THE JOURNAL OF COMPARATIVE NEUROLOGY 359:412–423 (1995)

Afferent Influences on Brainstem Auditory Nuclei of the Chicken: Regulation of Transcriptional Activity Following Cochlea Removal

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ABSTRACT

Neuronal survival in the cochlear nucleus of young animals is regulated by afferent activity. Removal or blockade of nerve VIII input results in the death of 20–40% of neurons in the cochlear nucleus, nucleus magnocellularis (NM), of the 10–14 days posthatch chick. Neuronal death in NM is preceded by complete failure of protein synthesis and degradation of ribosomes. In addition, there is a biphasic change in the immunoreactivity of ribosomes for a monoclonal antiribosomal RNA antibody, Y10B. Initially, the entire population of afferent-deprived NM neurons loses Y10B immunoreactivity, but, after 6 or 12 hours of afferent deprivation, lack of Y10B immunoreactivity specifically marks dying NM neurons. Whether RNA synthesis is also altered in afferent-deprived NM neurons has not previously been studied. To determine whether RNA synthesis in NM neurons is regulated by loss of afferent activity, we injected chicks with ^{3}H-uridine following unilateral cochlea removal and measured the incorporation of RNA precursor with tissue autoradiography. As early as 1 hour after cochlea removal, there was a significant decrease in ^{3}H-uridine incorporation by afferent-deprived NM neurons. After longer periods of afferent deprivation (6 or 12 hours), the majority of dying NM neurons (marked by loss of Y10B immunoreactivity) fail to incorporate RNA precursor. Six or 12 hours following cochlea removal, the subpopulation of surviving NM neurons incorporates ^{3}H-uridine at increased levels over those observed 1 or 3 hours after cochlea removal. These findings suggest that nuclear function is regulated by afferent synaptic activity and that failure of RNA synthesis occurs early in the cell death process.

Indexing terms: deafferentation, RNA synthesis, deprivation, neuronal death, ribosomes

In several neuronal populations, developmental or physiologic cell death involves active cellular mechanisms, including changes in gene expression (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990; Ellis et al., 1991). The evidence for an active neuronal death program during development, or after loss of target-derived neurotrophic factors, has been recently reviewed by Johnson and Deckwerth (1993). Neuronal survival in developmentally susceptible populations also depends on afferent synaptic input (Guillery, 1973; Kalil, 1980; Trune, 1982; Born and Rubel, 1985; Clarke and Egloff, 1988; Hashisaki and Rubel, 1989). It is not currently known whether loss of afferent input induces the same cellular mechanisms leading to neuronal death as those induced by loss of neurotrophic factors or other developmentally programmed signals.

Excitatory afferent input to brainstem auditory neurons can be interrupted by removal of the cochlea or placement of tetrodotoxin (TTX) in the cochlear-vestibular labyrinth. Following loss of afferent synaptic activity, a subset of neurons in the immature primary auditory brainstem nucleus of both mammals and birds undergoes cell death (Born and Rubel, 1985, 1988; Hashisaki and Rubel, 1989). The role of nerve VIII activity in the metabolic changes preceding cell death has been extensively investigated (Born and Rubel, 1988; Hyson and Rubel, 1989; Born,

Accepted January 12, 1995.

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Durham and Rubel, 1991; Pasic and Rubel, 1991) The cascade of intracellular events preceding cell death in the neuronal population of the avian primary auditory nucleus, n. magnocellularis (NM), includes a biphasic change in ribosome structure and function. During the initial response to afferent deprivation, reduced protein synthesis and loss of immunoreactivity for the monoclonal antiribosomal RNA (rRNA) antibody Y10B are observed throughout the population of NM neurons. One hour following loss of afferent activity, auditory neurons demonstrate a significant decrease in protein synthesis, as measured by ^{3}H-leucine incorporation (Steward and Rubel, 1985; Born and Rubel, 1988; Sie and Rubel, 1992). By 3 hours after cochlea removal, a parallel loss of immunoreactivity for the Y10B antibody develops throughout NM (Garden et al., 1994). During this early phase, electron microscopy reveals only minimal alterations in ribosome structure in a small subset of NM neurons (Rubel et al., 1991).

During the second phase of the response to afferent deprivation (6–12 hours following cochlea removal) NM neurons segregate into two populations that differ with respect to three measures of ribosome structure or function. Approximately 20–40% of afferent-deprived neurons demonstrate arrest of protein synthesis (Steward and Rubel, 1985; Born and Rubel, 1988), loss of Y10B immunoreactivity (Garden et al., 1994), and significant ultrastructural evidence of ribosome destruction (Rubel et al., 1991). The remaining NM neurons, presumably those that ultimately survive the loss of afferent input, retain normal ribosomal ultrastructure and demonstrate only modest decreases in Y10B immunoreactivity and ^{3}H-leucine incorporation (Rubel et al., 1991; Garden et al., 1994).

The profound early metabolic and structural changes observed in afferent-deprived NM neurons suggest that active degradative mechanisms are induced by loss of afferent input. Neuronal death in this population appears to involve three consecutive steps, down-regulation of translation, alteration of ribosome structure, and ribosome degradation. However, between 3 and 6 hours of afferent deprivation, the majority of NM neurons appears to be able to overcome the initial response to cochlea removal, regaining Y10B immunoreactivity and maintaining normal ribosome ultrastructure. Cellular mechanisms that appear to promote neuronal survival, including up-regulation of mitochondrial density and oxidative function, are also induced by afferent deprivation (Hyde and Durham, 1994a). Thus, it seems that the ultimate fate of an afferent-deprived NM neuron is determined by the competing influence of cellular mechanisms, leading either toward neuronal survival or neuronal death.

The finding that neuronal populations, under the influence of either the cell survival or cell death programs, are marked by changes in ribosome function and structural modification of rRNA suggests that these programs may involve regulation of ribosome biogenesis. Ribosomes consist of both protein and RNA components and the rate-limiting step in ribosome biogenesis appears to be transcription of the rRNA gene (Sollner-Webb, 1991). Decreased transcription in afferent-deprived tissue has been previously noted by Bateson et al. (1972). However, this study provides no information on the levels of transcription following chronic afferent deprivation. Currently, several techniques can be employed to study the regulation of specific gene transcription, including nuclear run-off assays and in situ hybridization. However, these techniques are not suitable for examining general changes in RNA synthesis in afferent-deprived NM neurons. Nuclear run-off assays cannot provide histologic information on which cells have altered gene expression, and in situ hybridization provides information only about the quantity of a single RNA species, reflecting the rate of both synthesis and degradation. Alternatively, metabolic labeling with a radiolabeled RNA precursor, in combination with tissue autoradiography, provides an index of the general level of RNA synthesis within an individual cell. Therefore, to address the question of whether the general level of RNA synthesis in NM neurons is regulated by loss of afferent activity, RNA precursor incorporation was examined in young chicks following unilateral cochlea removal.

The experiments presented herein demonstrate that the overall level of RNA synthesis in NM neurons is regulated by afferent synaptic activity. Owing to the exclusively unilateral input of the nerve VIII terminals on NM neurons, the NM population on the side contralateral to cochlea removal serves as an intact intra-animal control. Transcription in NM neurons, measured by ^{3}H-uridine incorporation, is down-regulated within 1 hour following loss of afferent input due to cochlea removal. A subpopulation of afferent-deprived NM neurons appears to have arrested transcription. The number of neurons in this subpopulation increases with time until 6 hours following cochlea removal, subsequently remaining stable. The majority of the subpopulation demonstrating transcription arrest also exhibited immunohistochemical evidence of ribosome degradation, an independent marker of dying NM neurons. In those NM neurons that continue to synthesize new RNA 6 or 12 hours after cochlea removal, ^{3}H-uridine incorporation increases significantly over levels observed after 3 hours of afferent deprivation. These findings suggest that regulation of transcription is an important component of the early neuronal response to loss of afferent input.

MATERIALS AND METHODS

Animals

All experimental and control animals were 10–14-day-old white leghorn chickens. Fertilized embryos were supplied locally (H&N, Redmond, WA) and incubated in the University of Washington vivarium. Young chicks were housed in AAALAC-approved facilities, with food and water freely available at all times. Special housing within the laboratory was provided for experimental animals during the period of ^{3}H-uridine incorporation. All procedures were approved by the University of Washington Animal Care Committee.

Cochlea removal

The effects of afferent deprivation on neuronal RNA synthesis were investigated by subjecting experimental animals to unilateral cochlea removal according to the method of Born and Rubel (1985). Chicks were deeply anesthetized with ketamine (80 mg/kg body weight) and sodium pentobarbital (15 mg/kg body weight). Two small incisions were made in the right external ear canal to create a wider orifice. Using microforceps, the tympanic membrane was punctured, the middle ear ossicle removed, and the cochlea pulled through the oval window. The excised cochlea was examined under a dissecting microscope to ensure its complete removal. The ear cavity was filled with Gelfoam soaked in benzalkonium chloride solution to prevent debris from entering the ear canal. Cochlea removal

produces an immediate cessation of evoked and spontaneous electrical activity in both the primary auditory neurons of NM and eighth nerve ganglion cells, leaving ganglion cell bodies and proximal nerve VIII axons intact (Born and Rubel, 1985; Born et al., 1991). This intervention interrupts all interactions between the nerve VIII terminals and NM neurons that are initiated by electrical impulses and synaptic vesicle release, but it should not interrupt other nonsynaptic communications between these two cell types. NM neurons on the left side of the brainstem receive normal afferent input from the intact left cochlea and serve as an intra-animal control.

^{3}H-uridine incorporation

To assess the effect of afferent deprivation on RNA synthesis in NM neurons, chicks were metabolically labeled with ^{3}H-uridine 30 minutes prior to sacrifice. One, three, six, and twelve hours after cochlea removal, chicks received a single intracardiac injection of 0.5 mCi of ^{3}H-uridine (NEN, Boston, MA). Unoperated control animals were labeled with ^{3}H-uridine 1 hour after undergoing pentobarbital/ketamine anesthesia. Thirty minutes after receiving ^{3}H-uridine, all animals were deeply anesthetized with sodium pentobarbital (30–50 mg/kg) and transcardially perfused with 0.9% saline for 5 minutes, followed by 4% paraformaldehyde for 10 minutes.

High-dose ^{3}H-uridine incorporation

Significant loss of incorporated ^{3}H-uridine occurs during the long aqueous incubations of the immunohistochemistry procedure. To maintain sufficient labeling of the neurons following immunohistochemistry, some animals received a higher dose of ^{3}H-uridine (2.5 mCi) 30 minutes prior to sacrifice. Tissue from these high-dose animals was processed following the same procedures as low-dose animals and prepared for immunohistochemistry.

Histology

The heads from paraformaldehyde-perfused chicks were postfixed in a large volume of 4% paraformaldehyde for 12–18 hours. The brainstems were dissected, dehydrated in graded alcohols, cleared in methyl salicylate, and embedded in paraffin. Paraffin-embedded brainstem tissue was serially sectioned on a rotary microtome at 6 μm. All sections were mounted on chrome-alum-coated glass slides and deparaffinized with xylene prior to processing.

Autoradiography

Autoradiography was performed according to standard methods described previously (Steward and Rubel, 1985). Sections from animals radio labeled with ^{3}H-uridine were deparaffinized, hydrated, and dipped in Kodak NTB-2 emulsion diluted 1:1 with distilled water. After drying for a minimum of 4 hours, emulsion-coated slides were sorted into light-proof boxes and usually stored at 4°C for 4–6 weeks. Some tissue was allowed to expose for 4–6 months in order to determine whether longer exposures would reveal labeling in cells classified as unincorporating. Emulsion was developed according to manufacturer recommendations, followed by a light thionin counterstain. Tissue double labeled for Y10B immunohistochemistry and ^{3}H-uridine autoradiography was not counterstained.

Immunohistochemistry

To determine the relationship between loss of transcriptional activity and eventual cell death, NM neurons were double labeled for ^{3}H-uridine incorporation and Y10B immunohistochemistry. To reduce the loss of uridine label in aqueous solutions, the immunohistochemistry procedure was modified from previously described methods (Garden et al., 1994). Deparaffinized sections from animals treated with high-dose ^{3}H-uridine were mounted on glass slides, rehydrated through a series of graded alcohols, and incubated in a 4% normal horse serum-blocking solution for 1 hour. Tissue sections were then incubated for 2 hours in a 1:500 dilution of the Y10B antibody within a humidified chamber at room temperature. Sections were washed (3 × 10 minutes) in phosphate-buffered saline (PBS) and incubated for 1 hour with a 1:200 dilution of biotinylated horse antimouse secondary antibody (Vector Labs, Burlington, CA). Secondary antibody was removed with three washes of PBS (3 × 10 minutes). Sections were then incubated with avidin-peroxidase conjugate (Vectastain ABC Kit; Vector) in PBS. After washing in PBS (3 × 10 minutes), diaminobenzidine (0.25 mg/ml) with 0.1% hydrogen peroxide in Tris buffer (pH 7.6) was applied to the sections for 10 minutes. The reaction was stopped by washing with PBS (3 × 10 minutes). Slides were placed in a humidified chamber at 60°C for 1 hour, dried overnight at 36°C, dipped for autoradiography, and developed according to the procedures described above.

Data analysis

The relative amount of RNA synthesis in afferent-deprived NM neurons was quantified by measuring the nuclear silver grain density in all NM neurons on both sides of the brainstem in each section analyzed. Grain density (GD) was measured using the BioQuant Image Analysis System (R&M Biometrics, Nashville, TN). One tissue section from each animal was selected for quantitative analysis. Each section contained 50–80 NM neurons, in which the nucleus was clearly visible, on each side of the brainstem. In all cases, the analyzed section was located between 30% and 70% of the NM rostrocaudal axis. Every NM neuron with a visible nucleus in the selected section was viewed under a ×100 objective on a Leitz Aristoplan microscope. A video camera (Dage-MTI, Michigan, IN) transferred the image to the computer for digitizing and then to a video monitor. Optimal light and contrast settings were determined for each section analyzed to allow clear demarcation of silver grains from counterstained nuclear material. A threshold level was determined that would recognize only those regions of the image darkly labeled by autoradiographic silver grains. The nuclear area of each cell was outlined on the video image, and the BioQuant program calculated the density of the silver grains present in the nucleus of each neuron, using methods described previously (Hyson and Rubel, 1989; Garden et al., 1994).

To confirm that the concentration of ^{3}H-uridine available to both the control and the experimental NMs was not affected by loss of synaptic activity, measurements of nucleotide incorporation in glial cells and background GD measurements were made. Twenty glial cells were randomly selected from the area surrounding NM neurons for grain density measurements on both sides of the brainstem. Twenty background GD measurements were obtained from each side of the brainstem by measuring the GD in a fixed

33.5 × 60 μm area of neuropil adjacent to NM that was free of cellular elements.

Grain density scores could not be compared directly between animals due to interanimal differences in the level of ^{3}H-uridine incorporation. To account for these differences, GD scores for each NM neuron were normalized for analysis according to the following two procedures. First, the percentage decrease in the mean GD of afferent-deprived NM neurons, from the mean GD of control NM neurons on the opposite side of each tissue section, was calculated for each animal [(control − experimental/control) × 100]. This calculation gave a measurement of the mean percentage decrease in RNA synthesis. Second, each afferent-deprived NM neuron was categorized as either incorporating or unincorporating, based on a cutoff GD measurement less than or equal to mean background GD plus 1 standard deviation of the background measurement for that side of the brainstem. Neurons with grain densities less than background plus 1 standard deviation were characterized as unincorporating.

RESULTS

Uridine incorporation

Several observations support the use of ^{3}H-uridine incorporation as a representative measure of RNA transcription in the chick nervous system. First, cells with identifiable nuclei throughout the brainstem appeared to incorporate ^{3}H-uridine into newly synthesized RNA during the 30 minute labeling period (Fig. 1). Second, differences in grain density reflect presumed varied rates of transcription between cell types, with dense labeling observed over some large neurons and the lowest grain densities over glial nuclei. Third, the nuclear localization pattern of silver grains in NM neurons suggests that ^{3}H-uridine autoradiography specifically labels newly transcribed RNA. Transport of newly synthesized RNA into the cytoplasm requires a longer time course (90–120 minutes) than the 30 minute period of uridine incorporation employed in these experiments (Edström, 1973). In all brainstem tissue analyzed, the subcellular localization of silver grains was confined to the nuclear area of neurons and glial cells, while the density of silver grains located over cytoplasm and neuropil did not differ from nontissue background. This pattern of silver grain localization would be expected for a general marker of transcription due to the previously described kinetics of RNA metabolism. These findings support the use of this technique as an accurate indicator of relative amounts of RNA synthesis.

Transcription in afferent-deprived NM neurons

Metabolic labeling of transcriptional activity in NM neurons using ^{3}H-uridine incorporation was carried out 1, 3, 6, and 12 hours following cochlea removal. Only those NM neurons with a distinct nucleus identified by Nomarski optics were considered in the analysis. The general pattern of ^{3}H-uridine autoradiography in normally innervated NM neurons is shown in Figure 2A. Control NM neurons, from the unlesioned side of the brainstem, demonstrate a normal distribution of nuclear silver grains. Every NM neuron receiving normal synaptic input, for which there was an identifiable nucleus, appeared to be able to incorporate ^{3}H-uridine into new RNA.

NM neurons responded to loss of afferent input with alterations in transcription that appeared to occur in two distinct phases. During the initial phase, 1–3 hours following cochlea removal, afferent-deprived NM neurons demonstrated reduced ^{3}H-uridine incorporation compared to control NM neurons on the contralateral side of the same tissue section (Figs. 2A,B). By 3 hours after cochlea removal, the level of ^{3}H-uridine incorporation was clearly reduced in most afferent-deprived NM neurons compared to those receiving normal afferent input on the opposite side of the tissue section. During the second phase of the response to afferent deprivation (6–12 hours after cochlea removal), two distinct patterns of ^{3}H-uridine incorporation appeared among NM neurons (Fig. 3). The majority of afferent-deprived NM neurons regained grain densities to within the normal range of densities observed in control NM neurons (thick arrows). This observation suggests that many NM neurons showed some recovery in the ability to synthesize RNA after 6–12 hours of afferent deprivation. The remaining subpopulation (approximately 30%) failed to incorporate enough ^{3}H-uridine into new RNA to produce silver grain densities that were reliably above background grain densities. Tissue sections, in which the emulsion was exposed for 4–6 months, had roughly the same number of deprived NM neurons that were devoid of silver grains above the nucleus, in spite of very dense labeling over all of the remaining neurons. It appeared that uridine incorporation had been totally arrested in this subpopulation.

To quantify the effect of afferent deprivation on RNA synthesis, nuclear silver grain density measurements were made using the BioQuant image-analysis system. Grain density measurements, from nuclei of glial cells located within NM, showed no significant effect of afferent deprivation ($P > 0.05$). In addition, background grain density levels, taken from NM neuropil regions on the afferent-deprived and control sides of the brainstem, were not significantly different ($P > 0.05$). These data suggest that availability of ^{3}H-uridine for incorporation into NM neurons is not regulated by synaptic activity and is, therefore, present at equal levels on both sides of the brainstem.

Due to variation in the general level of ^{3}H-uridine incorporation between animals, grain densities from afferent-deprived NM neurons and control NM neurons on the opposite side of the same tissue section were plotted for each individual animal. Representative distribution plots for the initial response (3 hour survival) and secondary response (12 hour survival) are shown in Figure 4. Grain density measurements from NM neurons on the unlesioned side of the brainstem were normally distributed. Early after cochlea removal (3 hours), the grain density distribution plot suggests that afferent-deprived NM neurons developed a generalized and dramatic reduction in ^{3}H-uridine incorporation. At later time periods (e.g., 12 hours), some of the NM neurons showed very low levels of ^{3}H-uridine incorporation, while many other afferent-deprived NM neurons regained transcriptional activity within the range of control NM neurons.

The percentage decrease in mean grain density was calculated for all experimental and unoperated control animals (Fig. 5A). Among three unoperated control animals, no significant difference in grain density between the two sides of the brainstem was observed. All experimental time periods showed a large decrease in RNA synthesis on the afferent-deprived side, with the peak reduction occurring 3 hours after cochlea removal. One-way ANOVA

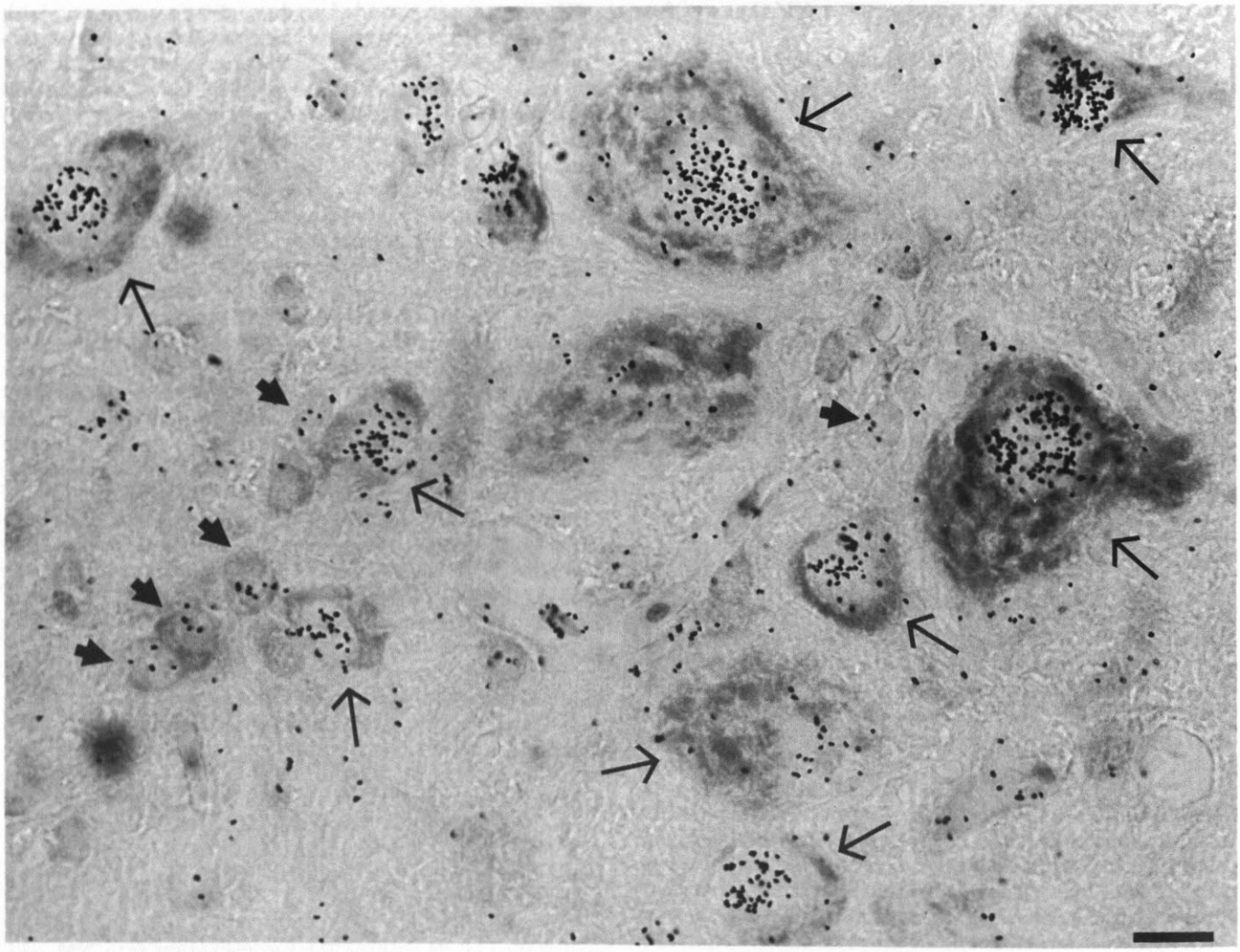

Fig. 1. Photomicrograph demonstrating the general pattern of ^{3}H-uridine incorporation in brainstem tissue. Neurons (long arrows) and glia (short arrows) from the region of the ventral brainstem (nucleus vestibularis descendans) are pictured with autoradiographic silver grains over the nucleus of each cell. The density of silver grains varies among the different cell types pictured. Scale bar = 10 μm.

revealed a significant effect of cochlea removal on ^{3}H-uridine incorporation ($P < 0.003$), and post hoc comparisons (Fisher's protected LSD) confirm that the decrease in grain density at each of the four survival times is significantly different from that in unoperated controls ($P < 0.01$).

The number of NM neurons demonstrating transcription arrest was assessed in the following way. Grain density was measured from 20 randomly selected areas of neuropil within afferent-deprived and control NM. All NM neurons demonstrating grain densities less than or equal to the mean background grain density, plus 1 standard deviation, were considered "unincorporating." Figure 5B presents the mean percentage of unincorporating NM neurons at each survival time. Unincorporating cells were not observed in unoperated controls. Two-way ANOVA revealed that cochlea removal significantly increased the percentage of afferent-deprived NM neurons classified as unincorporating ($P < 0.001$), and the proportion of NM neurons showing arrest in transcription significantly increased with longer survival times ($P < 0.01$). The mean percentage of unincorporating NM neurons increased significantly ($P < 0.01$) from the 1 hour survival time to the 6 and 12 hour survival times (Fisher's protected LSD). These data suggest that, as early as 3 hours after cochlea removal, a subpopulation of afferent-deprived NM neurons has undergone a drastic shift in metabolism, leading to the cessation of RNA synthesis.

Although the number of unincorporating NM neurons increased from 1 hour to 6–12 hours after cochlea removal, some recovery in the amount of uridine incorporation was observed in the subpopulation of cells that showed GD measurements greater than 1 SD above background. To determine the degree of recovery in RNA synthesis, the subpopulation of unincorporating cells was removed from the analysis, and the mean percentage decrease between the remaining population of NM neurons and the control NM neurons was compared (Fig. 5C). One or three hours after cochlea removal, afferent-deprived NM neurons, continuing to synthesize new RNA, did so at a rate significantly decreased from intact NM neurons on the opposite side of the brainstem ($P < 0.01$). However, after longer periods of afferent deprivation, 6 or 12 hours, there was no significant difference ($P > 0.05$) from control in the mean percentage

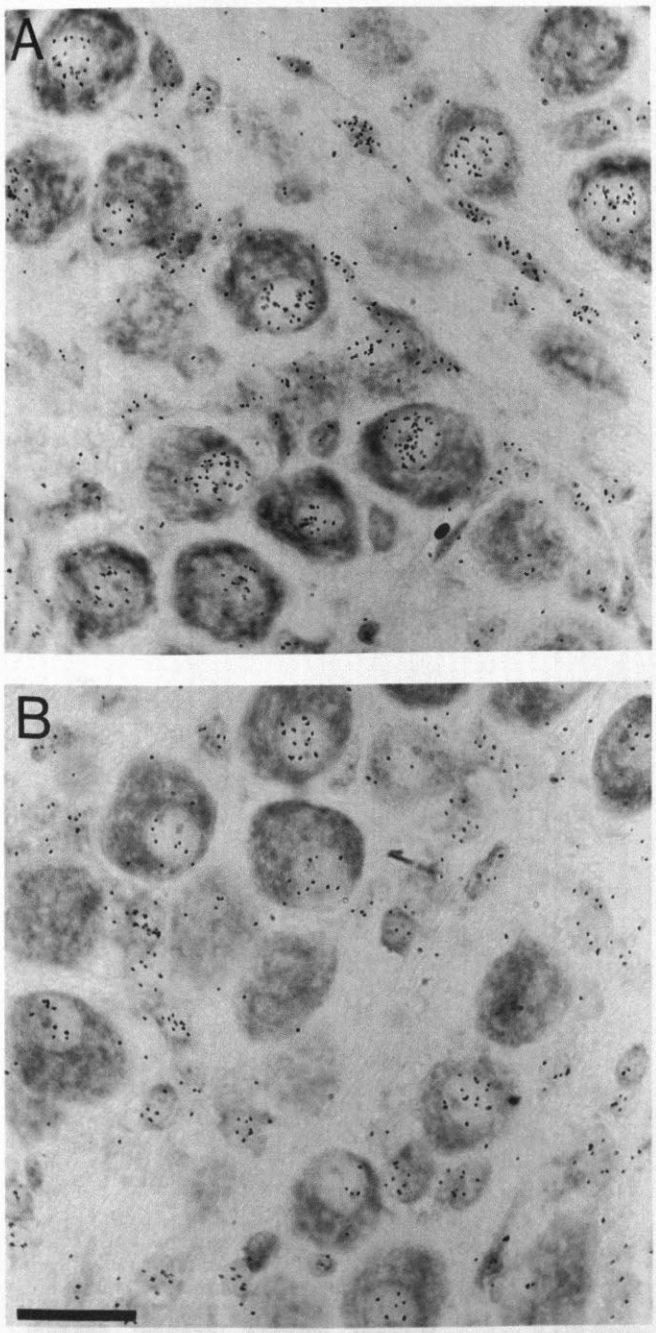

Fig. 2. Autoradiographic image of nucleus magnocellularis (NM) neurons from an animal labeled by a single ^{3}H-uridine injection 1 hour after cochlea removal and killed 0.5 hour later. The two panels are from the opposite sides of the same tissue section. **A:** NM neurons from the side of the brainstem contralateral to cochlea removal, showing a normal pattern of ^{3}H-uridine incorporation with fairly uniform density of silver grains over the nucleus of each neuron. **B:** Afferent-deprived NM neurons, demonstrating a generalized decrease in nuclear grain density. Scale bar = 20 μm.

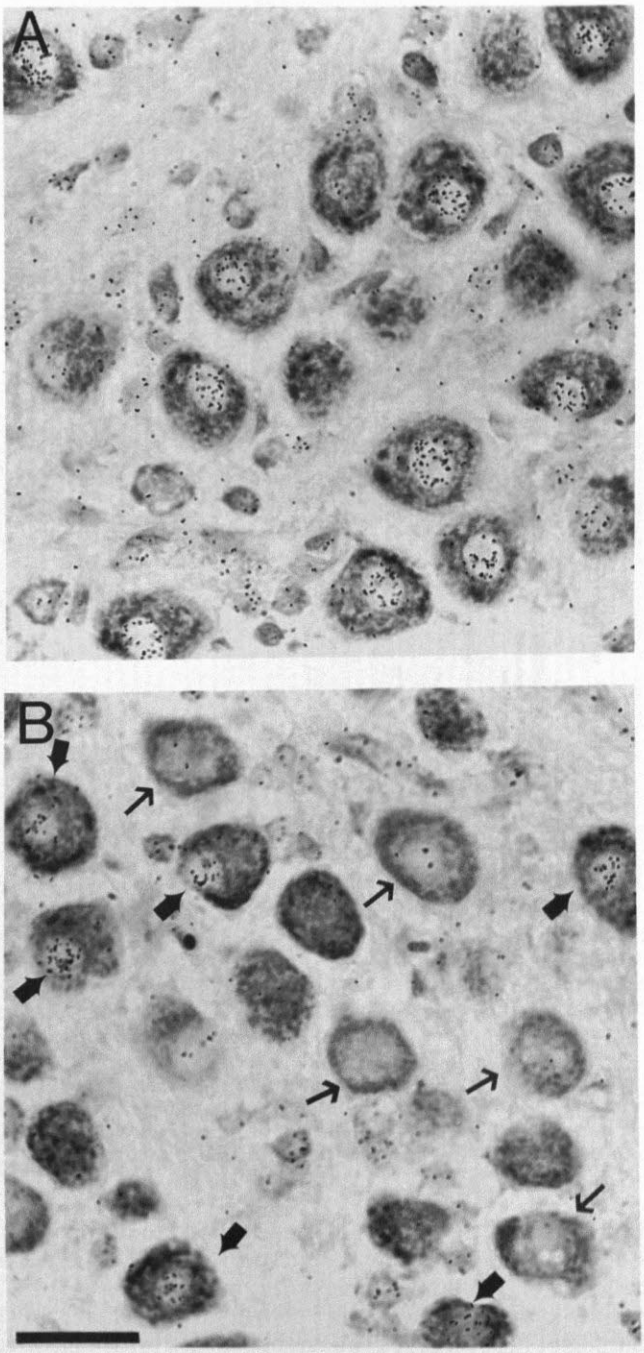

Fig. 3. NM neurons labeled by a 0.5 hour pulse of ^{3}H-uridine beginning 6 hours after unilateral cochlea removal. **A:** Normally innervated NM neurons from the side of the brainstem contralateral to cochlea removal. Note uniform density of nuclear silver grains. **B:** Afferent-deprived NM neurons from the same tissue section displayed in A demonstrate two different profiles of ^{3}H-uridine incorporation. One group of neurons demonstrates failure of RNA synthesis (thin arrows), while the remaining neurons (thick arrows) appear to have nuclear grain densities only slightly below those observed on the contralateral side. Scale bar = 20 μm.

decrease in ^{3}H-uridine incorporation among NM neurons with sustained transcriptional activity. In addition, the mean decrease in grain density, observed 1 or 3 hours after cochlea removal, was significantly greater ($P < 0.02$) than that observed after 12 hours of afferent deprivation. These results suggest that those neurons remaining transcriptionally active have increased levels of RNA synthesis compared to levels observed at the early survival times (1 or 3 hours).

RNA synthesis and ribosome degradation

The relationship between transcription arrest and eventual cell death was investigated using an antibody to rRNA,

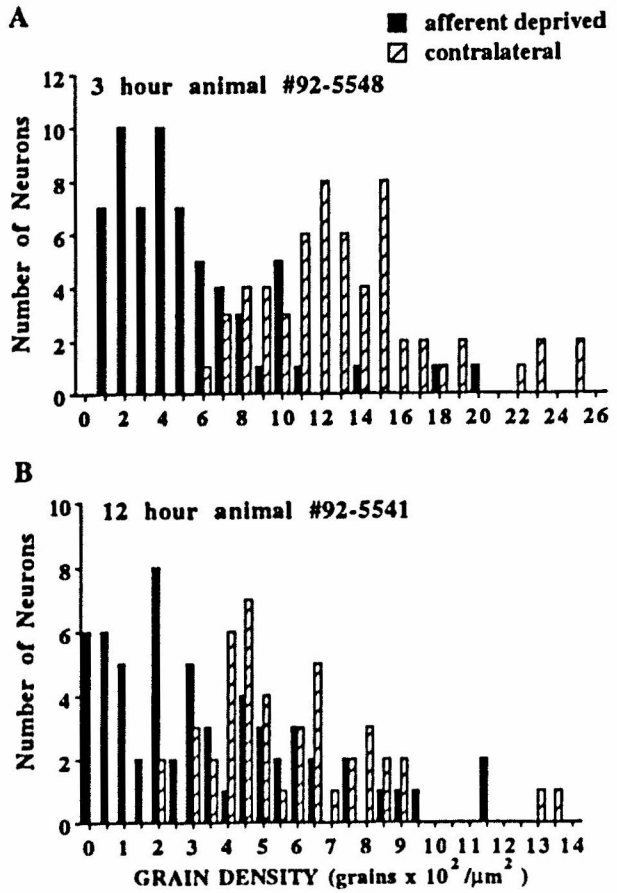

Fig. 4. Grain density distribution plots from an animal killed 3 hours after cochlea removal (A) and an animal killed 12 hours after cochlea removal (B) demonstrate the pattern of grain densities observed in afferent-deprived (solid bars) or contralateral (hatched bars) NM neurons. In A, the distribution of grain densities among afferent-deprived NM neurons is generally shifted to grain densities lower than those observed among contralateral NM neurons. B: The grain densities of a large portion of afferent-deprived NM neurons are within the contralateral distribution. Another subset of the afferent-deprived NM neurons has no measurable nuclear silver grains, indicating failure to incorporate ^{3}H-uridine during the 0.5 hour pulse.

Y10B, to mark those NM neurons destined to die. Previous studies have shown that ribosome degradation, as indicated by loss of Y10B immunoreactivity, marks dying NM neurons 6 hours after cochlea removal (Rubel et al., 1991; Garden et al., 1994). Tissue double labeled with high-dose ^{3}H-uridine (2.5 mCi) autoradiography and Y10B immunohistochemistry was used to investigate the relationship between changes in RNA synthesis and this marker of dying NM neurons 6 hours after cochlea removal. Positive or negative staining for Y10B was determined by visual inspection of the tissue and, again, only NM neurons in which a nucleus was observable were included in the analysis. In double-labeled tissue, all NM neurons on the control side of the brainstem maintained both Y10B immunoreactivity and ^{3}H-uridine incorporation. Among the 6 hour afferent-deprived NM neurons, each of four possible combinations of Y10B immunohistochemistry and ^{3}H-uridine incorporation was observed. Cell counts were made for each combination of Y10B immunoreactivity and ^{3}H-uridine incorporation. The most prevalent patterns of colocalization in double-labeled tissue (Fig. 6) included normal Y10B immunoreactivity with near-control levels of

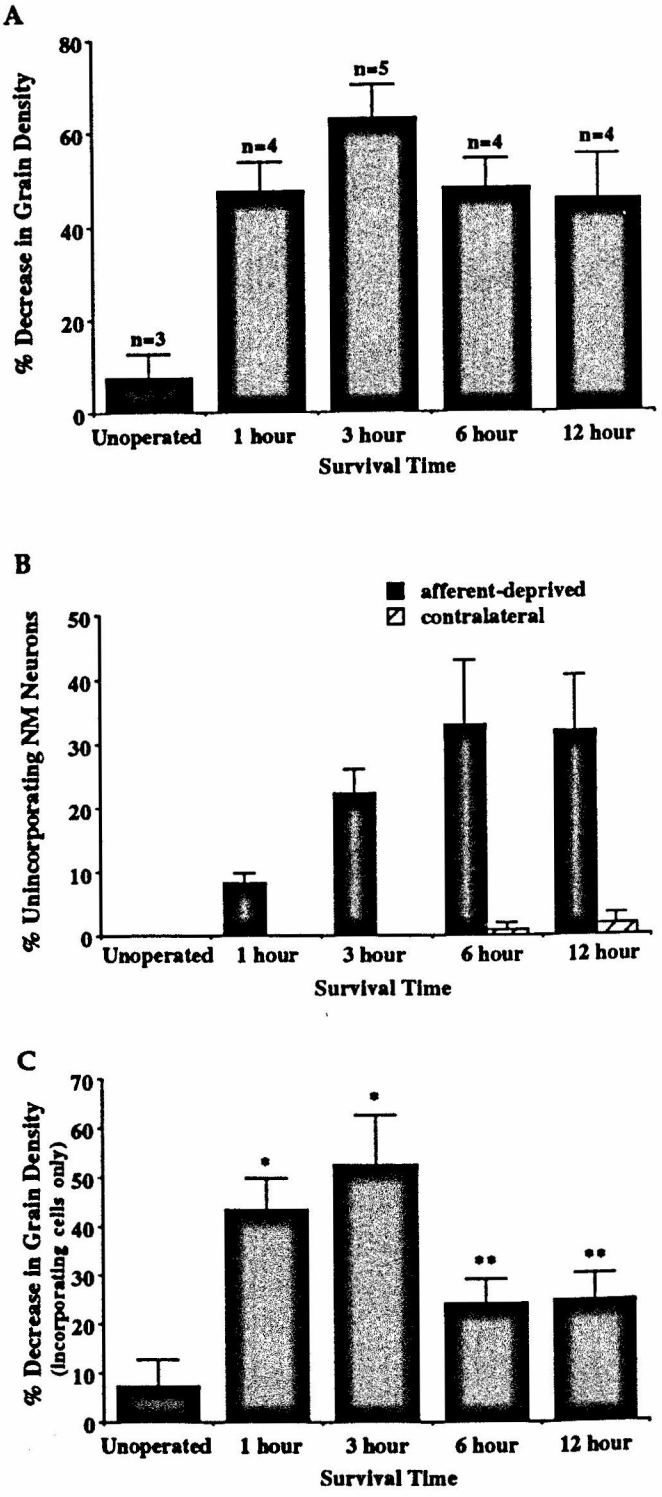

Fig. 5. A: Mean percentage decrease in nuclear grain density is shown for the five experimental groups. B: Mean percentage of NM neurons considered to be unincorporating (based on grain density less than mean + 1 standard deviation from the mean background grain density measured for each section) from each survival group. Solid bars represent afferent-deprived NM neurons, and hatched bars represent unincorporating neurons observed in the contralateral NM. C: Mean percentage decrease in nuclear grain density among those NM neurons continuing to incorporate ^{3}H-uridine. One asterisk indicates a significant difference from unoperated controls ($P < 0.01$). Two asterisks indicate a significant difference from the 3 hour survival time ($P < 0.02$). Error bars represent 1 SEM; n, number of experimental animals in each group.

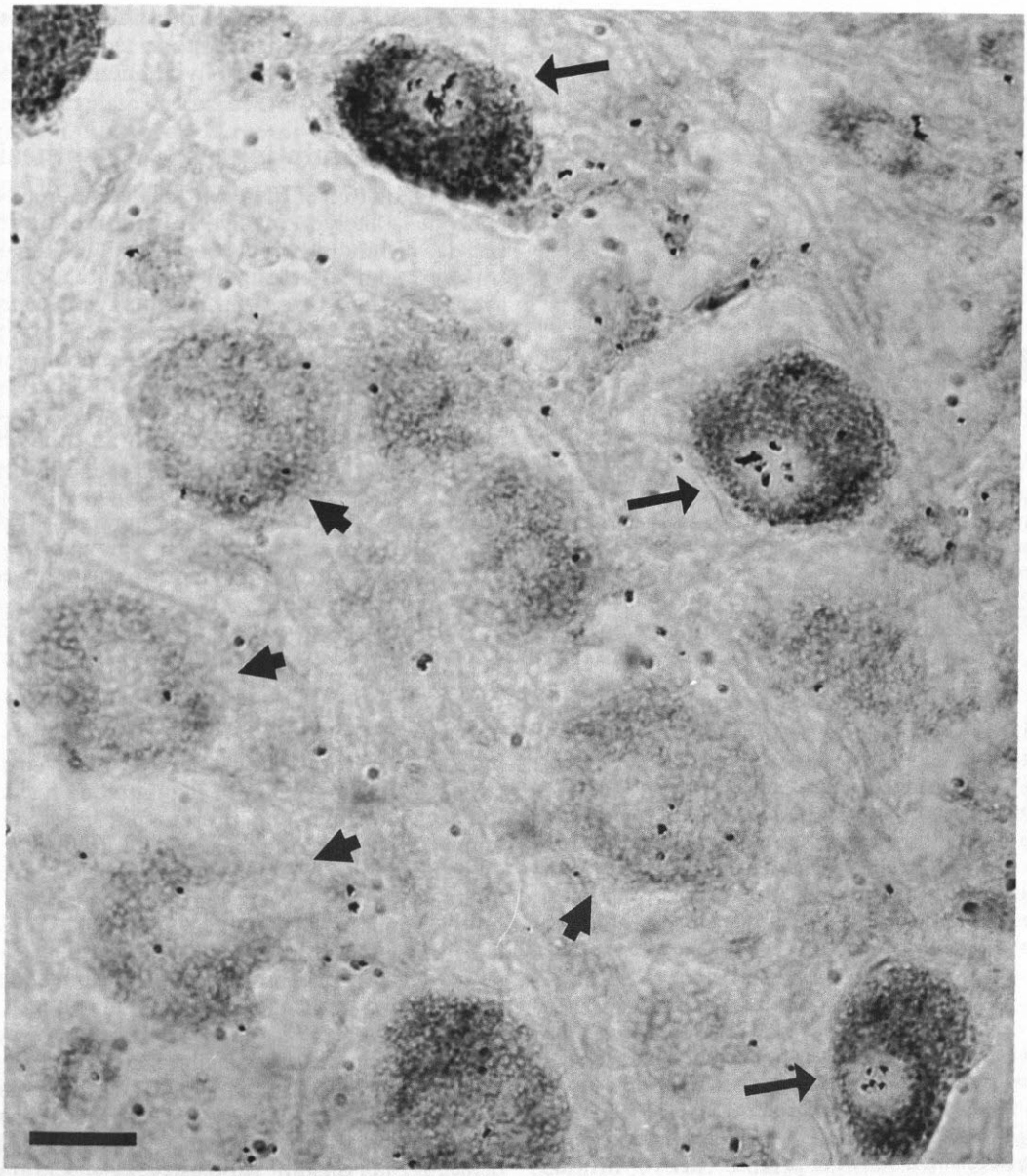

Fig. 6. Photomicrograph of afferent-deprived NM neurons from an animal killed 6 hours after cochlea removal and double labeled for ^{3}H-uridine incorporation and Y10B immunoreactivity. Neurons with intact Y10B immunoreactivity continue to incorporate ^{3}H-uridine (long arrows); those without Y10B label demonstrate transcription arrest (short arrows). Scale bar = 10 μm.

uridine incorporation, or loss of Y10B immunoreactivity, and no detectable ^{3}H-uridine labeling. Two smaller populations exhibited either normal Y10B labeling with negative uridine incorporation or complete loss of Y10B immunoreactivity with maintained ^{3}H-uridine incorporation. These neurons may represent a group of afferent-deprived NM neurons that have not yet committed to a program of cell survival or cell death. The mean percentages of afferent-deprived NM neurons demonstrating each of the four possible combinations of Y10B immunoreactivity and ^{3}H-uridine incorporation are shown in Table 1.

TABLE 1. Mean Percentage of Afferent-Deprived NM Neurons Observed in Each of the Four Categories From Brainstem Tissue, Double-Labeled With Y10B Immunohistochemistry and ^{3}H-Uridine Autoradiography, of Three Animals Killed 6 Hours After Cochlea Removal

	Y10B positive (% of total ± SD)	Y10B negative (% of total ± SD)
^{3}H-uridine positive	75.3 ± 8.56	4.65 ± 1.78
^{3}H-uridine negative	9.45 ± 7.77	12.27 ± 5.64

The mean grain density of the Y10B-positive and Y10B-negative populations was calculated from three animals sacrificed 6 hours after cochlea removal and compared to that of control neurons on the opposite side of the tissue sections. The mean percentage decrease in grain density of Y10B-positive and Y10B-negative afferent-deprived neurons was compared to the normal variation in uridine incorporation observed in unoperated animals (Fig. 7). A large decrease in grain density (80%) was observed among NM neurons unlabeled by the Y10B antibody. This finding suggests that dying NM neurons sustain a significant reduction in transcriptional activity. The reduction in uridine incorporation observed in Y10B-negative NM neu-

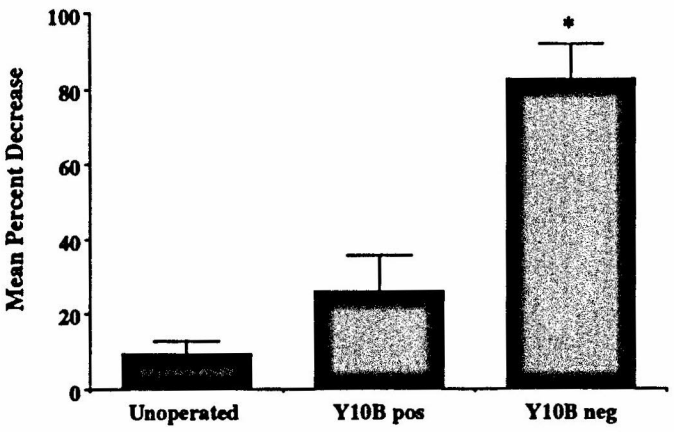

Fig. 7. Mean percentage decrease in nuclear grain density for neurons labeled or unlabeled by the Y10B antibody from the intact intraanimal control NM compared to the normal variation in grain density measured between the right and the left sides of the brainstem in unoperated control animals. Dying (Y10B-unlabeled) neurons demonstrate a significantly greater reduction in uridine incorporation than surviving (Y10B-labeled) NM neurons ($P < 0.003$). Error bars represent 1 SEM; asterisk indicates significant difference from unoperated controls.

rons was significantly greater ($P < 0.001$) than the normal variation measured in unoperated control animals (one-factor ANOVA and post hoc comparisons with Fisher's projected LSD). Afferent-deprived NM neurons with intact Y10B immunoreactivity (surviving cells) did not show a significant decrease ($P > 0.1$) in ^{3}H-uridine incorporation. These data suggest that loss of RNA synthetic function is associated with the process of cell death in afferent-deprived NM neurons.

DISCUSSION

Previous studies have demonstrated that short-term afferent deprivation produces many structural and metabolic changes in NM neurons (Rubel et al., 1990, 1991; Garden et al., 1994). In this series of experiments, we identified one additional cellular process, RNA synthesis, that is rapidly altered following loss of afferent input to auditory brainstem neurons. Initially (1–3 hours following cochlea removal), a significant decline in the amount of RNA synthesis occurs in afferent-deprived NM neurons. After longer periods of afferent deprivation (6 or 12 hours), NM neurons demonstrate two distinct patterns of RNA synthesis. The majority of afferent-deprived NM neurons continue to synthesize RNA at levels closer to control NM neurons than to levels observed after shorter periods (1 or 3 hours) of afferent deprivation. The remaining neurons (approximately 30% of the population) fail to incorporate any detectable ^{3}H-uridine during the 30 minute labeling period. The experiments described herein demonstrate that the synthetic function of the nucleus is affected very early following loss of afferent activity to NM neurons, adding one more element to the cascade of events initiated during afferent deprivation-induced neuronal death in this system.

These findings raise several questions about the process of neuronal death following loss of afferent synaptic activity. First, by what mechanism might loss of synaptic input to NM neurons result in reduced RNA synthesis? Second, how do the findings presented here relate to other examples of cell death? Finally, how do the alterations in nuclear function described here relate to the previously described cascade of events observed in NM neurons following loss of afferent synaptic activity?

Regulation of RNA synthesis

The regulation of RNA synthesis in afferent-deprived NM neurons is likely to involve an alteration in the enzymatic activity or binding properties of proteins involved in RNA transcription. In eukaryotic cells, total RNA synthesis is carried out by three separate enzyme complexes. RNA polymerase I transcribes only one gene, the preribosomal RNA containing three RNA components of mature ribosomes, the 18S, 5.8S, and 28S rRNAs. RNA polymerase II synthesizes messenger RNAs, and RNA polymerase III is involved in the synthesis of tRNA and the 5S rRNA. In a typical eukaryotic cell, over 50% of RNA synthesis is carried out by RNA polymerase I (Sollner-Webb, 1991). Therefore, any major alteration in the general rate of RNA synthesis is likely to involve regulation of this enzyme.

The activity of RNA polymerase I is regulated by growth phase (Schnapp et al., 1991), differentiation state (Larson et al., 1993), and extracellular hormones (Mahajan and Thompson, 1991; Weber et al., 1991). The mechanism of regulation, in each of these cases, involves alteration of transacting transcription factors that are required for initiation of RNA polymerase I activity. Evidence is mounting that the transcription factors involved in promoting rRNA gene expression are regulated by a signal transduction scheme involving protein phosphorylation. For example, transcriptional activity of RNA polymerase I is stimulated by ATP-γS, a substrate for protein kinases (Schnapp et al., 1991), and phorbol esters, activators of protein kinase C (Weber et al., 1991). It is possible that loss of synaptic input to NM neurons results in a change in the activity of protein kinases that eventually leads to reduced transcription of the rRNA gene.

Alternatively, it is possible that a more global event, impacting the activity of all three RNA polymerases, can account for the reduction in RNA synthesis observed in afferent-deprived NM neurons. Two mechanisms that could alter general transcriptional activity in response to a cell death signal include proteolytic degradation of enzymes involved in RNA synthesis and alteration of the DNA substrate required for transcription. Specific proteolytic inactivation of all three RNA polymerase complexes is induced by the poliovirus protease 3C, resulting in a generalized inhibition of transcription in infected cells (Clark et al., 1991, 1993; Rubinstein et al., 1992). It is possible that loss of synaptic activity stimulates induction or activation of an endogenous protease capable of inactivating either RNA polymerases or the transcription factors required for their activity.

In addition to transacting transcription factors, the activity of RNA polymerases is also regulated by cis-acting DNA elements (i.e., promoter and enhancer regions) contained within each gene. Inactivation of these regions, through protein blockade by inducible transacting factors or endonucleolytic damage, could result in decreased transcriptional activity. Endonucleolytic DNA degradation has been commonly observed in many systems of programmed cell death, including some neuronal cells (Arends et al., 1990; Edwards et al., 1991). However, the breakdown of DNA through the activity of a Ca^{2+}/Mg^{2+}-dependent endonuclease is usually a relatively late event in the cell death

process. Because the initial reduction of RNA synthesis observed in afferent-deprived NM neurons occurs within 1 hour of loss of afferent activity, it seems unlikely that DNA degradation could explain decreased transcriptional activity in this population. However, a recent study, employing more sensitive methods for identifying DNA damage, demonstrates that DNA degradation is observed as early as 3 hours following stimulation of cell death in PC12 cells (Batistatou and Greene, 1993). If an endonuclease activated by a cell death signal has specificity for those regions of DNA involved in the regulation of gene expression, very small and difficult-to-detect amounts of DNA damage could still have a profound impact on the rate of transcription.

RNA synthesis in cell death

The finding of a reduced level of general RNA synthesis in afferent-deprived NM neurons demonstrates that loss of synaptic activity rapidly elicits alterations in cellular metabolism and nuclear function. Whether the cascade of events leading to NM neuronal death following loss of afferent input is recapitulated in other cell types following a physiologic cell death signal has not been determined. Many investigators studying physiologic cell death in a variety of cell types have attempted to consolidate their findings into the category of apoptosis. This definition of programmed cell death, originally described in lymphocytes and epithelial cells (Wyllie et al., 1980), has also been applied to neurons undergoing developmental neuronal death (Wood et al., 1993) and neuronal death in vitro in response to withdrawal of trophic factors (Edwards et al., 1991; Batistatou and Greene, 1993).

One frequently noted feature of apoptosis is the requirement for new gene expression to carry out the cell death program (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990). Therefore, many investigators have looked for specific new mRNAs generated in response to the extracellular signal that activates a cascade of events culminating in cell death (Buttyan et al., 1988; Bettuzzi et al., 1989; Schwartz et al., 1990; Martin et al., 1992; Owens and Cohen, 1992). Despite the fact that many investigators are searching for the up-regulation of specific genes, a reduction in the general level of RNA synthesis has been reported in one well-studied cell death system. Lymphocytes exposed to glucocorticoid undergo a well-described apoptotic death and demonstrate decreased activity of RNA polymerases within 1 hour of exposure to steroid (Bell and Borthwick, 1976). It is possible that the loss of RNA synthesis in glucocorticoid-treated lymphocytes either is secondary to DNA damage by the endonucleolytic mechanism mentioned previously or results from a primary event regulating the activity of RNA polymerases. If transcriptional down-regulation results from DNA damage, future studies aimed at determining if and when DNA damage occurs in afferent-deprived NM neurons may aid in answering the question of whether common cellular mechanisms are initiated during apoptosis and afferent deprivation-induced cell death. Alternatively, if RNA polymerase activity is regulated by cell death signals in more accessible tissue (e.g., lymphocytes or other cultured cells), the signal transduction scheme leading to that regulation could be elucidated more easily than in an in vivo system such as afferent-deprived NM neurons.

RNA synthesis and transneuronal degeneration in NM neurons

Previous studies of transneuronal degeneration in NM neurons have revealed extensive changes in neuronal cytoplasm (Rubel et al.,1990). The earliest metabolic response to loss of synaptic activity observed prior to this series of experiments was a generalized reduction in protein-synthetic activity. The pattern of change in protein synthesis mirrors the changes observed in RNA synthesis, demonstrating decreased amino acid incorporation in all afferent-deprived NM neurons 1 hour after cochlea removal and a subpopulation of nonincorporating NM neurons 6 hours after cochlea removal (Steward and Rubel, 1985; Born and Rubel, 1988). It could be hypothesized that the initial reduction in translation is a direct result of decreased transcription. For example, a 50% reduction in uridine incorporation might contribute to the early reduction in protein synthesis, through either a large decrease in the available rRNA for ribosome biogenesis or a complete cessation of mRNA synthesis, with an eventual reduction in translational activity. However, without proposing concurrent induction of RNA degradation, this cannot fully explain the early reduction in protein synthesis; the half-lives of the majority of mRNAs and rRNAs are considerably longer than 1 hour. An alternative hypothesis involves concurrent down-regulation in the activity of both ribosomes and RNA polymerases. One possible mechanism for coregulation of transcription and translation may involve a signal transduction scheme that results in the posttranslational modification (i.e., phosphorylation) of enzymes that carry out protein and RNA synthesis.

Six hours following loss of afferent input, autoradiographic methods suggest that cessation of transcription and protein synthesis occur in a subpopulation of NM neurons. Detailed electron microscopic analysis demonstrated that those NM neurons failing to incorporate ^{3}H-amino acid into protein 6 hours following cochlea removal also exhibit ribosome and polysome degradation (Rubel et al., 1991). Several lines of evidence indicate that those NM neurons with profiles of ribosome degradation and cessation of protein synthesis after 6 hours of afferent deprivation are destined to die. First, the number of neurons in the subpopulation failing to make protein 6 hours following cochlea removal approximates the neuronal population eventually lost from the nucleus (Born and Rubel, 1985, 1988; Steward and Rubel, 1985). Second, cessation of protein synthesis correlates with loss of Y10B immunoreactivity after 6 hours of afferent deprivation (Garden et al., 1994). Third, chloramphenicol treatment, a manipulation that inhibits mitochondrial protein synthesis and results in increased neuronal death (Hyde and Durham, 1994a), also increases the number of cells demonstrating ribosome degradation and loss of Y10B immunoreactivity 6 hours following cochlea removal (Hartlage-Rübsamen et al., 1992; Garden et al., 1994). The present finding, loss of Y10B immunoreactivity correlates with transcription arrest, supports the hypothesis that transcription arrest is a new functional marker of the neuronal death process.

Two interesting points are raised by the possibility that those afferent-deprived NM neurons demonstrating transcription arrest are destined to undergo cell death. First, whereas the subpopulation demonstrating transcription arrest 6 hours after cochlea removal approximates the number that will eventually die, a smaller subpopulation

fails to incorporate ^{3}H-uridine at the shorter survival times. This finding suggests that cells within the population of dying NM neurons may be marked by a metabolic change that occurs between 1 and 3 hours following cochlea removal. If this were indeed the case, it would represent the earliest-appearing marker of dying neurons described thus far. Second, we have previously suggested that the ultimate fate of an afferent-deprived NM neuron depends on the competing influence of metabolic changes that promote either cell survival or cell death (Garden et al., 1994). Up-regulation of oxidative metabolism and expansion of mitochondrial density are observed in afferent-deprived NM neurons (Durham and Rubel, 1985; Hyde and Durham, 1990; Durham et al., 1994; Hyde and Durham, 1994b). Preventing this oxidative response with the mitochondrial protein synthesis inhibitor chloramphenicol results in a large increase in cell death (Hyde and Durham, 1994a). Currently, it is not known whether the initial reduction in RNA synthetic activity contributes to the cell death process or is part of the cellular attempt to prevent cell death. It is possible that reduced RNA synthesis may inhibit the transcription of genes necessary for the cell death process. However, the finding that transcription arrest correlates with loss of Y10B immunoreactivity suggests that this event may be part of, or secondary to, the cascade of events culminating in cell death. Thus, it seems more likely that the energy requiring cell survival mechanism, which appears to turn on between 3 and 6 hours following cochlea removal (Hyde and Durham, 1990; Hyde and Durham, 1994b), may facilitate the restoration of transcriptional activity observed in the majority of NM neurons after 6 hours of afferent deprivation.

ACKNOWLEDGMENTS

This work was supported by NIH grant DC00520 to E.W R., and G.A.G. was supported by NIH training grant GM07266. We thank Drs. D. Lurie, L. Adams, and J. Stone for helpful comments on previous versions of the manuscript. Dr. W. Lippe provided advice on statistical analysis, and Paul Shwartz and Janet Clardy provided photographic assistance.

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