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Astrocyte Proliferation in the Chick Auditory Brainstem Following Cochlea Removal

DIANA I. LURIE AND EDWIN W RUBEL
Virginia Merrill Bloedel Hearing Research Center, Department of Otolaryngology-Head and Neck Surgery, University of Washington School of Medicine, Seattle, Washington 98195

ABSTRACT

Astrocytes in the central nervous system (CNS) respond to injury and disease by proliferating and extending processes. The intermediate filament protein of astrocytes, glial fibrillary acidic protein (GFAP) also increases in astrocytes. These cells are called "reactive astrocytes" and are thought to play a role in CNS repair. We have previously demonstrated rapid increases (< 6 hours) in GFAP-immunoreactive and silver-impregnated glial processes in the chick cochlear nucleus, nucleus magnocellularis (NM), following cochlea removal or activity blockade of the eighth nerve. It was not known whether these changes were the result of glial proliferation, glial hypertrophy, or both. The present study examined the time course of astrocyte proliferation in NM following cochlea removal.

Postnatal chicks received unilateral cochlea removal and survived for 6, 12, 18, 24, 36, 48, and 72 hours. Bromodeoxyuridine was used to label proliferating cells. The volume and number of labeled cells in NM was calculated for both the experimental and control sides of the brains for experimental animals was well as for unoperated control animals.

A subset of astrocytes continuously divide in the normal posthatch chick brainstem. The percentage of labeled nuclei increases within NM 36 hours following cochlea removal and is robust by 48 hours. This increase is due to astrocyte proliferation within, rather than migration to, NM. These results indicate that rapid increases in GFAP following reduced activity are independent of cell proliferation. The time course of astrocyte proliferation suggests that cellular degeneration within the nucleus may play a role in upregulating astrocyte proliferation.

Key words: **glia, nucleus magnocellularis, deafferentation, bromodeoxyuridine, glial fibrillary acidic protein (GFAP)**

Astrocytes frequently respond to injury and disease by proliferating and extending processes. This usually results in the formation of an astroglial scar and these events are referred to as "reactive gliosis" (Reier and Houle, 1988; Reier et al., 1983). Reactive astrocytes typically display an increase in glial intermediate filament protein, as demonstrated by an increase in glial fibrillary acidic protein (GFAP) immunoreactivity. These events are associated with an increase in the number of astrocytic cells and their processes as well as an increased appearance of intermediate filaments within processes.

For example, transection of the facial nerve in rat leads to an increased expression of GFAP in astrocytes of the facial nucleus within 2–3 days following the lesion (Graeber and Kreutzberg, 1986, 1988). Increased synthesis of GFAP (as demonstrated by ^{35}S-methionine incorporation) occurs in this nucleus 24 hours after the facial nerve transection, preceding the increased immunohistochemical expression (Tetzlaff et al., 1988). GFAP mRNA has also been shown to increase as early as 6 hours following a mechanical injury to the rat cortex (Condorelli et al., 1990) and levels of GFAP mRNA have been shown to increase rapidly (12 hours) within the hippocampus following unilateral lesions of the entorhinal cortex (Steward et al., 1990). Electrically induced seizures in rat hippocampus also lead to a rapid increase in GFAP mRNA at the stimulation site as well as in areas synaptically activated by the seizures (Steward et al., 1991). Other central nervous system (CNS) lesions including nerve crush (Tetzlaff et al., 1988), cortical aspiration (Singh and Mathew, 1989), ibotenic acid lesions to the dorsal lateral geniculate cortex in rat (Hajos et al., 1990),

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Address reprint requests to Edwin W Rubel, Department of Otolaryngology, Hearing Development Laboratories, RL-30, University of Washington, Seattle, WA 98195.

transection of the olfactory bulb (Anders and Johnson, 1990), and cerebral ischemia in rat (Petito et al., 1990) all lead to increases in GFAP immunoreactivity within 1–7 days postlesion.

The various CNS injury paradigms can be divided into two major groups: (1) brain regions which receive injuries resulting in neuronal death. In these areas, glial proliferation (astrocytic or microglial) occurs; and (2) brain regions affected by an injury that does not produce neuronal death in that area. In these regions, GFAP immunoreactivity increases but there is no cell proliferation (Streit and Kreutzberg, 1988; Petito et al., 1990).

We have previously demonstrated a rapid increase in GFAP-immunoreactive and silver-impregnated glial processes in the chick cochlear nucleus, nucleus magnocellularis (NM), following activity loss of the eighth nerve (Canady and Rubel, 1992; Rubel and MacDonald, 1992). Both unilateral cochlea removal and unilateral eighth nerve activity blockade (achieved through intralabyrinthine injection of tetrodotoxin, TTX) result in increases in GFAP immunoreactivity as early as 1 hour following the manipulation. This immunoreactivity steadily increases during the subsequent 72 hours.

The present study was undertaken to document the time course and position of astrocyte proliferation in NM following unilateral cochlea removal and to determine whether glial proliferation plays a role in the increase in GFAP immunoreactivity seen at early times ($<$ 6 hours) following activity loss. Chicks received a unilateral cochlea removal and were then injected with bromodeoxyuridine (BRDU), a thymidine analog incorporated into dividing cells (deFazio et al., 1987; Tapscott et al., 1989). Animals were sacrificed 6, 12, 18, 24, 36, 48, and 72 hours later and the BRDU label visualized with immunohistochemical techniques. A second group of animals received a unilateral cochlea removal and 48 hours later received a single injection of BRDU. This group of animals was killed 6 hours after the pulse of BRDU in order to determine the location of astrocyte proliferation.

Because NM neurons in the chick brainstem receive their sole excitatory input via the eighth nerve, removal of the basilar papilla (avian cochlea) eliminates all extracellularly recorded action potentials in NM ipsilateral to the manipulation (Born and Rubel, 1988; Born et al., 1991) but leaves neuronal activity in the contralateral NM intact. Thus, NM cells (neurons and glia) ipsilateral to the cochlear removal were directly compared to contralateral cells within the same tissue section.

We found that a small percentage of astrocytes proliferates throughout chick brainstem in the unoperated animal. Astrocyte proliferation increases within the deafferented NM approximately 36 hours following cochlear removal and by 72 hours has tripled compared to the unoperated side (control NM). These results indicate that rapid increases in GFAP following reduced activity are independent of cell proliferation. The time course of the astrocytic proliferation suggests that cellular degeneration within the nucleus may play a role in upregulating astrocyte proliferation.

MATERIALS AND METHODS

Subjects

Posthatch chickens (7–14 days old) were used for all experiments. White leghorn eggs were obtained from a local supplier (H&N Farms, Redmond, WA) and incubated in the University of Washington vivarium in AAALAC-approved facilities. Animals were maintained in warm brooders and given free access to food and water at all times.

Surgical procedures

Chicks were deeply anesthetized with a combination of ketamine (80 mg/kg body weight i.m.) and sodium pentobarbital (15 mg/kg body weight i.p.). The procedure for removal of the basilar papilla (cochlea) has been previously described (Born and Rubel, 1985; Durham and Rubel, 1985). Briefly, the feathers around the ear canal are removed, the tympanic membrane reflected, and the columella removed from the middle ear. The cochlea is then extracted through the oval window with a pair of fine forceps. The cochlea is examined under a dissecting microscope to ensure complete removal. The cavity of the oval window is filled with Gelfoam to prevent bleeding and the incision sealed with cyanoacrylate glue. All procedures are carried out under aseptic conditions. Following the cochlea removal, all animals received an injection of BRDU subcutaneously (50 mg/kg). Those surviving for 12 hours (4 chicks) and 18 hours (5 chicks) received additional BRDU injections every 6 hours. Animals surviving for 24 hours (5 chicks) received a total of 3 injections, and those surviving for 36 hours (5 chicks), 48 hours (5 chicks) and 72 hours (5 chicks) received injections of BRDU twice daily. Unoperated control animals (n = 5) received a single injection of BRDU and were killed 6 hours later. Another group of animals (n = 5) received a unilateral cochlea removal and 48 hours later received a single injection of BRDU. This group of animals was sacrificed 6 hours after the BRDU injection. Finally, an additional group of animals received a unilateral cochlea removal and were given BRDU injections twice daily 48 hours and 72 hours after deafferentation. These animals were allowed to survive for 1 week following the final injection of BRDU.

Immunohistochemistry

At 6, 12, 18, 24, 36, 48, or 72 hours after cochlea removal, chicks were reanesthetized and transcardially perfused with chick Ringer's (154 Mm NaCl, 6 Mm KCl, 8.4 Mm $MgCl_2$, 5 mM HEPES, 8 Mm glucose, 1 Mm EGTA) for approximately 3 minutes and the brains removed and postfixed in a modified Carnoy's fixative (6 parts ethanol, 2 parts chloroform, 1 part glacial acetic acid, and 1 part 10× chick Ringer's) at 4°C for 6 hours. The brains were then rinsed in 70% ethanol, left in 70% overnight, and embedded in paraffin the following day. Ten micron sections were cut, and a 1 in 4 series mounted onto poly-lysine-coated glass slides, and deparaffinized. Sections were then processed for BRDU histochemistry. Sections were immersed in ddH_2O for 10 minutes, immersed in 1 N HCl for 20 minutes, and then washed in phosphate-buffered saline, pH 7.4 (PBS). Sections were blocked with 4% normal horse serum for 20 minutes. This and all other immunocytochemical reagents (except for the ABC reagent) were prepared in 1% bovine serum albumin (BSA)/0.1% sodium azide in PBS. The sections were incubated overnight in mouse monoclonal anti-BRDU (Becton Dickinson, San Jose CA; 1:300) at room temperature in a humidified chamber. The next day, sections were washed in PBS, incubated in 1:250 biotinylated horse anti-mouse serum (Vector Labs, Burlingame CA) for 1 hour, washed in PBS and then incubated in an avidin-biotin complex (Vectastain ABC elite kit, Vector Labs). The chromogen used was diaminobenzidine (0.25 mg/ml; Sigma, St. Louis MO) with 0.08% nickel chloride and 0.1% hydrogen peroxide in Tris buffer, pH 7.6. The sections were then counterstained with eosin, dehydrated and coverslipped with DPX (BDH Limited, Poole, England). Alternate sections from selected animals were stained for thionin or processed for OX42 histochemistry (complement receptor 3) in order to label microglia (Robinson et al., 1986; Rinamen et al., 1991; Lassman et al., 1991; Shigomatsu et al., 1992). Sections labeled with anti-OX42 (Harlan Bioproducts for Science, Indianapolis IN; 1:300) were processed similarly to those for BRDU histochemistry except that: (1) Tris buffer (pH 7.4) was used instead of phosphate-buffered saline; (2) sections were not immersed in 1 N HCl; (3) DAB was not intensified with nickel chloride, and (4) sections were not counterstained with eosin. Selected sections from animals receiving BRDU injections on days 2 and 3 after deafferentation and surviving 1 week thereafter, and selected sections from animals receiving one BRDU injection 48 hours after deafferentation and surviving 6 hours thereafter were double-labeled for BRDU and GFAP. Briefly, sections were processed for BRDU histochemistry as described above. The sections were not counterstained with eosin but were double-labeled with anti-GFAP (DAKO, Carpinteria, CA) and processed similarly to those for OX42 histochemistry.

Tissue analysis

Brainstem sections were viewed with a Zeiss Universal microscope at a final magnification of 260×. The number of BRDU-labeled cells within control and experimental NM was analyzed by two different methods. Only astrocytes took up the BRDU label (see Results). These cells were identified by their morphological features, including their large, pale nuclei.

Method 1. The border of NM was defined as the border around NM neurons. The total number of labeled nuclei within both ipsilateral and contralateral NM was counted. All BRDU-labeled nuclei were counted in every fourth section through the entire nucleus on both sides of the brainstem. The number of labeled astrocytes in experimental NM vs. control NM within the same tissue section was expressed as the ratio (number of cells ipsi/number of cells contra). In this way, it was possible to determine when the number of BRDU-labeled glia increased on the experimental vs. the control sides of NM. However, this traditional method of counting cells yields no information about absolute volumes or absolute numbers of glia. Since correction factors such as the Abercrombie correction (Abercrombie, 1946) were not applied because the glial counts were expressed as a ratio, increases or decreases in absolute numbers of BRDU-labeled astrocytes following deafferentation cannot be determined from these counts. In addition, such correction factors have been called into question and many authors suggest using volumetric measurements as demonstrated in Method 2 (Gunderson et al., 1988; West and Gunderson, 1990; Pover and Coggeshall, 1991; Tandrup, 1993).

Method 2. The Cavalieri method of estimating volumes was used to determine the total volume of labeled astrocyte nuclei within NM (ipsilateral and contralateral) as well as the total volume of NM itself. This method yields absolute volume measurements so that we could compare the mean total volume of BRDU-labeled nuclei within deafferented NM with the mean total volume of BRDU-labeled nuclei within control NM. The Cavalieri method could be employed here because systematic sections were collected through NM (Gunderson and Jensen, 1987).

With a random start, every eighth section through NM was analyzed. Slides were viewed with a microscope to which was attached a Dage MTI model 68 video camera connected to a MAC IIcx computer. A Datatranslation QuickCapture board (Datatranslation, Marlboro, MA) was used to capture the image from the microscope. NIH Image 1.45 (image analysis software) allowed analysis of the digitized images. A test grid of points was superimposed over the captured image on the computer screen. For the Cavalieri estimate of total NM volume, a test grid of points spaced 2 cm apart was used and all points falling on NM (magnification 260×) were counted. For the volume estimate of labeled glia, a 0.25 cm test grid was used and all points which fell on BRDU-labeled astrocytes (magnification 260×) were counted. The total volume of the structure (in this case, NM or the labeled astrocytes) was then calculated by the following formula:

$$V_{(volume)} = \Sigma P_{(organ)} \cdot A_{(point)} \cdot \bar{t}_{(slice)}$$

where V is the volume of the organ, EP is the sum of all the points on the organ, A is the area of the point on the test grid, and $\bar{t}$ is the thickness of the slice (Gunderson and Jensen, 1987). The coefficient of error (CE) for each volume calculation was calculated by using the equation:

$$CE(\Sigma P) = \frac{\sqrt{(3A + C - 4B)/12}}{\Sigma P}$$

where $A = (P \times P)$, $B = (P \times P) + 1$, and $C = (P \times P) + 2$. In practice, we used the computer program entitled "QM2000" developed by Dr. Robert Bolender at the University of Washington. Once EP was calculated for each NM and the astrocyte nuclei within NM, the numbers were entered into an IBM XT and the QM 2000 program calculated both the volume and the CE for each structure. Almost all of the calculated volume data had CEs ≤ 0.5. Significant differences in both the volume of NM and the volume of labeled astrocyte nuclei within NM were determined by a one-way ANOVA and for individual comparisons we compared values on the experimental (deafferented) side with those for the control side of the brain by using Dunnett's test. The underlying assumption of both Methods 1 and 2 is that the volumes of individual glial nuclei are comparable between control and experimental treatment groups. Although we did not sample large numbers of labeled glial nuclei, we found that the diameter of BRDU-labeled nuclei was similar in control and deafferented NM (unpublished observations).

RESULTS

Control animals

Five unoperated control chicks were injected with a single injection of BRDU and killed 6 hours later. Cells throughout the entire chick brainstem as well as within NM were labeled with BRDU (Fig. 1A). These non-neuronal cells were identified as astrocytes on the basis of their morphology and nuclear size (Fig. 1B). These cells possess oval/round nuclei approximately 10 μm in diameter which stain lightly with thionin (Fig. 1C). Unfortunately, the antibody to GFAP (an astrocytic marker) does not label all

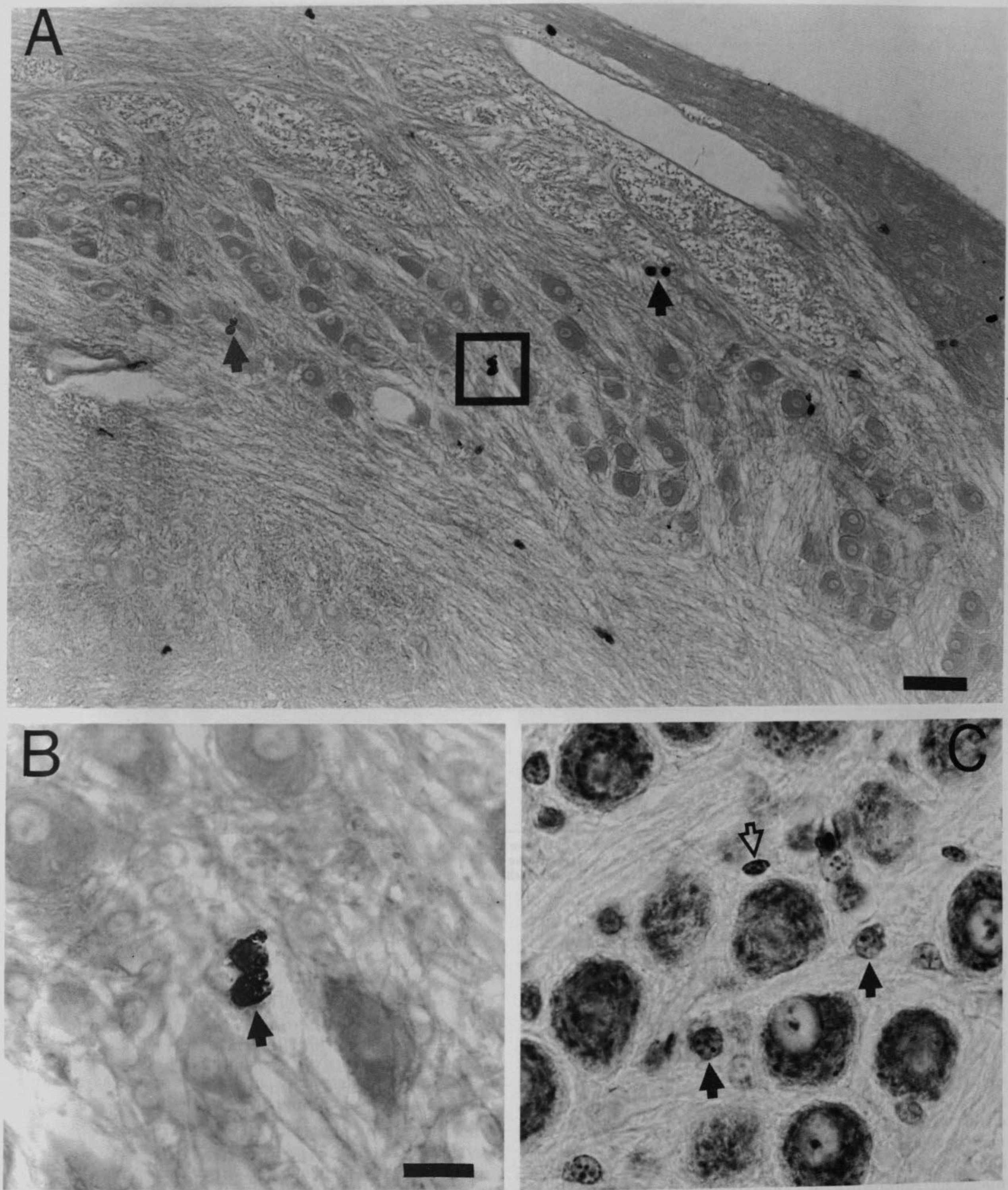

Fig. 1. Unoperated control chick nucleus magnocellularis (NM) labeled with a single injection of bromodeoxyuridine (BRDU) and sacrificed 6 hours later. **A:** Low power micrograph of NM. BRDU-labeled glial nuclei (arrows) located throughout the brainstem, including NM. **B:** Two BRDU-labeled glial nuclei (from the box in A) are shown at increased magnification (arrow). Their large, oval/round nuclei are characteristic of astrocytic nuclei. **C:** Alternate brainstem section of NM stained with thionin. The large, round, pale nuclei (dark arrows) are characteristic of astrocyte nuclei while the smaller, dark, elliptical nuclei (open arrow) are characteristic of oligodendrocytes. Note the BRDU-labeled nuclei in B resemble the large, pale astrocyte nuclei shown in C. Bars = 40 μm for A; 10 μm for B and C.

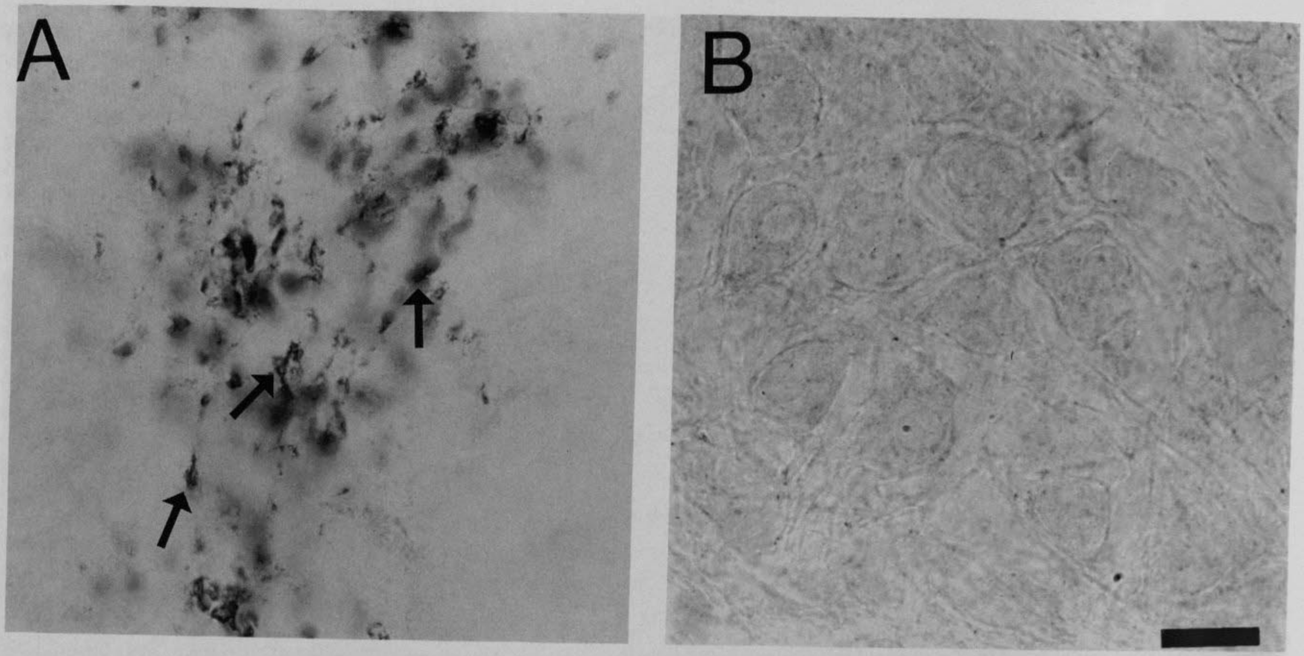

Fig. 2. Unoperated control chick NM immunolabeled with the OX42 antibody to microglia. **A:** The only OX42-positive cells in unoperated controls were located along the midline of the brainstem. The immunopositive cells are small (approximately 3 μm in diameter) and are triangular in shape (arrows). **B:** No OX42-immunolabeled cells were found in the unoperated, control NM. Bar = 10 μm for A and B.

TABLE 1. Analysis of Glial Proliferation Following Cochlea Removal[1]

	Control	No. of hours 6	12	18	24	36	48	72
Mean ratio of no. of BRDU-labeled nuclei ipsi/contra ± S.E.M. (Method 1)	0.98 ± 0.11	1.36 ± 0.12	1.05 ± 0.05	0.94 ± 0.04	1.04 ± 0.1	1.4 ± 0.1	1.5 ± 0.12	3.02 ± 0.4*
Mean volume BRDU-labeled nuclei ipsi/contra ± S.E.M. (Method 2)	1.0 ± 0.13	1.14 ± 0.06	1.14 ± 0.10	0.98 ± 0.05	1.08 ± 0.09	1.48 ± 0.12	1.92 ± 0.31*	3.38 ± 0.47*

[1]Comparison between two methods of analysis of glial proliferation. Top: Cell counts were made and then expressed as the ratio no. of nuclei in ipsi NM/no. of nuclei in contra NM. Bottom: Estimate of total volume of BRDU-labeled nuclei in ipsi NM/contra NM using the Cavalieri method of estimating volumes. Number expressed is mean among all animals at each time point. There is a significant increase in labeled glial nuclei at 48 and 72 h after cochlea removal by using volume estimates and a significant increase in labeled nuclei only at 72 h by using the ratio of labeled nuclear counts.
*Dunnet's test, P ≤ 0.05).

astrocytes in the chick brainstem (unpublished observations); thus, identification of these cells as astrocytes cannot be accomplished by GFAP immunostaining. The labeled cells are unlikely to be oligodendrocytes, as ongoing proliferation has not been observed once myelination has begun (Ludwin, 1988). In addition, brainstem sections stained with thionin revealed that most of the non-neuronal cells in NM possessed the lighter, round nuclei characteristic of astrocytes rather than the smaller, darker nuclei typical of oligodendrocytes (Fig. 1C). Immunohistochemical staining for the OX42 antibody which labels microglia (Robinson et al., 1986) revealed cells located along the midline of the brainstem which were much smaller than those labeled with BRDU (Fig. 2A). These cells were triangular and approximately 3 μm in diameter. No OX42-immunolabeled cells were found in NM (Fig. 2B) of unoperated animals. Thus, it appears that the non-neuronal cells which incorporate BRDU are astrocytic. No neurons within NM incorporated the BRDU label.

There was no significant difference in the volume (number) of labeled astrocyte nuclei between the two sides of NM in normal animals. The mean volume of labeled nuclei in NM ipsilateral to the cochlea removal was 2.6×10^{-4} mm^3, while the mean volume of labeled nuclei contralateral to the cochlea removal was 2.7×10^{-4} mm^3 (Fig. 5; Table 1). In other words, there are equal numbers of labeled astrocytes on both sides of NM in the unoperated controls. The BRDU-labeled astrocyte nuclei occupied approximately 0.42% of the total volume of NM.

Deafferentation

The total volume of proliferating glial nuclei within NM begins to increase 36 hours following cochlea removal. Deafferentation of the eighth nerve (cochlea removal) does not breach the blood-brain barrier. Therefore, it is unlikely that any BRDU-labeled cells found within NM are derived from sources outside the nervous system. Figures 3A,B, and 5 demonstrate that during the 24 hours following cochlea removal, there are no signs of increased glial cell proliferation within the deafferented nucleus. The number (volume) of labeled astrocyte nuclei appears to increase 36

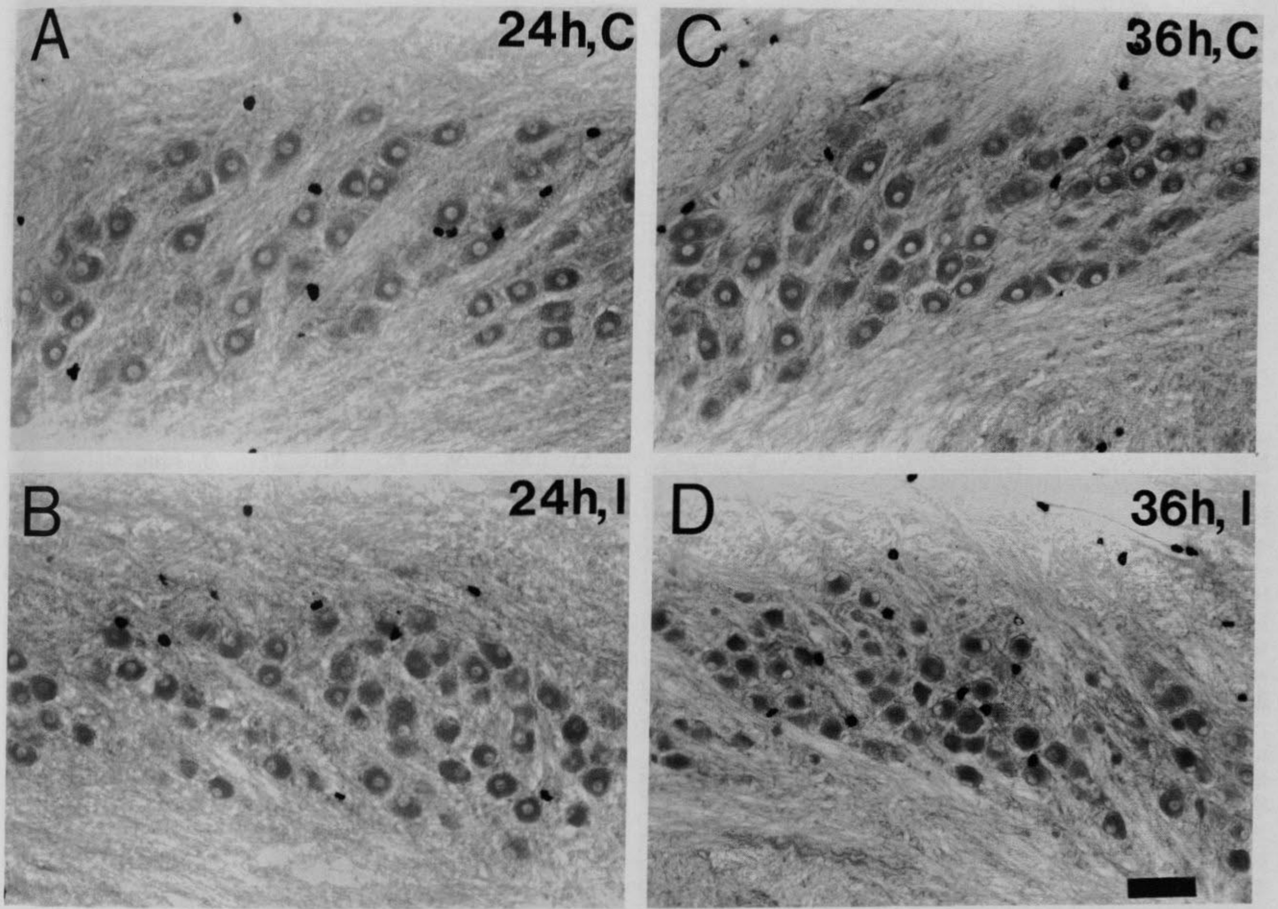

Fig. 3. Time course of astrocyte proliferation in NM after cochlea removal. Tissue sections are from animals sacrificed 24 hours (**A,B**) and 36 hours (**C,D**) after cochlea removal. A and C are control sides of the tissue sections, contralateral to the cochlea removal and contain few BRDU-labeled cells. B and D are from the same tissue sections, but on the side ipsilateral to the cochlea removal. There are no signs of increased astrocyte proliferation within NM 24 hours after deafferentation (B). By 36 hours after cochlea removal, the number of BRDU-labeled cells begins to increase (D). Bar = 40 μm.

hours after cochlea removal (Figs. 3C,D; 5) and becomes statistically significant by 48 hours after cochlea removal (Figs. 4A,B; 5). By three days following deafferentation, glial proliferation has tripled in the affected nucleus (Figs. 4C,D; 5).

Figure 5 shows mean volumes of BRDU-labeled nuclei within the ipsilateral and the contralateral NM for control animals and at each survival time examined. Note that the increase in the mean total volume of BRDU-labeled glial nuclei over time within the contralateral (control) NM reflects the small amount of ongoing glial proliferation which normally occurs in the unoperated chick brainstem. The longer the exposure to BRDU, i.e., 12 hours vs. 3 days, the more of these glial nuclei will be labeled. The increase in cell proliferation following deafferentation appears to be confined to the region containing NM and NL. Observations of BRDU-labeled cells in the rest of the brainstem do not show an increase in proliferation on the side of the brain ipsilateral to the cochlea removal.

The increase in glial proliferation appears to originate within NM itself. A group of five chicks received unilateral cochlea removals and then were allowed to recover for 48 hours. At that time, they were given a single injection of BRDU and killed 6 hours later (pulse-fix protocol). By 48 hours, increased glial proliferation is well underway in NM and a single pulse of BRDU should label the pool of cells which is in the process of completing S-phase of the cell cycle. If glial cells are proliferating outside the nucleus and then migrating into NM, BRDU-labeled cells would be observed in areas outside the nucleus following the pulse of BRDU. This was not the case. The single pulse of BRDU 48 hours after cochlea removal labeled many astrocytes within NM. A pool of proliferating cells outside the nucleus was not observed (Fig. 6). At no time following cochlea removal were BRDU-labeled neurons observed within NM or within the entire brainstem section.

The increased cell proliferation following cochlea removal initially involves the astrocyte population. Immunostaining with the OX42 antibody revealed a very small number of positive cells in NM 72 hours after deafferentation (Fig. 7B). However, thionin-stained alternate sections at 3 days demonstrated a greatly increased number of round pale glial (astrocytic) nuclei (Fig. 7A) and these type of glial cells accounted for the majority of glial cells observed in NM at this time.

Double-label experiments with antibodies against BRDU and GFAP were carried out to address two issues. First, by pulse labeling with BRDU and killing the animal shortly

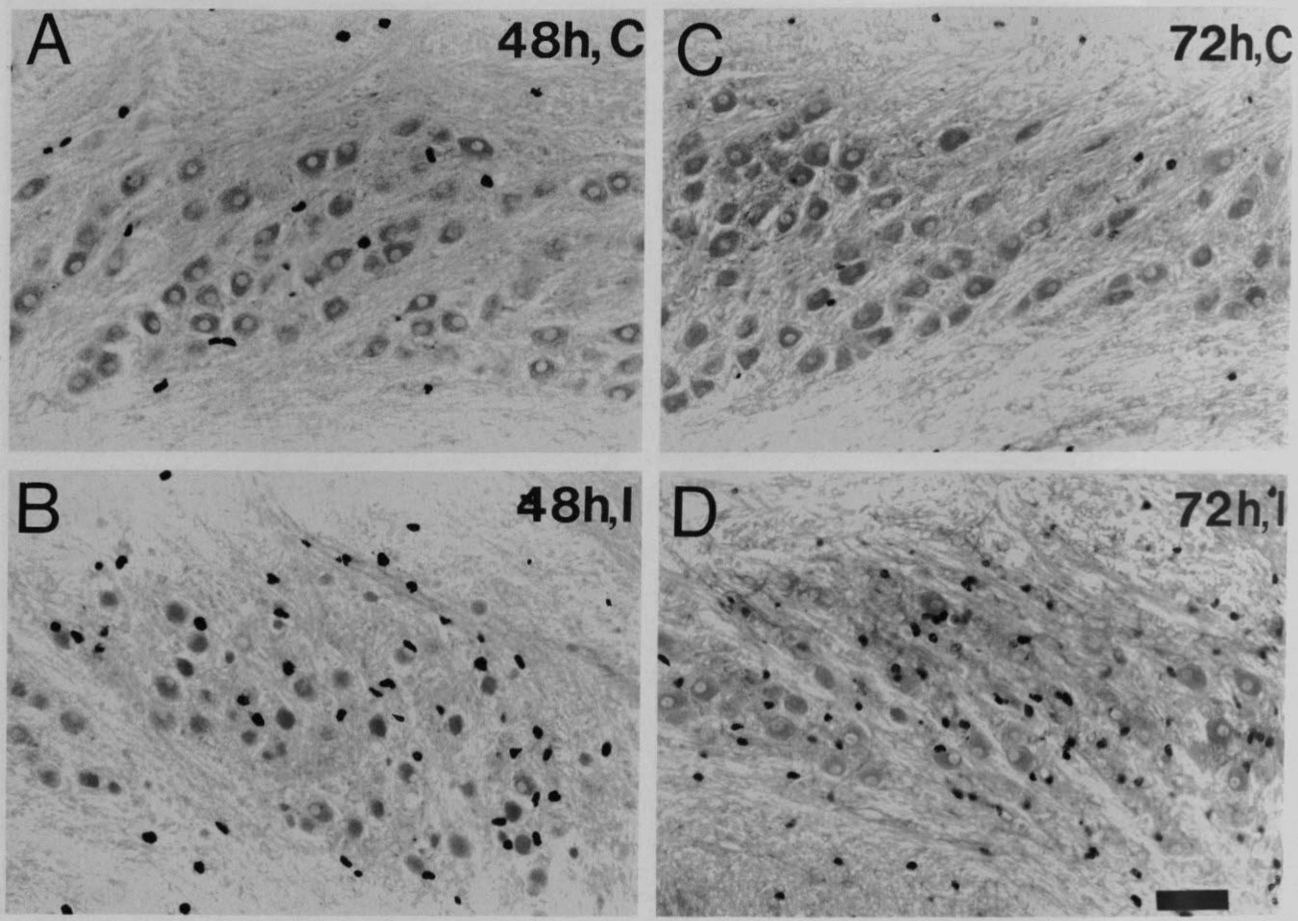

Fig. 4. Time course of astrocyte proliferation in NM after cochlea removal. Tissue sections are from animals sacrificed 48 hours (**A,B**) and 72 hours (**C,D**) after cochlea removal. A and C are contralateral to the cochlea removal and contain few labeled cells. B and D are from the side ipsilateral to the cochlea removal in the same tissue sections. By 48 hours after deafferentation, there are many more labeled cells (B), and glial proliferation triples in deafferented NM 72 hours after cochlea removal. Bar = 40 μm.

thereafter (6 hours survival), we sought to determine if proliferating cells could be identified as astrocytes by the presence of GFAP. Figure 8A shows a cell positive for both BRDU and GFAP in an animal which received a pulse of BRDU 48 hours after deafferentation and was sacrificed 6 hours later. However, these double-labeled cells were not frequently observed. It is likely that when astrocytes dedifferentiate and divide, they lose much of their immunoreactivity for GFAP. The second issue addressed by double-labeling experiments is the phenotype of the newly produced cells. To address this problem, a group of chicks received a unilateral cochlear removal and were then given BRDU injections twice daily 48 and 72 hours later. These animals were allowed to survive for a week following the last BRDU injection. This protocol allowed time for the cells, which were dividing during the 2 day pulse of BRDU, to differentiate. Brain sections from these animals were double-labeled for BRDU and GFAP. Careful inspection of this tissue revealed that the majority of BRDU-labeled nuclei were surrounded by a thin rim of GFAP-positive cytoplasm or had GFAP-positive processes extending outward. Examples are shown in Figure 8B. Therefore, the majority of BRDU-labeled cells are astrocytes.

Comparison of quantitative methods

In the present study, we employed two different methods to analyze the number of astrocytes labeled with BRDU. It is impossible to determine from the 10 μm sections whether the labeled nuclei are completely within the section. For example, only half of the nucleus may be present but it would be counted as one cell. Therefore, counting all labeled nuclei will result in an overestimate of the total number of proliferating astrocytes. In order to solve this problem, total cell counts were made and then expressed as a ratio of number of nuclear profiles in ipsilateral NM/number of nuclear profiles in contralateral NM. This was possible due to the fact the both the experimental and control NM come from the *same* tissue sections. When cell counts were expressed in this way, the increase in glial proliferation did not become statistically significant ($P \leq 0.05$) until 72 hours following deafferentation (Table 1), even though an increase was apparent by examining the tissue at 36 hours, and the ratio of the two sides averaged 1.4 at 36 hours.

A more accurate way to assess glial proliferation is to estimate the total volume of labeled cell nuclei within NM. It should be emphasized that the volume of NM occupied by all labeled astrocyte nuclei is being estimated, not the

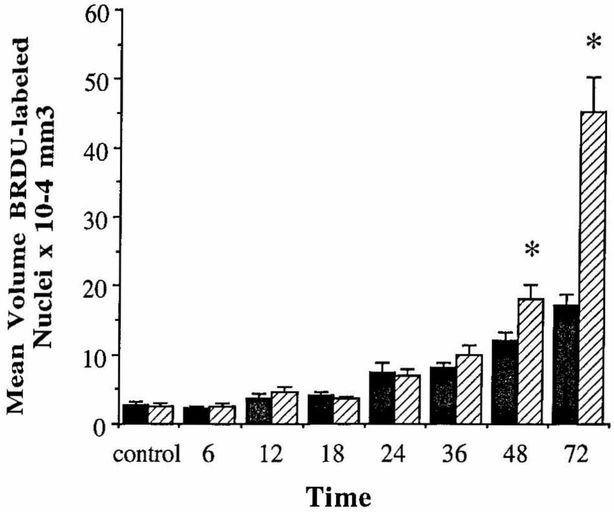

Fig. 5. Mean volumes of BRDU-labeled nuclei in ipsilateral NM compared to the contralateral NM for control animals and animals sacrificed 6, 12, 18, 24, 36, 48, and 72 hours after cochlea removal. The dark bars represent the mean of the volume of BRDU-labeled nuclei in NM contralateral to cochlea removal. The striped bars represent the mean volume of BRDU-labeled nuclei within the deafferented NM. There is a significant increase in the volume of BRDU-labeled nuclei in deafferented NM at 48 hours after cochlea removal. Error bars indicate standard error of the mean. Asterisk, one-way ANOVA, $P \leq 0.05$.

volume of an individual labeled astrocyte. (The volume of an individual astrocyte remains constant during proliferation.) This is a more accurate way of determining when proliferation increases because it gives quantitative information about three-dimensional structures (Gunderson and Jensen, 1987; Gunderson et al., 1988). When the total volume of labeled nuclei in ipsilateral NM was compared to the total volume of labeled nuclei in contralateral NM, we found that astrocytic proliferation was significantly increased at 48 hours after cochlea removal. This result could be explained by the fact there was less variability among our volume estimates than among our counts of the number of labeled astrocytes.

Volume of NM

Using the Cavalieri method of estimating volumes, we determined the volume of control vs. deafferented NM over time. The mean volume of NM was approximately $6.49 \pm 0.85 \times 10^{-4}$ mm^3 in control animals (data not shown). The mean total volume of the ipsilateral NM decreased by approximately 20% ($P \leq 0.05$) by 36 hours following cochlea removal compared to the contralateral NM. By 72 hours following deafferentation, the volume of the ipsilateral NM compared to the contralateral NM had only decreased by an additional 6% as compared to the volumes at 36 hours. Therefore, the increase in glial cell proliferation occurs when the ipsilateral NM is shrinking in volume compared to the contralateral side.

DISCUSSION

We have demonstrated that there is a basal rate of astrocyte proliferation in the posthatch chick brainstem. This proliferation appears to increase in nucleus magnocellularis approximately 36 hours after cochlea removal and is statistically significant by 48 hours. Concomitant with the increase in glial proliferation is a decrease in the volume of the deafferented NM relative to the control side. This volume decrease is significant at 36 hours after activity loss. The increase in GFAP immunoreactivity which has been observed in NM as early as one hour after activity loss (Canady and Rubel, 1992; Rubel and MacDonald, 1992) does not appear to be the result of increased glial proliferation. While increased GFAP immunoreactivity is triggered by activity loss, increased glial proliferation in this auditory brainstem nucleus appears to be correlated with the volume decrease in NM, which occurs many hours after deafferentation.

Identification of dividing cells and their progeny

The major goals of the present study were to examine the timing of proliferative activity after deafferentation of NM and the position of cells which became mitotically active. It is also of interest, however, to identify the precursor cell population and the cell types(s) of the differentiated progeny. These problems have been addressed (with variable success) by combining BRDU labeling with cytological markers thought to be specific for particular types of non-neuronal cells. Where immunologically specific cellular markers have proven insufficient, we have had to draw upon established histological criteria for identifying cell types (Peters et al., 1991).

Identification of the precursor population has proven difficult. A large number of studies have shown that astrocytes are capable of proliferation in response to CNS injury or when isolated in vitro (Cavanagh, 1970; Latov et al., 1979; Janeczko, 1988; Topp et al., 1989; Lillien and Raff, 1990; Malhotra et al., 1990). Oligodendrocytes are thought to proliferate in cases of CNS diseases such as multiple sclerosis (Raine et al., 1981; Prineas et al., 1989; Ludwin, 1988) and chronic relapsing experimental allergic encephalomyelitis (Raine et al., 1988). Wallerian degeneration of the rat optic nerve is also associated with a limited amount of oligodendrocyte proliferation (Skoff and Vaughn, 1971; Skoff, 1975). Microglial proliferation has been well documented by a number of investigators (Watson, 1965; Streit et al., 1988; Streit and Kreutzberg, 1988; Graeber et al., 1988). In addition, in vitro experiments have shown that an undifferentiated population of cells exists in embryonic optic nerve which can be activated to produce glial cells (Lillien and Raff, 1990). A precursor population of astroblasts in adult brain has also been described in vivo. These cells continuously divide throughout the life of the animal and give rise to astrocytes and oligodendrocytes (McCarthy and Leblond, 1988). It has also been suggested that such glial precursor cells in the mature brain give rise to at least some reactive astrocytes following trauma (Norton and Farooq, 1989).

While we have been unsuccessful at unequivocally identifying the precursors of the deafferentation-induced proliferation seen in NM, several lines of evidence suggest that the mitotically active cells are astrocytes. First, a 6 hour BRDU pulse, followed immediately by fixation in animals 48 hours after cochlea removal, demonstrates that a small number of S-phase (or G_2 phase) cells are immunopositive for GFAP. That most BRDU-labeled cells were not GFAP-positive may be due to the fact that many astrocytes in NM are not normally reactive to GFAP and others may lose immunoreactivity while in the dedifferentiation process of rounding-up and entering the mitotic cycle. Thus, GFAP

Fig. 6. Increased glial proliferation after deafferentation originates within NM. Tissue section containing deafferented NM from an animal which received a unilateral cochlea removal, was injected 48 hours later with a single injection of BRDU, and sacrificed 6 hours later. The single pulse of BRDU labeled many proliferating astrocytes within NM (arrows). A pool of proliferating astrocytes outside NM was not observed. Bar = 40 μm.

immunoreactivity may not be expected to reliably identify astrocytes which are in the process of undergoing cell division. The second reason we believe the proliferating cells are astrocytes is that in both normal tissue and deafferented tissue, the non-neuronal cells in NM display staining properties characteristic of astrocytes including large, pale, rounded nuclei. Finally, observations of NM at the electron microscopic level have shown that the majority of non-neuronal cells within NM have the characteristic morphology of astrocytes, in control brainstem sections as well as sections taken from 6 hours and 3 days postdeafferentation tissue (unpublished observations). These characteristics include: large cell body, round or oval nuclei, lucent cytoplasm containing sparsely distributed organelles, elongated mitochondria, occasional filaments, and small processes which form laminae around NM neurons and their synapses (Peters et al., 1991).

We do not think that oligodendrocytes proliferate in NM following deafferentation. Oligodendrocytes are not thought to proliferate in uninjured, fully myelinated tissue (Ludwin, 1988). Thus it is unlikely that the small population of proliferating cells observed in the unoperated NM are oligodendrocytes. In addition, very few non-neuronal cells possessing the dark, elongated nuclei of oligodendrocytes were observed at the light microscopic level in NM in either the control or the experimental sides of the brain 3 days after cochlea removal. Similarly, very few cells were observed at the electron microscopic level in deafferented NM which displayed the dark cytoplasm and clumped nuclear chromatin characteristic of oligodendrocytes. On the other hand, oligodendrocytes as well as other types of glia have been shown to proliferate as early as 3 days after CNS injury (Ludwin, 1984, 1985). Unfortunately, we were unable to label oligodendrocytes immunohistochemically. Antibodies including those to myelin basic protein, transferrin, gal C, and oligodendrocytes (Chemicon) failed to cross-react with the chick. Thus, we cannot definitively rule out the possibility that oligodendrocytes may contribute to the glial proliferation observed following cochlea removal.

Microglial proliferation does not appear to occur to any great extent in NM during the initial 72 hours following destruction of the cochlea. Antibody staining with OX42, a microglial marker (Robinson et al., 1986), revealed only a few labeled cells with very small, triangular shaped nuclei within NM three days after cochlea removal. Finally, we cannot rule out the possibility of an uncommitted precursor cell population which proliferate after cochlea removal.

When glial cells which had incorporated BRDU were given time to differentiate, the great majority of BRDU-positive cells were also found to be positive for GFAP. This was demonstrated by labeling glial cells with BRDU on days 2 and 3 after cochlea removal. These animals survived for 1 week following the last BRDU injection, allowing the astrocytes which had incorporated BRDU at the time of the

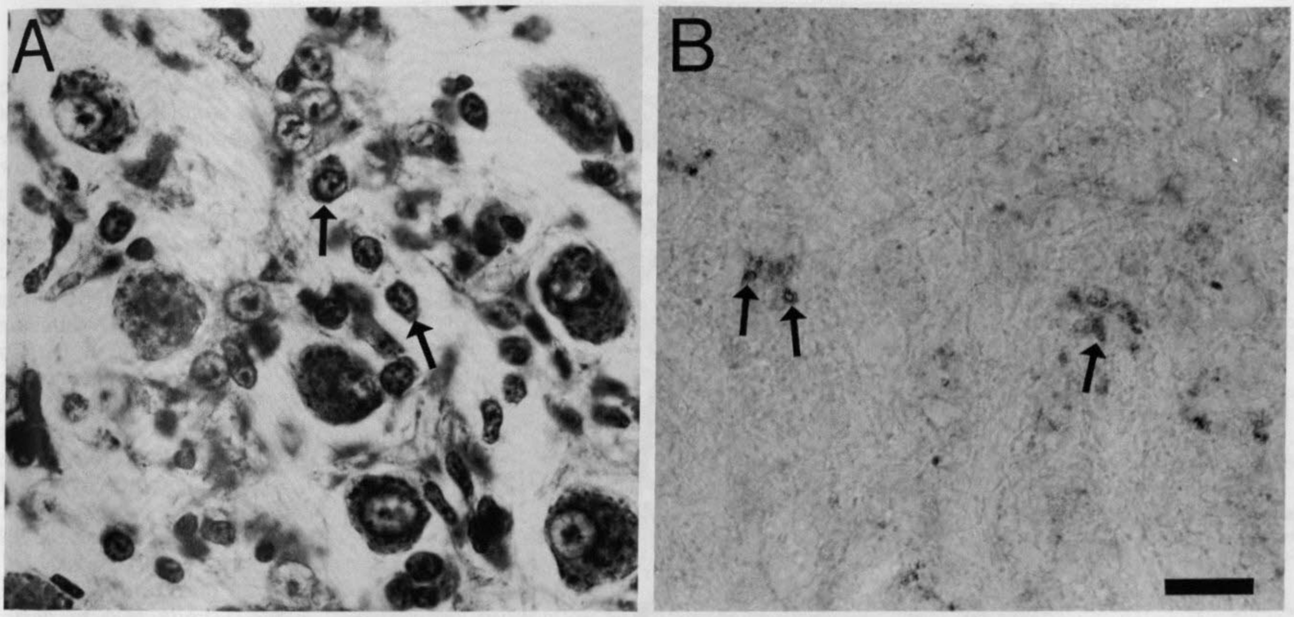

Fig. 7. The increased cell proliferation following cochlea removal involves the astrocyte population. **A:** Thionin-stained deafferented NM 3 days after cochlea removal. The majority of glial cells possess the round, pale nuclei characteristic of astrocyte nuclei (arrows). **B:** Alternate tissue section immunolabeled with the OX42 antibody. There are a small number of small triangular immunopositive cells within NM 3 days after cochlea removal (arrows). These cells resemble microglia. Bar = 10 μm.

injections to differentiate and express GFAP. Therefore, we do know that the majority of cells which result from cell division induced by deafferentation, differentiate into GFAP-positive astrocytes.

Methods of analysis

In the current study, two different morphometric methods were employed to document the increase in glial cell proliferation in NM following deafferentation. The first method involved simply counting the number of BRDU-labeled nuclei in NM and then expressing this number as a ratio of: number of labeled nuclei in ipsi NM/number of labeled nuclei in contralateral NM. The second method utilized stereological procedures to determine the total volume of BRDU-labeled nuclei within NM. The second method, utilizing the principles of quantitative morphology, appears to be a more powerful way to analyze the increase in proliferation. Observations of the tissue suggested that the number of BRDU-labeled nuclei begins to increase at 36 hours in some animals, and by 48 hours, the increase is quite obvious in all animals even to the casual observer. However, the increase in proliferation was only statistically significant at 72 hours, employing conventional counting techniques and parametric statistics. By measuring the volume of BRDU-labeled nuclei within NM, we found that the increase was statistically reliable by 48 hours. This result also concurred more closely with our qualitative observations of proliferation at this time. Although measuring volumes appears more indirect than actually counting the number of labeled cells, this method appeared to result in less variability, thereby yielding more consistent results. However, Method 1 would seem to be a valid indicator of when there is a reliable *change* in the number of proliferating cells. This is confirmed by the relative similarity in the time course of changes in proliferation observed using the two methods (Table 1).

Timing of astrocyte proliferation

There appears to be a small number of precursor cells in the undamaged posthatch chick brainstem which are normally proliferating at the ages we examined. In the young chick, the brain is still growing in size and while the neuronal cell population has attained its adult distribution, the glial cell population is increasing. The increasing number of glial cells must therefore contribute to the increasing brain size. Astrocytes as well as oligodendrocytes have been shown to proliferate according to a steady state system in 14-day-old rat (cortex, corpus callosum, nucleus caudatus putamen, and commissura anterior; Korr et al., 1983). However, this group also found that the glial proliferation was accompanied by continuous cell loss. While the current study did not examine cell loss, pyknotic nuclei are rarely observed in chick brainstem (unpublished observations).

The increase in proliferation observed in NM following deafferentation appears to involve cells that reside within the nucleus, rather than migration of postmitotic cells into NM. When a single pulse of BRDU is administered 48 hours after cochlea removal and the tissue is fixed 6 hours later, labeled cells are observed within and around NM. A large pool of proliferating cells outside of NM which would subsequently migrate into NM is never observed. This finding is consistent with observations of trauma to rat cortex and hippocampus, where puncture wounds result in astrocyte proliferation within the target area (Topp et al., 1989; Janeczko, 1991). On the other hand, *nondividing* glia have been reported to migrate into deafferented rat hippocampus while glial proliferation remained confined to the hippocampus (Gall et al., 1979).

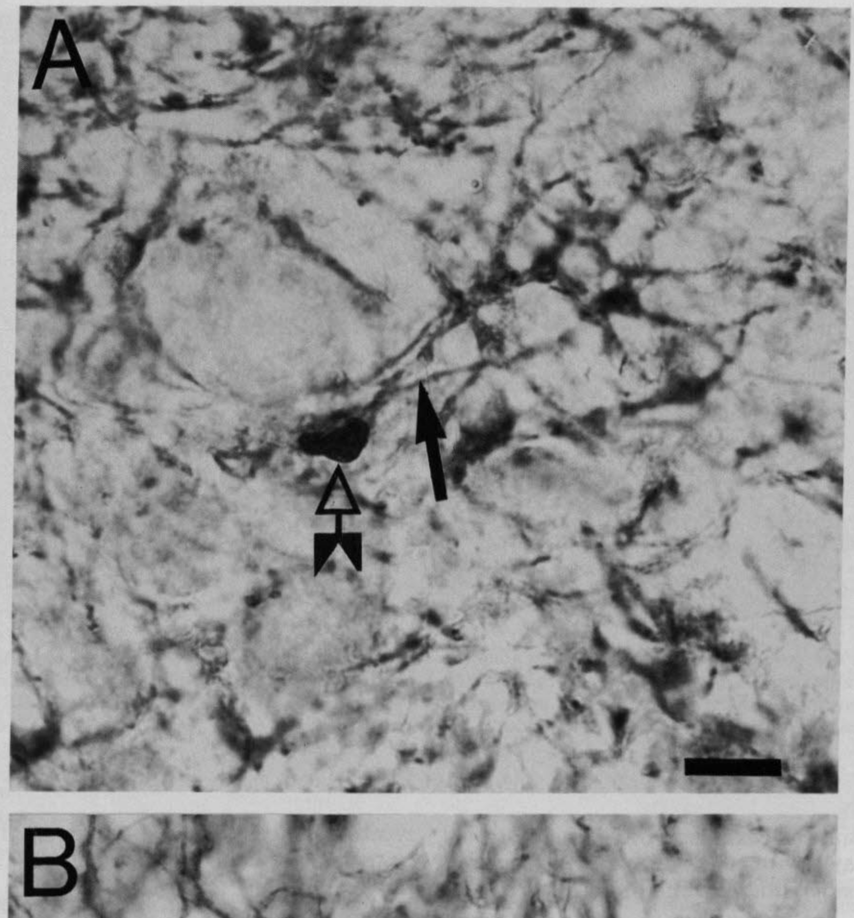

Fig. 8. BRDU-labeled glial cells also express glial fibrillary acidic protein (GFAP). **A:** Tissue section containing deafferented NM from an animal which received a unilateral cochlea removal, injected 48 hours later with a single injection of BRDU, and sacrificed 6 hours later. A few dividing glia (BRDU-positive, black and white arrow) also labeled with GFAP (black arrow). One such cell is shown. **B:** Tissue section containing deafferented NM from an animal which received a unilateral cochlea removal, injected twice daily with BRDU at 48 and 72 hours, and killed 1 week after the last injection. Many glial cells are labeled with both BRDU (black and white arrows) and GFAP (black arrows). Bars = 10 μm.

Potential astrocyte mitogens

The signal or signals which cause an increase in glial cell proliferation in NM following deafferentation remain unknown. Interestingly, recent studies suggest that injuries which result in neuronal death promote glial proliferation, whereas less severe trauma which does not result in neuronal loss, results in GFAP immunoreactivity increases with no acceleration of cell proliferation (Streit and Kreutzberg, 1988; Petito et al., 1990). In fact, deafferentation lesions of the rat hippocampus (Rose et al., 1976; Gall et al., 1979), spinal cord (Murray et al., 1990), and facial nucleus (Graeber and Kreutzberg, 1986) have demonstrated hypertrophy and possible migration of resident astrocytes rather than substantial proliferation. The dominant proliferating glial cell type in this injury paradigm appears to be microglia, with increased proliferation as early as 20 hours postlesion (Gall et al., 1979; Streit et al., 1988). In contrast, lesions which breach the blood-brain barrier such as a stab wound to the rat cortex (Cavanagh, 1970; Latov et al., 1979; Janeczko, 1988) and hippocampus (Topp et al., 1989), result in substantial astrocyte hypertrophy and proliferation within the lesion site. Why deafferentation of NM should result in increased production of macroglia rather than microglial cell proliferation is not known.

The control of astrocyte proliferation has been studied mainly in vitro and several compounds which have a positive stimulatory effect on astroblasts and astrocytes have been identified. These include the cytokines interleukin (IL)-1 (Guilan and Lachman, 1985), tumor necrosis factor a (TNF) and IL-6 (Selmaj et al., 1990), as well as epidermal growth factor (Westermark, 1976) and acidic and basic fibroblast growth factors (Morrison and DeVellis, 1982; Perraud et al., 1988; Petroski et al., 1991). Condorelli et al. (1989) have recently demonstrated that the addition of quisqualate (a glutamate analog) and glutamate reduced ^{3}H-thymidine incorporation and cell proliferation in primary cultures of rat cortical astrocytes. In the chick brainstem, the neurotransmitter at the synapse between the eighth nerve fibers and NM cells is thought to be an excitatory amino acid, specifically glutamate (Nemeth et al., 1985; Martin, 1985; Jackson et al., 1985; Rubel et al., 1990; Raman and Trussell, 1992). There is a very high rate of spontaneous activity in the eighth nerve fibers and subsequently in NM neurons (Rubel and Parks, 1988). An intriguing possibility is that the continuous release of glutamate from eighth nerve terminals onto NM neurons serves to keep proliferation of astrocytes at low levels. Following deafferentation, when the eighth nerve falls silent, the lack of glutamate release may allow astrocyte precursors to reenter the cell cycle. This possibility remains to be examined by using pharmacological blockade of eighth nerve activity (Born and Rubel, 1988) rather than cochlea destruction.

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