Rapid Regulation of Cytoskeletal Proteins and Their mRNAs Following Afferent Deprivation in the Avian Cochlear Nucleus

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ABSTRACT
During development, removal of neuronal input can lead to profound changes in postsynaptic cells, including atrophy and cell death. In the chicken brainstem cochlear nucleus, the nucleus magnocellularis (NM), deprivation of auditory input via unilateral cochlea removal or silencing the eighth nerve with tetrodotoxin leads to a loss of 25–30% of the neurons and the atrophy of surviving neurons. One intracellular component that may be involved in both cell atrophy and cell death is the cytoskeleton. The degradation of the cytoskeleton following deafferentation could potentially lead to either atrophy or death of NM neurons. However, little is known regarding the role of neuronal input on the cytoskeletal structure of NM neurons and whether changes in the cytoskeleton are responsible for cell death following deafferentation.

The present study examined whether changes in the cytoskeleton of NM neurons occurred following cochlea removal. Several components of the cytoskeleton were analyzed following unilateral afferent deprivation. Levels of immunostaining for tubulin, actin, and microtubule-associated protein 2 (MAP-2), and levels of β-tubulin and β-actin mRNAs were assessed in NM neurons following cochlea removal. Our results revealed that afferent deprivation results in a rapid decrease in immunostaining for all three cytoskeletal proteins examined. These decreases were observed as early as 3 hours after cochlea removal and persisted for up to 4 days. In addition, these changes occurred in all deafferented NM neurons at the early time points, indicating that both dying and surviving NM neurons undergo a similar change in their cytoskeletons. In contrast to the decreases in immunostaining, levels of β-tubulin and β-actin mRNAs were not noticeably altered by deafferentation. Our findings indicate that the cytoskeleton is altered or degraded following deafferentation but that this process is not regulated at the transcriptional level. J. Comp. Neurol. 389:469–483, 1997.

It has long been recognized that afferent activity plays an important role in the survival of postsynaptic neurons in the developing nervous system (for review, see Linden, 1994). However, it is difficult to study the effects of afferent deprivation on postsynaptic neurons, because most neurons receive more than one type of excitatory input. In many systems, the effects of removal of a single afferent projection are complicated by the presence of the remaining projections, which may sprout or alter their input. The chicken auditory system provides an excellent model for studying the effects of afferent deprivation. One subdivision of the chick cochlear nucleus, the nucleus magnocellularis (NM), receives a single excitatory input, which is from the basilar papilla (cochlea). Unilateral cochlea removal or perilymphatic injection of tetrodotoxin (TTX) leads to an immediate loss of excitatory afferent input to...
the ipsilateral NM (Born and Rubel, 1985; Born et al., 1991). A dramatic series of events takes place in the activity-deprived NM neurons that results in the death of approximately 25–30% of the NM neurons within the first 2 days and the atrophy of the remaining neurons (Born and Rubel, 1985, 1988). Because only a portion of the cells die in response to activity loss, this system provides an opportunity to study the events that occur in neurons that die in response to activity deprivation and in those that survive.

Some of the early changes that take place in NM neurons in response to activity deprivation include decreases in transcription and protein synthesis (Steward and Rubel, 1985; Garden et al., 1995) and increases in intracellular calcium (Zirpel et al., 1995; Zirpel and Rubel, 1996). In addition, NM neurons that are destined to die show polyribosome degradation (Rubel et al., 1991; Hartlage-Rubsamen and Rubel, 1996). Levels of transcription and protein synthesis, assessed by using [3H]uridine and [3H]leucine incorporation, respectively, have been found to decrease uniformly as early as 1 hour following cochlea removal (Steward and Rubel, 1985; Garden et al., 1995). However, by 6 hours following cochlea removal, approximately 70% of NM neurons have regained relatively normal levels of incorporation. The remaining 30% of NM neurons have not, indicating that transcription and protein synthesis are substantially reduced or absent in this population of cells.

Another bimodal change in deafferented NM neurons occurs in the integrity of polyribosomes. By using an anti-ribosomal antibody (Y10B), Garden et al. (1994) have demonstrated that, within the first several hours following deafferentation, all NM neurons exhibit decreased Y10B staining. However, from 6 to 12 hours following deafferentation, NM neurons segregate into two groups: one group that continues to exhibit very low levels of Y10B immunostaining and a second group that regains nearly normal levels of immunostaining. In addition, electron microscopic analysis of polyribosomes has shown that, by 6 hours following deafferentation, two populations of NM neurons are distinct: one with intact polyribosomes and the other with highly dissociated polyribosomes (Rubel et al., 1991; Hartlage-Rubsamen and Rubel, 1996). Neurons that show a loss of Y10B staining and degradation of polyribosomes are the same as those that exhibit dramatically reduced protein synthesis.

Recent studies from our laboratory suggest that increases in intracellular calcium may play a key role in these cytoplasmic changes in deafferented NM neurons. Experiments utilizing brainstem slice preparations have demonstrated that removal of eighth nerve input, both in vivo and in vitro, leads to dramatic increases in intracellular calcium concentrations ([Ca\textsuperscript{2+}]i) in NM neurons. In brainstem slices, unilateral stimulation of the eighth nerve maintains normal levels of intracellular calcium in the NM neurons ipsilateral to the stimulation, whereas the [Ca\textsuperscript{2+}]i in the contralateral, unstimulated NM neurons rises over twofold by 1 hour (Zirpel and Rubel, 1996). Furthermore, when brainstem slices are prepared from animals that have received a cochlea removal in vivo, levels of [Ca\textsuperscript{2+}]i in deafferented NM neurons rise to approximately three times the levels in control NM neurons by 3 hours (Zirpel et al., 1995). Together, these data suggest that afferent activity is crucial to the maintenance of normal calcium levels in NM neurons, and deprivation of afferent activity may produce increases in calcium that can regulate downstream signaling events.

One structure that may provide a link between initial changes in the deafferented NM neurons and the atrophy or death of these cells is the cytoskeleton. Increased calcium has been demonstrated to cause the breakdown of a number of cytoskeletal elements in neurons and in many other cell types (for reviews, see Choi, 1992; Saida et al., 1994; Siesjo, 1994; Trump and Berezesky, 1995). Changes in the cytoskeleton can also result from decreases in the availability of cytoskeletal mRNAs and protein monomers that may occur as a result of decreases in transcription and protein synthesis.

In the present study, we have examined both the immediate and the long-term effects of afferent deprivation on the cytoskeleton of NM neurons. Both immunocytochemical techniques and in situ hybridization were used to examine levels of cytoskeletal proteins and their mRNAs following cochlea removal. Our results indicate that deafferentation causes a loss of immunostaining for several cytoskeletal proteins that is apparent by 3 hours and continues up to 4 days following cochlea removal. Unlike many of the events previously characterized in deafferented NM neurons, these changes appear to occur in all NM neurons. In addition, we find that these decreases in immunostaining are not accompanied by decreases in cytoskeletal mRNA levels. Portions of this work have been presented previously in abstract form (Seftel et al., 1986; Kelley et al., 1995).

**MATERIALS AND METHODS**

**Experimental animals**

Posthatch White Leghorn chickens (7–12 days old) were used in the present study. Eggs were obtained from local suppliers (H and N, Redmond, WA; or The Heatwole Hatchery, Harrisonburg, VA) and were incubated and hatched in the University of Washington or the University of Virginia AAALAC-approved animal care facilities. Animals were kept in heated brooders and had free access to food and water at all times. All procedures were approved by the University Animal Care Committees.

**Cochlea removals**

Unilateral cochlea (basilar papilla) removals were performed as described previously (Born and Rubel, 1985). Briefly, chickens were anesthetized with ketamine hydrochloride (80 mg/kg i.m.) and sodium pentobarbital (15 mg/kg i.p.). The feathers surrounding the external auditory meatus were removed, and the ear canal was widened with two 5-mm incisions. The tympanic membrane was opened with Dumont no. 5 forceps, and the columella and the cochlea were removed through the oval window. Each cochlea was examined under a dissecting microscope to ensure its complete removal. The middle ear was filled with gelfoam, and the external incisions were closed with cyanoacrylate adhesive. Animals were kept in a heated cage until they regained consciousness; then, they were returned to the animal care facilities. At various postlesion survival times (ranging from 30 minutes to 5 weeks following cochlea removal), animals were euthanized as described below, and brain tissue was prepared for immunocytochemistry or in situ hybridization.
Immunocytochemistry
Twenty-five animals received unilateral cochlea removals. Birds were euthanized 30 minutes (n = 5), 3 hours (n = 4), 6 hours (n = 4), 2 days (n = 4), 4 days (n = 5), and 5 weeks (n = 3). Four unoperated animals served as controls. Animals were deeply anesthetized with sodium pentobarbital and were perfused transcardially with 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde for 8–12 hours. Thirty-micrometer-thick coronal Vibratome sections were collected serially through the entire NM in each brainstem. Care was taken to orient each tissue block such that symmetrical sections were obtained. Free-floating sections were stored in phosphate-buffered saline (PBS), pH 7.4, for 2–10 hours and were incubated for 1 hour in a blocking solution containing 0.2% saponin and 1% normal horse serum. Blocking buffer and all subsequent solutions were prepared in PBS. Sections were incubated in polyclonal primary antibodies for tubulin (Miles Laboratories, Naperville, IL), actin (Miles Laboratories), or microtubule-associated protein 2 (MAP-2; a gift of Richard Vallee, Worcester Foundation) for 18–24 hours at room temperature. Antisera and antibodies were prepared at a 1:1,000 dilution. Control sections were processed identically to other sections, except that primary antibody was excluded. Sections were washed in PBS, incubated in biotinylated anti-rabbit secondary antibodies (Vector Laboratories, Inc., Burlingame, CA), washed in PBS, and incubated in an avidin-biotin complex (Vector Laboratories, Inc., Burlingame, CA) overnight at 4°C. Sections were rinsed extensively and were incubated in a solution containing 0.2 µg of β-tubulin or β-actin antisense or sense cRNA probe per slide. Following hybridization, sections were rinsed and treated briefly (5–10 minutes at room temperature) in RNase A (0.002% in a 0.01 M Tris/0.5 M NaCl buffer, pH 8.0). Slides were rinsed and incubated in high stringency buffer (composed of 0.1 × SSC, 1 mM EDTA) for 1 hour at 60°C. Following the stringency wash, slides were rinsed in 0.5 × SSC followed by PTw. Sections were blocked in a solution containing 0.1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated in a 1:1,000 dilution of antidigoxigenin antibody (Boehringer Mannheim Biochemicals Inc., Indianapolis, IN) overnight at 4°C. Sections were rinsed short and were incubated in a solution containing 3-nitro blue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 3–8 hours at room temperature (depending on the probe). Slides were rinsed in PBS, rapidly dehydrated, cleared, and coverslipped in DPX.

RNA isolation and Northern hybridization
To confirm probe specificity, RNA was isolated from pre- and posthatch chickens, separated on agarose/formaldehyde gels, and transferred to nylon membranes for blotting with the digoxigenin-labeled probes. RNA from both embryonic and posthatch chickens were used for these experiments. Embryonic RNA was analyzed because it had been used to characterize the original β-tubulin and β-actin probes (Cleveland et al., 1980); RNA from posthatch chickens was also used because posthatch chickens were used in the present experiment. Embryonic day 15 (E15) chickens were decapitated, and whole brains were removed; 7-day-old posthatch chicks were given a lethal dose of sodium pentobarbital, decapitated, and their brainstems were removed. All tissue was immediately homogenized in Trizol reagent (Gibco BRL Inc., Grand Island, NY); RNA was isolated following a Trizol/chloroform extrac-
tion and an isopropanol precipitation. Concentrations of total cellular RNA were assessed by using OD$_{260}$ values.

Agarose (1.5%)/formaldehyde (0.66 M) gels were prepared in a 1×3-[N-morpholino] propanesulfonic acid (MOPS) buffer. RNA was denatured for 2 minutes at 95°C in a standard loading buffer containing formaldehyde, formamide, MOPS, and glycerol; 15 µg of RNA were applied to each lane. Gels were run in a 1×MOPS buffer and were transferred to a Nytran plus nylon membrane (Schleicher and Schuell Inc., Keene, NH) by using an overnight capillary transfer. Membranes were rinsed and baked (80°C for 1 hour) to immobilize RNA. Identical membrane strips were incubated in either methylene blue (to detect total RNA), β-actin antisense RNA, or β-tubulin antisense RNA. Strips used for hybridization were incubated first in a hybridization buffer (without probe) identical to that used for in situ hybridization for 3 hours at 60°C. Probes were added at a concentration of 200 ng/ml of hybridization buffer. Membranes were incubated in probe overnight at 60°C, rinsed, treated with RNase A (0.002%), and incubated again at 60°C in a high-stringency buffer. Membranes were rinsed in PTw and blocked in a PBS/BSA/Triton solution (identical to that used for in situ hybridization). Membranes were incubated in antidigoxigenin antibody (1:1,000) overnight at 4°C, rinsed, and developed in NBT/BCIP solution for 30 minutes to 1 hour at room temperature.

Data analysis

Relative levels of tubulin immunostaining and β-tubulin hybridization were quantified by using computerized densitometry. Criteria for inclusion into the analysis were similar for immunostaining and hybridization. One (for immunostaining) or two (for in situ) representative sections were selected from each animal that were symmetrical, uniformly labeled, and from the middle (30–70% of the rostrocaudal axis) of the nucleus magnocellularis. All NM neurons in the selected sections that possessed both a clear boundary and a visible nucleus were included in the analysis. Typically, from 35 to 90 neurons in each NM were selected for analysis of immunostaining; from 25 to 60 neurons in each NM were selected for analysis of in situ hybridization. However, in a few of the animals, fewer than 15 neurons were analyzed in a particular NM. These animals were typically those at the 36-day survival point, at which time a substantial number of the cells had died, and many others had atrophied to a degree that prevented their use for densitometry analysis.

Levels of tubulin immunostaining were quantified by using a Leitz Ergolux microdensitometer (Wetzlar, Germany). Neurons were visualized under a 40× dry objective, and the optical density (OD) of cytoplasmic staining was quantified. Because the staining between sections and between animals was variable, OD levels in deafferented NM neurons could not be compared directly with the OD levels of NM neurons in other sections. For this reason, mean OD levels for deafferented and control NM neurons were determined for each individual section. Mean levels of staining in each animal were determined by averaging the density of staining across all neurons from a single NM (either control or deafferented side of the brain). Levels of staining in the deafferented NM were expressed as a percentage of the levels in the control NM from the same section. These values were then averaged for all of the animals in a particular group. In addition to determining levels of staining in NM neurons, levels of immunostaining were also determined for the reticularis pars caudalis (RP), a group of neurons that does not receive input from the eighth nerve. These measurements served as an internal control for each group.

Levels of β-tubulin hybridization were quantified by using the NIH image-analysis system (NIH Image, version 1.57; public domain software from the National Institute of Mental Health, Bethesda, MD). Neurons were visualized under a Zeiss 63× Planapo oil objective (NA 1.4; Thornwood, NY) on a Zeiss Photomikroskop II. Images were captured for analysis with a Dage 68 camera (Dage-MTI, Michigan City, IN) and digitized into a Macintosh PowerPC 8100 with a QuickCapture board (Data Translation, Inc., Marlboro, MA). The NIH image-analysis program was used to quantitate densities of β-tubulin hybridization on the digitized images. The cytoplasm of each neuron conforming to the criteria was outlined, and the OD of staining within this area was quantified. Because the hybridization across sections and across animals was also variable, OD levels in deafferented NM neurons could not be compared directly with the OD levels of NM neurons in other sections. For this reason, mean OD levels for deafferented and control NM neurons were determined for each individual section. Mean OD levels in deafferented NM neurons are represented as a percentage of the mean OD levels in control NM neurons in the same tissue section. These values were then averaged for all of the animals in a particular group.

Because two different systems were used to provide quantification of the immunostaining and the in situ hybridization, it is important to note that the sensitivity of these two systems is comparable. Both the Ergolux and the NIH Image systems are eight-bit (256 gray levels) systems, and each system was carefully calibrated for the most sensitive part of the range, that is, the neurons on the control side were set at the center of the dynamic range of the OD range in order to assure that neither floor nor ceiling effects were encountered. Because the scores for each animal were determined by the ratios of the ODs of the control and experimental neurons, these procedures are valid and are reasonably equally sensitive for detecting changes by using either measurement system.

RESULTS

Immunocytochemistry: Qualitative assessments

Unoperated animals. To examine relative levels of cytoskeletal proteins, animals were euthanized from 30 minutes to 5 weeks following unilateral cochlea removal. Animals were perfused with 4% paraformaldehyde, and tissue was prepared for immunocytochemistry. The appearance of the staining of tubulin, actin, and MAP-2 in normal (control) NM neurons is shown in Figure 1. Neurons in both the nucleus magnocellularis (NM) and the nucleus laminaris (NL), second- and third-order nuclei of the avian auditory system, show strong immunostaining for all three proteins. These findings are consistent with the characteristic labeling patterns of antibodies to cytoskeletal proteins in neurons in the mammalian central nervous system (CNS; Bloom and Vallee, 1983, Caceres et al., 1984).

Deafferentation. Qualitative assessments indicate that dramatic decreases in tubulin and actin immunostaining are detectable by 3 hours following cochlea removal;
Fig. 1. A–F: Appearance of cytoskeletal immunostaining in chicken brainstem auditory nuclei. A, C, and E show tubulin, actin, and microtubule-associated protein 2 (MAP-2) immunostaining, respectively, in control nucleus magnocellularis (NM; arrows) and in the third-order brainstem auditory nucleus, the nucleus laminaris (NL; arrows). Staining patterns with all three antibodies are similar, with cytoplasm of both NM and NL neurons staining darkly. Although NM neurons do not possess dendrites, both apical and basal dendrites of NL neurons are strongly stained with all three antibodies. B, D, and F show tubulin, actin, and MAP-2 immunostaining, respectively, in NM neurons under high magnification and Nomarski optics. Arrows indicate staining of the axon. Scale bars = 100 µm in E (also applies to A, C), 10 µm in F (also applies to B, D).
MAP-2 immunostaining was not examined extensively at 3 hours. By 6 hours following deafferentation, substantial decreases in immunostaining for all three antibodies are apparent (Fig. 2) throughout the extent of the NM. Importantly, at these early times points, deprived NM neurons showed relatively uniform decreases in immunostaining within each individual animal. That is, a clearly bimodal distribution was never observed, which suggests that all NM neurons undergo similar cytoskeletal changes in response to activity deprivation. Changes in tubulin immunostaining at 30 minutes, 3 hours, and 5 weeks following cochlea removal are shown in Figure 3. Although qualitative changes in tubulin immunostaining were not observed at 30 minutes following cochlea removal, clear decreases were apparent by 3 hours. Levels of staining remained low at 6 hours, 2 days, and 4 days following cochlea removal. By 5 weeks, levels had returned to nearly normal levels in the surviving neurons. It should be noted that 5 weeks is a long recovery time compared with other changes that occur in deafferented NM neurons. Events such as decreased transcription and protein synthesis return to approximately normal levels within the first 96 hours following deafferentation (Garden et al., 1995).

**Immunocytochemistry: Quantitative assessments**

Quantitative measurements were performed on the tubulin-stained tissue in order to confirm the decreases in immunostaining we observed. The tubulin immunostaining was selected for analysis because it was representative of the qualitative changes observed in both the actin and the MAP-2 staining. To determine relative levels of tubulin immunostaining, the mean density of tubulin staining in both the deafferented (ipsilateral to cochlea removal) and the control NM was assessed in individual sections. Levels of staining were expressed as a ratio of the mean staining density for deafferented NM neurons to the mean staining density of the control NM neurons in the same section. Thus, the staining from each animal was assessed relative to its own internal control. Quantification of the tubulin immunostaining revealed a dramatic decrease in levels of tubulin immunostaining as early as 3 hours following cochlea removal (Fig. 4). The levels remained low for at least 4 days following cochlea removal but returned to nearly normal levels by 5 weeks.

**In situ hybridization: Qualitative assessments**

To examine relative levels of cytoskeletal mRNAs, animals were euthanized from 30 minutes to 5 weeks following unilateral cochlea removal. Animals were perfused with 4% paraformaldehyde, and tissue was prepared for in situ hybridization. Hybridization using digoxigenin-labeled β-tubulin and β-actin antisense probes revealed a similar pattern of staining for both probes. In situ hybridization data are not shown for MAP-2, because the MAP-2 probe that was available to us did not hybridize well in chick tissue. Staining in control NM neurons for β-tubulin and β-actin appeared to be specific for neuronal cell bodies, with little background staining. Levels of β-tubulin and β-actin hybridization in control (Fig. 5A,C) and deafferented (Fig. 5B,D) NM neurons 6 hours following cochlea removal are shown in Figure 5. Levels of both β-tubulin and β-actin hybridization in deafferented NM neurons were comparable to levels in control NM neurons at 6 hours. Interestingly, slight but consistent increases in β-actin hybridization outside NM neurons were apparent from 3 hours to 3 days following cochlea removal and are indicated by arrowheads in Figure 5D. Although the source of these increases was not investigated, it is possible that these extraneuronal increases in actin occurred in NM astrocytes. Our laboratory has previously observed increases in density of glial fibrillary acidic protein-labeled or Golgi-stained glial processes by 1–3 hours following deafferentation or afferent blockade by TTX (Canady and Rubel, 1992; Rubel and MacDonald, 1992).

No dramatic differences in β-tubulin or β-actin hybridization were observed in deafferented NM neurons in any area of the NM compared with control neurons at any of the time points examined. The levels of tubulin hybridization at 30 minutes, 3 hours, and 5 weeks following cochlea removal are shown in Figure 6. The levels of actin hybridization in NM neurons also appeared to be comparable in control and deafferented NM neurons at all time points examined (data not shown).

**In situ hybridization: Quantitative assessments**

Hybridization with the β-tubulin probe was used for quantitative analysis to provide a comparison with quantitative measurements of tubulin immunoreactivity. Levels of hybridization are expressed as a ratio of the mean staining density for deafferented NM neurons to the mean staining density of the control NM neurons in the same section. Thus, the staining from each animal is assessed relative to its own internal control. Quantitative analysis of relative levels of β-tubulin hybridization indicated that levels of tubulin mRNA were not substantially different between control and deafferented NMs at any of the time points examined, as shown in Figure 7. The only time point when there appeared to be any difference in β-tubulin hybridization between control and deafferented NM neurons was at 6 hours, when the average level of β-tubulin hybridization was approximately 8% lower in the deafferented NM (compared with the control NM). However, these differences were not striking by qualitative observation and were not comparable to the dramatic loss of tubulin immunostaining observed at both 3 and 6 hours following cochlea removal.

**Specificity of digoxigenin-labeled cRNA probes**

Specificity of probe binding was confirmed in two ways: hybridization with a sense cRNA probe and with Northern blot hybridization. In situ hybridization with β-tubulin sense cRNA probes revealed a very low level of background labeling across sections (Fig. 8); levels were not comparable to those observed with binding of the antisense cRNA probes. This finding indicates that the labeling observed with the antisense probe was not due to nonspecific binding.

In addition, specificity of probe binding was confirmed by evaluating binding to total cellular RNA from 7-day posthatch chicken brainstem (Fig. 9). Northern blots probed with the β-tubulin probe revealed that the probe binds to two bands of approximately 1.8 kb and 3 kb (Fig. 9, T). Blots probed with the β-actin probe revealed that the probe binds to a band of approximately 2 kb (Fig. 9, A). Identical bands were labeled in blots containing RNA from E15 embryonic brain (data not shown). These findings are
Fig. 2. A–F: Appearance of tubulin, actin, and MAP-2 immunostaining in control and deafferented NM neurons at 6 hours following cochlea removal. A, C, and E show tubulin, actin, and MAP-2 immunostaining, respectively, in control NM neurons. B, D, and F show immunostaining in deafferented NM neurons. Note the dramatic decrease in immunostaining for all three antibodies at 6 hours following cochlea removal. In addition, with all three antibodies, the levels of immunostaining appear uniform across deafferented NM neurons. Scale bar = 20 µm.
Fig. 3. Time course of changes in tubulin immunostaining following cochlea removal. Levels of tubulin immunostaining are shown in control (A,C,E) and deafferented (B,D,F) NM neurons at 30 minutes (A,B), 3 hours (C,D), and 5 weeks (E,F) following cochlea removal. Note that a dramatic decrease in immunostaining is apparent in deafferented NM neurons at 3 hours following cochlea removal but not at 30 minutes or at 5 weeks. Scale bar = 20 µm.
in agreement with previous studies using radiolabeled probes prepared from these cDNAs (Cleveland et al., 1980).

**DISCUSSION**

Deafferentation in the chick leads to a rapid and dramatic decrease in cytoskeletal immunostaining in NM neurons. This decrease appears to be relatively uniform across all NM neurons and can be seen as early as 3 hours following cochlea removal. These data suggest that all deafferented NM neurons undergo similar cytoskeletal changes in response to activity deprivation, which contrasts with many of our earlier findings in which two populations of neurons appeared in the deafferented NM by 6 hours: one population that exhibited changes that could be associated with cell death and another population that exhibited changes consistent with cell survival.

Unlike the decreases observed in cytoskeletal immunostaining, levels of mRNA for the cytoskeletal proteins β-tubulin and β-actin did not change significantly following deafferentation. These results indicate that the dramatic changes in the cytoskeletons of deafferented NM neurons are not explained by substantial decreases in the levels of mRNAs for these proteins. In the following discussion, we will consider some technical limitations of our experiments, several possible interpretations of our results, and the implications of our results for the study of cell death and cell survival.

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*Fig. 4. Quantification of the relative density of tubulin immunostaining. Levels of immunostaining in deafferented nucleus magnocellularis (NM) neurons are expressed as a percent of levels in control NM neurons. The dashed line represents values where deafferented and control NM neurons would be identical. A: The density of tubulin immunostaining in deafferented NM neurons appears to decrease dramatically by 3 hours following cochlea removal and remains depressed until 5 weeks following cochlea removal, when levels return to near normal. Although the levels of immunostaining within each individual animal were relatively uniform, some variability in the staining of deafferented NM neurons was observed across different animals. Reticularis pons caudalis (RP) neurons (a group of neurons quantified as a control population) were not affected by the cochlea removal, which explains the lower variability of staining observed in this population. B: Immunostaining at early time points following cochlea removal shown on an expanded time scale. Error bars indicate S.E.M.*
Technical considerations

An important caveat of these experiments is that the densitometry measurements of the immunostaining were used to confirm the qualitative observations of staining levels rather than to indicate an absolute level of protein or of antibody binding. Thus, the quantitative measurements do provide support for our qualitative observations that levels of immunostaining for tubulin did decrease by 3 hours following cochlea removal and remained low for several days thereafter.

Similarly, the quantification of tubulin hybridization provides a confirmation of our qualitative observations rather than an absolute measure of mRNA levels. However, a precise determination of mRNA levels is not critical to the overall conclusions of these studies. First, it is clear from both the qualitative observations of hybridization levels and the quantification of hybridization that levels of tubulin hybridization do not decrease in deafferented NM neurons with a comparable time course or extent to decreases in immunostaining. In addition, levels of tubulin hybridization appear to be comparable in all neurons within the deafferented NM of each individual animal. These data confirm that 1) the observed changes in tubulin immunoreactivity cannot be explained by changes in tubulin mRNA levels; and 2) within individual animals, NM neurons respond similarly to activity deprivation.

It is important to note in considering the results from the in situ hybridization that the level of color reaction product resulting from nonradioactive in situ hybridization may not be linearly related to absolute amounts of mRNA at all levels of color development. However, for these experiments, slides were examined throughout the color reaction to monitor the development of reaction product. Qualitative differences in hybridization between control and deafferented NM neurons were not observed in any animals at any point during the colorization reaction.

Fig. 5. A–D: Appearance of β-tubulin and β-actin hybridization in control and deafferented NM neurons at 6 hours following cochlea removal. A and C show tubulin and actin hybridization, respectively, in control NM neurons. B and D show hybridization in deafferented NM neurons. Levels of tubulin and actin hybridization in NM neurons appeared unchanged at 6 hours. However, actin hybridization outside deafferented NM neurons (neurons are indicated by arrows; hybridization outside neurons is indicated by arrowheads) was apparent from 3 hours to 3 days following cochlea removal but was not apparent at 30 minutes or at 5 weeks. Scale bar = 20 µm.
Fig. 6. Time course of changes in β-tubulin hybridization following cochlea removal. Levels of tubulin hybridization are shown in control (A,C,E) and deafferented (B,D,F) at 30 minutes (A,B), 3 hours (C,D), and 5 weeks (E,F) following cochlea removal. Note that, unlike levels of tubulin and actin immunostaining, the levels of tubulin hybridization in deafferented NM neurons appear comparable to levels in control neurons at all of the time points examined. Scale bar = 20 µm.
These observations indicate that it is unlikely that substantial decreases occurred in the deafferented NM that were not detected with our nonradioactive in situ hybridization.

Causes of cytoskeletal changes

The decreases observed in cytoskeletal immunostaining could result from a number of different events that have been shown to occur in deafferented NM neurons. First, it has been demonstrated that intracellular levels of calcium increase dramatically in deafferented NM neurons compared with control NM neurons (Zirpel et al., 1995). These increases in intracellular calcium may activate calcium-sensitive proteases, which, in turn, can degrade the cytoskeleton (Choi, 1992; Saido et al., 1994; Siesjo, 1994; Trump and Berezovsky, 1995). An important point is that all evidence thus far indicates that calcium levels increase uniformly in deafferented NM neurons (Zirpel et al., 1995). If increases in calcium were linked to changes in the cytoskeleton, then we would predict that the changes in the cytoskeleton should also occur uniformly across NM neurons. We have demonstrated in the present study that decreases in cytoskeletal immunostaining do occur in all deafferented NM neurons to a similar extent: Bimodal changes in cytoskeletal immunostaining were not observed at any of the time points examined. These results are consistent with the possibility that calcium increases that occur following deafferentation are related to the observed decreases in cytoskeletal immunostaining.

Second, we cannot exclude the possibility that the decreases in immunostaining we observed are the result of alterations of the cytoskeleton that prevent antibody binding rather than a degradation of the cytoskeleton. These alterations might involve structural changes in the cytoskeleton, which could be mediated by intermediate proteins that bind to the cytoskeleton. Calcium may also be involved in these cytoskeletal alterations, because many cytoskeletal-associated proteins are regulated by changes in intracellular calcium (i.e., spectrin, MAPs, etc.; Fifkova, 1985; Bamburg and Bernstein, 1991). Similarly, it is
Possible that antibody epitopes are simply masked by binding of calcium (Winsky and Kuznicki, 1996) or cytoskeletal-associated proteins in the absence of structural alterations or cytoskeletal degradation. However, immunostaining for some cytoskeletal proteins, like neurofilament and tau, does not decrease following deafferentation (D. Lurie and E. Rubel, unpublished observations). Therefore, it seems unlikely that a general masking of cytoskeletal epitopes by calcium or other proteins is occurring in deafferented NM neurons. In addition, it is important to note that the main conclusions of this study are that the cytoskeleton is rapidly altered by the loss of afferent activity, that these changes occur in all deafferented NM neurons, and that these changes are not the result of alterations in mRNA levels. None of these conclusions is critically dependent on the nature of the changes that take place in the cytoskeleton.

A third possibility is related to the finding that dramatic decreases in both overall transcription and protein synthesis have been shown to occur in deafferented NM neurons within the first few hours following cochlea removal (Steward and Rubel, 1985; Garden et al., 1995). It is possible that decreases in transcription of cytoskeletal mRNAs could lead to lower levels of cytoskeletal proteins, which, in turn, may produce decreases in immunoreactivity. However, our results indicate that only slight decreases in tubulin mRNA levels occur in deafferented NM neurons, and these changes take place after substantial decreases in cytoskeletal immunostaining have already occurred. If decreases in transcription of cytoskeletal mRNAs were the cause of the decreased immunostaining, then dramatic decreases in the mRNA levels should have been observed prior to the decreases in immunostaining.

Finally, decreases in protein synthesis could affect the cytoskeleton if levels of cytoskeletal proteins were prohibitively low, and the cytoskeleton could not be maintained. However, the life span of cytoskeletal protein monomers, such as tubulin monomers, is approximately 20 hours (Alberts et al., 1994), so it is unlikely that decreases in synthesis of cytoskeletal proteins could be reflected in decreased cytoskeletal immunostaining as early as 3 hours following cochlea removal. Furthermore, if decreases in overall protein synthesis were responsible for the changes we have observed in cytoskeletal immunostaining, then we would expect to observe similar changes in all cytoskeletal proteins. However, unpublished data suggest that levels of neurofilament staining (D. Lurie, unpublished observations) and levels of tau protein staining (E. Rubel, unpublished observations) are not altered in deafferented NM neurons at 6 hours following cochlea removal. Therefore, it is unlikely that the changes we have observed in cytoskeletal immunostaining were due to an overall decrease in synthesis of cytoskeletal proteins.

Cytoskeletal changes and cell survival

Previous studies from our laboratory have demonstrated that initial deprivation-induced decreases in transcription and protein synthesis occur in the entire population of NM neurons. However, by 3–6 hours, approximately 70% of the neurons show recovery toward normal levels, whereas, while in about 30% of the neurons, macromolecular synthesis appears to cease, creating a distinctly bimodal population (Born and Rubel, 1985, 1988; Steward and Rubel, 1985; Rubel et al., 1991; Garden et al., 1994, 1995). These data suggest that, between 3 and 6 hours following cochlea removal, deafferented NM neurons select either a cell death pathway (which may include prolonged decreases in transcription, protein synthesis, and breakdown of polyribosomes) or a cell survival pathway (which may involve a restoration of normal levels of transcription and protein synthesis and the reassociation or preservation of polyribosomes). This choice may be either an active decision on the part of the neuron or the selection of a default pathway. If the observed decreases in cytoskeletal immunostaining are evidence of a detrimental change to the cytoskeleton that is directly linked to the death of NM neurons, then we) between 3 and 6 hours following cochlea removal, approximately 70% of NM neurons would be expected to regain more normal levels of cytoskeletal immunostaining while the remaining 30% should continue to show low levels of immunostaining; and 2) neurons that survive longer periods of deafferentation should show relatively normal levels of immunostaining.

In the present study, we have demonstrated that dramatic decreases in cytoskeletal immunostaining precede the death of NM neurons. However, within individual animals, all activity-deprived NM neurons exhibit relatively uniform decreases in immunostaining for cytoskeletal proteins at both 3 hours and 6 hours following cochlea removal. These data strongly suggest that all deafferented NM neurons, whether they are destined to die or to survive in response to activity deprivation, exhibit similar cytoskeletal changes. In addition, if low levels of immunostaining are indicative of detrimental changes to the cytoskeleton that lead to cell death, then surviving neurons would not be expected to continue to exhibit low levels of immunostaining. However, comparable decreases in immunostaining are observed at 3 and 6 hours, when no NM neurons...
have died in response to deafferentation, and at 2 days following cochlea removal, when approximately 30% have died (Born and Rubel, 1985). In fact, levels of cytoskeletal immunostaining in the surviving neurons do not begin to return to normal until 4 days, and they do not reach normal levels until sometime between 4 days and 5 weeks following deafferentation. It is also important to note that these surviving NM neurons may have atrophied as much as 30% in volume. Thus, our calculated density levels of staining in these neurons probably overestimate the absolute level of cytoskeletal proteins in the cells compared with controls. Together, these data indicate that the loss of cytoskeletal immunostaining and the changes in the cytoskeleton that it represents are not directly linked with the death of NM neurons. Instead, we suggest that these changes may be involved in a cell survival mechanism.

Effects of cytoskeletal changes

The types of changes we have observed in the cytoskeleton suggest that it is structurally altered or destroyed in deafferented NM neurons. Although damage to the cytoskeleton would seem to be detrimental to NM neurons, several studies have demonstrated a possible role for cytoskeletal breakdown in cell survival. One protective role of cytoskeletal breakdown in neurons exposed to environmental stress has been suggested by the work of Mattson and colleagues. They have demonstrated that the destabilization of the actin cytoskeleton in cultured hippocampal neurons leads to protection of neurons from insults ranging from exposure to amyloid β-peptide (Furukawa and Mattson, 1995) to glutamate excitotoxicity (Furukawa et al., 1995). In our model, the alteration or destruction of the actin and tubulin components of the cytoskeleton in deafferented NM neurons might enhance cell survival following deafferentation.

In a separate series of studies, a second mechanism has been proposed in which the cytoskeleton may mediate protection of cells from detrimental increases in calcium. It has been shown that stabilization of the actin and tubulin cytoskeleton with agents, such as taxol and phallolidin, can reduce calcium channel inactivation (Ohson and Byerly, 1993, 1994). Conversely, agents that destabilize the cytoskeleton reverse the effects of taxol and phallolidin on calcium channel inactivation (Ohson and Byerly, 1994). These data suggest that the cytoskeleton may interact directly with calcium channels to modulate calcium influx. More specifically, these findings are consistent with the possibility that, when calcium enters a cell and alters the stability of the cytoskeleton, a conformational change in the calcium channels can occur that reduces subsequent influx of calcium (Ohson and Byerly, 1993). It is possible that a similar relationship between calcium channels and the cytoskeleton exists in NM neurons. In deafferented NM neurons, dramatic increases in intracellular calcium could lead to a substantial destabilization or degradation of the cytoskeleton, which would explain the decreased levels of tubulin and actin immunostaining observed in these cells. The degradation of the cytoskeleton, in turn, could lead to the inactivation of calcium channels and a further reduction in calcium influx. Therefore, the cytoskeletal changes that we have observed in deafferented neurons may play a role in protecting these cells from detrimental rises in intracellular calcium levels.

At present, we do not fully understand the interrelationships among the myriad changes that occur in activity-deprived NM neurons. However, a recent review by Hesketh (1994) suggests an interesting mechanism that might explain some aspects of the relationship between changes in the cytoskeleton, decreased protein synthesis, and the loss of polyribosomes. Evidence presented from several laboratories indicates that polyribosomes are frequently associated with the cytoskeleton, which suggests that the interaction of ribosomes with the cytoskeleton may be essential for the proper translation of particular mRNAs into proteins (Hesketh, 1994). Although the majority of these analyses were conducted on cell lines, similar associations may be present in neurons in vivo as well. If the breakdown of the cytoskeleton in NM neurons begins earlier than is detectable with immunocytochemistry, protein synthesis could be severely compromised at very early times following deafferentation. This could provide an explanation of why protein synthesis initially decreases in all NM neurons following cochlea removal. In addition, these results may explain why polyribosomes completely dissociate in some activity-deprived NM neurons. Further experiments will be required to determine whether a relationship exists between the cytoskeletal changes and the decreases in macromolecular synthesis in this system.

Relationship to other models of cell death

It is interesting to note that changes very similar to the ones observed in activity-deprived NM neurons have also been observed in other instances of cell death. For example, axotomy of CNS neurons frequently results in retrograde degeneration and death of the injured neurons. This atrophy and death is preceded by decreases in both RNA and protein synthesis (for review, see Barron, 1989). These events are similar to those observed in our model of deafferentation-induced atrophy and death. Similarly, cellular atrophy and decreases in macromolecular synthesis have been observed in cultured sympathetic neurons deprived of nerve growth factor (Deckwerth and Johnson, 1993). In this system, atrophy was apparent within the first 24 hours following trophic factor removal. In addition, total protein content and protein synthesis (as measured by [35S]methionine incorporation) decreased substantially within 12–24 hours (Deckwerth and Johnson, 1993). Finally, RNA synthesis (as measured by [3H]uridine incorporation) also decreased dramatically in these neurons within the same time frame (Deckwerth and Johnson, 1993). It is possible that these changes are part of a conserved mechanism in the damaged CNS cells that down-regulates systems that consume large portions of the cell’s energy. For example, energy that is conserved by the down-regulation of protein synthesis may be directed to fuel cell survival mechanisms. Evidence from Ratan et al. (1994), which indicates that energy from down-regulated protein synthesis is shunted to production of beneficial compounds, such as glutathione, is consistent with this hypothesis. Similarly, the atrophy of surviving neurons may also benefit these cells by decreasing their energy needs. Although these cell death/cell survival changes have not been carefully compared across many systems, the similarities do suggest that a survival mechanism involving modifications in the cytoskeleton and decreased macromolecular synthesis may be present in many CNS neurons.
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LITERATURE CITED


