Cochlear nucleus cell size is regulated by auditory nerve electrical activity

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Accumulating evidence suggests that sensorineural hearing loss in animals is rapidly followed by degenerative changes in central auditory neurons. For example, cochlear removal in birds and mammals results in a reduction in central auditory neuron cell size within 48 hours. A similar decrease in cell size after pharmacologic blockade of auditory nerve electrical activity with tetrodotoxin has been reported. In the present study, we evaluate the reversibility of central auditory changes after a profound sensorineural hearing loss caused by blockade of auditory nerve action potentials. Tetrodotoxin, which blocks voltage-sensitive sodium channels, was embedded in a slow-release vehicle and placed next to the round window membrane of gerbils. Tetrodotoxin diffused into perilymph and unilaterally blocked electrical activity in auditory nerve axons. Electrical activity blockade was confirmed with recordings of auditory brainstem response. Animals were killed immediately after 24 hours of electrical blockade or 7 days after a transient 24- or 48-hour blockade. Large spherical cells of the anteroventral cochlear nucleus ipsilateral to manipulation were measured and compared to large spherical cells on the opposite, unmanipulated side of the brain. Animals killed immediately after a 24-hour blockade of electrical activity showed a mean decrease of 16% in cell size ipsilateral to the blockade ($p < 0.05$). In animals allowed to recover for 7 days after blockade for 24 or 48 hours, cell size returned to previous levels. There was no longer a consistent difference in cell size between the two sides of the brain in these animals ($p > 0.05$). Thus changes in size of anteroventral cochlear nucleus large spherical cells were reversible after recovery of electrical stimulation. These data provide evidence that electrical activity is involved in the regulation of cell size in mammalian central auditory neurons. Additionally, cochlear nucleus neurons can return to their former size after brief periods without stimulation. (OTOLARYNGOL HEAD NECK SURG 1991;104:8.)

Electrical stimulation of the cochlea by means of implantable electrodes is now possible for the audiologic rehabilitation of some patients with profound sensorineural hearing loss.[1-3] This strategy has the advantage of bypassing missing receptor cells to directly stimulate auditory nerve fibers. However, correction of deafness with implantable cochlear electrodes requires intact and potentially functional auditory nerves and central auditory pathways.

Accumulating evidence suggests that the viability and maintenance of central auditory neurons are dependent on continuing afferent input. For example, experimental manipulations of the peripheral auditory system that cause a hearing loss in young gerbils, mice, rats, or chickens are associated with a decrease in the size and number of central auditory neurons.[4-7] Additionally, neurons that survive the loss of cochlear input demonstrate decreased levels of protein synthesis, decreased glucose uptake, and alterations in enzyme activity.[8-10] Thus, it is possible that cochlear damage that causes a profound hearing loss may be associated with a secondary compromise of central auditory system structure and function.

Previous work has indicated that the electrical activity in auditory nerve fibers and subsequent activation of postsynaptic receptors may be the biologic signal that regulates the metabolism of neurons in the cochlear

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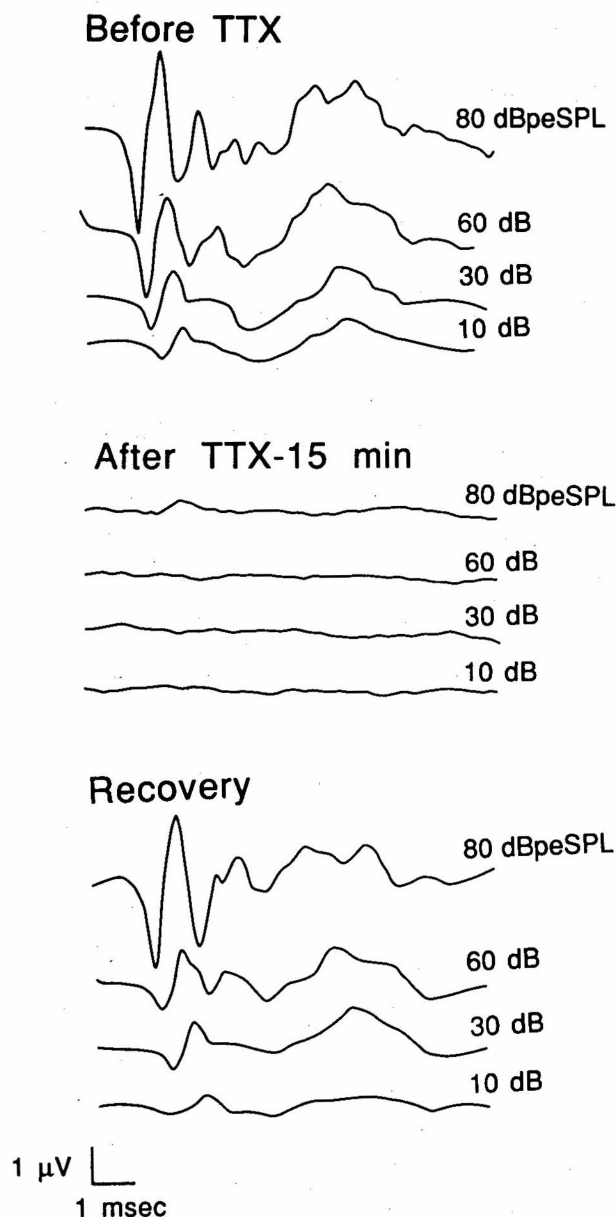

Fig. 1. A series of ABR recordings is presented at decreasing stimulus intensities in a normal animal, in the same animal after TTX blockade of auditory nerve action potentials, and after recovery from TTX. A reproducible waveform is not obtainable during TTX exposure. An intensity series similar to the initial series is obtained after recovery from action potential blockade. *peSPL,* Peak equivalent SPL.

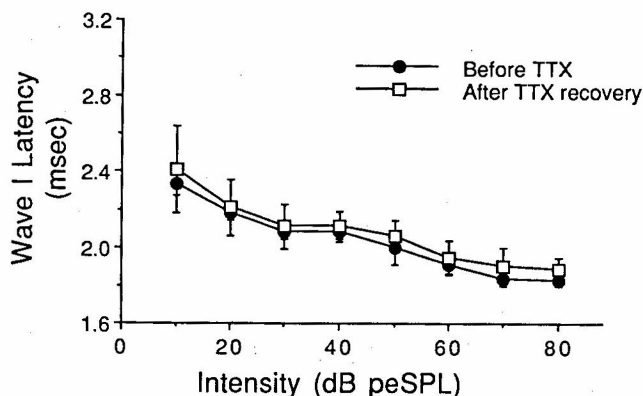

Fig. 2. The mean latency ± SEM of ABR wave I is shown at increasing stimulus intensities before auditory nerve electrical activity blockade and after recovery from activity blockade ($n = 5$). There is no significant difference in latency/intensity series. This indicates that a full functional recovery from TTX-induced electrical activity blockade has occurred. *peSPL,* Peak equivalent SPL.

nucleus.[11-13] Spontaneous and evoked electrical activity in the auditory nerve can be blocked by infusion of tetrodotoxin (TTX) into the perilymph of the inner ear.[11] In gerbils, anteroventral cochlear nucleus (AVCN) cell size significantly decreases within 48 hours of unilateral surgical ablation of the cochlea. Pharmacologic blockade of auditory nerve electrical activity produces the same change in cell size as cochlea ablation. These findings are consistent with the hypothesis that net electrical activity in the auditory nerve acts as a regulatory signal to maintain the metabolic and structural integrity of cells in the central auditory nervous system.[14]

It is unknown if such changes in cellular properties are permanent or reversible, a distinction that may determine the ultimate utility of electrical auditory stimulation in humans after prolonged hearing loss. If cochlear nucleus neurons are not capable of recovery on return of afferent stimulation, neurologic constraints may be imposed on audiologic rehabilitation. However, if cochlear nucleus neurons are capable of recovery after a temporary loss of input, the full potential of the central auditory system may be retained. Additonally, if central auditory neurons can recover, they will have shown a degree of plasticity dependent on auditory nerve activity not previously appreciated.

We unilaterally blocked auditory nerve electrical activity in gerbils for 24 hours. We then compared the size of AVCN large spherical cells on the side of the brain ipsilateral to electrical activity blockade with cell size on the contralateral, unmanipulated side of the brain. With other animals we allowed electrical activity to resume after 24 or 48 hours of activity blockade. One week after resumption of electrical activity, we measured auditory brainstem response (ABR) thresholds and compared the sizes of AVCN large spherical cells on the two sides of the brain.

METHODS AND MATERIAL

Subjects. Mongolian gerbils *(Meriones unguiculatus)* were obtained from Tumblebrook Farms

Table 1. AVCN large spherical cell cross-sectional area

	Ipsilateral (μm^2)	Contralateral (μm^2)	Difference (%)*	*p* Value
After TTX for 24 hours	184.1 ± 20.0	226.6 ± 20.6	18.7	<0.001
	184.8 ± 21.7	225.5 ± 22.5	18.7	<0.001
	193.6 ± 21.9	219.8 ± 31.6	11.9	<0.001
	226.2 ± 27.4	278.4 ± 43.5	18.8	<0.001
	231.9 ± 33.9	269.5 ± 39.9	14.0	<0.001
After TTX for 24 hours and 7 days without TTX	170.6 ± 27.6	177.9 ± 28.9	4.1	>0.1
	186.8 ± 24.7	192.2 ± 27.1	2.8	>0.1
	202.1 ± 31.2	194.7 ± 30.2	−3.9	>0.1
	217.8 ± 28.9	223.4 ± 25.7	2.5	>0.1
	293.7 ± 37.2	302.5 ± 39.9	2.9	>0.1
	293.9 ± 43.2	294.7 ± 39.7	0.3	>0.1
After TTX for 48 hours and 7 days without TTX	193.8 ± 22.4	203.5 ± 31.7	4.8	>0.05
	197.7 ± 42.2	194.3 ± 31.5	−1.7	>0.1
	219.6 ± 23.8	225.8 ± 29.5	2.7	>0.1
	276.9 ± 38.7	275.5 ± 46.6	−0.5	>0.1
	283.3 ± 48.7	275.3 ± 35.2	−2.9	>0.1

*Negative value reflects larger ipsilateral value.
Values expressed as mean ±SD.

(West Brookfield, Mass.) or the University of Washington breeding colony derived from this supplier. Animals were given free access to food and water. Sixteen animals between 4 and 6 weeks of age were used in the study. For surgery and ABR recordings, the gerbils were anesthetized by intramuscular injection with ketamine (75 mg/kg) and xylazine (5 mg/kg). Supplemental doses were given as required to maintain anesthesia. Animal care was conducted in accordance with "The Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Three experimental groups were studied. One underwent unilateral auditory nerve electrical blockade for 24 hours, one underwent unilateral auditory nerve electrical blockade for 24 hours followed by 1 week without TTX, and one underwent unilateral auditory nerve electrical blockade for 48 hours followed by 1 week without TTX. In all animals the cochlea and cochlear nucleus contralateral to manipulation were used as a within-animal control. Previous studies[4,12] have shown that after unilateral ablation or TTX-induced activity blockade of the cochlea, cell size in the opposite (contralateral) AVCN is unaffected.

TTX preparation. Methods for the preparation of controlled-release TTX have been previously described.[12] Briefly, Elvax, an ethylene vinyl-acetate copolymer (DuPont Co., Wilmington, Del.) was dissolved in dichloromethane at 37° C to make a 10% weight/volume solution. TTX crystals (Sigma Diagnostics, St. Louis, Mo.) were dissolved in distilled water, added to the Elvax solution, and shaken to make a suspension. The suspension was poured into a glass Petri dish cooled on dry ice, then stored for 2 days at −20° C. The frozen disk was placed in a lyophilizer for 4 days. Plugs of TTX/Elvax were cut from the disk for placement in the round window niche.

Surgical manipulation. After adequate anesthesia was obtained, the mastoid bulla was exposed through a 2-cm incision posteroinferior to the external auditory canal. A 3-mm hole was created to expose the round window niche in the middle ear. A small piece of TTX/Elvax (0.1 mg) was then placed in the round window niche, resting against the round window membrane. The skin incision was closed with cyanoacrylate glue. Control disks of Elvax without TTX do not influence ABR thresholds or AVCN cell size.[12]

Physiologic analyses. With the animal under anesthesia, the pinna was removed and ABR data were collected. The stimulus was an unfiltered click presented at a rate of 20 Hz. A 0.1-msec, 1-volt computer-generated stimulus was viewed on an oscilloscope and sent to a 2-Hz to 2000-Hz bandpass filter (Krohn-Hite 3550, Krohn-Hite Instruments, Avon, Mass.). The audio signal was passed through an attenuator (model 350C; Hewlett-Packard Co., Palo Alto, Calif.), amplified, further attenuated, and delivered to a Beyer DT-48 earphone (Eugen-Beyer, Heilbronn, F.R.G.). The insulated metal housing of the earphone was connected to a tapered plastic adapter sealed against the external auditory meatus of the subject. Recordings from sub-

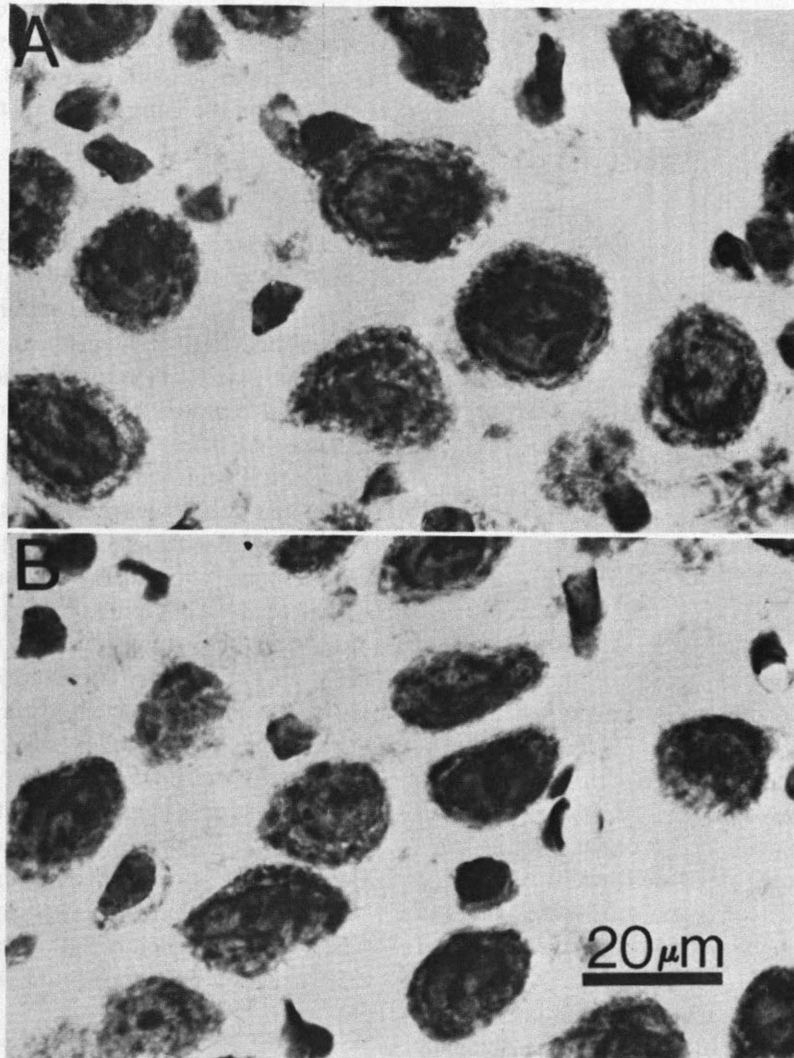

Fig. 3. Photomicrographs of thionin-stained cochlear nucleus large spherical cells **(A)** contralateral and **(B)** ipsilateral to auditory nerve blockade with tetrodotoxin for 24 hours. Cells ipsilateral to blockade are significantly smaller than are cells contralateral to manipulation.

dermal electrodes at the occiput, anterior neck, and caudal back were amplified, viewed on an oscilloscope, and sent to a signal averager. A 10-msec response from 512 alternating rarefaction/condensation stimuli was averaged at each stimulus intensity. Stimuli were presented at 10-dB steps from 80-dB peak equivalent sound pressure level (dB peak equivalent SPL) to near-threshold values. Threshold was defined in 5-dB steps as the lowest stimulus intensity associated with reproducible response, as assessed by visual inspection.

After an intensity series and threshold were obtained, an Elvax pellet containing TTX was placed in the round window niche. All animals underwent two physiologic testing sessions: one before and immediately after the TTX plug was placed in the round window niche and one just before being killed. Action potential blockade was documented by loss of reproducible waveforms at approximately 80-dB peak equivalent SPL above previously determined thresholds. Recovery from action potential blockade was documented by re-obtaining an intensity series and threshold and comparing these to previously obtained values for each animal.

Cell-size analysis. After the experimental manipulation was complete and ABR data were collected, all animals were deeply anesthetized and transcardially perfused with 10% buffered formalin or 4% buffered paraformaldehyde. Brains were postfixed in the same solution for 3 days, dissected from the head, blocked, and embedded in paraffin. A one-in-four series of 10-µm thick transverse sections was mounted on chrome-alum treated slides and stained with thionin to detect Nissl substance. The cross-sectional area of AVCN

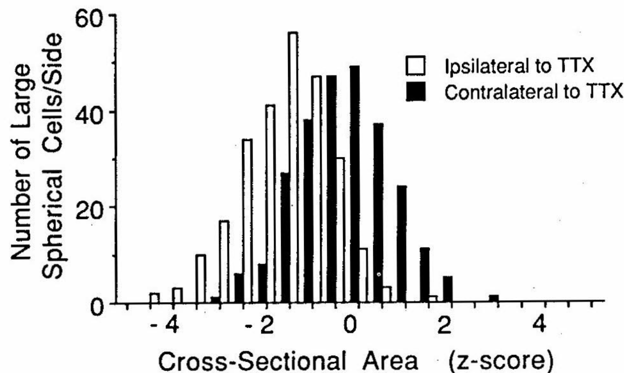

Fig. 4. Cochlear nucleus large spherical cell cross-sectional area was measured ipsilateral and contralateral to auditory nerve electrical activity blockade for 24 hours (n = 5). Values were converted to z scores with respect to the contralateral cell size. There is a significant decrease in cell size ipsilateral to auditory nerve action potential blockade.

large spherical cells on both sides of the brain was measured with the aid of a Zeiss Videoplan interactive image-analysis system (Carl Zeiss Inc., Thornwood, N.Y.), Zeiss photomicroscope and × 100 planapochromatic objective lens. Cross-sectional area was determined by outlining the largest diameter of neurons satisfying the previously described criteria for large spherical cells.[12,15-18] The somatic cross-sectional area of approximately 100 cells was measured in each animal (50 cells on each side of the brain).

Data analysis. Auditory thresholds and latency/intensity functions for wave I of the ABR were obtained before TTX exposure, during TTX exposure, and after the 7-day recovery period. A two-tailed Student's t test was used to compare mean values for auditory threshold.

The difference between manipulated and unmanipulated sides of the brain in large spherical cell size was expressed in square microns or as the percent difference between the two sides of each brain according to the formula (Unmanipulated − Manipulated)/Unmanipulated × 100. A two-tailed Student's t test for means was applied to differences in mean somatic cross-sectional area between sides of the brain.

Cell-size z scores were also calculated to normalize values and compare results between animals. The difference between the size of a given cell and the mean cell size on the unmanipulated side of the brain in that animal was divided by the standard deviation of the cell sizes on the unmanipulated side of the brain, yielding the cell's z score. In this way, the mean cell-size z score on the unmanipulated side of the brain was 0 and the standard deviation was 1 (in other words, a standard normal distribution). The changes in cell size on the manipulated side within an experimental group could then be combined and compared to the distribution of cell sizes on the control side of the brain.

RESULTS

Physiology

A representative series of ABR waveforms before placement of the TTX is shown in Fig. 1. Waveforms show decreasing amplitude and increasing latency with decreasing stimulus intensity. In contrast, shortly after TTX was placed in the round window niche in the same animal, a reproducible waveform was no longer obtainable. We believe the blockade of sound-evoked electrical activity in auditory nerve fibers, as manifested by the loss of ABR waveforms, is paralleled by a blockade of spontaneous electrical activity.[11]

In animals allowed to recover for 1 week after removal of the TTX plug, the intensity series obtained closely resembled that found in the baseline condition (Fig. 1). The auditory threshold to unfiltered click stimuli before TTX placement averaged 1-dB peak equivalent SPL. After recovery from TTX treatment, the threshold averaged 5-dB peak equivalent SPL. There is no statistically significant difference between these values ($p > 0.05$). Similarly, the latency/intensity series obtained before TTX placement and after recovery from blockade were not significantly different (Fig. 2). Thus, TTX completely and reversibly blocked sound-evoked action potentials in the auditory nerve.

Cochlear Nucleus Cell Size

Cell size ipsilateral to TTX blockade was measured and compared to cell size contralateral to manipulation (Table 1). The difference in cell size between sides was expressed as the percent change compared to contralateral cell size. In animals exposed to only 24 hours of TTX-induced electrical blockade of auditory nerve fibers, large spherical cells ipsilateral to manipulation averaged 16% smaller than contralateral cells ($p < 0.05$ for each animal). Examples of changes in cell size after 24 hours of auditory nerve blockade are presented in Fig. 3.

To compare cell-size changes in each group of animals, cell sizes were normalized by converting the raw data to z scores, resulting in a mean of 0 and a standard deviation of 1 for AVCN cell sizes contralateral to manipulation. Data from the ipsilateral side of the brain can then be pooled, with minimal effect of interanimal differences on cell-size distribution. The resulting histogram of cell sizes for all animals that underwent 24 hours of unilateral auditory nerve blockade is shown in Fig. 4. There is a striking downward shift in the dis-

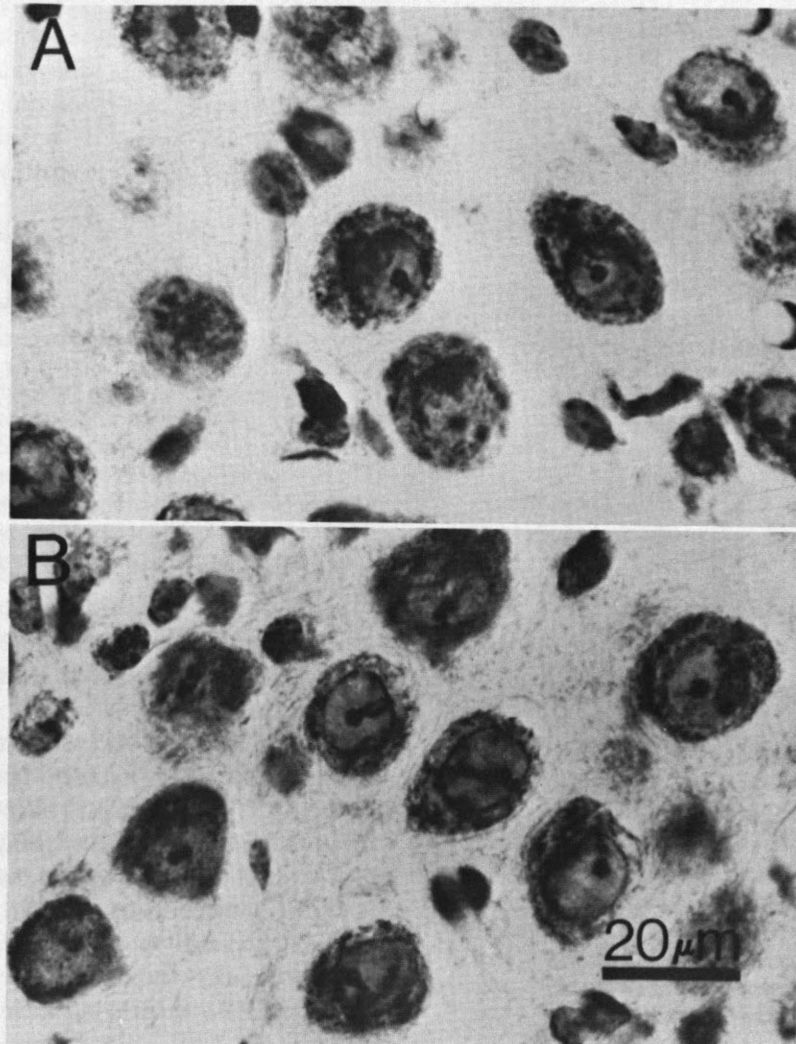

Fig. 5. Photomicrographs of thionin-stained cochlear nucleus large spherical cells 1 week after a 48-hour auditory nerve electrical activity blockade **(A)** contralateral and **(B)** ipsilateral to the manipulation. In contrast to findings showing a difference in cell size immediately after a 48-hour blockade, there is no longer a difference in cell size after return of electrical activity.

tribution of large spherical cell sizes ipsilateral to electrical blockade compared to that of cell sizes contralateral to manipulation.

Other animals received either 24 or 48 hours of auditory nerve blockade followed by 1 week of restored electrical stimulation. There was no longer a significant difference in cell size between the two sides of the brain after this recovery period (Fig. 5, $p > 0.05$ for each animal). Cumulative histograms of cell size do not show a difference in cell size 1 week after 24 or 48 hours of electrical activity blockade (Fig. 6).

AVCN large spherical cells ipsilateral to auditory nerve action potential blockade for 24 hours are significantly smaller than are control cells on the opposite side of the brain. These changes are completely reversible. When electrical stimulation is restored, large spherical cell size returns to previous levels and there is no longer any difference in cell size between the two sides of the brain (Fig. 7).

DISCUSSION

There has long been an interest in the role of experience in the development and maintenance of neurons in the central nervous system.[19-21] This interest has given rise to experimental paradigms in which neurons are isolated from their inputs and then examined. Conclusions can thus be drawn about the relative contribution of afferent stimulation to development. These studies have shown that the structural and functional integrity of neurons are markedly affected by isolation from inputs.[22,23]

The recent development of cochlear stimulation de-

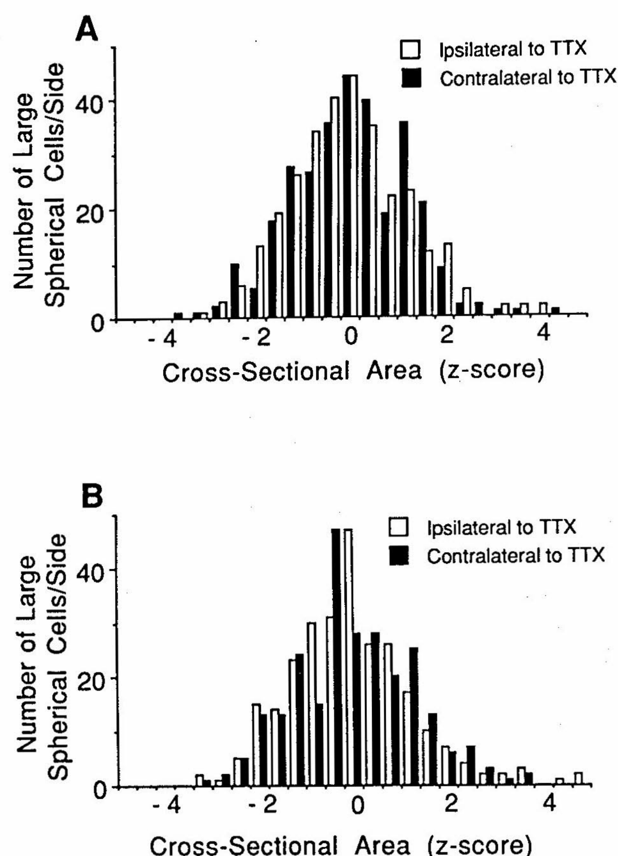

Fig. 6. Cochlear nucleus large spherical cell size measured ipsilateral and contralateral to auditory nerve blockade for **(A)** 24 hours in six animals or **(B)** 48 hours in five animals followed by 1 week without blockade. Cell sizes are presented as *z* scores. There is no significant difference in cell sizes between TTX-exposed and unmanipulated sides.

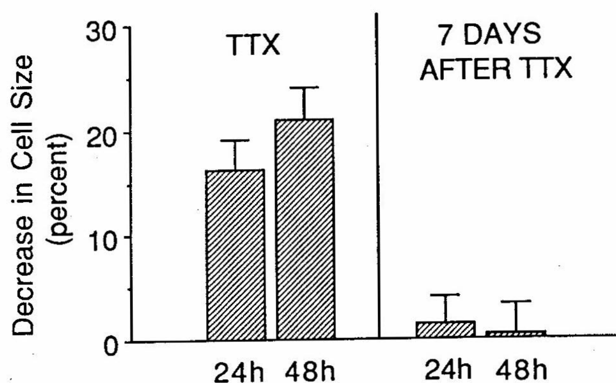

Fig. 7. Histogram summarizes the relative loss and subsequent recovery of cell size. A decrease in ipsilateral cell size in comparison to cell size on the opposite side of the brain is shown as a positive value. Cell size decreases after a 24- or 48-hour auditory nerve electrical activity blockade. Cell size returns to previous levels after a 1 week of recovery of electrical activity, so that there is no longer a difference in cell size between sides of the brain.

vices for persons with profound hearing loss provides a set of circumstances clinically similar and relevant to these studies. In the auditory system, manipulations that cause a loss of either conductive hearing (such as with ear plugs) or sensorineural hearing (such as with cochlear ablation) have been used as models for this clinical situation. However, these manipulations have usually lacked the specificity required to adequately define the signals responsible for the changes observed, especially when the relevant signals may be associated with electrical activity. For example, a conductive hearing loss is often frequency dependent, may not produce the same loss in each subject, and may not affect spontaneous electrical activity in the auditory nerve.[24] Although cochlear ablation reliably eliminates all auditory nerve electrical activity,[25] it also disrupts axons, causing axonal degeneration. Results seen after cochlear ablation could thus be caused by pathologic reactions to auditory nerve injury rather than by physiologic changes in auditory stimulation. Interpretation of results from such studies should therefore be made with these qualifications in mind.

TTX specifically blocks the voltage-sensitive sodium channels responsible for the propagation of action potentials.[26] TTX has recently been used in the study of the auditory system.[11,12] Extracellular electrical recordings in the cochlear nucleus of the chick after perilymphatic TTX injection demonstrate complete elimination of all evoked and spontaneous electrical activity. Simultaneous near- and far-field recordings in birds have shown that the electrical blockade can be reliably monitored by the ABR measurement. With ABR monitoring, we have found the auditory threshold to a broad band click to be unobtainable during blockade of auditory nerve action potential with TTX. The threshold and the latency/intensity function for wave I return to normal after a 1-week recovery from electrical activity blockade. On the basis of these data, we believe that TTX exposure completely and reversibly blocks electrical activity in the auditory nerve. This allows us to test the hypothesis that electrical activity is a biologically important regulatory signal that can bidirectionally influence neuronal cell size.

The cross-sectional area of neurons has been used as a parameter of cellular integrity in previous studies.[4-7,12] Decrease in cell size has been interpreted as an aberration in normal cellular maintenance. In the case of auditory nerve electrical activity blockade, spiral ganglion cells, but not cochlear nucleus cells, are manipulated. Therefore any changes in cochlear nucleus cell size do not reflect a toxic effect of TTX but rather the relative importance of auditory nerve stimulation. Recovery of cell size is interpreted as a return to the

previous state of regulation. The recovery of cell size after transient auditory nerve activity blockade demonstrated in this study may also indicate that AVCN neurons retain their full functional potential during periods without stimulation. These anatomic findings are in agreement with the full physiologic recovery of ABR threshold and wave-I latencies.

The loss of AVCN electrical stimulation followed by restoration of this stimulation is similar to the sequence of events experienced by patients with profound hearing loss who undergo cochlear electrode implantation. Although we do not claim to have modeled this clinical situation, we do believe that similarities between reversal of electrical activity blockade and electrical stimulation in patients with a profound sensorineural hearing loss are present. We believe that we have at least provided evidence that restoration of electrical stimulation can reverse one of the many central auditory changes that have been documented in mammals after loss of input to AVCN neurons. The direct clinical relevance of this study to the treatment of patients with cochlear implants remains to be determined.

We conclude that cochlear nucleus neurons atrophy in response to a loss of electrical stimulation and fully recover after restoration of stimulation. Additionally, auditory nerve electrical activity is a dynamic biologic signal regulating the size of postsynaptic central auditory neurons.

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