The award winning paper provides the first insight into the cellular events which lead to neuronal death. The main finding is that following deafferentation a rapid destruction of the mechanism of protein synthesis in neurons occurs. The cessation of protein synthesis causes the death of the affected neurons. The rapidity of this process, which causes the destruction of ribosomes, suggests the existence of a killer enzyme. The natural evolution of this research will be that of recognizing such an enzyme and the ways to control its action.

In addition to the Award winning work, the present issue of Brain Dysfunction contains other papers which were submitted for the Prize and were selected by the Committee due to their scientific interest and relevance. This group of papers covers different aspects of neural plasticity. The paper by Kolb and Wishaw demonstrates differences in recovery between neonatal- and adult-lesioned rats (retrosplenial cingulate lesions). The work by Takeuchi et al. investigates the effects of unilateral 6-hydroxydopamine treatment on

striatal serotonin in neonatal rats. The paper by Sharma et al. shows that rearing at high ambient temperature induces tolerance to heat stress in young rats and this is related to functional plasticity. The paper by Xu et al. investigates the influence of cerebral ischemia on cholecystokinin octapeptide release from brain cortex and the effects of GM1 on this release and regional blood flow. Finally, the paper by Hachinski et al. systematically studies stroke in the elderly and its relations with age.

We hope that this issue will be helpful for those involved in research on neural plasticity by providing them an overview of the different aspects of this field. This effort will continue with forthcoming issues devoted to the same topic and containing selected contributions to the 2nd International Symposium on Brain Dysfunction, 'Neural Plasticity in Brain Development', which was held in Troina on September 23–26, 1991.

R. Ferri, Troina
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A Cellular Mechanism Underlying Activity-Dependent Transneuronal Degeneration: Rapid but Reversible Destruction of Neuronal Ribosomes

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Abstract. The present study defines an initial step in the cellular cascade leading to transneuronal degeneration of neurons following disruption of their afferent input. Previous studies have shown that neurons in the cochlear nucleus of the chick, which undergo transneuronal degeneration after removal of the cochlea, cease protein synthesis entirely within hours after the cessation of afferent activity. The present experiments define the cellular mechanisms underlying this cessation of neuronal protein production. Neurons which had ceased producing protein after cochlea removal were identified using autoradiographic techniques. The same neurons were then evaluated at the electron microscopic level in thin sections adjacent to those prepared for autoradiography. The ultrastructure of neurons that were not synthesizing protein was compared with other neurons in the same section which exhibited normal levels of protein synthesis. Neurons that were inactive in protein synthesis exhibited a profound destruction of cellular ribosomes. By 6 h after removal of the cochlea, some neurons contained essentially no indentifiable ribosomes. In the place of ribosomes was an electron-dense debris that resembled the remains of ribosomes digested with ribonuclease. Other organelles in the affected neurons (including microtubules and mitochondria) were not obviously affected at this time. Disruption of neuronal ribosomes could be detected as early as 1.5 h after removing the cochlea, and ribosome destruction was evident in some neurons by 3 h. The cessation of protein synthesis and the destruction of ribosomes also occurred following blockade of eighth nerve activity by tetrodotoxin. Surprisingly, these early stages of neuronal degeneration were reversible if activity was restored (by eliminating the tetrodotoxin blockade). These results are the first to demonstrate a rapid and profound alteration in neuronal cytoplasmic organelles as a result of the cessation of afferent activity which could account for the rapid transneuronal degeneration of neurons in developing animals.

Introduction

Interactions between neurons and their synaptic targets can be categorized to the time course of their effects. The first, and best-understood interactions are those involved in the transmission of information through synaptic networks. Information transmission involving chemical synapses occurs when chemical substances (transmitters or modulators) are released from presynaptic endings and bind to a postsynaptic receptor ion channel or indirectly modify an ion channel through a second messenger. Alternatively, in electrical coupling between synapses, current may flow directly from the pre- to the postsynaptic element. There is considerable information about the cellular and molecular events involved in these forms of information transmission; many transmitters and modulators have been identified, release mechanisms have been defined, and postsynaptic receptor events have been characterized. These events take place in the time domain of milliseconds, sometimes extending to seconds or minutes (in the case of second-messenger-mediated events).

The second broad category of intercellular interactions between pre- and postsynaptic elements involves long-term regulation of cell phenotype and intercellular connectivity (so-called 'trophic interactions'). Trophic interactions are less well understood at the cellular and molecular level, but are known to have some unique properties. First, trophic interactions may be either orthograde or retrograde [1, 2]. Retrograde trophic interactions have been extensively investigated and are thought to involve target-derived trophic substances. On the other hand, orthograde interactions are less well understood. Second, both orthograde and retrograde trophic interactions

are often age dependent. Young animals often show greater susceptibility to the loss of inputs than adults [1]. However, in at least some systems, developing neurons are actually less sensitive to the loss of normal targets than adult neurons [3]. Third, the time domain over which trophic signals operate is from minutes to days and even years. In fact, the effects of trophic interactions during early development can persist throughout the life of the organism. This is the case because circuits whose development is regulated by trophic interactions often become independent of the trophic signals once established [4 – 6].

In this paper we shall be concerned with one form of trophic interaction – afferent regulation of target neuron metabolism and survival. It has repeatedly been shown that decreases in the amount or pattern of synaptic activity impinging on a neuron can lead to either atrophy or death of the postsynaptic neuron. These changes in individual neurons lead to permanent changes in the transmission of information through neuronal networks. While such demonstrations are important, there are only a few systems that have been amenable to analysis of the cellular events underlying these forms of intercellular regulation. Studies of the cellular events that occur following dramatic changes in afferent input also provide a catalog from which to study more subtle forms of synaptic plasticity that may be involved in experience-dependent modifications.

In some systems it has been shown that transneuronal degeneration or atrophy are linked to presynaptic activity [see for example ref. 7]. Thus, atrophy or degeneration can be set into motion either by direct injury to presynaptic inputs or by manipulations which decrease activity without directly damaging the presynaptic element.

The cellular events underlying transneuronal degeneration can be roughly divided into three areas of investigation: (1) What are the presynaptic and intercellular signals that are responsible for maintaining postsynaptic neurons? (2) What are the intracellular cascades that cause a cell to undergo transneuronal atrophy or death when presynaptic input is disrupted? (3) What are the biological factors underlying age differences in the susceptibility of neurons to disruptions of afferent input? While considerable attention has been focused on defining the presynaptic and intercellular signals [8], little is known regarding the latter two questions. The present experiments seek to identify the intracellular events that may be responsible for transneuronal cell death and to determine the presynaptic signals that set these events into motion.

An especially useful system in which to study orthograde trophic effects is the brain stem auditory pathway of the chicken [9, 10]. In this system, the survival of the auditory relay neurons depends upon presynaptic activity. Action potentials over eighth nerve axons can be permanently eliminated by removing the cochlea (leaving the cochlear ganglion cells and central processes intact) or reversibly eliminated by injecting tetrodotoxin (TTX) into the perilymph. Several nuclei in the brain stem receive direct input from eighth nerve axons, including nucleus magnocellularis (NM), which is the avian homolog of the anteroventral cochlear nucleus of mammals, and nucleus angularis which is analogous to the posteroventral and dorsal cochlear nuclei in mammals. The sole excitatory input to NM neurons is from the ipsilateral eighth nerve, and following its inactivation the NM cells are electrically silent. After removal of the cochlea or injections of TTX into the perilymph of

young chicks, about 30% of the neurons in NM undergo rapid transneuronal degeneration and disappear by about 2 days. The remainder of the NM neurons atrophy, but survive indefinitely. In adult animals, little or no neuronal loss or atrophy occurs [11].

In previous studies investigating the cellular mechanisms that may underlie transneuronal degeneration in NM, we found that the earliest change that allows us to discriminate between neurons that are destined to die and those that will survive is a cessation of protein synthesis [12]. Protein synthesis appears to cease completely in about 30% of the NM neurons within about 6 h after cochlea removal or elimination of activity over the eighth nerve. Other neurons in the nucleus initially exhibit large decreases in protein synthesis and then partially recover; the surviving neurons maintain a reduced level of synthesis that correlates with their reduced size. Thus, in the ipsilateral NM there is a bimodal distribution in the pattern of autoradiographic labeling. The average grain density over unlabeled neurons was 3 – 5 standard deviations below the average grain density over neurons from the contralateral side. These unlabeled neurons die within 2 – 3 days. The remaining two thirds of the neurons show only a 20 – 30% reduction in grain density as compared with neurons on the contralateral side; these neurons survive. These decreases in protein synthesis and the ensuing cell death are dependent upon changes in the level of activity over the afferents. Blockade of cochlear nerve activity with TTX results in the same pattern of decreases in protein synthesis, cell atrophy, and cell death as destruction of the cochlea [13]. An especially interesting feature about cellular events that occur following TTX blockade is that the changes in protein synthesis are reversible. Normal levels of synthesis are reestablished

when the TTX blockade is reversed, as is normal cell size [13]. Importantly, cells in which protein synthesis has completely ceased can apparently completely recover if activity is restored in time. Thus, at least some of the changes that occur following deafferentation do not invariably lead to the death of the affected neurons.

In the present study we demonstrate that the cessation of protein synthesis which occurs within hours of activity blockade is the result of complete destruction of ribosomes in the affected cells. This observation is of considerable importance because it reveals an early event in the active intracellular cascade that is set into motion by the cessation of activity and which leads to transneuronal cell death. On a priori grounds, it seemed unlikely that a cell could recover after total destruction of its protein-synthesizing machinery. However, our studies of the consequences of reversible TTX blockade of eighth nerve activity revealed that the postsynaptic neurons can recover and remain viable if normal activity is restored. These results reveal one of the key intracellular events which are regulated by this activity-dependent orthograde trophic influence.

Methods

Cochlea Removal

The experiments were carried out on 2- to 3-week-old white leghorn chicks. Eggs were obtained from a local supplier and incubated and hatched in our laboratory. The basilar papilla (cochlea) was removed unilaterally, while chicks were anesthetized with a combination of ketamine hydrochloride (80 mg/kg i.m.) and Nembutal (20 mg/kg i.p.). The procedures and controls for this operation have been previously described in detail [11]. This operation unilaterally eliminates action potentials in the eighth nerve and in the ipsilateral NM, but does not directly destroy

the ganglion cell bodies which give rise to the axons in the eighth nerve. Cells in the contralateral NM maintain normal eighth nerve input and normal activity. We examined neurons in NM on both sides of the brain; the ipsilateral NM cells that were electrically silenced due to removal of the receptor were compared amongst themselves and with normally active NM cells on the contralateral side of the same brain.

Sixty minutes (n = 3), 3 h (n = 3), or 6 h (n = 5) after removal of the cochlea, the chicks were injected intracardially with ^{3}H-leucine (0.5 mCi) and were allowed to survive for 30 min. The chicks were then deeply anesthetized and perfused with 2% glutaraldehyde/2% paraformaldehyde in cacodylate buffer (pH 7.2). The brain stems were allowed to postfix for 12–24 h and then sectioned in the transverse plane using an Oxford vibratome. Blocks containing the auditory relay nuclei were dissected free, osmicated, stained en bloc, and embedded in Marglass. Paired semithin (1 μm) and ultrathin (60 nm) sections were prepared from each block. The 1-μm (semithin) sections were mounted on glass slides and coated with Kodak NTB-2 emulsion and processed for autoradiography [12]. Ultrathin sections were collected on formvar-coated slotgrids for electron microscopic evaluation.

^{3}H-leucine that has been injected systemically is rapidly incorporated into protein by neurons that are actively synthesizing protein. Virtually all of the free amino acid that is not incorporated into protein is washed out by the fixation and embedding procedure. With a 30-min pulse, there is not sufficient time for significant transport of the recently synthesized protein from the site of synthesis. Thus, in the autoradiographic preparations, the number of silver grains over individual cell bodies is proportional to the amount of protein synthesis by those cells. Since the NM neurons whose afferent input was silenced (ipsilateral NM) and control NM cells (contralateral NM) were on the two sides of the same tissue sections and processed identically, the relative grain densities over the neurons are indicative of relative amounts of protein synthesis.

The first goal of the present study was to characterize the ultrastructural correlates of the complete cessation of protein synthesis 6 h after the removal of the cochlea. For this purpose, neurons that had ceased protein synthesis were identified from the autoradiograms prepared from the 1-μm sections, and the same neurons were then identified in the ultrathin sec-

tions. Electron micrographs of the cytoplasm (at a magnification of ×20,000–30,000) of these neurons were then compared with those from adjacent cells which were actively synthesizing protein as well as with randomly selected micrographs from neurons in the contralateral NM (control side).

Previous studies have shown that 1.5 and 3 h after the cochlea removal the cessation of protein synthesis was not yet fully developed. In particular, neurons on the affected side did not fall into dichotomous populations. Thus, at these time points, electron micrographs were taken of all neurons in NM in 2–4 sections both ipsilateral and contralateral to the cochlea removal. These micrographs were then evaluated for the cytoplasmic changes that were identified in the above analysis as characteristic of unlabeled neurons at 6 h (specifically, the changes in ribosomes).

In addition to these qualitative analyses, in 2 animals from each survival time, we evaluated the grain density over all neurons in a single 1-μm section and then quantitatively rated the integrity of ribosomal complexes in electron micrographs of each neuron. The procedures for measuring grain density over individual neurons have been described previously [13]. Briefly, tissue was viewed through a ×100 planapochromatic objective (NA = 1.3) on a Zeiss Universal microscope. This image was digitized at 512 × 512 pixels on a Gould-Deanza system interfaced to a PDP 11/70 computer. From the digitized image, the perimeter of each neuron sectioned through the nucleus was outlined with a cursor, and an automated algorithm computed the number of silver grains over the cytoplasm. Simple division of the silver grain count by the cross-sectional area of the neuron yielded a measure of grain density. To evaluate ribosomal complexes, high-power electron micrographs (×30,000–40,000) were then taken of the cytoplasm of each neuron. All micrographs (n = 131) from experimental and control sides of both animals were randomized, and each was assigned a coded number. Each micrograph was scored blind by two of the authors (E.W.R. and O.S.), rating ribosomes as intact or disrupted. Ratings were made without knowledge of whether the micrographs were from the experimental or control side of the brain and without knowledge of the grain density over the neuron. Ratings were on a 6-point scale, with '1' indicating complete absence of ribosomes and '6' indicating a completely normal-appearing cytoplasm. The micrographs were then decoded, allowing us to relate grain density to our

rating of ribosomal integrity on a cell-by-cell-basis. This analysis provides numerical data on the relationship between the ultrastructure of each neuron and its protein synthetic activity (as measured by grain density).

TTX Blockade of Eighth Nerve Activity

An additional 13 chicks of the same age sustained unilateral blockade of eighth nerve activity without damage to the cochlea or eighth nerve axons. In previous studies we have shown that a fully reversible total blockade of eighth nerve action potentials can be achieved by small injections of TTX into the perilymph of the saccule [13]. In the present study, animals were anesthetized as described above and then a small hole was made in the skull overlying the right saccule. A single dose (1 μl) of 0.3 mM TTX in saline was injected into the perilymph, or 1 μg of TTX in polyvinyl alcohol was placed on the saccule; the latter procedure results in a slow release of TTX into the perilymph. Physiological studies have revealed that the consequences of the two modes of administration are similar; both techniques block all eighth nerve action potentials for at least 6 h. Subsequently, activity returns as a result of the metabolism of the TTX or replacement of the perilymph, allowing full recovery of cochlear and neural function. In all of the animals reported here, the effectiveness of the TTX blockade was monitored behaviorally (observing vestibular dysfunction) and by recording brain stem auditory evoked potential thresholds and input-output functions. In animals allowed to recover from the activity blockade (see below), the effectiveness of the blockade was confirmed at 6 h and the recovery of normal auditory function was confirmed 7 days later using brain stem evoked potentials. In earlier studies the effectiveness of the TTX blockade and the recovery was confirmed by recording action potentials from the brain stem auditory nuclei [13].

Four animals were euthanized during unilateral eighth nerve blockade. These animals were allowed to survive either 3 h (n = 2) or 6 h (n = 2) after TTX injection, at which time the animal was anesthetized, and electrophysiological techniques were used to confirm the blockade of eighth nerve activity. The animals were perfused transcardially with the EM fixative, and brain stems were processed for electron microscopy as described above, except that protein synthesis was not evaluated autoradiographically. This tissue was used to determine if the polyribosomal changes seen

after cochlea removal were entirely attributable to a decrease in neuronal activity, or if other processes, resulting from damage to the nerve, are required to initiate the destruction of ribosomes.

An additional 9 animals treated unilaterally with TTX were allowed to recover for 7 days. The auditory function was then assessed electrophysiologically; all recovered to within 10 dB of normal threshold. The animals were then anesthetized and perfused with 4% paraformaldehyde, and the brain stems were sectioned for analysis by light microscopy. Neuronal size and neuron number in NM on both sides of the brain stem were evaluated as described previously [11]. The purpose of these animals was to determine if NM neurons could fully recover from the ultrastructural changes that we see after 6 h of afferent activity blockade.

Results

Rapid Destruction of Ribosomes following Cochlea Removal

We have previously shown that at 6 h following cochlea removal or activity blockade, a population of NM neurons can be identified which have apparently ceased synthesizing new proteins, as indicated by incorporation of ^{3}H-leucine [12, 13]. Over the ensuing 2 days these neurons die, while the deafferented remaining neurons atrophy but survive. The animals in this study that were sacrificed 6 h after cochlea removal allowed us to compare the ultrastructural properties of the neurons destined to degenerate with those of the neurons destined to survive.

As we have found in previous studies, cochlea removal leads to complete cessation of protein synthesis in some neurons in NM (fig. 1A, upper cell). The neurons that ceased producing protein entirely were obvious in the autoradiograms because they had only background numbers of grains overlying their cytoplasm. Other neurons in the nucleus exhibit a somewhat reduced level of synthesis

(fig. 1A, lower cell) in comparison to neurons on the control side. On the control side, every neuron was labeled (fig. 1B).

Figure 1C illustrates the appearance of the cytoplasm of the unlabeled neuron shown in figure 1A, and figure 1D illustrates the appearance of the cytoplasm of the nearby labeled neuron. Figure 1E illustrates the appearance of the cytoplasm of the neuron on the control side. The striking and consistent finding was that unlabeled neurons contained essentially no intact ribosomes (either on the stacks of endoplasmic reticulum or free in the cytoplasm). In contrast, all neurons that were actively synthesizing protein possessed ribosomes, polyribosome clusters, and rough endoplasmic reticulum.

The loss of ribosomes in the affected cells can best be appreciated by comparing the stacks of endoplasmic reticulum. In normal cells, these stacks were studded with ribosomes (fig. 2A). In the cells that were not synthesizing protein, the stacks of endoplasmic reticulum were entirely bare (fig. 2B). Instead,
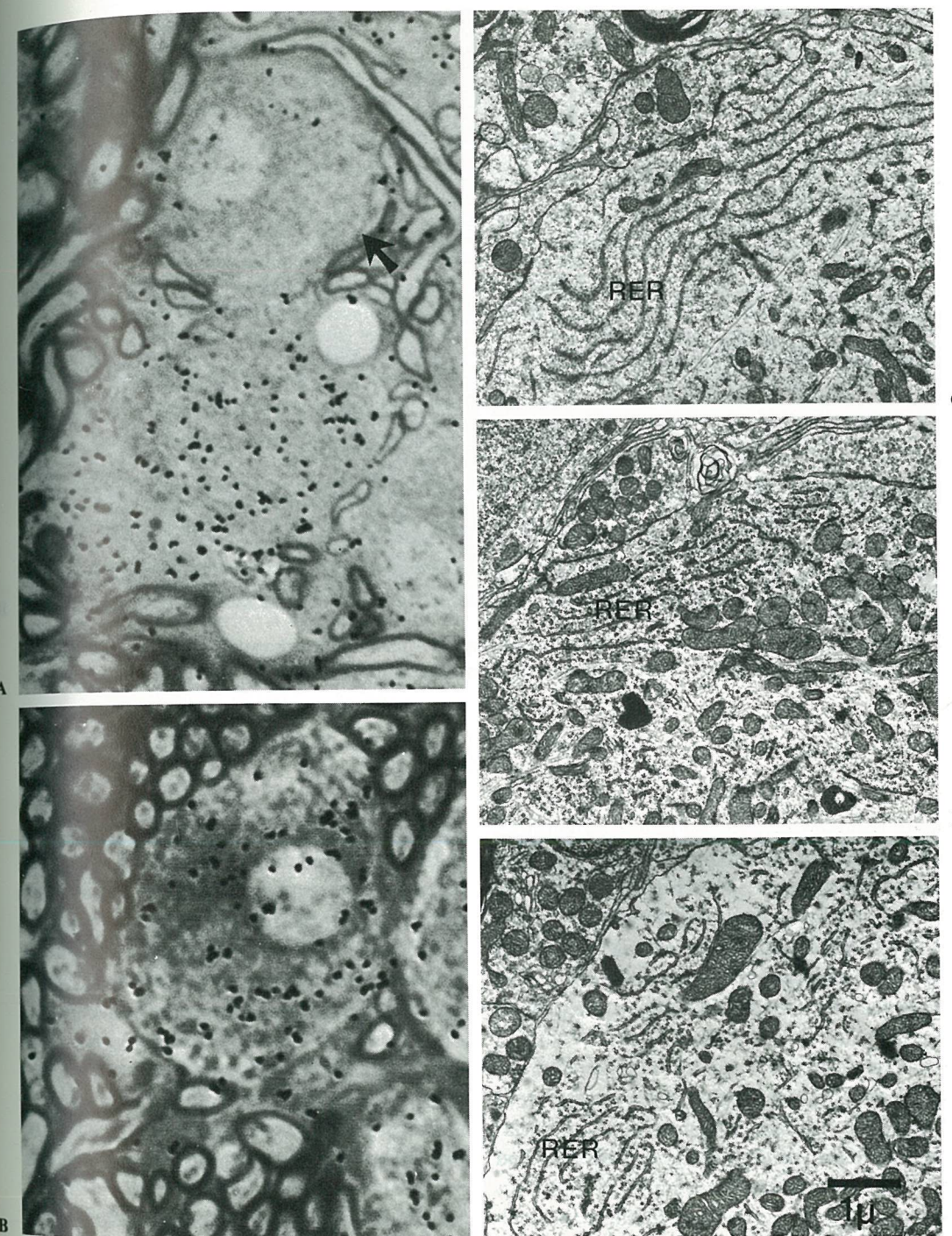

Fig. 1. Representative light microscope autoradiographs and electron micrographs of neurons in NM 6 h after cochlea removal. **A** Autoradiograph of NM neurons ipsilateral to the cochlea removal. An unlabeled cell is evident in the upper portion of the field (arrow), and a labeled cell is immediately adjacent. The ultrastructural appearance of the cytoplasm of these two cells is illustrated in **C** and **D** respectively. Note the absence of ribosomes in the unlabeled cell (especially the absence of ribosomes from the stacks of endoplasmic reticulum) and the apparently normal appearance of ribosomes and rough endoplasmic reticulum (RER) in the labeled cell. Other cytoplasmic elements and synapses upon the unlabeled neuron appear normal. **B, E** A labeled NM neuron from the contralateral side of the same brain.
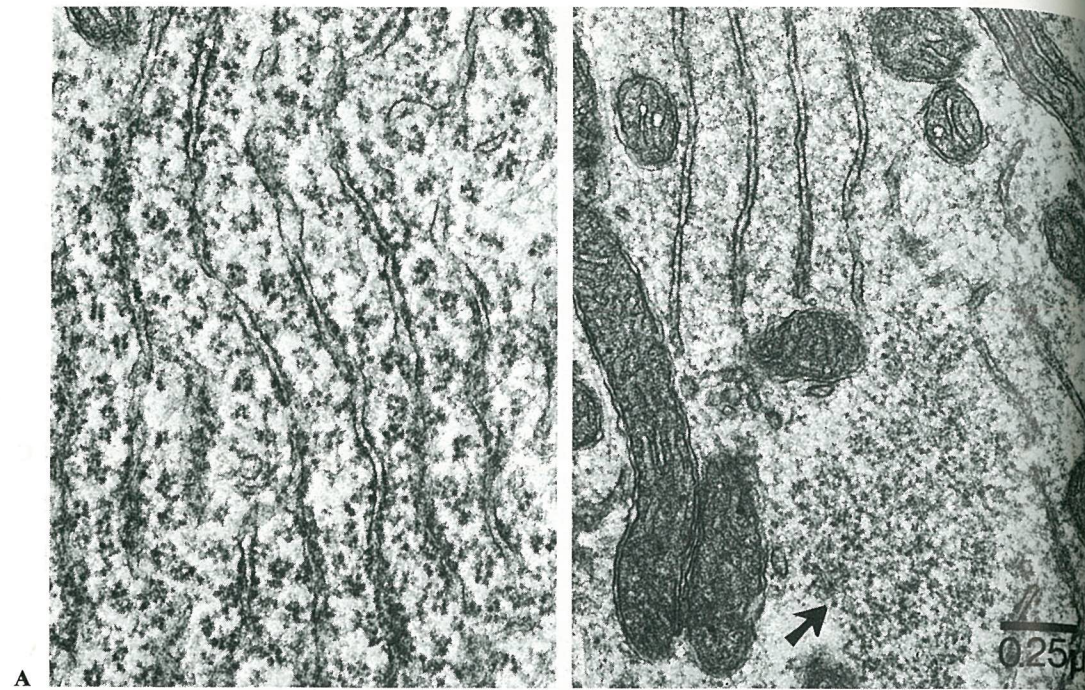
Fig. 2. High-power electron micrographs illustrating ribosomes and rough endoplasmic reticulum in normal NM neurons (**A**) and in a neuron that was not synthesizing protein (**B**).

Fig. 3. Three-dimensional graphs illustrating the relationship between the extent of labeling and the rating of polyribosome integrity from electron micrographs of the same neurons. The Y axis shows the number of neurons in each category, the X axis shows the grain density over the neurons, and the Z axis indicates the rating of ribosome integrity. **A** This relationship for NM neurons ipsilateral to the cochlea removal, **B** The relationship for the NM neurons on the contralateral (control) side.

the stacks ere surrounded by an amorphous electron-dense material which bears a striking resemblance to ribosomal remains that can be seen in tissue treated with RNAse [14]. For this reason, we believe that the electron dense material represents the remnants of ribosomes that were rapidly destroyed by some process taking place in the affected cells.

There was no evidence of a general degradation of cellular ultrastructure, as can be seen by comparing synapses, mitochondria, microtubules, endoplasmic reticulum, and other organelles in affected and normal cells. However, there were some features that distinguished the unlabeled cells other than the

changes in ribosomes. For example, stacks of endoplasmic reticulum near the cell periphery appeared more prominent in the unlabeled neurons. In addition, the affected neurons also contained electron-dense granular material in the cytoplasm that was not apparent in normal cells or in cells that were active in producing protein on the deafferented side. Typically, the granular material occupied a portion of cytoplasm $1-2$ μm in diameter. Other organelles were generally absent from the area occupied by these electron-dense granular accumulations. The granular material appeared different from the flocculent electron-dense material surrounding the en-
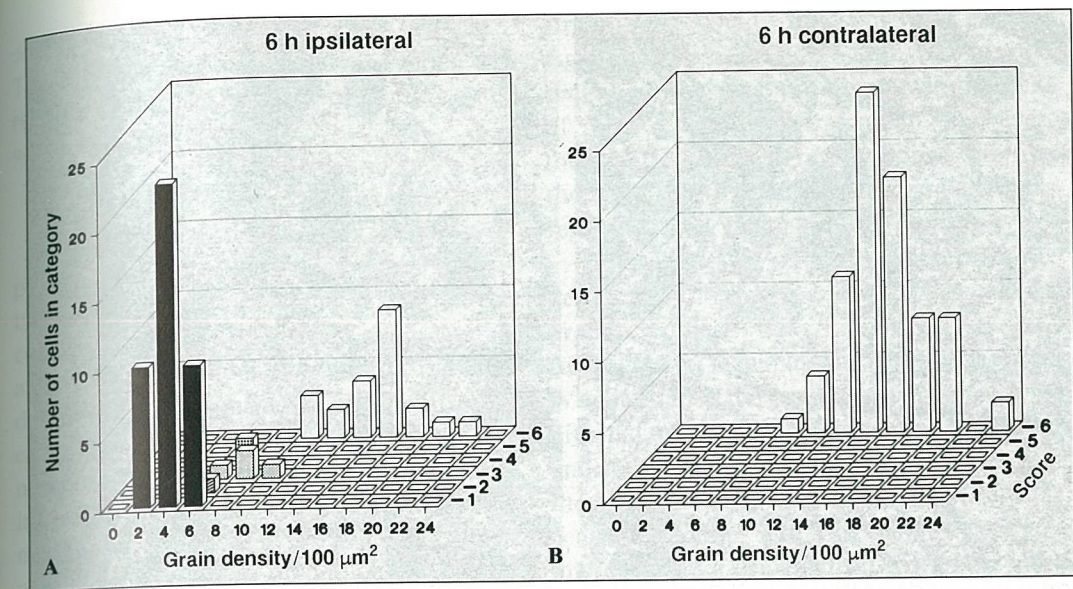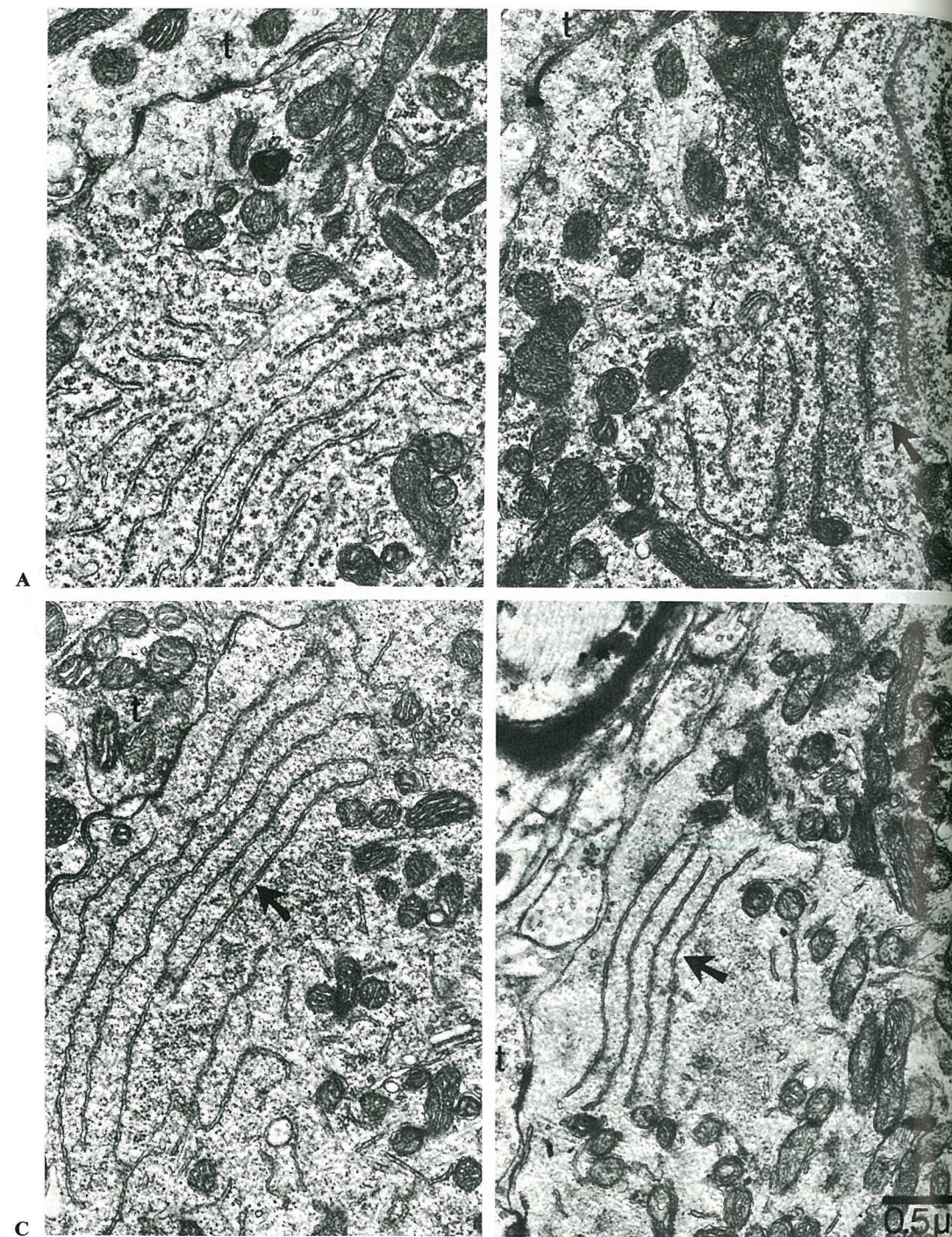
doplasmic reticulum that we believe represents ribosome debris.

To assess the quantitative relationship between the cessation of protein synthesis and the destruction of ribosomes, a detailed analysis was carried out in 2 animals. From a single section midway through the rostrocaudal extent of NM we determined grain density over every neuron on the experimental and on the control side and correlated these values with our ratings of the ribosomal integrity for each neuron. Figure 3 reveals the results of this analysis for an animal killed 6 h after the cochlea removal. In every neuron from the control (contralateral) side of the brain (n =

63), the ribosomal integrity was rated as normal (score = 6). The grain densities for these neurons ranged from 24 to 8 grains/100 μm². On the side of the brain ipsilateral to receptor removal (fig. 3A), the distributions of grain densities and ribosomal ratings were bimodal. Of the 68 neurons evaluated, 52 were rated as having either no ribosomes (score = 1) or normal ribosomes (score = 6). The grain density over cells rated as '1' was 6 grains/100 μm² or less, whereas the grain density over cells receiving a rating of '6' was over 12 grains/100 μm². Sixteen neurons from the experimental side exhibited intermediate levels of ribosomal degradation; these had grain

Fig. 4. Time course of ribosome destruction in NM neurons. **A** Control side, **B** 1 h after cochlea removal, **C** 3 h after cochlea removal, **D** 6 h after cochlea removal. Note the progressive loss of ribosomes and the appearance of a fine electron-dense 'dust' which we interpret as the debris from ribosomes. Apparently intact synaptic terminals can be seen contacting the NM neurons (t).

counts ranging from 4 to 16 grains/100 μm^2. The Pearson product-moment correlation relating grain density to cytoplasmic rating was r = 0.92.

Course of Ribosome Destruction

The bimodal distribution of the levels of protein synthesis (as revealed by grain density) was not apparent 1 and 3 h after the cochlea removal [12]. Instead, there was an apparently continuous distribution of grain densities. No neurons could be identified as completely unlabeled at 1 h and only a few neurons were entirely unlabeled at 3 h. Thus, instead of attempting to identify unlabeled neurons at 1 and 3 h we evaluated every neuron represented in the individual section and searched for cytoplasmic abnormalities, paying particular attention to the neuron's ribosomes and rough endoplasmic reticulum.

As illustrated in figure 4B, neurons could be found that exhibited the signs of ribosome destruction even 1 h after cochlea removal. Often the only regions showing ribosome destruction at 1 h were within parallel stacks of rough endoplasmic reticulum. The endoplasmic reticulum would be free of ribosomes, and an electron-dense material would be apparent between the layers. Elsewhere within the neurons, ribosomes appeared unaffected. At 3 h (fig. 4C), the extent of ribosome de-

struction appeared to be somewhat greater. Again, the ribosome destruction that was present at 3 h was most prominent within stacks of rough endoplasmic reticulum. However, there were still only a few neurons exhibiting the complete ribosome destruction that was regularly observed at 6 h.

To evaluate the relationship between the destruction of ribosomes and the decreases in protein synthesis, quantitative analyses were carried out on 2 animals at each survival time. We determined the grain density over each individual neuron in a single section ipsilateral and contralateral to the cochlea removal. We then identified the same neurons in the adjacent thin sections and took sample electron-micrographs of each neuron. All of the electron-micrographs were encoded and mixed together. The scorers were blind with respect to the animal's history, survival time, grain density measurements, and whether the micrograph was from the NM ipsilateral or contralateral to the cochlea removal. In each brain 80–100 neurons from each side were evaluated in this manner for a total of approximately 750 cells.

As in the animals killed 6 h after cochlea removal, the neurons on the control side were uniformly rated as ultrastructurally near normal (scores 5–6). In 3 of the animals, all of the cells on the control side received a score of 6. In the other animal, 8 out of 106 neurons from the control side received a score of 5, while the remainder received a score of 6. These results confirm that the abnormalities we observed even at very short times after cochlea removal are indeed restricted to the functionally deafferented NM neurons.

Neurons on the deafferented side received scores ranging from 1 to 6. There were fewer neurons at the lower end of the scale at 1 and 3 h than was the case at 6 h. One of the two
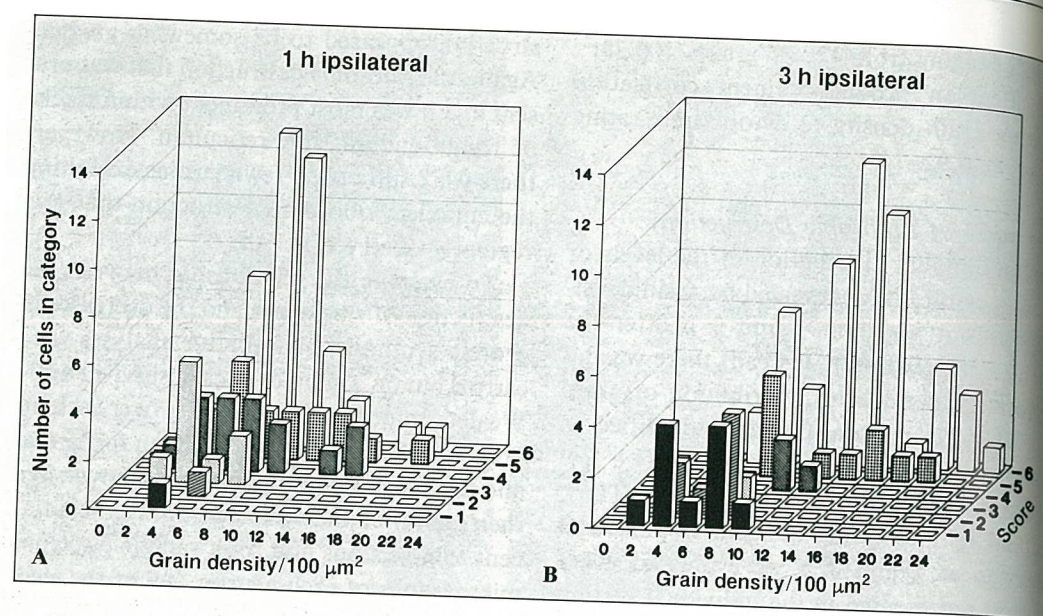
Fig. 5. Relationship between grain density and polyribosome integrity in NM neurons 1 (**A**) and 3 h (**B**) after cochlea removal. See figure 3 for details.

Fig. 6. Ultrastructural appearance of the cytoplasm of NM neurons ipsilateral (**A**) and contralateral (**B**) to blockade of eighth nerve activity by perilymphatic application of TTX. Again, note the profound loss of ribosomes from the rough endoplasmic reticulum (RER). The unlabeled arrow indicates an accumulation of electron-dense material in the cytoplasm. t = Intact synaptic terminal. Bar = 1 μm.

animals prepared 3 h after the cochlea removal (chick No. 3-1) exhibited a very small number of ultrastructurally abnormal neurons, and the autoradiographs from this animal showed uniformly low levels of labeling. Thus, the relationship between grain density and ultrastructural appearance could not be meaningfully evaluated. In 1 of the animals prepared at 1 h all cells ipsilateral to the cochlea removal (chick No. 1-1) showed an almost complete cessation of protein synthesis (average 3 grains/100 μm^2) while the control NM neurons showed normal levels (average 12 grains/100 μm^2). This overall marked reduction was common at very short survival intervals, but prevented us from relating grain counts to ribosomal integrity in the experimental NM of this animal.

Figure 5 shows the relationships of grain density to ribosomal ratings for NM cells on the ipsilateral side in the 2nd animal at 1 h (chick No. 1-2) and the 2nd animal at 3 h after cochlea removal (chick No. 3-2). There was a clear relationship between the level of labeling and the score assigned based on ultrastructural appearance. In general, neurons exhibiting the lowest levels of labeling were also the ones exhibiting the most severe ribosome destruction. However, the relationship was not as dramatic as that seen at 6 h. At 1 h there was no indication of the sort of bimodal distribution that was obvious at 6 h, whereas at 3 h, there was only a slight indication that two distinct populations were emerging. The correlations relating grain density to the ultrastructural ratings were r = 0.34 and r = 0.65 for the 1-hour and 3-hour animals, respectively. Both are highly reliable (p < 0.001).
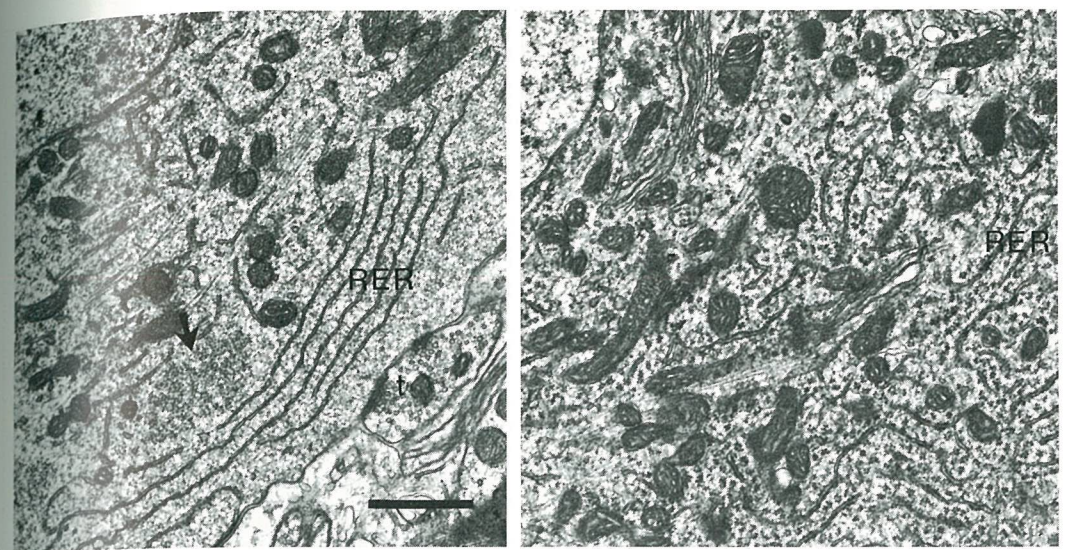
Destruction of Ribosomes and Neuron Viability following TTX Blockade of Eighth Nerve Activity

Several facts led us to believe that the ribosomal changes observed at short survival times following cochlea removal are due to the elimination of eighth nerve action potentials and the associated decrease in synaptic activation of NM neurons. These include: (1) removal of the cochlea initially spares the ganglion cells [11]; (2) signs of degeneration of synaptic endings in NM are not observed at the light microscope level until after 18 h [15] or at the electron microscopic level until 15 – 18 h [unpubl. observations]; (3) changes in protein synthesis, cell size, and cell number observed following cochlea removal are all observed to a similar extent following unilateral TTX injections into the cochlea which does not permanently alter eighth nerve action potential thresholds or input/output functions, and (4) the protein synthesis changes in NM neurons can be prevented by synaptic activation of NM neurons [16]. Nevertheless, it was important to directly determine if the changes in ribosomal integrity are regulated by changes in the activity impinging on NM neurons and if these neurons can recover after such massive changes in cytoplasmic integrity.

As illustrated in figure 6, there was clear evidence for ribosome destruction in animals prepared for electron microscopy after unilateral injections of TTX into the perilymph. At 6 h there were many cells in which ribosomes were totally destroyed. In fact, the extent of ribosome destruction appeared to be comparable to that following cochlea removal.

In addition, neurons exhibiting ribosome destruction also exhibited the unusual stacks of endoplasmic reticulum and electron-dense, flocculent material that were characteristic of neurons exhibiting low levels of synthesis following cochlea removal. The results from the 2 animals sacrificed 3 h following TTX injection also were identical to those discussed above following cochlea removal.

The massive destruction of ribosomes in a large complement of NM neurons just 6 h after stopping afferent excitatory activity suggests that these neurons may have become committed to a series of changes culminating in cell death. For this reason, we thought it unlikely that cells could recover after the apparent total destruction of ribosomes. The ability to elicit this cellular change in NM neurons by temporary blockade of the eighth nerve action potentials allowed us to directly address the question of recovery.

In 9 animals we blocked action potentials in one ear for 6–12 h by TTX injections and then allowed the animals to survive for 7 days. We then counted the number of neurons and determined the average cell size in NM on both sides of the brain. The rationale was that if the cells that had lost their ribosomes during 6–12 h of TTX blockade were committed to die, we would see a consistent decrease in cell number similar to that seen after cochlea removal or after long-term TTX treatment.

Table 1 compares the percentage of NM neurons that die by 7 days after cochlea removal, the percentage that die after 48 h of TTX treatment, and the percentage that are lost after 6–12 h of TTX treatment followed by 7 days of recovery.

There was no apparent cell loss in NM 7 days after transient (6–12 h) blockade of eighth nerve activity. This is in contrast to the consistent cell loss seen 7 days after cochlea

Table 1. Mean proportion of cells dying after each manipulation

Treatment	n	Mean % lost (±1 SD)[a]
Cochlea removal (7-day survival)[b]	4	29 (10)
TTX (48 h)[c]	6	20 (11)
TTX (6–12 h) + 7 days[d]	9	−4 (6)

[a] Computed by determining the percent decrease in NM cell number ipsilateral to the manipulation for each brain and then averaging these numbers.
[b] Data from Born and Rubel [11].
[c] Data from Born and Rubel [13].
[d] Data from Born [34].

removal or after 48 h of blockade of eighth nerve activity. The recovered NM cells were also equivalent in average cross-sectional area to the contralateral NM cells.

Thus, it appears that the massive changes seen in the cytoplasm of some NM neurons after 6 h of deafferentation *do not* irreversibly condemn the cell. These neurons can apparently reform their protein-synthesizing machinery and remain structurally and functionally viable.

Discussion

The long-term goal of the research described in this paper is to understand the cellular mechanisms through which the pattern or amount of activity in a neural circuit influences the organization of neural elements. Previous studies have shown that modulation of activity over the eighth nerve profoundly affects neurons in the cochlear nucleus [7]. Decreasing the normal level of activity (by removing the cochlea or blocking action potentials in eighth nerve axons) leads to short-term changes in neuronal metabolism, to transneuronal atrophy of most neurons in NM, and to transneuronal degeneration of about 30% of the neurons in NM. Changes in cell size and cell number are fully developed by 2 days after elimination of afferent activity. However, the metabolic changes, such as decreases in protein synthesis and increased mitochondrial enzyme activity, are marked during the first 24 h, and some changes are fully developed within only a few hours.

The overall level of protein synthesis in NM neurons has served as an excellent indicator of the eventual fate of these neurons. By 1 h after elimination of afferent activity, protein synthesis decreases by about 50%. By 6 h, two populations of neurons can be distinguished; in one population of neurons protein synthesis ceases, and these cells will die; in the other population protein synthesis is reduced (by about 25–30%), and the neurons survive in an atrophied state [12, 13]. We have proposed that it is the cessation of protein synthesis that actually leads to the death of the doomed neurons. Thus, understanding the cellular mechanisms underlying the cessation of protein synthesis will reveal the mechanisms through which one type of orthograde trophic influence operates.

The present results provide important new insights into the cellular mechanisms that lead to the cessation of protein synthesis. Early in the course of the neurons' response to decreased activity, some process is initiated that leads to the destruction of the neurons' protein synthetic machinery (ribosomes). Given the extreme nature of the destruction, it is no surprise that these neurons cease protein synthesis. These results will be discussed in relation to (1) trophic regulation of neuronal structure; (2) cellular mechanisms underlying transneuronal and embryological cell death, and (3) ontogenetic factors influencing the reactions to brain injury.

Trophic Influences

Retrograde and orthograde trophic interactions have been shown to play an extremely important role in determining the development of neural systems. These influences can determine survival, differentation, and functional properties of the systems in which they operate.

Retrograde trophic interactions are comparatively well understood. Both the intercellular signals and the intracellular processes regulated by these signals are at least partially defined. For example, a number of target-derived trophic factors have been identified which serve as the signals between targets and the presynaptic inputs [17]. In general, retrograde trophic factors are thought to be synthesized by target cells, released, actively taken up by the presynaptic axons, and then transported retrogradely to the cell body of the presynaptic neuron where the factor regulates the neuron's gene expression. These types of factors are known to be important for cell survival, cell differentiation, and perhaps for the regulation of the functional attributes of the presynaptic neuron (i.e., neurotransmitter type). The intracellular cascades that play a role in retrograde trophic regulation are beginning to be defined [18].

The present study focuses on *orthograde trophic influences*, defined as the influence of presynaptic elements on target cells, orthograde trophic influences are less well understood than the retrograde influences. The signalling process for orthograde trophic in-

fluences often seems to involve presynaptic activity. (Or at least the signalling covaries with presynaptic activity.) The evidence for this claim is that manipulations that affect activity often have the same overall effect as manipulations that lead to the physical loss of the inputs. These types of influence are especially interesting because they provide a potential means through which interactions with the environment (experience) can influence development, differentiation, and long-term function of neural systems.

Chronic manipulations of afferent activity in developing systems have a variety of long-term consequences on brain development. These range from modifications in the overall size of a brain region to cellular and subcellular modifications of neuronal and glial structure, connectivity, and metabolism. In most studies the consequences of manipulations have been studied days, weeks, or months after the initiation of the treatments which alter activity. Such studies identify the phenotypic consequences of altered orthograde trophic effects, but do not reveal the cellular processes leading to the phenotypic variations. The few studies that have evaluated short-term consequences of altered activity have focused on the regulation of synthetic or metabolic processes in the postsynaptic neurons. However, the causal relationships between these short-term effects and the long-term changes in neuronal structure and function are not clear.

In the present study, we have identified a very early consequence of altered activity which may mediate the long-term consequences of the manipulations (atrophy and death of NM neurons). The destruction of ribosomes within 6 h of activity blockade would certainly lead to the cessation of protein synthesis in the affected neurons which

in turn would lead to their eventual death. However, two features of the present observations are remarkable: (1) the disruption of protein synthetic machinery in affected neurons occurs within minutes after the cessation of activity, and there is complete destruction of ribosomes in some neurons within hours, and (2) the apparently complete destruction of ribosomes is reversible if the activity is restored. We will comment on each of these points below.

To our knowledge, the changes in ribosome structure described here are the most dramatic example of an activity-dependent transneuronal regulation of cellular ultrastructure that has been reported. Previously, such drastic changes in cellular ultrastructure have been reported only after direct damage to the cell, as a consequence of ischemic insult, or as a result of the introduction of a cytotoxic agent in the immediate extracellular environment. Because changes in protein synthesis can be observed within 30 min after the destruction of the cochlea, it is likely that disruption in ribosomes actually begins within 5 min, before any changes are evident at the ultrastructural level. The destruction of ribosomes is particularly striking given the fact that ribosomes are much more stable intracellular elements than many other organelles which appear to be unaffected by the decreased presynaptic activity (microtubules, endoplasmic reticulum, and mitochondria).

The second remarkable aspect of the present results is that the apparently complete destruction of ribosomes is not necessarily terminal. Restoration of activity after 6 h of blockade rescues cells which would otherwise die (and in which ribosomes are presumably destroyed). Presumably, neurons exhibiting complete ribosome destruction can reassemble the machinery for protein synthesis (by

synthesizing new ribosomes or reassembling subunits) and resume normal metabolic functions. Thus, increases in afferent activity (at least from a depressed baseline) can induce the formation of new ribosomes, leading to the restoration of protein synthesis. This is a particularly dramatic example of an orthograde trophic effect of neuronal activity. It seems likely that increases in afferent activity from a normal baseline could also enhance the synthesis of neuronal proteins.

It is important to note that in vitro studies have revealed that antidromic activation of NM neurons does not prevent the decreases in neuronal protein synthesis that occur after deafferentation [16]. Thus, protein synthesis by NM neurons is regulated via synaptic activation by eighth nerve axons, and not as a result of action potentials in the NM neurons themselves. This result suggests that activity-dependent regulation of the ribosome complement in NM neurons occurs via some receptor-dependent second messenger which is yet to be identified.

Does a Similar Generalized Disruption of Cellular Gene Expression Occur during Other Forms of Cell Death?

The present results define a plausible mechanism underlying at least one form of transneuronal atrophy and degeneration. It remains to be determined whether this mechanism operates in other settings. In the sections below, we will consider whether similar mechanisms operate during the types of transneuronal degeneration that occur in other systems and during other forms of cell death.

It is well established that extensive cell death occurs during normal development [19–22]. This naturally occurring cell death can be markedly increased by reducing the available targets for a given set of presynap-

tic neurons [23] or by eliminating afferents to a set of postsynaptic targets [7, 9, 21]. It is commonly believed that embryonic cell death is one mechanism for numerical matching between inputs and targets.

Recently, there has been considerable interest in defining the cellular processes which lead to cell death. Clarke [20] provides an outstanding current review of this literature, defining four types of embryological cell death based on the morphological appearance of the dying cells. Loss of ribosomes and displacement of ribosomes from the rough endoplasmic reticulum have been reported in what Clarke [20] has termed type I cell death or 'apoptosis' [24–26]. However, these changes in ribosomes have not been reported to occur before other changes (such as nuclear disruption). Thus, from the literature, it is difficult to determine the exact sequence of cytoplasmic and nuclear events in other cells undergoing cell death. We have not observed the early nuclear changes which are described as one of the earliest indications of apoptosis, however. Thus, our results indicate that the destruction of ribosomes is one of the earliest steps in the intracellular cascade leading to the death of at least one type of neuron.

Given that ribosome destruction is an early step in the cascade leading to cell death, the question becomes, what are the earlier steps in the process? The selective destruction of ribosomes (and the preservation of other cellular organelles which are usually thought to be much more delicate than ribosomes) persuasively argues that this form of cell death is not due to a generalized deterioration of the cell through a passive process such as tissue necrosis. It is also certain that the loss of ribosomes cannot be explained by a cessation of ribosome synthesis (leading to loss due to

turnover). The normal half-life of ribosomes in the brain is on the order of 6 days [27, 28], so very few ribosomes would be lost within a few hours, if ribosome biogenesis ceased completely. Thus, the rapidity of the effect, its dependence on a physiological signal, and its reversibility, argue that the process is active and highly regulated.

Our results are most consistent with the postulation that some active process leads to the rapid destruction or disassembly of the ribosomal particle (some sort of 'suicide factor' which is held in check by normal afferent activity). The nature of this putative suicide factor is open to speculation. Clearly, one possibility is some suicide enzyme which is newly synthesized or activated in response to decreases in activity. The fine electron-dense ribosome debris is strikingly similar to the debris observed in tissue treated with ribonuclease [14]. Thus, the induction of a powerful ribonuclease is clearly one possibility. The notion that neuronal death is an active process involving either the activation or new synthesis of a suicide enzyme has received considerable support from recent studies on the effect of protein synthesis inhibitors on induced embryonic cell death [29, 30].

Ontogenetic Factors

One of the most fascinating aspects of transneuronal atrophy and cell death is the wide variation across species and systems in the ontogenetic restrictions on such changes. That is, in most animals there is an ontogenetic period during which elimination of afferent input causes particularly severe cell loss and atrophy of postsynaptic elements. This period is often referred to as the 'sensitive period'. Little is known about the biological mechanisms underlying the sensitive period or the reasons why some animals and some

systems show clearly defined sensitive periods and others do not. In the chick auditory system, we have previously shown that the sensitive period extends beyond 6 weeks of age, well beyond the period of functional and structural maturation [11, 12]. At 1 year of age, cochlea removal has little or no effect on cell size or cell number. In the gerbil auditory system and the cat visual system, on the other hand, ablation of the receptor organ causes differential effects on postsynaptic cell size and neuron number as a function of age; there is a sensitive period for regulation of neuron number, but the neuron size is influenced by presynaptic integrity throughout life [31, 32]. Finally, in some systems, such as the olfactory system and barrel field of the rat forebrain, deprivation of afferent input appears to influence maturation only during a very sharply defined period early in postnatal development.

Unfortunately, the vast majority of studies have only examined the long-term consequences of deafferentation or deprivation. Thus it is impossible to ascertain whether the initial metabolic consequences of these manipulations are similar across species and neural systems. This question becomes particularly relevant when the results of the present study are considered along with those of Rubel et al. [7] and Hyde and Durham [33], who have shown that rapid increases in mitochondria are responsible for the survival of deafferented neurons. These results suggest the interesting hypothesis that differential vulnerability of neurons as a function of age or species may reflect differences in a neuron's ability to metabolically equilibrate its internal milieu following deafferentation rather than differences in the initial changes brought about by deafferentation or deprivation. To our knowledge this hypothesis has not been adequately evaluated.

Elucidation of the Mechanism of Transneuronal Cell Death May Provide an Opportunity to Reduce Injury Induced Neuronal Death

One important aspect of our study and of others which define why neurons die is that it may be possible to devise treatments to reduce cell death that would otherwise occur after brain injury. Clearly, transneuronal degeneration is a common consequence of lesions in developing animals. In addition, this study and others clearly indicate that this degeneration results from the loss of afferent activity. Because antidromic activation of the postsynaptic neuron cannot replace afferent activity [16], it must be the synaptic activation by the afferents which serves as the orthograde trophic signal. Given this, it may be possible to devise treatments which would replace this orthograde trophic signal. Because most neurons are sensitive to the loss of inputs only during a particular developmental period, even temporary measures may be sufficient to rescue neurons that would otherwise die after injury, thus contributing to the sparing of functions normally mediated by these circuits.

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