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Progenitor cell cycling during hair cell regeneration in the vestibular and auditory epithelia of the chick

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Summary

We investigated nucleotide-labeling patterns during ongoing hair cell regeneration in the avian vestibular epithelium and during drug-induced regeneration in the avian auditory epithelium. For utricle experiments, post-hatch chicks received an injection of bromodeoxyuridine (BrdU) and were allowed to survive from 2 hours to 110 days after the injection. Utricles were fixed and immunoreacted to detect BrdU. The number of BrdU-labeled nuclei in the hair cell and support cell layers of the utricular sensory epithelium changes significantly between 2 hours and 110 days post-BrdU. At 2 hours, most labeled cells are isolated, while by 5–10 days, the majority of labeled cells are organized in pairs that are most frequently composed of a hair cell and a support cell. Pairs of labeled cells are seen as late as 110 days. Clusters of more than 3 labeled cells are uncommon at all time-points. The total number of labeled cells increases approximately 1.5-fold between 5 and 60 days post-BrdU. This increase is due primarily to a rise in the number of labeled support cells, and it is likely that it represents additional rounds of division by a subset of cells that were labeled at the time of the BrdU injection. There is a significant decrease in labeled nuclei in the hair cell layer between 60 and 110 days post-BrdU, suggesting that hair cells die during this period. To investigate support cell recycling in the drug-damaged auditory epithelium, we examined nucleotide double labeling after separate injections of BrdU and tritiated thymidine. A small number of support cells that incorporate BrdU administered at 3 days post-gentamicin treatment also label with tritiated thymidine administered between 17 and 38 hours later. We conclude that a small population of support cells recycles during regeneration in both the normal utricle and the drug-damaged basilar papilla.

Introduction

Hair cells (HCs) are sensory receptors that transduce mechanical stimuli into neural impulses. Their morphology and function are maintained across a wide range of vertebrate classes. Loss of HCs leads to auditory and/or vestibular sensory deficits. While many cold-blooded animals possess the ability to produce new HCs throughout life, mature mammals appear to have a limited capacity for HC regeneration (reviewed in Stone *et al*. (1998). Birds appear to be the only warm-blooded animals that demonstrate a robust HC regenerative response post-embryologically. Jørgensen and Mathiesen (1988) first demonstrated that ongoing proliferation of sensory HCs occurs in the vestibular epithelium of the adult budgerigar. This regenerative process does not result in an increase in the total number of BrdU-labeled cells in the post-hatch bird over time because there is spontaneous apoptosis of vestibular HCs on an ongoing basis (Jørgensen, 1991). Ongoing vestibular HC death is postulated to drive the production of new HCs (Kil *et al*., 1997). Spontaneous HC death does not appear to occur in the normal avian auditory epithelium (basilar papilla) (Mason *et al*., 1995). There, HCs regenerate only in response to experimentally induced HC injury or death (Cotanche, 1987a; Cruz *et al*., 1987; Corwin & Cotanche, 1988; Ryals & Rubel, 1988).

The progenitor to new HCs is not well characterized, but it is known to exist among the support cell (SC) population in the avian inner ear (Girod *et al*., 1989; Raphael, 1992; Hashino & Salvi, 1993; Stone & Cotanche, 1994; Tsue *et al*., 1994; Warchol & Corwin, 1996) as well as in other species. Support cells reside amongst HCs in sensory epithelia, and they have many diverse functions in the mature ear besides being HC progenitors, including production of the overlying extracellular

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components (e.g. the tectorial membrane; Cotanche (1987b) and Cotanche (1992)) and maintenance of structural integrity. In most non-mammalian vertebrates, there is no molecular or ultrastructural component that delineates sub-populations of SCs in either the vestibular or auditory end organs (Duckert *et al*., 1996; Presson *et al*., 1996). Therefore, it is not clear if distinct functional or morphological sub-types of SCs exist in non-mammalian vertebrates, as they do in mammals.

The main goal of the current study was to further characterize SC proliferation during HC regeneration in the avian inner ear. We chose to study both the undamaged vestibular organ and the drug-damaged auditory organ, because the environmental influences on SC proliferation are quite distinct in each case. In the undamaged utricle, regeneration of individual HCs occurs in a field of normal tissue, while in the damaged basilar papilla, a large number of HCs is regenerated in an area that is devoid of HCs. We gave normal, undamaged chicks a single injection of BrdU and examined labeled cells in the utricle over a 110-day period. In addition, we used nucleotide double labeling in the drug-damaged basilar papilla to further test the hypothesis that SCs divide multiple times during regeneration (Stone & Cotanche, 1994). Our findings indicate that, in both organs, SC recycling is not a major strategy employed during HC regeneration.

Methods

SUBJECTS

Fertilized White Leghorn (Gallus *domesticus*) eggs were obtained from a local supplier (H & N International, Redmond WA) and incubated at 37°C. Post-hatch chicks were maintained in the University of Washington's animal care facility prior to experimental use at post-hatch day 7 (P7). All experimental protocols were reviewed and approved by the University of Washington Animal Care Committee.

EXPERIMENTS ON NORMAL UTRICLES

P7 chicks received a single intraperitoneal (IP) injection of 5′-bromo-2′-deoxyuridine (BrdU; 100 mg/kg body weight; Sigma) dissolved in sterile PBS. Chicks were deeply anesthetized with sodium pentobarbital (IP; 100 mg/kg) and decapitated at 2 or 6 hours, or 2, 5, 10, 20, 30, 60, 90, 100, or 110 days after the BrdU injection ($n = 5$ chicks per timepoint) (Fig. 1A). Utricles were removed via the middle ear and placed in chilled Hank's balanced salt solution. Otoconia were removed, and utricles were fixed with buffered 4% paraformaldehyde (pH 7.4) for 30 minutes to 1 hour at room temperature.

To detect BrdU-labeled cells, we followed the protocol established for cochlear whole-mounts by Raphael (1992) and modified by Stone & Cotanche (1994). Rinses with 10 mM phosphate buffered saline (PBS; pH 7.4) were performed between most steps. Procedures were conducted at room temperature, and 0.05% Triton X-100 in PBS served as the dilutant for solutions, unless otherwise indicated. Whole-mounts of utricles were placed in 2 N HCl for 20 minutes at 37°C, in 0.5%

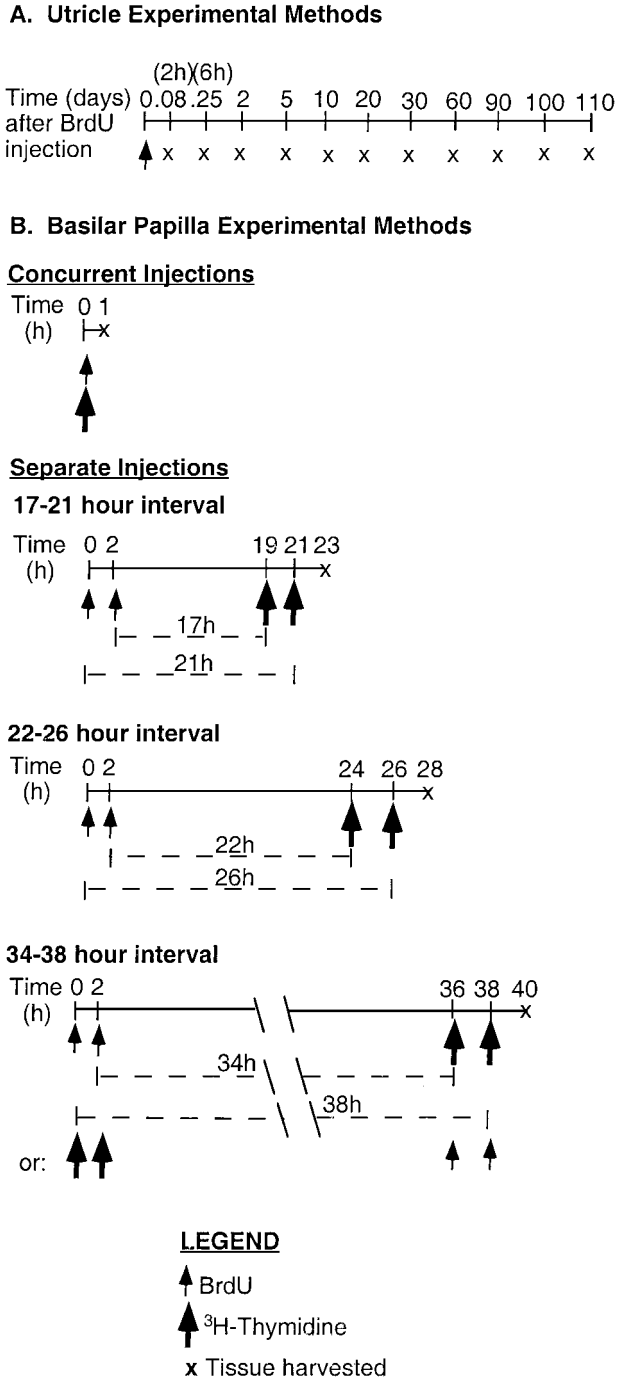

Fig. 1. Methods for nucleotide labeling. (A) Methods used in experiments on normal utricles. (B) Methods used in experiments on drug-damaged basilar papillas.

hydrogen peroxide in PBS for 15 minutes and 5% normal goat serum for 20 minutes. Whole-mounts were then incubated with mouse anti-BrdU monoclonal antibody (1 : 100, Becton Dickenson) overnight at 4°C. Biotinylated horse anti-mouse IgG (1 : 200, Vector Laboratories) was applied for 30 minutes. The avidin-biotin-horseradish peroxidase complex (1 : 500, Vector Laboratories) was applied for 1 hour. Tissue was placed in 50 mM Tris/HCl buffer (pH 7.6) and then in 0.04% 3,3′-diaminobenzidine and 0.05% H_2O_2 diluted in Tris/HCl buffer for 3–5 minutes. Tissue was rinsed, mounted onto slides in

90% glycerol in PBS, and cover-slipped. The following analyses were performed on immunoreacted whole-mounted utricles on a Leica Aristoplan microscope with Nomarski optics.

Quantification of BrdU-positive nuclei

BrdU-labeled nuclei were counted throughout the entire sensory epithelium of utricles from chicks at all time-points after the BrdU injection ($n = 5$–7 utricles per time-point, except for 110 days, where $n = 2$ utricles) using a 10×10 square eyepiece reticule and 400× magnification. Labeled nuclei were assigned to the hair cell layer (HCL) or support cell layer (SCL). No nuclei from the stromal connective tissue were counted. The nuclear shape, packing density, and depth within the epithelium distinguish each histological compartment. Nuclei in the HCL are large and round, loosely packed, and most superficial (Fig. 2B). Nuclei in the SCL are small and round, tightly packed, and located in the intermediate layer (Fig. 2C). Nuclei in the stroma are oval or fusiform, loosely packed, and most basal (Fig. 2D).

Analysis of grouping of BrdU-positive nuclei

To determine how BrdU-labeled nuclei group over time following a single BrdU injection, labeled nuclei were examined in the utricular epithelium at 2 hours, 10 days, 60 days, and 110 days post-BrdU. Labeled nuclei were classified as follows: mitotic figure; single nucleus (the nearest labeled nucleus is greater than 3 SC nuclear diameters away [in the lumenal plane] and the nucleus is not in M phase); pairs of nuclei (two labeled nuclei located within 3 SC nuclear diameters of each other); or clustered nuclei (three or more labeled nuclei located within 3 SC nuclear diameters of each other). The location of each labeled nucleus in the epithelium (i.e. HCL versus SCL) was determined. Seven 90,000 μm^2 regions with 2–10 cells per region were randomly chosen in each utricle for analysis at 400× with a 10×10 eyepiece reticule. Three utricles were analyzed per time-point.

Statistical analyses

Data were subjected to one- or two-factorial analyses of variance (ANOVA) using Statview 4.1 (Abacus Concepts). Differences across groups were considered significant if the confidence level was equal to or exceeded 95%. Fisher-PLSD post-hoc analyses were conducted to determine between-group differences.

EXPERIMENTS ON DRUG-DAMAGED BASILAR PAPILLAS

Chicks at P7 received a single subcutaneous injection of gentamicin (Fujisawa) at 400 mg/kg body weight and recovered in the animal care facility following the injection. All injected chicks exhibited systemic toxic effects of the gentamicin, and 0–25% died as a result of this toxicity despite artificial hydration.

To determine the number of SCs in S phase at different periods after gentamicin treatment, we performed BrdU pulse/fix experiments. Chicks at 1, 2, 3, 4, 5, 6, 7, or 9 days post-gentamicin injection received two injections of BrdU (100 mg/kg) spaced 4 hours apart and were killed 2 hours after the last injection. Chicks were euthanized, and cochlear ducts were dissected as described above for utricle experiments with the following additional steps: the tegmentum vasculosum was dissected with fine microforceps, and the tectorial membrane was removed by enzymatic and mechanical means (Stone *et al.*, 1996). BrdU immunohistochemistry was performed as described above for utricles. BrdU-labeled cells in the lesioned area were counted at 400× using Eutectics Neuron Tracing System (Sun Microsystems) ($n = 3$ basilar papillas, except for 1 day post-gentamicin, where $n = 1$ basilar papilla).

We also examined SC recycling in the drug-damaged basilar papilla. At 3 days post-gentamicin, chicks were separated into two experimental groups: concurrent nucleotide injections or separate nucleotide injections (Fig. 1B). For concurrent injections, 3 chicks received an IP injection of BrdU (100 mg/kg; 2×10^{-5} moles per animal) and a subcutaneous (SQ) injection of ^{3}H-thymidine (10 μCi/g; 6×10^{-9} moles per animal), spaced a few seconds apart. Chicks were euthanized 1 hour after the last nucleotide injection. For separate injections, chicks received 2 injections of BrdU (IP, 100 mg/kg, spaced 2 hours apart). Then, at different times following the BrdU injection series, the same chicks received 2 injections of ^{3}H-thymidine (SQ, 10 μCi/g), spaced 2 hours apart. The time intervals between the two sets of nucleotide injections were 17–21 hours, 22–26 hours, or 34–38 hours ($n = 3$–4 chicks per group). A second group of chicks received the nucleotides in the reverse order. For this group, we examined only the 34–38 hour interval ($n = 4$ chicks). All chicks were euthanized 2 hours after the last nucleotide injection.

Cochlear ducts were dissected, fixed and immunoreacted to detect BrdU as described above. Following immunohistochemistry, tissue was processed for autoradiography as described in Stone *et al.* (1996). Briefly, tissue was post-fixed for 20 minutes with 4% paraformaldehyde and embedded in Spurr's plastic. The proximal 1/3–1/2 of the epithelium was serially sectioned at 2–3 μm. Slides were dipped in Kodak NTB2 radiographic emulsion and exposed at 4°C for 2 months, on average.

For analysis, we selected basilar papillas that were intact and had extensive BrdU labeling throughout the lesioned area (proximal 1/3–1/2 of the epithelium). We counted labeled nuclei in the lesioned region using 100× magnification and Nomarski optics on a Leica Aristoplan microscope. Nuclei were considered BrdU-positive if they had a strong brown precipitate filling them and ^{3}H-thymidine-positive if they had at least 10 autoradiographic grains over them. For the concurrent nucleotide injections, the number of BrdU-positive and/or ^{3}H-thymidine-positive nuclei was determined in every 10th section ($n = 3$ basilar papillas). For the separate injections, the number of double-labeled cells (BrdU-positive and ^{3}H-thymidine-positive) per lesion was determined by examining every section through the lesioned area ($n = 3$ basilar papillas for each interval group).

Results

BrdU LABELING IN THE NORMAL UTRICLE

BrdU is a thymidine analog that is incorporated into nuclei during the DNA synthesis (S) phase of the cell cycle and passed on to daughter cells during mitosis. We chose to use BrdU rather than ^{3}H-thymidine to study SC proliferation because it is a rapid, non-radioactive method (Gratzner, 1982) that can be used

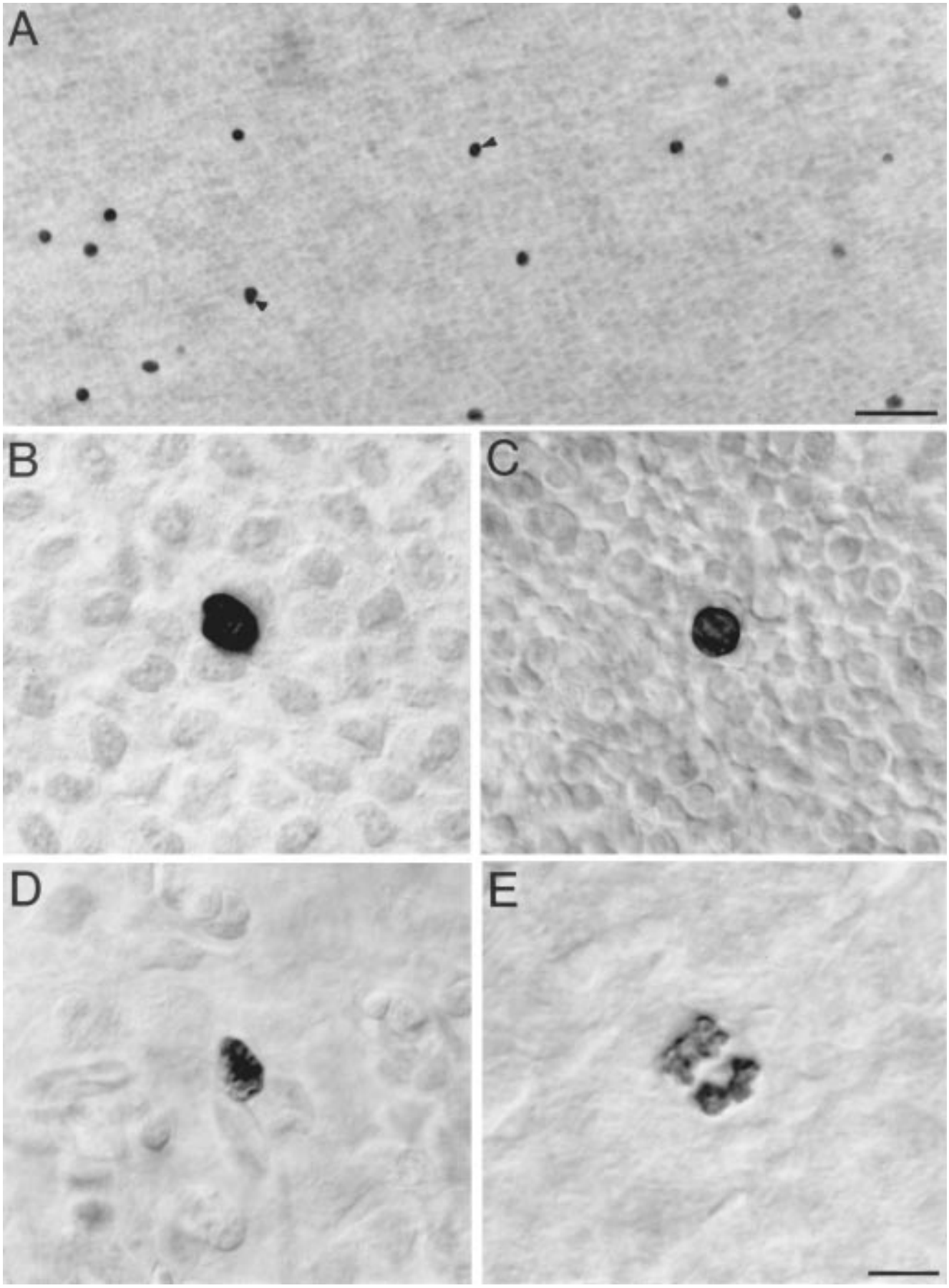
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Table 1. Grouping of BrdU-labeled cells in normal utricle over time.

Time-point (*post-BrdU*)	*Unpaired*		*Paired*				
	HCL[a]	*SCL*[b]	*2HCL*[c]	*2SCL*[d]	*Split*[e]	*Mitotic*	*Clustered*
2 hours	8 (±3)	38 (±8)	0 (±0)	3 (±1)	0.3 (±1)	1 (±1)	0.3 (±1)
	15%	69%	0%	11%	1%	2%	2%
	84%			**12%**		**2%**	**2%**
10 days	5 (±3)	3 (±2)	2 (±1)	11 (±7)	45 (±9)	0 (±0)	6 (±5)
	3%	2%	3%	15%	62%	0%	15%
	5%			**80%**		**0%**	**15%**
60 days	3 (±1)	10 (±2)	1 (±1)	10 (±6)	37 (±7)	0 (±0)	6 (±2)
	2%	8%	2%	16%	58%	0%	14%
	10%			**76%**		**0%**	**14%**
110 days	7 (±1)	12 (±4)	1 (±1)	6 (±1)	20 (±5)	0 (±0)	2 (±1)
	9%	15%	2%	15%	51%	0%	8%
	14%			**68%**		**0%**	**8%**

Numbers of BrdU-positive cells that are unpaired, paired, clustered or mitotic observed in 7 regions (90,000 μm^2 in size) per utricle, and location of BrdU-positive cells in each category. Three utricles were analyzed per time-point. Values represent mean (±SD) or mean% of analyzed cells for each time-point. Bolded% values represent mean% for each category. [a]HCL = Nucleus in hair cell layer. [b]SCL = Nucleus in support cell layer. [c]2HCL = Both nuclei in HCL. [d]2SCL = Both nuclei in HCL. [e]Split = One cell in HCL, one cell in SCL.

in whole-mount preparations of inner ear tissues, allowing one to visualize the three-dimensional array of labeled cells (e.g. Hashino & Salvi, 1993; Lanford *et al*., 1996; Raphael, 1992; Stone & Cotanche, 1994).

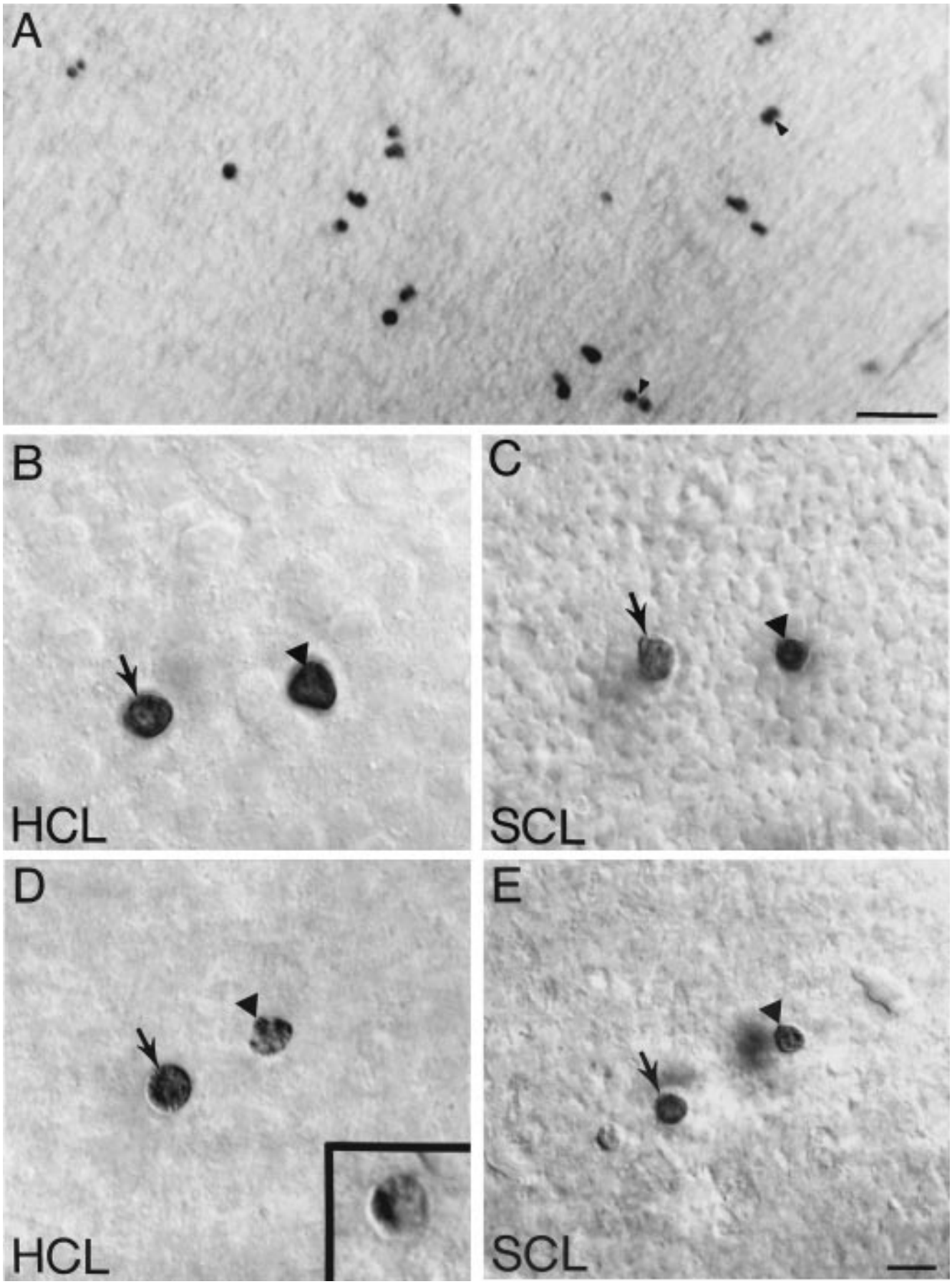
We examined utricles from normal chicks at different times after a BrdU injection at P7. Histological criteria were employed (see Methods) to ensure that our analyses involved labeled nuclei in the epithelium and not the stromal connective tissue and to determine if labeled nuclei are located in the hair cell layer (HCL) or support cell layer (SCL) of the epithelium (Fig. 2B–D). BrdU-labeled nuclei are found in all utricles in our study and do not appear to be concentrated in any particular region at any time-point. A similar finding has been reported in the normal chick utricle (Roberson *et al*., 1992; Tsue *et al*., 1994; Kil *et al*., 1997) and the normal fish saccule (Lanford *et al*., 1996). We did not detect labeled nuclei in the process of being expelled from the epithelium at any time-point.

GROUPING OF BrdU-LABELED CELLS CHANGES OVER TIME

Quantitative estimates of the grouping of labeled nuclei were undertaken to determine if labeled cells are isolated, organized in pairs, or form clusters at 2 hours, 10 days, 60 days, and 110 days after the BrdU injection. Data are presented in Table 1. At 2 hours post-BrdU, 84% of BrdU-labeled nuclei are unpaired (i.e., located farther than 3 nuclear lengths from another labeled nucleus). The majority of unpaired cells are located in the SCL (see Fig. 2C). This finding was expected since most labeled utricular SCs are pre-mitotic, in S or G2 phase, at 2 hours after the BrdU injection (Tsue *et al*., 1994). At 2 hours post-BrdU, 12% of labeled nuclei are paired (i.e. located within 3 nuclear lengths of each other). We interpreted such pairs to be adjacent proliferative SCs rather than post-mitotic sibling cells, because we rarely saw labeled mitotic figures at this time. While very few mitotic figures are seen at 2 hours post-BrdU they are readily apparent throughout the epithelium at 6 hours post-BrdU (approximately 4–12 per epithelium), (Fig. 2E). Surprisingly, a similar relatively high number of labeled mitotic figures are seen at 2 days post-BrdU (data not shown). In both cases, labeled mitotic figures are not found adjacent to other labeled cells. Only very rarely are mitotic figures detected at later time-points. By 10 days post-BrdU and later, the majority of labeled cells are paired (Table 1; Fig. 3A), and relatively few labeled cells are unpaired or clustered (i.e. grouped by more than 2 cells).

Data were subjected to one-factorial ANOVAs (grouping × #cells) and two-factorial ANOVAs (grouping

Fig. 2. BrdU labeling in the normal utricle at early time-points. (A) BrdU-labeled nuclei (arrowheads) in the utricular epithelium at 2 hours post-BrdU. Labeled cells are evenly distributed in the epithelium and mostly isolated (i.e. located greater than 3 nuclear lengths from another labeled nucleus). (B)–(D) BrdU-labeled nuclei in the HCL at 2 hours post-BrdU (B), in the SCL at 6 hours post-BrdU (C), and in the stromal connective tissue at 2 hours post-BrdU (D). It is easy to distinguish the layer in which a BrdU-labeled nucleus resides due to differences in cell densities and shapes in each layer and the depth of the nucleus (not shown) in the epithelium. (E) Many mitotic figures are detectable at 6 hours and 2 days post-BrdU; a sample from 6 hours post-BrdU is shown. Scale bars: in (A) = 50 μm; in (B)–(E) = 10 μm.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and time × #labeled cells), and post-hoc comparisons (Fisher-PLSD) of individual experimental groups were conducted. At 10, 60, and 110 days post-BrdU, there are significantly fewer labeled unpaired cells than at 2 hours post-BrdU ($p < .001$ for 10, 60 and 110 days). In contrast, there are significantly more paired cells at times later than at 2 hours post-BrdU ($p < .001$ for 10 and 60 days, $p < .05$ for 110 days). For all groupings, there is no significant change in the number of labeled cells between 10 and 60 days post-BrdU ($p = .391$ for single cells, .233 for pairs, and .547 for clusters). The number of labeled single or clustered cells does not change significantly between 10 and 110 days post-BrdU ($p = .059$ for single cells and .075 for clusters). However, there is a trend toward a decrease in labeled clustered cells and an increase in labeled single cells between 10 and 110 days post-BrdU. Finally, there is a significant decrease in the total number of labeled pairs between 60 and 110 days post-BrdU ($p < .05$).

To summarize, our data demonstrate that there is a significant change in the grouping of BrdU-labeled cells over time in the normal utricle following a single BrdU injection. Our statistical analyses show a very strong interaction between time and cell grouping ($p < .001$) with respect to the number of labeled cells. The number of unpaired BrdU-labeled cells decreases sharply by 10 days post-BrdU, and it does not change significantly by 110 days post-BrdU. The number of labeled pairs increases between 2 hours and 10 days post-BrdU, stays approximately level between 10 and 60 days, then decreases between 60 and 110 days. Finally, the percentage of labeled cells that are clustered increases between 2 hours and 10 days post-BrdU and stays the same until 110 days.

ONE HAIR CELL AND ONE SUPPORT CELL COMPOSE A PAIR

At time-points later than 2 hours post-BrdU, paired cells commonly have similar densities and patterns of nuclear BrdU labeling (Fig. 3B–E). Cells in such pairs are presumed to be siblings that are generated from one mitotic event. In most pairs, one cell is located in the HCL and the other is located in the SCL (Fig. 3B–E), suggesting that most sibling pairs generated during ongoing regeneration in the chick utricle differentiate asymmetrically, as a HC and a SC. Our grouping analysis showed that the number of sibling pairs that differentiate asymmetrically (i.e. one cell in the SCL and one cell in the HCL) is considerably higher than the number of sibling pairs that differentiate symmetrically (i.e. both cells in the SCL or both cells in the HCL) (Table 2). This trend is the same at 10 days, 60 days, and 110 days post-BrdU, although there appears to be a small increase in the percentage of sibling pairs that are asymmetric over time. The relatively uncommon symmetric pairs are usually comprised of two SCs rather than two HCs.

Table 2. Ratio of asymmetric to symmetric pairs of labeled cells in normal utricles.

	10 d p-BrdU	*60 d p-BrdU*	*110 d p-BrdU*
Asym prs[a]	45 (±9)	37 (±7)	20 (±5)
Sym prs (2 HCs)[b]	2 (±1)	1 (±1)	1 (±1)
Sym prs (2 SCs)[c]	11 (±7)	10 (±6)	6 (±1)
$\frac{\text{Total prs}}{\text{Asym prs}} \times 100$	78%	86%	91%

Average numbers (mean ± SD) of symmetric and asymmetric pairs of BrdU-positive cells observed in 7 regions (90,000 μm^2 in size) per utricle. Three utricles were analyzed per time-point.
[a] Asym prs = Asymmetric pairs, composed of 1 HC (hair cell) and 1 SC (support cell).
[b] Sym prs = symmetric pairs, composed of 2 SCs.
[c] Sym prs = symmetric pairs, composed of 2 HC.

We found morphological changes in some BrdU-labeled nuclei at 60 days post-BrdU and later that are typical of apoptotic cells (Fig. 3D and inset). Specifically, we noted that the labeling in some cells in the HCL appears to become condensed or confined to one quadrant of the nucleus, resembling the marginalization of nuclear chromatin seen in apoptotic cells (Jørgensen, 1991; Wyllie *et al.*, 1981). We did not see apoptotic-like cells in the SCL at these later times nor anywhere in the epithelium at earlier times.

CHANGES IN THE NUMBER OF BrdU-LABELED CELLS OVER TIME

At each survival time, we determined the number of BrdU-labeled nuclei per utricle. Data were categorized as follows: the total labeled nuclei (HCL plus SCL), the labeled nuclei in the HCL, and the labeled nuclei in the SCL (Fig. 4). The number of labeled cells in each of these categories changes significantly between 2 hours

Fig. 3. BrdU labeling in the normal utricle at later time-points. (A) BrdU-labeled nuclei (arrowheads) in the utricular epithelium at 10 days post-BrdU. Labeled cells are evenly distributed and mostly paired. (B) and (C) Both panels show the same field of utricular epithelium at 10 days post-BrdU. (B) is focused on the HCL, and (C) is focused on the SCL. Two pairs of sibling cells are shown; arrowheads point to cells in one pair, and arrows point to cells in the second pair. (D) and (E) Both panels show the same field of utricular epithelium at 110 days post-BrdU. (D) is focused on the HCL, and (E) is focused on the SCL. Two pairs of sibling cells are shown; arrowheads point to cells in one pair, while arrows point to cells in the second pair. Arrowhead in (D) points to an apoptotic-like nucleus in the HCL at 110 days post-BrdU. Inset in (D) shows an apoptotic-like nucleus in the HCL at 60 days post-BrdU. Scale bars: in (A) = 50 μm; in (B)–(E) = 10 μm.

and 110 days post-BrdU ($p < .001$). When we examined the early versus late periods of post-BrdU survival, we noted several interesting differences.

The data for the total number of labeled cells and the number of labeled cells in the SCL show similar trends. There is no significant difference in the number of labeled cells in either category between 2 hours and 2 days post-BrdU (for total $p = .436$, for SCL $p = .770$). The number of labeled cells approximately doubles between 2 and 5 days and increases approximately 1.5 times between 5 and 60 days. The total number of labeled cells decreases significantly between 60 and 110 days post-BrdU ($p < .05$), while the number of labeled cells in the SCL does not change significantly during this period ($p = .319$).

The number of labeled cells in the HCL increases significantly between 2 hours and 2 days post-BrdU ($p < .05$). This was expected, as nuclei migrate from the SCL into the HCL during mitosis (Katayama & Corwin, 1993; Tsue *et al.*, 1994). The number of cells in the HCL grows approximately 1.5-fold between 5 and 60 days post-BrdU. There is a significant decrease in the number of labeled cells in the HCL between 60 and 110 days ($p < .01$).

NUCLEOTIDE INCORPORATION IN THE DRUG-DAMAGED BASILAR PAPILLA

A previous study suggests that SC recycling may occur in damaged avian HC epithelia (Stone & Cotanche, 1994). This study reported an increase in the number of BrdU-labeled cells forming clusters over time after noise damage in the basilar papilla, which suggested clonal growth from single progenitor cells. We decided to re-examine this phenomenon in the regenerating basilar papilla using a different method than the one employed by Stone and Cotanche (1994).

First, we performed BrdU pulse-fix experiments to determine the peak time of cell proliferation following a single gentamicin injection at 400 mg/Kg. Very few cells are in S phase at 1 and 2 days post-gentamicin (Fig. 5). There is a dramatic increase in the number of BrdU-labeled cells at 3 days post-gentamicin, and the number of labeled cells falls rapidly by 5 days post-gentamicin. A very low rate of cell proliferation then persists as late as 9 days post-gentamicin. Our finding is similar to data reported in previous studies of rates of cell proliferation in the chick basilar papilla following a single aminoglycoside injection at a lower dose (Bhave *et al.*, 1995; Cotanche & Messana, 1997).

Next, we gave drug-damaged chicks an injection of one nucleotide (either BrdU or ^{3}H-thymidine) at 3 days post-gentamicin, the peak time of proliferation. Then, at various times after the first injection, the same chicks received an injection of a different nucleotide. The rationale for this experimental approach is that a cell that incorporates the two different nucleotides must be in S phase when each nucleotide is available for uptake. If the intervals between the nucleotide administrations are greater than the length of the cell's S phase and the nucleotides are cleared rapidly from the tissues, then that cell must have entered a second S phase by the time the second nucleotide was available for uptake. In other words, that cell has entered

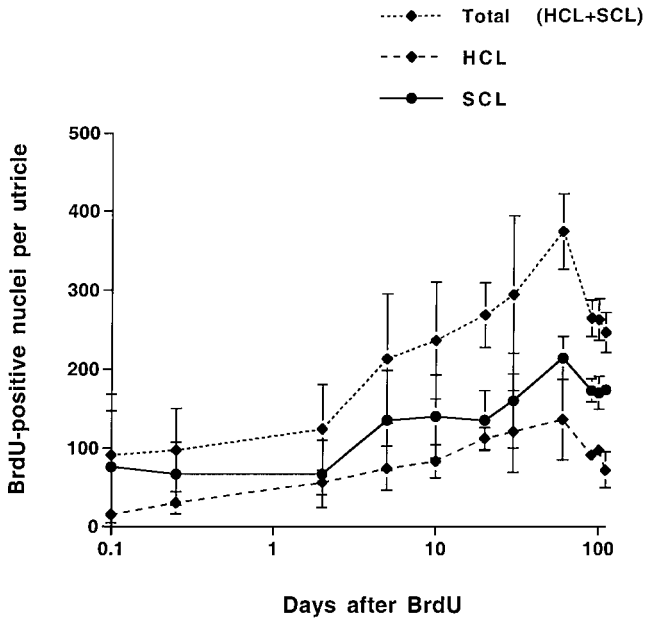

Fig. 4. Quantification of BrdU-labeled cells in normal utricles over time. The number of BrdU-labeled cells per utricle was determined in normal chicks killed at different times after a single BrdU injection at P7. $n = 5$–7 utricles, except for 110 days, where $n = 2$ utricles. Error bars = S.D.

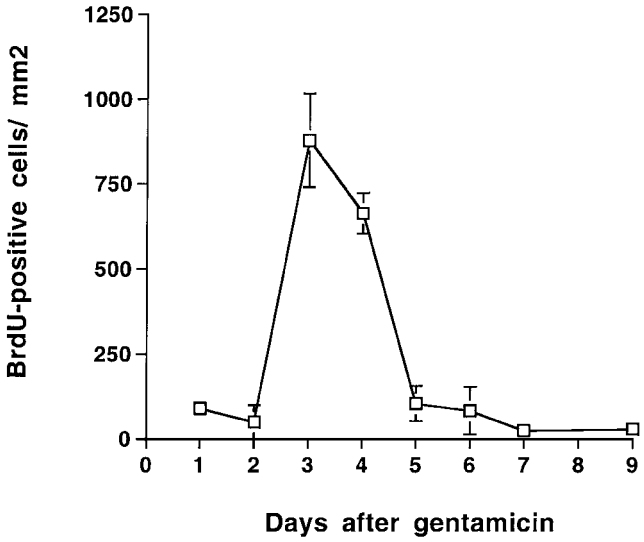

Fig. 5. Quantification of BrdU-labeled cells in drug-damaged basilar papillas after pulse/fix labeling. The number of BrdU-labeled cells per mm^2 was determined in chicks at different times after gentamicin treatment using a pulse/fix labeling paradigm. $n = 3$ basilar papillas, except for 1 day, where $n = 1$ basilar papilla. Error bars = S.D.

the cell cycle two times. This method may also yield estimates of the S phase-to-S phase duration (or the cell cycle time). The intervals between nucleotide injections that we chose are 17–21 hours, 22–26 hours, and 34–38 hours. These groups represent the duration between the two series of injections, not the duration between the two periods when each nucleotide is available for uptake. We opted to define the groups in this manner because we do not know exactly how long a significant amount of each nucleotide is available for uptake after it is injected. A good guess is between 2 and 6 hours after injection. Studies in the embryonic chick have shown that most ^{3}H-thymidine is cleared from the plasma by 2 hours after intravascular injection (Katayama & Corwin, 1993). In addition, BrdU appears to be cleared from the cochlear compartment of mature chicks by 6 hours after intraperitoneal injection (Stone & Cotanche, 1994). Because of some lag in clearance after the injection, the duration between the two periods of availability is longer than that between the two injections. Therefore, at best, this method allows us to make a liberal estimate of the cell cycle length. We used traditional methods of delivering each nucleotide. BrdU was delivered intraperitoneally and ^{3}H-thymidine was delivered intramuscularly. The two nucleotides were administered at different doses (BrdU > ^{3}H-thymidine, see Methods). Finally, in a control group, we gave concurrent injections of ^{3}H-thymidine and BrdU to chicks at 3 days post-gentamicin; chicks were killed 1 hour after the injections.

We felt confident that this approach would be effective for double-labeling recycling cells because BrdU and ^{3}H-thymidine labeling methods have been performed successfully on inner ear tissues, individually (e.g. Oesterle *et al*., 1993; Stone & Cotanche, 1994) and in a double-labeling paradigm (Wilkins *et al*., 1999). However, we do not know how long S phase is for SCs. Our methods make a conservative assumption that S phase is shorter than 17 hours (see Discussion), which is the shortest interval between each series of injections.

In the concurrent injectable group, many double-labeled cells are detected in the lesioned area of the basilar papilla (Fig. 6A). Fifty-six percent of labeled cells are positive for both nucleotides, 43% of labeled cells are BrdU-positive only, and 1% are ^{3}H-thymidine-positive only. Therefore, two times as many cells incorporated BrdU as 3H-thymidine. Nonetheless, these results indicate that our methods enable visualization of both BrdU and ^{3}H-thymidine when incorporated by a single cell.

We examined incorporation of the nucleotides into cells in the lesioned area when the nucleotides were administered with the following intervening times (with BrdU given first and ^{3}H-thymidine given second): 17–21 hours, 22–26 hours, 34–38 hours. Data are shown in Table 3. On average, we detected 1 double-labeled cell per papilla in the 17–21 hour group (range = 0–1 cell), 2 double-labeled cells per papilla in the 22–26 hour group (range = 1–3 cells), and 0 double-labeled cells per papilla in the 34–38 hour group (range = 0–1 cell). Double-labeled cells (Fig. 6B) are present in samples from each of the three time-interval groups. Thus, it was not possible to deduce the inter-S phase time for SCs based on our findings. We reversed the order of the nucleotides (^{3}H-thymidine, then BrdU)

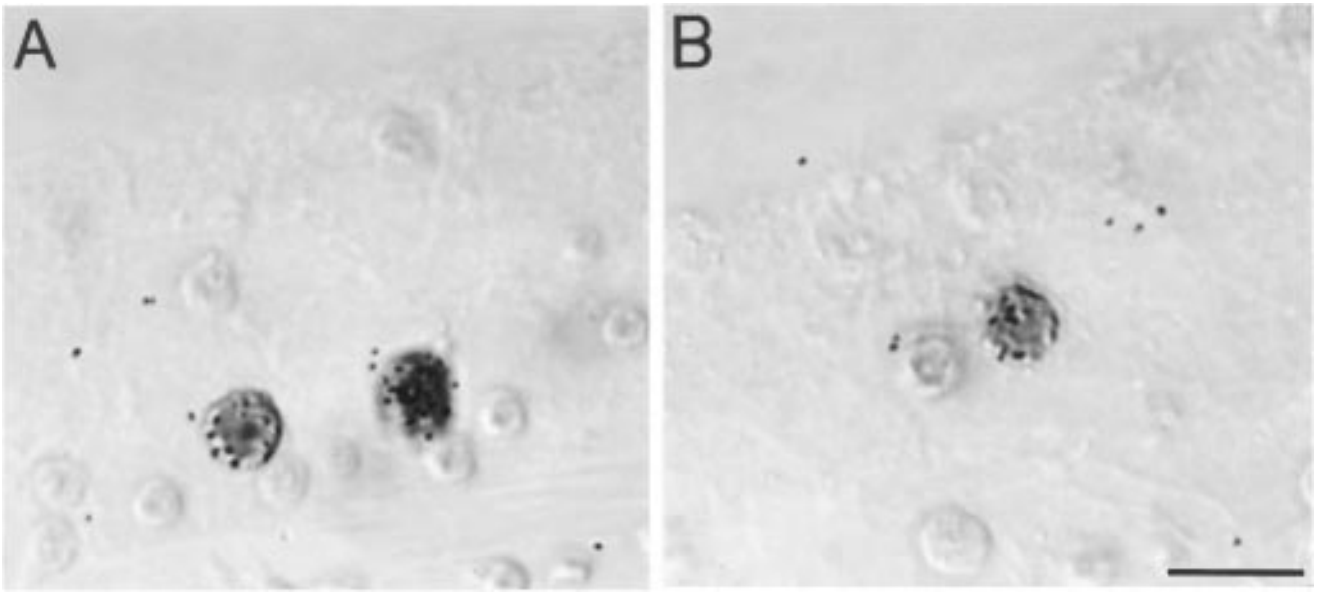

Fig. 6. ^{3}H-thymidine and BrdU double-labeling in the regenerating basilar papilla. (A) Cells are double-labeled for BrdU (gray label in nucleus) and ^{3}H-thymidine (black granules over nucleus) in the basilar papilla by 1 hour after the concurrent injection of both nucleotides at 3 days post-gentamicin. (B) A small number of cells are double-labeled for BrdU (gray label in nucleus) and ^{3}H-thymidine (black granules over nucleus) following separate injections of the nucleotides. A section from the 22–26 hour group (BrdU then ^{3}H-thymidine) is shown. Scale bar = 10 μm.

Table 3. Number of double-labeled cells per basilar papilla.

	17–21 h *B/T*[a]	*22–26 h* *B/T*	*34–38 h* *B/T*	*34–38 h* *T/B*[b]
Mean	1	2	0	1
Range	0–1	1–3	0–1	0–1

[a]B/T = BrdU injection first, $n = 3$ basilar papillas per time-point. [b]T/B = ^{3}H-thymidine injection first, $n = 3$ basilar papillas per time-point.

to see if the number of double-labeled cells change significantly, and we found that it does not.

Discussion

In this study, two nucleotide-labeling strategies were employed to examine SC cycling in the regenerating avian inner ear. First, we examined BrdU labeling at 11 survival times after a single BrdU injection in utricles of undamaged chicks. Second, we employed double labeling with two different nucleotides, BrdU and ^{3}H-thymidine, to further test the hypothesis that SCs recycle during damage-induced auditory HC regeneration.

THE NORMAL UTRICLE

Our studies of the undamaged chick utricle showed that the total number of BrdU-labeled cells does not increase significantly between 2 hours and 2 days post-BrdU, but nearly doubles between 2 days and 5 days. We had initially expected the number to double shortly after 6 hours post-BrdU, since avian SC mitosis is known to occur approximately 6 hours after BrdU uptake (Stone & Cotanche, 1994). One possible explanation for this unanticipated result is that mitosis is delayed among a subset of cells in the utricle as late as 2 days after BrdU uptake. Our data support this explanation, since we detected relatively high numbers of BrdU-labeled mitotic figures in utricles at 2 days post-BrdU and at 6 hours post-BrdU, but not at later times. We have considered three other explanations for our observation. First, the labeled mitotic figures that we saw at 2 days may represent cells that are dividing a second time after the BrdU injection. We feel this is not likely, because labeled mitotic figures are rarely seen in close association with single labeled cells, which would be the case were a second round of division occurring in one daughter cell. Second, BrdU may remain available for uptake 2 days after its injection. While there have been no direct studies of BrdU clearance in the chick utricle, a study in the chick basilar papilla suggests that BrdU is cleared in under 6 hours (Stone & Cotanche, 1994). Third, one of the daughter cells may die shortly after it is born. Such a strategy for maintaining cell number or type has been reported in the mature mammalian olfactory epithelium (Carr & Farbman, 1993), which continually regenerates neurons. Our data do not fully support this explanation. The death of a daughter cell after many division events should result in a relatively high number of single cells at 10 days post-BrdU. On the contrary, only a small proportion (5%) of BrdU-labeled cells is unpaired at this time.

We favor the interpretation that some SCs become arrested in G2 phase of the cell cycle, which would result in SC mitosis at variable times after BrdU incorporation. It is unclear why, in the normal chick utricle, a large number of progenitor cells would become arrested in G2. Interestingly, there is evidence that G2 arrest occurs among some SCs in the regenerating chick basilar papilla as well (Bhave *et al.*, 1995). G2 arrest serves as a checkpoint, permitting repair of DNA in cells that are damaged by external stimuli, such as irradiation, and it may lead to cellular suicide if DNA damage is severe (reviewed in Mercer (1998) and O'Connor (1997)). In the absence of an external damage stimulus, however, it seems unlikely that G2 arrest in HC progenitors is due to DNA damage. Our observation of labeled mitotic figures at 2 days post-BrdU injection suggests that most cells that become arrested in G2 arrest go on to divide rather than die. G2 arrest has been documented in very early developing cells in many animals: for example, fruit fly (Edgar & O'Farrell, 1990) and limpet (van der Kooij *et al.*, 1998). While the role of complexity in cell cycle patterns is not well understood, G1 arrest among a population of cells may be important for coordinating the production of specific cell types (e.g. Ready *et al.*, 1976; Thomas *et al.*, 1994). Further, cell cycle status may determine a cell's responsiveness to certain environmental cues (McConnell & Kaznowski, 1991). Accordingly, G2 arrest in the chick utricle may serve to restrict the number of progenitor cells that divides and thereby prevent overproduction of HCs or SCs. The cellular mechanisms that could direct this type of regulation remain to be characterized.

The total number of labeled cells increases approximately 1.5-fold between 5 and 60 days post-BrdU. This increase suggests that between 25 and 50% of labeled SCs divide at least one more time after the initial division or that a few SCs divide several times. The lack of exponential growth in labeled cells suggests that the majority of SCs do not recycle continuously between 10 and 110 days after the BrdU injection. This interpretation is corroborated by our observation that there is no significant increase in the number of BrdU-labeled cells forming clusters in the epithelium over time, which should occur as a result of clonal growth. This interpretation is further supported by a recent study in the chick utricle (Wilkins *et al.*, 1999) that used a nucleotide double-labeling paradigm to show that progenitor recycling does not occur within 3, 4, or 7 days in the normal chick utricle. Future studies should test directly whether SCs undergo more than one round of division.

Such studies could employ retroviral lineage analysis to examine clonal growth patterns (Fekete *et al*., 1998) or quantitative ^{3}H-thymidine analysis to examine changes in the mean and variability of HC and SC labeling densities over time.

Between 10 and 60 days post-BrdU, the majority of BrdU-labeled cells are organized in discrete pairs that appear to be stably present in the epithelium. We assumed that such pairs are sibling pairs based on their proximity and the similarity of their BrdU labeling patterns. Analysis of sibling pairs showed that most differentiate asymmetrically, as a HC and a SC. Previous studies indicate that asymmetric cell production (generating a HC and a SC) occurs more commonly than symmetric cell production (generating two like cells) in the undamaged post-hatch utricle. Roberson *et al*. (1992) noted in sectioned chick utricles that pairs of ^{3}H-thymidine-labeled cells at 11–12 days after the ^{3}H-thymidine is administered are most frequently composed of a HC and a SC. Stone and Rubel (1999) found that pairs of BrdU-labeled cells at 8–12 days after BrdU incorporation are most frequently composed of a calretinin-positive cell (a HC) and a calretinin-negative cell (a SC). In addition, asymmetric HC production predominates during development of the chick basilar papilla (Fekete *et al*., 1998), as well as during regeneration of the damaged lateral line organ of the salamander (Jones & Corwin, 1996). In contrast, mitotic events are equally likely to produce any combination of daughter cells (i.e. 2 HCs, 2 SCs, or one HC and one SC) during regeneration in the drug-damaged basilar papilla (Stone & Rubel, 2000). The cellular mechanisms underlying different cell fate decisions across organs and developmental stages are unknown.

There is a statistically significant decrease in the total number of BrdU-labeled cells, as well as the number of labeled cells in the HCL, between 60 and 90 days post-BrdU. The number of pairs of labeled cells also declines significantly. Additionally, we saw BrdU-labeled cells in the HCL that had apoptotic features at 60 days post-BrdU and at later time-points. These findings suggest that most HCs die between 60 and 110 days after they are formed. This conclusion does not agree with those of two previous studies, which postulated significantly shorter life spans for utricular HCs in the chick. Kil *et al*. (1997) estimated the HC life span to be approximately 20 days, taking into account the rate of S phase entry and the total number of HCs in the sensory epithelium. Wilkins *et al*. (1999) found that approximately 50% of BrdU-labeled cells are expelled from the utricular epithelium by 7 days post-BrdU injection and postulated that many post-mitotic HCs die at this early time. We conducted our own estimates based on what we feel are more conservative predictions about progenitor cells in the chick utricle, and they differ significantly from those of Kil and colleagues. The calculations that Kil and colleagues employed to determine the number of HCs born per day (based on the number of BrdU-labeled cells following a 2 hour pulse/fix paradigm) assumed that S phase is instantaneous or very short. The critical difference in our approach was to view S phase as a protracted process, lasting for several hours (see below). If a group of cells is labeled after a 2 hour BrdU pulse at one time, a subset of those cells will also become labeled following a 2 hour pulse at an earlier time or a pulse at a later time. Given this sampling redundancy, it is not sufficient to simply multiply the number of labeled cells after a 2 hour pulse by 12 to determine the number of cells that are labeled in a 24 hour period. In fact, the relationship between the number of labeled cells after a pulse and the number of cells in S phase each day depends on three variables: the length of S phase, the time that BrdU is available for uptake, and the amount of time after the injection that is required for SC labeling. The relationship among these factors leads to the following formula for determining the number of cells that enter S phase in a day; presumably, all of those cells eventually go on to divide.

$$\#\text{ of cells in S phase per day} = \frac{\#\text{ of cells labeled in pulse} \times 24\text{ hours}}{H}$$

$$H = \#\text{ of hours S phase overlaps with BrdU availability} = S + A - 2(L)$$

where $S = \#$ of hours of S phase, $A = \#$ of hours of BrdU availability, and $L = \#$ of hours required for SC labeling.

We saw an average of 91 BrdU-labeled cells at 2 hours after a single BrdU pulse. Since we do not know the exact values of S, A, or L, we used a range of conservative estimates of those values to calculate the number of cells that divide in a day. For these calculations, $S = 6$, 8, or 10 hours, $A = 2$ hours, and $L = 15$ minutes or 1 hour. We chose the values of S phase length (S) based on the following factors. While there is considerable variation in the length of S phase among eukaryotic cells, the length is on the order of several hours (Ford and Pardee, 1998). For example, S phase length is estimated to be 4 hours for both the embryonic day (E) 14 mouse cerebral cortex (Takahashi *et al*., 1993) and the E1 chick retina (Dutting *et al*., 1983). S phase lengths for cells in the nervous system increase after birth: for example, S phase is estimated to be 16 hours in the P1 mouse retina (Young, 1985) and 9 hours in the adult rat olfactory epithelium (Huard & Schwob, 1995). We have the following unpublished evidence that S phase in cochlear SCs in the chick is between 4 and 10 hours in duration (J. S. S., S. M. N. W. & E. W. R., unpublished observations). We gave chicks at 3 days post-gentamicin a single injection of BrdU followed by a single injection of ^{3}H-thymidine either 4 or 10 hours later, then killed the chicks 2 hours after the ^{3}H-thymidine injection. The number of double-labeled cells in basilar papillas in the 10 hour group was

Table 4. Estimates for number of hair cells born per day in control utricles.

	S phase length (S)		
L[a]	*6 h*	*8 h*	*10 h*
15 mins	291	230	190
1.0 hrs	364	273	218

[a] L = Number of hours requred for SC labeling with BrdU.

approximately 25% of that observed in the 4 hour group. This observation suggests that S phase is between 4 and 10 hours in auditory SCs. With respect to *A* (the time of BrdU availability), there are no concrete data on how long BrdU is present in the cochlear compartment of a mature chick. We estimated that some BrdU is available for uptake for 2 hours after injection, since this is the time it takes ^{3}H-thymidine to be cleared from the blood in embryonic chicks (Katayama and Corwin, 1993). Finally, we estimated the time after injection required for BrdU labeling (L) to be 15 minutes or 1 hour. We have shown here that BrdU is detectable in SCs by 1 hour after injection. Also, we have seen BrdU in some SCs by 30 minutes after injection (J.S.S., S.M.N.W. & E.W.R, unpublished observations). Thus, it seems likely that BrdU may be incorporated into cells by even shorter periods after injection, such as 15 minutes.

The results of the calculations are shown in Table 4. Using our model and the chosen values for the three variables, we estimate that between 190 and 364 cells (mean = 261 cells) enter S phase in one day. Since HC production is asymmetric in the normal early post-hatch utricle (Stone & Rubel, 1999), the number of HCs that are produced daily is also 261. Accordingly, approximately 11 HCs are born per hour. If there are 28,000 HCs in the mature utricle at any given day (M. E. Warchol, unpublished observations; reported in Kil *et al*. 1997), then 2500 hours (or 105 days) are needed to replace all HCs in the utricle. Therefore, our calculations estimate that the approximate life span of a HC is 105 days and the half-life of a HC is about 52 days.

We acknowledge that this estimate is based on assumed values for the three variables. Interestingly, though, this estimate is in agreement with our observations of apoptotic-like HCs, and a decrease in BrdU-positive cells in the HCL, between 60 and 90 days post-BrdU. We are not sure how to interpret our findings relative to those of Wilkins *et al*. (1999). They found that approximately 50% of BrdU-labeled cells are expelled from the utricular epithelium by 7 days post-BrdU injection and postulated that many post-mitotic HCs die at this early time. We did not see BrdU-labeled cells in the process of being extruded.

It is important to note that we also detected a decline in the number of BrdU-labeled cells in the SCL between 60 and 110 days. While this change was not statistically significant, we cannot rule out that some SCs also die by 110 days after they are born.

THE DRUG-DAMAGED BASILAR PAPILLA

Previous studies suggest that HC regeneration is accomplished by clonal growth of sensory epithelial cells. In the noise-damaged basilar papilla of the chick, clusters of BrdU-positive cells appear to grow over time after a single BrdU injection (Stone & Cotanche, 1994). Support cells in the oscar saccule incorporate both ^{3}H-thymidine and BrdU when they are administered individually with an intervening 7 day period (Wilkins *et al*., 1999), suggesting that many SCs divide at least twice during a single week. In addition, direct observation of the salamander lateral line organ following HC ablation has revealed that some SCs divide at least two times during regeneration (Jones & Corwin, 1996).

We found that, in the drug-damaged basilar papilla, only 1–3 cells per basilar papilla incorporate both BrdU and ^{3}H-thymidine when the nucleotides are injected with an intervening latency of 22–26 hours. We did not see a marked difference in the number of double-labeled cells when we changed the duration between nucleotide injections or reversed the order of nucleotide delivery.

We used these data and data from single nucleotide labeling experiments to estimate the proportion of the progenitors that recycle during regeneration. On average, 221 SCs in the basilar papilla label with a 3 hour pulse of BrdU at 3 days post-gentamicin (Stone & Rubel, 2000). Relative to 3 days post-gentamicin, we found that the number of cells entering S phase decreases approximately 25% by 24 hours after 3 days post-gentamicin. Thus, we estimate that approximately 166 cells should label after administration of a second nucleotide 24 hours after the first nucleotide. Using our double-labeling methods, BrdU is two times as likely as ^{3}H-thymidine to become incorporated into cells in the cochlear epithelium by 1 hour after administration. This discrepancy is most easily explained by the fact that BrdU is more rapidly delivered to the cochlear fluids than ^{3}H-thymidine because it is administered intraperitoneally and at a higher molar dose than ^{3}H-thymidine. Alternatively, it is possible that there is some quality intrinsic to the BrdU molecule that makes it easier for delivery and uptake. Based on these findings, we would expect to see as many as 83 (166/2) double-labeled cells per basilar papilla using the 22–26 hour labeling interval, if all cells were recycling. However, we see only 1–3 double-labeled cells per basilar papilla, indicating that only 1–4% of progenitor cells divide more than once in this period. The fact that the majority of SCs do not re-enter the cell cycle after 3 days post-gentamicin is mirrored by the rapid decrease in cells in S phase between 3 and 5 days post-gentamicin. We must conclude

that the vast majority of cells that become labeled during the 17–38 hours after the first nucleotide injections are either newly recruited to the cell cycle or are cells that divided initially before the time of the first nucleotide injection, at 3 days post-gentamicin. It is also possible that the length of the SC cycle is considerably shorter or longer than the durations that we chose to analyze (i.e. <17 hours or >38 hours). In this case, we would have underestimated the number of recycling cells.

We noted an average of 1–2 double-labeled cells in most of the time-interval groups that we examined, which made it impossible to pinpoint the length of the cell cycle (i.e. the S phase-to-S phase duration). The reason for this finding is unknown, but we can provide three possible explanations. First, the double-labeled cells that we documented may not be homogeneous, but rather may represent several different types of cells with different cell cycle lengths (e.g. SCs, and macrophages, leukocytes, or microglia-like cells, which proliferate in the basilar papilla following drug damage (Warchol, 1997; Bhave *et al*., 1998). Second, all of the double-labeled cells may be SCs, each with a fixed cell cycle time, but the durations between the two periods when the nucleotides are available for uptake may be considerably longer than we estimated and therefore may overlap each other. For example, if both nucleotides take 2 hours to be cleared, then the 17–21 hour duration becomes 15–23 hours and the 22–26 hour duration becomes 20–28 hours. In this case, SCs with a 20 hour cell cycle would be double-labeled in both groups. If the clearance time exceeds 8 hours, then there would also be overlap between the two longest duration groups. Third, the length of the SC cycle may change over the course of regeneration. Subsequent studies that strive to pinpoint the time of the cell cycle will need to more carefully define the clearance time and choose durations between the injections accordingly.

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