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Rapid Changes in Cochlear Nucleus Cell Size Following Blockade of Auditory Nerve Electrical Activity in Gerbils

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ABSTRACT

Large spherical cells of the mammalian anteroventral cochlear nucleus (AVCN) receive direct excitatory input from auditory nerve axons. Transsynaptic regulation of neuronal cell size and cell number after cochlear ablation has been previously demonstrated in neonates of several vertebrate species, including the gerbil. Such changes may be related to loss of spontaneous or evoked auditory nerve electrical activity or to loss of activity-independent factors. We have developed a method to chronically, yet reversibly, block auditory nerve electrical activity without violating the integrity of the inner ear. Tetrodotoxin (TTX) was embedded in an ethylene-vinyl acetate copolymer resin (Elvax). A small piece of Elvax containing TTX was placed next to the round window membrane, which allowed TTX to diffuse into the inner ear. As a measure of the effectiveness of manipulation, the onset, duration, and magnitude of the auditory threshold shift were measured by the auditory brainstem response. The sound-evoked response was abolished within 10 minutes of placement of TTX on the round window membrane. The duration of threshold shift was dose-dependent and lasted 24–46 hours. Implants of Elvax without TTX did not produce a significant threshold shift. TTX, which blocks voltage-gated sodium channels, did not abolish the potassium-based cochlear microphonic response.

The consequence of blocking afferent electrical activity on gerbil AVCN large spherical cells was examined by measuring their cross-sectional area after each of four manipulations: unilateral auditory nerve action potential blockade with TTX; unilateral surgical cochlear ablation; ipsilateral TTX exposure/contralateral cochlear ablation; and unilateral sham operation (Elvax without TTX). Large spherical cells ipsilateral to cochlea TTX exposure were 21% smaller than contralateral large spherical cells. Cells ipsilateral to cochlear ablation were 25% smaller than contralateral cells. There was not a significant difference between the effect of cochlear ablation and TTX exposure on AVCN cell size and there was not a reliable effect of sham operation. These findings are consistent with previous work in the avian auditory system and support the hypothesis that electrical activity or the sequelae of electrical activity is a major factor in transneuronal regulation of cell size.

Key words: TTX, ABR, neural activity, deafferentation

Deafferentation studies in vertebrate auditory and visual systems have consistently indicated that an intact and functioning peripheral receptor is required for normal development and maintenance of its associated central neurons (reviewed by Cowan, '70; Globus, '75). In the mammalian and avian auditory systems, cochlea removal abolishes a major source of excitatory afferent input to brainstem auditory

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nuclei and is associated with significant changes in first- through fourth-order auditory neurons. Neuron number and cross-sectional area decrease after manipulations intended to reduce auditory nerve electrical activity in the mouse (Trune, '82a; Webster, '83), rat (Coleman and O'Connor, '79), gerbil (Hashisaki and Rubel, '89), and chicken (Born and Rubel, '85). Glucose uptake (Woolf et al., '83), amino acid incorporation (Steward and Rubel, '85), metabolic enzyme activity (Durham and Rubel, '85), and dendritic arborization (Benes et al., '77; Trune, '82b; Deitch and Rubel, '84) may also serve as criteria of interneuronal regulation and are affected by cochlea removal. These transsynaptic influences may result from loss of net electrical activity in auditory nerve axons, loss of sound-evoked electrical activity, or loss of an activity-independent signal delivered to central auditory neurons by their presynaptic elements.

Previous experiments in the avian auditory system designed to distinguish among these possibilities have indicated that the average or net amount of electrical activity is a transneuronal signal regulating neuron cell size (Tucci et al., '87). Recently, specific pharmacologic blockade of auditory nerve spontaneous electrical activity with tetrodotoxin (TTX) has successfully reproduced many of the effects of cochlea removal in chickens (Born and Rubel, '88). These findings are consistent with the hypothesis that deafferentation affects transneuronal regulation to the extent that the manipulation affects the total amount of presynaptic electrical activity (Rubel et al., '84).

The gerbil auditory system provides desirable developmental, physiological, and anatomical features for the study of transneuronal regulation (Finck et al., '72; Frisina et al., '82; Ryan et al., '82; Woolf and Ryan, '84, '85; Dolan et al., '85; Schwartz and Ryan, '86; Sanes and Rubel, '88). In addition, the round window membrane of the gerbil is recessed in the round window antrum and allows the secure placement of a slow-release vehicle for noninvasive drug delivery to the perilymph.

In the present set of experiments we test the hypothesis that reversible blockade of auditory nerve action potentials can produce transneuronal atrophy of mammalian auditory system neurons similar to that produced by complete destruction of the inner ear. Our manipulation unilaterally, chronically, and reversibly abolishes action potentials in auditory nerve axons without affecting the anatomical relations of the cochlea. We use cross-sectional area of large spherical cells in AVCN as a measure of transneuronal regulation. The results suggest that rapid changes in soma size following elimination of auditory nerve action potentials are comparable to those following unilateral destruction of the inner ear.

MATERIALS AND METHODS

TTX preparation

Methods for preparation of the controlled release of TTX were derived from procedures developed by Langer et al. ('85). Ethylene vinyl-acetate copolymer resin (Elvax, DuPont) was washed for at least 2 hours at 37°C in distilled water five times, in 95% ethanol ten times, and in 100% ethanol five times to remove impurities. Washed Elvax is noninflammatory (Niemi et al., '85). The absorbance of wash solution was measured at 230 nm to confirm wash efficacy. Final readings were less than 0.02 absorbance units. Washed Elvax was dissolved in dichloromethane (Fisher Scientific) at 37°C to make a 10% weight/volume solution. TTX crystals (Sigma, St. Louis, MO) 0.25–0.50 mg were dissolved in distilled water, added to the Elvax solution to make a 15% volume/volume suspension, and stirred on a vortex for 60 seconds. The suspension was immediately poured into a 16 or 32 mm-diameter Petri dish cooled on dry ice. After 2 days at −20°C the TTX-embedded Elvax disc was removed from the Petri dish and lyophilized at less than −85°C and less than 30 mtorr for 4 days. Discs were subsequently stored at −20°C and thawed at room temperature for 1–2 hours before use. Plugs of TTX-Elvax weighing 0.5 mg each and containing 250–750 ng of TTX were cut from the disc by using a 17-gauge stub adaptor. Elvax discs without TTX were also made for use in control animals.

Subjects

Mongolian gerbils were obtained from Tumblebrook Farms (West Brookfield, MA) and given free access to food and water. Fourteen animals between 4 and 6 weeks of age were used for physiological studies and 12 animals of similar age were used for anatomical studies. Animals were anesthetized with ketamine (75 mg/kg, IM) and xylazine (5 mg/kg, IM) prior to all physiological or surgical procedures. Supplemental doses were given as required to maintain anesthesia. Body temperature was maintained at 38°C by a heating pad during physiological testing.

Physiological analysis

Under anesthesia, the pinna was removed and auditory brainstem response (ABR) data were collected. The stimulus was an unfiltered click presented at a rate of 20 Hz. A 0.1 msec/1.0 V stimulus from a pulse generator (Systron-Donner model 100A) was viewed on an oscilloscope and sent to a 2 Hz–200 kHz bandpass filter (Krohn-Hite model 3550). The audio signal was passed through an attenuator (Hewlett-Packard model 350C), amplified (Crown D-75 or Dynaco Stereo 70), further attenuated, and delivered to a 2-inch speaker (Telephonics TDH-49P). The insulated metal housing of the speaker was connected to a tapered plastic adaptor that was sealed against the external auditory meatus of the subject. Recordings from subdermal electrodes at the occiput, anterior neck, and caudal back (ground) were amplified (Grass model P511J), viewed on an oscilloscope, and sent to a signal averager (Nicolet model 1174). A 10 msec duration response from 512 alternating rarefaction/condensation or only rarefaction click stimuli was averaged at each stimulus intensity. Stimuli were presented at 10 dB steps from 80 dB peak equivalent sound pressure level (peSPL) to near-threshold values. The time between stimulus onset and the peak of wave I (wave I latency) was measured at each stimulus intensity. Threshold was defined at 5 dB steps as the lowest stimulus intensity that was associated with a reproducible response by visual inspection. Animals were excluded from further study if a threshold difference of 15 dB or greater was detected between ears prior to any manipulation.

Half of the animals underwent unilateral cochlea ablation after initial ABR data were collected and at least 24 hours prior to TTX placement. Through a perforation in the tympanic membrane, the maleus was removed with watchmaker's forceps and the projection of the cochlea into the middle ear was identified. The cochlear walls and modiolus were fractured with a fine sharpened probe and the modiolus was removed. Postoperatively, ABR data collection was repeated and a reproducible waveform was not obtainable 80 dB above the previous threshold on the operated side.

All animals then underwent placement of Elvax plugs containing 0, 250, 500, or 750 ng TTX (n = 3, 4, 4, 3). The temporal bulla was exposed through a 20 mm incision posteroinferior to the external auditory meatus. A 3 mm hole in the bulla was created with an electric drill and a plug of Elvax was placed in the round window antrum resting against the round window membrane. The skin incision was closed by use of cyanoacrylate glue. ABR data were obtained from stimulation of the Elvax-implanted ear at 5-minute intervals until the threshold was unobtainable (i.e., no reproducible waveform at approximately 80 dB above baseline threshold) and then at 8–12 hour intervals until a threshold was reobtained. In animals with a normal contralateral ear, the threshold to click stimuli presented to the normal ear was obtained at a time when the threshold in the TTX-implanted ear was unobtainable in order to assess for a systemic effect of TTX. Finally, all gerbils that underwent placement of Elvax or TTX/Elvax plugs underwent ABR data collection 7 days after return of sound-evoked neural activity.

Analysis of cell size

In another group of subjects, initial ABR data were obtained as described above followed by unilateral cochlear ablation (n = 3), unilateral Elvax/TTX 750 ng (n = 4), unilateral Elvax without TTX (n = 3), or Elvax/TTX in one ear and contralateral cochlea ablation (n = 2). At 24 and 48 hours after TTX placement animals underwent ABR testing to verify loss of evoked response. At 24 hours, all implants were replaced with similar fresh TTX/Elvax or Elvax plugs. Forty-eight hours after the initial manipulation all animals were deeply anesthetized and transcardially perfused with 10% buffered formalin. After 3 days postfixation in formalin, brains were 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-coated slides and stained for Nissl substance with thionin. The cross-sectional area of large spherical cells of the anteroventral cochlear nucleus (AVCN) on both sides of the brain was measured with the aid of a Zeiss Videoplan interactive image analysis system by using a Zeiss photomicroscope and 100× planapochromatic objective (N.A. 1.3). Large spherical cell soma area was determined by outlining the largest diameter of neurons satisfying the following criteria: cytoplasmic shape, nuclear position, and cell location consistent with large spherical cells in AVCN (Harrison and Irving, '65; Osen, '69; Brawer et al., '74) and clearly identifiable cytoplasmic, nuclear, and nucleolar borders (Born et al., '87). A technician blinded to the manipulation and side of the brain also measured cell size in several animals. There were no consistent differences between the measurements obtained by the technician and the investigator. Approximately 270 cells were measured in each animal; a total of 3,238 cells were measured.

Data analysis

Physiology. The onset of action potential blockade was determined by serial ABR recordings every 5 minutes after TTX placement. The endpoint of action potential blockade was defined as the time midway between the last unobtainable evoked neural response and the first obtainable evoked neural response. The mean and standard error of the duration and thresholds were calculated for each group.

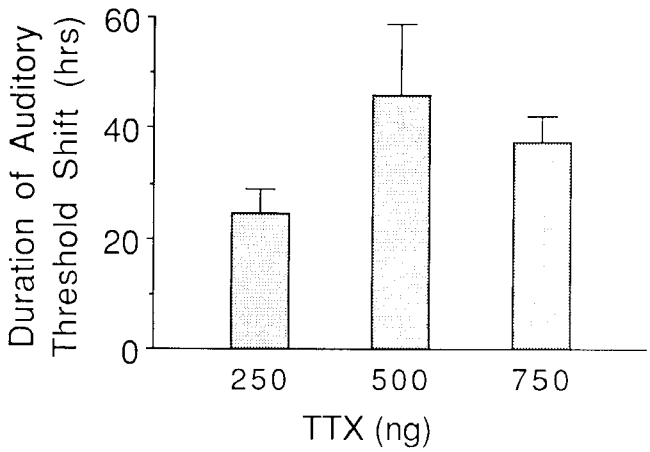

Fig. 1. Mean duration of sound-evoked action potential blockade (±SEM) after TTX placement measured by auditory brainstem response recordings in 11 subjects. Three doses of TTX embedded in Elvax were placed in the round window niche.

Cell size. The difference in large spherical cell size between manipulated and unmanipulated sides of the brain was expressed in square microns or percent difference ((Unmanipulated-Manipulated)/Unmanipulated × 100) between the two sides of the brain. The data were tested against a null hypothesis of no difference. A two-tailed Student's t-test for means was applied to data from ipsilateral TTX/contralateral cochlear ablation animals and Elvax control animals. A one-tailed Student's t-test was used for all other comparisons.

RESULTS

TTX effects

The mean ABR threshold to unfiltered click stimuli in 32 ears was −4.2 dB peSPL. This threshold is slightly lower than, although consistent with, best-frequency behavioral (Ryan, '76) and single-unit thresholds (Woolf and Ryan, '85) previously reported in the gerbil. Cochlea ablation reliably eliminated the ABR measured at an intensity approximately 80 dB greater than threshold values. Placement of a 0.5 mg Elvax pellet containing 250–750 ng TTX was associated with a similar loss of the ABR within 10 minutes in nine of ten animals and within 20 minutes in the remaining animal. The duration of evoked neural activity blockade ranged from 24 to 46 hours and is summarized in Figure 1. The auditory threshold upon initial recovery averaged approximately 15 dB higher than pre-exposure thresholds. By 7 days after return of evoked potentials, the thresholds were within 2–5 dB of pre-exposure levels (Fig. 2). Additionally, the baseline wave I latency-intensity function was not significantly different from that obtained 1 week after recovery (Fig. 3). This suggests that TTX does not permanently damage the auditory periphery but temporarily eliminates evoked neural activity. Auditory responses from the ear contralateral to TTX exposure were not systematically altered during action potential blockade in the ipsilateral ear. Examination of ABR data in animals receiving Elvax plugs without TTX showed that neural thresholds were not significantly different from preplacement values at any time after manipulation.

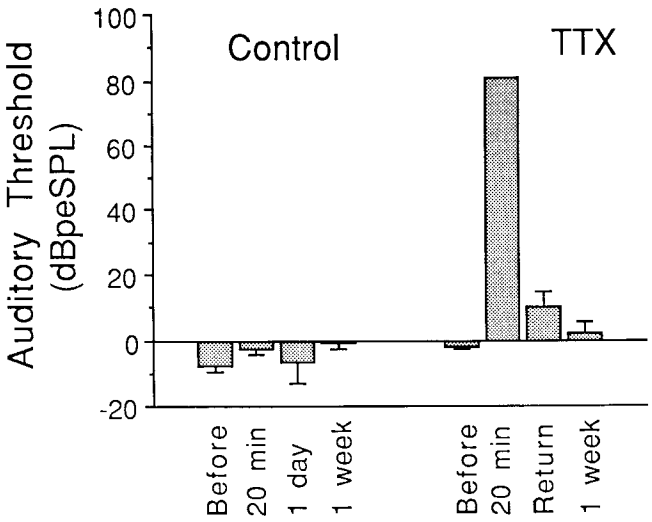

Fig. 2. Auditory threshold measured in the ear ipsilateral to either TTX-Elvax (TTX; n = 11) or Elvax alone (control; n = 3) before and at various times after manipulation. Elvax implants alone do not significantly affect the auditory threshold at any time tested. TTX-Elvax is associated with a rapid and reversible threshold shift. The duration of auditory threshold shift (return) was dose dependent.

Whereas cochlea ablation eliminated the neural and cochlear microphonic (CM) responses to auditory stimuli, TTX exposure eliminated only the auditory nerve compound action potential (N1 component) and the subsequent neural responses (Fig. 4). The CM persisted in TTX-

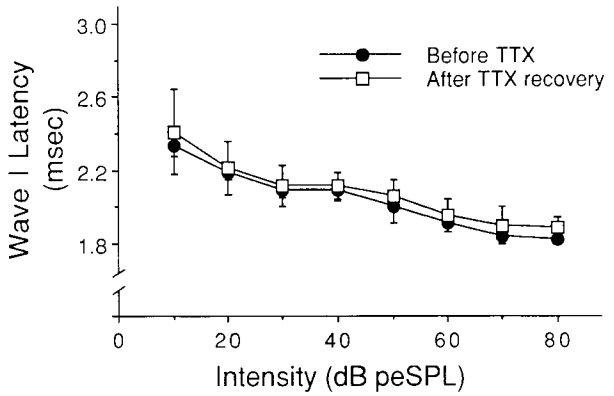

Fig. 3. Mean latencies of ABR wave I (±SEM; n = 4) measured in milliseconds before TTX placement and 1 week after recovery of sound-evoked responses. There is no consistent difference between latency-intensity functions.

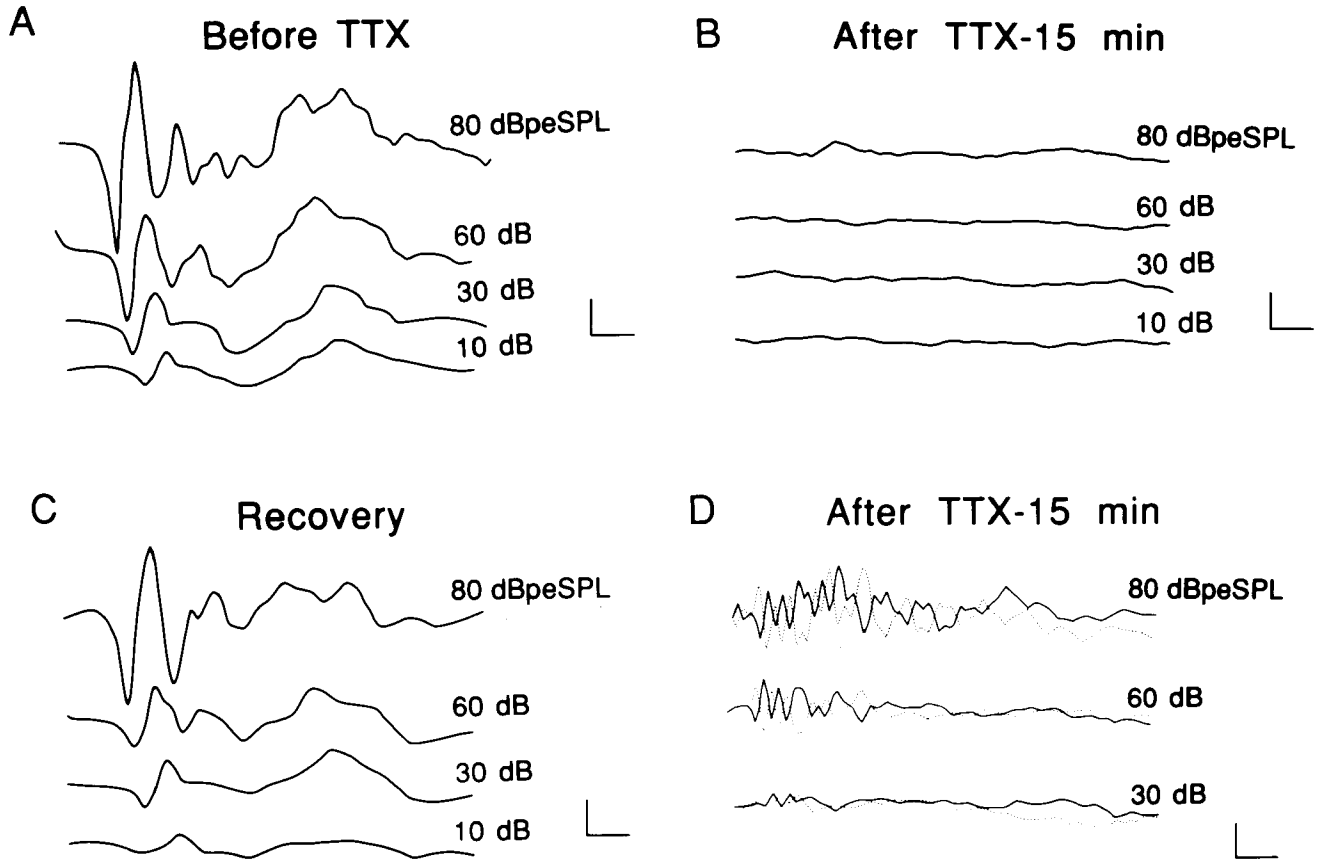

Fig. 4. Representative intensity series of averaged ABR waveforms from 512 alternating rarefaction/condensation click stimuli (A–C) and rarefaction or condensation stimuli alone (D). **A:** Before TTX in a normal ear there is an increasing wave latency with decreasing stimulus intensity. **B:** Fifteen minutes after TTX-Elvax is placed in the round window niche no neural response is present. **C:** Return of neural activity shows waveforms similar to those prior to manipulation. **D:** Cochlear microphonic response from rarefaction (solid line) or condensation stimuli (dashed line) 15 minutes after placement of TTX-Elvax. There is no latency shift with decreasing stimulus intensity. The waveform is dependent upon stimulus polarity. The neural response is blocked and the cochlear microphonic response is "unmasked." Scale bar 1 msec/1 μV (A–C) and 1 msec/0.5 μV (D).

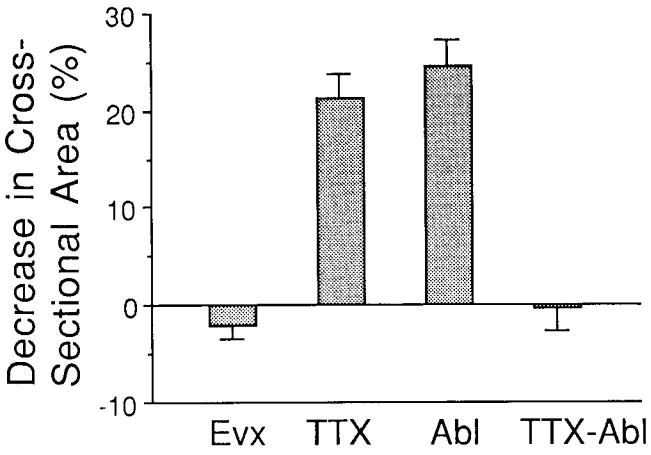

Fig. 5. Mean difference in AVCN large spherical cell size between the manipulated ipsilateral and unmanipulated contralateral side of the brain. Positive values reflect smaller ipsilateral mean cross-sectional areas. Evx, Elvax alone; TTX, tetrodotoxin; Abl, cochlea ablation; TTX-Abl, ipsilateral TTX and contralateral cochlea ablation.

exposed animals and was differentiated from the neural response in two ways. First, the neural response was characterized by increasing latency of waves with decreasing stimulus intensity, whereas CM latency was not intensity dependent. Second, neural waveform polarity was independent of stimulus polarity whereas the CM was not. An ABR waveform from a normal hearing ear showed only a neural response to alternating rarefaction/condensation sound stimuli and a neural and CM response to rarefaction or condensation stimuli alone. A TTX-exposed ear showed no response to alternating stimuli because the neural response was blocked. However, stimulus dependent CM waveforms were easily elicited by rarefaction or condensation stimuli alone (Fig. 4D). These differences enabled us to verify that a neural response was not obtainable at a time when the CM response was obtainable.

Soma cross-sectional area

The mean soma cross-sectional area of large spherical cells in the AVCN ipsilateral and contralateral to each manipulation is presented in Table 1 for each animal. Within 48 hours of unilateral exposure to TTX, the cross-sectional area of large spherical cells is 21% smaller ipsilateral to manipulation ($P < .001$ for each animal; Fig. 5). A photomicrograph from a representative animal is shown in Figure 6 and the distribution of large spherical cell cross-sectional areas ipsilateral and contralateral to TTX exposure is presented in Figure 7. Additionally, large spherical cells ipsilateral to cochlear ablation were 25% smaller than cells on the nonmanipulated side within 48 hours ($P < .001$ for all animals). The animals that received cochlea ablation on one side and TTX exposure of the other ear showed no reliable differences between the two sides of the brain ($P > .20$ for all animals). Finally, there was no reliable effect of Elvax without TTX on cell size ($P > .10$ for all animals).

In Table 1 it can be noted that although there is not a reliable difference in cell size in the ipsilateral TTX/contralateral cochlear ablation group, the absolute cross-sectional area of neural soma appears larger than on the manipulated side in the other groups. However, the TTX and cochlea

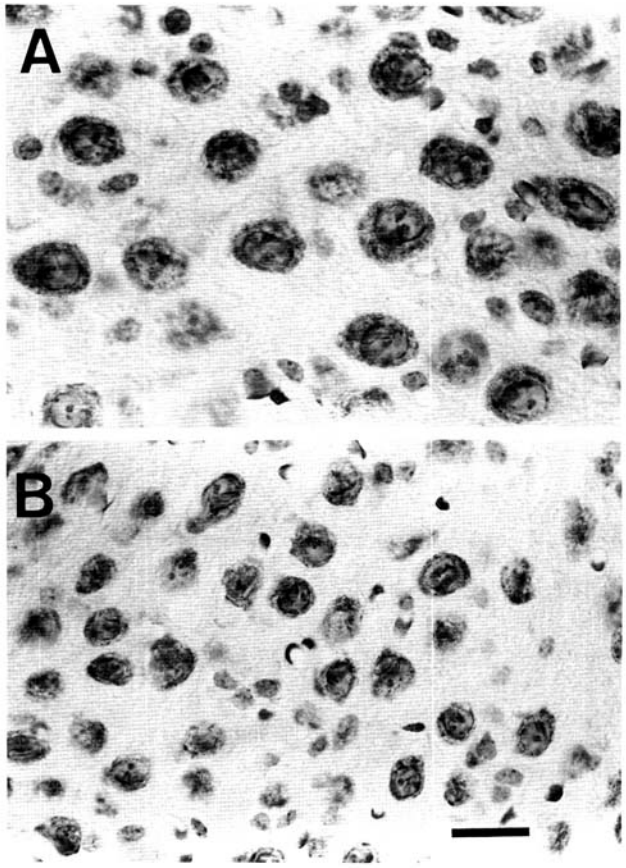

Fig. 6. Large spherical cells of gerbil anteroventral cochlear nucleus on the normal contralateral side (**A**) and ipsilateral (**B**) to auditory nerve electrical activity blockade with TTX for 48 hours. Scale bar = 20 μm.

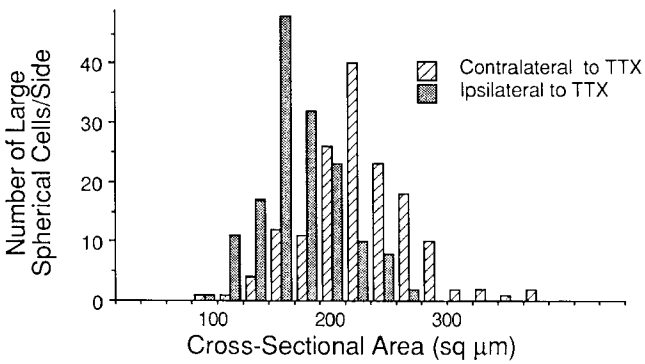

Fig. 7. Histogram showing distribution of large spherical cell soma cross-sectional areas ipsilateral (shaded bars) and contralateral (striped bars) to TTX exposure in a representative animal.

ablation groups both show a significant effect on cell size when compared to the nonoperated side. Age, duration of tissue perfusion or postfixation, and temperature and pressure during paraffin embedding may all increase the variance of soma cross-sectional area measurements between animals (Kalil, '80). Therefore, size comparisons are most meaningful when made between sides of the brain of the same animal.

TABLE 1. Mean Neural Soma Cross-Sectional Area of AVCN Large Spherical Cells for Subjects in Each Group (±SEM)

Group contralateral/ipsilateral	Contralateral	Ipsilateral	df/t[1]	P value
Unmanipulated/TTX	170.57 (3.62)[2]	140.45 (2.82)	124/6.56	<0.001
	189.21 (2.62)	152.86 (1.87)	303/11.92	<0.001
	181.26 (2.68)	136.53 (1.88)	302/13.68	<0.001
	288.51 (4.12)	219.91 (2.71)	252/13.67	<0.001
Unmanipulated/ablated	183.02 (2.16)	144.60 (1.85)	302/13.46	<0.001
	189.48 (2.25)	146.60 (1.85)	296/15.42	<0.001
	245.35 (3.11)	172.53 (2.24)	305/19.01	<0.001
Unmanipulated/Elvax	172.44 (2.12)	172.81 (2.58)	314/0.11	>0.4
	172.47 (2.90)	172.96 (2.92)	208/1.63	>0.1
	190.92 (2.17)	195.63 (2.80)	311/1.33	>0.1
Ablated/TTX	237.85 (2.38)	244.32 (3.41)	293/0.64	>0.5
	258.43 (2.91)	253.34 (2.88)	228/1.24	>0.2

[1]Degrees of freedom/Student's t-value.
[2]Square microns.

DISCUSSION

Tetrodotoxin has been used to block electrical activity in the auditory nerve of the gerbil. Large spherical cell cross-sectional area is significantly smaller ipsilateral to TTX exposure and has been observed after only 48 hours of neural activity blockade. A similar decrease is seen 48 hours after cochlear ablation.

Sustained release TTX

The method of slowly releasing TTX has been developed for the purpose of selectively, chronically, and reversibly blocking action potentials in mammalian auditory nerve axons (Langer et al., '85; Reh and Constantine-Paton, '85). This manipulation does not compromise the integrity of the inner ear. The kinetics of TTX release are indirectly described by auditory threshold testing. Manipulation of release kinetics through changes in TTX drug loading or concentration may result in longer durations of action potential blockade than we have currently achieved.

We have documented the loss of sound-evoked neural electrical activity after TTX exposure by using an averaged far-field recording technique. The relation between evoked and spontaneous electrical activity of the auditory nerve with far-field or near-field recordings has been described in the chick auditory system and probably applies to the gerbil auditory system as well (Born and Rubel, '88). First-order central auditory neurons in nucleus magnocellularis of the chick are characterized by a spontaneous firing rate that is totally abolished after the peripheral receptor is either removed or exposed to TTX. Along with the loss of spontaneous activity there is a parallel loss of reproducible ABR waveforms at previously determined suprathreshold intensities. Single-unit recordings in the gerbil brainstem have shown spontaneous electrical activity in cochlear nucleus neurons (Woolf and Ryan, '85). Large spherical cells in AVCN receive their major excitatory input from large auditory nerve axon terminals and are silenced after cochlea destruction in the mammal (Koerber et al., '68). Thus, we believe that the ability to block auditory nerve action potentials with TTX, as documented by the ABR, also results in a dramatic decrease or total elimination of spontaneous and evoked action potentials in auditory nerve axons and large spherical cells of the gerbil AVCN. Further evidence of the effect of TTX on neural activity is found in studies of the feline visual system in which retinal ganglion cell spontaneous and evoked electrical activity were simultaneously eliminated after intraocular injections of TTX (Dubin et al., '86; Stryker and Harris, '86).

We have noted a persistent CM response following TTX exposure. This finding is consistent with known mechanisms of the CM response which include the mechanical modulation of nonspecific cation channels in or near hair cell stereocilia. Potassium is the major cation in the endolymph surrounding hair cells. Since TTX specifically blocks voltage-gated sodium channels, the CM response persists after TTX exposure. This "unmasking" of the CM after TTX exposure may prove to offer advantages in the study of some of its properties.

Cell size changes

Previous studies have shown a decrease in size and number of certain AVCN cell types ipsilateral to cochlea ablation. For example, Nordeen et al. ('83) killed gerbils 4–12 months after neonatal cochlea ablation and found near-complete AVCN atrophy in half of the animals and severe cell loss in the remaining half. In mice that had survived 39 days after cochlear ablation a decrease in the size of octopus, multipolar, globular, and small spherical cells was found (Trune, '82a). Although there was marked loss of large spherical cells ipsilateral to cochlea ablation, those that remained were not significantly different in size from controls. Webster ('83) killed mice 41 days after unilateral removal of the external auditory meatus. Large spherical cell size was 18% smaller ipsilateral to manipulation compared to the contralateral side. Finally, there was a 21% difference in rat AVCN large spherical cell size between the two sides of the brain 34–40 days after ossicle removal (Coleman and O'Connor, '79).

The preceding paper (Hashisaki and Rubel, '89) describes significant decreases in cell number and cell size 48 hours after unilateral cochlea ablation in young gerbils. In addition to replicating some of these results, we have found that the blockade of action potentials between the cochlea and AVCN can fully account for the rapid changes in cell size seen after cochlea ablation. These results agree with those found in the avian auditory system (Born and Rubel, '88). They are consistent with the hypothesis that the trophic effects of the auditory periphery on central auditory neurons are at least qualitatively associated with neuronal activity levels (Rubel et al., '84). Thus, electrical activity in large spherical cells (either spontaneous or sound evoked) is required for maintenance of soma cross-sectional area. Investigations of the role of electrical activity in the development and maintenance of visual pathways have led to similar conclusions (Archer et al., '82; Schmidt and Edwards, '83; Riccio and Matthews, '85; Reh and Constantine-Paton, '85).

We believe that to the extent TTX selectively blocks electrical activity in auditory nerve axons, we have shown that the regulatory signal influencing AVCN large spherical cell size is activity-dependent. The relative importance of presynaptic and postsynaptic electrical activity in AVCN and the quantitative relationship between activity and cell atrophy are less well understood. For example, the effect of activity on cell size may be associated with specific ion flux, receptor binding, or the co-release of peptides and/or neurotransmitter. Direct quantitative manipulation of pre- and postsynaptic electrical activity has been achieved in the chick auditory brainstem in vitro (Hyson and Rubel, '80). These results suggest that presynaptic electrical activity is a biologically important neuroregulatory signal.

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