Synchronization of hair cell regeneration in the chick cochlea following noise damage

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Summary

Pure-tone overstimulation for prolonged time leads to hair cell death in frequency-specific regions of the cochlear epithelium. Unlike mammals, birds replace missing hair cells by stimulating mitosis in an uncharacterized precursor cell. Regenerated hair cells, initially identifiable by their immature stereocilia and small surface areas, differentiate into mature cells in a manner which parallels embryonic development. In the current study, we examined whether hair cell regeneration is initiated during noise exposure or after the end of acoustic trauma. We exposed 7- to 15-day-old chicks to a 1500 Hz pure tone at 120 dB SPL (re 20 μPa) for 4, 12, and 24 hours and examined the recovering cochlear epithelium with scanning electron microscopy to determine when regenerated hair cells were first identifiable. The earliest evidence of new hair cells appeared roughly 96 hours after the onset of 4-, 12-, and 24-hour exposures. Our previous studies initially identified new hair cells 96 hours after the start of a 48-hour exposure. Therefore, hair cell regeneration follows a similar time course relative to the onset of noise exposure, regardless of the ultimate duration of exposure. Since we estimate that hair cells take at least 48 hours after their genesis to form immature stereocilia, the signal which induces hair cell precursors to re-enter the cell cycle and to divide probably has its initial effects very early during the exposure period. (A previous report of these data was given at the 1991 American Society for Cell Biology conference.)

Key words: auditory, noise damage, cell cycle.

Introduction

The sensory epithelium of the cochlea consists of two primary cell populations: hair cells and supporting cells. Hair cells, the auditory receptors, have an apical bundle of stereocilia that is arranged in a staircase configuration and anchored to the overlying tectorial membrane. The coupling of hair cell stereocilia and the tectorial membrane mediates the transduction of mechanical disturbances within the fluid of the scala media into neural signals (Lowenstein and Wersäll, 1959; Hudspeth and Corey, 1977). Hair cells in different regions of the cochlear epithelium are responsive to specific frequencies of sound, and this tonotopy is reflected in the systematic gradation in hair cell morphology along the length of the epithelium (Lim, 1980; Tilney and Saunders, 1983). Each hair cell is surrounded by several supporting cells, which have apical microvilli and secrete a portion of the tectorial membrane during development (Cohen and Fermin, 1985; Sheil and Cotanche, 1990).

Hair cells are traumatized as a result of acoustic overstimulation and exposure to ototoxic drugs. Following noise exposure, some hair cells sustain only a mild degree of structural damage and display contraction of their apical surfaces and/or splaying of the stereociliary bundle. Other hair cells are severely damaged and are expelled from the epithelium shortly after exposure to noise (Cotanche et al., 1987; Cotanche, 1987a; Cotanche and Dopyera, 1990). Balloon-like structures protruding from the cochlear epithelium into the scala media are identifiable as hair cell remnants because they have stereocilia (Cotanche et al., 1987). The tectorial membrane dissociates from hair cells in the damaged region and retracts toward the superior edge (Cotanche, 1987b; Cotanche et al., 1991). In mammals, lost hair cells are not replaced, and permanent hearing deficits ensue (Engstrom et al., 1966; Bohne, 1976; Hawkins and Johnsson, 1976; Bohne and Rabbit, 1983). However, birds and other submammalian vertebrates reconstitute the appropriate number and morphology of hair cells in the damaged region, and threshold shifts recover (Cotanche, 1987a; Cruz et al., 1987; Henry et al., 1988; McFadden and Saunders, 1989; Duckert and Rubel, 1990; Tucci and Rubel, 1990). Tritiated thymidine studies which compared normal and regenerating avian cochleae demonstrated that the precursors to new hair cells undergo
DNA synthesis and divide prior to differentiating into recognizable hair cells, but post-embryonic mitotic activity is not routine (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Girod et al., 1989; Lippe et al., 1991). Thus, with the appropriate stimulus, cell division is triggered in the mature, normally quiescent cochlear epithelium. The precursor to new hair cells, which has not been identified, probably exists in the epithelium or in its vicinity in a growth-arrested (G₀) state. In order to produce two daughter cells, this precursor must re-enter the cell cycle at the gap 1 (G₁) phase and proceed through the DNA synthesis (S) phase, the gap 2 (G₂) phase, and the mitotic (M) phase. Unlike some regenerating organs, such as the epidermis or the gastrointestinal tract, there does not appear to be a local population of stem cells poised to divide in the cochlea. Therefore, it is likely that a relatively differentiated cell leaves the growth-arrested state, re-enters the cell cycle, and generates new hair cells. These cells then develop into mature hair cells in a precise, stepwise manner that is morphologically and temporally similar to that for embryos (Cotanche, 1987a; Corwin and Warchol, 1991; Cotanche et al., 1991).

The specific mechanisms that regulate hair cell regeneration have not been characterized. However, the duration and intensity of noise during exposure clearly determine the extent of hair cell damage and recovery; longer exposure periods and higher intensities induce larger areas of damage and greater numbers of regenerated hair cells (Rubel and Ryals, 1982; Cotanche et al., 1987; Cotanche and Dopyera, 1990; Cotanche et al., 1991). Until recently, it was generally reasoned that hair cell regeneration cannot occur while acoustic trauma or ototoxic drug damage is in progress. However, results from three recent studies provide convincing, although indirect, evidence that precursor cells divide during the insult. Immature bundles of stereocilia were evident 24 hours after a 5-day treatment with an ototoxic drug, gentamicin (Duckert and Rubel, 1990). Taking into account our knowledge of the embryonic development of hair cells, this observation suggests that hair cell regeneration begins during gentamicin treatment. In the embryo, the first terminal mitoses occur in many regions of the cochlea by embryonic day 4 (Katayama and Corwin, 1989). Hair cells are first identifiable at embryonic day 6 in the distal end, since they have formed an immature bundle of stereocilia (Cotanche and Sulik, 1984). Thus, hair cells require approximately 48 hours after their genesis to differentiate and complete cell-specific profiles. It follows that the cell division necessary to yield new hair cells must be completed before this 48-hour period, during gentamicin treatment. Two different studies involving noise exposure also support the notion that hair cell regeneration is initiated during trauma. New hair cells were first identifiable between 90 and 96 hours after the onset of acoustic overstimulation periods of either 18 or 48 hours, respectively (Cotanche, 1987a; Girod et al., 1989).

In the current study, we show that the earliest evidence of newly regenerated hair cells exists 96 (± 6 hours) after the onset of three exposures of different durations (4, 12, and 24 hours). We present the hypothesis, based on this finding, that hair cell precursors re-enter the cell cycle at a similar time regardless of the final duration of acoustic overstimulation. In addition, it is highly likely that, during exposures as long as 24 hours, the cell cycle is initiated and partially completed in precursors while noise exposure is in progress.

Materials and methods

Noise exposure

One-day-old White Leghorn chicks were received from Spafas, Inc. (Norwich, CT) and housed in a thermoregulated coop with adequate food and water until noise exposure experiments were initiated. Chicks between 7 and 14 days of age were exposed in pairs to a 1500 Hz, pure-tone stimulus at an intensity of 120 dB SPL (sound pressure level re 20 μPa) for 4, 12 or 24 hours. After exposure, the birds were either killed immediately or allowed to recover for periods equivalent to 1, 2, 3, 4, 5 or 6 days after the onset of exposure (Table 1). In previous studies, we characterized the recovery period by the time elapsed from the end of exposure. However, since we were interested in knowing when new hair cells first appear after different exposures, we wanted equivalent recovery times for this study. Therefore, we measured recovery from the onset of exposure, to standardize the time scale for each exposure period. For each experiment, two chicks were placed in a chicken-wire cage in the center of a sound booth (IAC) equipped with a large mid-range loudspeaker. Following the exposure period, each pair of birds was removed from the sound booth and either killed immediately or returned to the coop for recovery. Age-matched chicks that did not undergo the noise exposure served as controls.

### Table 1. Description of chicks examined in this study

<table>
<thead>
<tr>
<th>Recovery time</th>
<th>No. of samples</th>
<th>Length of exposure</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>4 hours</td>
</tr>
<tr>
<td>No recovery</td>
<td>Chicks</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Cochlea</td>
<td>4</td>
</tr>
<tr>
<td>Time after onset of exposure (days)</td>
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<tr>
<td>1</td>
<td>Chicks</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cochlea</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Chicks</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cochlea</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Chicks</td>
<td>1</td>
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<tr>
<td></td>
<td>Cochlea</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Chicks</td>
<td>2</td>
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<tr>
<td></td>
<td>Cochlea</td>
<td>4</td>
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<td>5</td>
<td>Chicks</td>
<td>3</td>
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<td></td>
<td>Cochlea</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Chicks</td>
<td>2</td>
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<tr>
<td></td>
<td>Cochlea</td>
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The number of chicks that were exposed and the number of cochleae that were examined are listed. Some cochleae were damaged during the phases of preparation for scanning electron microscopy, and thus we did not always examine two cochleae per chick.
Preparation for scanning electron microscopy
Following the recovery period, cochleae were processed for scanning electron microscopy. Each chick was killed by intraperitoneal injection of urethane (ethyl carbamate) and decapitated, and the temporal bones were isolated in oxygenated Hank's buffered saline solution (4°C, pH 7.4). Much of the bone and cartilage around each cochlea was removed, thus exposing the tegmentum vasculosum. The remaining tissue was immersed in 0.005% protease (Sigma Type XXVII) in oxygenated phosphate buffered saline for 2 minutes to loosen the tectorial membrane from the hair cells and supporting cells. The cochlea was then fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (4°C, pH 6.4) for 1 hour, rinsed in distilled water, and dehydrated in a graded ethanol series to 70% ethanol. At this point, the tectorial membrane was removed from the sensory epithelium with microdissection forceps, exposing the cochlear epithelium. The cochlea was dehydrated, to 100% ethanol, critical-point-dried, mounted on adhesive stubs, and sputter-coated with gold/palladium.

Assessment of cellular damage and hair cell regeneration
The luminal surfaces of cochlear epithelia were analyzed with a scanning electron microscope (Amray 1000B) at an accelerating voltage of 20 kV at different stages of recovery after the three exposures in order to track the progress of recovery from noise damage and to determine the earliest time point that regenerated hair cells first appeared. New hair cells were distinguished by their smaller surface areas and shorter, less differentiated stereociliary bundles. We counted the new hair cells in five cochleae from each exposure group (4, 12, and 24 hours) on day 4 or 5 after the onset of noise exposure. Since new hair cells have never been seen in chicks that are not overstimulated, we did not count new hair cells in controls. For 4-hour exposures, all 5 cochleae were from chicks that recovered 5 days after the onset of overstimulation. For 12-hour exposures, all 5 cochleae were from chicks that recovered 4 days after the onset of overstimulation. And, for 24-hour exposures, 2 out of 5 cochleae were from chicks that recovered for 4 days after the onset of overstimulation, and the remainder recovered in 5 days. This variation in sample recovery time was necessary because we could only quantify new hair cells in cochleae that were not damaged during processing and that had the regenerated tectorial membrane thoroughly removed by protease treatment. To count the new hair cells, we spanned the damaged area in each cochlea at x5000 by sweeping from the superior edge to the inferior edge in columns roughly 35 μm wide and counted the cells which fit the morphological criteria of new hair cells. The number of new hair cells per cochlea was compared among chicks exposed for 4, 12, and 24 hours by one-factorial analysis of variance. Differences between exposure groups were considered significant if confidence levels exceeded 99% (P<0.05).

Results
The normal cochlear epithelium consists of a mosaic of hair cells and supporting cells. Hair cells are hexagonally arranged, and several supporting cells surround each hair cell (Fig. 1A,B). In most of the chicks we examined, this cellular mosaic was disrupted in a specific region of the epithelium following acoustic overstimulation. The morphological changes that occur in the basilar papilla immediately after noise exposure have been documented in detail in previous papers (Cotanche et al., 1987; Cotanche, 1987a; Cotanche and Dopyera, 1990; Cotanche et al., 1991), but they are briefly reviewed here for comparison to the events during recovery. After a 24-hour exposure, the damaged region was semicircular in shape and extended from the inferior edge of the epithelium halfway toward the superior edge (Fig. 1C). The damage centered around a mid-proximal point (between 25 and 30% of the total length of the cochlear epithelium, when the proximal end is considered to be 0%). On the proximal and distal sides of the damaged region, the structural integrity of the epithelium was retained. Hair cells that had been traumatized and were protruding from the epithelium looked like cytoplasmic balloons with stereocilia, and supporting cells displayed expanded surfaces (Fig. 1D). Damaged hair cells with splayed stereocilia and smaller than normal apical surface areas were also present in the damaged region. Following a recovery period equivalent to 15 days after the onset of exposure, little evidence of noise damage persisted. In fact, it was difficult to locate the noise-damaged region at low magnifications (Fig. 1E). However, with closer scrutiny, a few abnormal supporting cells and hair cells were still evident. In addition, new hair cells with well-developed stereociliary bundles occupied the once-damaged region (Fig. 1F) (see Cotanche et al., 1991).

Chicks that were acoustically overstimulated for 4 or 12 hours and examined after no recovery also had damage that was centered around a point between 25 and 30% of the length of the cochlear epithelium. However, the regions of damage were smaller immediately following these shorter exposures than after 24-hour exposures (see Cotanche and Dopyera, 1990). In chicks that received 4-hour exposures, noise damage was confined to a few small finger-like areas which projected from the inferior edge of the epithelium 1/4 of the way to the superior edge (Fig. 2A). Despite this diminished area of injury, the degree of damage which was sustained by cells within the region resembled that seen after longer, 24-hour exposures. Supporting cells with expanded surface areas formed large multicellular rings around the weakened hair cells (Fig. 2B). Severely damaged hair cells appeared as large blebs, occasionally with stereocilia, among the expanded supporting cells. Hair cells with limited degrees of damage, such as shrunken surface areas and disrupted stereociliary bundles, were also found in the damaged areas. The damaged region was larger immediately after 12 hours of overstimulation (Fig. 2C) than after 4-hour exposures, but it was smaller than after 24 hours of overstimulation. Supporting cells and hair cells within the damaged region resembled cells after 24-hour and 4-hour exposures and no recovery (Fig. 2D).

One goal of this study was to determine whether the damage seen immediately after the end of 4 hours of noise exposure is a representation of the full extent of damage the basilar papilla incurs, or if the damage increased throughout the first few hours of recovery. We examined the structural changes that occurred in
Fig. 1. Noise damage and recovery after 24-hour noise exposure. (A,B) Scanning electron micrograph of the 1500 Hz region of an unexposed, control epithelium at low and high magnifications. Note the normal mosaic of hair cells and supporting cells. A. Bar, 100 μm. B. Bar, 10 μm. (C) The 1500 Hz region of epithelium of a chick exposed for 24 hours and sacrificed immediately after the end of exposure exhibits a crescent-shaped area of noise damage (arrowheads). In this region, dying hair cells protrude into the luminal space. Bar, 100 μm. (D) Immediately after 24 hours of exposure, the normal, orderly array of the epithelium in the frequency-specific region is altered, the supporting cells appear expanded (arrowheads), and the hair cell apical surfaces are constricted (arrow). Bar, 10 μm. (E) Fifteen days after the onset of a 24-hour exposure, the characteristic organization of the normal epithelium is partially recovered in the 1500 Hz region. Bar, 100 μm. (F) Regenerated hair cells (arrowheads) with smaller surface areas and supernumerary stereocilia appear in the noise-damaged region 15 days after the start of a 24-hour noise exposure. Bar, 10 μm.
Synchronized hair cell regeneration

Fig. 2. Noise damage after 4- and 12-hour noise exposures. (A) Noise damage following 4 hours of exposure and no recovery involves several thin, finger-like areas of epithelium in the 1500 Hz region (arrowheads). Bar, 100 μm. (B) Ballooned hair cells (arrowhead), hair cells that have reduced surface areas or possess disrupted stereocilia, and expanded supporting cells (arrows), are found within the thin regions of damage seen immediately after 4-hour noise exposures. Bar, 10 μm. (C) Noise damage after 12 hours of exposure and no recovery consists of a semicircular region of epithelium in the 1500 Hz region (arrowheads). Bar, 100 μm. (D) The degree of cellular disruption that is apparent after 12-hour noise exposures is similar to that seen after 4 hours of exposure. Arrowheads indicate ejected hair cells. Arrows show expanded supporting cells. Bar, 10 μm.

As hair cells were lost from the epithelium, expanded

These results rule out the possibility that the trauma caused by a 4-hour exposure would lead to the same extent of damage as a 24-hour exposure if the 4-hour exposure were allowed to recover for 20 hours.

We counted ballooned hair cells at the end of the three exposures in cochleae that were completely intact (n=1 cochlea for each age). Despite this very small sample size, there was a difference in the number of ballooned hair cells relative to the length of exposure. The fewest ballooned cells (n=5) were seen at the end of 4-hour exposures, and many more ballooned cells (n=11) were present after 12-hour exposures. The most ballooned hair cells (n=28) were present after 24-hour exposures. This correlation between the number of ballooned hair cells and the length of exposure was maintained throughout recovery.

As hair cells were lost from the epithelium, expanded
supporting cells came into contact with one another. Thus, large areas that were devoid of hair cells and composed of several adjacent supporting cells became prevalent as recovery progressed. As early as 2 days after the onset of the three different exposures, the epithelium began to show distinct signs of recovery. Mildly damaged hair cells were more uniformly aligned toward the inferior edge of the epithelium, and their stereociliary bundles appeared less disrupted. New tectorial membrane was evident between 2 and 3 days after the onset of exposure in the samples in which it remained intact. However, the old and new components of the tectorial membrane were usually removed with protease treatment.

Very young regenerated hair cells were described by Cotanche (1987a) to have small apical surface areas and immature stereocilia that are slightly thicker and taller than the microvilli found on the surrounding supporting cells. He first detected new hair cells among the expanded supporting cells 96 hours after the start of a 48-hour noise exposure (equivalent to 48 hours after the end of the 48-hour exposure) (see Figs 3 and 5 of Cotanche, 1987a). In the current study, we searched for the first concrete evidence of regenerated hair cells in the epithelia of chicks that were exposed for time periods of 4, 12, and 24 hours. Regardless of the length of exposure, the first new hair cells were detected 96 hours (±6 hours) after the onset of the exposure (Fig. 3). The length and thickness of the stereocilia of these new hair cells were comparable among the chicks that were exposed for different times. Young hair cells had stereocilia that were between 0.5 and 1.0 μm in height, and were taller than the microvilli of supporting cells, which are roughly 0.5 μm high. Stereocilia on regenerated hair cells also appeared thicker than the supporting cell microvilli. New hair cells were distinguishable from the hair cells that survived noise trauma, since the tallest stereocilia on surviving cells are between 2.5 and 3.0 μm high (Tilney and Saunders, 1983). The new hair cells resembled hair cells in the mid-proximal region of the cochlea at embryonic day 8 (Cotanche and Sulik, 1984), when they first become easily distinguishable from the surrounding supporting cells. Most of the new hair cells that were identified within each cochlea were synchronized with respect to the degree to which their stereociliary bundles had differentiated.

A few important differences in regenerated hair cells following the various exposures should be noted. The apical surface areas of the regenerated hair cells appeared larger following longer exposures. In addition, more new hair cells were present following longer exposures. We examined the basilar papillae of chicks exposed for 4, 12, and 24 hours at 4-5 days after the onset of overstimulation. Chicks exposed for 4 hours had an average of 5 new hair cells, chicks exposed for 12 hours had an average of 15 new hair cells, and chicks exposed for 24 hours had an average of 34 new hair cells (Fig. 4). There was a significant difference in the number of new hairs between chicks exposed for 4 or 24 hours (P<0.05%). However, the differences between 12- and 24-hour exposures and 12- and 4-hour exposures were not significant according to analysis of variance measures (P>0.05%). The position of the new hair cells also varied with the different exposure durations. Young hair cells were apparent only in the inferior-most region of the epithelium after 4 hours of exposure, but the majority of new hair cells were found in the superior region, near where the superior fibrocartilaginous plate ends, after 12- and 24-hour exposures.

Newly regenerated hair cells continued to differentiate 4 and 6 days after the onset of 4-, 12-, and 24-hour exposures (Fig. 5). Over this recovery period, hair cell differentiation followed a similar course, regardless of the length of noise exposure. New hair cells began to elongate a couple rows of stereocilia by 5 days after the onset of exposure, thereby initiating the formation of the steplike stereociliary bundle. These new rows of stereocilia were approximately 1 μm in height. This stage of hair cell differentiation normally occurs in the proximal region in chick embryos around day 10 (Cotanche and Sulik, 1984; Tilney et al., 1988). New hair cells gradually increased their apical surface areas over the course of recovery.

Discussion

It has been the general belief that hair cell regeneration is initiated after the end of exposure to intense noise and ototoxic drugs. This belief was challenged when new hair cells were seen as early as 48 hours after noise exposure ended (Cotanche, 1987a,b) and 24 hours after the termination of gentamicin treatment (Duckert and Rubel, 1990). It is highly unlikely that cell division and differentiation, processes which require at least 24 hours each, could occur within these short recovery periods. The results of this study reinforce the idea that hair cell regeneration begins during noise exposure and ototoxic drug treatment rather than after these forms of trauma have ceased. In addition, our results lend credence to the notion that the stimulus initiating cell division of the precursors is present early during the exposure period.

We observed that newly regenerated hair cells were first apparent 96 hours (or 4 days) after the onset of exposure, regardless of whether the exposure period was 4, 12, or 24 hours. At this time of recovery, new hair cells were synchronized in their degree of differentiation, even across the different exposure conditions. In fact, all of the new hair cells we saw at 96 hours resembled developing hair cells when they first appear in the mid-proximal region of the cochlea at embryonic day 8 (Cotanche and Sulik, 1984). During embryonic development, it takes hair cells roughly 48 hours to become identifiable after cell division. Since regeneration mimics normal development, it is likely that differentiation of the new hair cells starts roughly 48 hours before they first appear in the epithelium. Therefore, hair cell precursors must exit the growth-arrest state and undergo cell division sometime during the 48-hour period preceding differentiation (Fig. 6).
Fig. 3. Synchronized appearance of new hair cells after different exposures. (A) Four days after the onset of a 4-hour exposure, several small areas of damage are still apparent in the 1500 Hz region (arrowheads). Bar, 10 μm. (B) Within the damaged region, new hair cells (arrowheads) first appear as small cells with immature stereocilia 4 days after the onset of a 4-hour noise exposure. Bar, 5 μm. (C) The area of damaged epithelium is larger 4 days after the onset of a 12-hour exposure than at the equivalent recovery time from a 4-hour exposure (arrowheads). Bar, 10 μm. (D) Regenerated hair cells first appear 4 days after the onset of a 12-hour noise exposure (arrowheads). Bar, 5 μm. (E) One portion of noise-damaged epithelium 4 days after the onset of a 24-hour exposure is shown. The area of damage (arrowheads) is usually larger than the damaged area present after shorter exposures of 4 and 12 hours. Bar, 10 μm. (F) Newly regenerated hair cells are first apparent 4 days after the onset of a 24-hour noise exposure (arrowheads). Bar, 5 μm.
Length of exposure

Fig. 4. Quantification of new hair cells. The mean number of regenerated hair cells present on days 4 or 5 after the onset of 4, 12, and 24 hours of noise exposure are graphed. Five cochleae were analyzed for each different exposure. The number of new hair cells increased with the length of noise exposure. This difference was statistically significant according to analyses of variance between 4- and 24-hour exposures, but not between 4- and 12-hour exposures or 12- and 24-hour exposures. Error bars represent standard error of the mean.

Our preliminary investigations of cell division in the recovering cochlear epithelium confirm this estimate (Stone and Cotanche, unpublished results). We injected bromodeoxyuridine (BrdU), an S phase marker, into chicks at various stages of recovery from 4- and 24-hour noise exposures and performed anti-BrdU immunohistochemistry on whole-mount preparations of the cochleae. Cells entered S phase as early as 28 hours after the onset of 24-hour exposures. Our future experiments will further define the S phase period for the various exposures in order to determine whether this stage of the cell cycle is truly synchronized in epithelia recovering from noise exposures of different lengths.

From our observations, it is likely that the events leading to cell division occur during acoustic overstimulation and ototoxic drug treatment when acoustic trauma lasts for long periods, such as 24 and 48 hours. Interestingly, the degree of cellular damage which is sustained during our method of acoustic injury does not hamper the capacity of hair cell precursors to divide. Therefore, the acoustic trauma primarily affects hair cells, which are mechanically equipped to directly respond to acoustic stimulation. Nonsensory cells may not be damaged by the noise levels used in this study. However, levels of higher intensities induce more widespread damage and injure the nonsensory, supporting cells.

The fact that the first new hair cells appear at a synchronized time - 4 days after the onset of 4-, 12-, and 24-hour exposures - suggests that hair cell regeneration is initiated at a similar time despite different exposure durations, perhaps as early as 4 hours after the start of acoustic overstimulation. This theory is currently only speculative and relies heavily on the assumption that the genesis of new hair cells by cell division and

Fig. 5. Synchronized differentiation of new hair cells after different exposures. (A) Regenerated hair cells (arrowheads), 5 days after the onset of a 4-hour exposure. Stereocilia elongate and apical surface areas appear to increase between 4 and 5 days after the onset of noise exposure. Bar, 5 μm. (B) New hair cells (arrowheads), 5 days after the onset of a 12-hour exposure. Bar, 5 μm. (C) New hair cells (arrowheads), 5 days after the onset of a 24-hour exposure. Bar, 5 μm.
differentiation follows a similar time course every time it is initiated in the precursor cells.

We were surprised to see new hair cells after 4 hours of exposure, since our previous efforts with the same exposure conditions failed to induce hair cell regeneration (unpublished observations). Four-hour exposures are the shortest pure-tone stimuli shown, to date, to induce hair cell regeneration. Our preliminary studies of BrdU uptake after 4-hour exposures have shown that cell division does precede the appearance of new hair cells. Nonetheless, our studies have not ruled out the possibility that hair cells which regenerate after such short noise exposures may arise from transformation or induction of hair cells which are not the precursors to new hair cells. Supporting cells serve as the precursors to new hair cells and may contribute different glycoprotein components to their intracellular junctions and form a heterophilic bond. When supporting cells appose each other during recovery from noise damage, homophilic contacts are lost. These changes may stimulate cell division of the supporting cell.

What factors could stimulate cell division at an early point in noise exposure? The loss of hair cells may play a regulatory role in hair cell regeneration by releasing an inhibitory influence on precursor cells and thereby allowing them to re-enter the cell cycle (Cotanche, 1987a,b; Corwin and Warchol, 1991; Corwin et al., 1991; Raphael and Altschuler, 1992). In this proposed model, supporting cells serve as the precursors to new hair cells. They are stimulated to divide when they lose contact with hair cells and appose only other supporting cells, such as when hair cells die and are ejected from the epithelium during noise damage. The maintenance of heterophilic cell-cell interactions is one factor which may hold precursor cells in the growth-arrest state (Corwin et al., 1991). Hair cells and supporting cells may contribute different glycoprotein components to their intracellular junctions and form a heterophilic bond. When supporting cells appose each other during recovery from noise damage, homophilic contacts are created and potentially inhibitory heterophilic contacts are lost. These changes may stimulate cell division of the supporting cell.

In our current study, hair cells were being ejected from the epithelium immediately after the end of 4-, 12-, and 24-hour exposures, suggesting that similar mechanisms induced cell loss during the three overstimulation periods. Also, the number of ejected hair cells closely paralleled the quantity of regenerated hair cells, and both varied proportionately with the duration of the noise exposure. Thus, our results support the role of missing hair cells in stimulating hair cell regeneration. Our data also suggest that the number of ejected hair cells determines the number of precursor cells that re-enter the cell cycle and ultimately yields new hair cells. This is an interesting relationship which will be addressed in future studies.

Other potential influences on cell proliferation in the regenerating cochlea during recovery from acoustic or ototoxic trauma are locally or humorally secreted growth factors. Corwin et al. (1991) suggested that locally secreted growth factors play a role in regeneration of hair cells in the lateral line organ of salamanders. One putative source of these growth factors is phagocytes that exist within the damaged region. We have not yet identified phagocytes in the regenerating cochlea, but it is possible that they or another cell type secrete a substance in response to the stress of acoustic trauma.

Despite the general similarities that have been noted between hair cell differentiation during regeneration and embryonic development, no study has directly addressed the question of whether they are identical processes. An important future goal of this laboratory will be to pinpoint the time at which cells undergo
various stages of the cell cycle and differentiate cell-specific characteristics after noise exposures of different durations. We hope these efforts will determine, as the current study suggests, that the precursor cells are stimulated to re-enter the cell cycle, divide, and differentiate in a synchronized pattern regardless of the length of noise exposure.

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