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MAIN ARTICLE

Effect of early postnatal air-conduction auditory deprivation on the development and function of the rat spiral ganglion

F WANG[1,2], X GAO[2,3], J CHEN[2], S-L LIU[2], F-Y WANG[2], R-Y HEI[2], Y CHEN[2], J-H QIU[2]

[1]*Department of Otolaryngology, General Hospital of Air Force, Beijing,* [2]*Department of Otorhinolaryngology, Head and Neck Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, and* [3]*Department of Otorhinolaryngology, Head and Neck Surgery, General Hospital of Second Artillery, Beijing, People's Republic of China*

Abstract

Objective: To evaluate the effect of early postnatal air-conduction auditory deprivation on the development and function of the rat spiral ganglion.

Study design: Randomised animal study.

Methods: Sixty neonatal Sprague–Dawley rats were randomly divided into two groups: controls ($n = 30$) given regular chow and water ad libitum; and study animals ($n = 30$) fed within a soundproof chamber. Auditory brainstem response testing was conducted in both groups on postnatal day 42.

Results: Auditory deprivation between postnatal days 12 and 42 resulted in an increased hearing threshold and reduced auditory brainstem response amplitudes, together with degeneration of type I spiral ganglion neurons and the presence of apoptotic cells.

Conclusion: Non-invasive auditory deprivation during a critical developmental period resulted in numerous changes in rat cochlear function and morphology.

Key words: Cochlea; Rats; Spiral Ganglion; Sensory Deprivation; Neuron-Specific Enolase; Hearing

Introduction

A number of animal species have a critical period in the development of their sensory systems, during which the structure and function of the central nervous system (CNS) may be significantly influenced by the removal or alteration of neural afferent impulses. The auditory deprivation effect was first proposed in 1996 by the Eriksholm group (15 audiologists), and was defined as the gradual decrease in auditory function resulting from decreased acoustic information.[1]

In clinical practice, otologists have increasingly observed that auditory deprivation has harmful effects on the auditory function of hearing-impaired patients. Research has shown that a hearing-impaired ear which is unaided tends to lose its remaining ability to hear and to understand, whereas if a hearing aid is worn then that ear retains its ability to interpret sounds over a prolonged time period. Patients wearing two hearing aids keep both ears active. In addition, wearing one hearing aid when two are required may result in greater deterioration of hearing loss in the unaided ear than if no hearing aid were worn at all.[2] Effects seen include an increase in hearing threshold and a decrease in speech discrimination scores.[3]

At present, the anatomical and physiological changes in the brain which result from auditory deprivation have been studied primarily in animal models. Cochlear ablation, which damages the integrity of the cochlear hair cells, is a common method used to establish an auditory deprivation animal model. Cochlear ablation results in undetectable auditory function, as measured by auditory electrophysiological tests, as well as dysfunction of the organ of Corti, the initiation site for the auditory pathway.

In the current study, we used earplugs to non-invasively reduce afferent sound signals to the auditory system during the critical period of development in immature rats. This method maintained the normal structure of the cochlea; we were thus able to examine the effect of auditory deprivation on the development of the spiral ganglion, within the auditory system.

Materials and methods

Reagents

Rabbit anti-rat neuron-specific enolase polyclonal antibody and streptavidin-biotin complex immunostaining kits were purchased from Boster Wuhai Biological

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Technology (Wuhan, China). The DeadEnd™ Colorimetric Tunel system was obtained from Promega (Madison, Wisconsin, USA). All other chemicals used were of analytical reagent grade.

Animal model and groups

Neonatal Sprague–Dawley rats (male and female) were provided by the Experimental Animal Center of the Fourth Military Medical University. All studies were performed with the approval of the experimental animal committee at our university.

The animals were randomly divided into two groups: a control group ($n = 30$) given regular chow and water ad libitum; and an auditory deprivation group ($n = 30$) fed separately within a soundproof chamber.

In the latter group, bilateral earplugs were inserted on postnatal day 12 (in rats, hearing begins to function approximately on this day) and removed on postnatal day 42 (at the end of the critical period of auditory development extending from postnatal day 30 to 45 (with postnatal day 0 being the day of birth)). The earplugs consisted of a polymeric-based hearing aid impression compound. They were used to fill the concha after the opening of the external meatus, and were attached at the conchal margin using cyanoacrylate adhesive. The external auditory canal was sutured to avoid removal of the plug by the animal. Plugs were inspected daily and changed as often as was necessary to maintain a tight seal (Figure 1).

Auditory brainstem response testing

Auditory brainstem response (ABR) testing was carried out in both groups on postnatal day 42, using auditory electrophysiological apparatus (Biologic; Traveler Express E, Chicago, Illinois, USA), as previously described.[4]

Briefly, rats were anaesthetised with an intraperitoneal injection of 2 per cent pentobarbital sodium. The recording electrode was inserted underneath the scalp at the intersection point of the sagittal suture and the line connecting the two external auditory canals; the reference stimulating electrode was placed subcutaneously post aurem; and the grounding electrode was placed subcutaneously at the base of the tail. An alternating click paradigm was employed, with a 0.1 millisecond duration, 100–2000 Hz band-pass, 10 millisecond scanning time, 512 stacking time, and a sound source 1 cm away from the external auditory canal. Five waves were observed within the rat ABR, with waves II and III predominating. Clearly defined initial waves were selected from which to calculate the ABR hearing threshold, latency and amplitude. Auditory brainstem response testing was conducted in a double-layer shielded chamber with environmental noise of 40 dB sound pressure level (SPL) or less.

Electron microscopy

To prepare electron microscopy slides, animals were sacrificed with an overdose of pentobarbital, and then perfused with a mixture of 4 per cent paraformaldehyde and 1.5 per cent glutaraldehyde for 2 hours. Following decapitation, the temporal bones were removed and the cochleae harvested. After removal of the stria vascularis and bone cover, cochleae were dissected out and fixed in 3 per cent glutaraldehyde for more than 2 hours, followed by cold 2 per cent osmium acid for 2 hours. The cochleae were then dehydrated through graded acetone solutions and embedded in Epon (Hexion Specialty Chemicals, Ennis, TX, USA) using standard procedures. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Hitachi H600 transmission electron microscope (Hitachi, Tokyo, Japan).

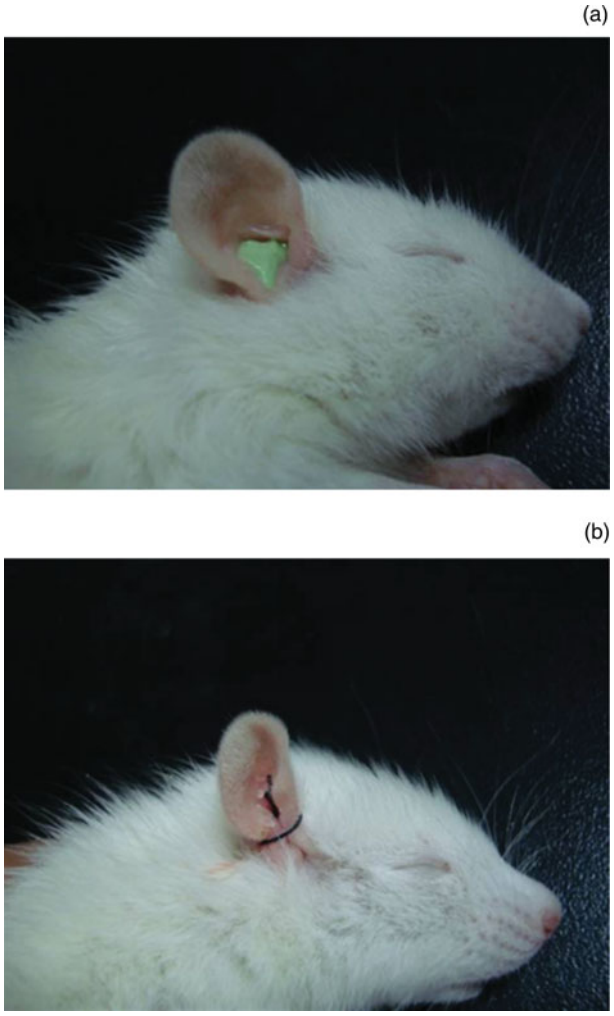

FIG. 1

Air-conduction auditory deprivation procedure. (a) Bilateral earplugs were inserted and fixed in place using cyanoacrylate adhesive. (b) The external auditory canal was sutured to prevent the animal removing the earplugs.

Immunohistochemical staining

To prepare samples for immunohistochemical staining, animals were sacrificed using an overdose of pentobarbital on postnatal day 42, and then transcardially perfused with normal saline. Following decapitation, tympanic bullae were isolated and fixed in 4 per cent paraformaldehyde overnight, followed by decalcification via

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immersion in 10 per cent ethylene diamine triacetic acid for 10 days. Specimens were cryoprotected by incubation in 30 per cent sucrose in phosphate-buffered saline, and then subsequently embedded in Tissue-Tek OCT for cryostat sections. Embedded tissues were sectioned (at 10 μm) and mounted on pretreated glass slides.

Spiral ganglion neuron-specific enolase staining was performed using the streptavidin-biotin complex kit. Sections were treated with 0.3 per cent H_2O_2 in methanol for 20 minutes, washed with phosphate-buffered saline, and then blocked with 5 per cent bovine serum albumin in phosphate-buffered saline for 30 minutes. Sections were incubated overnight at 4°C with the anti-rat neuron-specific enolase polyclonal antibody (1:300 diluted in blocking buffer), followed by biotinylated goat anti-rabbit immunoglobulin G for 40 minutes at 37°C and the pre-prepared streptavidin-biotinylated alkaline phosphatase complex for 20 minutes. Following rinsing with phosphate-buffered saline, sections were immersed in 3,3′-diaminobenzidine solution for 5 minutes. Sections were then counterstained for 20 seconds with Mayer's haematoxylin solution, dehydrated through an ethanol series and coverslipped. The primary antibody was omitted in the negative controls.

Sections containing the second turn of the cochlea were selected for spiral ganglion cell counting. Sixty high-power fields were randomly selected for counting.

Tunel assay

Tunel assays were performed using the DeadEnd Colorimetric Tunel system, according to the manufacturer's instructions. Briefly, sections containing the brainstem and otocyst were fixed in 4 per cent paraformaldehyde for 15 minutes and treated with 20 μg/ml proteinase K for 20 minutes at room temperature. Sections were treated with 3 per cent H_2O_2 for 5 minutes to inactivate endogenous peroxidases, and then incubated with a reaction buffer containing terminal deoxynucleotidyl transferase enzyme for 1 hour at 37°C, to label DNA at the 3′ ends with biotin-deoxyuridine-5′-triphosphate. Sections were then incubated with a streptavidin-horseradish peroxidase conjugate for 30 minutes at room temperature, to detect biotinylated nucleotides. 3,3′-Diaminobenzidine was added to the labelled samples to generate an insoluble, coloured substrate at the site of DNA fragmentation. Negative control sections were processed identically, except that the terminal deoxynucleotidyl transferase was omitted. Sections were mounted with 100 per cent glycerin.

Statistical analysis

All data were expressed as means ± standard deviations (SDs), and analysed using the Statistical Package for the Social Sciences version 11.0 for Windows software program (SPSS Inc, Chicago, Illinois, USA). One-way analysis of variance was used to determine statistically significant differences between groups. The significance of variance between two different groups was assessed using Student's *t*-test. A p value of less than 0.05 was considered statistically significant.

Results

Auditory brainstem response changes

We monitored ABR thresholds on postnatal day 42. Auditory brainstem response thresholds were significantly higher in the auditory deprivation group compared with the control group (being 28.4 ± 5.5 and 18.6 ± 3.89 dBSPL, respectively; $p < 0.01$). There were no significant differences in the latencies of waves I to V ($p > 0.05$), comparing the two groups (Figure 2a). However, the amplitudes of waves II to V differed significantly between the two groups ($p < 0.05$; Figure 2b). Wave V exhibited the most stable ABR with the highest amplitude.

The interval between wave I and wave V is also termed the 'brainstem transmission time' or 'CNS transmission time'. Values for this parameter were 3.98 ± 0.43 milliseconds in the control group and 3.63 ± 0.63 milliseconds in the auditory deprivation groups; this difference was statistically significant ($p < 0.001$).

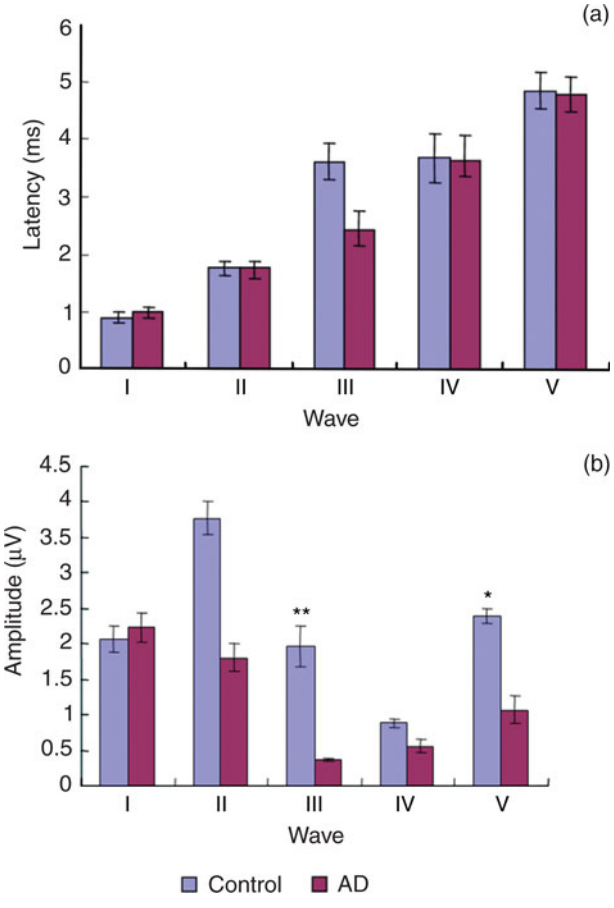

FIG. 2

Changes in auditory brainstem response (ABR) following auditory deprivation: (a) ABR wave latencies; (b) ABR wave amplitudes. $n = 26$. Results represent means with standard deviations indicated by outliers. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs* control group.

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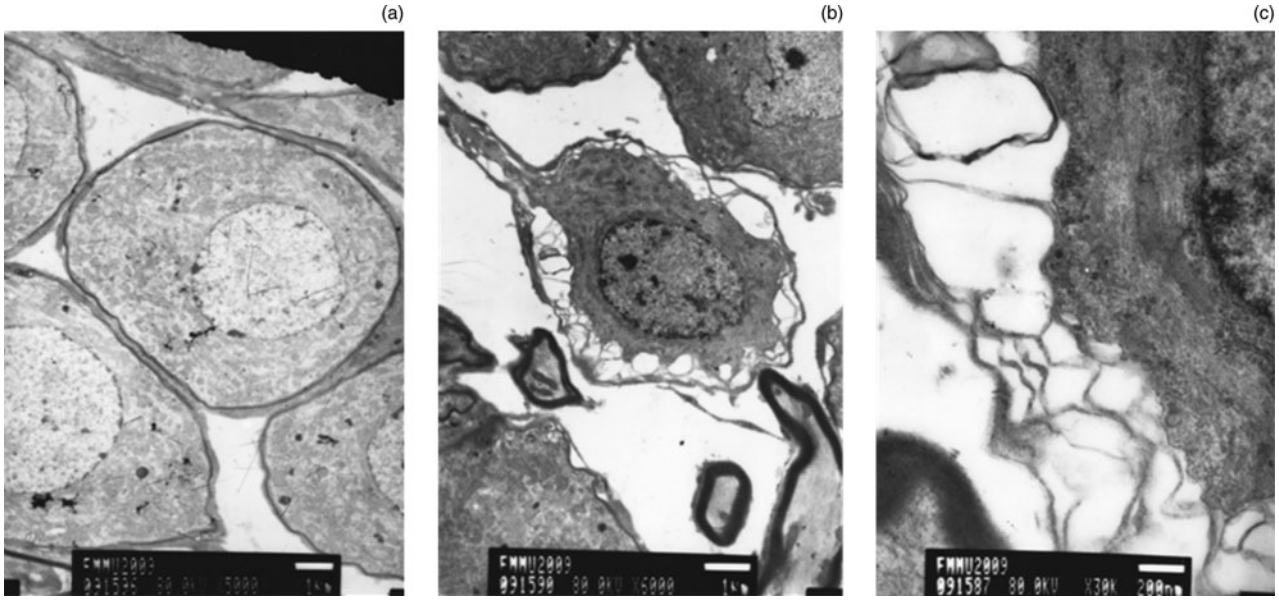

FIG. 3

Transmission electron photomicrographs of spiral ganglion neurons, demonstrating the morphological consequences of auditory deprivation. (a) Control group neurons, showing plump, normal organelles (original magnification ×5000). (b) Auditory deprivation group neurons, showing markers of degeneration: decreased cellular volume, increased intercellular space and increased cytoplasmic membranous structures (original magnification ×6000). (c) Further magnified region from part (b), showing cytoplasmic membranous structure, lack of myelin sheathing and degenerate organelles (original magnification ×30 000).

Spiral ganglion changes

In the control group, transmission electron microscopy showed the type I spiral ganglion neurons to be encased in a myelin sheath, with a plump morphology, small intercellular space and well developed satellite cells. In these neurons, the intracellular rough endoplasmic reticulum was gathered into piles, and the mitochondrial structure was normal.

In contrast, the type I spiral ganglion neurons in the auditory deprivation group exhibited numerous signs of degeneration: a significantly decreased volume, increased intercellular space, cytoplasmic concentration, increased electron density, increased heterochromatin in the nucleus, increased cytoplasmic membranous structures close to the cell membrane, poorly developed satellite cells, no myelin sheath, fewer and degenerated organelles, and immature mitochondria (Figure 3).

Neuron-specific enolase immunoreactivity

In the control group, most neuron-specific enolase immunoreactivity was located in neurons with a normal appearance: normal cytoplasm, an obviously stained cell body, a lightly stained nucleus, and a certain density of organelle arrangement. The two types of neurons (I and II) could not be distinguished based on morphology. Neuron-specific enolase immunoreactivity was lighter in nerve fibres extending from the cell body than in the cell body itself.

In the auditory deprivation group, neuron-specific enolase immunoreactivity appeared reduced, compared with the control group, with fuzzy cell morphology and disordered arrangement (Figure 4). Nerve fibre staining in this group was nearly normal (Figure 4). The number of neuron-specific enolase positive neurons in the auditory deprivation group was significantly lower than that in the control group (7.9 ± 0.99 versus 10.6 ± 1.1, respectively; $p < 0.05$).

Spiral ganglion cell apoptosis

To analyse pathological changes associated with hearing loss, we performed the Tunel reaction to detect apoptosis within the spiral ganglion. Apoptotic cells were not detected in the spiral ganglion cells of the control group (Figure 5a); however, Tunel-stained apoptotic cells were observed in the auditory deprivation group (Figure 5b).

Discussion

In the present study, ABR testing indicated that the hearing threshold in the auditory deprivation group was approximately 10 dB higher than that in the control group, with lower amplitudes. During ABR testing, wave I served as a reference for the other waves, and was particularly important. The amplitude of wave V was the least influenced by changes in given noise repeatability and decreasing sound intensity. Wave V persisted after the disappearance of the other waves. Because of the importance of the interval between waves I and V, we statistically analysed the value of this parameter in the control and auditory deprivation groups; a statistically significant difference was seen ($p < 0.01$).

These results demonstrate that rat auditory function is adversely affected by auditory deprivation. We hypothesise that such deprivation has deleterious

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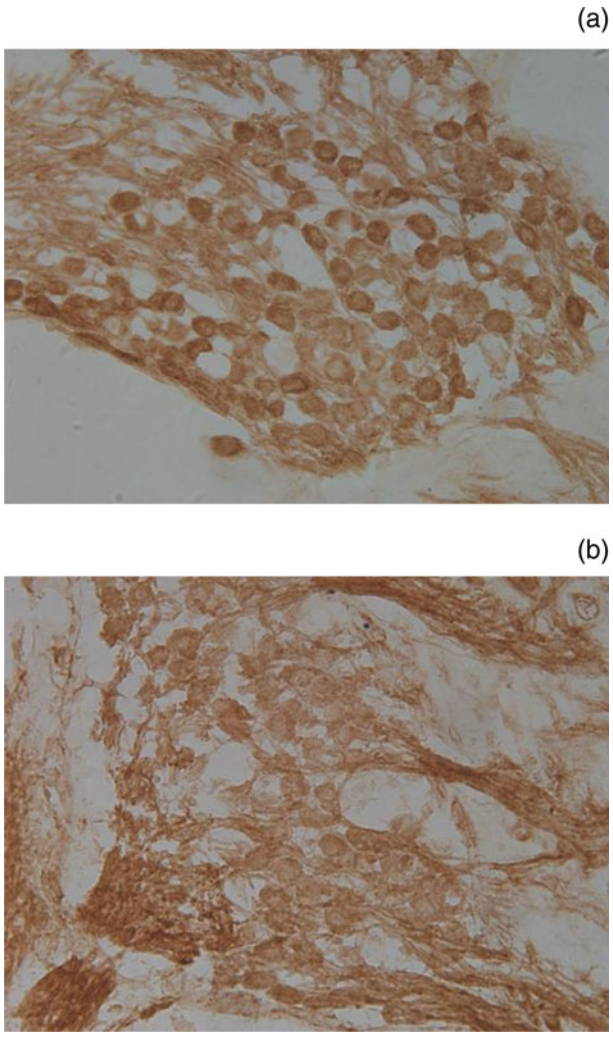

FIG. 4

Photomicrographs illustrating alteration of neuron-specific enolase immunohistochemistry in spiral ganglion cells in the two groups. (a) In the control group, cytoplasmic neuron-specific enolase immunoreactivity clearly delineates the cell body, with lighter nuclear staining. (b) In the auditory deprivation group, neuron-specific enolase immunoreactivity shows unclear cell body morphology and a lighter intensity of staining; neuronal processes exhibited normal staining. (Original magnification ×60)

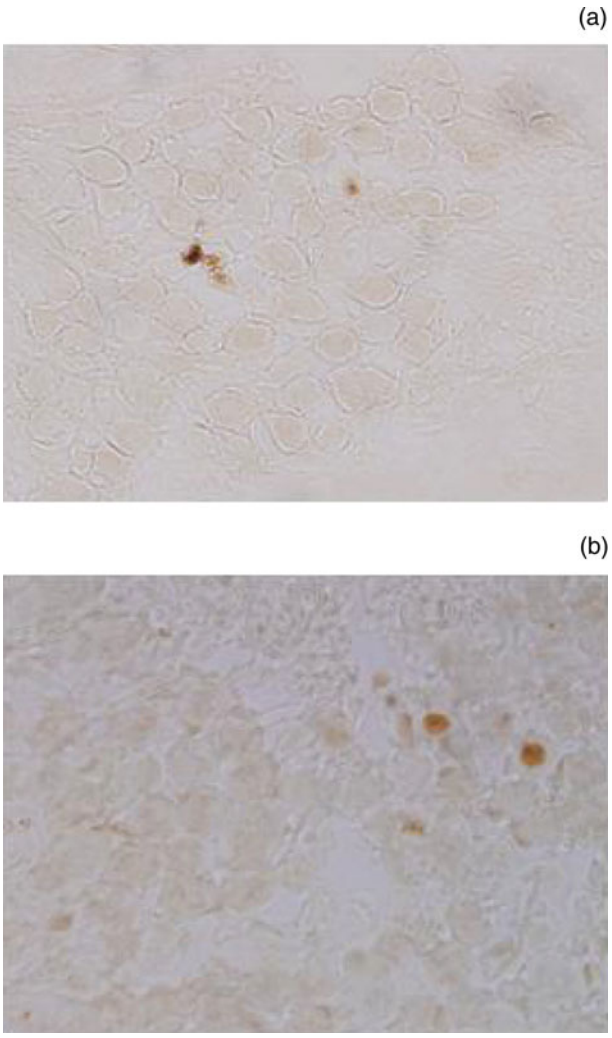

FIG. 5

Photomicrographs illustrating spiral ganglion cell apoptosis following auditory deprivation. (a) In the control group, Tunel staining detects no apoptotic cells. (b) In the auditory deprivation group, Tunel staining identifies scattered apoptotic cells (with brown-yellow stained nuclei) in the spiral ganglion. (Original magnification ×60)

effects on the function of various components of the auditory pathway.

In 1958, Deol and Kocher described the sequence of histopathological changes in the ears of mutant mice carrying a deafness gene.[5] At birth, the inner ear structures of these mice appeared normal. However, degeneration of the organ of Corti commenced on postnatal day 10 for Deiter's cells, on day 15 for hair cells, and was well under way by day 21 for all scala media structures. The spiral ganglion cells remained apparently normal until postnatal day 50, after which they also underwent progressive degeneration. At postnatal day 90, degeneration of both the organ of Corti and the spiral ganglion was nearly complete.

The present study observed apoptotic cells within the spiral ganglion of the auditory deprivation group, but not in the control group. It is likely that this apoptosis was triggered by decreased external sound stimuli. Complete absence of auditory input (due to cochlear damage) results in greater regional spiral ganglion degeneration than partial auditory deprivation.[6] The air-conduction auditory deprivation paradigm does not produce complete deprivation; therefore, the quantity of apoptotic cells may not be large. In the present study, transmission electron microscopy revealed that the spiral ganglion neurons in the auditory deprivation group exhibited multiple signs of degeneration.

Neuron-specific enolase immunohistochemical analysis in the auditory deprivation group clearly indicated lighter 3,3′-diaminobenzidine staining than in the control group, with cells exhibiting poorly delineated morphology and a disordered arrangement. In the auditory deprivation group, neuron-specific enolase immunoreactivity in the nerve fibres was similar to that in the control group; however, the number of neuron-specific enolase positive neurons was significantly reduced.

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Enolase is a key enzyme in glycolysis, being active at all stages of glycolysis metabolism. Neuron-specific enolase is an enolase isozyme mainly found in the cytoplasm of neurons and neuroendocrine cells.[7] The amount of neuron-specific enolase present in peripheral nerves is 10 to 100 times lower than that in the CNS.

In the current study, the less intense 3,3′-diaminobenzidine staining of neuron-specific enolase immunoreactive neurons seen in the auditory deprivation group indicates that reduced afferent auditory input may lead to cell energy metabolism dysfunction which induces down-regulation of neuron-specific enolase gene expression. Decreased glycolysis and adenosine triphosphate production would then impair the normal functioning of the neuron.

The development and maturation of the neural network structure and function involved in the olfactory, visual and auditory systems can be influenced by altering the relevant afferent signals.[8–10] Auditory deprivation has been shown to result in delayed development of inhibitory synapses in the lateral superior olive neurons in rats. Sanes *et al.* showed that activity-dependent positioning of axons and dendrites relevant to the inhibitory synapse can be blocked by cochlear ablation in rats at postnatal week 1.[11] Likewise, in immature Wistar rats the morphological development of inhibitory synapses, and of lateral superior olive neuron dendrites, can be blocked by unilateral cochlear ablation.[12] Overall, it seems that the effect of auditory deprivation depends largely on the age of onset. In this respect, a critical period is defined as a time period during which the action of a specific condition is required for normal development of a system. If development is disturbed, the effect seems largely irreversible. In the rat, ABR testing indicates that auditory function has begun to occur on postnatal day 12 and has reached near-adult thresholds on approximately postnatal day 22.[13] Therefore, the postnatal critical auditory developmental period in rats is generally considered to be from postnatal days 12 to 22.[14] Notably, the rat external auditory meatus is fully developed and opened at postnatal day 12. In the present study, auditory deprivation was commenced towards the beginning of the rat auditory system maturation process. Deprivation was maintained from postnatal days 12 to 42, thereby reducing the input of sound signals to the auditory system during this critical period. Webster observed that postnatal auditory deprivation in mice resulted in incomplete maturation of most brainstem auditory neurons, and also that acoustic stimulation had a more pronounced effect on neuronal maturation before 45 days of age compared with the same stimulation given between 45 and 90 days.[15,16] Auditory stimulation during the maturation period may thus lead to accelerated development, reflected by decreasing ABR latencies and thresholds.[17]

The absence of auditory stimuli leads to degenerative effects. Morphological changes have been described, for example in sound-deprived mice and rats.[18,19] Such changes may then lead to impaired development of ABRs, with consequently longer latencies. Several studies have showed prolongation of ABR latencies in sound-deprived guinea pigs and rats.[20,21]

Numerous studies have shown that the health and overall number of spiral ganglion cells play a profoundly important role in hearing. Pathological studies have shown that auditory hair cells and spiral ganglion cells can be damaged by a variety of auditory system insults, ranging from noise to ototoxins and infection. Thus, in the present study we anticipated that auditory deprivation would affect the development of the spiral ganglion during the rat auditory system maturation period.

- **This study assessed the effects of auditory deprivation during the critical period of postnatal rat auditory development (postnatal days 12 to 42)**
- **Auditory brainstem responses (ABRs) were analysed and cochlear spiral ganglia histology determined**
- **Auditory deprivation caused increased hearing thresholds and reduced ABR wave amplitudes, together with apoptosis and degeneration of type I spiral ganglion neurons**

The auditory process is very complex, and involves cooperation between auditory receptors and the auditory pathway. It is difficult to determine which part of the auditory system will be influenced by auditory deprivation during the critical period, and it is possible that multiple components of the system are involved. Further studies are needed to assess whether the spiral ganglion cell degeneration and ABR disturbances induced by auditory deprivation are reversible. It is important to elucidate the complex mechanisms that underlie hearing loss resulting from auditory deprivation.

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Address for correspondence:
Dr Yang Chen,
Department of Otorhinolaryngology, Head and Neck Surgery,
Xijing Hospital, The Fourth Military Medical University,
Xi'an, Shaanxi 710032, P R China

Fax: +86 29 83224749
E-mail: chendoc@fmmu.edu.cn

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