Afferent Regulation of Neurons in the Brain Stem Auditory System

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INTRODUCTION

Structural and functional reorganization of neural circuits involves the back-and-forth communication between presynaptic and postsynaptic elements. Presumably, in a stable state each element is being regulated through both intracellular regulatory machinery and by the interaction of these processes with the extracellular environment. Various investigators have elegantly demonstrated pieces of intracellular regulatory machinery and, in some cases, the actual kinetics involved. For example, the energy metabolism of a cell is maintained by a dynamic system of intracellular feedback loops, and the intracellular machinery involved in protein synthesis has been described in detail. Intercellular regulatory systems appear less well understood, but the literature is replete with examples of their importance. For example, it is well appreciated that the levels of circulating steroids, particularly during development, have marked influences on neuronal and musculoskeletal systems as well as on the reproductive system (Beyer and Feder, 1987; Funder and Sheppard, 1987). Other substances circulating in the body fluids, as well as those produced and excreted by local elements (e.g. growth factors or extracellular matrix molecules), play equally important roles in establishing and maintaining the dynamic equilibrium between neural elements and their targets (Berg, 1984; Edelman, 1984).

One of the greatest challenges facing modern neurobiology is to understand these *intercellular* regulatory events. How is a "stable state" maintained; what changes in one element are necessary and/or sufficient to bring about changes in other elements; what is the cellular chain of events and what are the kinetics of any intercellular regulatory circuit? While the literature is full of examples of normal developmental or experimentally induced changes in neurons or neuronal circuits, we have relatively few answers to the above questions.

The research program summarized in this review represents an attempt to begin answering these questions for one type of intercellular communication, the interaction of presynaptic excitatory afferents with postsynaptic neuronal elements (either the soma or individual dendritic trees). The intercellular events underlying afferent regulation of postsynaptic elements, although just one class of the myriad of cellular interactions, are of obvious interest regarding how an organism's external environment influences the nervous system. That is, through intermediate elements (receptors and their associated structures) the environment must exert chronic influences on neural structure and function through changes in the pattern or amount of activity in particular neuronal circuits. We have hoped that by studying the neuronal events surrounding changes in the integrity or pattern of activity of excitatory afferents in one relatively simple system we can further understand this class of interactions.

For these investigations we have chosen to study the brain stem auditory pathways, primarily in the chick but, more recently, in the gerbil as well. These preparations are chosen because of their relative simplicity, the ability to isolate our manipulations to a single type of excitatory afferent on a given postsynaptic surface, and the possibilities for direct manipulation of the integrity and activity of excitatory afferents in a variety of ways. While most investigations of afferent transneuronal regulation have concentrated on the long-term effects (weeks or months), we have concentrated on the short-term changes (minutes, hours, or days). Long-term effects demonstrate the capacity

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of the nervous system for alteration of the "final product." On the other hand, it is our feeling that detailed investigations of the short-term events preceding permanent structural change is the only way to understand the cellular dynamics of interneuronal regulation.

Finally, since most of this volume is devoted to the topic of "competitive interactions" between neurons we should indicate how our research relates to this topic. Guillery (1981) critically reviews the concept of competition. While this essay is now almost 10 years old, most of his points are still relevant. First, he points out that competition between two separate populations of nerve fibers (Type II competition; Guillery, 1981) is usually defined by the outcome of an experimental manipulation which, in some way, is thought to "weaken" one population. This in itself, points out Guillery, is not sufficient evidence to conclude that competition plays a role in normal development. Second, Guillery notes that it is often difficult to distinguish between competition and the ontogeny of site-specific markers as the mechanism for axonal sorting. Finally, underlying such confusion is the lack of consensus about the definition of the term "competition" and our failure to understand the underlying cellular and molecular events.

The pathways we are examining provide two excellent examples of situations in which deafferentation experiments alone might suggest the occurrence of competitive interactions, but where careful examination of normal development reveals no evidence of competition nor a need to invoke such a concept. The first example involves the innervation of the cochlear nuclei of the chick. Normally, nucleus magnocellularis (NM) receives its sole excitatory input from the ipsilateral auditory nerve (see below). Jackson and Parks (1988) removed one otocyst on embryonic day 3, thereby preventing the formation of the auditory nerve. This deafferentation resulted in a massive ectopic projection from the normal NM to the deafferented contralateral NM by embryonic day 11, which persisted through hatching. This projection might suggest that the auditory nerve and contralateral NM fibers *compete* during development for innervation of these neurons. Young and Rubel (1986), however, have shown that early in the ontogeny of the chick brain stem there is a very minute connection between the cochlear nuclei; only about 1 in 100 fibers from magnocellularis on one side of the brain send a collateral to nucleus magnocellularis on the opposite side. These collaterals are reabsorbed or degenerate prior to embryonic day 14. Thus it is unlikely that a truly competitive interaction takes place during normal ontogeny.

The second example of how the concept of competition may be inappropriately applied is in the development of the projection from NM to nucleus laminaris (NL) of the chick. The normal projection from NM to the ipsilateral and contralateral NL is exquisitely segregated onto separate dendritic surfaces (see below). When one cochlea is removed in young hatchling chickens, however, an ectopic projection to the "wrong" dendritic tree can be observed (Rubel, Smith, and Steward, 1981). This might suggest that the ipsilateral and contralateral inputs somehow *compete* for membrane surface area on the different dendrites. Careful analysis of the development of this projection, however, shows that the segregation of innervation is apparent throughout normal ontogenesis (Young and Rubel, 1986). Thus, as in the above example, observation of the experimental data may have invoked competition as a process guiding normal ontogeny, but careful analysis of normal development reveals no evidence for such a process.

To a great extent the work described in this review largely avoids the issues brought forth by Guillery because we have chosen to manipulate afferents that are highly segregated at the time of the manipulation. It is only in this situation that the response of the postsynaptic element to elimination or "weakening" of its afferents can be studied independent of other, potentially competitive, afferents. Since most competitive interactions are defined on the basis of responses to deafferentation, it is of some interest to understand the metabolic interactions between presynaptic and postsynaptic elements in the absence of competition. For example, we might not expect competition between afferents for postsynaptic space if elimination of one afferent causes rapid reregulation of membrane surface area.

The remainder of this paper is divided into two sections. First, we discuss and provide examples of the time course of postsynaptic events following deafferentation. In the second section we review experiments attempting to examine the nature of the presynaptic signal that is regulating these postsynaptic events and experiments beginning to look at the mechanisms involved at the interface between presynaptic and postsynaptic elements. Although some new data are presented (e.g., regulation of cytoskeletal proteins and subcellular organelles), most of the information is summarized from previous publications, where detailed descriptions of the methods and database can be found. More detail on the normal ontogeny and

effects of manipulations early in ontogenesis can be found in Rubel and Parks (1988).

CELLULAR RESPONSE TO AFFERENT MANIPULATION

Figure 1 is a schematic diagram of the auditory neurons in the chick brain stem. In avian species the basilar papilla (cochlea) is a relatively flat membrane lying within the cochlear duct. Hair cells are innervated by the peripheral processes of eighth nerve ganglion cells, whose cell bodies also lie within the cochlear duct. The central processes of the eighth nerve ganglion cells enter the brain stem and bifurcate. One branch innervates neurons in nucleus angularis (NA) the avian homologue of the dorsal and posteroventral cochlear nuclei. The other central branch synapses with large, calyx-like endings (the end bulbs of Held) on neurons in nucleus magnocellularis (NM). NM neurons are homologous to the spherical cells in the mammalian anteroventral cochlear nucleus (AVCN). Each NM neuron, which has few if any dendrites, receives two or three end bulbs, which cover about two thirds of the surface of NM somata (Hackett, Jackson, and Rubel, 1982; Parks, 1981). The eighth nerve input provides the only excitatory input to NM neurons and is strictly ipsilateral. A second type of synaptic ending is seen on NM neurons, small boutons that are GABAergic (Code, Burd, and Rubel, 1989) and thus probably inhibitory in nature. Axons from NM neurons bifurcate and project bilaterally to third-order neurons in nucleus laminaris (NL). The dendrites of NL neurons are spatially segregated into dorsal and ventral domains, and input from NM neurons likewise is segregated. One branch of each NM axon innervates the dorsal dendrites of the ipsilateral NL neurons, and the other branch crosses the midline in the crossed dorsal cochlear tract (XDCT) to innervate the ventral dendrites of the contralateral NL neurons. NM axons provide the exclusive excitatory input to the NL soma and dendrites.

The sources of excitatory input to both NM and NL are relatively easily manipulated. All excitatory input to NM can be eliminated by cochlea removal, which severs the peripheral processes of the eighth nerve ganglion cells but leaves their cell bodies intact. These cell bodies and their central processes remain intact for at least 12 hours and then begin to degenerate. Following cochlea removal, action potentials recorded in NM cease within minutes, and eventual degeneration of end bulbs of Held results. Excitatory input to NL can

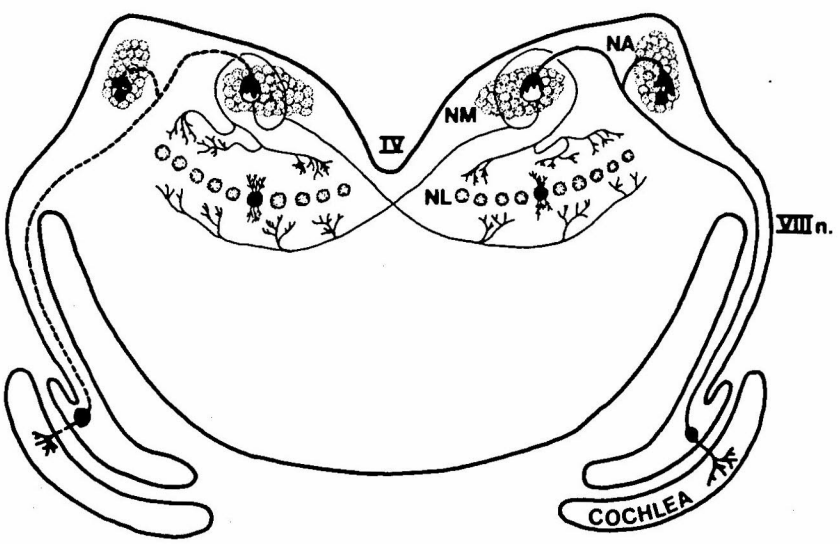

Figure 1 Schematic diagram showing the organization of the chick brain stem auditory nuclei. The basilar papilla (cochlea) is innervated by the peripheral processes of eighth nerve ganglion cells. The central processes (VIII n.) bifurcate and synapse in the second-order nucleus magnocellularis (NM) and nucleus angularis (NA). Axons from NM bifurcate and project bilaterally to third-order neurons in nucleus laminaris (NL). NL neurons are arranged in a monolayer sheet and possess dendrites spatially segregated into domains dorsal and ventral to the cell body lamina. The projection from NM is also segregated; axons from the ipsilateral NM terminate on dorsal NL dendrites and cell bodies, and axons from the contralateral NM terminate on ventral NL dendrites and somata. Abbreviation: IV, fourth ventricle. From Rubel and Parks (1988). Reprinted by permission of John Wiley and Sons.

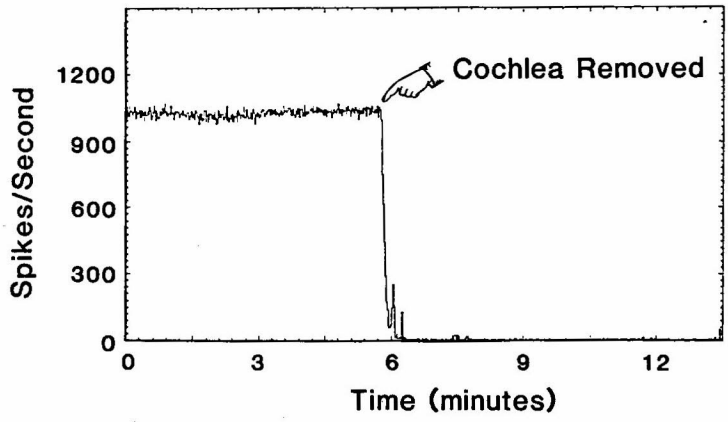

Figure 2 Time course of changes in neuronal discharges recorded in nucleus magnocellularis during cochlea removal. Extracellular recordings were made with a tungsten microelectrode. Spikes were counted by setting the level of a pulse height discriminator at a level twice that found with the electrode just above the brain stem. The spike rate calculated by computer from the output of the pulse height discriminator is plotted as a function of time. There is a steady, high level of activity recorded in nucleus magnocellularis even when no specific acoustic stimulus is presented. Immediately following cochlea removal, the spike rate precipitously falls such that within 15–30 sec no more discharges are recorded. No change in the level of activity was found for up to 6 h after cochlea removal. From Born (1986).

be *partially* eliminated by removing one cochlea or by severing the XDCT (tract cut). The latter manipulation totally denervates only the ventral dendritic regions of NL neurons. Cell death of some neurons in NM following cochlea removal produces partial denervation of specific dendritic regions in both ipsilateral and contralateral NL. Both of these manipulations offer the opportunity to examine the effects of specific afferent manipulation on a well-defined group of neurons. In addition, NM neurons on the side of the brain contralateral to cochlea removal or NL dendrites whose innervation remains intact can serve as a within-animal control. In the following sections we will describe the postsynaptic consequences of afferent manipulation, first on NM and then NL. For reasons discussed above, we will concentrate on early cellular events.

NM Response to Afferent Manipulation

The early events after afferent manipulations are of particular interest in understanding the mechanism of afferent control of postsynaptic targets. We present first a time course of the events occurring in NM following unilateral cochlea removal, followed by brief discussion of two specific aspects of the response to cochlea removal. One potentially confusing consequence of presenting the time course of cellular events is that not every postsynaptic response to cochlea removal has been examined at exactly the same points. Every effort will be made to make clear whether or not a particular response has been examined. In each case given, the effects were observed in NM ipsilateral to cochlea removal unless otherwise stated.

Early Events (Up to 1 h after Cochlea Removal). The most rapid postsynaptic event examined in NM following cochlea removal is the cessation of electrical activity. As shown in Fig. 2, extracellular recordings made in NM before, during, and after cochlea removal show a cessation of action potentials occurring within 1 min after removal of the cochlea (Born and Rubel, 1984). Because of the resolution limits of extracellular recording, it is not known whether subthreshold EPSPs occur in NM neurons after eighth nerve input has been eliminated. As might be expected, glucose uptake also rapidly decreases in NM as measured with the 2-deoxyglucose technique (Heil and Scheich, 1986; Lippe, Steward, and Rubel, 1980). The earliest time point measured is 1 h after cochlea removal, but it seems likely that glucose uptake may decrease much sooner.

Experiments using the ^{14}C-iodoantipyrine method (Sakuroda, Kennedy, Jehle, Brown, Carbin, and Sokoloff, 1978) show a 30% decrease in blood flow in NM 30 min after cochlea removal (Richardson and Durham, 1989). A 50% decrease in the incorporation of amino acids into proteins is observed 30 min after cochlea removal, as measured by uptake of ^{3}H-leucine (Steward and Rubel, 1985). It is tempting to conclude that changes in amino acid incorporation are a consequence of the blood flow changes. However, recent work using

an in vitro slice preparation, in which labeled precursor is not limited by blood flow, indicates that the decrease in amino acid incorporation is independent of decreases in blood flow (Hyson and Rubel, 1989). Finally, changes in nonneuronal elements are also occurring within 1 h of cochlea removal. Immunocytochemical staining for glial fibrillary acidic protein (GFAP) shows a striking increase in the number of immunopositive glial fibers within NM (Rubel and MacDonald, 1987). Experiments using a silver impregnation stain suggest that new glial processes are being produced, as opposed to increased GFAP production in existing fibers (MacDonald and Rubel, 1989).

Three to 6 h after Cochlea Removal. At this time, a number of processes observed earlier are still occurring. Blood flow, 2-DG uptake, and electrical activity remain decreased by the same magnitude as seen immediately after cochlea removal. In-

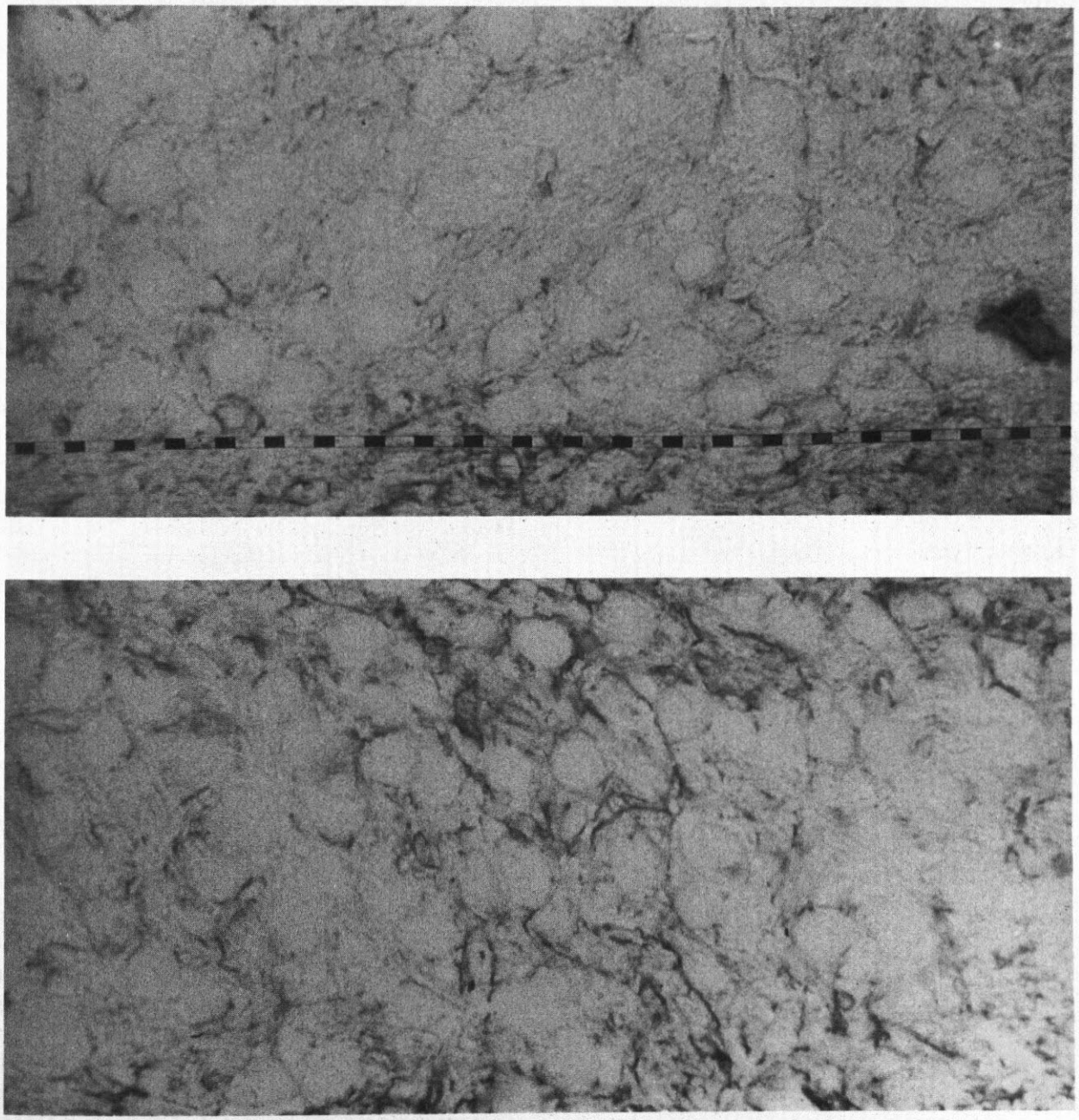

Figure 3 Photomicrographs of NM glial cells from an animal sacrificed 6 h after cochlea removal. NM contralateral (top) and ipsilateral (bottom) to cochlea removal are shown from a single tissue section stained with an antibody to GFAP. Dotted line in top panel indicates border of NM. Stained glial processes can be seen in both panels; the number of these stained fibers is greatly increased on the side of the brain ipsilateral to cochlea removal. (See color plate section at end of issue.)

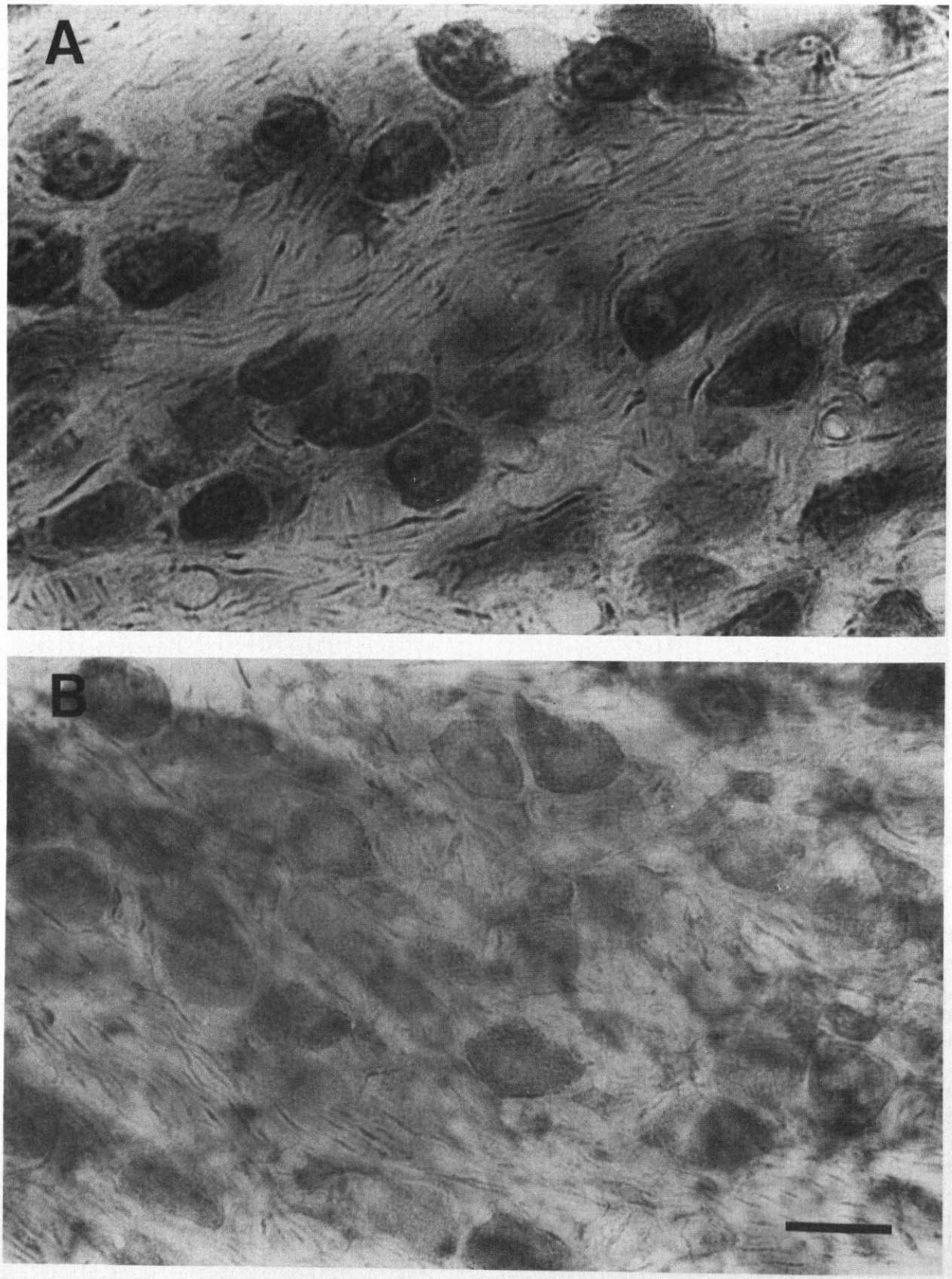

Figure 4 Photomicrographs of NM neurons from an animal sacrificed 6 h after cochlea removal. NM contralateral (A) and ipsilateral (B) to cochlea removal are shown from a single 30 μm tissue section stained with an antibody to actin. Note staining in cytoplasm of all neurons and absence of staining in nucleus. Neurons ipsilateral to cochlea removal appear more lightly stained than neurons on the contralateral side of the brain. Scale bar = 20 μm.

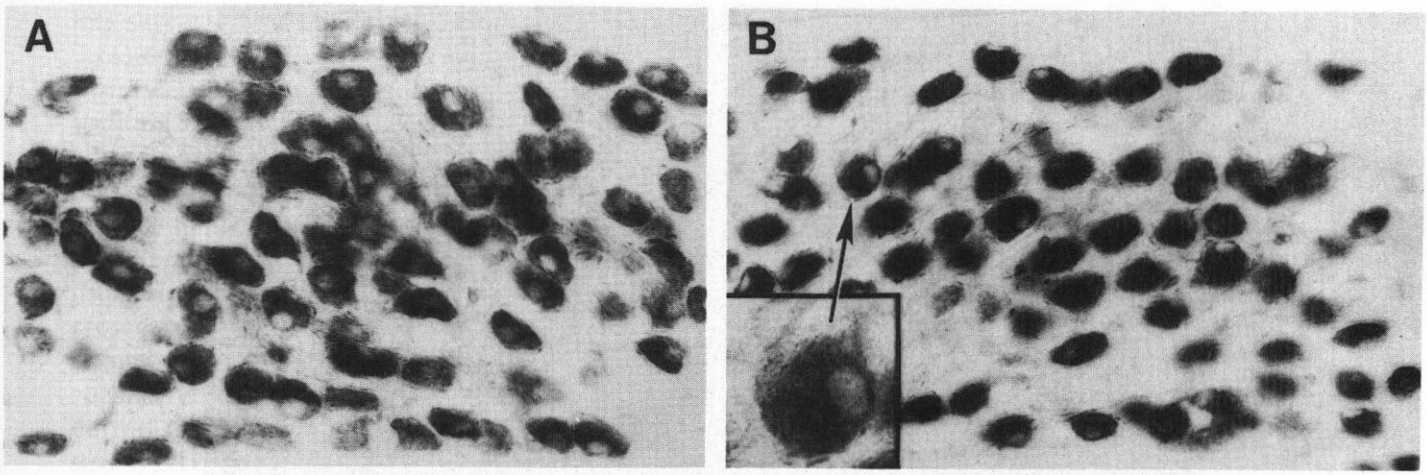

Figure 5 Photomicrographs of NM neurons stained for CO from chickens sacrificed 6 h after cochlea removal. NM contralateral (A) and ipsilateral (B) to cochlea removal are shown from a single 25 μm tissue section. In NM neurons ipsilateral to cochlea removal, note the darker CO reaction product as compared with neurons on the opposite side of the brain. The inset in B shows a neuron with an eccentric nucleus, increased CO-staining in the perinuclear region, and lighter CO staining in the pole of the cell opposite the nucleus.

creases in glial processes are more pronounced than those observed earlier (Fig. 3).* Changes in structural proteins within NM neurons are now apparent as well. Immunocytochemical staining for three structural proteins [tubulin, actin and microtubule associated protein 2 (MAP2)] show a decrease beginning 3 h after cochlea removal (Fig. 4). The decrease in staining appears to occur in all NM neurons (Seftel, Deitch, and Rubel, 1986). No changes in cell size have been observed at this time point.

Metabolic changes other than glucose uptake begin to occur between 4 and 6 h after cochlea removal. *Increases* in the activity of two Krebs' cycle enzymes, succinate dehydrogenase (SDH) and malate dehydrogenase (MDH), have been described in individual NM neurons using histochemical staining (Durham and Rubel 1985a,b). Changes in MDH activity have been confirmed with biochemical measurements from similarly prepared tissue (Durham, Rubel, and Matschinsky, 1985). These *increases* in oxidative enzyme activity are surprising considering the decrease in glucose uptake observed with 2-DG and the decrease in blood flow. Recent evidence suggests that these metabolic increases also are observed in the oxidative phosphorylation pathway. Light microscopic histochemical staining for cytochrome oxidase (CO) demonstrates increases in CO activity in the cytoplasm of individual NM neurons beginning between 3 and 6 h after cochlea removal (Hyde and Durham, 1989a) (Fig. 5). The purpose of this apparent metabolic burst is not yet clear.

In addition to an increase in the density of CO reaction product in NM neurons following cochlea removal, a change in the distribution of CO reaction product within the cytoplasm is evident (inset, Fig. 5). Less CO reaction product is seen at the edges of each NM neuron, suggesting that the mitochondria in which the CO is localized have been redistributed. To examine this possibility, Hyde and Durham (1989b) prepared tissue to examine CO at the electron microscopic level. A striking increase in the surface density of mitochondria is observed 6 h after cochlea removal (Fig. 6). In addition, mitochondria in ipsilateral NM neurons appear more branched and more heavily stained for CO. Qualitative observations also suggest that changes in other cellular components such as lipid vacuoles are occurring. It is not known as yet whether these ultrastructural changes occur earlier than 6 h after cochlea removal.

Within several days after cochlea removal approximately one third of the ipsilateral NM neurons will die (see below). Several lines of evidence suggest that by 6 h after cochlea removal 2 populations of neurons within NM can be discerned and that they correspond to neurons that will eventually live or die. First, in vivo studies examining uptake and incorporation of ^{3}H-leucine demonstrate two populations of NM neurons ipsilateral to cochlea removal (Fig. 7). One group, approximately one third of all cells, shows virtually no labeling, indicating they have essentially ceased protein synthesis. The remaining neurons show a

* See color plate section at end of issue.

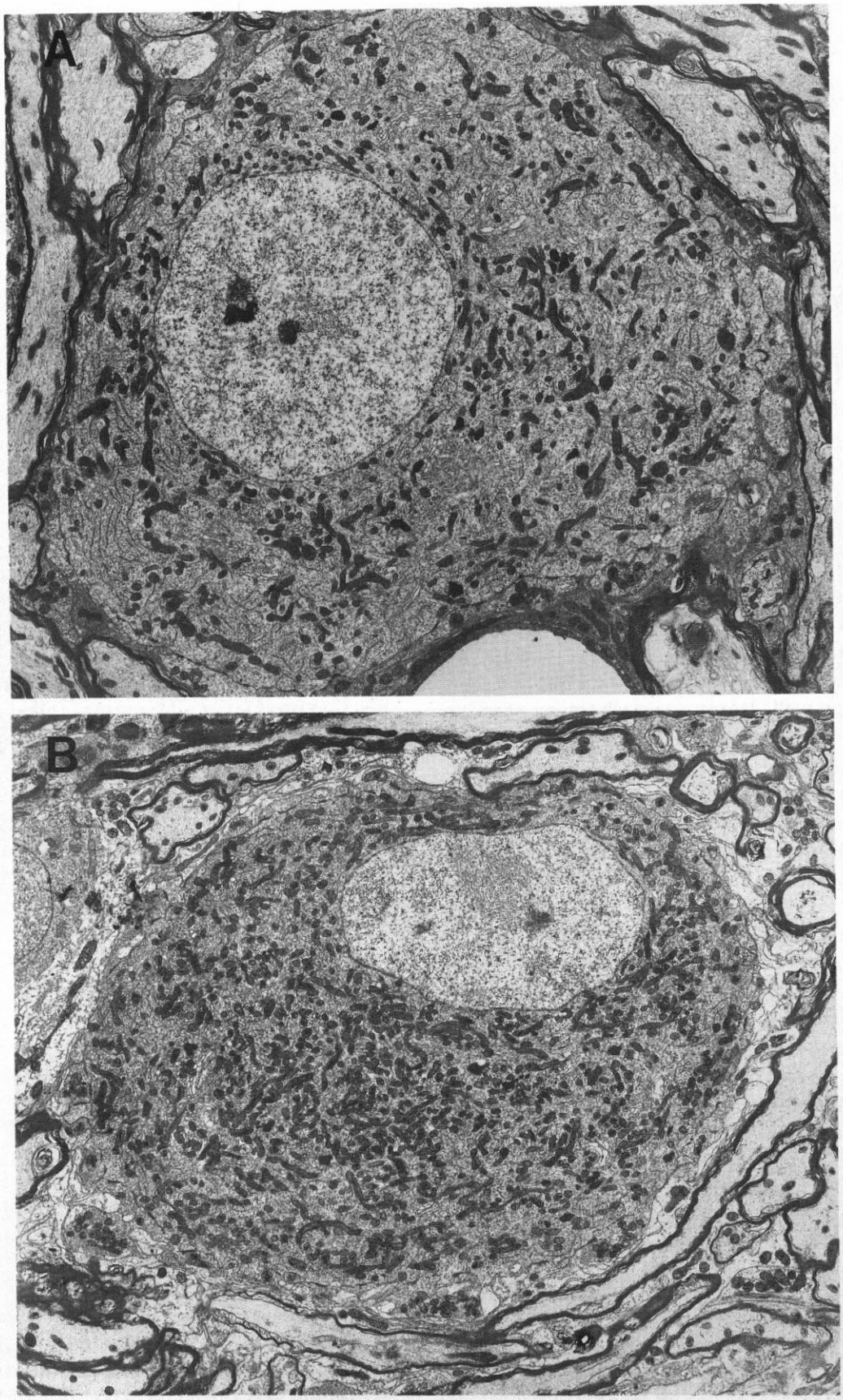

Figure 6 Two nucleus magnocellularis neurons from a chicken sacrificed 6 h after cochlea removal. The top neuron (A) is representative of "control" neurons on the side of the brain contralateral to cochlea removal. The bottom cell (B) is representative of the ipsilateral "deaf-

less severe but highly significant (approximately 20%) decrease in labeling compared with control neurons on the opposite side of the same tissue section (Steward and Rubel, 1985). Second, pulse labeling experiments, in which birds are given a pulse of ^{3}H-leucine 6 h after cochlea removal and allowed to survive 3–6 days, indicate that the unlabeled cells seen in Fig. 7 do not survive. Third, using alternate sections prepared for electron microscopy and autoradiography, Rubel and colleagues (1988) were able to show that at 6 h after cochlea removal, the unlabeled neurons are totally devoid of polyribosomes and show other ultrastructural changes that separate them from labeled neurons on the side of the brain ipsilateral to cochlea removal (Fig. 8). Finally, although at the light microscopic level histochemical staining for metabolic enzymes has never revealed two populations of NM neurons in either control animals or following cochlea removal, preliminary data suggest that NM neurons ipsilateral to cochlea removal that show degenerative changes in ribosomes also show neither the increase in mitochondria nor increases in CO staining seen in other neurons ipsilateral to cochlea removal (Hyde and Durham, unpublished observations). The ability to distinguish at the ultrastructural level which NM neurons are destined to die will better allow investigations of the events leading to neuronal death.

One to 3 Days after Cochlea Removal. At this time, both electrical activity and glucose uptake are decreased to the same extent as seen at earlier time points. Gross morphological changes in NM neurons now become apparent. A 20% decrease in cross sectional neuronal area can be measured either in Nissl-stained material (Born and Rubel, 1985) or in CO-stained material viewed with Nomarski optics (Hyde and Durham, 1989a). By 2 days an apparent 30% loss of neurons is observed in Nissl-stained material (Born and Rubel, 1985). This cell loss is due to the loss of Nissl substance in approximately one third of the NM neurons. These "ghost neurons" are unlabeled after ^{3}H-leucine injections and presumably are the neurons that eventually will die [see above and Steward and Rubel (1985) for a fuller discussion of this issue]. Degeneration of eighth nerve fibers is first observed two days after cochlea removal (Parks and Rubel, 1978). Between 2 and 4 days after cochlea removal, recovery in the density of staining for cytoskeletal proteins in the remaining neurons begins. Between 1 and 3 days following cochlea removal the increase in glial fibers seen immediately after cochlea removal is more pronounced; there is a fivefold increase in the number of GFAP-stained glial fibers in ipsilateral NM.

One day after cochlea removal the activity of SDH and CO are still elevated in NM neurons ipsilateral to cochlea removal (Durham and Rubel, 1985a; Hyde and Durham, 1989a). However, activity then begins to decrease in deafferented NM neurons relative to control such that by 3 days after cochlea removal ipsilateral neurons show less histochemical reaction product than control neurons. MDH activity reverses even more quickly; by 1 day after cochlea removal NM neurons on the 2 sides of the brain show similar enzyme activity, and ipsilateral NM neurons are less heavily stained by 3 days (Durham, et al., 1985).

Long-Term Changes. While our emphasis has been on early events following cochlea removal, some aspects of NM neurons have been examined several weeks following the surgery. No additional changes are observed in neuron size or number between 3 and 26 days after cochlea removal (Born and Rubel, 1985). Both CO and SDH remain decreased at 2 weeks, and SDH remains decreased as long as 90 days following cochlea removal (Durham and Rubel, unpublished observations). By 35 days after surgery the density of staining for cytoskeletal proteins is the same on the 2 sides of the brain, suggesting that ipsilateral NM neurons have reregulated levels of cytoskeletal proteins (Seftel, et al., 1986).

All of the changes reported so far occur on the side of the brain ipsilateral to cochlea removal. One contralateral change has been observed, however, involving terminals on NM neurons which use GABA as their neurotransmitter (Code, Durham, and Rubel, 1988). Immunocytochemical staining with an antibody to GABA has been shown to label these terminals in normal animals

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ferented" neurons. The darkly stained organelles which are so much more abundant in the ipsilateral neuron are mitochondria. Stereological measurements of mitochondrial surface density show a 53% increase in mitochondria in ipsilateral NM neurons 6 h after cochlea removal. Note also the irregular shape of the nucleus in the ipsilateral neuron. Magnification 4950×.

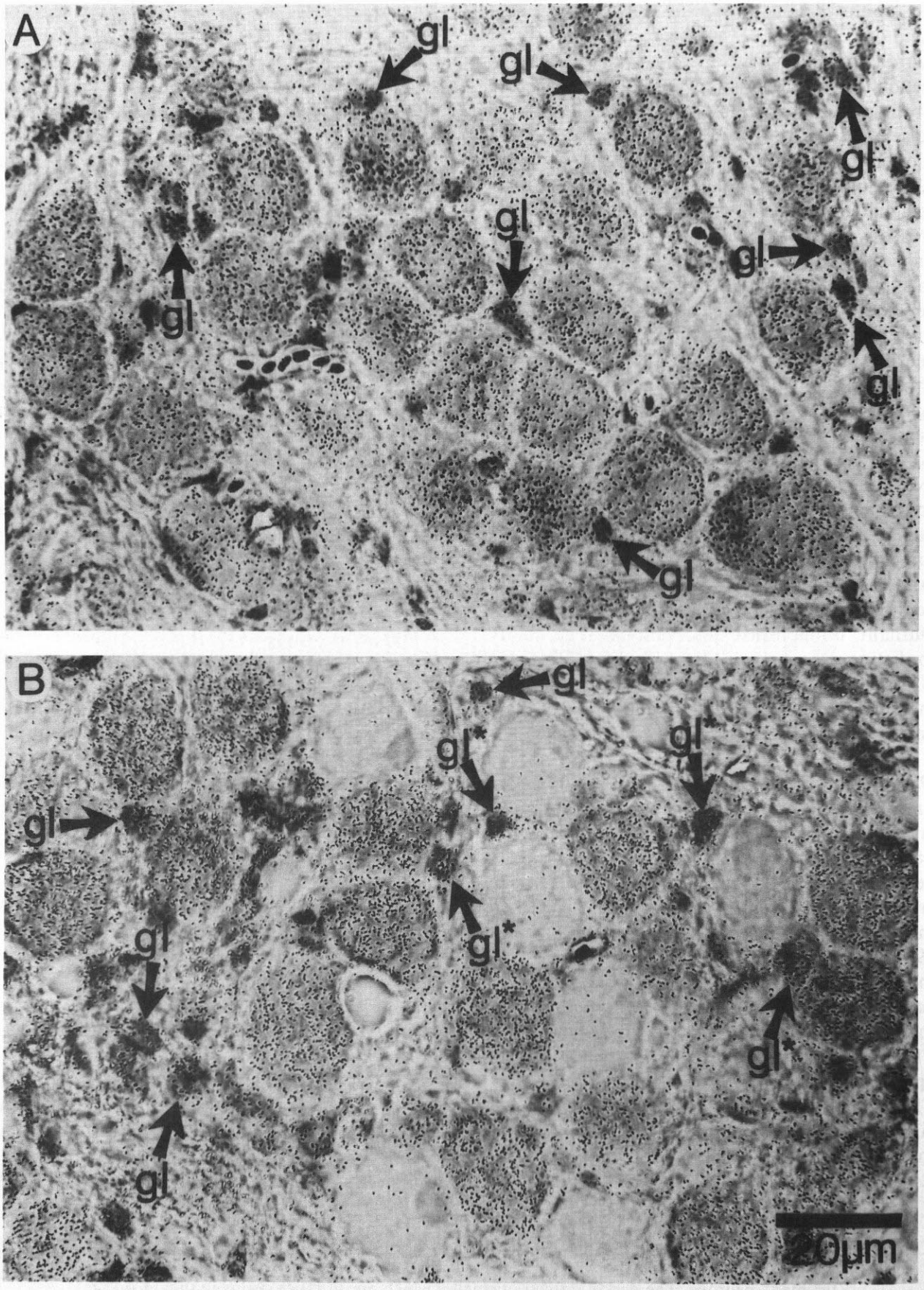


Figure 7 Phase contrast photomicrographs of 10 μm Nissl-stained paraffin sections through NM prepared for emulsion autoradiography from chicks sustaining unilateral cochlea removal. ^{3}H-leucine was injected 12 hours after cochlea removal and birds were sacrificed 30

(Code et al., 1989). Following cochlea removal, a slight increase in the density of these terminals ipsilateral to cochlea removal occurs, due to the decrease in NM volume occurring after this manipulation. However, a surprising decrease in the density of GABA terminals also occurs on the side of the brain contralateral to cochlea removal, even though no direct connection exists between the eighth nerve and the contralateral NM (Fig. 9).* Parallel studies on similarly prepared animals stained with an antibody to glutamic acid decarboxylase (GAD) show no differences in the density of terminals on the two sides of the brain. The interpretation of these immunocytochemical results regarding absolute levels of either substance is preliminary, since no direct biochemical measurements of GABA or GAD have been made, but they suggest the possibility of independent regulation of the levels of the enzyme (GAD) and its product (GABA). In addition, it is interesting to note that changes in these terminals were not observed ipsilateral to cochlea removal, where no other terminals compete for synaptic space; changes are only observed on neurons for which the other input is still intact.

Comments on Cochlea Removal and NM

One of the most intriguing questions about the consequences of cochlea removal is why only one third of the NM neurons die, even though all NM neurons lose their excitatory input. None of the parameters examined so far, such as innervation patterns, response properties, cell size, baseline staining for any oxidative enzyme, or staining for cytoskeletal proteins suggest that normal NM neurons can be divided into two populations. This inability may reflect both the subtlety of the differences in these parameters as well as the sensitivity of the assays employed to detect them. The use of ^{3}H-leucine incorporation, the best marker to date to label neurons that will eventually die after cochlea removal, has provided information regarding ultrastructural changes involved in neuron death (Rubel et al., 1988). These ultrastructural changes may provide clues as to whether any parameters can be detected in normal NM neurons that separate them into classes based on the probability that they will "succumb" to cochlea removal. At least they can provide clues for which parameters should be studied with time-intensive stereological techniques. A further extension of this reasoning asks, if such a characteristic exists, whether it is a constant feature of an individual neuron, or whether it is a feature that regularly varies in each neuron, and only those neurons "caught" expressing that characteristic at the time of removal of excitatory input will eventually die.

Figure 10 is a composite, showing the time courses of changes in several parameters in ipsilateral NM following cochlea removal. The most interesting features are the rapidity of the response as well as the unusual *increase* in oxidative markers at a time when we can determine which neurons will live and which will die.

One must ask if the rapid response of NM neurons and glia is representative of what is seen in other parts of the brain or is specific to this system. If representative, why haven't similarly rapid and dramatic effects of deafferentation been seen in other systems? Several lines of evidence suggest to us that these responses to deafferentation are characteristic of the events occurring in any *immature* system following deafferentation. First, similar events with a similar time course have been seen in the gerbil cochlear nucleus following deafferentation or eighth nerve activity blockade (Hashisaki and Rubel, 1989; Pasic and Rubel, 1989a; Sie and Rubel, 1989). Second, while most studies have only examined long-term changes, those that have looked for short-term effects have found rapid changes in cell size and cell number (e.g., Kalil, 1980). Finally, NM neurons are unusual in the sense that they appear to have only a single source of excitatory afferents, whereas most other neurons have several. Thus, in other systems, the metabolic influence of removing a single source of excitatory afferents may be less dramatic because of continued maintenance by other afferents as well as the influence brought about by synaptic

* See color plate section at end of issue.

min. later. (A) The control side contralateral to the surgery; (B) the side of the brain ipsilateral to cochlea removal. Neurons on the contralateral side of the brain are heavily labeled with silver grains. On the ipsilateral side, however, some neurons are labeled and others are virtually unlabeled. Note that the small cells (presumably glia, gl) appear to be at least as heavily labeled on the side ipsilateral to the removal of the cochlea (B) as on the control side (A). A similar pattern of labeled and unlabeled ipsilateral neurons is seen 6 h after cochlea removal. From Steward and Rubel (1985).

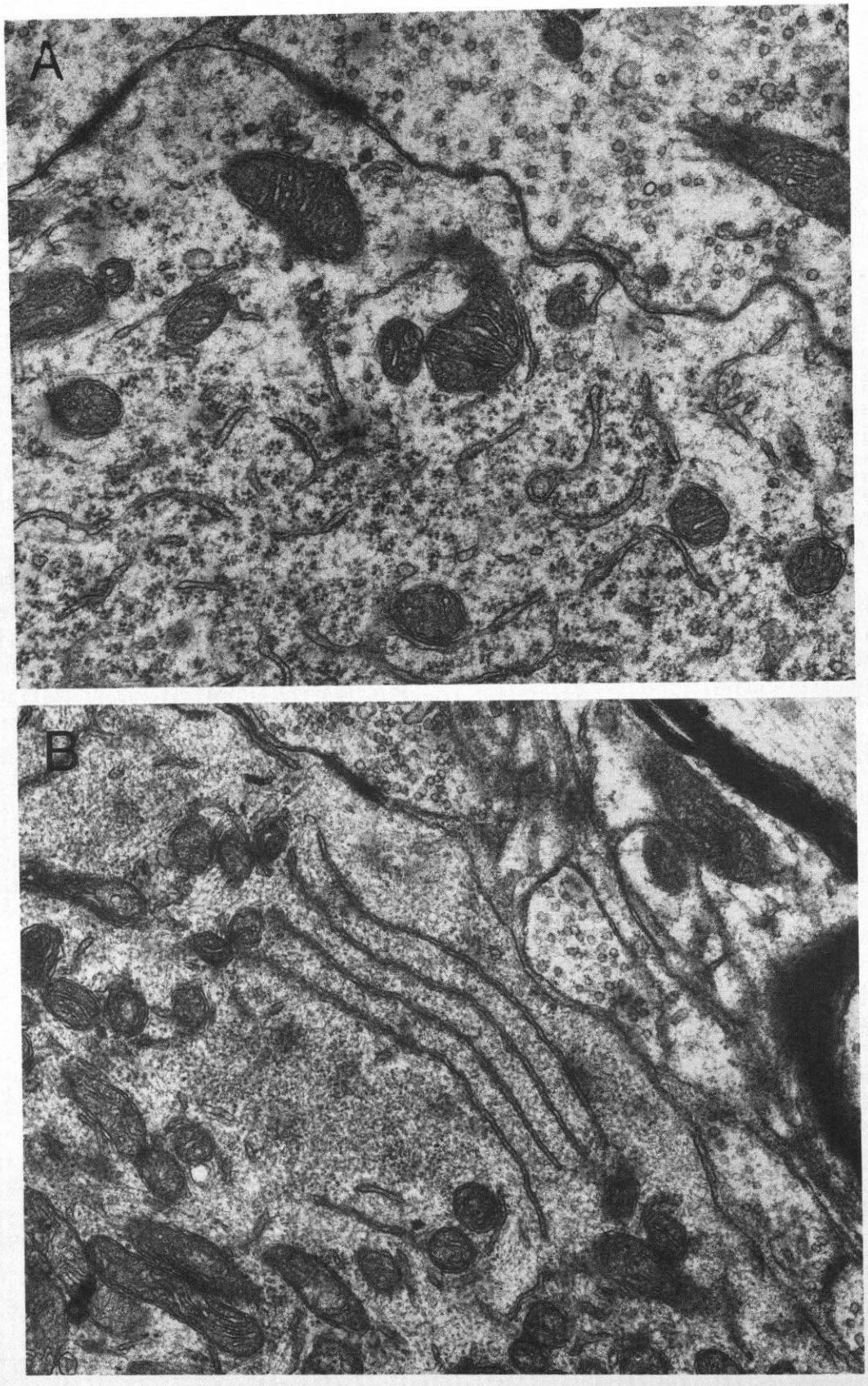


Figure 8 Electron micrographs of NM neurons from an animal sacrificed 6 h after cochlea removal and prepared for ^{3}H-leucine autoradiography. Examination of 1 μm sections which were adjacent to the sections from which these photographs were taken and were prepared for emulsion autoradiography showed that the neuron in panel A was labeled (still undergoing protein synthesis) and the neuron in panel B was unlabeled (no protein synthesis, destined to die). Note the normal appearance of ribosomes in the neuron in A. Endoplasmic reticulum in neuron shown in B is devoid of ribosomes, and no polyribosomes are present.

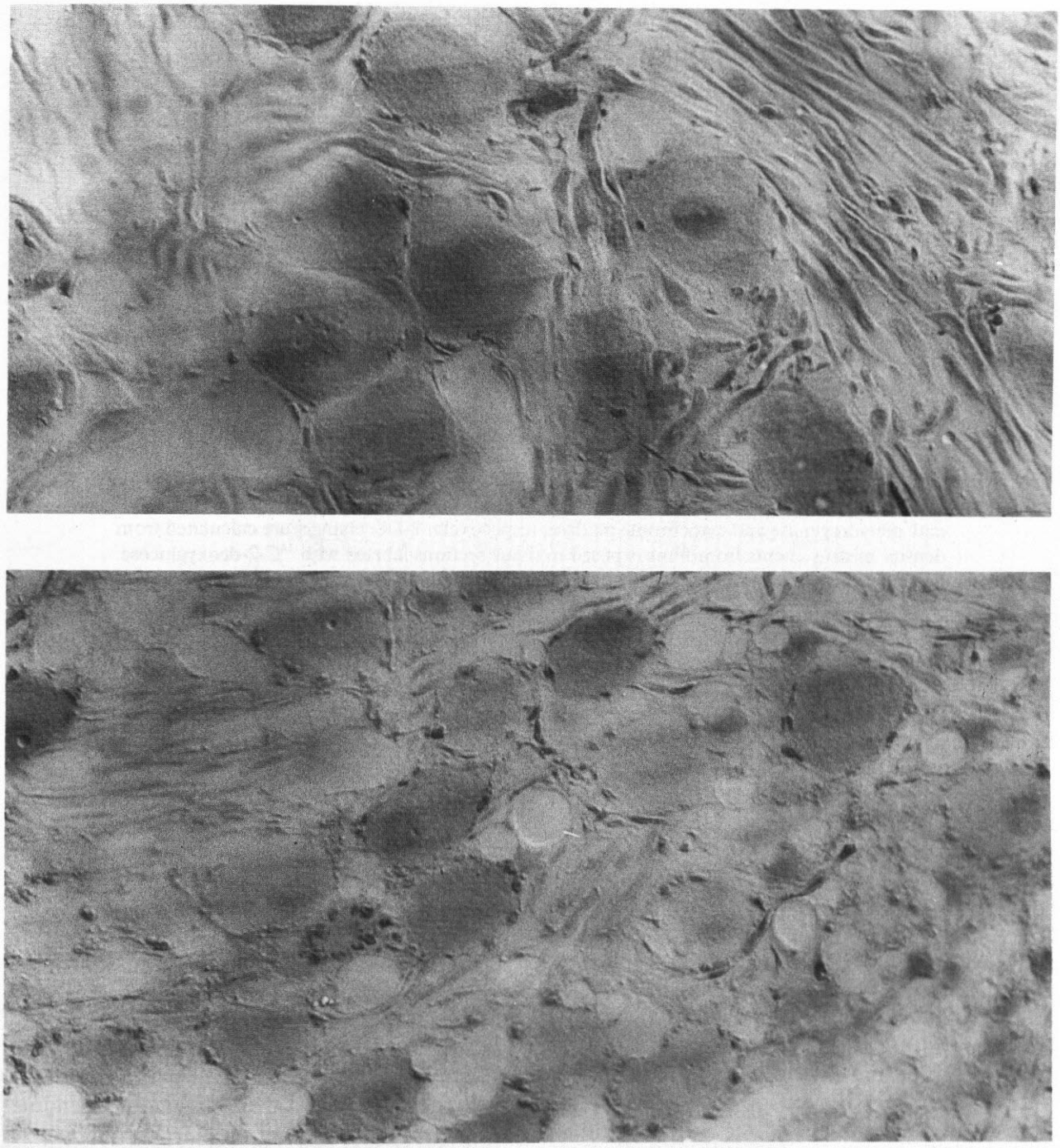


Figure 9 Photomicrographs of NM from a single tissue section stained with an antibody to GABA from an animal sacrificed 14 days after cochlea removal. Neurons in the top panel are from NM contralateral to cochlea removal, and those in the bottom panel are from NM ipsilateral to cochlea removal. Small, discrete patches of label are GABA terminals. NM cytoplasm shows some background staining. Note the marked reduction in the number of GABA terminals in NM contralateral to cochlea removal. (See color plate section at end of issue.)

reorganization, i.e., competition for synaptic sites or sprouting.

Another characteristic of the response to cochlea removal in NM neurons may be useful in answering the question of how these neurons can be differentiated as to their susceptibility to the deleterious effects of cochlea removal. It is well known in other sensory systems as well as the auditory system that the age of the animal at the time of the afferent manipulation affects the magnitude and even the existence of a response (Globus, 1975; Guillery, 1973; Kalil, 1980). A similar situa-

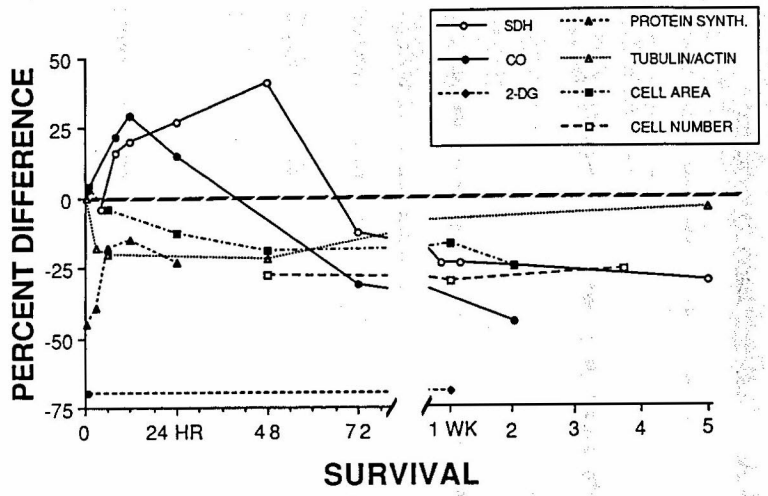


Figure 10 Time course of changes in various aspects of cellular metabolism in NM neurons after cochlea removal. The percent difference of any given measure is calculated as 100× (mean contralateral − mean ipsilateral)/mean contralateral. SDH and CO changes are calculated from optical density measurements of tissue sections histochemically stained for succinate dehydrogenase and cytochrome oxidase, respectively. 2-DG changes are calculated from density measurements from films exposed to tissue sections labeled with ^{14}C-2-deoxyglucose. Protein synthesis changes were calculated from grain density measurements of autoradiograms following ^{3}H-leucine incorporation. Tubulin/actin changes were calculated from optical densities of immunostained tissue sections. Cell area and cell number measurements were made on Nissl-stained tissue sections.

tion exists in the response of NM neurons to cochlea removal. Many of the changes seen in NM only occur when cochlea removal is done in young birds. For example, no changes in neuron size, number, SDH activity, or ^{3}H-leucine incorporation occur in NM when the cochlea is removed in adult birds (Durham and Rubel, 1985a; Steward and Rubel, 1985; Born and Rubel, 1985; Hyde and Durham, 1989a). Our evidence suggests, however, that decreased electrical activity and glucose utilization do occur in adult animals following cochlea removal (Durham, Born, and Rubel, 1984). It would appear that older animals have somehow uncoupled these postsynaptic metabolic events from their input. It will be of interest to determine what differences exist between young and adult bird physiology, metabolism, or morphology that make NM neurons seemingly immune to the deleterious effects of deafferentation. An interesting comparison is the difference between adult bird neurons and the young bird neurons that survive following cochlea removal—do they have the same or different strategies for cell survival?

NL Response to Afferent Manipulation

As mentioned above, afferents to third-order neurons in nucleus laminaris (NL) also can be manipulated, by either partially or totally denervating one set of dendrites. The postsynaptic consequences of each of these manipulations will be considered, followed by comments regarding the possible role of competition in the neuronal response to deafferentation in these neurons.

Tract Cut: Complete Deafferentation of Half a Cell. As shown schematically in Fig. 1, NL dendrites are separated into dorsal and ventral domains. Within the nucleus, a gradient of dendritic length for both sets of dendrites exists along a rostromedial to caudolateral axis, identical to that of the tonotopic representation in the nucleus (Smith and Rubel, 1979). This gradient can be used to predict the length of a given NL neuron's dendrites based on its position in the nucleus; the axis for dendritic length is the same for dorsal and ventral dendritic fields. Dorsal and ventral NL dendrites receive excitatory input almost exclusively from ipsilateral or contralateral NM axons respectively (Parks and Rubel, 1975). The input to the ventral dendrites on both sides of the brain can be easily removed by cutting the axons as they cross the midline. This manipulation deafferents only the ventral dendritic region for each NL neuron, allowing us to examine the subcellular spatial resolution of the response to deafferentation. Early electron microscopic morphometric analysis suggested that enormous reductions in dendritic volume density were occurring within 1–4 days after tract cut and were confined to the ventral (deafferented)

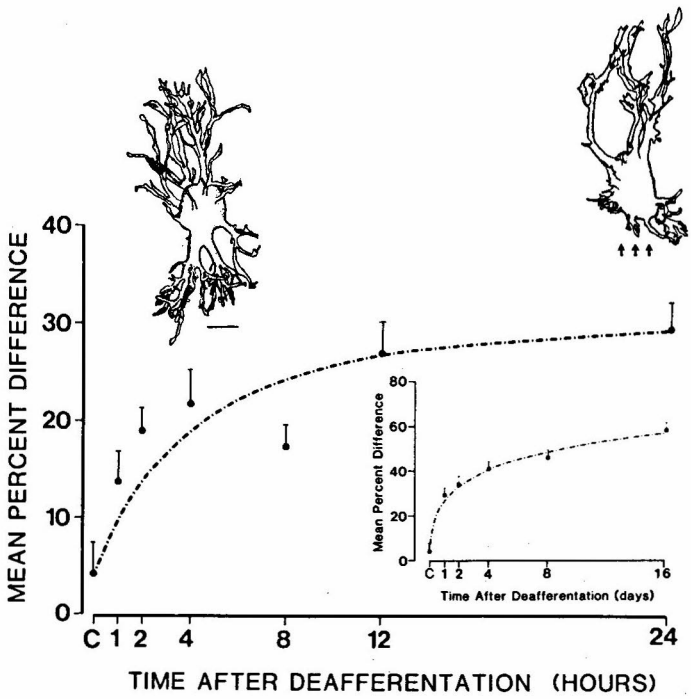


Figure 11 Cells at top show a normal NL cell and an NL cell 16 days after deafferentation of the ventral dendrite. The amount of ventral dendrite lost as a function of time after tract cut is shown in the graphs. The amount of dendrite lost from each cell is estimated by the difference between the length of the dorsal dendrites (which predicts the normal ventral dendritic length) and the length of the atrophied ventral dendrites, taken as a percentage of the dorsal dendritic population. Large graph: mean percentage difference over the first 48 h after deafferentation. Inset: mean percentage difference as a function of days after surgery. Note that there is a very rapid loss of the ventral dendrites; they are 14% shorter than the dorsal dendrites after 1 h and 20% shorter after 2 h. The atrophy continues at a slower rate throughout the 16-day interval examined. From Deitch and Rubel (1984).

neuropil (Benes, Parks, and Rubel, 1977). A later study using the Golgi method determined that, in fact, very rapid reductions in dendritic volume occur in the deafferented dendrites of NL following tract cut (Deitch and Rubel, 1984). Within just 2 h, ventral dendrites were 20% shorter than those on the dorsal side of the same neurons or the ventral dendrites in control animals. Loss of ventral dendrites continues rapidly, such that by 16 days after tract cut 60% of the ventral dendrites have disappeared (Fig. 11). Ventral dendritic loss is also evident in tissue stained with an antibody to MAP-2 (Fig. 12).* The percentage of ventral dendrite lost is the same all along the frequency (and dendritic length) gradient in NL; thus, the absolute amount of dendrite lost varies as a function of dendritic length. Comparison with control animals also shows that the dorsal dendrites remain normal following deafferentation of the ventral dendrites; thus the cellular response is confined to the dendritic surface which is directly deafferented.

Ultrastructural study of the early time periods following tract cut, designed to examine the subcellular correlates of dendritic atrophy, revealed a number of changes in the subcellular organization of NL neurons (Deitch and Rubel, 1989b). Within 4 h of tract cut a marked reduction in microtubule density at the base of the ventral dendrites was observed which became more pronounced with time. By 12 h after tract cut neurofilament density at the base of ventral dendrites decreases as well. Subsequently, a lucent gap appears at the base of the ventral dendrites, which becomes more pronounced with time. Surprisingly, no evidence of degeneration of dendritic plasma membrane was evident until 2 days following tract cut. Examination of the soma, however, revealed an *increase* in volume within hours of tract cut, which increased in magnitude up to 8 days following the lesion (Deitch and Rubel, 1989a). These results suggest a resorption of dendritic membrane as the mechanism of at least the early decrease in ventral dendritic length.

As was the case following cochlea removal in NM, removal of the exclusive excitatory input to

* See color plate section at end of issue.

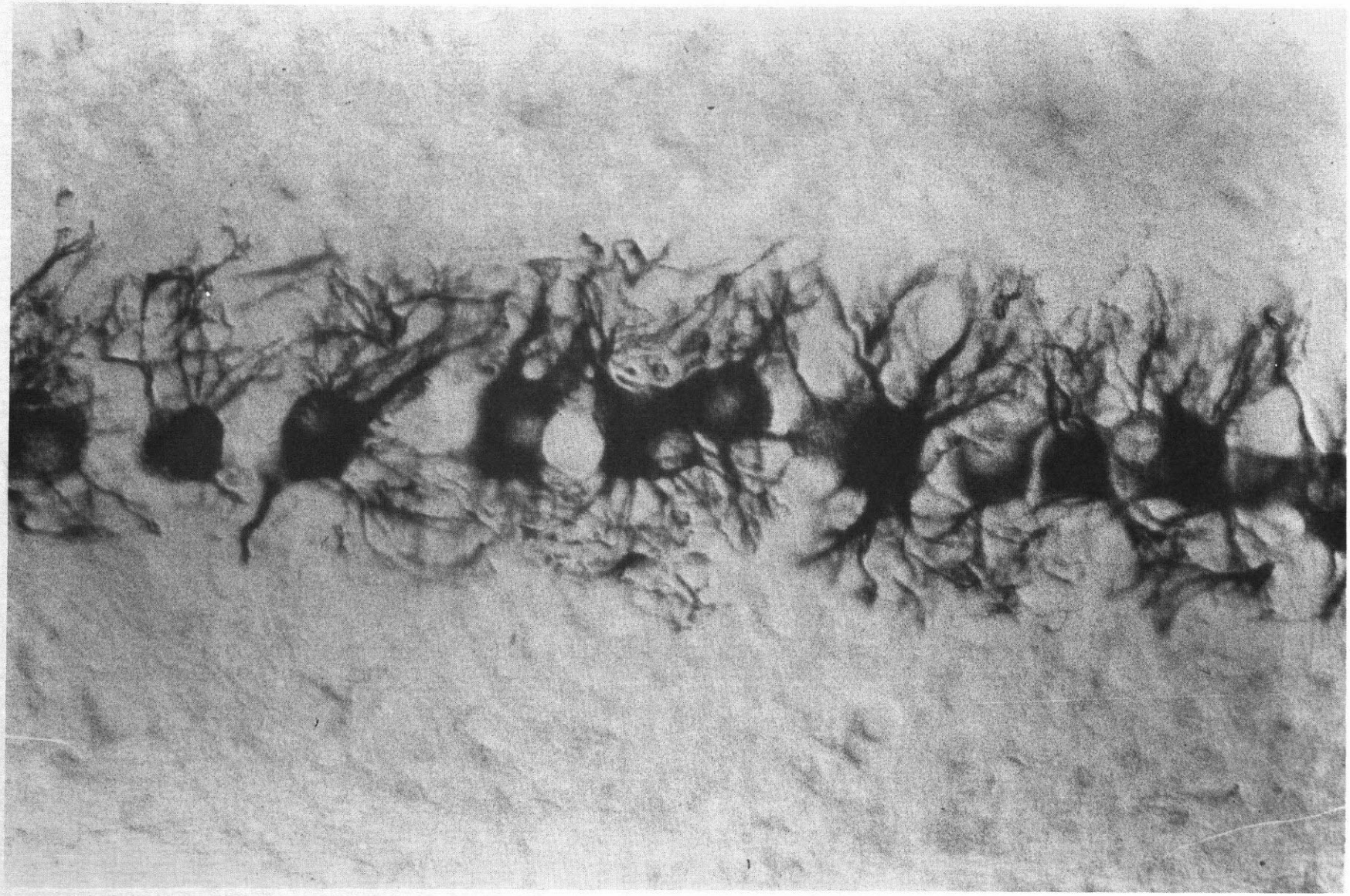


Figure 12 Photomicrograph of NL neurons stained with an antibody to the cytoskeletal protein MAP2 from an animal sacrificed 12 h after tract cut. Note staining of cell somata and dendritic processes, which are segregated into dorsal (top) and ventral (bottom) dendritic domains. Note short ventral dendritic processes. (See color plate section at end of issue.)

NL dendrites results in rapid and dramatic morphological changes in NL neurons. The unique morphology and synaptic connections in NL allowed the additional demonstration that afferent excitatory input can independently regulate different parts of the same neuron. While such regulation is undoubtedly occurring in other systems, NL provides a convenient system in which to examine this phenomenon more precisely.

Cochlea Removal: Partial Deafferentation of a Set of Dendrites. Because cochlea removal results in the death of approximately one third of NM neurons, and thus degeneration of NM axons, cochlea removal partially deafferents ipsilateral dorsal and contralateral ventral NL dendrites. Unlike the results of tract cut, however, little dendritic atrophy is observed following cochlea removal (Rubel et al., 1981). These results suggest that the remaining input is sufficient to maintain the dendritic surface integrity. Changes in oxidative enzymes are observed in NL neuropil after cochlea removal, however. Within 3 days of cochlea removal, both CO (Hyde and Durham, 1989a) and SDH (Durham and Rubel, 1985a) decrease in the NL neuropil receiving input from the deafferented NM neurons. Due to the limitations of light microscopic analysis, it is not known, however, whether the changes are occurring in NM axons or NL dendrites.

Up to this point, we have not seen examples of synaptic reorganization in the chick auditory system that can be attributed to competition between afferent terminals for a target structure such as vacant postsynaptic space on a neuron, a gland, or a muscle fiber. As noted above, GABAergic terminals do not appear to proliferate after denervation of NM. In addition, we see no evidence of sprouting by NM terminals from the opposite side of NL after one dendrite is completely deafferented. Instead, there is rapid and profound loss of the deafferented dendrite and, therefore, of vacated membrane sites. Examination of NL dendrites following cochlea removal indicates that although there is massive degeneration of the NM axons onto one side of NL, the dendrite remains nearly normal. Thus a "vacated" membrane surface exists which should support sprouted NM afferents from the opposite side of NL.

This idea was tested directly by comparing the amount of sprouting occurring in NL following either total deafferentation of one set of dendrites (tract cut) or partial deafferentation (following cochlea removal) (Rubel et al., 1981). At long (45 day) survival periods following either manipulation, the extent of sprouting by the NM axons innervating the opposite NL dendrites was determined using degeneration techniques. Only in the case in which partial deafferentation was the initial insult is sprouting seen to occur. Thus, it would seem that sprouting depends on the amount of membrane space preserved. Taken alone, these results could be interpreted as evidence for a process in which topographic boundaries are maintained by competitive interactions. However, as noted earlier, if we accept the definitions of Guillery (1981) this can not be classified as an example of competition since there is no evidence for its role in normal development.

INTERCELLULAR SIGNAL FOR AFFERENT REGULATION

Activity

Since cochlea removal results in an immediate cessation of activity in NM and changes in NM neuronal metabolism can be observed within as little as 0.5 h after cochlea removal, one logical postulate is that the alteration in afferent activity is responsible for this metabolic regulation. Cochlea removal, however, also damages the distal processes of the eighth nerve fibers and results in eventual degeneration of most of the ganglion cells. Thus, it is possible that the transneuronal effects on NM neurons and glia are a result of reaction to this trauma. To convincingly demonstrate that *activity* is an important regulating factor, one must eliminate afferent activity without damaging the nerve fibers. Born and Rubel (1988) succeeded in doing just this. They injected tetrodotoxin (TTX) into the perilymph of the inner ear at the level of the saccule. TTX blocks voltage-dependent sodium channels, thereby preventing the generation of action potentials in the eighth nerve. Injection of TTX into the perilymph results in a virtually immediate blockade of all activity in the ipsilateral NM (Fig. 13). Evidence that this treatment does not result in permanent damage to the cochlea or eighth nerve fibers is provided by two control experiments: (1) spike activity in NM returns to normal levels (if time is allowed for the TTX to wear off); (2) evoked potential thresholds in response to acoustic stimuli return to normal.

The effects of eighth nerve activity blockade on protein synthesis are identical to those observed after cochlea removal and are displayed in Fig. 14. After 1 h of activity blockade, protein synthesis is reduced by approximately 40% in the ipsilateral NM. TTX administered in this way, however, has a limited duration of action, with activity beginning

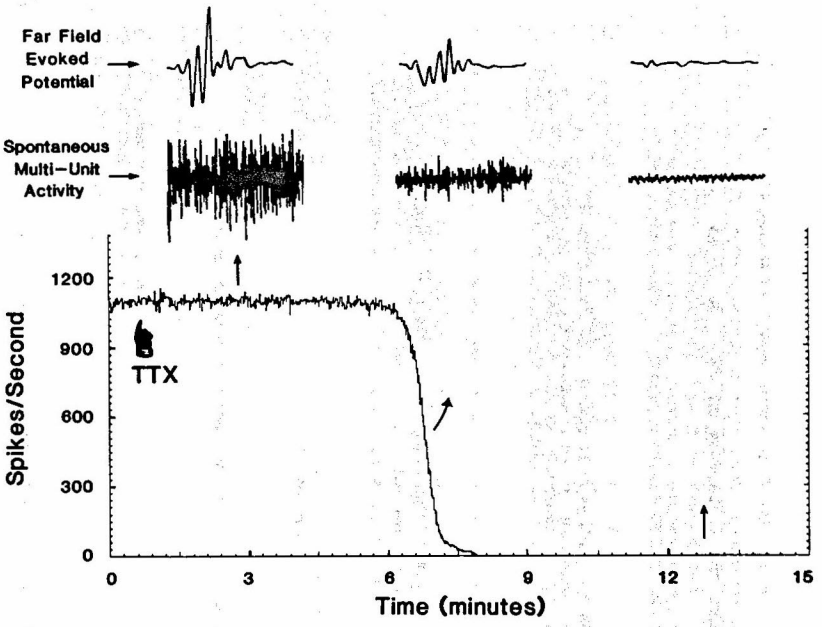


Figure 13 Spike rate in nucleus magnocellularis (NM) as a function of time. Above the graph are samples of the microelectrode output during the periods indicated (arrows). Above the microelectrode traces are far-field evoked potential averages recorded from the same animal at the same time, using subdermal electrodes (averages of 200 repetitions). The time of the TTX injection is indicated by the pointer. In this animal, the contralateral cochlea was removed prior to TTX injection. Approximately 1100 spikes/s are recorded in NM under these conditions. Approximately 6 min after a 0.1 μL injection of a 3 m*M* TTX solution (pointer), there is a precipitous drop in the spike rate. Within a minute, no spikes are recorded. These spontaneous neural potentials are seen to decrease to a level similar to the electrical noise of our recording system. Elimination of the evoked potential coincides with the decrease in action potentials. The small peaks that are preserved in the evoked potential recording are the cochlear microphonic, which is maintained after TTX injections. From Born and Rubel (1988).

to return in approximately 6 h. As seen in Fig. 14, protein synthesis in the ipsilateral NM shows a corresponding return to normal levels within 24 h after a single injection of TTX. Longer duration blockade of activity was achieved by multiple injections of TTX. The results of these experiments again replicate the effects of cochlea removal (see Fig. 15). After 6 h of continuous activity blockade, neurons in NM can be dissociated into two populations: one which appears to have completely ceased making protein and one which continues synthesis but at a reduced level. Again, if afferent activity is allowed to return to normal levels, the cells completely recover. If however, activity of the eighth nerve is blocked for 48 h, cell size and cell number changes mimic those seen following cochlea removal. These data not only confirm that changes in *activity* are responsible for the alterations in cellular metabolism and cell structure following cochlea removal, but also suggest that a dynamic relationship exists between afferent activity and the metabolism of the postsynaptic NM neuron; protein synthesis can be down-regulated by a reduction of presynaptic activity and then up-regulated by allowing activity to return to normal levels.

Similar effects of blocking eighth nerve activity are observed in the mammalian brain stem. Pasic and Rubel (1989a) used TTX to block activity of the eighth nerve in gerbils and examined subsequent changes in the size of large spherical cells in the anteroventral cochlear nucleus (AVCN). They produced a continuous blockade of activity by embedding TTX into a slow-release copolymer (Elvax). A pellet of this compound was placed adjacent to the round window. The TTX released from the compound passed through the round window into the perilymph, which bathes the processes of eighth nerve fibers. The effectiveness of the TTX-induced activity blockade was assessed by measuring sound-evoked auditory brain stem responses (ABRs). Within 15 minutes after the TTX pellet is in place, ABRs are abolished (Fig. 16). An ABR threshold shift remains for 24–48 h, as long as the pellet is in place. After this time, or after removal of the pellet, ABR thresholds return to normal levels. Figure 16 also shows cochlear

microphonic (CM) responses which are "unmasked" after blocking neural activity with TTX. The persistence of the CM after TTX administration suggests that this treatment does not damage the inner ear; outer hair cells, which are believed to generate the CM, are still functional.

The effect of TTX-induced blockade on the cross-sectional area of spherical cells in the AVCN is shown in Fig. 17. Blockade of auditory nerve activity with TTX for 48 h results in a decrease in the size of large spherical cells in the ipsilateral AVCN. Control subjects receiving implantation of the Elvax vehicle alone show no difference in cell size on the two sides of the brain. This decrease in cell area after TTX blockade of activity is as great as the decrease observed after total destruction of the cochlea. When animals received a cochlea ablation on one side and TTX administered to the other ear, there is no difference in the size of AVCN large spherical cells. Thus, it appears that changes in afferent *activity* can completely account for the alterations in cell size observed after cochlea removal. Pasic and Rubel (1989b) have gone on to show that a dynamic relationship exists between afferent activity and postsynaptic cell size. After 24 or 48 hours of unilateral activity blockade, they removed the Elvax-TTX pellets and allowed animals to survive an additional 7 days, during which time normal levels of activity returned. They found that the decrease in cell size normally found after 24 or 48 hours of activity blockade is not observed after this recovery period. Thus, neuronal cell size in the gerbil can be down-regulated by a decrease in presynaptic activity and then up-regulated if activity returns to normal levels.

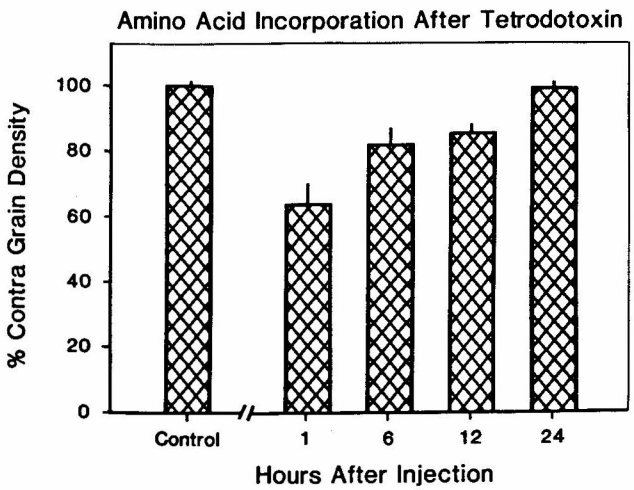


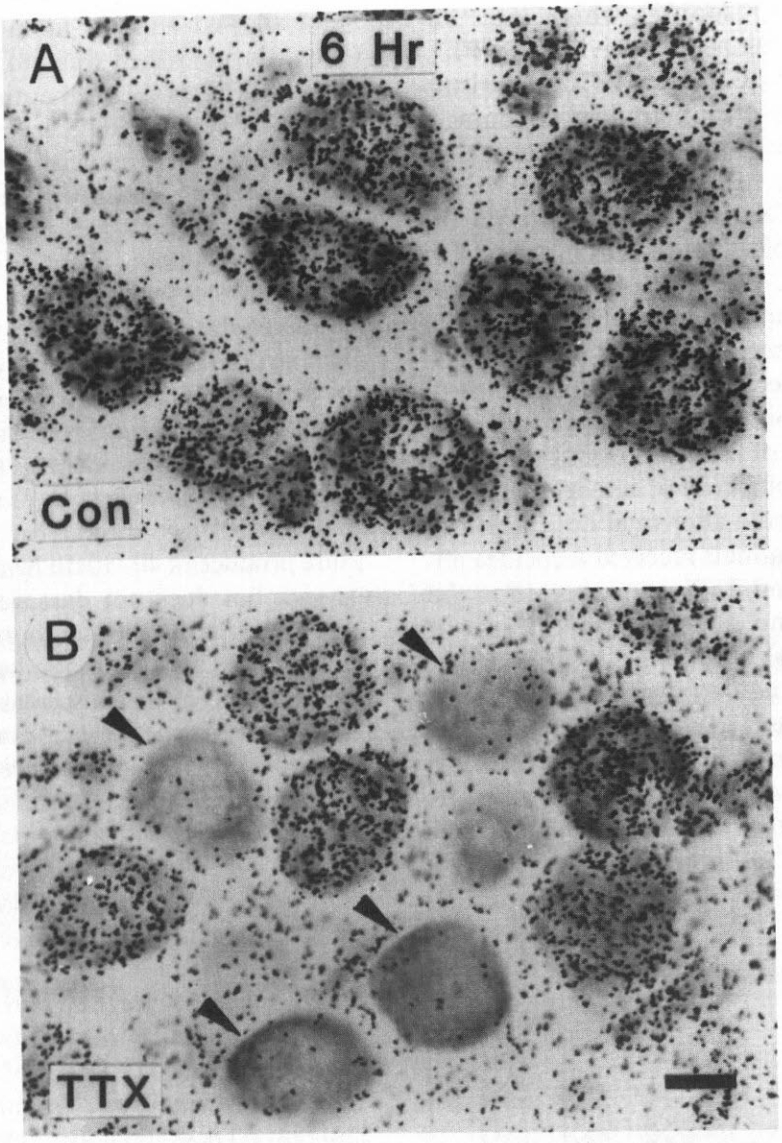
Figure 14 Time course of changes in ^{3}H-leucine incorporation by NM neurons after a single injection of TTX. For each animal the average grain density on the side ipsilateral to the TTX injection is expressed as a percentage of the grain density over neurons in the contralateral NM. The group means are plotted as a function of time after the TTX injection. One hour after a single injection of TTX, ^{3}H-leucine incorporation ipsilateral to the injection reduces to 60% of that found on the contralateral side. There is a gradual return of amino acid incorporation to control levels over time. By 24 h after a single injection, there is no difference between the two sides of the brain. Bars indicate SEM. From Born and Rubel (1988).

Given the dramatic changes in NM neurons following elimination of activity by cochlea removal or activity blockade, Tucci and Rubel (1985) reasoned that perhaps more subtle manipulations would have similar effects. Thus, they produced a conductive hearing loss by removing the columella (the bird's single middle ear ossicle). This procedure produces a 40–50 dB hearing loss for airborne sound, but does not damage the cochlea, as assessed by thresholds for bone-conducted sound. Surprisingly, this dramatic hearing loss has absolutely no effect on neuron size in NM (Fig. 18). Measurements of electrophysiological activity in NM after this manipulation resolved the dilemma. Figure 19 displays the changes in spike rate observed after various manipulations of the ear. Although columella removal results in a threshold shift for airborne sound, it does not result in any detectable reduction in the overall spontaneous activity recorded in NM.

These data suggest that the overall level of activity, rather than the information about the external environment received by NM neurons, is important for transneuronal regulation of neuronal structure. These data also illustrate an important point for experiments examining the roles of "experience" in development. Investigators often make assumptions as to how a particular manipulation affects afferent activity. This experiment points to the importance of directly examining the effects of each experiential manipulation; it is critical that one define the effect of that manipulation on activity in the region under study (see also Globus, 1975).

In Vitro Analyses

The experiments of Born and Rubel (1988) clearly show that the activity of afferents is important for transneuronal regulation of cellular metabolism. They do not address, however, what aspect of activity is important, the nature of the trans-synaptic signals, or the postsynaptic cellular events involved. To address these questions, Hyson and Rubel (1988, 1989) have utilized an in vitro slice preparation of the chick brain stem auditory system containing portions of the eighth nerve, NM,


A
6 Hr
Con
B
TTX
C
Tetrodotoxin
Contralateral
NUMBER OF NEURONS
0
10
20
30
40
50
60
70
80
−7.5
−6.0
−4.5
−3.0
−1.5
0
1.5
3.0
NORMALIZED GRAIN DENSITY

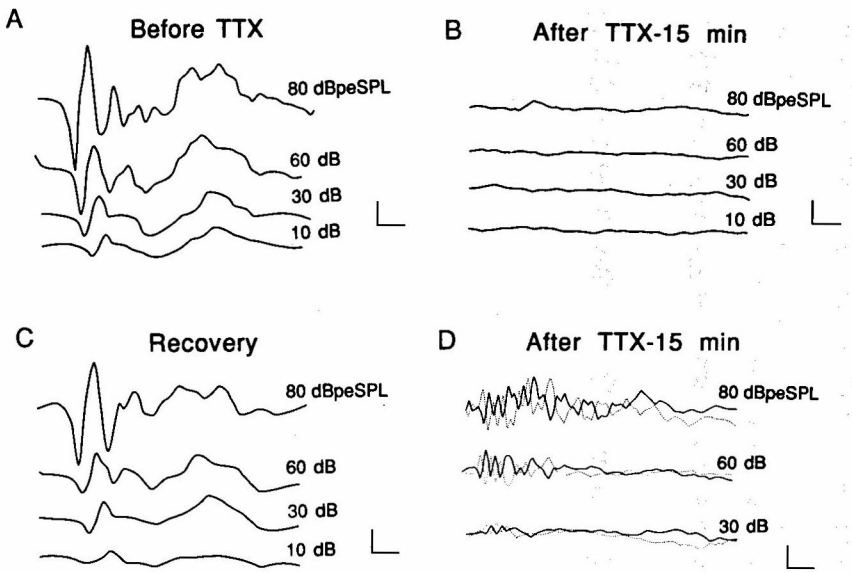


Figure 16 Representative intensity series of average ABR waveforms from 512 alternating rarefaction/condensation click stimuli (A–C) and rarefaction or condensation stimuli alone (D). (A) Before TTX there is an increase in wave latency with decreasing stimulus intensity. (B) Fifteen minutes after TTX-Elvax is placed in the round window niche, no ABR is present. (C) Return of neural activity shows waveforms similar to those observed prior to manipulation. (D) Cochlear microphonic response from rarefaction (solid line) or condensation stimuli (dashed line) 15 min after placement of TTX-Elvax. There is no latency change with decreasing stimulus intensity. The waveform is dependent upon stimulus polarity. The neural response is blocked and the cochlear microphonic response is "unmasked." Scale bar = 1 ms/1 μV (A–C) and 1 ms/0.5 μV (D). From Pasic and Rubel (1989a).

and NL bilaterally. This preparation provides the advantages of having direct control over the amount and pattern of afferent activity as well as allowing manipulation of the external ionic and chemical environment of the neurons.

The first question to be addressed using this preparation was whether afferent regulation of neuronal metabolism requires some signal released from the presynaptic auditory nerve terminal or if the activity of the postsynaptic neuron per se is sufficient to maintain normal neuronal metabolic activity. This is a question that has been fruitfully addressed in analyses of the influence of motoneuron activity on muscle. Damaging or stopping the activity of the motoneuron affects the acetylcholine receptor system, sodium conductance mechanisms, and resting membrane potential of the deafferented muscle (Guth, 1968; Harris, 1980). Some of these changes, however, can be prevented or attenuated by electrically stimulating the deafferented muscle (Lomo and Rosenthal, 1972; Lomo and Westgaard, 1975). The same general strategy was used for investigating the role of presynaptic versus postsynaptic activity in regulating neurons in NM (Hyson and Rubel, 1989).

Figure 15 Photomicrographs of autoradiograms and distributions of normalized grain densities from an animal that received 3 injections of TTX during a 6-h interval prior to being injected with ^{3}H-leucine. (A) Autoradiogram from NM contralateral (Con) to the injections, showing a uniformly high level of labeling over individual NM neurons. (B) NM on the side ipsilateral to TTX injection. A noticeable paucity of labeling is seen over a portion of the neurons (arrowheads). The remaining neurons appear to be slightly less labeled than those on the contralateral side. (C) Graph below shows distribution of normalized grain densities over NM neurons from six animals. The standard scores on both sides of each brain were calculated on the basis of the mean and standard deviation of grain densities on the contralateral normal side. The distribution of grain densities of NM neurons on the side ipsilateral to TTX injection (closed bars) can be divided into two populations. One population, with grain densities more than four standard deviations below the mean on the normal side, represents "unlabeled" samples. These neurons constitute about 20% of the neurons. Scale bar = 10 μm.

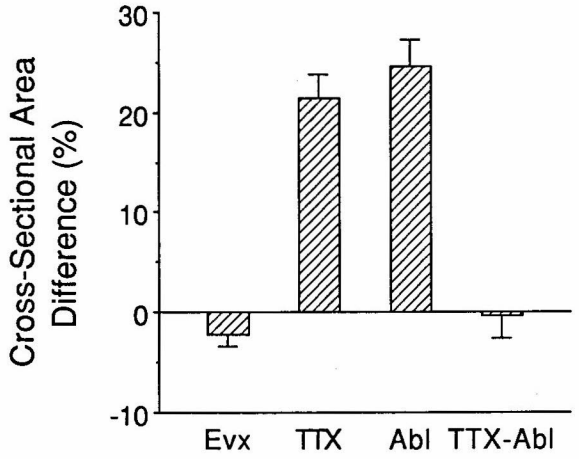


Figure 17 Mean percent difference in AVCN large spherical cell size between the manipulated ipsilateral and unmanipulated contralateral side of the brain. Positive values indicate smaller ipsilateral mean cross-sectional areas. Evx = Elvax alone; TTX = tetrodotoxin; Abl = cochlea ablation; TTX-Abl = ipsilateral TTX and contralateral cochlea ablation. From Pasic and Rubel (1989a).

In all of the in vitro experiments summarized here, protein synthesis, as measured by amino acid incorporation, has been used as the dependent variable. It will be recalled from the preceding section that this dependent variable is a rapid predictor of the final changes in neuron number and neuron size seen in NM. We, therefore, have made the assumptions that the regulation of protein synthesis seen in vitro reflects the same cellular events and intercellular communication process as observed after cochlea removal or eighth nerve activity blockade in vivo. Eventually, we will have to evaluate these assumptions by discovering other short-term indicators which predict the long-term events or by conducting long-term chronic stimulation experiments on cultured brain stem slice preparations. For the present description, however, it is important to understand that the goal of these experiments is to begin unraveling the *intercellular* signals involved in transneuronal structural and metabolic regulation rather than studying protein synthesis per se. That is, protein synthesis is used as a marker for postsynaptic change and our goal is to understand the transneuronal events leading up to such changes.

In the brain slice, the cochleae on both sides of the brain have been removed. In order to mimic the condition of a unilateral cochlea removal, the eighth nerve on one side of the slice is electrically stimulated (Fig. 20). After 1–3 h of unilateral orthodromic stimulation, tritiated amino acid is added to the bathing medium for ½ h, and protein synthesis is subsequently assessed by autoradiography. As in the in vivo experiments, levels of protein synthesis in neurons on the stimulated (analogous to cochlea intact) and unstimulated (analogous to cochlea removal) sides of the brain are compared.

Unilateral stimulation in vitro produces the same results as unilateral cochlea removal in vivo: stimulated neurons show greater protein synthesis. An example of this effect is shown in Fig. 21. This effect appears to require synaptic release since preventing release by maintaining the slice in a low Ca^{+2}/high Mg^{+2} medium also prevents the differ-

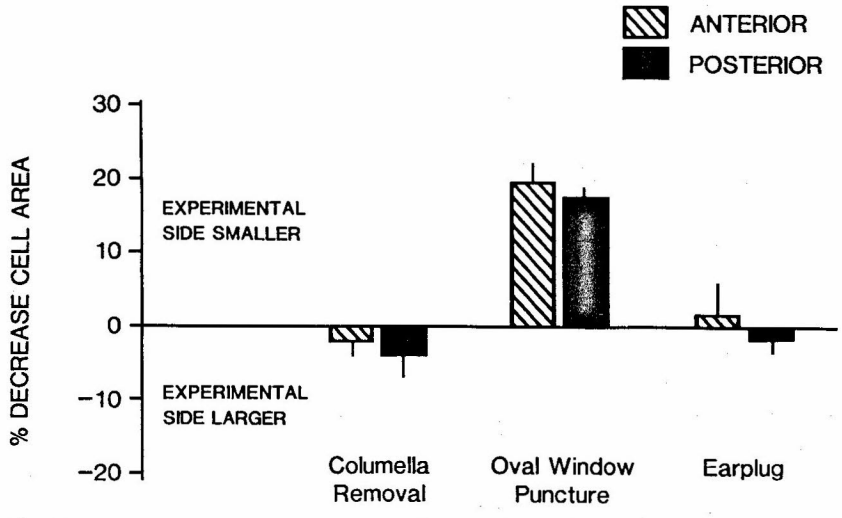


Figure 18 Mean percentage decrease in cell area following columella removal and oval window puncture in animals that survived 60 days. Percent decrease in cell area is 100 × (area contralateral − area ipsilateral)/area contralateral; positive numbers indicate that ipsilateral cells are smaller than those contralateral to manipulated ear. Mean scores are given for anterior and posterior portions of NM. Bars indicate SEM. From Tucci and Rubel (1985).

ence in protein synthesis between the "stimulated" and unstimulated sides.

To assess whether activity of the postsynaptic neuron is sufficient to up-regulate protein synthesis, NM neurons were antidromically activated by stimulating their axons as they approach midline (see Fig. 20). Thus NM neurons on one side of the slice are electrically active but are deprived of synaptic transmission from the eighth nerve. If action potentials of the postsynaptic neuron are sufficient to regulate protein synthesis, then one would expect that antidromically stimulated neurons would make more protein than unstimulated neurons. Surprisingly, antidromic activation actually resulted in reliably less synthesis by the stimulated cells (see Fig. 21). The mechanism responsible for this reduction in synthesis is unknown, but these data clearly indicate that postsynaptic action potentials are not responsible for the up-regulation of synthesis observed after unilateral orthodromic stimulation.

Together, these results suggest that afferent activity regulates the metabolic properties of the postsynaptic neuron through the action of some substance released from active auditory nerve terminals. The next question, then, is what is the nature of this substance?

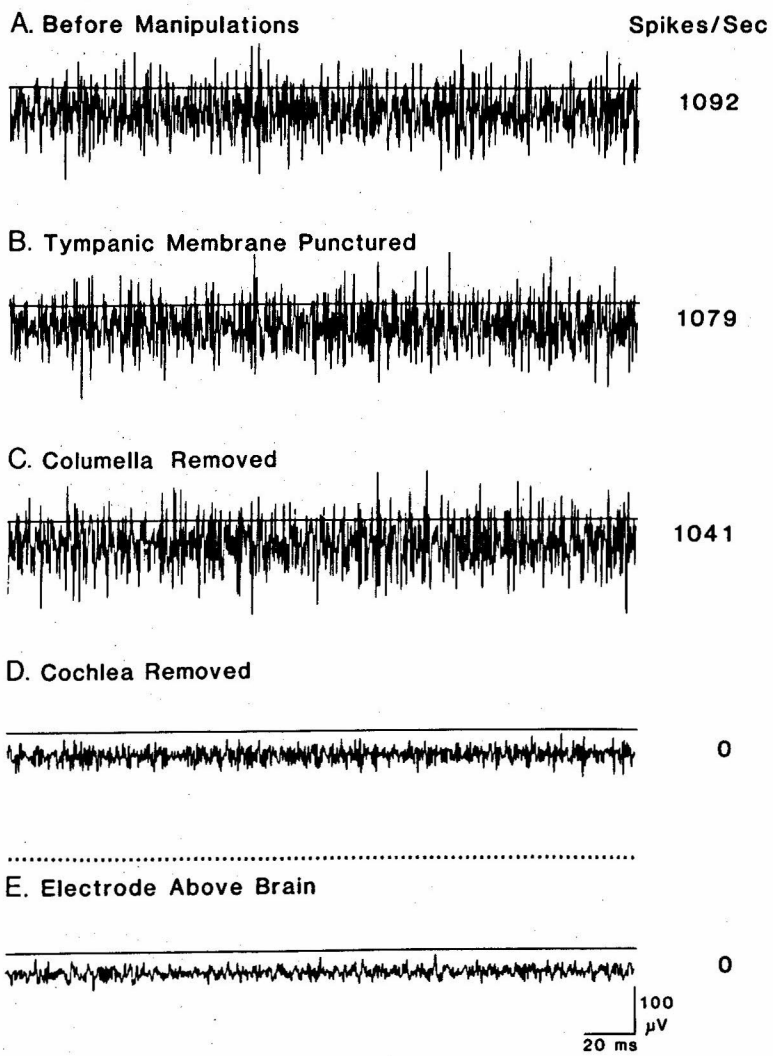


Figure 19 Recordings made in nucleus magnocellularis with a tungsten microelectrode. On the right are spike rates determined by counting the triggers from the pulse height discriminator. The trigger level is shown as the solid horizontal line in each trace. Each plot is of "spontaneous" activity, defined as activity recorded in a sound-attenuating room with sound delivery tubes in place. The traces show recordings after each procedure in one animal. (A) The plot of activity recorded in NM before any experimental manipulation. After puncturing the tympanic membrane (B) or removing the columella (C), there is no change in the level of spontaneous activity. By 1 min after cochlea removal (D) neuronal discharges recorded in NM cease. The potentials recorded following cochlea removal are of similar magnitude to those above the brain (E). From Born (1986).

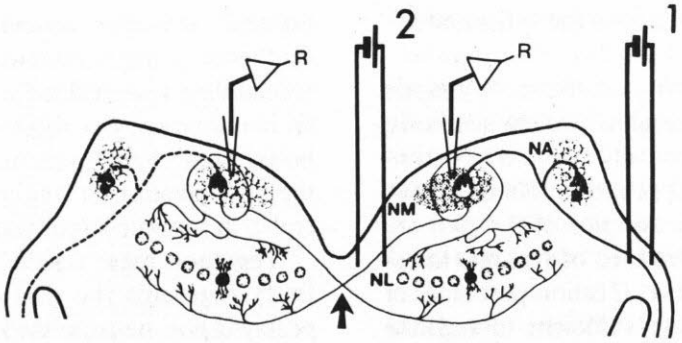


Figure 20 Schematic of the brain stem auditory system of the chick displaying the methodology for in vitro experiments. Neurons in NM are stimulated either orthodromically (1), via activation of the auditory nerve, or antidromically (2), via activation of their axons as they approach midline. Recording electrodes (R) are periodically placed on NM to monitor evoked activity. From Hyson and Rubel (1989).

One substance that could be involved in the metabolic coupling of presynaptic and postsynaptic elements is the neurotransmitter. The transmitter for the auditory nerve-NM neuron synapse is believed to be an excitatory amino acid (Jackson, Nemeth, and Parks, 1985; Martin, 1985; Nemeth, Jackson, and Parks, 1983; Nemeth, Jackson, and Parks, 1985). To assess if the action of excitatory amino acids is necessary for transneuronal regulation of protein synthesis, excitatory amino acid

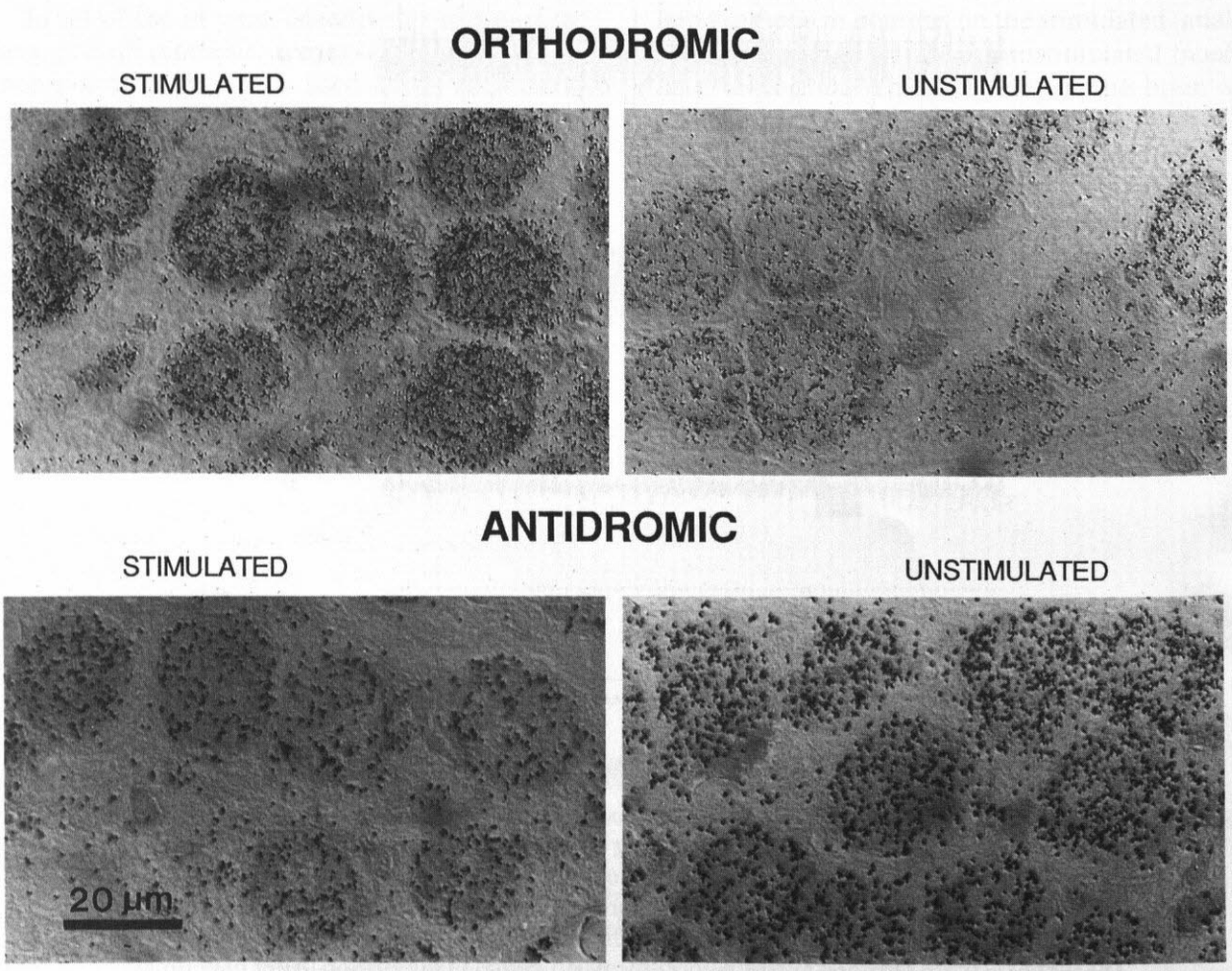


Figure 21 Autoradiographs of stimulated and unstimulated NM neurons. The top photomicrographs are taken from a slice stimulated orthodromically for 1.5 h; the bottom photomicrographs are taken from a slice stimulated antidromically for 1.5 h. In both cases, ^{3}H-leucine was added to the bath during the last 0.5 h. From Hyson and Rubel (1989).

receptors were blocked with the receptor antagonist kynurenic acid. Kynurenic acid blocks synaptic transmission in this system and also blocks the difference in protein synthesis between the stimulated and unstimulated sides of the slice (Hyson and Rubel, 1988). Thus, it appears that activation of some excitatory amino acid receptor(s) is necessary for this transneuronal regulation of cellular metabolism.

Although it is clear that excitatory amino acids are necessary signals for this form of transneuronal regulation, it is not known if they are sufficient. Additionally, kynurenic acid blocks both the NMDA and non-NMDA subtypes of excitatory amino acid receptors (Elmslie and Yoshikami, 1985; Ganong, Lanthorn and Cotman, 1983). Thus, we do not know the relative contributions of these different receptor types. Finally, it is not known what postsynaptic cellular events occur after receptor activation to produce these changes in cellular metabolism. The antidromic stimulation experiments suggest that the generation of action potentials has a negative effect on protein synthesis. Thus, some cellular event(s), requiring receptor activation, must overcome depression of synthesis resulting from the generation of action potentials and enhance synthesis beyond the level observed in unstimulated neurons.

SUMMARY

We have reviewed a series of experiments which begin to examine the cellular events underlying afferent regulation of neuronal structure. Our initial interest in such experiments stemmed from a desire to understand the cellular nature of experiential influences on brain development. While this remains a long-range goal, it's elusive nature has become increasingly apparent; how will we know when such a goal is achieved? On the other hand, it has become increasingly clear that by approaching this question as a subset of the larger problem of tissue interactions regulating nervous system structure and function, some progress is possible. In this respect, understanding afferent regulation is part and parcel of understanding "competition." Both exemplify the fact that we are dealing with a dynamic system, where changes in the *balance* of extracellular factors result in a cascade of events defining a new "steady state." Unfortunately, most of our methods are limited to taking "snapshots" of a few parameters and attempting to reconstruct an epic.

Our analyses of the postsynaptic events following cochlea removal have only scratched the surface. They are beginning to reveal myriad cellular processes that are dramatically altered by changing the balance of synaptic activity, or "synaptic drive," in a neuronal system. We have been continually struck by the rapidity of these postsynaptic changes when the manipulations are performed on immature animals. While the kinetics of metabolic and structural events we have studied do not yet match those of ionic events involved in information transmission, the two classes of intercellular communication are coming much closer. Some neuromodulators can alter synaptic currents for up to many seconds, and we have shown that altering afferent activity can cause changes in protein synthesis within a few minutes. The merging of these two classes of phenomena should come as no surprise since our studies and many others have definitively linked a variety of metabolic and structural events to changes in the synaptic drive between two neurons. On the other hand, this progress does highlight the need for increased attention to the short-term changes following manipulations of afferent activity. Hopefully such studies will lead to an understanding of the *intracellular chain of events* responsible for the regulation of neuronal form.

A second area of interest has been the age restrictions on the events we have studied. While it is well known that a variety of manipulations affect young animals more severely than adults, the age restrictions, or "sensitive periods," are usually assumed to be correlated with the maturation of synaptic connections or their "stabilization" (e.g., see Cowan, 1970). Our studies do not support this idea. It is true that the age at which we are first able to detect postsynaptic morphological changes following cochlea removal corresponds to the development of synaptic transmission (embryonic day 11–13; see Rubel and Parks, 1988). However, the sensitive period extends well past the maturation period of the auditory system. Chicks hear with adult sensitivity by the first week after hatching (Rubel and Parks, 1988) but the postsynaptic metabolic changes we have observed are as pronounced in 6-week-old birds as in newly hatched animals. Although the "sensitive period" extends well past the period of auditory system maturation, it does not extend into adulthood (recall that adult animals are largely immune to such effects). At this time we have little understanding of the cellular events responsible for the termination of this sensitive period but they may be systemic (i.e. related to puberty) rather than localized to specific neural structures. In addition, why neurons in adult animals are insensitive, or relatively insensitive, to deafferentation-induced

changes remains a mystery. Both of these problems provide important avenues for future research.

As noted above we have begun to generate a large list of metabolic and structural processes that are dynamically regulated by the afferent activity impinging on neurons or their individual dendrites. How these events and others are coupled in order to regulate cell death, cell size, dendritic growth, etc., also remains to be explored. Equally important is to begin evaluating the interaction of nonneuronal elements in processes such as transneuronal regulation and competitive interactions. The influence of activity on local blood flow has been demonstrated by our work and by others, but we still do not know the spatial or temporal constraints on this interaction. We have recently discovered rapid and dramatic changes in astrocyte processes in NM which appear to be regulated by the activity of eighth nerve axons or the postsynaptic neurons (Canady and Rubel, 1989). The proximal signals controlling glial cell structure and the interactions of glia cells with structural metabolic changes in neuronal elements remain unknown. It is clear, however, that these nonneuronal elements are serving a variety of regulatory functions in the development and maintenance of neural structure and function. The bidirectional interaction between these structures needs much more attention.

An area in which significant progress is being made involves the nature of signals underlying afferent regulation (see also Miller et al., 1989). It is clear from the experiments discussed above that the total amount of excitatory synaptic activity impinging on a cell is one regulatory event. Neither the time domain (i.e., the period over which the cell "averages") nor the shape of this function are known, however. In other situations, i.e., some competitive interactions, it appears that the temporal balance (or cross-correlation) of firing patterns between two or more afferents is critical. Again, the exact temporal kinetics are not well understood and are important in that they may shed light on the postsynaptic events that are being regulated. We have demonstrated that the cascade of events involved in synaptic transmission—calcium-activated release of transmitter, binding to a postsynaptic receptor, etc.—are involved in the transneuronal regulation of cochlear nucleus neurons; antidromic stimulation alone does not mimic orthodromic stimulation. Given these advances, the molecules, receptor structures, and second messenger systems underlying activity-regulated metabolic interactions should be readily accessible.

How, then, might the processes we are investigating, or those involved in competitive interactions, be involved in the dynamic regulation of nervous tissue underlying normal behavior? At this point we can only draw analogies between the results of manipulations we impose and the alterations of environmental events an organism experiences. Hopefully, integration of the cellular events underlying transneuronal interactions with a thorough understanding of the neuronal circuits underlying the behavioral repertoire will provide meaningful solutions. We are reminded of the sage advice given to Alice by the White Queen:

> "Why sometimes I've believed as many as six impossible things before breakfast"
>
> From *Through the Looking Glass,*
> by Lewis Carroll
> (Grossett and Dunlap, New York, 1983, p. 222).

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