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Research report

Activity-dependent regulation of a ribosomal RNA epitope in the chick cochlear nucleus

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Abstract

Elimination of auditory nerve activity results in rapid metabolic changes, cell atrophy, and cell death in nucleus magnocellularis (NM), the cochlear nucleus of the chick. The transneuronal signals involved in the activity-dependent regulation of NM neurons are not well understood. One of the most rapid transneuronal effects is alteration in protein synthesis by NM neurons. Previous studies using an in vitro preparation of the brain stem auditory system suggested that up-regulation of protein synthesis in NM neurons requires the action of some trophic substance released by active auditory nerve fibers. Here, similar results were obtained when measuring changes in immunoreactivity using a monoclonal antibody (Y10B) that recognizes ribosomal RNA. This immunolabeling assay has advantages over the global protein synthesis assay in that it is not sensitive to possible changes in specific activity of the precursor pool or possible differences in the uptake of the labeled amino acids. Unilateral stimulation of the auditory nerve for 1 h resulted in greater immunolabeling of NM neurons on the stimulated side of the slice. This is consistent with previous in vivo results after unilateral deafferentation. Blockade of synaptic transmission by maintaining the slice in a low-Ca^{2+}/high Mg^{2+} medium prevented the stimulation-induced difference in immunolabeling. Electrical stimulation of the postsynaptic NM neurons alone (antidromic stimulation, via electrical stimulation of NM neuron axons) did not result in greater immunolabeling. Rather, antidromically stimulated neurons tended to show lighter labeling. Thus, the transneuronal regulation of ribosomes in NM neurons appears to require some substance released from the active auditory nerve. Further, blockade of protein synthesis using cycloheximide did not prevent the activity-dependent difference in immunolabeling. Thus, the activity-dependent regulation of ribosomes does not appear to require new protein synthesis during the period of differential activity.

Keywords: Cell death; Auditory system; Nucleus magnocellularis; Y10B antibody; Protein synthesis; Afferent deprivation; Brain slice

1. Introduction

Removal of afferent input often results in the atrophy or death of cells in the target structure [8,17,18,34]. In the central nervous system, transneuronal effects after manipulations of afferent activity have been observed in every sensory system thus far examined [4,10,15,16,25,29,42], particularly when these manipulations are performed in young animals. Although it is clear that afferent neurons can regulate the structure and survival of postsynaptic neurons, relatively little is known about the transneuronal signals involved in this regulation.

One model system for examining transneuronal effects after manipulations of afferent activity is the brain stem auditory system of the chick [25,38]. Neurons in the avian cochlear nucleus, nucleus magnocellularis (NM), receive their sole excitatory input from the ipsilateral auditory nerve [35]. If auditory nerve activity is eliminated, a portion of NM neurons degenerate and

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the surviving neurons decrease in size [2]. Alterations in the metabolic activities of NM neurons can be observed much earlier after removal of afferent activity. Of particular interest for the present report are rapid changes in protein synthesis [1,39]. Changes in protein synthesis can be observed as early as one hour after removal of auditory nerve activity. Within 3–6 h after the deafferentation, NM neurons appear to segregate randomly into two distinct populations: one population showing a complete cessation of protein synthesis and one population that continues to synthesize proteins, but at a reduced level compared to normally innervated neurons on the opposite side of the brain. The neurons that completely cease making proteins are the neurons that will eventually degenerate unless activity is restored within the first 12–24 h. Thus, rapid alterations in protein synthesis appear to be among he early events leading toward cell death in this system, although it does not mark a 'commitment' to cellular degeneration.

To examine the signals by which afferent activity regulates protein synthesis in NM neurons, Hyson and Rubel [21] used an in vitro slice preparation of the brain stem auditory system. NM neurons were activated on one side of the slice and the amount of protein synthesis by individual NM neurons was measured by autoradiographic analyses of [^{3}H]leucine incorporation. Orthodromic activation of NM neurons, via electrical stimulation of the auditory nerve, resulted in more protein synthesis by NM neurons on the stimulated side of the slice. Thus, just as is observed in vivo, electrical activity of the auditory nerve regulated the amount of protein synthesis in the postsynaptic NM neurons in the in vitro preparation. The greater level of protein synthesis produced by orthodromic stimulation appears to require synaptic release since the difference in protein synthesis was not observed if synaptic transmission was prevented by maintaining the slice in a medium containing low-Ca^{2+} and high-Mg^{2+} concentrations. To determine if electrical activity of the postsynaptic NM neuron alone was sufficient to upregulate protein synthesis, NM neurons on one side of the slice were antidromically activated by electrical stimulation of their axons. Although this resulted in the generation of action potentials that could be recorded at the soma, this form of stimulation did not result in greater protein synthesis in activated neurons. Unexpectedly, antidromic stimulation, if anything, decreased the level of protein synthesis in stimulated neurons. Together, these results suggest that transneuronal regulation of protein synthesis in NM neurons requires the calcium-dependent release of some trophic molecule(s) from the presynaptic auditory nerve and activation of postsynaptic receptors.

Although protein synthesis provides a good assay for examining transneuronal regulation of cellular metabolism, the measurement of overall protein synthesis using [^{3}H]leucine incorporation is somewhat non-specific. A change in the incorporation of amino acids does not identify what aspect of the chain of events leading to protein synthesis is disrupted. In general, changes in protein synthesis could result from changes in the rate of transcription of the genetic code into mRNA, or from changes in translation of message into protein. In support of the latter hypothesis, electron microscopic analyses of NM neurons after deafferentation revealed that the neurons that cease to make proteins also show a marked dissociation of polyribosomes [37]. This destruction of the major protein synthetic machinery of the cell would likely account for the cessation of protein synthesis observed in these neurons.

Recently, a method has been developed by which to investigate ribosomal function at the light microscopic level. This method makes use of a monoclonal antibody, Y10B, which binds to ribosomal RNA [24]. Garden et al. [13] examined changes in Y10B immunolabeling after eliminating auditory nerve activity in the chick. Their results parallel the changes observed when examining protein synthesis after deafferentation. Specifically, within 3 h after silencing the auditory nerve, immunoreactivity was reduced in NM neurons on the deafferented side of the brain. Within 6–12 h after deafferentation, neurons in the deafferented NM could be divided into two distinct populations based on the level of immunoreactivity.

The present series of experiments begin to examine the signals by which afferent activity regulates ribosomal function. As a first step, the types of potential signals can be divided into 4 general categories: (1) action of a trophic substance released from active presynaptic terminals; (2) the action of some trophic substance or ionic change resulting from active afferents, but not requiring synaptic release; (3) events associated with the generation of action potentials in the postsynaptic neuron; or (4) recurrent processes subsequent to activation of the postsynaptic neuron. These possibilities were dissociated by examining Y10B immunolabeling after stimulating NM neurons in a slice preparation of the chick brain stem. Our results show that regulation of ribosomal structure, as measured by Y10B immunolabeling, follows the same trends as previously observed when assaying ribosomal function (protein synthesis). In both cases, it appears that transneuronal regulation requires the activity-dependent release of some trophic substance from the auditory nerve. Further, blockade of protein synthesis using cycloheximide did not prevent the activity-dependent difference in immunolabeling. This indicates that activity-dependent regulation of ribosomes does not require new protein synthesis during the period of differential activity.

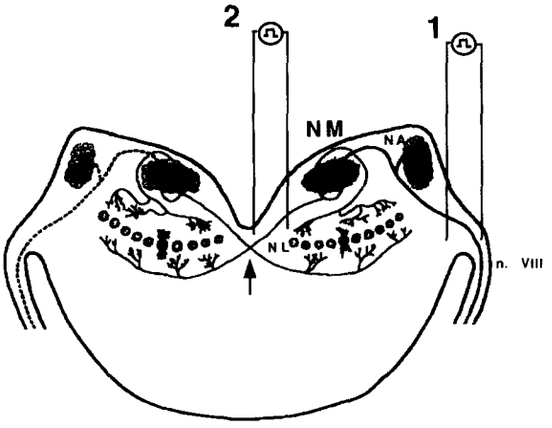

Fig. 1. Schematic of the brainstem auditory system of the chick depicting the stimulation conditions. Nucleus magnocellularis (NM) neurons on one side of the brain slice were stimulated either orthodromically (1), via stimulation of the auditory nerve (n. VIII), or antidromically (2), via stimulation of their axons as they approach midline in the crossed dorsal cochlear tract. The axons were severed at midline (arrow) to restrict antidromic activation to NM neurons on only one side of the slice. To control for the possible effects of axotomy, these axons were also severed in a portion of the slices receiving orthodromic stimulation. The effectiveness of stimulation was periodically monitored by recording field potentials in NM.

2. Materials and methods

2.1. Brain slice preparation

The procedure for obtaining and maintaining slices of the brain stem auditory system has been fully described previously [21]. All slices were obtained from 7–14-day posthatch chicks that were hatched and reared at the University of Washington (White Leghorn) or at the Florida State University (Ross × Ross). Chicks were decapitated, the brainstem was removed from the cranium and a 300 μm coronal slice was cut using a vibratome. Dissection and slicing were performed while the brainstem was immersed in oxygenated artificial cerebrospinal fluid (ACSF). After the slice was cut it was transferred to a recording chamber where it was superfused with warmed (34°C), oxygenated ACSF. Standard ACSF consisted of (in mM): NaCl 130, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, D-glucose 10. For low-calcium/high-magnesium experiments the ACSF contained 0.5 mM $CaCl_2$ and 5 mM $MgCl_2$ with other concentrations remaining the same as the standard solution. For the experiments investigating the effects of protein synthesis blockade, the standard ACSF contained 100 mg/l cycloheximide.

2.2. Stimulation treatments

The different types of treatments are outlined in Fig. 1. Different groups of slices received either unilateral orthodromic stimulation or unilateral antidromic stimulation of NM using a bipolar stimulating electrode constructed from Teflon-coated silver wire. Continuous stimulation was provided for one hour at a rate of 10 Hz using 50 V, 20 μs pulses. For orthodromic stimulation of NM, the stimulating electrode was placed on the auditory nerve of one side of the slice. For antidromic stimulation, the stimulating electrode was placed on the axons of NM neurons as they approached midline in the crossed dorsal cochlear tract. To restrict antidromic activation to NM on only one side of the slice, a cut was made in the crossed dorsal cochlear tract at midline and the stimulating electrode was placed on one side of the cut. To assure that differences between the results obtained after orthodromic stimulation as compared to antidromic stimulation were not attributed to the severing of NM fibers, an identical (control) cut was made at midline for a portion of the orthodromically stimulated slices. The severing of NM axons had no reliable effect on the results obtained using orthodromic stimulation.

The effectiveness of stimulation was periodically monitored in each slice using a glass microelectrode filled with ACSF. Previous experiments have shown that the orthodromic and antidromic stimulation procedures are equally effective at driving NM neurons [21].

2.3. Anatomical procedures

After stimulation for one hour, the slice was fixed by immersion in 4% paraformaldehyde or a modified Carnoy's solution containing Ringer's solution (154 mM NaCl, 6 mM KCl, 8.4 mM $MgCl_2$, 5 mM HEPES, 8 mM glucose). Some fixed slices were immersed in 30% sucrose in phosphate buffered saline (PBS) before freezing and sectioning using a cryostat. Other slices were dehydrated and embedded in paraffin. Sections were cut at 10 μm and mounted on chrome–alum subbed slides. Endogenous peroxidase activity was quenched by treating the tissue with 0.03% H_2O_2 in absolute methanol for 20 min. After rinsing with PBS, sections were preincubated in 4% normal horse serum for 1 h and then incubated in 1:5000 Y10B primary antiserum overnight. Following washing with PBS, the sections were incubated in 1:200 biotinylated horse anti-mouse secondary antibody for 2 h. The sections were then washed and incubated in an avidin-biotin complex (Vectastain ABC Kit, Vector Labs, Burlingame, CA) and reacted using 0.25 mg/ml diaminobenzidine and 0.1% H_2O_2 in Tris buffer (pH 7.6). All immunoreagents were diluted in 1% bovine serum albumin in PBS.

2.4. Data analysis

Immunolabeled sections were analyzed using a digital image analysis system (Bioquant, R&M Biometrics). The video image of the tissue observed under a 40× objective was digitized and displayed on a computer monitor. First, the light and contrast setting were adjusted to normalize the distribution of gray levels in the digitized image. Once these settings were adjusted, they remained constant for all measurements of a given section. The average gray level of individual NM neurons was measured for 20–40 neurons on either side of the same tissue section and 1–3 sections were measured from each slice. These densitometric measurements were used to compare the intensity of immunolabeling for the NM neurons on the stimulated and unstimulated side of the same slice and section.

2.5. Protein synthesis

To determine the role of new protein synthesis in the activity-dependent regulation of immunolabeling, cycloheximide was used to block protein synthesis during the stimulation period. The effectiveness of the protein synthesis blockade was evaluated by measuring incorporation of [^{3}H]leucine. Slices were incubated for 1 h in 0.1 mCi [^{3}H]leucine/25 ml ACSF either with or without 100 mg/l cycloheximide. After the incubation period, the whole slice was homogenized in 200 μl of 0.05 M phosphate buffer containing 0.75% desoxycholate (pH 7.1). The homogenate was centrifuged for 10 min at 12,500 rpm. [^{3}H]leucine and protein content were measured on different aliquots of the supernatant. [^{3}H]Leucine content was measured by scintillation counting of a TCA precipitant of the aliquot (25 or 50 μl of supernatant). Protein was precipitated in 500 μl of 5% TCA, and the sample was centrifuged (10 min at 12,500 rpm). The pellet was resuspended in TCA and centrifuged once more. The resulting pellet was then dissolved in 500 μl 5% NaOH, and a 100 μl sample was added to scintillation fluid and counted. To measure

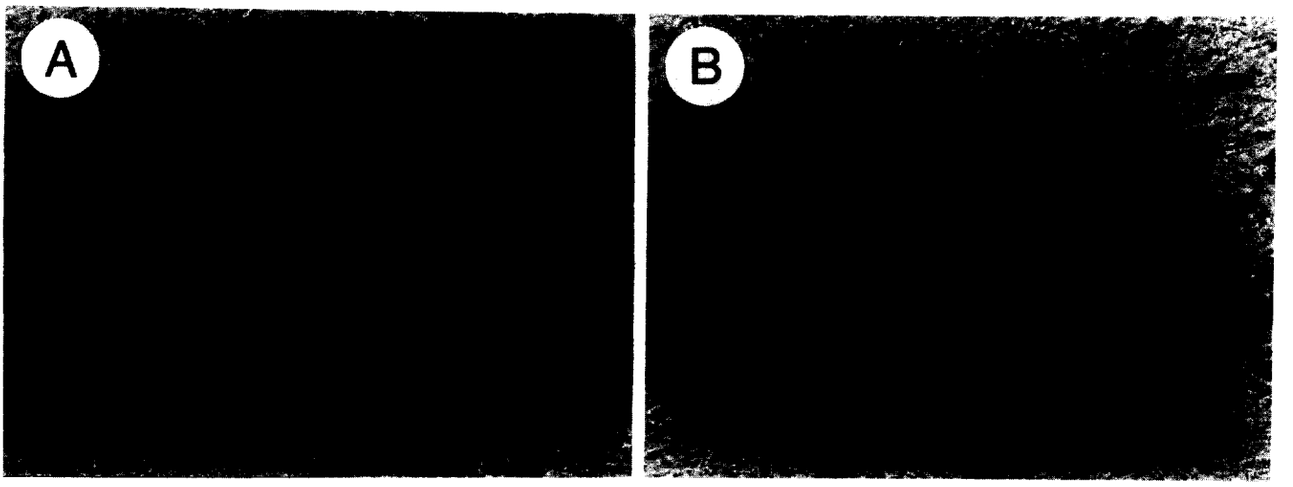

Fig. 2. Representative micrographs of neurons in NM immunolabeled using a monoclonal antibody (Y10B) that recognizes ribosomal RNA. This slice received unilateral stimulation of the auditory nerve. NM neurons on the stimulated side of the section (A) show darker cytoplasmic labeling than those on the unstimulated side of the same section (B). Scale bar = 20 μm.

protein content a 60 μl aliquot of supernatant was diluted 1:10 in 0.1 M HEPES buffer. Protein content of each slice was measured in triplicate on 10, 15 and 20 μl samples of the supernatant/buffer using a Bio-Rad protein assay kit according to manufacturer's instructions. Dilutions of bovine plasma γ-globulin (0–40 μg) were used for standards. Normalizing the [^{3}H]leucine counts to the amount of protein showed that, by this assay, cycloheximide reduced protein synthesis up to 85% over the 1 h incubation period ($\bar{x}$ = 75%, n = 3). Previous autoradiographic analysis of the effects of the same dose of cycloheximide on leucine incorporation showed a 95% reduction in synthesis during the last 0.5 h of a 1.5 h incubation period [19].

3. Results

3.1. Orthodromic stimulation

Unilateral orthodromic stimulation resulted in greater Y10B immunolabeling in NM neurons on the stimulated side of the slice. An example of this effect can be seen by comparing panels A and B of Fig. 2. Objective analysis of the intensity of immunolabeling using the video image analysis system confirmed these impressions. In all 6 slices receiving orthodromic stimulation, the average intensity of labeling (gray level) of NM neurons on the stimulated side of the slice was significantly greater (darker) than the intensity of labeling of neurons on the unstimulated side of the same section (t-tests; $P < 0.05$). Fig. 3A shows a representative distribution of immunolabeling intensity (gray level) of neurons on opposite sides of the same tissue section. Gray level data from individual sections were transformed into z-scores based on the mean and standard deviation of the gray level scores for neurons on the unstimulated side of the same slice [(gray level of cell − average gray level of unstimulated cells)/standard deviation of gray levels of unstimulated cells]. This was necessary because absolute gray levels can be quite variable between sections and the average difference in gray level between the two sides can be influenced by the light level and contrast settings of the camera and imaging system. Fig. 3B shows the distribution of z-

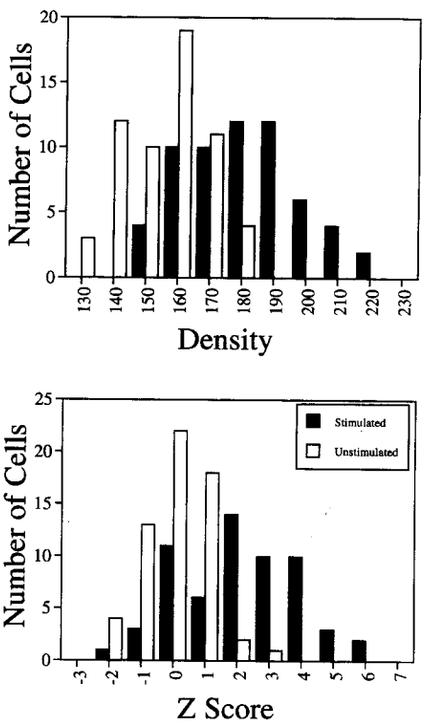

Fig. 3. Distributions of gray scale densities of Y10B immunolabeled NM neurons after unilateral orthodromic stimulation. A: distributions of average gray scale values of individual neurons show that the stimulated neurons are more darkly labeled than unstimulated neurons on the opposite side of the same tissue section. B: distributions of the same data transposed into z-scores based on the mean and standard deviation of the distribution of gray levels of the unstimulated neurons.

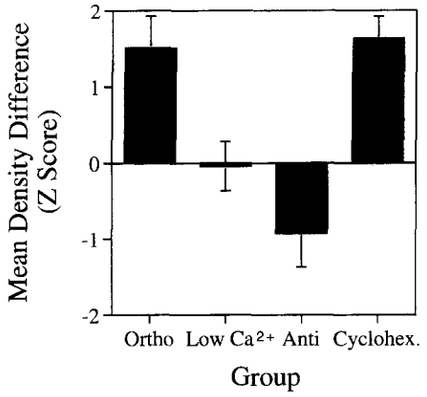

Fig. 4. Average z-score of stimulated neurons for slices receiving either unilateral orthodromic stimulation (Ortho), unilateral orthodromic stimulation while maintaining the slice in a low-Ca^{2+}/high-Mg^{2+} medium (Low-Ca^{2+}), unilateral antidromic stimulation (Anti), or unilateral orthodromic stimulation with the protein synthesis inhibitory, cycloheximide, added to the medium (Cyclohex). Positive numbers indicate that stimulated neurons were more darkly labeled than unstimulated neurons. Bars represent standard error of the mean.

scores for the raw data shown in Fig. 3A. By definition, the z-score distribution for cells on the unstimulated side has a mean of zero and a standard deviation of 1. The distribution of z-scores for the cells on the stimulated side of this section maintains the same general shape as the distribution of raw scores. In this case, the average z-score for cells on the stimulated side is 2.1. That is, the average gray level of cells on the stimulated side of the slice was 2.1 standard deviations above the mean gray level of cells on the unstimulated side of the slice. For statistical analyses, the average z-scores of individual sections were pooled to produce an average z-score for each slice and each slice's average z-score was considered as one data point in the group. The average z-scores for each group are presented in Fig. 4. For slices that received unilateral orthodromic stimulation, the average z-score was significantly greater than zero (t-test, $P < 0.05$), indicating that staining density is reliably higher on the stimulated side of these slices.

3.2. Orthodromic stimulation in low-calcium / high-magnesium medium

When slices were maintained in a medium having low-Ca^{2+} and high-Mg^{2+} concentrations, synaptic transmission from the stimulated auditory nerve to NM was blocked (see also [21]). This treatment also prevented the difference in immunolabeling between NM neurons on the stimulated and unstimulated sides of the slice. Analysis using the video imaging system revealed that none of these slices ($n = 3$) showed significantly greater labeling of NM neurons on the stimulated side of the slice and the mean z-score of −0.04 did not differ from zero ($P > 0.9$).

3.3. Antidromic stimulation

Stimulation of the NM neurons' axons results in antidromically driven action potentials that can be recorded at the soma (see also [21]). In contrast to the effects of orthodromic stimulation, antidromic stimulation did *not* result in greater immunolabeling of the stimulated neurons. If anything, antidromic stimulation resulted in *lighter* immunolabeling of the stimulated neurons compared to unstimulated neurons on the opposite side of the same tissue section. Densitometric analyses revealed that the stimulated NM neurons were significantly lighter than the unstimulated neurons in 3 of the 4 slices (t-tests, $P < 0.05$). Analysis of the z-scores, however, revealed that the mean score of −0.92 for this group was not reliably lower than zero ($P > 0.4$).

3.4. Between-group comparisons

The z-scores were also used for making between group comparisons of the effects of the various stimulation conditions. A one-way analysis of variance on these scores showed a reliable effect of Group ($F_{2,10} = 8.9$, $P < 0.01$). Post-hoc pairwise comparisons (Fisher's PLSD, $P < 0.05$) revealed that the orthodromically stimulated group had a higher z-score than either the low-Ca^{2+}/high-Mg^{2+} group or the antidromic stimulation group, which did not reliably differ from each other.

3.5. Protein synthesis blockade

To determine if the effects of unilateral orthodromic stimulation on immunolabeling requires protein synthesis during the stimulation period, slices ($n = 4$) were unilaterally stimulated for 1 h in ACSF containing the protein synthesis inhibitor, cycloheximide. Stimulated NM neurons still showed greater immunoreactivity in these slices (see Fig. 4). The mean z-score of 1.628 for stimulated neurons in these slices was statistically reliable (t-test, $P < 0.05$). The z-scores for the cycloheximide treated group did not differ reliably from the group receiving orthodromic stimulation in normal ACSF (t-test, $P > 0.8$).

4. Discussion

4.1. Requirements for afferent regulation of ribosomes

Antigenicity for Y10B was higher in NM neurons that were stimulated for 1 h via electrical activation of

their afferents in the auditory nerve. This finding agrees with the in vivo results reported by Garden et al. [13]. In their experiments, activity of one auditory nerve was eliminated either by removal of the cochlea or by injection of TTX into the inner ear. In both cases, Y10B immunolabeling was higher on the active side of the brain. Our results also agree in that the difference in antigenicity does not appear to require new protein synthesis during the period of differential activity.

In vitro, one can begin to dissect out the important features for the activity-dependent regulation of the postsynaptic neuron. When driven orthodromically: (1) the presynaptic fiber is active; (2) substances are released from the presynaptic terminal; (3) the postsynaptic neuron is activated; and (4) the postsynaptic neuron then relays activity to other neurons, which could potentially have recurrent influences on the cell, either through recurrent connections or the release of trophic molecules. Any of these events, either alone or in combination, could potentially influence ribosomal activity within the postsynaptic neuron.

In the present experiments, when the auditory nerve was stimulated but synaptic transmission was prevented by maintaining the slice in a low-Ca^{2+}/high-Mg^{2+} medium, there was no difference in the degree of immunolabeling between the stimulated and unstimulated sides of the slice. This suggests that changes in the ionic milieu as a result of presynaptic nerve action potentials is not sufficient for the regulation of ribosomes in the NM neuron or the Ca^{2+} influx from the extracellular environment is necessary for the maintenance of Y10B staining. Electrical activation of the postsynaptic neuron in the absence of synaptic transmission (antidromic stimulation) also was not sufficient to up-regulate antigenicity for Y10B. Thus the events associated with action potentials in the postsynaptic cell and any possible recurrent effects as a result of the propagation of those action potentials are also not sufficient for the regulation of ribosomes in these neurons. Together, these results suggest that the regulation of ribosomal function requires the action of some substance released from active presynaptic terminals.

The trends of these results are identical to those obtained when the assay for afferent regulation was a measure of overall protein synthesis. Hyson and Rubel [21] measured incorporation of [^{3}H]leucine by NM neurons using the identical stimulation procedures as used in this report. In that study, orthodromic stimulation resulted in greater protein synthesis in stimulated neurons and this effect was blocked by maintaining the slice in a low-Ca^{2+}/high-Mg^{2+} medium. Additionally, antidromic stimulation, if anything, resulted in less protein synthesis by stimulated neurons.

The incorporation of leucine into protein is a measure of ribosomal function whereas Y10B immunolabeling can be considered a measure of ribosomal structure. The change in immunolabeling indicates a change in the binding epitope. Without afferent stimulation this epitope is either no longer present, it is blocked by some other molecule, or it has undergone some conformational change. Regardless of the cause for the change in antigenicity, the parallel results of protein synthesis and Y10B binding suggest that the change indicated by Y10B binding is of functional significance. A decreased level of Y10B binding appears to correspond to a lower capacity of the ribosome to synthesize protein.

The parallel change in protein synthesis and Y10B binding is also important in that it suggests that Y10B immunolabeling is an appropriate assay for examining the signals that may be necessary for afferent regulation of neuronal metabolism. This immunolabeling assay has advantages over the global protein synthesis assay in that it is not sensitive to possible changes in specific activity of the precursor pool or possible differences in the uptake of the labeled amino acids.

4.2. Alterations in ribosomal structure and function

In the present experiments, the assay for examining the effects of afferent regulation of postsynaptic neurons was an alteration in antigenicity using a ribosome-specific antibody. This assay was chosen because of the critical role of ribosomal integrity in the function and survival of cells and because one of the earliest changes in NM neurons after removing afferent activity is a change in protein synthesis. The cause of the changes in protein synthesis is presumably due to a disruption of ribosomes based on the observed rapid changes in immunolabeling with the Y10B antibody [13] and EM analyses showing polyribosome dissociation within a few hours after deafferentation [37]. Examination of changes in ribosomal integrity is particularly important because the destruction of ribosomes appears to be an early step in the sequence of cellular events leading toward the cell death which will occur in a portion of NM neurons after a prolonged period of deafferentation. In addition to cell death in NM neurons after deafferentation, destruction of polyribosomes is also observed in the early stages of cell death induced by transient cerebral ischemia [9,40] and excess glutamate [11] in neurons, deprivation of EGF in cultured embryonic cells [36], and light-induced damage of rod photoreceptors [30]. The chain of events leading toward the destruction of the ribosome and eventual cell death is not fully understood in any of these instances.

The onset of changes in the ribosomes after deafferentation is quite rapid. Within 1–3 h after deafferentation, a dramatic reduction in protein synthesis [39] and a corresponding loss in Y10B antigenicity [13] is observed. Comparison of the time course of changes

after deafferentation shows that changes in the ribosome are apparent by Y10B immunolabeling [13] well before obvious changes in the polyribosomes are observed by EM analyses of the tissue [37]. Additionally, recent evidence has shown the activity-dependent alterations in Y10B antigenicity are not observed if the tissue is treated with a protease prior to labeling [14]. This suggests that ribosomes have not been rapidly destroyed, but rather, a protein has either blocked the epitope or bound to the ribosome so as to change the conformation of the epitope. Since the change in Y10B antigenicity is still observed when new protein synthesis is blocked, the protein involved in changing the ribosomal epitope must already be present in the neuron and is not newly created as part of a 'suicide' sequence.

4.3. Possible signals for afferent regulation of neuronal metabolism

When muscle fibers are denervated, they undergo transynaptic changes in the acetylcholine receptor system, sodium conductance mechanisms and resting membrane potential [17,28]. These changes in the muscle, however, can be reversed or prevented by electrical stimulation of the denervated muscle [26]. In contrast, electrical activity of NM neurons does not counteract the effects of deafferentation. Rather, these neurons appear to be dependent on some trophic substance released from active auditory nerve fibers. The nature of this trophic substance is unknown, but the known neurotransmitter systems in NM provide some obvious potential candidates.

One possible trophic substance is the neurotransmitter released from the auditory nerve terminal. This neurotransmitter is known to be an excitatory amino acid [22,27,31,32,43,44]. Preliminary studies have shown that complete blockade of excitatory amino acid receptors with a high concentration of kynurenic acid prevents the activity-dependent regulation of [^{3}H]leucine incorporation in NM neurons [20], suggesting that excitatory amino acids play a role in this form of transneuronal regulation. Both physiological [27] and receptor binding data (Hyson, unpublished observations) suggest that both NMDA and non-NMDA types of excitatory amino acid receptors are present in NM. In addition, biochemical assays have shown that a type of metabotropic receptor is also present in this nucleus [45]. Glutamate stimulation of the metabotropic type of receptor results in activation of the phosphoinositide second messenger cascade. Any of these receptor subtypes could play a role in the regulation of ribosomal activity either through activation of a second messenger and/or through the influx of Ca^{2+}.

A second neurotransmitter system known to be present in NM is GABA. GABAergic terminals [6] and $GABA_A$ receptors [7] are present in NM, but the physiological effects of GABA appear to have an unusual phenotype [19]. Activation of these GABA receptors depolarizes the neuron and this action appears to be insensitive to the $GABA_A$ antagonist, bicuculline, and is not mimicked by the $GABA_B$ agonist, baclofen. Activation of GABA receptors on the NM neuron could result in activation of some second messenger system or, since the response is depolarizing, it could result in an influx of Ca^{2+}. The GABAergic input into NM arises from neurons located in the superior olive [23] and from scattered neurons located between NM and NL [41], but the circuit by which the GABAergic neurons are activated is not known. If the GABAergic input is somehow activated after stimulating the auditory nerve, then GABA could play a role in the afferent regulation of the NM neuron. However, if this is the case, then either these GABAergic inputs are not activated when NM neurons are antidromically stimulated, or some other signal from the active auditory nerve is also required for transneuronal regulation of the NM neuron, since antidromic stimulation is not sufficient for the up-regulation of either Y10B antigenicity or protein synthesis.

One prominent event in the regulation process could be an alteration in Ca^{2+} levels inside the NM neuron. Changes in Ca^{2+} levels could result from activation of either the glutamate or the depolarizing GABAergic receptors. A role of Ca^{2+} has been postulated for various types of neuronal cell death [33]. For example, excess Ca^{2+} is toxic to the cell and may be an important factor in cell death resulting from excess glutamate [5]. In cases such as deafferentation, however, where it appears that electrical stimulation is necessary to support survival of the cell, it is the elimination, not excessive stimulation, of the glutamatergic input that results in cell death. Similarly, for neurons maintained in cell culture, survival can be *promoted* by chronic depolarization [12]. Franklin and Johnson [12] have suggested that this survival-enhancing effect of depolarization is the result of a sustained Ca^{2+} influx and they have postulated a set-point hypothesis for the role of Ca^{2+} in neuronal survival. According to this hypothesis, cell death will result if Ca^{2+} levels are either too low or too high. In a midrange of Ca^{2+} levels, they suggest that the neuron may also need some other form of trophic support to survive, but they also postulate that there is a range of Ca^{2+} levels that can promote survival in the absence of other trophic influences.

Within this framework, if the transneuronal regulation of ribosomes in NM is solely dependent on Ca^{2+} levels, then orthodromic and antidromic stimulation must have different effects on intracellular Ca^{2+} concentrations. Calcium imaging data suggest the antidromic stimulation does, in fact, result in higher

intracellular Ca^{2+} levels in NM neurons than is observed with orthodromic stimulation (Zirpel and Rubel, unpublished observations). This might possibly lead to the adverse effects on ribosomal integrity that are observed with antidromic stimulation. Afferent input not only prevents the destruction of ribosomes which occurs as a result of denervation, but also overcomes or prevents the apparent detrimental effect of postsynaptic activity alone. If this is accomplished solely through the regulation of Ca^{2+} levels, then some factor associated with orthodromic stimulation must keep the Ca^{2+} levels from rising to the high levels produced by the generation of action potentials. This could be possible through the known influence of afferent activity on calcium binding proteins, such as calretinin [3], or perhaps by transneuronal activation of a second messenger system [45] which interacts with some calcium homeostatic mechanism. Alternatively, it is possible that a different form of trophic support is provided when the auditory nerve is stimulated, and the increase in Ca^{2+} levels after antidromic stimulation is merely a byproduct of some degenerative process.

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