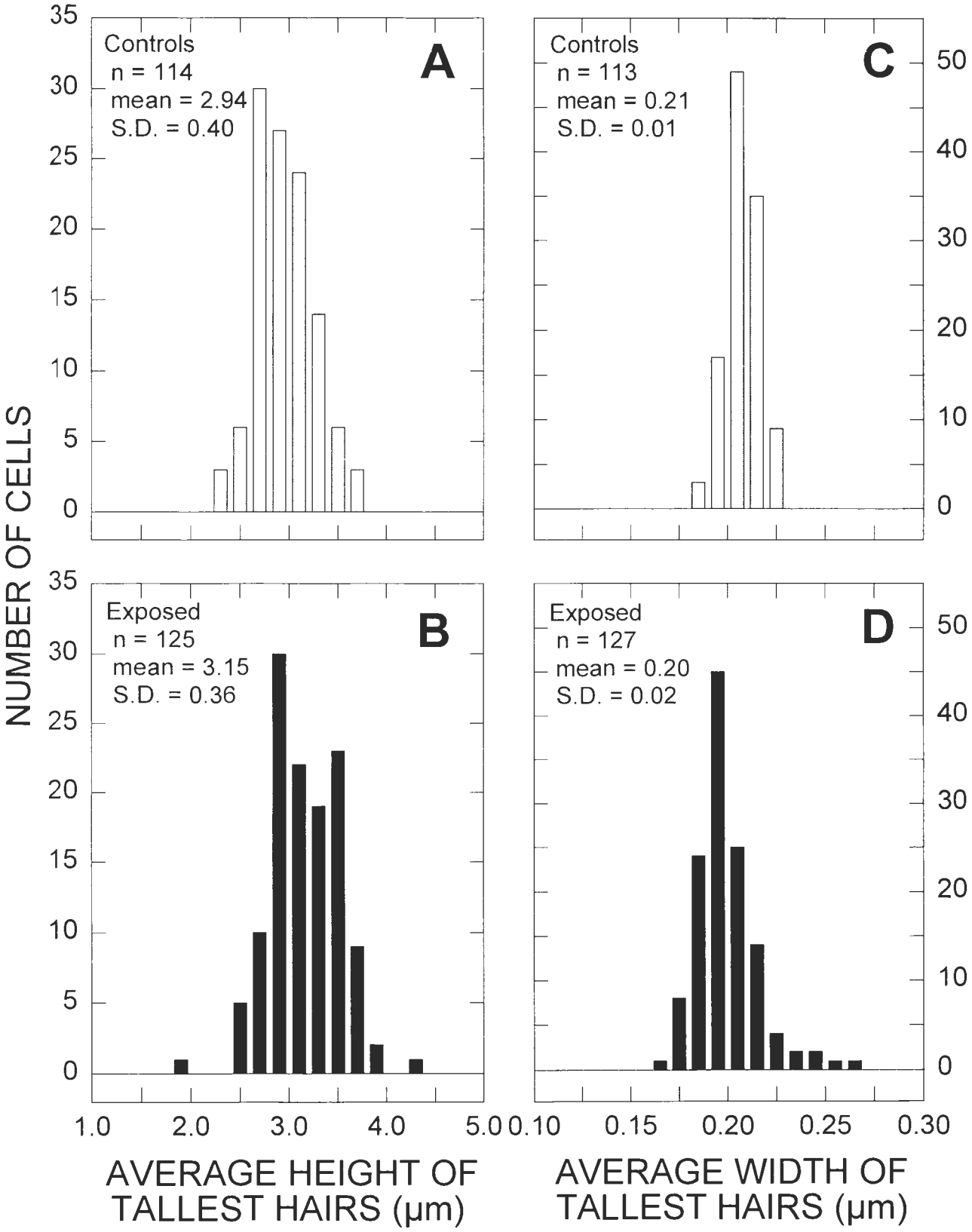

Figure 2. Figures **2A** and **2B** show frequency histograms of stereocilia height for the tallest row of hairs on control and exposed cells. Figures **2C** and **2D** illustrate the same results for hair thickness or width in the tallest row of hairs.

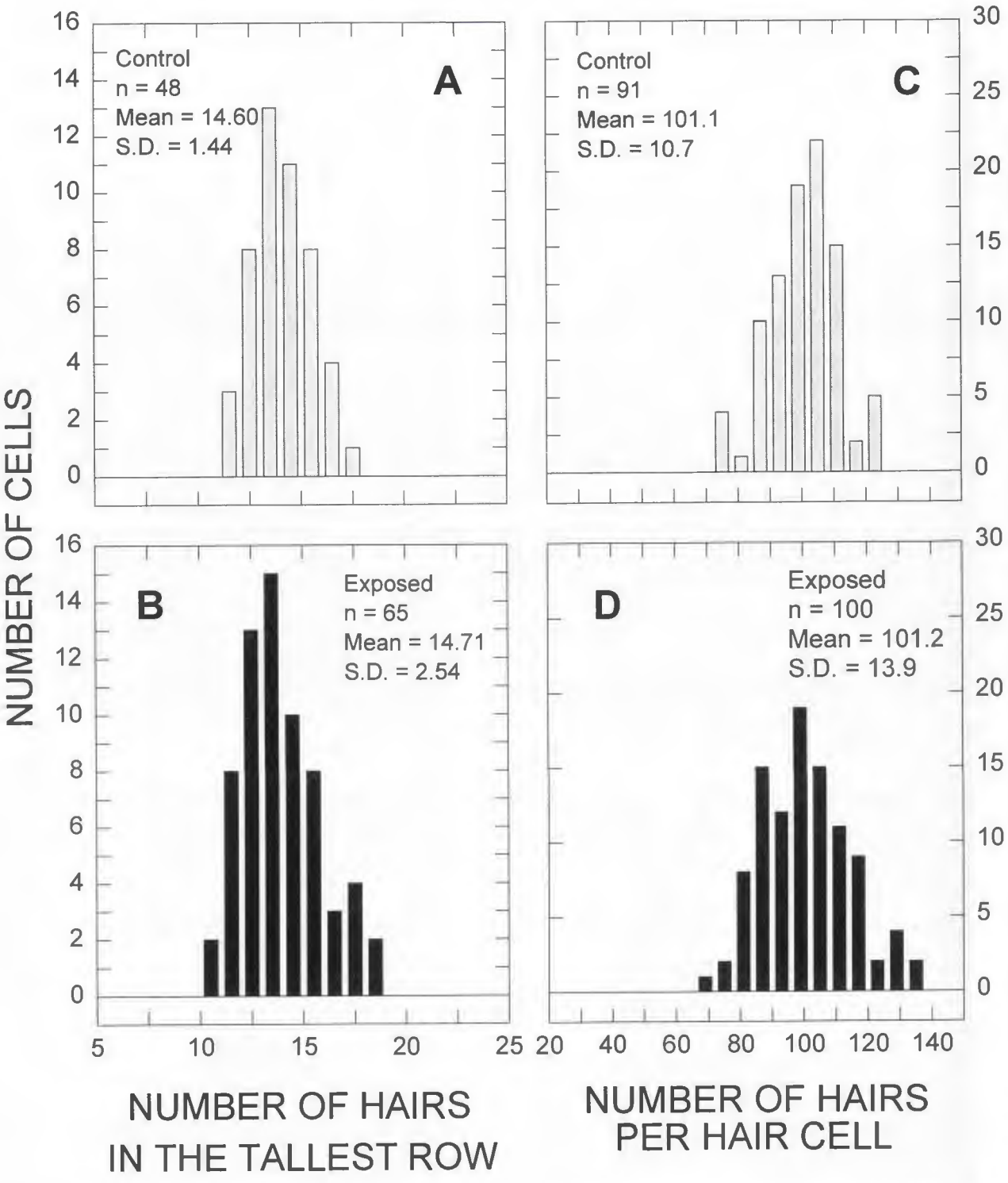

Figure 3. Figures **3A** and **3B** show histograms of the total number of hairs on the tallest row of control and exposed hair bundles. The total number of hairs on bundles in control and exposed cells is presented in Figures **3C** and **3D**.

1B, 1D and 1F came from exposed papillae. The appearance of the stereocilia in Figure 1B is typical of most exposed cells in the patch. The hairs on these cells had a wavy appearance and a membrane surface that appeared rough with the presence of spots or protrusions on the hair surface. We do not know if this latter observation represented some sort of blister on the membrane surface or cellular debris from the degenerating hair

cells or the destroyed portion of the tectorial membrane. In Figure 1D, the apical and basal perimeters of the lesion were defined by the presence of cellular damage on the papilla surface. In this example, the tectorial membrane was not removed and the extent of its disappearance defined much of the lesion perimeter. Finally, Figures 1E and 1F show the surface of a control and surviving short hair cell. A great reduction in surface area is obvious in Figure 1F, as is the increased size of the surrounding supporting cell. The perimeter of many of these surviving hair cells was quite irregular as seen in this example.

Figure 2 presents frequency histograms of hair bundle height in control (Fog. 2A) and exposed cells (Fig. 2B) fixed immediately after removal from the exposure. The mean height of the tallest hairs in control cells was 2.94 μm while in exposed cells they were 3.15 μm. A two-tailed t-test for independent samples revealed that the 0.21 μm increase (a 7% difference) in exposed hair height was statistically significant ($t = 4.37$, $df = 237$, $p < 0.01$). Figures 2C and 2D present the results for tallest hair thickness. The control hairs (Fig. 2C) were 0.207 μm in diameter while the exposed hairs (Fig. 2D) were 0.199 μm in diameter. The 0.008 μm difference between groups represented a 4% reduction in exposed hair width, and although small, this change was statistically reliable ($t = 4.78$, $df = 237$, $p < 0.01$).

The number of hairs in the tallest row of stereocilia is presented in Figure 3. There were an average 14.60 hairs on the control cells (Fig. 3A) and 14.71 hairs on the exposed cells (Fig. 3B). A t-test revealed that the 0.11 hair difference was due to chance sampling ($t = 0.25$, $df = 111$, $p > 0.05$). The total number of hairs in the bundle are also illustrated in Figure 3. There were 101.1 and 101.2 hairs on the control (Fig. 3C) and exposed (Fig. 3D) cells, respectively, and the difference in these measures was also due to chance sampling.

The analysis of hair bundle width yielded the results in Figure 4. The exposed hair bundles (Fig. 4B) were 0.39 μm narrower than the control hair bundles (Fig. 4A), and this difference was statistically reliable ($t = 7.49$, $df = 220$, $p < 0.01$).

Figure 5 presents changes in the hair cell surface area and perimeter. Both the surface area (Fig. 5A) and perimeter (Fig. 5B), when compared to the control papilla, significantly decreased in the exposed ears. Surface area decreased in exposed cells by 61% while the perimeter shortened by 31%. Both of these changes in area and perimeter were significant.

The contour plots of the patch lesion in the six exposed papilla are seen in Figure 6. We purposely made the resolution of the contour lines very great, and while each line actually represents a change in density of 0.1 of a cell, it is not meant to convey a quantitative

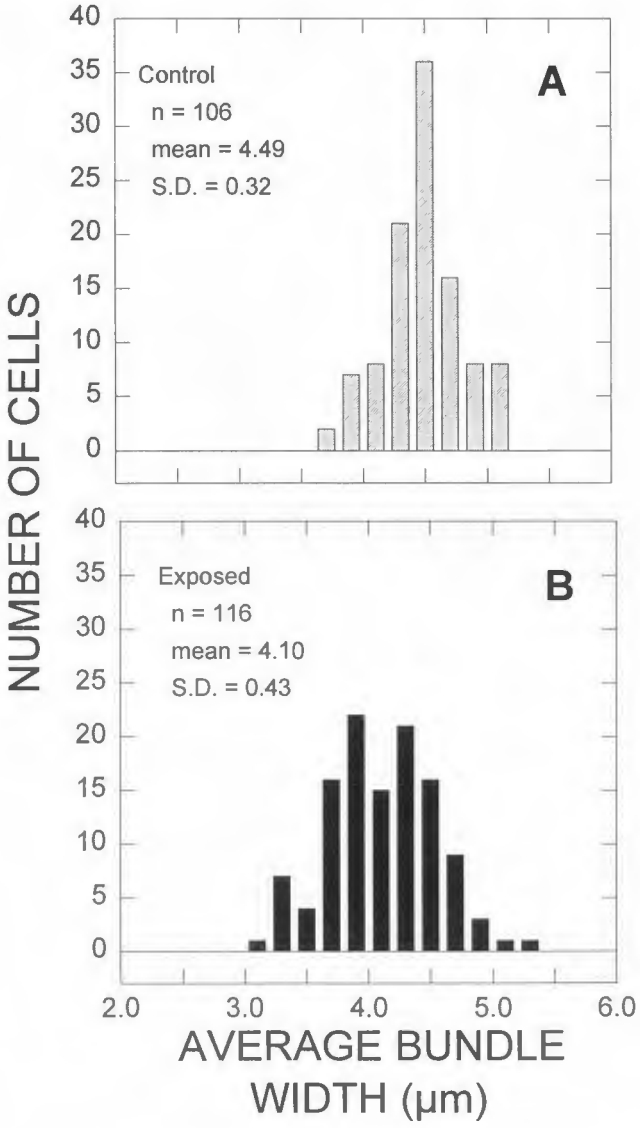

Figure 4. Histograms of hair bundle width on control and exposed hair cells are presented in Figures **4A** and **4B**.

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dimension. Rather, these representations of the patch lesion need to be seen as areas in which the cell count was normal (e.g., greater than 2.5 cells per square) or reduced. The white areas within the body of the patch represent that area of the lesion where the hair cell count was normal (see Fig. 6E). The dark areas within the patch (where the contour lines are most numerous) represent those regions of the lesion where there were missing hair cells (e.g., hair cell density was less than 2.5 cells per square). Thus, the contour lines in these reconstructions serve only to highlight areas of decreased hair cell density. The contours appear smooth because of the interpolation routines used by the graphic program

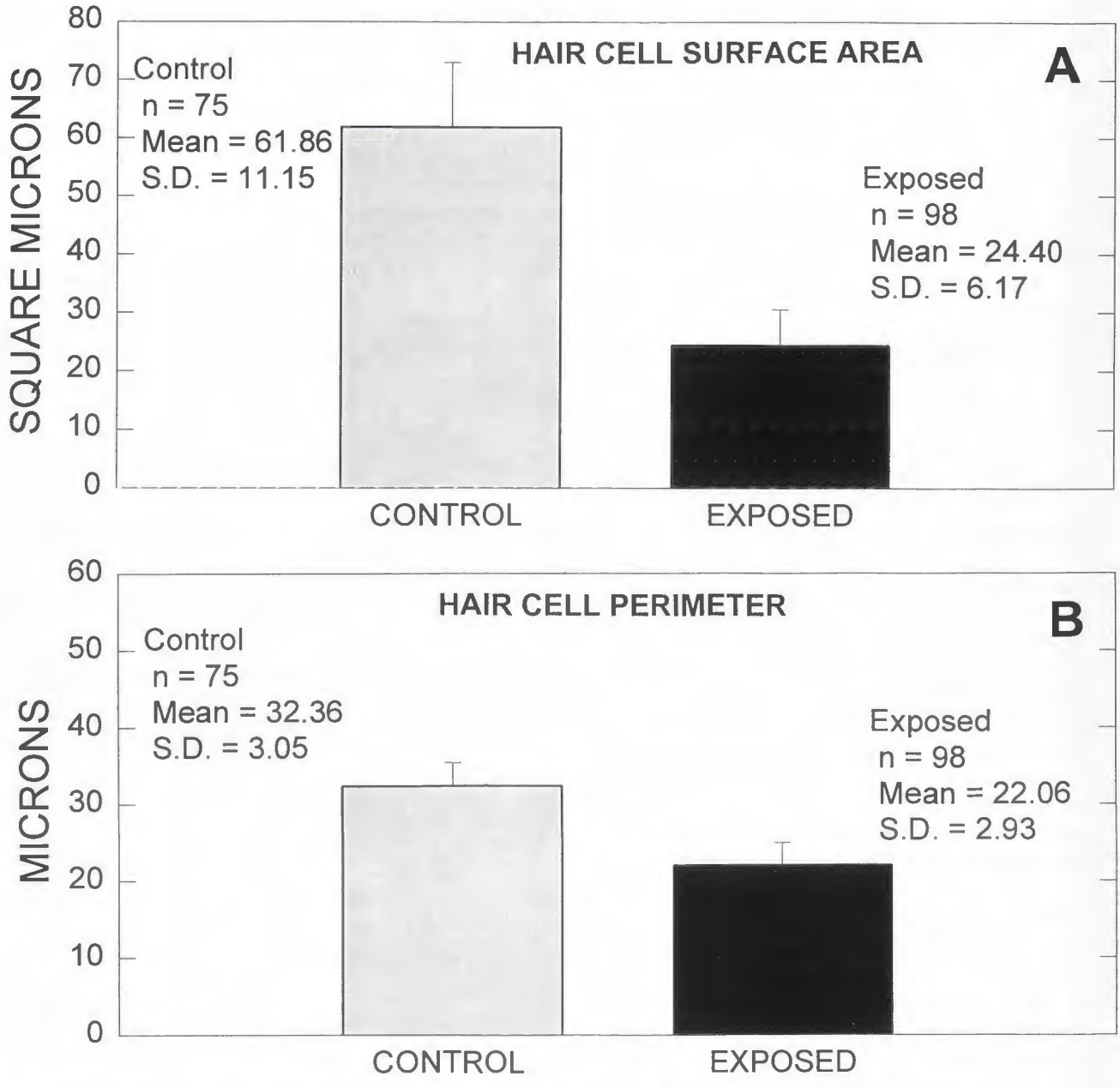

Figure 5 (above). The changes in hair cell surface area (Fig. **5A**) and perimeter (Fig. **5B**) are compared in control and exposed cells. The vertical bars represent one standard deviation above the mean.

Figure 6 (on the facing page 1135). Contour plots of six different exposed papilla are presented. The region contained within the contour lines represents cell densities less than 2.5 cells per grid square. The white areas within the boundary of the lesion has hair cell density greater than 2.5 cells per grid square. Because of the way in which cell density was measured, the outer-most contour line represents a cell density of zero. The apical portion of the lesion is to the left and the top of the plot faces the neural or superior edge of the papilla.

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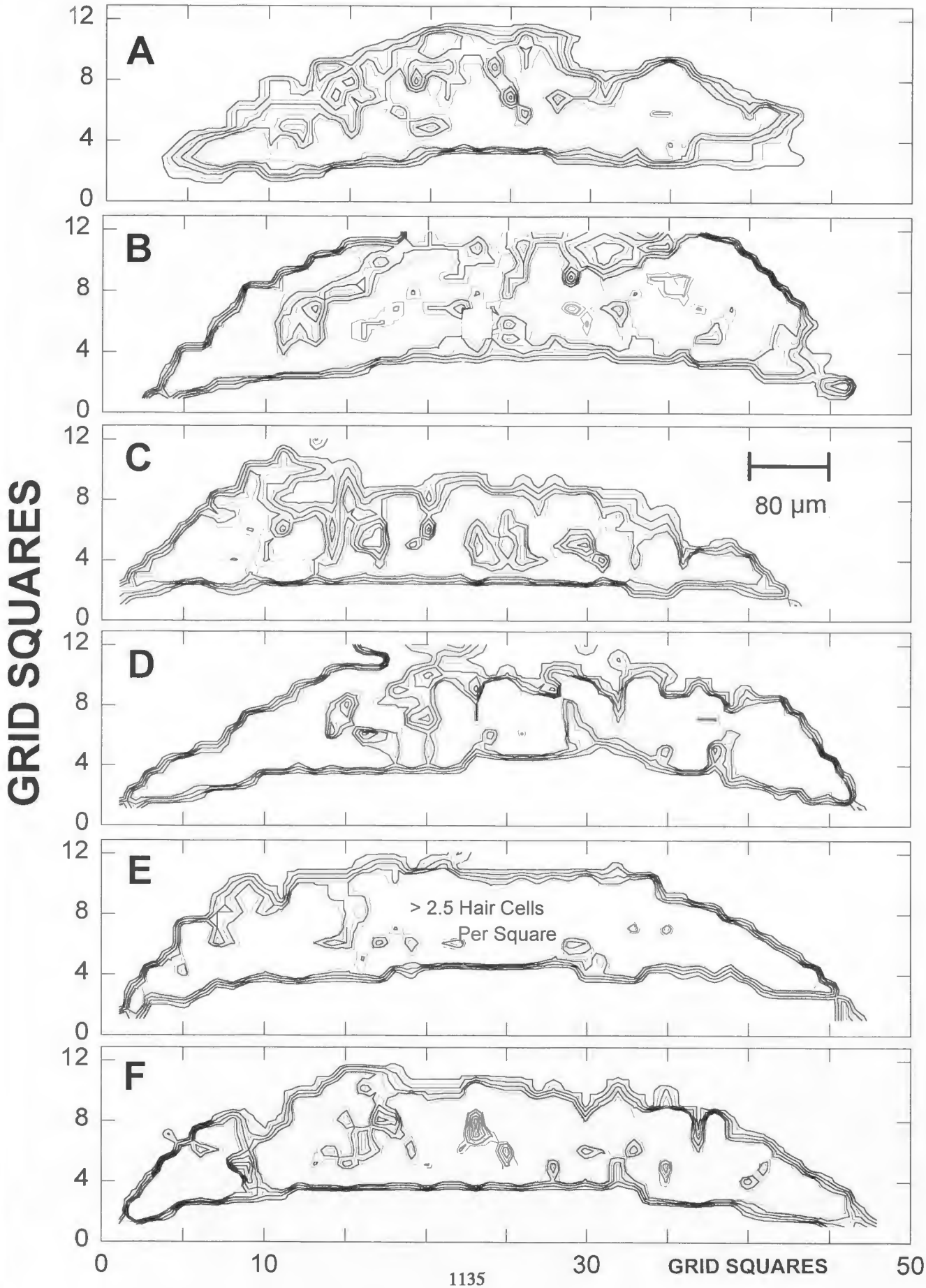
to create these graphs. In each panel of this figure (Figs. 6A-6F), fingers can be seen extending downward from the neural (top) edge of the lesion. These are the "wedges" described first by Cotanche *et al.* (1987). Also scattered throughout the six samples were islands of missing hair cells (see, for example, Fig. 6F). The inescapable conclusion from these figures is that the pattern of damage was unique to each patch. Moreover, these plots indicate that the 30-35% hair cell loss in the patch was not widely distributed throughout the lesion,


A
B
C
D
E
F
12
8
4
0
80 µm
> 2.5 Hair Cells
Per Square
GRID SQUARES
0
10
30
50
GRID SQUARES

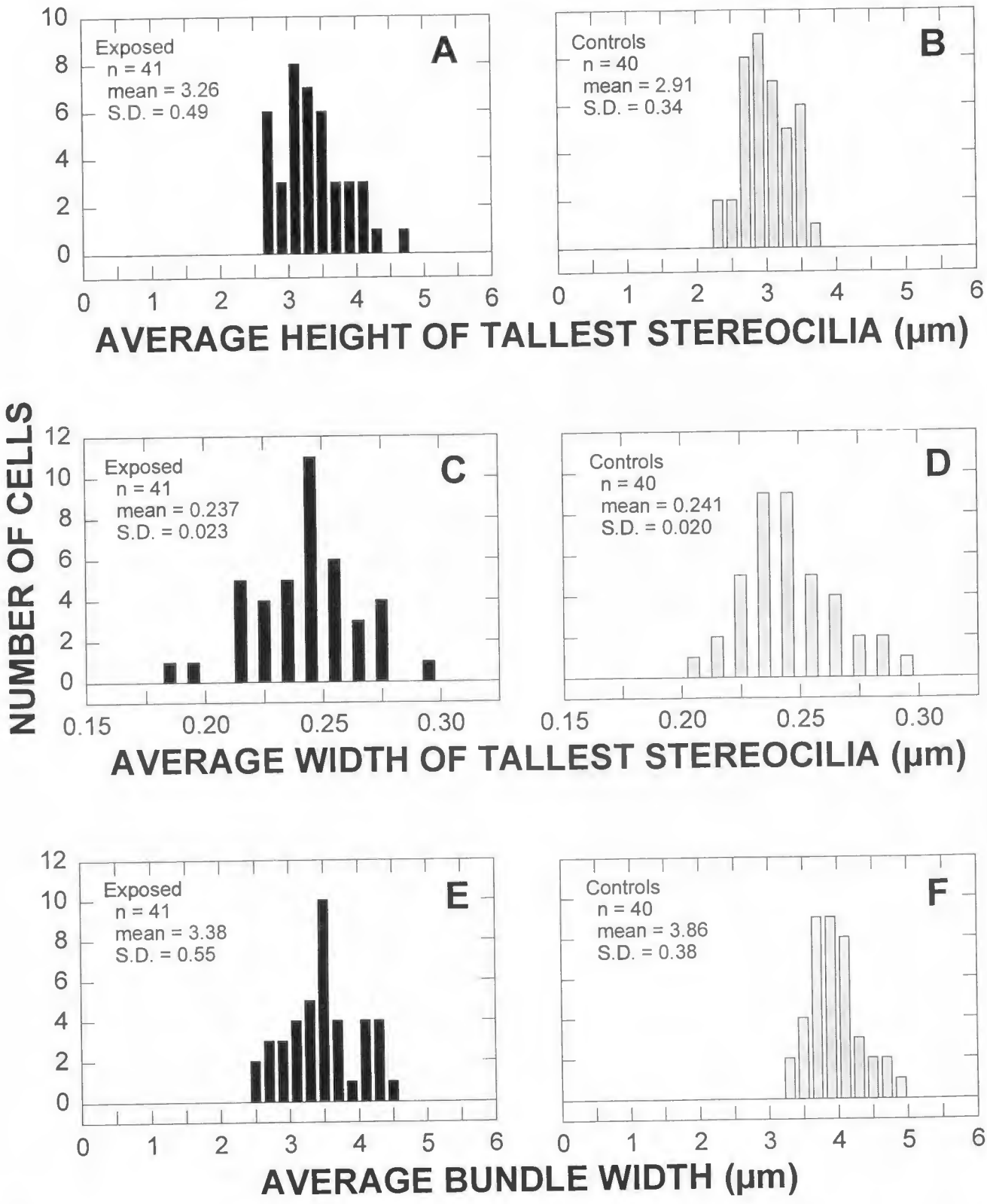


Figure 7. Histograms for the 12-day recovery and control groups are presented. Figure **7A** and **7B** show data for the height of the tallest hairs, Figures **7C** and **7D** describe the width of the tallest hairs, and Figures **7E** and **7F** illustrate the bundle width.

but was localized to the wedges or islands which seemed to be randomly distributed throughout the patch from one specimen to the next.

Figure 7 shows the results on hair height, width, and hair bundle width from exposed and age-matched control ears after 12 days of recovery. The figures in the right column (Figs. 7B, 7D and 7F) are for data from control cells, while those in the left column (Figs. 7A, 7C and 7E) are from cells in 12-day recovered ears. Figures 7A and 7B show hair height which averaged 2.91 μm and 3.26 μm, respectively, in the control and recovered samples. The 0.35 μm difference between these samples was statistically reliable ($t = 3.73$, $df = 79$, $p < 0.01$). Figures 7C and 7D show the hair thickness which was nearly identical in both groups, while Figures 7E and 7F show hair bundle width. The 0.48 μm difference in bundle width between groups was reliable ($t = 4.53$, $df = 79$, $p < 0.01$).

Discussion

The findings in the present study revealed that intense sound exposure altered the morphology of surviving hair cells and their sensory hair bundles. Most of our measures, with the exception of the tallest hair height, showed a size reduction in the exposed ears. Since the morphologic changes caused by the exposure were for the most part small, we made an effort to increase the sample size as much as possible to validate our observations.

The stereocilia responses to intense sound reported here involved a slight increase in height and reduction in diameter of the tallest hairs in the bundle. Other changes have been observed in the stereocilia of surviving chick hair cells (e.g., floppy hairs, fused hairs, missing hairs, splayed bundles, etc.). These aspects of the surviving hair cells were not examined in this paper because of their relatively infrequent occurrence (Saunders and Tilney, 1982; Saunders *et al.*, 1985).

The tallest row of hair cell stereocilia on the chick papilla are in contact with the tectorial membrane (Tanaka and Smith, 1975; Tilney and Saunders, 1983). The tectorial membrane is partially destroyed and disappears altogether over the patch lesion during the exposure (Cotanche, 1987b, 1992, Cotanche and Dopyera, 1990). Cotanche (1987a,b) felt that tectorial membrane destruction might uproot some of the tallest hairs out of the hair cell. If this happened, then the number of sensory hairs on the exposed bundles should be less than that counted on control cells. As a consequence, the average height of the tallest row of hairs in the bundle would be smaller on the surviving cells. Cotanche (1987a) examined surviving hair cells in the patch lesion and reported missing hairs in the tallest row of stereocilia, as well as a reduction in the height of the tallest hairs on surviving hair cells. His estimates of tallest row hair height, however, were based on a very small sample of cells. The observation of hair loss came from a larger sample of cells, but it appeared to be be more of a qualitative than quantitative assessment. The current results, from a much larger sample of cells, showed that the hairs increased in height in the exposed ears (Fig. 2B). Moreover, our counts of the number of hairs in a bundle (Fig. 3) showed no difference between exposed and control short hair cells. We cannot account for the differences between Cotanche's (1987a) and our observations, but they might be related to the exposure frequency which was 1.5 kHz in his study and 0.9 kHz in this one.

Others have reported shrinkage in the apical surface of surviving hair cells at 0-days of recovery (Cotanche, 1987a; Henry, *et al.*, 1988; Marsh, *et al.*, 1990; Raphael, 1993). The shrinkage is accompanied by an equally dramatic increase in the surface area of supporting cells (Cotanche and Dopyera, 1990). Many published scanning electron micrographs of the 0-day recovered papilla show what appears to be hair cells in the process of being extruded from the sensory surface (Cotanche and Dopyera, 1990; Cotanche *et al.*, 1994; Saunders *et al.*, 1992). Similarly, other cells appeared to have the cuticular plate and hair bundle "blown out" of the top of the hair cell (Cotanche, 1987a; Cotanche and Dopyera, 1990; Henry *et al.*, 1988; Marsh *et al.*, 1990). One possible explanation for this hair cell destruction is that the expanding supporting cells produce increased pressure on the plasma membrane of the hair cells which causes them to shrink and/or be "blown out" of the epithelium (Saunders *et al.*, 1992). Increased intracellular pressure might be expected to push cytosolic fluid up into the sensory hairs causing them to increase in both height and width. The present results indicated a 7% increase in height, but a 4% decrease in width (see Fig. 2). The shrinkage and expansion of the hair cell and supporting cell might also arise from osmotic imbalances due to the infusion of endolymph into the intracellular spaces of the damaged papilla (Poje *et al.*, 1995; Saunders *et al.*, 1996a). After 48 hours of exposure, it is possible that a sufficient number of hair cells have been extruded from the sensory surface thus relieving the pressure on the surviving hair cells. Nevertheless, these observations raise the interesting question as to whether or not the volume of the tallest hairs remained the same in the exposed and control ears.

If the tallest hairs in the bundle are modeled as a cylinder, then the volume of control and exposed hairs can be compared using the following equation (1):

$$\pi r_c^2 h_c = \pi r_e^2 h_e \qquad (1)$$

where r_c and r_e represent the radius of the control and exposed stereocilia, and h_c and h_e are their respective heights (see panels C and D in Fig. 2). The ratio of these two equations can be determined and expressed as a percent difference, and when this was done, the volume of the tallest hairs in control and exposed papillae differed by only 1.35%. This small difference suggested that hair volume was conserved in the two groups. We speculate that the changes in stereocilia height and width may be due to alterations in the paracrystalline structure within the core of the chick sensory hair (DeRosier *et al.*, 1980; Saunders *et al.*, 1985; Tilney and Tilney, 1986). It has been shown that excessive sound stimulation is capable of depolymerizing this paracrystalline array throughout the core of the hair or in the rootlet region (Liberman and Dodds, 1984; Mulroy, 1986; Tilney *et al.*, 1982), and these changes in the hair could be related to the observed alterations in height and width.

The decrease in bundle width by 9% is most likely related to the dramatic reduction (60%) in the apical surface area of the hair cell (Fig. 5). This reduction in surface area was probably accompanied by a compaction or shrinkage of the cuticular plate and a reduction in the inter-hair spacing at the base of the stereocilia. We tried to predict the changes in hair bundle width given the observed reduction in hair cell surface area and perimeter. However, as Figures 1E and 1F show, the apical shape of the hair cell was complex, and predicting a change in the length of a line segment (the bundle width) on the cell surface was difficult to model. Nevertheless, since bundle width at the base of the hairs was reduced, it is reasonable to expect that the hairs in the bundle should splay outwards. Figure 1B, which was typical of all exposed cells, indicates that the tallest hairs on the exposed bundle remained perpendicular to the apical surface of the hair cell. This result could be due to the reduction in hair width as seen in Figure 2D, as well as a reduction in the spacing between the individual hairs. Hair bundle width dropped by 0.39 μm on the exposed cells (Fig. 4). With an average reduction in individual hair width of 0.008 μm and an average of 14.71 hairs in the tallest row (see Figs. 2 and 3B), only 0.117 μm or 30% of the reduced bundle width could be accounted for by the reduction in hair width.

It is possible to calculate the spacing between the hairs in the bundle from various aspects of our data and then compare this inter-hair spacing in control and exposed cells. This was accomplished by using the following equation (2):

$$E_s = [b_w - (\#h_t \cdot h_w)] / (\#h_t - 1) \qquad (2)$$

where E_s is the extracellular space between adjacent tall hairs, b_w is the bundle width on exposed cells as reported in Figure 4B, $\#h_t$ is the number of hairs in the tallest row (Fig. 3B), and h_w is the average width of the tallest hairs (Fig. 2D). Solving for the extracellular space in the control ears yielded a value of 0.108 μm between hairs, while in the exposed ears it was only 0.085 μm. This 21% reduction accounts for a substantial portion of the changing width of the hair bundle. Moreover, this damage to the extracellular glycocalyx, consisting of the surface cell coat (Santi and Anderson, 1987) and the so-called side-to-side, lateral, and tip links (Csukas *et al.*, 1987; Hackney *et al.*, 1988; Pickles *et al.*, 1984), is what may account for the disarrayed appearance of the hair bundle on exposed cells. The integrity of this linkage among hairs is what is thought to maintain the cohesive appearance of the bundle. It is known that fixation of the basilar papilla for SEM affects all structures of the sensory epithelium (Adler, 1995). However, fixation artifact may not account for differences in the appearance of exposed and control hair bundles since both were prepared in the same manner.

The results in Figure 7 for the 12-day recovered ears present a somewhat confusing picture of recovery. The heights of the 0-day and 12-day control hairs were approximately the same (2.94 and 2.91 μm, respectively), while the widths of the 0-day and 12-day control hairs were different (0.207 and 0.241 μm, respectively). Similarly, the average widths of the hair bundle were 4.49 and 3.86 μm in the 0-day and 12-day control samples. During the 12-day recovery period, the length of the basilar papilla on both control and exposed ears expanded (Ryals *et al.*, 1984). Since the total number of hair cells on the papilla is constant during this time (Tilney *et al.*, 1986), there is reason to suspect that the size of individual hair cells, and perhaps their hair bundles, are undergoing developmental changes. Thus, there may be changes in the parameters of stereocilia morphology between the 0-day and 12-day control groups. The comparison between 12-day exposed and control hair cells, however, might reveal if recovery has occurred. This analysis, unfortunately, was compromised by the fact that the newly regenerating hair cells had not fully matured, and so the results in Figures 7A, 7C, and 7E are confounded by measures obtained from both surviving hair cells and new hair cells. Thus, it is not surprising, for example, that the width of the bundle in the 12-day recovered cells was smaller than at 0-days of recovery. The issue of recovery may be more clearly resolved if longer post-exposure durations (e.g., 24 days) are used in the future.

The last issue to be discussed, and perhaps the most important to consider, is whether or not the current observations resulted from actual changes in the structure of the stereocilia themselves or are the result of subtle sampling errors. These latter errors might arise because

the data obtained in control cells included measures from both surviving cells and cells that would be destroyed by intense sound in the exposed ears. In the exposed ears, however, only the actual surviving hair cells were sampled. The question is whether or not hair bundle morphology on surviving and destroyed hair cells was the same prior to exposure. We recognized this problem and sought to achieve some resolution by performing the contour analysis of the exposed papilla. We reasoned that if some consistent pattern of hair cell loss could be identified on the exposed ears, then it might be possible to identify hair cells on the control ears that would be earmarked for destruction with sound exposure. Stereocilia morphology on these cells could then be analyzed independently and compared to the morphology on the remaining hair cells in the area. Unfortunately, the results in Figure 6 revealed that there was no consistent pattern of damage within the lesion area, and the distribution of wedges and islands varied unpredictably from specimen to specimen. At the moment, this problem cannot be resolved, and so caution is needed in interpreting the changes in hair bundle morphology. In defense of the possibility that these changes reflect acoustic damage to the hair bundles themselves rather than a sampling error is the fact that all the hair cells examined were homogeneously distributed across the papilla surface in both the control and exposed samples. This does not exclude a sampling problem, but at least we know that certain regions of the lesion were not excluded across all the ears sampled.

Hopefully, methods can be developed in the future that will make it possible to identify those cells targeted for destruction from exposure to intense sound. It may yet be that some unique morphological parameter of the hair bundle renders these cells more susceptible to acoustic injury than others.

Acknowledgements

This research was supported in part by awards from the NIDCD (DC-00710) and the National Organization for Hearing Research to J.C.S., and the Nassau Fund to J.S.E. The authors appreciate the critical comments of Daryl Doan, Keith Duncan, Orville Dyce, and Stefan Plontke, as well as the excellent technical assistance of Ms. Rachel Kurian. The editorial work of Mrs. Amy Lieberman is also appreciated. Portions of this research were presented at the Mid-Winter Meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, Florida (February, 1996), and the Scanning Microscopy meeting in Bethesda, Maryland (May, 1996). J.S.E. received the Rose Award from The University of Pennsylvania for undergraduate research for this work.

References

Adler HJ (1995) Comparative analysis of patch lesions in the chick inner ear following acoustic trauma: Optical versus scanning electron microscopy. Scanning Microsc. **9**, 825-831.

Adler HJ, Saunders JC (1995) Hair cell replacement in the avian inner ear following two exposures to intense sound. J. Neurocytol. **24**, 111-116.

Adler HJ, Poje CP, Saunders JC (1993) Recovery of auditory function and structure in the chick after two intense pure tone exposures. Hear. Res. **71**, 214-224.

Adler HJ, Kenealy JFX, DeDio RM, Saunders JC (1992) Threshold shift, hair cell loss, and hair bundle stiffness following exposure to 120 and 125 dB pure tones in the neonatal chick. Acta Otolaryngol. **112**, 444-454.

Cohen YE, Saunders JC (1993) The effects of sound overexposure on the spectral response patterns of nucleus magnocellularis in the neonatal chick. Exp. Brain Res. **95**, 202-212.

Corwin JT, Cotanche DA (1988) Regeneration of sensory hair cells after acoustic trauma. Science **240**, 1772-1774.

Cotanche DA (1987a) Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. Hear. Res. **30**, 181-196.

Cotanche DA (1987b) Regeneration of the tectorial membrane in the chick cochlea following severe acoustic trauma. Hear. Res. **30**, 197-206.

Cotanche DA (1992) Video-enhanced DIC images of the noise-damaged and regenerated chick tectorial membrane. Exp. Neurol. **115**, 23-27.

Cotanche DA, Corwin JT (1991) Stereocilia bundles reorient during hair cell development and regeneration in the chick cochlea. Hear. Res. **52**, 379-402.

Cotanche DA, Dopyera CE (1990) Hair cell and supporting cell response to acoustic trauma in the chick cochlea. Hear. Res. **46**, 29-40.

Cotanche DA, Saunders JC, Tilney LG (1987) Hair cell damage produced by acoustic trauma in the chick cochlea. Hear. Res. **25**, 267-286.

Cotanche DA, Lee KH, Stone JS, Picard, DA (1994) Hair cell regeneration in the bird cochlea following noise damage or ototoxic drug damage. Anat. Embryol. **189**, 1-18.

Csukas SR, Rosenquist TH, Mulroy MJ (1987) Connections between stereocilia in auditory hair cells of the alligator lizard. Hear. Res. **30**, 147-156.

DeRosier DJ, Tilney LG, Egelman E (1980) Actin in the inner ear: The remarkable structure of the stereocilia. Nature **287**, 291-296.

Hackney CM, Furness DN, Sayers DL (1988) Stereociliary cross-links between adjacent inner hair cells.

Hear. Res. **34**, 207-212.

Henry WJ, Makaretz MO, Saunders JC, Schneider ME, Vrettakos P (1988) Age dependent loss and recovery of hair cells in the chick following exposure to intense sound. Otolaryngol. Head Neck Surg. **98**, 607-611.

Liberman MC, Dodds LW (1987) Acute ultrastructural changes in acoustic trauma: Serial-section reconstruction of stereocilia and cuticular plates. Hear. Res. **26**, 45-64.

McFadden EA, Saunders JC (1989) Recovery of auditory function following intense sound exposure in the chick. Hear. Res. **41**, 205-216.

Marsh RR, Xu L, Moy JR, Saunders JC (1990) Recovery of the basilar papilla following intense sound exposure in the chick. Hear. Res. **46**, 229-238.

Mulroy MJ (1986) Permanent noise-induced damage to stereocilia: A scanning electron microscopic study of the lizard's cochlea. Scanning Electron Microsc. 1986; IV, 1451-1457.

Pickles JO, Comis SD, Osborne MP (1984) Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. Hear. Res. **15**, 103-112.

Poje CP, Sewell DA, Saunders JC (1995). The effects of exposure to intense sound on the DC endocochlear potential in the chick. Hear. Res. **82**, 197-204.

Pugliano FA, Pribitkin E, Adler HJ, Saunders JC (1993a) Growth of evoked potential amplitude in neonatal chicks exposed to intense sound. Acta Otolaryngol. **113**, 18-25.

Pugliano FA, Wilcox T, Rossiter J, Saunders JC (1993b) Recovery of auditory structure and function in neonatal chicks exposed to intense sound for eight days. Neurosci. Lett. **151**, 214-218.

Raphael Y (1993) Reorganization of the chick basilar papilla after acoustic trauma. J. Comp. Neurol. **330**, 521-532.

Ryals BM, Creech HB, Rubel EW (1984) Post natal changes in the size of the avian cochlear duct. Acta Otolaryngol. **98**, 93-97.

Santi PA, Anderson CB (1987) A newly identified surface coat on cochlear hair cells. Hear. Res. **27**, 47-66.

Saunders JC, Tilney LG (1982) Species differences in susceptibility to noise exposure. In: New Perspectives on Noise-Induced Hearing Loss. Hamernik RP, Henderson D, Salvi R (eds.)., Ravin Press, New York. pp. 229-248.

Saunders JC, Schneider ME, Dear SP (1985) The structure and function of actin in hair cells. J. Acoust. Soc. of Amer. **78**, 299-311.

Saunders JC, Adler HJ, Pugliano FA (1992) The structural and functional aspects of hair cell regeneration in the chick as a result of exposure to intense sound. Exp. Neurol. **115**, 13-17.

Saunders JC, Doan DE, Cohen YE, Adler HJ, Poje CP (1996a) Recent observations on the recovery of structure and function in the sound-damaged chick ear. In: Auditory Plasticity and Regeneration: Scientific and Clinical Implications. Salvi RJ, Henderson D, Fiorino F, Colletti V. (eds). Thieme, New York. pp. 62-82.

Saunders JC, Doan DE, Poje CP, Fisher KA (1996b) Cochlear nerve activity after intense sound exposure in neonatal chicks. J. Neurophysiol. **76**, 770-788.

Tanaka K, Smith CA (1975) Structure of the avian tectorial membrane. Ann. Oto. Rhino. Laryngol. **84**, 287-296.

Tilney LG, Tilney MS (1986) Functional organization of the cytoskeleton. Hear. Res. **22**, 55-75.

Tilney LG, Saunders JC (1983) Actin Filaments, stereocilia, and hair cells of the bird cochlea I. length, number, width and distribution of stereocilia of each hair cell are related to the position of the hair cell on the cochlea. J. Cell Biol. **96**, 807-821.

Tilney LG, Saunders JC, Egelman E, DeRosier DJ (1982) Changes in the organization of actin filaments in the stereocilia of noise damaged lizard cochleae. Hear. Res. **7**, 181-197.

Tilney LG, Tilney MS, Saunders JC, DeRosier DJ (1986) Actin filaments, stereocilia, and hair cells of the bird cochlea III. The development and differentiation of hair cells and stereocilia. Devel. Biol. **116**, 100-118.

Discussion with Reviewers

H.J. Adler: Does intense sound cause changes in the surface morphology of hair cells outside the patch area?
Authors: Our study only examined sensory hair morphology in control and exposed papilla in the region of the patch. An examination of hair properties outside of the patch might be an interesting control, however, we did not compare hair bundles outside of the patch area because there is no indication of overt hair cell damage in these areas. The possibility exists, however, that subtle stereocilia changes may occur in regions adjacent to the patch and that could be the subject of some future examination.

M. Mulroy: Would you comment on how you distinguish between degenerative morphological changes in progress, regenerative changes in progress and the completely recovered state of stereociliary tufts after the exposure.
Authors: The current study used a single "snapshot" evaluation of the hair bundles at 0-days and 12-days of recovery. Thus, the dynamic properties of hair bundle change in terms of the parameters reported here, as well

as their recovery, remain unknown. Obviously, we do not know if the observations reported here are on the "degenerative" or "regenerative" side of a maximum. At the gross morphologic level, the degeneration of hair cells and the re-emergence of new hair cells have been described as a function of exposure and recovery duration. As noted in the text, an evaluation at recovery periods longer than 12 days may be necessary to assure that all the hair bundles (new and surviving) have achieved a stable level of recovery or maturation.

M. Mulroy: Would you speculate about the possible effect of the taller, thinner stereocilia on the frequency tuning of the sound-exposed hair cell.
Authors: The taller, thinner hair bundles identified here in the sound-exposed chick were confined to the short hair cell system. These cells carry relatively few afferent fibers, and are more richly innervated by efferent inputs from the brainstem. Without the tectorial membrane, it is doubtful that these cells play any role in tuning. Recent evidence from cochlear ganglion recordings suggest that the distribution of characteristic frequencies of turning curves does not measurably change in the ear tested immediately after the removal from the exposure (Saunders *et al.*, 1996b).

J.O. Pickles: Is it possible to tell which bundles are original and which are regenerated, and therefore analyze the two groups separately? What portion of cells are thought to be new? Is there evidence to show whether the new hair cells have shorter or narrower bundles? Do you have information on the size of the developmental changes expected in normal chicks during this period in the absence of acoustic trauma, and how much of the observed change could account for that?
Authors: In the 12-day recovered papilla, it is possible to identify newly regenerated hair cells by their smaller apical surface relative to the original surviving short hair cells. We did not make a comparison among these two populations because the regenerating hair bundles were still in an immature condition. With longer recovery durations, it becomes increasingly difficult to identify the regenerated hair cells because they look more and more like the surviving hair cells. Nevertheless, the morphology of the mature regenerated hair cell, to our knowledge, has not been studied. The development of normal chick hair bundles has been traced in a series of studies by Tilney and his colleagues (Tilney and Tilney, 1986; Tilney and Saunders, 1983; Tilney *et al.*, 1982, 1986).

J.O. Pickles: Have the authors any suggestion as to the basis of the patterns of loss shown in Figure 6?
Authors: We wish we knew why the patterns of hair cell loss differ from ear to ear. Perhaps there are patterns of micro-overstimulation on the papilla surface based on highly localized mechanical properties of the sensory epithelium, and this leads to the destruction of hair cells at that location. There could also be unique physiologic properties of the hair cell, perhaps in the distribution of membrane ion channels, that render them more susceptible to acoustic injury. We have explored here (though unsuccessfully) the possibility of a unique hair bundle morphology that might render the hair cell more vulnerable. Finally, the distribution of hair cell destruction within the patch lesion may be a stochastic or random process. The problem of why some hair cells survive or are destroyed by acoustic overstimulation is just as much a mystery in the mammalian cochlea as it is in the bird cochlea. Nevertheless, any information that improves our understanding of why there is a micro-structure to hair cell destruction within the confines of the patch lesion may provide important insight to the mechanisms of hair cell loss.

C.M. Hackney: Is it possible that the apparent change in hair bundle morphology is the result of selective loss of cells bearing shorter, wider bundles on a greater apical area? If these cells were randomly disposed amongst cells with taller narrower bundles is it possible that no consistent pattern of hair cell loss would emerge but that selective acoustic trauma could be producing your results?
Authors: Dr. Hackney's question was in part the motivation for this study because such an observation would indicate that a particular hair bundle morphology renders the short hair cells more susceptible to destruction. While her suggestion is plausible, our results do not provide any supporting evidence.

C.M. Hackney: Because of the depth of field and thus the effects of perspective, it can be difficult to obtain accurate dimensional measurements from scanning electron micrographs. What steps did you take to calibrate your measurements? Was the possibility of making measurements from stereopairs or deliberately tilted specimen considered?
Authors: The use of stereopairs for making linear measures in SEM images does not gain a great deal of accuracy and indeed the trigonometric corrections necessary to calculate true hair heights would complicate matters greatly. Our viewing angle was estimated to be between 0° and 15° above the apical surface of each hair cell sampled. This would introduce a slight underestimation of hair height. This error would be very small, however, given the hair bundle height (around 3-4 μm) and the working distance of the microscope (13,000 μm). Since we believe the viewing angle was randomly distributed among the exposed and control

conditions, the observation of differences in our height measures remained valid estimates.

Y. Raphael: The authors regard stereocilia as passive structures directly influenced by physical forces. It would be fair to the cells not to deal with their "arms" and "legs" as if they were some detached and uncontrollable passive structure. It is very likely, for example, that distorted stereocilia are a response of the cell to trauma, manifested in the stereocilia, but not necessarily reflecting primary damage. Would the authors care to comment on this?
Authors: Dr. Raphael's point is very important, and perhaps being biased by prevailing concepts of acoustic trauma, we have viewed the hair bundle changes as a consequence of damage. However, the issue with regard to this question is how to experimentally distinguish an intrinsic response of the cell to overstimulation from one which reflects structural "damage" as a direct consequence of exposure.

Y. Raphael: The choice of methods needs to be considered. Because of significant shrinkage caused by SEM preparation, SEM may actually be a poor choice. We have shown that light microscopy combined with immunocytochemistry, using non-dehydrated material, reveals elongated stereocilia after trauma. The authors need to comment on the suitability of SEM to study stereocilia morphology.
Authors: The issue of method is, of course, always important. While other procedures may prove better, we believe the current method coupled with the experimental design yields valid results. Both control and exposed papilla were prepared identically. Unless there are really strange consequences of an interaction between exposure and SEM preparation, the only uncontrolled variable was the exposure. Perhaps the best way to conduct this study is with *in vitro* preparations where there is no fixation. Unfortunately, the accuracy of measurement in unfixed tissue evaluated by light microscopy may not be sufficient to reveal the subtle types of changes reported here.

Y. Raphael: A control to determine the influence of protease treatment and the delay of fixation was not done. How can the authors be sure that the changes observed were not due to the protease or due to the delay between sacrifice and fixation?
Authors: Since both control and exposed tissue were treated identically with regard to the application of protease or the interval of time between sacrifice and fixation, it is not clear how these variables could effect one group of cells differently from the other.

Y. Raphael: Hair cells tend to extend microvilli on their apical surface after trauma, especially near the edges. How can one define the border between hair cells and supporting cells using SEM after trauma?
Authors: In our preparations, the border of apical surface of the short hair cell was relatively easy to discern.