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## Afferent influences on brainstem auditory nuclei of the chick: nucleus magnocellularis neuronal activity following cochlea removal

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Elimination of presynaptic elements often results in marked changes, such as atrophy and death, in postsynaptic neurons in the central nervous system. These transneuronal changes are particularly rapid and profound in young animals. In order to understand the cellular events underlying transneuronal regulation it is necessary to explore changes in the local environment of neurons following manipulations of their afferents. In previous investigations we have documented a variety of rapid and marked cellular changes in neurons of the cochlear nucleus of neonatal chicks (n. magnocellularis) following cochlea removal. In adult chickens, however, these transneuronal changes are either absent or minor. The goals of the studies presented here were to examine changes in the electrical activity of nucleus magnocellularis cells and their afferents following removal of the cochlea and to determine if these changes were similar in adult and neonatal animals. Two measures of electrical activity were used; multiunit recording with microelectrodes and incorporation of radiolabeled 2-deoxyglucose (2-DG). Microelectrode recordings revealed high levels of spontaneous activity in n. magnocellularis and n. laminaris, the binaural target of n. magnocellularis neurons. Neither puncturing of the tympanic membrane nor removal of the columella causes significant changes in spontaneous activity, although the latter results in a profound hearing loss (40–50 dB). Removal of the cochlea, on the other hand, results in immediate cessation of all extracellular electrical activity in the ipsilateral n. magnocellularis. Recordings from the same location for up to 6 h failed to reveal any return of spontaneous activity. When the electrode tip was placed in n. laminaris, unilateral cochlea removal had no discernible effect on extracellularly recorded spontaneous activity, probably due to the high levels of excitatory input from the intact ear. Bilateral cochlea removal, however, completely eliminated activity in n. laminaris. 2-DG studies conducted 1 h to 8 days following unilateral cochlea removal revealed marked decreases in 2-DG incorporation in the ipsilateral n. magnocellularis and bilaterally in the n. laminaris target of the ablated cochlea. No compensatory return of 2-DG incorporation was observed for up to 8 days. Comparisons of adult and neonatal chicks failed to reveal significant differences in the effects of cochlea removal on multiunit activity or 2-DG incorporation, suggesting that age differences in transneuronal regulation are due to intrinsic biochemical differences in young and adult neurons rather than differences in the proportion of synaptic input that has been abolished.

### INTRODUCTION

When neurons in the central or peripheral nervous system are damaged, there are profound effects not only on the injured neurons, but also on the postsynaptic targets<sup>6,9,37</sup>. Some of the postsynaptic consequences of damage to presynaptic elements include neuron death, soma shrinkage, cytoskeletal disruption and altered oxidative enzyme levels<sup>10,15,28,43</sup>. In general, the severity of these transneuronal changes varies markedly with the age of the animal. When afferents are eliminated before functional synaptic connections are established, transneuronal degeneration and atrophy are not seen until the time at which normal synaptic function is established, and then are usually profound<sup>14,17,21</sup>. In young animals, when

synaptic connections are established but the nervous system is still immature, removal of the primary excitatory afferent results in rapid and profound transneuronal effects. In adult animals, however, the same manipulation usually produces less dramatic effects and often few or no transneuronal changes are observed by conventional methods<sup>3,12,15</sup>.

To determine what factors may be responsible for the postsynaptic changes, it would be useful to know what features of the neuron's environment change as a result of the injury to the presynaptic element and how these features may change as a function of age. One aspect of the presynaptic input thought to be altered by injury is the number or pattern of action potentials relayed to the postsynaptic neuron<sup>9</sup>. To examine how changes in action

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potentials reaching the postsynaptic target may contribute to the morphologic or metabolic changes, it is necessary to know the neuronal activity at the target at the time of the injury. This information is known only in limited cases for adult animals<sup>8,27</sup> and direct comparisons between adults and neonates have not been previously made. Thus it would be useful to examine neural activity reaching the postsynaptic neurons at the time of damage to their presynaptic innervation.

The primary approach to studying neuronal electrical activity is through the use of microelectrodes. Depending on the type of microelectrode and its location, this technique can detect voltage changes reflecting individual or summed action potentials, synaptic potential changes, or other subcellular potentials. This approach requires anesthesia and surgical preparation to gain access to the neurons of interest. The method is limited further by the small area and number of sites which can be examined.

Another indirect approach to studying changes in neuronal activity is the 2-deoxyglucose (2-DG) technique<sup>39</sup>. Since its introduction, this method has been used for mapping brain areas activated by sensory stimuli, providing information on patterns such as tonotopic organization<sup>34</sup> and visual columnar organization<sup>33</sup>. The technique provides these data without special preparation of the animal. However, 2-DG labeling provides information about only one, relatively long (45 min) time period and is thought to be best correlated with sodium pump activity, rather than action potentials, per se<sup>20</sup>.

Brainstem auditory neurons in the chick have been used to study the postsynaptic consequences of injuring or removing presynaptic neurons<sup>28</sup>. Removing the cochlea from hatchling chickens up to 6 weeks of age produces a dramatic reduction in protein synthesis, alterations in metabolic pathways and 30% neuron loss in second order neurons of nucleus magnocellularis<sup>3,7,40</sup>. Adult animals greater than one year of age do not show such morphologic or metabolic alterations after removing the cochlea.

The 2-DG technique demonstrated marked reductions in glucose uptake in nucleus magnocellularis (NM) following cochlea removal in hatchling birds<sup>13,19</sup> providing indirect evidence for decreased activity in NM. It is possible that NM neurons in adult birds respond differently to cochlea removal because activity changes are not the same as in hatchling birds. No data are available regarding 2-DG changes in adult birds, nor have any direct measurements been made of electrophysiological activity in NM in hatchling or adult birds during or after cochlea removal.

In the present study we examined neuronal activity recorded with microelectrodes during and after cochlea removal and 2-DG uptake after cochlea removal in the brainstem auditory nuclei of hatchling and adult birds.

Immediately following cochlea removal, neuronal activity in nucleus magnocellularis ceases. We also studied nucleus laminaris (NL), which is the only target of NM neurons<sup>23,45</sup>. The results in NL are consistent with our finding that NM neurons no longer generate action potentials after cochlea removal. The changes in electrical activity and 2-DG metabolism were similar in hatchling and adult birds. We conclude that all excitatory input to NM originates in the cochlea, and that NM is not spontaneously active.

## MATERIALS AND METHODS

### *Subjects*

White Leghorn chickens used for these studies were either 1–3 ( $n = 21$ ) or 66–68 ( $n = 12$ ) weeks of age. These animals will be referred to as 'hatchling' and 'adults', respectively. For the hatchlings, eggs were obtained from a local supplier (Truslow Farms, Rockingham, MD) and were incubated in a forced draft incubator in our laboratory. After hatching, chickens were housed in communal brooders with free access to food and water. The adults were purchased from the same supplier and housed in individual cages with free access to food and water.

### *Electrophysiological studies*

*Surgery.* Unilateral and bilateral cochlea removals were performed on anesthetized chickens while a microelectrode monitored neuronal electrical activity in brainstem auditory areas. Surgical preparations and electrophysiological recording procedures were similar to those described previously<sup>18,29,42</sup>. Briefly, birds were anesthetized with ketamine hydrochloride (80 mg/kg, i.m.) and Chloropent (1.5 ml/kg, i.p.; Fort Dodge Laboratories, Inc.) or sodium pentobarbital (0.5 mg/kg, i.p.). Atropine sulfate (0.01 mg/kg, i.m.) was administered to inhibit tracheal secretions. In addition, a tracheostomy was performed and a tube was placed in the trachea to maintain an adequate airway. The animal then was secured in a specially designed headholder within a double-walled sound-attenuating room (IAC). Body temperature was maintained at 40 °C. Supplementary doses of ketamine (30 mg/kg) and Chloropent (0.5 ml/kg) or pentobarbital (0.17 mg/kg) were given as needed to maintain a consistent level of anesthesia, indicated by the lack of nociceptive responses.

Exposure for recording was made by removing the skull overlying the cerebellum. In most cases the cerebellum was aspirated in order to expose the floor of the fourth ventricle. The eighth nerve fibers then could be seen coursing rostromedially over the medulla. The brainstem was covered with warm mineral oil. The skin and cartilage of the external ear were removed to permit access to the middle and inner ear structures. Hollow tubes, which were used to deliver sounds to the ears, were sealed to the external auditory canal.

*Recording procedures.* Recordings in nucleus magnocellularis and nucleus laminaris were made with glass-insulated tungsten microelectrodes (1–4 M $\Omega$  impedance). This electrode typically recorded from a small 'cluster' of cells and/or terminals. The output of the microelectrode, with reference to a grounded neck muscle, was amplified, filtered to pass 0.4–10 kHz, viewed on an oscilloscope, and led to a pulse height discriminator. The amplified signal was also recorded by a 4-channel tape recorder (Racal, Inc.) on magnetic tape for later analysis. The output of the pulse height discriminator was led to a PDP 11/23 computer for compiling spike counts. At the start of each experiment the pulse height discriminator was set such that the trigger level was immediately above the electrical noise level of our recording system and produced rare triggers. The pulse height discriminator level was then left at this level for the duration of the experiment in order to obtain an objective measure of

multiunit activity before, during, and after each manipulation. Electrical noise was defined as the potentials recorded with the microelectrode tip in the cerebrospinal fluid (CSF) above the brainstem.

Tones were delivered via a closed sound system attached to the hollow ear tubes<sup>42</sup>. The sound delivery system was calibrated on line at the beginning of each experiment through a probe tube inserted at the ear canal entrance. After setting the pulse height discriminator, the microelectrode was then lowered into the medulla and the auditory nuclei were located by their characteristic response to pure tones. Spontaneous discharges of the auditory neurons were monitored. Spontaneous activity was defined as the level of electrical activity recorded with the animal in the sound-attenuating room when no auditory stimulus was present. Spike rate was defined as the number of triggers generated by the pulse height discriminator set as described above. Spikes were counted in 1-s bins and the average number of spikes/s (spike rate) for 100 bins was calculated by the computer.

**Cochlea removal.** Neuronal discharges from auditory brainstem nuclei were measured before, during, and after: (1) unilateral tympanic membrane puncture; (2) columella removal; and (3) cochlea removal (see Fig. 1). First the tympanic membrane was punctured with forceps, exposing the columella. Next the columella was removed, permitting access to the oval window. Finally, the

cochlea (basilar papilla) was removed via the oval window using forceps. After each manipulation the spontaneous and sound-evoked spike rates were measured. In some animals the level of activity was monitored for extended periods following the cochlea removal. After 1–10 h, either a similar procedure was performed on the contralateral ear or the animal was treated as described below (Histological processing).

Changes in spontaneous spike rate were quantified by comparing the number of spikes/s before any manipulation to the spike rate following each manipulation. The pre-manipulation 'baseline' for each recording site was determined by averaging 3 samples, each of which was 100 s in duration.

**Histological processing.** At the end of each experiment, a small lesion was placed at the site of the recording electrode. The animal was then given an overdose of sodium pentobarbital and transcardially perfused with 10% buffered formalin. The head was placed in fix and after 1 week the brain was removed, dehydrated and embedded in paraffin. Coronal sections were cut at 10  $\mu$ m and mounted on slides. Thionin-stained sections were examined to verify the location of the lesion.

#### 2-DG studies

**Cochlea removals.** Cochlea removals were performed as previously described<sup>3</sup>. Chickens were anesthetized using ketamine

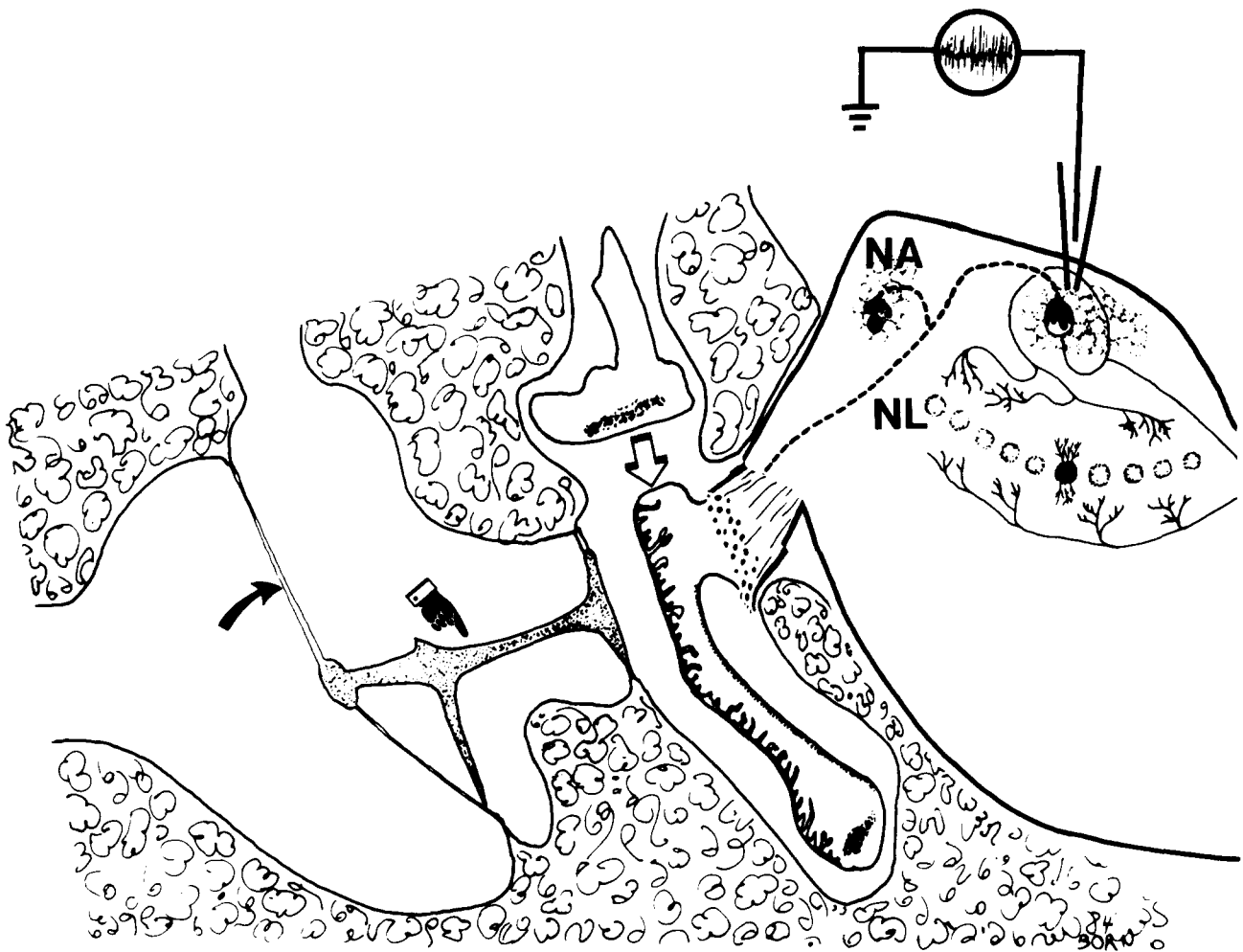


Fig. 1. Schematic depicting peripheral and brainstem auditory structures in the chicken. The electrode is indicated in nucleus magnocellularis (NM). Recordings were made to define the spike rate before any manipulation and then following puncturing the tympanic membrane (filled arrow), after removing the columella (hand pointer), and after removing the cochlea (open arrow). We also recorded in nucleus laminaris (NL), the only target of NM neurons, which receives bilateral input. NA, nucleus angularis.

hydrochloride (80 mg/kg, i.m.) and Chloropent (1.5 ml/kg, i.p.; Fort Dodge Laboratories, Inc.). Atropine (0.01 mg/kg, i.m.) was given to inhibit tracheal secretions. A small incision was made in the external ear and then in the tympanic membrane. The columella was removed using fine forceps, exposing the oval window. Finally the cochlea (basilar papilla) was removed using forceps. A piece of gelfoam was placed at the oval window to control any minor bleeding. The cochlea was floated on water and examined to verify complete removal.

**Isotope preparation and injection.**  $^{14}\text{C}$ -labeled 2-deoxyglucose (2-deoxy-D-[ $^{14}\text{C}$ ]glucose, spec. act. 45–55 mCi/mmol, New England Nuclear) was dried and reconstituted with normal saline to a concentration of 200  $\mu\text{Ci/ml}$ . At either 4–6 h or 1 or 8 days following cochlea removal, the 2-DG was administered. For hatchlings, the isotope was injected intraperitoneally (17  $\mu\text{Ci}/100\text{ g}$ ; 50  $\mu\text{Ci/ml}$  solution), and for adults isotope was injected intravenously (50  $\mu\text{Ci}/\text{kg}$ ; 200  $\mu\text{Ci/ml}$  solution). The animals were anesthetized prior to injection of isotope and remained somnolent during exposure to the controlled acoustic stimulus.

**Acoustic stimulation.** For 45 min after isotope injection the animals were placed in a double-walled sound-attenuating chamber and were exposed to tape-recorded music (Shostakovich's Fifth Symphony) at approximately 75 dB SPL. Stimulus intensity was determined at the position of the animal's head using a sound level meter (Bruel and Kjaer 2203) and a 1-inch microphone.

**Tissue preparation and autoradiography.** At the end of the acoustic stimulation period the animal was deeply anesthetized and briefly perfused transcardially with 10% phosphate buffered formalin (pH 7.4). The brain was rapidly removed and immediately frozen in heptane cooled in dry ice. Brains were stored at  $-80\text{ }^\circ\text{C}$  in closed vials until sectioning. Sections through the brainstem auditory nuclei were cut at 20  $\mu\text{m}$  on a cryostat at  $-22\text{ }^\circ\text{C}$  and thaw-mounted onto chrome alum-subbed coverslips at  $55\text{ }^\circ\text{C}$ . Coverslips were mounted onto cardboard and were exposed to X-ray film (Kodak SB5 or XTL) for 1 week to 3 months. X-ray films were developed with GBX developer (Kodak). After acceptable films were obtained, sections mounted on coverslips were stained with thionin, dehydrated, and affixed to slides with DPX.

**Autoradiographic analysis.** Film autoradiograms were analyzed using the Bioquant IV image analysis system (R&M Biometrics). For each brain, two sections at a point located 50% of the anterior-to-posterior extent of each nucleus were selected for measurement. Since nucleus magnocellularis lies more caudal than nucleus laminaris, the sections used for measurement were different for each nucleus. Nuclear boundaries and section edges were traced over the image of each thionin-stained section and this overlay image was stored in the computer. Using the section edges on the overlay, the overlay and the image of the corresponding film were aligned, and the average optical density (OD) of each outlined nucleus was determined. Optical density ratios were calculated for comparisons among animals<sup>35</sup>. For NM, comparisons were made between NM ipsilateral and contralateral to cochlea removal. For NL, the areas of termination of inputs dorsal and ventral to the cell body lamina were compared. To ensure that the intensity of the autoradiogram was within the linear range of the film, calibrated [ $^{14}\text{C}$ ]methylmethacrylate standards with a range of radioactivities were exposed to the film along with the brains. Only those densities that fell within the linear range of the standards were included in the analysis.

## RESULTS

### Electrophysiology

We recorded from both NM and NL before any experimental manipulation, subsequent to puncturing the tympanic membrane, after removing the columella, and after cochlea removal. From the histological reconstruc-

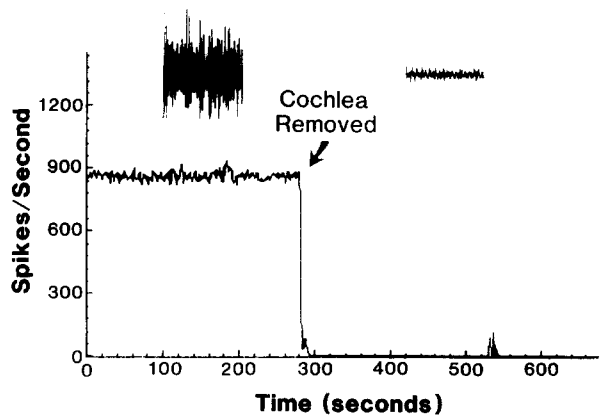


Fig. 2. Time course of changes in neural discharges recorded in NM during cochlea removal in a hatchling. The spike rate computed by the computer from the output of the pulse height discriminator is plotted as a function of time. Representative 100 ms oscilloscope traces are shown above the graph. There is a steady, high level of activity recorded in nucleus magnocellularis even when no specific acoustic stimulus is presented. Immediately following cochlea removal (arrow) the spike rate precipitously falls, such that within 15–30 s no more discharges are recorded. No change in the level of activity was found for up to 6 h after cochlea removal. The small fluctuation in spike rate at 530 s occurred when one of the investigators entered the sound-attenuated room.

tions the position of the microelectrode tip was determined. Data from chickens in which lesions were not located within the boundaries of NM or NL are not included in the analyses. In hatchlings, microelectrode lesions were located in NM in 7 birds, NL in 4 birds, and elsewhere in 3 birds. In adults, lesions were found in NM in 5 birds, in NL in 2 birds, and elsewhere in 2 birds. Recording sites within the auditory nuclei were located throughout their posterior-to-anterior extent. The characteristic frequencies found at these sites ranged from 900 to 3000 Hz and were consistent with the known tonotopic organization of the chick auditory nuclei<sup>29</sup>. The results from recording in NM will be presented first, followed by those from NL. In a final section we present the results from procedures used to examine the contribution of tissue damage caused by the electrode to the observed changes.

### Nucleus magnocellularis

In both hatchling and adult chickens there is a high level of spontaneous activity recorded in nucleus magnocellularis. Fig. 2 plots the 'spike rate' under these conditions and illustrates multiunit activity as recorded in one hatchling animal. Prior to any manipulation, the average multiunit spike rate ( $\pm$  S.D.) for hatchling animals was  $1127 \pm 71$  spikes/s. There was no reliable change in spontaneous spike rate following tympanic membrane puncture or columella removal. Fig. 4 shows these changes as a percent of the baseline activity. As

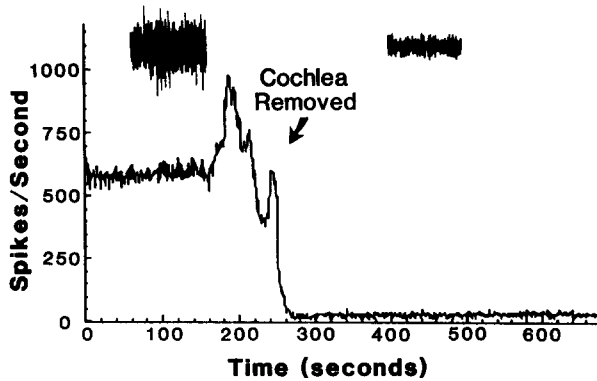


Fig. 3. Time course of changes in neuronal discharges recorded in NM during cochlea removal in an adult bird. All conventions as in Fig. 2. Note high level of spontaneous activity and precipitous fall following cochlea removal as in hatchlings. The large fluctuations in 'spontaneous rate' just prior to cochlea removal occurred when the forceps touched the oval window. This artifact was observed in approximately 25% of cases.

seen in Fig. 2, removing the cochlea instantly abolishes recordable unit activity in NM. One min after the cochlea was removed there was only a small difference between electrical traces recorded with the electrode tip in NM and those recorded with the electrode tip in CSF above the brainstem. This change represents a 99% decrease compared to baseline activity (Fig. 4). We recorded with the electrode in the same position for up to 6 h without any subsequent return of neural activity.

In adult chickens our manipulations prior to cochlea removal also produced no change in recorded electrical activity. The average multiunit spike rate for adult

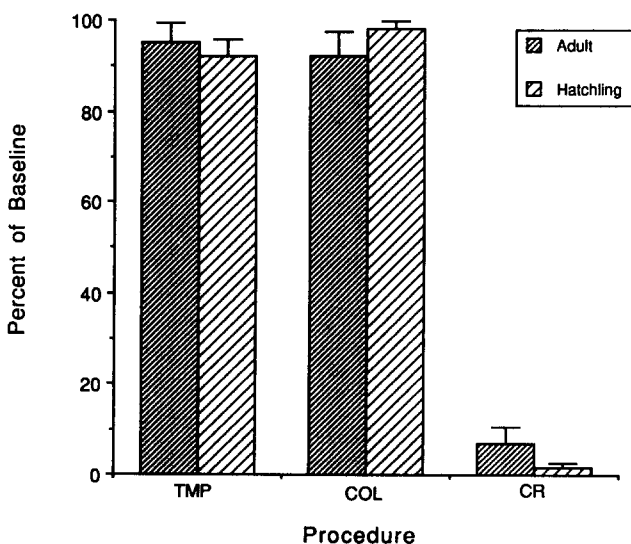


Fig. 4. Percent of pre-manipulation baseline spike rate in hatching and adult chickens following each of the manipulations indicated. Error bars indicate standard error of the mean. The baseline was the average of three 100-s intervals and after each manipulation a single 100-s interval is used. TMP, tympanic membrane puncture; COL, columella removal; CR, cochlea removal.

animals was  $1467 \pm 94$ . Fig. 3 illustrates our results for a single animal as was plotted for a hatchling chicken in Fig. 2. As was the case in hatchlings, removing the cochlea produced a dramatic reduction in the electrical activity. As shown in Fig. 4, for 3 animals the average change expressed as a percent of baseline was greater than 95%.

#### Nucleus laminaris

Nucleus laminaris is the only target of nucleus magnocellularis neurons<sup>23,45,46</sup> and each NL neuron receives input from NM on both sides of the brain (Fig. 1). Therefore we recorded in NL to assess whether changes in neuronal activity in NM after cochlea removal are reflected in NL activity. Representative results from one animal are shown in Fig. 5. Removal of one cochlea causes no change in the rate of spontaneous activity

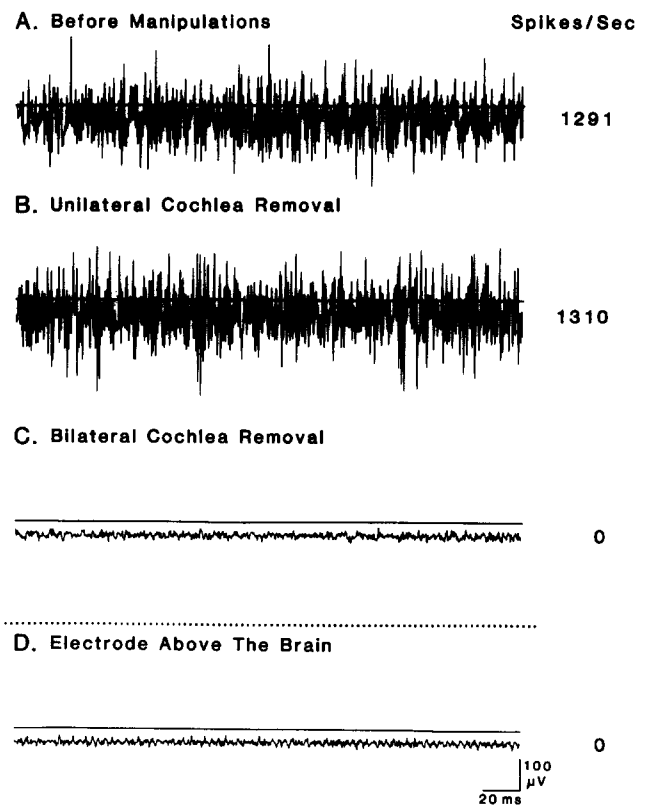


Fig. 5. Digitized recordings from NL of a neonatal chicken before any experimental manipulation and after removal of each cochlea. The spike rate compiled by the computer from the output of the pulse height discriminator is shown at the right. The solid line above each trace shows the discriminator voltage level. The neuronal activity in NL recorded under these conditions is a sustained high level of activity (A). Removing the ipsilateral cochlea causes no apparent change in the level of neuronal activity (B). Apparently the input from the intact contralateral cochlea sustains the level of recorded neuronal activity. When the contralateral cochlea is subsequently removed, neuronal discharges in NL cease (C). The level of the electrical activity is comparable to that seen with the tip of the electrode above the brain as described in Materials and Methods (D).

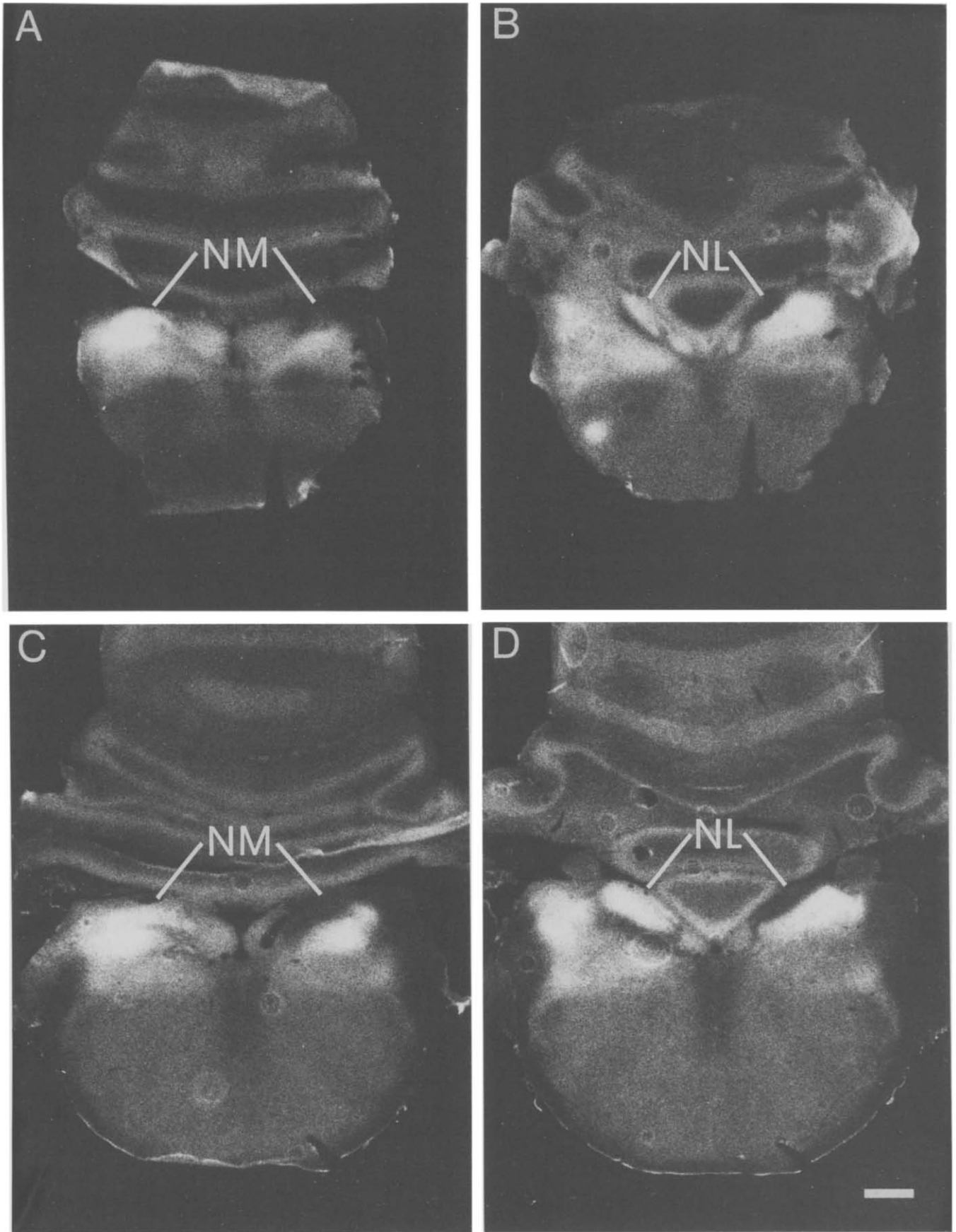


Fig. 6. Representative 2-DG autoradiograms from a hatchling (top) and an adult (bottom) animal undergoing 2-DG administration 4–6 h after cochlea removal. Images are reversed such that white areas indicate 2-DG label. Notch in right ventral brainstem indicates side of cochlea removal. Note decreased label in ipsilateral NM (A,C) and ipsilateral dorsal and contralateral ventral NL (B,D) in both animals. Boundary between high and low levels of label in NL occurs at the cell body lamina. Bar in D = 1.0 mm.

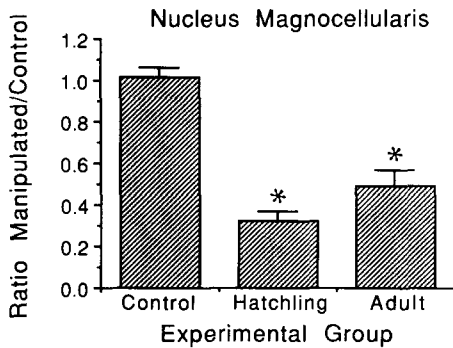


Fig. 7. Average ratios of optical density measurements ( $\pm$  S.E.M.) in ipsilateral (manipulated) to contralateral (control) NM for 3 groups. For unoperated control animals, ratio of right to left NM was used. Note similar decreases in hatchlings and adults following cochlea removal. Asterisks indicate that average ratios are reliably different from controls.

recorded in ipsilateral or contralateral NL (compare Fig. 5A and B). Neuronal activity in NM contralateral to cochlea removal probably accounts for the continued high level of activity in NL. Bilateral cochlea removal, however, has a dramatic effect on spontaneous and sound-evoked neuronal activity in NL. Immediately after removing the second cochlea, no spikes were recorded in NL (Fig. 5C) and the activity was comparable to that recorded with the electrode tip in the CSF above the brainstem. There was no further change in recorded potentials for up to 10 h after the bilateral cochlea removal.

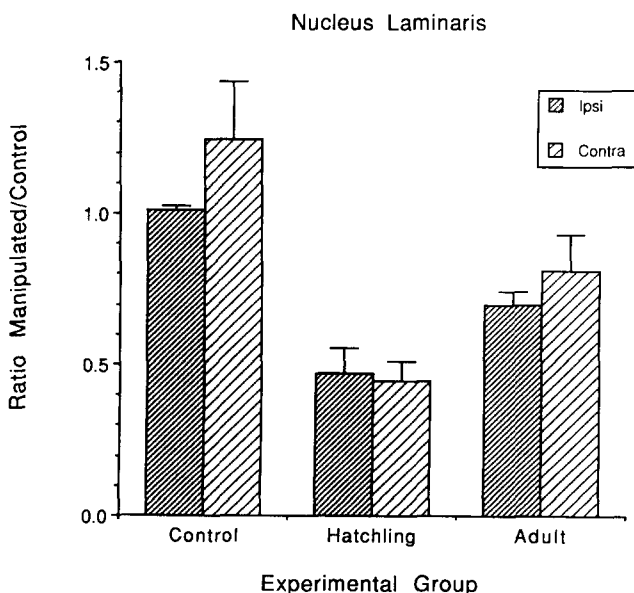


Fig. 8. Average ratios of optical density measurements in ipsilateral and contralateral NL after cochlea removal. Greater decreases are seen hatchlings than adults.

### Control procedures

We considered several alternative explanations for the decline in neuronal activity recorded in NM following cochlea removal. It is possible that movement of the animal relative to the electrode contributed to the change. In addition to results from steps prior to cochlea removal, where no change in spike rate was observed during manipulations of the ear, some specific control procedures were tested. The cochlea was removed while recording in the contralateral NM. This manipulation produced no change in spike rate in the 3 hatchlings and 1 adult chicken in which it was performed. It is also possible that tissue damage caused by the electrode contributed to the decreases in neuronal activity we observed. To examine this possibility, we recorded from NM for up to 12 h without performing any manipulations. The results of these long-term recordings revealed no change in activity during chronic electrode placement. For one animal in which activity was measured for 12 h before any experimental manipulation was performed, activity was found to vary between 834 and 1243 spikes/s, with a mean ( $\pm$  S.D.) of  $1010 \pm 163$ . Activity was found to increase somewhat during 3 h of recording in one animal, following columella removal, from 1566 to 1627 spikes/s, with an average spike rate of  $1579 (\pm 34.2)$  spikes/s during that period. Finally, at the end of each experiment, recordings made within a  $100\text{-}\mu\text{m}$  region on either side of the original recording site in NM or NL confirmed that activity changes, when observed, were not limited to the original recording site.

### 2-Deoxyglucose

The 2-DG method provides an alternative, although indirect, means of addressing changes in neuronal activity after cochlea removal. Fig. 6 shows representative autoradiograms of NM and NL from both hatchling and adult animals. As described previously<sup>13,19</sup>, cochlea removal in hatchlings causes a reduction in 2-DG labeling both in the ipsilateral NM (Fig. 6A) and in the neuropil regions of NL receiving input from denervated NM (Fig. 6B). Following cochlea removal in adult birds, reductions in 2-DG labeling qualitatively similar to those in hatchlings are observed both in NM (Fig. 6C) and NL (Fig. 6D). For both hatchlings and adults, the reductions in NM and NL labeling were similar for survival times either hours or days after cochlea removal.

In order to quantitatively compare these 2-DG changes among animals, for each animal we calculated the ratio of optical density (OD) in manipulated versus control regions for each nucleus and averaged these ratios among animals in a group. Results for NM are shown in Fig. 7. In control animals, no asymmetry in 2-DG uptake exists and OD ratios approach 1. Slightly

greater reductions in 2-DG uptake are observed in hatchlings than in adults. Statistical analysis of NM 2-DG ratios shows a significant effect of group (Kruskal–Wallis  $P < 0.02$ ). Post-hoc pairwise comparisons (Mann–Whitney  $U$ ) show that ratios of both hatchlings and adults are reliably lower than controls ( $P < 0.02$  and  $P < 0.01$ , respectively) and that the slight difference between hatchling and adult ratios following cochlea removal is reliable ( $P < 0.05$ ).

Ratios for NL ipsilateral and contralateral to cochlea removal are shown in Fig. 8. In control animals, no asymmetry in 2-DG uptake exists and OD ratios approach 1. In young animals, a similar decrease in labeling occurs on both sides of the brain in the neuropil region of NL receiving input from the deafferented NM. In adult animals, decreases in labeling are less pronounced than in hatchlings, but again they are similar in magnitude on the two sides of the brain. Statistical analysis of NL 2-DG ratios (Kruskal–Wallis) shows a significant effect of group both ipsilateral ( $P < 0.02$ ) and contralateral ( $P < 0.02$ ) to cochlea removal. Post-hoc pairwise comparisons (Mann–Whitney  $U$ ) in ipsilateral NL show that ratios are reliably lower than those in control animals for both hatchlings ( $P < 0.01$ ) and adults ( $P < 0.02$ ); differences between adults and hatchlings are not statistically significant ( $P > 0.10$ ). In contralateral NL, ratios in hatchlings are significantly different from controls ( $P < 0.01$ ) but those for adults only approach reliability ( $P < 0.07$ ). Ratios in young animals are reliably lower than those in adults ( $P < 0.02$ ).

## DISCUSSION

The principal finding of the present study is that removal of the cochlea leads to an immediate cessation of all action potentials recorded in the ipsilateral nucleus magnocellularis. We also recorded in nucleus laminaris, the only target of NM neurons. Unilateral cochlea removal causes no change in recorded electrical potentials in NL; however, bilateral cochlea removal reduced the recorded potentials to the level of the electrical noise. From these data, we conclude that action potentials in NM neurons are eliminated following cochlea removal. In the discussion that follows, we first consider several alternative explanations for these findings. Next, our data are examined in light of other anatomical and physiological properties of chicken brainstem auditory pathways. Finally, we compare our results to those from other studies examining the effect of cochlea removal.

### *Methodological considerations*

The results clearly indicate that cochlea removal produces an immediate, dramatic change in neuronal

activity. There are two alternative interpretations of this observation that must be considered before we can conclude that cochlea removal eliminates electrical activity in NM neurons. First, it is possible that neurons in nucleus magnocellularis continued to be active after cochlea removal but we failed to detect this activity. For example, this could happen if the recording electrode had been displaced from nucleus magnocellularis. Second, the elimination of activity in NM may have resulted from tissue damage caused by the electrode itself.

The recording procedures used in this study were chosen to minimize the possibility that we would fail to detect active neurons. The tungsten microelectrodes were constructed to record from a large volume of tissue rather than to isolate single units. Compared to the electrical noise level, the recordings in NM and NL were characterized by high amplitude, high frequency voltage changes characteristic of many presynaptic *and* postsynaptic elements. Given these properties, it is unlikely that we would fail to detect active neurons following cochlea removal.

Another alternative explanation for the failure to record neuronal activity in NM after cochlea removal is movement of the brain to the electrode. The headholder was designed to minimize movements of the head during the experiment. There were no detectable movements during any manipulation. In addition, some of our results are inconsistent with the possibility that movement of the animal's head contributed to the reduction in neural activity. Removing the columella is the most difficult procedure, yet there was no change in spontaneous activity at this stage of the experiment. Also, removal of the contralateral cochlea has as much potential for moving the animals' head as removal of the ipsilateral cochlea; however, ongoing activity and rate-intensity functions from NM were not altered by removal of the contralateral cochlea. Taken together, these results suggest that our electrode stayed in the original recording site. The second alternative explanation for the cessation of neuronal activity following cochlea removal is damage caused by the electrode itself. We tested this possibility by recording at one site for up to 12 h without removing the cochlea. No substantial changes in spontaneous or sound-evoked activity in NM were observed.

One final methodological consideration should be mentioned. When recording in NM with extracellular electrodes, the contribution of presynaptic and postsynaptic events cannot be distinguished. Our finding that *all* action potentials cease following cochlea removal suggests that action potentials have ceased in both the eighth nerve terminals and the postsynaptic NM neurons. However, it is possible that action potentials in NM neurons could be generated at a site remote from our



recording electrode. We examined the output of NM neurons by recording from the third order neurons in NL<sup>1,23,45</sup>. Bilateral cochlea removal completely silences this third order nucleus. This finding confirms our conclusion that NM neurons have ceased firing.

#### *Anatomical and physiological correlations*

Following cochlea removal all signs of neuronal activity in NM cease. This cessation of activity implies that all excitatory input to NM originates in the cochlea. Numerous studies indicate that the eighth nerve provides the major excitatory input to NM<sup>11,24,29,31</sup>. In the chicken<sup>23</sup>, blackbird<sup>31</sup>, and barn owl<sup>41</sup>, units in NM show primary-type response patterns to pure tone stimuli. These are similar to those found in the auditory nerve<sup>30</sup> and are characterized by an initial rapid increase in firing rate at the onset of a tone, followed by a decrease to a sustained level<sup>29</sup>. Considering how well the neurons of nucleus magnocellularis follow the pattern of activity in the eighth nerve, it seemed reasonable that the major excitatory input was from the eighth nerve. Supporting the findings made with extracellular microelectrodes *in vivo* are intracellular recordings made from NM *in vitro*<sup>11</sup>. Stimuli applied to the eighth nerve produce large amplitude, short latency excitatory postsynaptic potentials (EPSPs).

Although cochlea removal completely eliminates excitatory activity in NM, at least two other inputs to NM have been described in anatomical studies. Following cochlea removal the large primary endings, the end bulbs of Held, degenerate<sup>22</sup>. Two morphologically distinct terminals remain on NM neurons. One class of terminals is characterized by symmetrical synaptic densities and dense core synaptic vesicles. This morphology is generally attributed to inhibitory synapses<sup>25</sup>. Evidence from immunocytochemical studies using antisera to GAD and to GABA suggest that these terminals use GABA as their neurotransmitter<sup>5</sup>. It is possible that this input contributes some tonic inhibition following cochlea removal and may account, in part, for the complete lack of action potentials in NM. This possibility could be investigated by microiontophoretic application of the GABA antagonist bicuculline onto NM neurons following cochlea removal<sup>36</sup>.

The second distinct morphological class of terminals seen in electron microscopic analyses of NM are small round terminals with asymmetric synaptic densities and clear pleomorphic vesicles. There are relatively few of these terminals. Except for their smaller size and slightly higher density of vesicles, these terminals are similar to the terminals of the eighth nerve input. This morphology is usually associated with excitatory inputs<sup>25</sup>. This input may be active after cochlea removal but because of its

smaller amplitude EPSP does not lead to action potentials in NM neurons. The origin of this input is unknown.

The high level of spontaneous neuronal activity found in nucleus magnocellularis makes the loss of activity following cochlea removal particularly striking. The results of studies examining the eighth nerve and nucleus magnocellularis of several avian species indicate that there is normally a high rate of activity in NM when no defined stimulus is present<sup>18,29,31</sup>. Thus it was of interest to determine if the source of the spontaneous activity is endogenous to these neurons or reflects the influence of the cochlea. The results of the present study clearly demonstrate that the spontaneous activity recorded in nucleus magnocellularis arises from activation of the peripheral receptors.

On the other hand, our results do not help to explain the dramatic differences we and others have observed in the reaction of young and adult animals to deafferentation or other afferent manipulations. One hypothesis to explain the heightened susceptibility of young animals to afferent manipulations is developmental changes in the overall proportion of excitatory input that is being manipulated. For example, if receptor elimination or deprivation alters 80% of the excitatory input to a postsynaptic neuron in young animals but only 40% in adults, we might expect major differences in the long-term effects of such deprivation<sup>17</sup>. The results of this study, however, do not support such a hypothesis. Whereas NM neurons in adult chickens show little transneuronal change following cochlea removal<sup>3,7,40</sup>, we observed changes in NM activity following cochlea removal in adults that were comparable to those seen in neonates. At both ages cochlea removal completely eliminates extracellularly recorded action potentials in NM. This argument does not eliminate the possibility of new inputs providing trophic support of adult NM neurons. However, observations of the ultrastructure in NM of neonatal and adult chickens also fail to reveal significant changes (unpublished observations). A more viable explanation of the age difference is a fundamental difference in the biochemical composition of young and adult neurons that somehow renders the adult NM cells resistant to chronic changes in afferent input. It is tempting to speculate that young neurons express a set of molecules that allow adaptations to conform to variations in their local environment, whereas activation of these genes is suppressed in adults.

#### *Comparison with 2-deoxy-D-glucose studies*

The 2-DG technique for assessing glucose uptake has been used extensively to study changes in functional states in the nervous system<sup>38,39</sup>. Glucose uptake following cochlea removal in chickens has been measured in

NM and NL using this method<sup>13,19</sup>. There is a rapid (within 70 min) reduction in NM glucose uptake as measured by the autoradiographic distribution of grains. These autoradiographic results are consistent with the reduction in the level of electrical activity recorded with a metal microelectrode. The same pattern of glucose uptake was seen 8–9 days after cochlea removal, indicating that the changes in activity are long-lasting. Studies of the cochlear nucleus of mammals using the 2-DG technique have given results consistent with those found in NM. When the cochlea is ablated and the cochlear nucleus is examined 1–48 h later using the 2-DG technique, there is reduced 2-DG uptake in the anteroventral cochlear nucleus ipsilateral to cochlea ablation<sup>32,44</sup>.

#### Comparison with other electrophysiological studies

The elimination of NM neuronal activity following cochlea removal is comparable to that seen in the homologous anteroventral cochlear nucleus of mammals. The only previous study using microelectrodes to examine the consequences of cochlea removal during the manipulation examined single units in the cochlear nucleus of cats<sup>16</sup>. Following cochlea removal, the most prominent change in activity occurred in the ventral cochlear nucleus. In this area, active single units could not be found immediately after cochlea destruction. They were, however, able to record discharges from units in other nuclei of the cochlear nucleus. It is notable that in the present study one electrode location was found to be in nucleus angularis, a second order auditory nucleus

considered homologous to dorsal and posterior portions of the cochlear nucleus of mammals. Activity in nucleus angularis following bilateral cochlea removal was reduced only 70%.

Other neural systems have been the subject of numerous experiments characterizing the role of afferent input. To date, there are only a few studies attempting to characterize the physiological change during and subsequent to chronic manipulations of the afferents<sup>4,8,26,27</sup>. In one study microelectrode recordings were made in the lateral geniculate nucleus while a photocoagulation lesion was placed in the retina<sup>8</sup>. There was an immediate reduction in action potentials recorded, although there were occasional discharges in some neurons.

In conclusion, we have shown that cochlea removal immediately eliminates excitatory activity in nucleus magnocellularis. This result is important in light of the rapid changes in NM neurons following cochlea removal, including neuron death, soma shrinkage, and altered metabolic enzyme levels. The change in activity precedes any detectable morphological changes in the presynaptic terminals<sup>22</sup>.

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