THE JOURNAL OF COMPARATIVE NEUROLOGY 301:643–654 (1990)

Effects of Cochlea Removal on GABAergic Terminals in Nucleus Magnocellularis of the Chicken

REBECCA A. CODE, DIANNE DURHAM, AND EDWIN W RUBEL
Hearing Development Laboratories, University of Washington School of Medicine, Seattle, Washington 98195

ABSTRACT

The effects of unilateral cochlea removal on GABA-immunoreactive (GABA-I) terminals in nucleus magnocellularis (NM) of the chick were assessed by immunocytochemical (ICC) techniques. Posthatch chicks (5–8 days old) survived from 1–37 days following unilateral cochlea removal. In the ipsilateral NM, the density of GABA-I terminals appeared to increase relative to normal controls 10–37 days after cochlea removal. However, most of that increase could be attributed to a decrease in cell size, cell number, and volume of the nucleus as a result of deafferentation. In the contralateral NM, the density of GABA-I terminals decreased relative to the ipsilateral NM and to normal animals 1–21 days after cochlea removal. The number of GABA-I terminals per NM neuron also decreased in the contralateral NM while that in the ipsilateral NM was comparable to normal controls. To ascertain whether these changes represented changes in the number of terminals or in the amount of GABA contained within the terminals, we also examined these terminals using an antibody to glutamic acid decarboxylase (GAD), the biosynthetic enzyme for GABA. Following unilateral cochlea removal, there was no difference in the density of GAD-I terminals in NM between the two sides of the brain for any of the survival times. Similarly, bilateral cochlea removal had no discernible effect on the density of GABA-I terminals in NM. These data suggest that unilateral deafferentation may temporarily downregulate the biosynthesis of GABA in the contralateral NM.

Key words: deafferentation, GABA, GAD, auditory brainstem, cochlear nucleus

Changes in synaptic organization that occur as a result of altered sensory experience have intrigued neurobiologists because of the implication that such anatomical remodeling may form the basis for learning or recovery of function. Previous investigators have demonstrated that removal of one input to a nucleus in the brain may cause sprouting of other afferents (Raisman, '69; Schneider, '73; Steward and Vinsant, '78; Tsukahara, '81; Cotman et al., '81). Most of those studies involved complex nuclei receiving multiple heterogeneous inputs. Nucleus magnocellularis (NM), the avian homologue of the mammalian anteroventral cochlear nucleus, is a comparatively simple nucleus consisting almost exclusively of a single cell type (Jhaveri and Morest, '82). In addition, NM receives only two major inputs: the auditory nerve provides the only excitatory input to the ipsilateral NM (Parks, '81; Born and Rubel, '84). The other major input is provided by nerve terminals which contain gamma-aminobutyric acid (GABA), a putative inhibitory neurotransmitter (Carr et al., '89; Code et al., '87, '89a; Müller, '87). There also appears to be a minor glycinergic input to NM (Code et al., '89b; Code and Rubel, '89).

In the chick, removal of the basilar papilla (cochlea) causes the eventual degeneration of the auditory nerve and its nerve terminals (end-bulbs of Held) on neurons in NM (Parks and Rubel, '78). We wished to ascertain the effects of the elimination of this excitatory input on the presumably inhibitory GABA-containing terminals in NM. Two hypotheses were considered: 1) if the presence of auditory afferents plays some role in the maintenance of GABAergic terminals, then removal of the cochlea might decrease the number of GABAergic terminals in the ipsilateral NM, 2) conversely, cochlea removal might lead to an increase in the number of GABAergic terminals (sprouting) due to less competition for target cell membrane. In either case, we expected a change in the number of GABAergic inputs in

Accepted August 10, 1990.

A preliminary account of these results has been published in abstract form (Code et al., '88).

Address correspondence to Edwin W Rubel, Hearing Development Lab., RL-30, University of Washington School of Medicine, Seattle, WA 98195.

R.A. Code is now at Department of Anatomy and Cell Biology, USUHS, 4301 Jones Bridge Road, Bethesda, MD 20889.

© 1990 WILEY-LISS, INC.

the *ipsilateral* NM, either an increase or decrease, after cochlea removal.

Our findings show that unilateral cochlea removal results in little or no change in the immunocytochemical staining for GABA in terminals in the ipsilateral NM. In the *contralateral* NM, however, GABA immunocytochemistry suggests that cochlea removal causes a transient decrease in the density and number of GABA-containing terminals. However, this decrease is not due to degeneration of the GABAergic terminals since their presence can be demonstrated after cochlea removal by using an antibody to glutamic acid decarboxylase (GAD). Thus, cochlea removal appears to regulate differentially the levels of GABA and GAD contained in nerve terminals in NM.

MATERIALS AND METHODS

Animals

Inbred White Leghorn chickens (H and N International, Redmond, WA) between 5–8 days of age were used in these experiments. A total of 25 chicks underwent unilateral cochlea removal and were divided into seven survival groups: 1, 2, 5, 10, 14, 21, and 37 days. In addition, 16 chicks sustained bilateral cochlea removal and survived for 2–38 days. One or two age-matched unoperated control chicks were included in each survival group. Auditory brainstem tissue was processed for GABA immunocytochemistry (ICC). An additional 15 chicks underwent unilateral cochlea removal. After surviving 1–37 days, their auditory brain stems were processed for GAD ICC.

Surgical procedures

Unilateral basilar papilla (cochlea) removal was performed according to procedures which have been described previously (Born and Rubel, '85). Briefly, chicks were anesthetized with ketamine hydrochloride (Vetalar; 80 mg/kg) and sodium pentobarbital (Nembutal; 20 mg/kg). The right cochlea was removed with forceps, floated in a water-filled Petri dish and examined to ensure that it was removed in its entirety. Skin incisions around the external auditory canal were sealed with cyano-acrylate glue, and the animals were allowed to recover.

Tissue processing

Under deep anesthesia with Nembutal, the chicks were perfused intracardially with buffered saline followed by a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature (for GABA ICC) or cold 1% paraformaldehyde (for GAD ICC). The brains were removed from the skull, blocked in the transverse plane and placed in the fixative at 4°C overnight (for GABA ICC) or for 4 hours in fix, and then into 30% buffered sucrose overnight (for GAD ICC).

Immunocytochemical techniques were performed as described previously (Code et al., '89a). Standard ICC procedures were followed for the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., '81) with reagents from Vectastain kits (Vector Labs). Tissue processing techniques were carefully standardized to minimize variability among animals. Incubations in primary antiserum for GABA were performed at room temperature and those for GAD at 4°C. All other incubations were performed at room temperature. Transverse sections through NM were cut, either on a Vibratome for GABA or on a freezing microtome for GAD (30–40 μm thick). The sections were rinsed several times in phosphate buffer and then incubated for 1 hour in a pre-soak solution of 3% normal goat serum (NGS) in 0.5 M Tris buffer (pH 7.6) (for GABA ICC) or 3% normal horse serum (NHS) in phosphate-buffered 0.3% Triton-X (for GAD ICC). The sections were then incubated for 12–36 hours in either GABA antiserum (Immunonuclear, Inc.) diluted 1:2,000-1:4,000 or in GAD-2 antiserum (Gottlieb et al., '86), diluted 1:4,000. Each primary antiserum was diluted in its pre-soak solution. Following several buffer rinses, the sections were incubated for 1 hour in secondary antiserum consisting of biotinylated goat anti-rabbit IgG, diluted 1:200 in phosphate buffer (for GABA) or biotinylated horse anti-mouse IgG, 1:200 (for GAD). After four buffer rinses, the sections were incubated in ABC for 1 hour. They were rinsed again for 30 minutes and then reacted for 10–20 minutes in a 0.02% diaminobenzidine solution to which hydrogen peroxide was added at a final concentration of 0.03%. Following several buffer rinses, the sections were mounted onto slides coated with poly-L-lysine, dried, and coverslipped with DPX or Permount. Adjacent sections were stained for Nissl substance with thionin.

Controls

Control studies included tissue incubated in 3% NGS or NHS without the GABA or GAD antisera, which resulted in the absence of GABA-I or GAD-I staining. In addition, control experiments performed in a previous study confirmed that the GABA antiserum is highly specific and that it does not cross-react with structurally similar amino acids (Code et al., '89a).

Quantitative analyses

Counts of terminals. GABAergic terminals, as defined by established criteria (Code et al., '89a), were counted with the aid of a videocamera attached to a microscope and a Zeiss Videoplan imaging system. With the aid of a 100× objective (N.A. 1.3), an image of the tissue was displayed on a television monitor at a final magnification of 2,590×. GABA-labeled terminals in NM were counted in one focal plane on both the ipsilateral and contralateral sides of the same tissue section by using one of the two sampling techniques described below.

Standard sampling procedure. GABA-I terminals in NM were counted from both sides of the brainstem according to a sampling procedure that is fully described elsewhere (Code et al., '89a). Briefly, the total posterior-to-anterior (P-A) extent of NM was determined and the tissue section at the 50% P-A level was selected for analysis. If this section was not available because of tearing during sectioning or loss during ICC processing, the next closest usable section was selected. The sections used for analysis ranged from the 42–58% P-A level.

Within the section, three regions of NM, medial, central, and lateral, were chosen for analysis (Fig. 1). The medial and lateral regions were defined as the most medial and most lateral regions of the nucleus of that section, respectively, and the central region as halfway between them. In each region, the number of GABA-I terminals in three separate 2,500 μm^2 areas was counted and averaged together. This provided, for each animal, values of the mean density of GABA-I terminals (the number of terminals per unit area) in each of the three mediolateral regions of NM.

Area-weighted sampling. In the standard sampling method described above, the selection of areas within NM from which GABA-I terminals were to be counted was performed at a magnification at which the GABA-I termi-

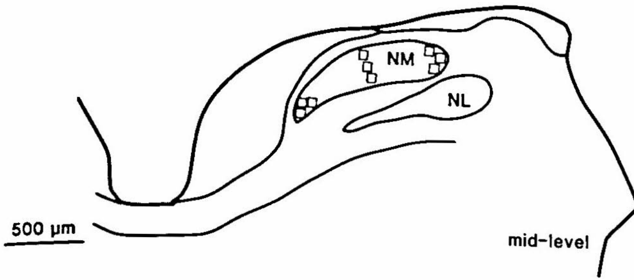

Fig. 1. Sampling areas in the medial, central, and lateral regions of NM at the 50% posterior-anterior (P-A) level of the nucleus. The medial and lateral regions were defined as the most medial and most lateral regions of NM, respectively, in that tissue section. The central region was defined to be halfway between the medial and lateral borders of NM in that section. Each square represents the approximate location of a 2,500 μm^2 sampling area (a square, 50 µm on a side) from which GABA-I terminals were counted. Data from the three squares in each of the three mediolateral regions depicted were averaged to obtain the mean density of GABA-I terminals.

nals could be easily observed. Thus, an element of bias may have been introduced during selection of sample areas. To determine whether this bias actually occurred with the standard sampling method, a second sampling procedure, the area-weighted sampling method, was adopted (Miles and Davy, '76). In this procedure, as in the first sampling method, GABAergic terminals in NM were counted on both sides of the brain with the Zeiss Videoplan imaging system. An eyepiece reticule with a 10 × 10 grid was placed in the microscope ocular. By using a 16× objective (N.A. 0.35), which did not permit visualization of the GABAergic terminals, the tissue section was arbitrarily placed under the grid so that the borders of NM fit entirely within the grid and such that one horizontal grid line bisected NM in a lateral-to-medial direction. At the intersection of every other vertical grid line with the horizontal grid line, the tissue was centered in the field, the objective was changed to 100×, and the field of view was displayed on the television monitor. The number of GABAergic terminals in a 2,500 μm^2 sampling area was counted as described above. Values from approximately four of these 2,500 μm^2 areas within one tissue section were averaged to give the mean density of GABAergic terminals for that particular posterior-anterior (P-A) level. The density of GABA-I terminals was determined at four different P-A levels of NM from one animal 10 days after cochlea removal. In addition, the density of GAD-I terminals in NM was determined in three animals, 10 days after cochlea removal, by means of the area-weighted sampling method. Results from the area-weighted sampling method were consistent with those obtained with the standard sampling method.

Counts of terminals per cell. The average number of GABA-I terminals surrounding NM neurons in cross-section was determined from two chicks that survived 10 days after unilateral cochlea removal, three chicks in the 14-day survival group, and one age-matched, control animal from each of these two survival groups. The number of GABA-I terminals surrounding an individual NM neuron in cross-section was counted directly from the microscope using a 63× oil objective (N.A. 1.4). Only terminals directly apposed to NM neuronal cell bodies which exhibited a nucleus were counted. If two cells lying next to each other appeared to share a labeled terminal, then only one of the cells was selected for analysis to preclude the possibility of counting the same terminal twice. For each animal, the number of GABA-I terminals was counted and averaged from approximately 50 neurons within NM at the 50% P-A level on each side of the brain.

Measurements of NM volume. The mean volume of NM was measured in two animals that had survived 10 days after unilateral cochlea removal and two age-matched, control animals. By using a 10× objective (N.A. 0.32) and a videocamera attached to the microscope, an image of the Nissl-stained tissue section, adjacent to the sections processed for GABA ICC, was displayed on a television monitor. The perimeter of NM on each side of the brainstem was outlined with the aid of a cursor and a digitizing pad (Zeiss Videoplan). Approximately 14 sections from the caudal to the rostral pole of NM were analyzed from each animal. The areas of NM from each section were added together then multiplied by the distance between the sections (90 µm) to obtain an estimate of the total volume of the nucleus.

RESULTS

GABA immunocytochemistry

We have demonstrated previously that the small, round, or oval structures that are within the neuropil and surrounding nerve cell bodies in NM of posthatch chicks and that are stained with the GABA antiserum are nerve terminals (Code et al., '89a). In the present report, the terms GABA-immunoreactive (GABA-I) and GAD-immunoreactive (GAD-I) are used to refer to terminals that are immunostained with the GABA and GAD antisera, respectively.

Figure 2 shows representative photomicrographs of GABA-I terminals in NM from the sides of the brain contralateral and ipsilateral to cochlea removal at three different survival times. One day after cochlea removal, approximately equal numbers of GABA-I terminals are present in contralateral and ipsilateral NM (A, B). However, 2 days after cochlea removal (not shown), there appears to be a decrease in the number of GABA-I terminals in contralateral compared to ipsilateral NM. This difference continues to increase in magnitude so that by 10 and 14 days postoperatively, there are relatively few identifiable GABA-I terminals in contralateral NM compared to ipsilateral NM (C, D). The number of GABA-I terminals in other, presumably unmanipulated, areas of the brain (for example, the adjacent medial vestibular nucleus) appears normal at this time; the number of terminals in the contralateral NM relative to the medial vestibular nucleus appears to be decreasing. By 21 days, the number of GABA-I terminals in the contralateral NM appears to increase, and by 37 days there does not appear to be any difference in the number of GABA-I terminals between the two sides of the brainstem (E, F).

GABA-I terminal density

Quantitative analyses confirmed the observations described above. Figure 3 shows the density of GABA-I terminals in three mediolateral regions of NM contralateral and ipsilateral to cochlea removal for one representative animal in each survival group. A decrease in the density of GABA-I terminals in the contralateral NM relative to the ipsilateral NM is apparent at 2, 10, 14, and 21 days after cochlea removal.

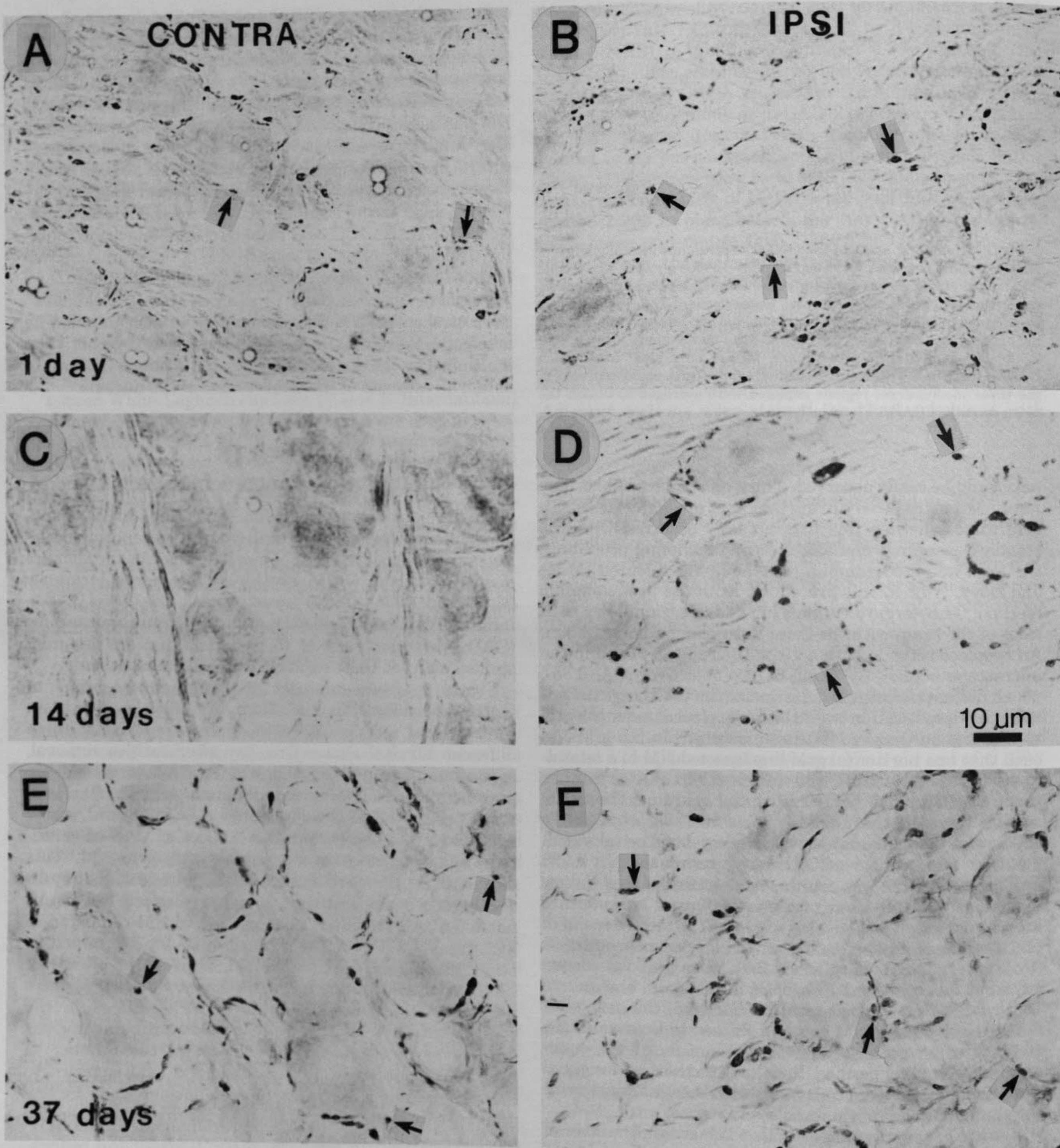

Fig. 2. GABA-labeled terminals (arrows) at similar P-A and mediolateral positions within NM from the contralateral side of the brain (left column) and from the side of the brain ipsilateral to cochlea removal (right column). **A,B.** One day after cochlea removal. No difference in the number of GABA-I terminals between the two sides. **C,D.** 14 days after cochlea removal. A dramatic decrease in the number of terminals on the contralateral side compared to the ipsilateral side. Scale bar applies to all panels. **E,F.** Thirty-seven days after cochlea removal. There is little difference in the number of terminals in NM between the two sides of the brain.

The area-weighted sampling method confirmed the difference in the density of GABA-I terminals in NM between the two sides of the brain in animals examined 10 days after cochlea removal (Fig. 4). At each of the four P-A levels within NM from one animal, there is a significant decrease in the density of GABA-I terminals in the contralateral NM compared to that in the ipsilateral NM. A two-way analysis of variance (ANOVA) (side of the brain X P-A level) revealed

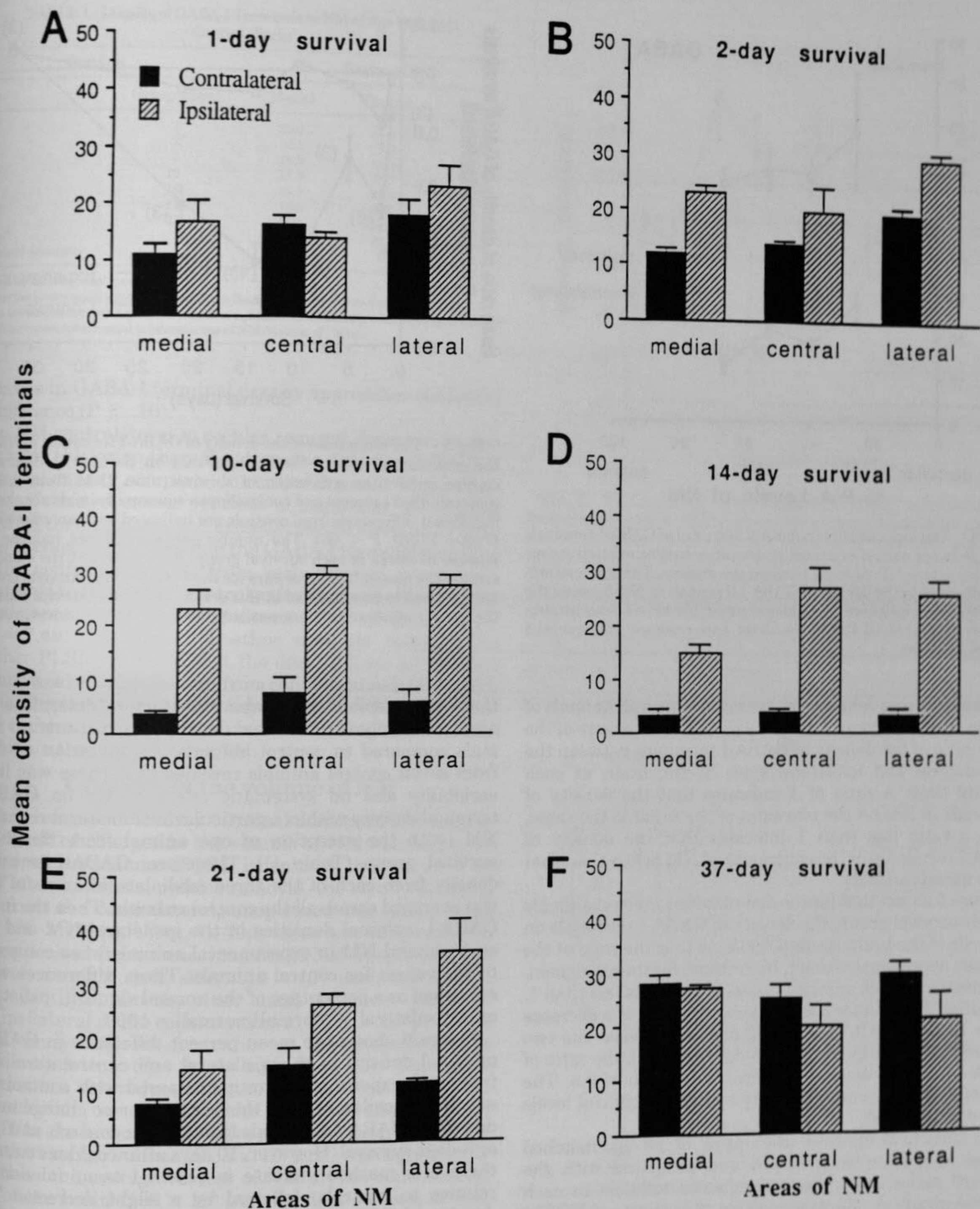

Fig. 3. Graphs of the average density (number/2,500 μm^2) of GABA-I terminals in three mediolateral areas of NM contralateral (black) and ipsilateral (hatched) to cochlea removal. Data from one representative animal is depicted for each survival group. Bars are the standard errors of the mean. Decreases in the density of GABA-I terminals in NM on the contralateral side of the brain versus the ipsilateral side occur at 2, 10, 14, and 21 days after cochlea removal.

a significant effect of side of the brain [$F(1, 24) = 96.06$; $P < .001$]. The effects of position and the interaction term were not significant.

The time course of the changes in GABA-I terminal density for all animals is shown in Figure 5. For each mediolateral region of NM in each animal, the average density of GABA-I terminals on the side of the brain contralateral to cochlea removal was divided by the average density on the ipsilateral side. The ratios were averaged across the three mediolateral regions of NM in each animal

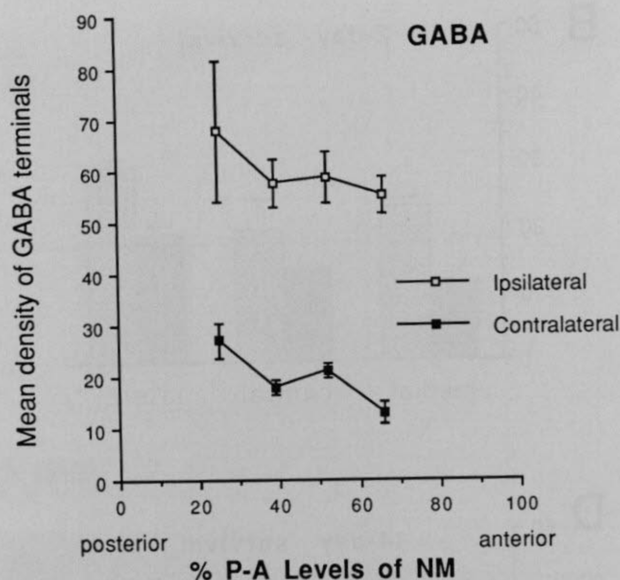

Fig. 4. The mean density (number/2,500 μm²) of GABA-I terminals in NM from one animal examined 10 days after cochlea removal at four posterior-anterior (P-A) levels through the nucleus. There is a significant difference in the density of GABA-I terminals in NM between the side of the brain ipsilateral to cochlea removal (black) and the contralateral side (white) at all P-A levels. Error bars represent the standard error of the mean.

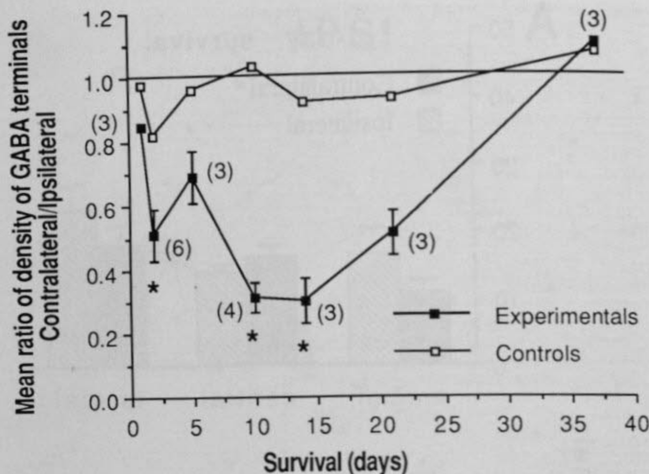

Fig. 5. The mean ratio of the density of GABA-I terminals in NM on the contralateral side of the brain to that on the side ipsilateral to cochlea removal as a function of survival time. Data from cochlea removal (filled squares) and control (open squares) animals are shown. Significant differences from controls are indicated with an asterisk (*) (Fisher PLSD, $P < .05$). The numbers in parentheses indicate the number of chicks in each survival group. Bars represent the standard error of the means. The error bars for the 1- and 37-day survival groups are too small to be visualized in this graph. There are no error bars for the control values since there was only one animal per survival group.

and the resulting values were averaged across all animals of a given survival group. This value provided a measure of the mean ratio of the density of GABA-I terminals between the contralateral and ipsilateral sides of the brain at each survival time. A ratio of 1 indicates that the density of terminals in NM on the two sides of the brain is the same, while a ratio less than 1 indicates that the density of GABA-I terminals in the contralateral NM is less than that in the ipsilateral NM.

Figure 5 shows that for the age-matched, control animals in each survival group, the density of GABA-I terminals on each side of the brain is comparable, so that the ratio of the densities approximates unity. In contrast, for the experimental animals, at most survival times, this ratio is less than 1. Beginning 2 days after cochlea removal, there is a decrease in the ratio of GABA-I terminal density between the two sides of the brain; by 10- and 14-days survival, the ratio of GABA-I terminal density reaches a minimum of 0.3. The ratio of GABA-I terminal density returns to control levels by 37 days survival.

For statistical analysis, the ratios of all age-matched control animals were averaged and compared with the averaged ratios from the experimental animals in each survival group. A one-way analysis of variance (ANOVA) revealed a significant effect of survival time [$F(6, 27) = 6.83$; $P < .001$]. Posthoc multiple comparisons (Fisher-PLSD) revealed that the ratios of GABA-I terminal density from the 2-, 10-, and 14-day survival groups were significantly different from that in the control group ($P < .05$). The 1-, 5-, 21-, and 37-day survival groups were not significantly different from controls ($P > .05$).

These differences in the ratios of the density of GABA-I terminals from the two sides of the brain could result from either a decrease in the contralateral NM or from an increase in the ipsilateral NM. To distinguish between these two possibilities, it was necessary to determine the *absolute* changes in terminal density in experimental animals compared to control animals. Examination of data from seven control animals revealed that there was little variability and no systematic effect of age on GABA-I terminal density within a particular mediolateral region of NM [with the exception of one animal from the 2-day survival group (Table 1)]. Therefore, GABA-I terminal density from each of the three mediolateral areas of NM was averaged across all the control animals. Then the mean GABA-I terminal densities in the ipsilateral NM and the contralateral NM in experimental animals were compared to the values for control animals. These differences were expressed as a percentage of the normal value: {[(ipsilateral or contralateral) − normal]/normal} × 100%.

Figure 6 shows the mean percent difference in GABA-I terminal density in the ipsilateral and contralateral NM from each experimental group compared with control values. In the ipsilateral NM, there is little or no change in the density of GABA-I terminals from that in controls at 1-, 2-, or 5-days survival. However, 10 days after cochlea removal, there is a marked increase in GABA-I terminal density relative to controls, followed by a slight decrease. The density of GABA-I terminals at 14 days survival is still above control values and remains relatively unchanged through 37 days postoperative.

For statistical analysis, the densities of GABA-I terminals in the ipsilateral NM from each survival group were compared to the densities of all control animals. A one-way analysis of variance (ANOVA) revealed a significant effect of group [$F(7,31) = 3.67$; $P < .01$]. Posthoc multiple comparisons (Fisher-PLSD) revealed that the density of GABA-I terminals in the ipsilateral NM was significantly different from control animals only for the 10-day survival group ($P < .05$). From 14–37 days after cochlea removal, the

TABLE 1. Density of GABA-I Terminals in NM of Age-Matched, Control Chicks[1]

Age	Survival Group (days)	Medial	Central	Lateral
P7	1	20.0	21.0	38.5
P8	2[2]	6.5	7.0	13.0
P12	5	18.3	15.2	25.9
P18	10	17.8	28.0	25.7
P19	14	15.7	23.0	30.2
P26	21	19.0	26.5	29.3
P45	37	23.0	38.0	37.8
Mean[3]		17.2	22.7	28.6
Standard deviation		5.2	9.85	8.61

[1]N = 1 for each survival group.
[2]These values are considerably different from those of the other control animals.
[3]Data are consistent with a previous report of a gradient in the density of GABAergic terminals which increases from medial to lateral areas of NM (Code et al., '89a).

increase in GABA-I terminal density approaches statistical significance ($P < .10$).

In NM contralateral to cochlea removal, however, by one day after the lesion there is a dramatic *decrease* in GABA-I terminal density compared to control animals. The magnitude of this decrease reaches a maximum at 14-days survival then recovers and exceeds control levels at 37 days after cochlea removal. When the GABA-I terminal densities in the contralateral NM from each survival group were compared to those of all control animals, a one-way analysis of variance revealed a significant effect of group [$F(7, 31) = 7.49$; $P < .0001$]. Posthoc multiple comparisons (Fisher-PLSD) revealed that the density from all survival groups was reliably different from control animals ($P < .05$). Thus, the most significant and consistent effect of unilateral cochlea removal is the decrease in GABA-I terminal density in the contralateral NM.

Changes in the volume of NM

The changes in GABA-I terminal density in NM may be the result of changes in the volume of NM; therefore, we measured the volume of NM. In animals that survived 10 days after unilateral cochlea removal, the mean volume of NM from the contralateral, unoperated side of the brain was 126×10^6 μm³ (Fig. 7). This value was comparable to values derived from measurements of NM in age-matched, control animals (left side, 119×10^6 μm³; right side, 128×10^6 μm³). On the other hand, the mean volume of NM ipsilateral to the cochlea removal (87.1×10^6 μm³) was significantly different from that on the unoperated side (Student's t-test; df = 2; t = 4.49; $P < .05$). This value represents a 31% reduction in the volume of ipsilateral NM.

In addition, we measured the volume of the ipsilateral NM from three animals 37 days postoperatively and found that the average volume (86.5×10^6 μm³) was significantly less than the average volume of NM on the contralateral side of the brain (196×10^6 μm³; Student's t-test; df = 4; t = 6.22; $P < .01$). The mean volume of the ipsilateral NM was 61% less than the mean volume of NM from the left and right sides of the brain from two age-matched control animals (220.5×10^6 μm³. The mean volume of NM ipsilateral to cochlea removal after 37 days survival was comparable to the mean volume of the ipsilateral NM from two animals in the 10-day survival group (87.1×10^6 μm³). Thus, the effect of cochlea removal on the volume of NM is the same at 10 and 37 days postoperatively.

Since the *volume* of NM is markedly decreased on the side of the brain ipsilateral to cochlea removal, the *density* of GABA-I terminals would increase even if there is no change

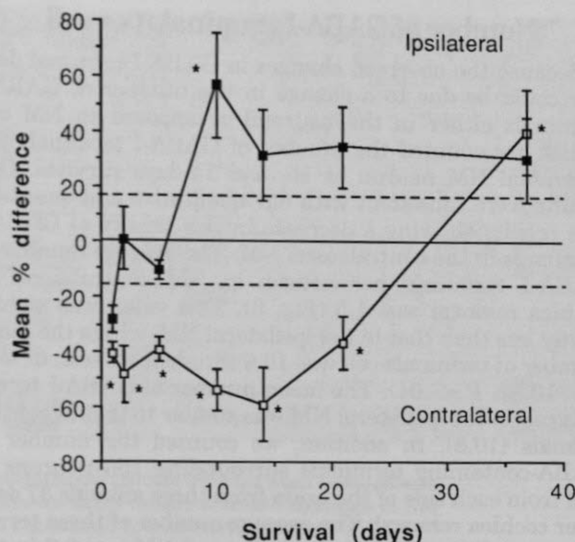

Fig. 6. The mean per cent difference in GABA-I terminal density from control levels in NM ipsilateral to cochlea removal (filled squares) and on the contralateral side (open squares) as a function of survival time. Error bars represent the standard error of the mean. Any value above the solid horizontal line represents an increase in the density of GABA-I terminals compared to control levels and any value below the line represents a decrease from controls. Values that are statistically different from control values ($P < .05$) are indicated with an asterisk (*). The dashed lines at ±16.5% represent values at the 95% confidence interval of the mean control values.

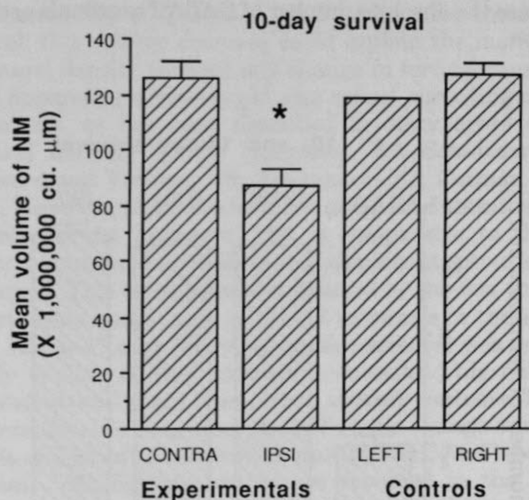

Fig. 7. The mean volume of NM on each side of the brainstem from two animals which survived 10 days after unilateral cochlea removal and from two age-matched, control animals. The mean volume of NM from the side of the brain ipsilateral to cochlea removal (IPSI) is significantly different from that on the contralateral side (CONTRA) and from normal controls (see text). Error bars represent the standard error of the mean. The error bar on the left side of the control group is too small to be visualized in this graph.

in the absolute number of terminals. Thus, at least a portion of the increase in density of GABA-I terminals in the deafferented NM can be attributed to the decrease in the volume of NM which occurs as a result of cochlea removal.

Number of GABA-I terminals per cell

Because the observed changes in GABA-I terminal density could be due to a change in the number of GABA-I terminals either in the neuropil or apposed to NM cell bodies, we counted the number of GABA-I terminals per individual NM neuron at 10- and 14-days survival. Our results were consistent with our qualitative and quantitative results showing a decrease in the density of GABA-I terminals in the contralateral NM. The average number of GABA-I terminals per neuron in NM contralateral to cochlea removal was 4.5 (Fig. 8). This value was significantly less than that in the ipsilateral NM, where the mean number of terminals/cell was 10.8 (Student's t-test; df = 8; t = 10.92; $P < .01$). The mean number of GABA-I terminals/cell in the ipsilateral NM was similar to that in control animals (10.8). In addition, we counted the number of GABA-containing terminals surrounding 150 neurons in NM from each side of the brain from three animals 37 days after cochlea removal. The average number of these terminals per cell was 10.2 in the ipsilateral NM and 9.7 in the contralateral NM. These results were not significantly different from each other nor from the average number of GABA-I terminals per cell from two age-matched control animals (9.6).

Thus, the increase in the density of GABA-I terminals in NM ipsilateral to cochlea removal at 10- and 14-days survival appears to be due to the decrease in volume of NM (see above) and not to an increase in the absolute number of GABA-I terminals/cell. On the contralateral side of the brain, however, cochlea removal not only decreases the density of GABA-I terminals in NM, but it also appears to decrease the absolute number of GABA-I terminals per NM neuron.

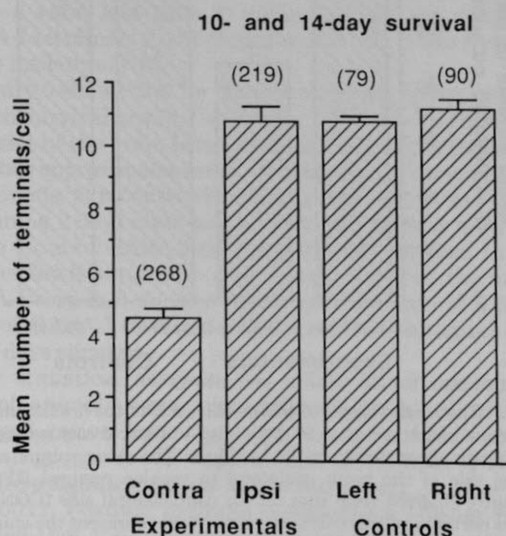

Fig. 8. The average number of GABA-I terminals per neuron in the contralateral and ipsilateral NM from five experimental chicks (10 or 14 days after cochlea removal), and the left and right NM from two age-matched, control animals. The number in parentheses is the total number of NM neurons from which surrounding GABA-I terminals were counted. The bars represent the standard error of the mean. There is a significant decrease in the number of GABA-I terminals/cell in the contralateral NM compared to that in the ipsilateral NM and normal controls.

GAD immunocytochemistry

There are two possibilities that may account for the observed decrease in GABA-I terminals in the contralateral NM: 1) the GABAergic terminals in NM on the contralateral side of the brain degenerated, or 2) these terminals stopped synthesizing GABA (or at least sufficient amounts of it to be detected by ICC). To distinguish between these two possibilities, another marker for GABAergic terminals, an antibody to glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA (Roberts and Kuriyama, '68), was used on auditory brainstem tissue following unilateral cochlea removal. Previous studies comparing GAD and GABA antisera suggest that they label the same terminals (Fex and Altschuler, '84; Fex et al., '86).

Results from the present study using GAD-2 antiserum (Gottlieb et al., '86) are consistent with these earlier reports. In control animals, the morphology of nerve terminals in NM labeled with GABA and GAD-2 are similar. In addition, the GAD-I terminals are distributed along the same gradient as the GABA-I terminals: highest in caudolateral regions of NM and lowest in rostromedial regions (Code et al., '89a). However, the absolute number of GAD-I terminals appears to be greater than that of GABA-I terminals (compare Figs. 10 and 3C). We believe this is due to the fact that the GAD-2 antibody is directed against chick GAD and thus is probably more sensitive than the GABA antiserum.

Ten days after cochlea removal, there are many GAD-I terminals in NM on both the contralateral and the ipsilateral sides of the brain (Fig. 9). The number of GAD-I terminals in the contralateral NM is strikingly greater than the number of GABA-I terminals (Fig. 2C), where virtually no GABA-I terminals are seen.

Quantitative analysis using the area-weighted sampling method confirmed this observation. There is no difference in the mean density of GAD-I terminals in NM between the two sides of the brain (Fig. 10). A two-way analysis of variance (ANOVA) (side of the brainstem X P-A level) revealed no reliable effect of side of the brain, but there was a reliable effect of P-A level [$F(3,22) = 3.87$; $P < .05$]. The density of GAD-containing nerve terminals is higher in posterior levels of the nucleus and lower in rostral levels.

GAD-I terminals were examined in NM on both sides of the brain at all the same survival times as the GABA-I terminals. At each survival time, neither the morphology, the distribution, nor the density of GAD-I terminals in NM appeared to change on either side of the brain.

Bilateral cochlea removals

Bilateral cochlea removal appeared to have no effect on the density of GABA-I terminals in NM on either side of the brain compared with control animals (qualitative observations). For each of the survival times (2, 5, 10, 14, 21, or 38 days), no change was detected in the density of GABA-I terminals in NM between the two sides of the brain.

DISCUSSION

Removal of the cochlea in hatchling chicks appeared to cause a transient decrease in the density of GABA-I terminals in the contralateral NM. These results were initially interpreted as the absence of terminals containing GABA. However, the presence of these terminals could still be

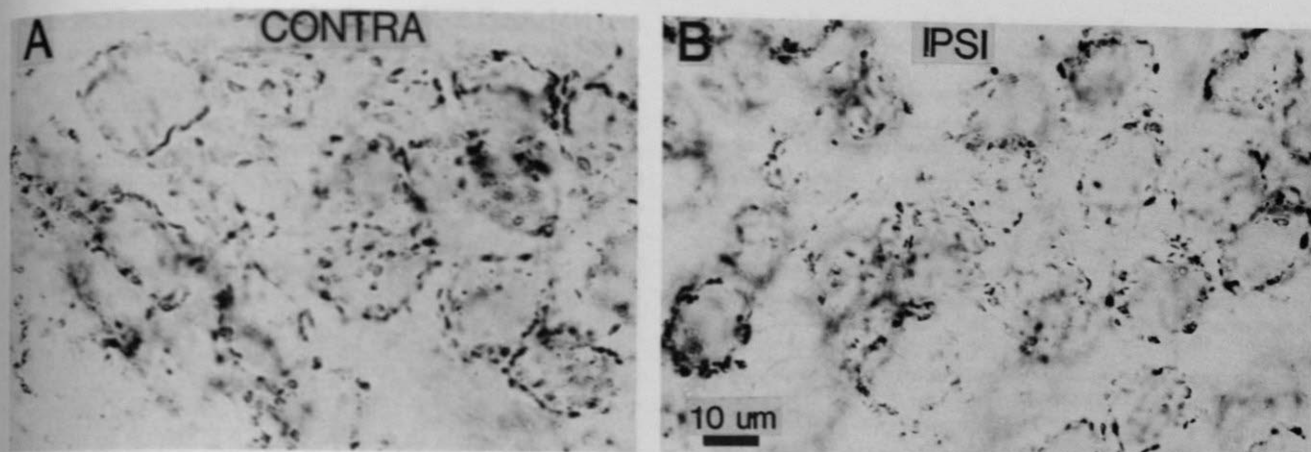

Fig. 9. GAD-I terminals in the contralateral (**A**) and the ipsilateral (**B**) NM from the same tissue section 10 days after cochlea removal. There appears to be no difference in the density of GAD-I terminals in NM between the two sides of the brain. Compare panel A with Fig. 2C and note the virtual absence of *GABA*-I terminals at approximately the same survival time. Scale bar applies to both panels.

demonstrated by using an antibody to GAD, the biosynthetic enzyme for GABA. We believe that the most parsimonious interpretation of these results is that cochlea removal somehow decreases the amount of GABA in nerve terminals in the contralateral NM without affecting their absolute number. We do not believe that the changes in density of GABA-I terminals are artifactual for the following reasons: 1) the number of animals in which these effects are observed suggests that it is unlikely that they can be attributed to chance, and 2) the two different sampling methods gave consistent results. The area-weighted sampling method also showed that the decrease in the density of GABA-I terminals in the contralateral NM occurred throughout the P-A extent of the nucleus and not only at the 50% level.

Inherent in our interpretation of the results of GAD and GABA ICC is the belief that ICC staining does reflect the *amount* of these substances actually present in nerve terminals in NM. However, caution must be exercised in the interpretation of data from immunocytochemical studies especially with regard to the possibility of false-negative staining. Although the presence of an immunostained nerve terminal is strong evidence that that terminal contains the antigen of interest, the absence of immunoreactivity does not unequivocally prove that the terminal is absent. The terminal may be present but longer contain sufficient amounts of the antigen to be detected by immunocytochemistry. The absolute amount of staining seen with ICC is dependent on such factors as fixation, penetration of the antibody, and linearity of the binding of antibody to antigen. It seems unlikely that any of these factors might be differentially affecting NM on the two sides of the brain. Experiments are currently being conducted to determine quantitatively the amounts of GABA and GAD in NM after cochlea removal using sensitive biochemical assays.

The effects of unilateral cochlea removal on GABAergic terminals in NM will be addressed, followed by a discussion of differential regulation of GABA and GAD in NM. A final section will comment on the gradient of GABAergic terminals after cochlea removal.

GABAergic terminals in ipsilateral NM after unilateral cochlea removal

Cochlea removal appears to increase the density of GABA-I terminals in the ipsilateral NM relative to control animals. This increase was statistically significant at 10 days postoperative and approached statistical significance 14–37 days postoperative. Concurrently, the volume of the ipsilateral NM is decreased 10–37 days after cochlea removal; this volume decrease could explain the increase in terminal density without any change in terminal number. The apparent increase could also reflect sprouting of new terminals, as has been described in other areas of the central nervous system (Raisman, '69; Schneider, '73; Steward and Vinsant, '78; Tsukahara, '81; Cotman et al., '81). However, since the number of GABA-I terminals per neuron in the ipsilateral NM is comparable to that in control animals, it is unlikely that significant sprouting has occurred. This conclusion is supported by the fact that the distribution and density of GAD-I terminals in the ipsilateral NM 1–37 days following cochlea removal was qualitatively similar to that in control animals. This result is consistent with data from other sensory systems. In the mammalian cochlear nucleus (CN), little change occurs in levels of GAD after cochlear lesions (Davies, '73; Fisher and Davies, '76). Similar results are reported in the visual system. After monocular enucleation in the kitten, GAD-immunoreactivity was essentially normal in the lateral geniculate nucleus (Bear et al., '85). Following unilateral eye removal in the rat, there was no decrease in the number of GAD-positive synaptic profiles in the superficial layers of the superior colliculus compared to controls (Houser et al., '83). Thus, deafferentation appears to have little effect on GAD-I terminals in first-order targets.

The present study also demonstrates that the cochlea can be eliminated as a significant source of the GABAergic input to the ipsilateral NM. Terminals containing GABA and GAD are present in the ipsilateral NM at every survival time. This conclusion is consistent with biochemical studies in the mammalian cochlear nucleus which suggest that GABA and GAD are not associated with auditory nerve

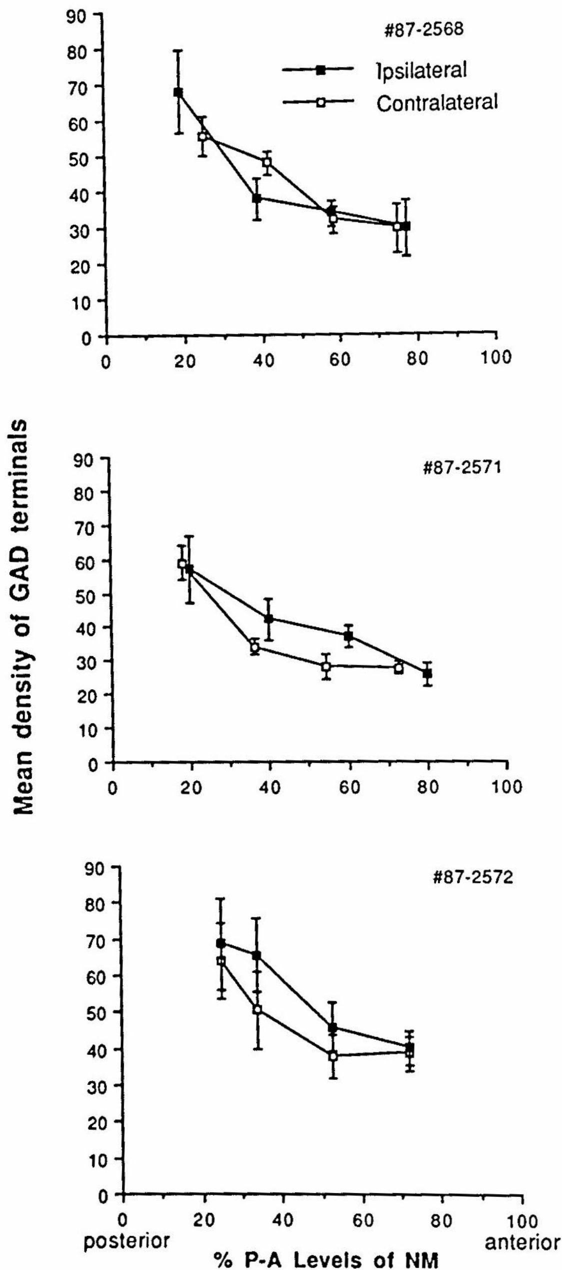

Fig. 10. The mean density (number/2,500 μm²) of GAD-I terminals in NM at four posterior-anterior (P-A) levels through the nucleus from three chicks 10 days after cochlea removal. There is no reliable difference in the mean density of GAD-I terminals between the side of the brain ipsilateral to cochlea removal (black) and the contralateral side (white). Error bars represent the standard error of the mean.

terminals (Davies, '73; Fisher and Davies, '76; Godfrey et al., '77; Wenthold, '79; Canzek and Reubi, '80).

GABAergic terminals in the contralateral NM after unilateral cochlea removal

Unilateral cochlea removal causes a significant decrease in the absolute density of GABA-I terminals in the contralateral NM. Since the volume of the contralateral NM did not differ from that in control animals, the decrease in the density of GABA-I terminals is probably a reflection of a decrease in the mean number of GABA-I terminals per cell.

The decrease in the number of GABA-I terminals does not appear to be the result of their degeneration. We base this conclusion on results using an antiserum to GAD. In control animals, the morphology and gradient of GAD-I terminals is similar to that of GABA-I terminals (Code et al., '89a,b), suggesting the two antisera label the same terminals. Although the density of GAD-I terminals was quantified only at 10-days survival, no difference in the density of GAD-I terminals in NM between the two sides of the brain was apparent at any time after cochlea removal. These data suggest that the decrease in the density of *GABA*-I terminals in contralateral NM is due to decreases in the level of GABA within these terminals.

That unilateral cochlea removal should have an effect on GABA-I terminals in contralateral NM is quite surprising and unexpected since there is no direct anatomical connection between the cochlea and the contralateral NM. Furthermore, an NM-to-NM projection has not been demonstrated in posthatch chicks, although there is evidence for such a transient projection in early embryos which expands and remains after early otocyst ablation (Young and Rubel, '86; Jackson and Parks, '88). By what mechanism, then, might the contralateral NM detect a change of sensory input to the ipsilateral NM? Although most of the GABAergic input to NM is believed to originate ipsilaterally from small, local neurons surrounding NM (von Bartheld et al., '89), the inputs to these GABAergic cells are not known. These GABAergic cells may receive descending projections from higher binaural centers in the auditory pathway or from the efferent olivocochlear system. Binaural centers might detect asymmetric levels of activity from the two ears as a result of cochlea removal and then signal GABAergic cells surrounding the contralateral NM to decrease their synthesis of GABA. If unequal activity in NM on the two sides of the brain is the signal for changes in GABA levels, no changes in GABA-I terminals would be expected after bilateral cochlea removal. Indeed, this is what we observe.

The present study documents a differential change in the density and number of GABA-I terminals contralateral to the side of sensory deprivation. Thompson et al. ('86) also showed a decrease in GABA-like immunoreactivity in the contralateral lateral vestibular nucleus after vestibular end-organ ablation in the monkey. In addition, other investigators have reported changes in the activities of various metabolic enzymes after sensory deprivation (Dietrich et al., '82). In the mouse, the facial whiskers project via several synapses to "barrels" in the contralateral cerebral cortex. After clipping all the whiskers on one side of the face, enzyme activities in the contralateral barrels were essentially normal but were significantly increased in the ipsilateral barrel field. These findings are similar to the present results in that changes in the activities of metabolic enzymes were not found on the side of the brain related to the reduced sensory input, but rather on the side which is related to the intact sensory periphery. An important distinction between their study and the present one is that clipping of the whiskers is an example of sensory *deprivation,* that is, without damage to the sensory receptors. Cochlea removal, on the other hand, is an example of sensory *deafferentation* in which the auditory receptors are removed and the auditory nerve degenerates.

Differential regulation of GAD and GABA in NM

The data reported here suggest that cochlea removal differentially regulates the biosynthetic activity of GAD and subsequent levels of GABA in NM. Additional evidence that sensory deafferentation can regulate the activity of GAD comes from studies in the mammalian cochlear nucleus (CN). Unilateral cochlear ablation results in a small but significant changes in the enzymatic *activity* of GAD in the ipsilateral CN even though its *concentration* remains unchanged (Fisher and Davies, '76). This implies that there would be no change in the amount of GAD or in the number of terminals containing GAD when observed immunocytochemically. However, if the activity of GAD is altered, then the levels of GABA might also be changed and possibly be reflected in the number of GABA-I terminals. Cochlea removal may also affect the activity of other enzymes which control GABA levels, such as GABA-transaminase (GABA-T). GABA-T is contained in many CN cells (Davies, '75) and its activity is reduced in ipsilateral CN after cochlear ablation (Fisher and Davies, '76). Currently little is known about the mechanisms which control the regulation of the activities of these enzymes (see Martin, '87 and Tunnicliff and Ngo, '86 for reviews). Thus, further speculation about the biochemical mechanisms underlying the phenomenon reported here seems unwarranted.

Gradient of GABAergic terminals

In normal posthatch chicks, the density of GABAergic terminals increases in a rostromedial-to-caudolateral direction across NM (Code et al., '89a). The density of GABA-I and GAD-I terminals in ipsilateral NM still maintains this gradient after cochlea removal (see Figs. 4 and 10). These data suggest that the organization of the GABAergic terminals in NM is not regulated by afferent input from the cochlea but may be regulated by sources intrinsic to the cochlear nucleus.

ACKNOWLEDGMENTS

GAD antiserum was generously supplied by Dr. David Gottlieb, Washington University. We would like to thank Dr. Robert Bolender, University of Washington, for his helpful comments and advice on the area-weighted sampling method. We are indebted to Dr. Rick Hyson for his help with the statistical analyses. We appreciate Dr. Bill Lippe's critical reading of this manuscript. This research was supported by NIH grants HD07239 and DC00393.

LITERATURE CITED

Bear, M.F., D.E. Schmechel, and F.F. Ebner (1985) Glutamic acid decarboxylase in the striate cortex of normal and monocularly deprived kittens. J. Neurosci. *5*:1262–1275.

Born, D.E., and E.W Rubel (1984) Cochlea removal eliminates physiological activity in brain stem auditory nuclei of the chicken. Soc. Neurosci. Abstr. *10*:843.

Born, D.E., and E.W Rubel (1985) Afferent influences on brain stem auditory nuclei of the chicken: Neuron number and size following cochlea removal. J. Comp. Neurol. *231*:435–445.

Canzek, V., and J.C. Reubi (1980) The effect of cochlear nerve lesion on the release of glutamate, aspartate, and GABA from cat cochlear nucleus, in vitro. Exp. Brain Res. *38*:437–441.

Carr, C.E., I. Fujita, and M. Konishi (1989) The distribution of GABAergic neurons and terminals is correlated with functional inhibition in the auditory system of the barn owl. J. Comp. Neurol. *286*:190–207.

Code, R.A., G.D. Burd, and E.W Rubel (1987) Development of GABA-immunoreactive terminals in chick brain stem auditory nuclei. Soc. Neurosci. Abstr. *13*:543.

Code, R.A., G.D. Burd, and E.W Rubel (1989a) Development of GABA-immunoreactivity in brain stem auditory nuclei of the chick: Ontogeny of gradients in terminal staining. J. Comp. Neurol. *284*:504–518.

Code, R.A., D. Durham, and E.W Rubel (1988) Effects of unilateral cochlea removal on GABA-immunoreactive terminals in nucleus magnocellularis of the chicken. Eleventh Midwinter Meet. Assoc. Res. Otolaryngol., Clearwater Beach, FL, Jan. 31–Feb. 4, pp. 43–44.

Code, R.A., D. Durham, and E.W Rubel (1989b) Comparison of glycine-, GABA-, and GAD-immunoreactivities in the auditory brain stem of the chick. Twelfth Midwinter Meet. of the Assoc. Res. Otolaryngol., St. Petersburg Beach, FL, pp. 33–34.

Code, R.A., and E.W Rubel (1989) Glycine-immunoreactivity in the auditory brain stem of the chick. Hearing Res. *40*:167–172.

Cotman, C.W., M. Nieto-Sampedro, and E.W. Harris (1981) Synapse replacement in the nervous system of adult vertebrates. Physiol Rev. *61*:684–784.

Davies, W.E. (1973) Glutamic Acid Decarboxylase Content of the Cochlear Nucleus after Cochlear Destruction, I.R.C.S. (Medical Science), 16-7-6.

Davies, W.E. (1975) The distribution of GABA-transaminase–containing neurones in the cat cochlear nucleus. Brain Res. *83*:27–33.

Dietrich, W.D., D. Durham, O.H. Lowry, and T.A. Woolsey (1982) "Increased" sensory stimulation leads to changes in energy-related enzymes in the brain. J. Neuroscience *2*:1608–1613.

Fex, J., and R.A. Altschuler (1984) Glutamic acid decarboxylase immunoreactivity of olivocochlear neurons in the organ of Corti of guinea pig and rat. Hearing Res. *15*:123–131.

Fex, J., R.A. Altschuler, B. Kachar, R.J. Wenthold, and J.M. Zempel (1986) GABA visualized by immunocytochemistry in the guinea pig cochlea in axons and endings of efferent neurons. Brain Res. *366*:106–117.

Fisher, S.K., and W.E. Davies (1976) GABA and its related enzymes in the lower auditory system of the guinea pig. J. Neurochem. *27*:1145–1155.

Godfrey, D.A., J.A. Carter, S.J. Berger, O.H. Lowry, and F.M. Matschinsky (1977) Quantitative histochemical mapping of candidate transmitter amino acids in cat cochlear nucleus. J. Histochem. Cytochem. *25*:417–431.

Gottlieb, D.I., Y.-C. Chang, and J.E. Schwob (1986) Monoclonal antibodies to glutamic acid decarboxylase. Proc. Natl. Acad. Sci. U.S.A. *83*:8808–8812.

Houser, C.R., M. Lee, and J.E. Vaughn (1983) Immunocytochemical localization of glutamic acid decarboxylase in normal and deafferented superior colliculus: Evidence for reorganization of γ-aminobutyric acid synapses. J. Neurosci. *3*:2030–2042.

Hsu, S.M., L. Raine, and H. Ganger (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. *29*:577–580.

Jackson, H., and T.N. Parks (1988) Induction of aberrant functional afferents to the chick cochlear nucleus. J. Comp. Neurol. *271*:106–114.

Jhaveri, S., and D.K. Morest (1982) Neuronal architecture in nucleus magnocellularis of the chick auditory system with observations on nucleus laminaris: A light and electron microscope study. Neuroscience *7*:809–836.

Martin, D.L. (1987) Regulatory properties of brain glutamate decarboxylase. Cell. Mol. Neurobiol. *7*:237–253.

Miles, R.E., and P.J. Davy (1976) Precise and general conditions for the validity of a comprehensive set of stereological fundamental formulae. J. Microsc. *107*:221–226.

Müller, C.M. (1987) γ-Aminobutyric acid immunoreactivity in brainstem auditory nuclei of the chicken. Neurosci. Lett. *77*:272–276.

Parks, T.N. (1981) Morphology of axosomatic endings in an avian cochlear nucleus: Nucleus magnocellularis of the chicken. J. Comp. Neurol. *203*:425–440.

Parks, T.N., and E.W. Rubel (1978) Organization and development of the brain stem auditory nuclei of the chicken: Primary afferent projections. J. Comp. Neurol. *180*:439–448.

Raisman, G. (1969) Neuronal plasticity in the septal nuclei of the adult rat. Brain Res. *14*:25–48.

Roberts, E., and K. Kuriyama (1968) Biochemical-physiological correlations in studies of the γ-aminobutyric acid system. Brain Res. *8*:1–35.

Schneider, G.E. (1973) Early lesions of superior colliculus: Factors affecting the formation of abnormal retinal projections. Brain, Behav. Evol. *8:*73–109.

Steward, O., and S.L. Vinsant (1978) Identification of the cells of origin of a central pathway which sprouts following lesions in mature rats. Brain Res. *147:*223–243.

Thompson, G.C., M. Igarashi, and A.M. Cortez (1986) GABA imbalance in squirrel monkey after unilateral vestibular end-organ ablation. Brain Res. *370:*182–185.

Tsukahara, N. (1981) Synaptic plasticity in the mammalian central nervous system. Annu. Rev. Neurosci. *4:*351–379.

Tunnicliff, G., and T.T. Ngo (1986) Regulation of γ-aminobutyric acid synthesis in the vertebrate nervous system. Neurochem. Int. *8:*287–297.

von Bartheld, C.S., R.A. Code, and E.W Rubel (1989) GABAergic neurons in brain stem auditory nuclei of the chick: Distribution, morphology and connectivity. J. Comp. Neurol. *287:*470–483.

Wenthold, R.J. (1979) Release of endogenous glutamic acid, aspartic acid and GABA from cochlear nucleus slices. Brain Res. *162:*338–343.

Young, S.R., and E.W Rubel (1986) Embryogenesis of arborization pattern and topography of individual axons in n. laminaris of the chicken brain stem. J. Comp. Neurol. *254:*425–459.