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The Influence of Inhibitory Afferents on the Development of Postsynaptic Dendritic Arbors

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ABSTRACT

The growth and maintenance of dendritic form is dependent on normally functioning excitatory afferents. We have now examined the development of dendritic arbors in the gerbil lateral superior olive (LSO), following contralateral cochlear removal at postnatal day 7, a manipulation that substantially eliminates driven inhibitory transmission. Previous studies have demonstrated that the morphology of LSO dendritic arbors varies with tonotopic position and becomes more restricted with age.

The presumed decrease of inhibitory transmission in the contralateral LSO resulted in a *hypertrophic* response. Quantification of Golgi-impregnated neurons revealed that dendrites had a significantly greater number of branch points, and their arbors were more spread out along the frequency axis compared to normal. This was especially apparent in the high frequency projection region where the glycine receptor density is known to be 4-fold higher than in the low frequency projection region. A measure of LSO nucleus size, cross-sectional area, was identical to control values, indicating no overt signs of degenerative phenomena. Cochlear ablation resulted in a significant atrophy of the ipsilateral LSO, with significant effects on dendritic structure.

We conclude that decreased inhibitory transmission during development does not lead to a net degenerative response. Rather, the postsynaptic neurons exhibit a hypertrophic phenotype that may be due to the persistence of an immature state. These results indicate that activity-dependent morphogenetic events are a consequence of both excitatory and inhibitory synaptic transmission.

Key words: auditory pathways, development, inhibition, lateral superior olive, dendrites

Decreasing excitatory transmission typically leads to degenerative changes in postsynaptic neuron morphology, particularly during development (Larsell, '31; Levi-Montalcini, '49; Matthews et al., '60; Wiesel and Hubel, '63; Globus and Scheibel, '66; Peusner and Morest, '77; Benes et al., '77; Parks, '81). These effects appear to be at least partially attributable to the transmitter-evoked regulation of postsynaptic protein metabolism (Steward and Rubel, '85; Durham and Rubel, '85; Hyson and Rubel, '89). To date, the influence of inhibitory synaptic transmission on the development or maintenance of neural elements has not been the subject of experimental analysis. We report here a *hypertrophic* response in lateral superior olivary dendrites following the functional deafferentation of their inhibitory synaptic input.

The lateral superior olive (LSO), a brainstem auditory nucleus, is a favorable system for determining the developmental influence of inhibition. The contralateral afferents to the LSO derive primarily from a set of glycinergic neurons in the medial nucleus of the trapezoid body (MNTB: Rasmussen, '46; Browner and Webster, '75; Moore and Caspary, '83; Glendenning et al., '85; Spangler et al., '85; Peyret et al., '87; Wenthold et al., '87; Zook and DiCaprio, '88; Wenthold et al., '90; but see Kitzes et al., '91). Since this predominant inhibitory system is activated by contralateral sound stimuli (Boudreau and Tsuchitani, '70; Caird and Klinke, '83; Harnischfeger et al., '85; Sanes and Rubel, '88), unilateral cochlear ablation leads to a relatively discreet removal of inhibition to the contralateral LSO. Unilateral cochlear ablation also disrupts the major excitatory afferent pathway to the ipsilateral LSO which originates in the ipsilateral ventral cochlear nucleus (Warr, '82; Cant and Casseday, '86). In this case, an extirpation at postnatal day 7 leads to a 50% cell death as well as soma shrinkage in the cochlear nucleus within 2 weeks (Hashisaki and Rubel, '89).

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The morphology of the LSO principal neuron dendrites has been shown to vary along the LSO tonotopic axis in adult gerbils, with high frequency neurons having a more restricted field (Sanes et al., '90). This dendritic pattern emerges during the third postnatal week as a result of regressive events (Sanes et al., '92). The functional development of the gerbil auditory system is largely occurring at the same time. Animals first become responsive to airborne sound on postnatal day 12, and an adult-like state is reached between days 18–30, depending on the electrophysiological assay (Finck et al., '72; Ryan and Woolf, '88; Harris and Dallos, '84; Woolf and Ryan, '84, '85; Sanes and Rubel, '88; Sanes et al., '89).

Several findings indicate that the inhibitory projection originating from the MNTB is somewhat dynamic during development. First, with a computer-aided morphometric system, we have recently determined that single inhibitory arborizations become successively more restricted during development (Sanes and Siverls, '91). Second, our in vivo electrophysiological data also point to a mutable system: sound-evoked inhibition is more efficacious in the low frequency region of the LSO of young animals (Sanes and Rubel, '88). Third, the distribution of glycine receptors in the lateral superior olive changes during development (Sanes and Wooten, '87). Finally, inhibitory postsynaptic potentials in the LSO are much more pronounced in length and amplitude during the early stages of maturation (Sanes, '92). These normal developmental modifications suggested that inhibitory afferents might also contribute to the maturation of postsynaptic neurons.

MATERIALS AND METHODS

Cochlear ablation

Gerbils (*Meriones unguiculatus*) aged 7 days postnatal were anesthetized with hypothermia so that respiration ceased and a nociceptive response could not be evoked. An incision was made ventral and slightly posterior to the pinna, and the soft tissue was retracted from the ventrolateral wall of the bulla. A small hole was made in the bulla with a forceps tip, and the middle ear mesenchyme was removed with an aspirator. A small hole was then made in the cochlear wall, and the contents were rapidly freed with a forceps and cleared with a needle aspirator. A piece of gel foam was placed in the vacant cochlear cavity, and the wound was closed with cyanoacrylate glue. The animals were gradually warmed on a heating pad and returned to the nest after respiration and motor activity resumed. The animals were reared for the following 2 weeks in the same conditions as normal animals.

Golgi impregnations

Tissue was prepared as described previously (Adams, '79; Sanes et al., '90). Briefly, gerbils aged 21 days were given a lethal dose of anesthetic (sodium pentobarbital; 80 mg/kg; i.p.) and perfused transcardially with 0.9% NaCl, 10% buffered formalin, and a potassium dichromate mordant. Following the perfusion, the brain case of each animal was microdissected to verify that the left cochlea was intact and that the right cochlea had been removed. Successful extirpations were quite evident, as the cochlear cavity was filled with the hardened piece of gel foam that had been inserted after the cochlea was removed. The brains were stored in mordant for 3 days and transferred to a silver nitrate solution for 3–4 days. Transverse vibratome sections at 150 μm were cleared in methyl salicylate and mounted for viewing.

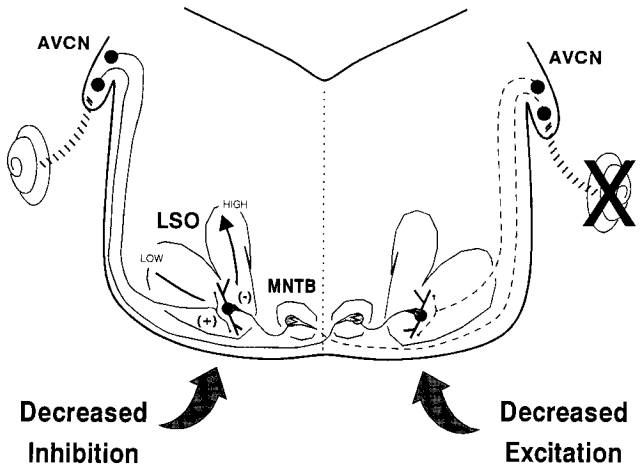

Fig. 1. Schematic of the experimental manipulation. The right cochlea was extirpated on postnatal day 7, and the lateral superior olive (LSO) was examined 2 weeks later. The cochlear ablation leads to decreased cell number in the ipsilateral cochlear nucleus (AVCN). Therefore, the ipsilateral LSO is deprived of both excitatory activity and AVCN afferents. The contralateral LSO is primarily deprived of inhibitory activity. MNTB, medial nucleus of the trapezoid body.

Image analysis and 3-D reconstruction

The morphometric analysis of Golgi impregnated neurons has been described previously (Sanes et al., '90; Sanes et al., '92). Briefly, principal neurons with distinguishable processes, and no significant cut ends were observed with a light microscope (Zeiss Standard 16; Planapo 40 oil) and displayed on a color monitor (MTI-Dage Series 68 Neuvicon Video Camera). A custom designed program was used for data acquisition, analysis, and 3-D reconstruction (Cellmate/Treemate, R & M Biometrics; Canaday et al., '90). The numerical analyses included the number of primary dendrites, the number of branch points, the total dendrite length, the tonotopic position, the dendrite spread along the tonotopic axis, and the LSO cross-sectional area. The dendrite spread along the tonotopic axis was obtained from a calibrated plot of each neuron. A rectangle was drawn around the dendritic field with the long axis oriented parallel to the presumed isofrequency contour (Sanes et al., '89), and the dimension perpendicular to the isofrequency contour was measured.

Explanation of data groups

The level of analysis necessitates that certain definitions be made clear from the outset. Figure 1 shows a schematic of the experimental paradigm. The data from animals with unilateral cochlear ablations were divided into neurons in the LSO ipsilateral to the ablations (CA_I), and those contralateral to the ablation (CA_C). All parametric measures were pooled for neurons with a position $< 50\%$ from the apical most projection region or $\geq 50\%$ from the apical most projection region. The rationale for dividing the data into low and high frequency regions is based on our original report of dendritic form (Sanes et al., '90), and several recent anatomical findings in the gerbil LSO (Sanes et al., '87; Schwartz and Hockfield, '89; Schwartz and Eager, '91; Hafidi et al., '91, '92). These two groups will hereafter be

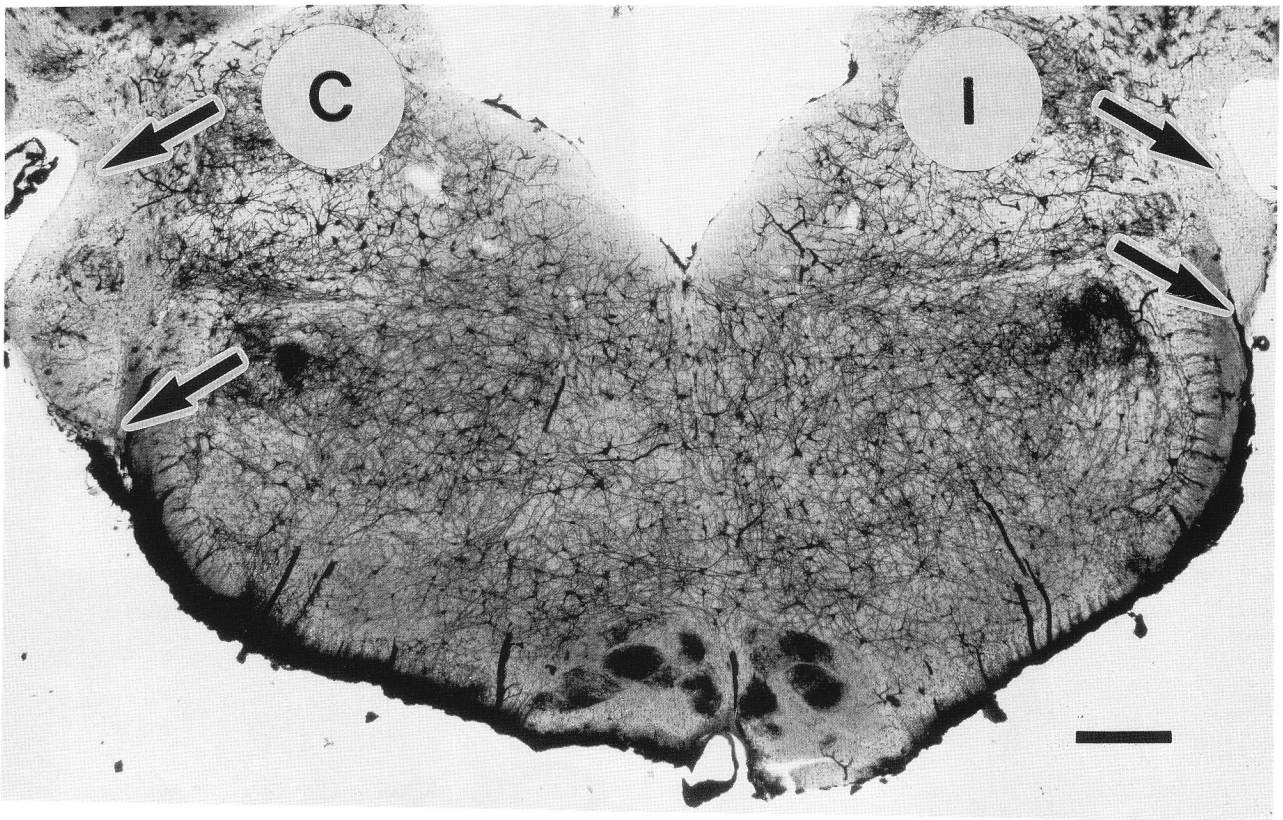

Fig. 2. Low power photomicrograph of a Golgi section through the brainstem of an experimental animal at 21 days postnatal. The ventral cochlea nucleus ipsilateral to the ablated cochlea (I) has atrophied, whereas the ventral cochlear nucleus contralateral to the ablated cochlea (C) remained of normal size. The ventral cochlear nucleus on both sides is demarcated by two arrows. Bar, 400 μm.

referred to as the *low frequency* neurons and *high frequency* neurons, respectively.

RESULTS

The quantitative results of this study are drawn from 109 neurons taken from 21 Mongolian gerbil brains sectioned in the transverse plane. In addition, we compared values previously obtained from normal 21 day animals (Sanes et al., '92). The global consequences of unilateral cochlear ablation are illustrated in Figure 2. The ipsilateral cochlear nucleus was much decreased in size compared to the contralateral side. A measure of LSO size, maximum cross-sectional area, was obtained as a means of comparing the LSO ipsilateral and contralateral to the ablated cochlea. The value from normal animals was 0.284 ± 0.02 mm^2 ($\overline{X}$ ± SE), significantly greater than the LSO ipsilateral to the manipulation, 0.173 ± 0.01 mm^2 ($t = 5.10$; $P < 0.0001$; $df = 29$). The value for the LSO contralateral to the manipulation 0.275 ± 0.01 mm^2 was not significantly different from normal ($t = 0.435$; $P > 0.5$; $df = 34$).

Figure 3 shows representative neurons from a normal 21 day animal, and from the LSO contralateral to a cochlear ablation. These photomicrographs indicate a relative proliferation of processes in the neuron that had developed with decreased inhibitory transmission. There were a number of neurons that displayed a unique morphological trait: dendrites were found to reverse their direction of growth (Fig. 4). This characteristic was not previously recognized in neurons from adult animals, nor during development (Sanes et al., '90; Sanes et al., '92).

Consequences of presumed decrease of inhibitory transmission

The greatest parametric difference between normal 21 day LSO neurons and those from the LSO contralateral to the ablated cochlea was in branch point number. The value for CA_C high frequency neurons, 14 ± 1 branches per neuron ($\overline{X}$ ± SE), was significantly greater than that from normal animals, 7 ± 1 branches ($t = 5.55$; $P < 0.0001$; $df = 56$). This change was also found for low frequency neurons, but the disparity was not as great (Fig. 5). For both high and low frequency neurons, the enhancement of branch number was associated with a significant spread of dendritic arbors across the frequency axis (Fig. 6). For example, CA_C high frequency dendrites spread 80 ± 5 μm along the frequency axis, whereas the value from neurons in control animals was 49 ± 4 μm ($t = 4.86$; $P < 0.0001$; $df = 57$).

There were two significant differences between normal and CA_C neurons that were only found in the high frequency region. First, the total dendritic length for CA_C neurons, 1,086 ± 58 μm, was greater than in normals, 865 ± 47 μm ($t = 2.99$; $P < 0.005$; $df = 57$). Second, the mean number of primary dendrites for CA_C neurons, 3.7 ± 0.3, was less than in normals, 4.5 ± 0.2 ($t = 2.35$; $P < 0.05$;

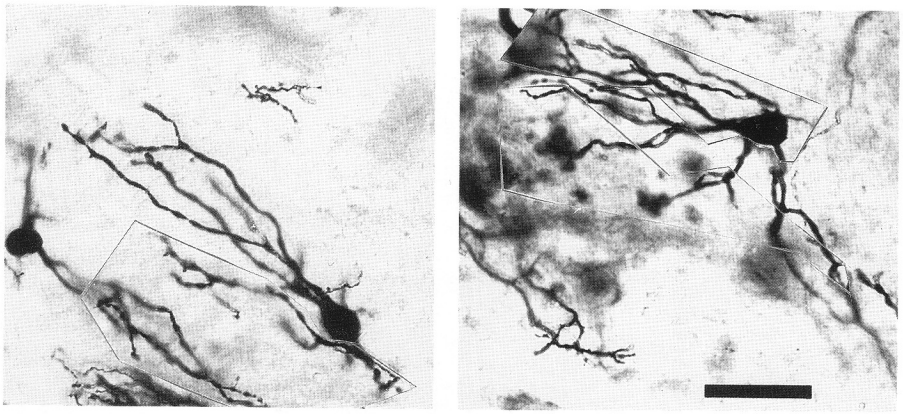

Fig. 3. Photomicrographs of Golgi-stained LSO neurons in a normal animal (left), and contralateral to a cochlear ablation (right) at 21 days postnatal. The branching pattern of the neuron contralateral to the ablated cochlea is more exuberant than that of the neuron from a normal animal. In neither case is the entire dendritic arborization entirely in focus. Bar, 50 μm.

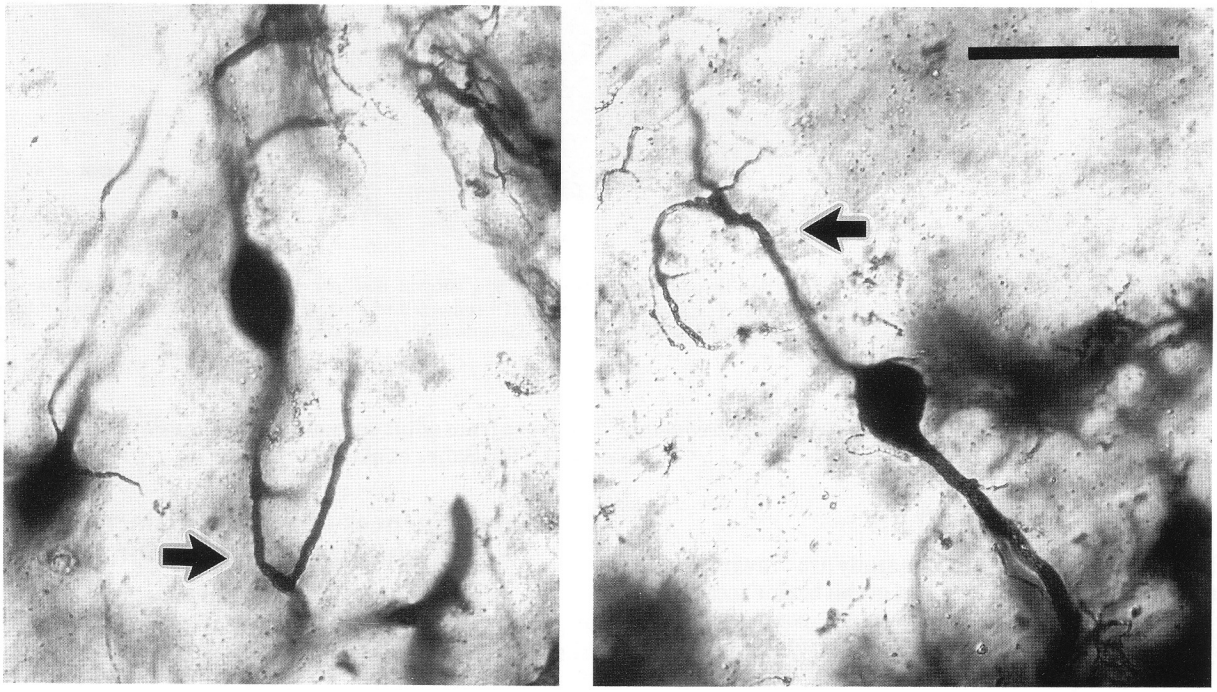

Fig. 4. Photomicrographs of two neurons from the LSO contralateral to a cochlear ablation (left), and ipsilateral to a cochlear ablation (right). In both cases, one of the dendritic processes is seen to have grown back in the opposite direction (arrows). Bar, 50 μm.

df = 57). There were no significant changes to low frequency neurons for either of these two parameters.

Consequences of presumed decrease of excitatory transmission

The LSO ipsilateral to the ablated cochlea was much decreased in size, presumably due to the decreased ipsilateral cochlear nucleus afferent projection (Hashisaki and Rubel, '89). We did not determine whether there was a decrease in LSO neuron number. For both low and high frequency CA_I neurons there was a modest, but significant increase in branch point number compared to normal (Fig. 5). High frequency CA_I neurons showed a large increase in dendritic spread across the frequency axis with a value of 98 ± 9 μm, compare to the normal value of 49 ± 4 μm (Fig. 6). There was a significant difference between high frequency CA_C and CA_I neurons for both the number of branch points ($t = 3.28$; $P < 0.005$; $df = 55$), and the total dendritic length ($t = 2.93$; $P < 0.01$; $df = 55$). However, the total dendritic length of both high and low frequency CA_I neurons was not significantly different than that of normals.

DISCUSSION

We have demonstrated an effect on dendrite morphology following a manipulation that presumably inactivates the

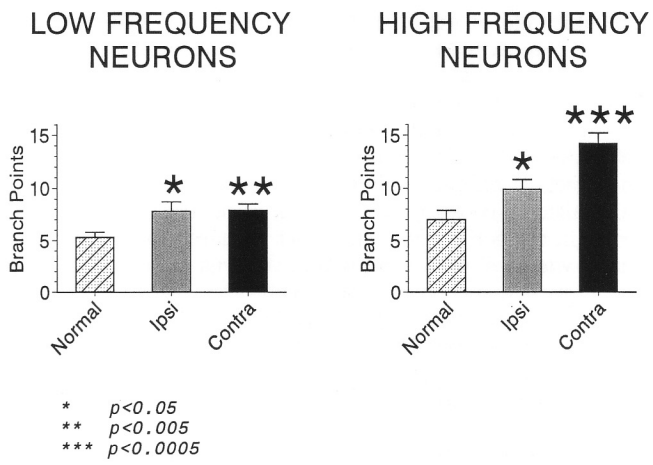

Fig. 5. The mean number of branch points per LSO neuron in normal and experimental 21-day animals. For low frequency neurons there was a modest increase in branch points ipsilateral (Ipsi) and contralateral (Contra) to the ablated cochlea. For high frequency neurons there was a dramatic increase in branch point number contralateral to the ablated cochlea.

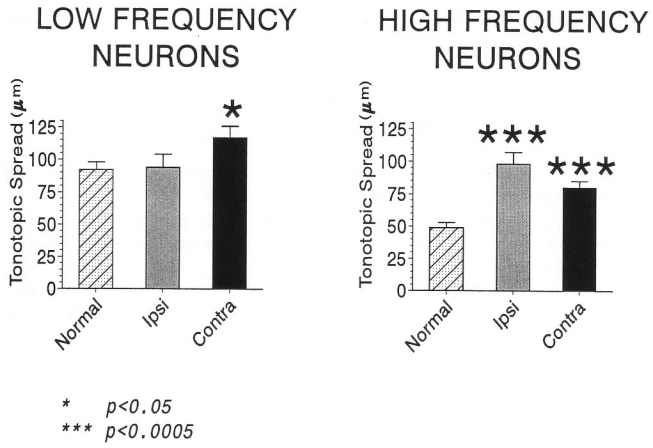

Fig. 6. The spread of dendritic arbors across the LSO tonotopic axis in normal and experimental 21-day animals. For low frequency neurons there was a significant increase in dendritic arbor spread contralateral (Contra) to the ablated cochlea. For high frequency neurons there was a highly significant increase on both sides (Ipsi and Contra).

predominant inhibitory afferent population to the LSO, the glycinergic projection from the MNTB. The largest changes were found for high frequency neurons contralateral to the cochlear ablation. There was a significant increase in dendrite branch points, total dendritic length, and the distance that these dendrites spread across the frequency axis (Figs. 5 and 6). The effect was much less pronounced in the low frequency projection region. This result is consistent with the known distribution of glycinergic afferents to the gerbil LSO. We have previously reported that there is a 4-fold greater concentration of ^{3}H-strychnine binding sites in the high frequency region (Sanes et al., '87; Sanes and Wooten, '87), and that approximately 70% of the MNTB neurons project to the high frequency region (Sanes and Siverls, '91). Therefore, the region of the LSO that should be most affected by decreased inhibitory transmission is the high frequency region.

The effects of afferent activity on the central auditory system

The development of dendrite morphology is well correlated with afferent activity in the central nervous system (Valverde, '68; Rakic, '72; Rakic and Sidman, '73; Pinto Lord and Caviness, '79; Berry et al., '80; Vaughn et al., '88; Harris and Woolsey, '81). In the central auditory system, manipulations that lead to reduced excitatory activity and deafferentation are associated with degenerative effects. These include cell death and reductions in neuron size (Levi-Montalcini, '49; Murphey et al., '75; Parks, '79, '81; Coleman and O'Connor, '79; Webster and Webster, '79; Conlee and Parks, '81; Feng and Rogowski, '80; Trune, '82; Blatchley et al., '83; Webster, '83; Deitch and Rubel, '84; Born and Rubel, '88; Moore and Kowalchuk, '88; Hashisaki and Rubel, '89). There is evidence that the underlaying mechanisms include abnormal protein metabolism (Steward and Rubel, '85), and a decline in tubulin polymerization (Deitch and Rubel, '89).

Although degenerative sequelae are the norm for such manipulations, there are two reports showing increased dendrite size in the central auditory system following unilateral cochlear ablation or sound attenuation. Smith and co-workers ('83) found that low frequency neuron dendrites in the chicken brain stem were larger following a period of monaural conductive hearing loss. The results were interpreted to reflect an increase in low frequency-evoked activity as a result of enhanced bone conduction. An increase in dendrite length was also found for nonpyramidal neurons in the rabbit auditory cortex following neonatal cochlear ablation and was hypothesized to be a consequence of afferent reorganization (McMullen et al., '88).

To the extent that the present results, showing an increase in dendrite branching and length, have any general applicability, it would be necessary to investigate whether there was a specific change of inhibitory transmission in the two experimental situations where dendrite length increased. For example, the density of GABAergic terminals was quantified in the chicken nucleus magnocellularis and found to be significantly greater in the low frequency projection region (Code et al., '89). These authors also illustrated a greater density of GABA immunoreactive terminals in the lateral region of nucleus laminaris (Code et al., '89; Fig. 4E,F), which is more closely associated with the low frequency projection region than the medial portion of the nucleus (Rubel and Parks, '75). Therefore, it is possible that monaural conductive hearing loss led to a net reduction of inhibitory transmission, primarily in the low frequency projection region of nucleus laminaris, leading to enhanced dendritic growth.

Comparison between ipsilateral and contralateral LSO

In the present experiment, it is likely that the unilateral cochlear ablation led to qualitatively different manipulations at the ipsilateral and the contralateral LSO. Following cochlear ablation at postnatal day 7 the ipsilateral anteroventral cochlear nucleus exhibits a marked cell death and atrophy (Hashisaki and Rubel, '89). Therefore, the ipsilateral LSO would be expected to have a much decreased excitatory afferent projection. Indeed, we found that the size of the ipsilateral LSO is much smaller than normal, and this atrophy may well include cell loss in the ipsilateral LSO. There is no such global change in LSO nucleus size

contralateral to the ablation. Although there is no information on cell loss in the contralateral MNTB, the inhibitory afferent population to the contralateral LSO, there was no obvious difference in nucleus size compared to the undeprived MNTB. However, it has previously been found that cochlear ablation leads to a reduced soma size in the contralateral MNTB (Webster and Webster, '79). Given the general atrophic condition of the ipsilateral LSO, the dendritic changes reported here are difficult to interpret.

We must also consider the other, less defined, synaptic inputs to the LSO. There is electrophysiological evidence for an ipsilateral inhibitory projection to the cat LSO (Brownell et al., '79), and immunocytochemical evidence for GABAergic synaptic endings in the gerbil (Roberts and Ribak, '87). In addition, a direct projection from the contralateral cochlear nucleus to part of the low frequency region of the LSO has recently been described in the gerbil (Kitzes et al., '91). While it is clear that neither experimental side is an elementary case of excitatory or inhibitory deprivation, the contralateral LSO more closely approximates the ideal, particularly in the high frequency region. However, a rigorous examination of the potential afferent influences underscores the difficulty in interpreting all experimental paradigms of this sort.

Interpretation of inhibitory deprivation

A decrease in synaptic inhibition in the LSO could have both direct and indirect effects on the postsynaptic neuron. Normal MNTB-evoked synaptic transmission would be expected to gate chloride channels in the LSO (Coombs et al., '55; Barker and McBurney, '79; Moore and Caspary, '83; Borman et al., '87), and electrical stimulation of the MNTB elicits hyperpolarizing synaptic potentials even at 3 days postnatal (Sanes, '92). The level of spontaneous activity prior to ear canal opening is not known, but results from other systems suggest that some level of maintained discharge is present (Lippe, '84; Sanes, '84; Fitzgerald, '87; Galli and Maffei, '88). Therefore, a direct consequence of unilateral cochlear ablation would be an average decrease in Cl^- levels within the contralateral LSO neurons. Since the regulation of internal pH is known to be dependent on internal Cl^- levels (Thomas, '82), this may constitute a secondary cellular effect of decreasing glycinergic transmission. Another possibility is that decreased inhibitory transmission influences the production of a trophic factor by the postsynaptic population (Zafra et al., '91). Although such effects do not necessarily occur following cochlear ablation, they are advanced to suggest that decreased inhibitory synaptic transmission may influence the postsynaptic neurons metabolism.

A second potential consequence of decreased inhibition would be a relative increase in postsynaptic depolarization. With fewer spontaneous and evoked hyperpolarizing events, the excitatory pathway from the AVCN would be expected to bring the LSO neuron to threshold more often. The relative enhancement of the excitatory synaptic efficacy may lead directly to postsynaptic growth, or may allow the excitatory afferents to establish a larger terminal field, and support more postsynaptic membrane. Results from in vitro experiments suggest that inhibitory transmission can modulate process outgrowth (Michler-Stuke and Wolff, '87; Mattson and Kater, '89). Finally, the functionally denervated MNTB neurons appear to alter their axonal arborizations within the LSO (Sanes, preliminary observations), as has been shown for visual excitatory projections following the blockade of action potentials (Reh and Constantine-Paton, '85; Stretavan et al., '88). The aberrant growth of any afferent pathway to the LSO would be expected to influence postsynaptic form.

The present results demonstrate that both synaptic excitation and inhibition must be considered when interpreting the effects of afferent activity. Decreased inhibitory transmission failed to constrain the growth of principal neuron dendrites in the gerbil LSO. The effect appeared to be specific in that it was most prominent within the area of the LSO having the greatest density of glycine receptors (Sanes et al., '87). We have recently tested this hypothesis by pharmacologically blocking the glycine receptor with sublethal doses of strychnine during the postnatal period, and the results are fully consistent with those reported above for contralateral cochlear ablation (Sanes and Chokshi, '92). Finally, the spread of dendritic arbors across a greater distance of the tonotopic axis noted in this report may allow us to experimentally test the hypothesis that postsynaptic dendritic dimensions influence frequency selectivity (Sanes et al., '90).

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