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Audiometric Outcomes of Topical IGF1 Treatment for Sudden Deafness Refractory to Systemic Steroids

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Objective: To examine the effects of topically applied recombinant human insulin-like growth factor 1 (IGF1) via gelatin hydrogels on alterations in pure-tone audiometry (PTA) in patients with sudden sensorineural hearing loss (SSHL) refractory to systemic steroids.
Study Design: Retrospective chart review.
Setting: A single university hospital.
Patients: Twenty-five patients with SSHL refractory to systemic steroids who received topical IGF1 treatment.
Intervention: Single topical application of IGF1 to the round window niche using gelatin hydrogels.
Main Outcome Measures: The primary outcome was alterations in PTA thresholds at frequencies of 0.25, 0.5, 1, 2, and 4 kHz after topical IGF1 application. Secondary outcomes included differences in final improvements in PTA thresholds among frequencies tested and cumulative numbers of patients showing 10- or 20-dB recovery in PTA during the observation period.
Results: Topical IGF1 application via gelatin hydrogels significantly altered PTA thresholds at each frequency tested and at the average frequency. The numbers of patients with a 10-dB recovery in PTA increased until 4 weeks after treatment and then stabilized, whereas those patients showing 20-dB recovery gradually increased during the observation period.
Conclusion: Topical IGF1 application via gelatin hydrogels contributes to the recovery of PTA levels in patients with SSHL refractory to systemic steroids. Major recovery of PTA levels occurs within 4 weeks after treatment. **Key Words:** Drug delivery system—Growth factor—Local application—Sudden sensorineural hearing loss.
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Sudden sensorineural hearing loss (SSHL) is the most common cause of acute sensorineural hearing loss and is one of the few types that can be cured, with spontaneous recovery occurring in 30% to 60% of cases (1,2). Most recoveries occur within the first 2 weeks of onset, and after this time, it can be difficult to detect further improvement in hearing. The most common therapy for SSHL is the systemic application of glucocorticoids (3,4), but approximately 20% of patients do not respond to this treatment (5). In addition, no therapy has been demonstrated to be effective according to the evidence criteria (6).

Various experimental studies have been performed to identify alternative therapeutics for acute sensorineural hearing loss including SSHL, and of several potential candidates, insulin-like growth factor 1 (IGF1) was shown to be crucial for the development and maintenance of the cochlea (7); its clinical use also has been permitted, so it is a reasonable treatment for us to focus on. IGF1 delivery to the cochlear fluid at appropriate concentrations for sufficient durations has proved difficult, however, because most drugs, particularly growth factors, require sustained delivery for their appropriate actions. Topical application can increase drug concentrations in the cochlear fluid, but simple tympanic injection does not achieve sustained delivery (8). To overcome this, we have used gelatin hydrogels for the sustained release of proteins or peptides in topical cochlear treatment (9,10).

Based on our preclinical studies (11–13), we performed a prospective clinical trial to test the safety and efficacy of topical IGF1 application via gelatin hydrogels for SSHL refractory to systemic steroids (14). The main outcome measures of this clinical trial were the proportions of patients showing hearing improvement, as determined by hearing recovery of 10 dB or above in the mean level of 5 frequencies tested in pure-tone audiometry (PTA)

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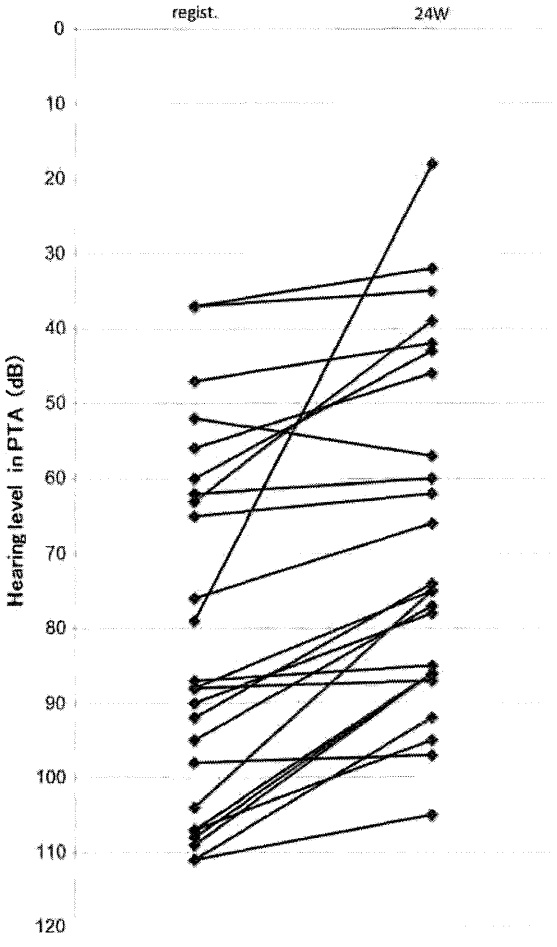

FIG. 1. Alterations in hearing levels in PTA between at registration (regist.) and 24 weeks after topical IGF1 treatment in each patient.

at 12 and 24 weeks after the test treatment. The null hypothesis was that 33% of patients would show hearing improvement, as was reported for a historical control after hyperbaric oxygen therapy (15). At 12 weeks after treatment, 48% of patients showed hearing improvement, and this proportion increased to 56% at 24 weeks, which was statistically significant (14). These findings indicate that topical IGF1 application may be efficacious for hearing recovery in patients with SSHL refractory to systemic steroids. However, details of audiometric alterations after topical IGF1 treatment have not yet been elucidated and might prove crucial for understanding mechanisms of IGF1 therapeutic effects on SSHL and for the design of a consecutive randomized trial. The present study therefore aimed to understand these details.

MATERIALS AND METHODS

Patients

Medical records were reviewed of 25 patients (13 female and 12 male subjects, of median age of 49 yr at registration [range, 23–72 yr]) with SSHL who received topical IGF1 treatment in our previous clinical trial (14). Briefly, the main inclusion criteria of our previous clinical trial were as follows: diagnosis of SSHL within 29 days of onset at the Department of Otolaryngology, Head and Neck Surgery of Kyoto University Hospital, Kyoto, Japan, and hearing recovery of less than 10 dB for an average of PTA thresholds at 5 frequencies (0.25, 0.5, 1, 2, and 4 kHz) after systemic steroid treatment. We excluded patients with active chronic otitis media, acute otitis media, otitis media with effusion or dysfunction of the auditory tube, malignant tumors, severe liver dysfunction (aspartate aminotransferase, >100; and alanine aminotransferase, >100), uncontrolled diabetes (hemoglobin A1C, >10), pituitary or adrenal dysfunction, severe systemic illness that affected life expectancy, a history of severe drug allergy, a history of alcohol or drug dependence within the past 1 year, and pregnant or lactating women. All patients registered in this study had no previous history of noise exposure. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and approved by the ethical committee of the Graduate School of Medicine, Kyoto University (registered number, C165). Each patient gave written, informed consent to participate in this study.

Intervention

Patients received topical IGF1 treatment using gelatin hydrogels at a median of 23 days (range, 15–32 d) after the onset of SSHL, between 2007 and 2009 at Kyoto University Hospital. Gelatin hydrogels were made from porcine skin gelatin (Nitta Gelatin Inc., Osaka, Japan) in a clean room at the Department of Pharmacy, Kyoto University Hospital. Topical IGF1 treatment procedures were performed in the Day-Surgery Unit of Kyoto University Hospital. Mecasermin (recombinant human IGF1, Somazon, 10 mg for injection; Astellas Pharma Inc., Tokyo, Japan) was dissolved in physiologic saline at a final concentration of 10 mg/ml. A 30-μl sample of mecasermin solution was mixed with 3 mg gelatin hydrogels 60 minutes before application. The single treatment involved placing hydrogel containing 300 μg mecasermin in the round window niche of the middle ear after tympanostomy under local anesthesia with 1% lidocaine. Patients were hospitalized for 4 days after the surgical procedure, and their general and local conditions were examined at the outpatient clinic of the Department of Otolaryngology, Head and Neck Surgery, Kyoto University Hospital, for 24 weeks after the test treatment.

Audiometric Examination

PTA was performed on the day of registration and at 1, 2, 4, 12, and 24 weeks after the test treatment. Thresholds in air conductance at frequencies of 0.25, 0.5, 1, 2, and 4 kHz were determined.

Outcome Measures

A retrospective chart review was performed with a primary outcome measure of alterations in PTA thresholds for each frequency tested and for the average of 5 frequencies during the observation period. Secondary outcome measures included differences in final improvements of PTA thresholds among frequencies tested and cumulative numbers of patients showing 10- or 20-dB recovery in the average of PTA thresholds at each time point after treatment.

Statistical Analysis

Alterations in PTA thresholds during the observation period and differences in final improvements of PTA thresholds among

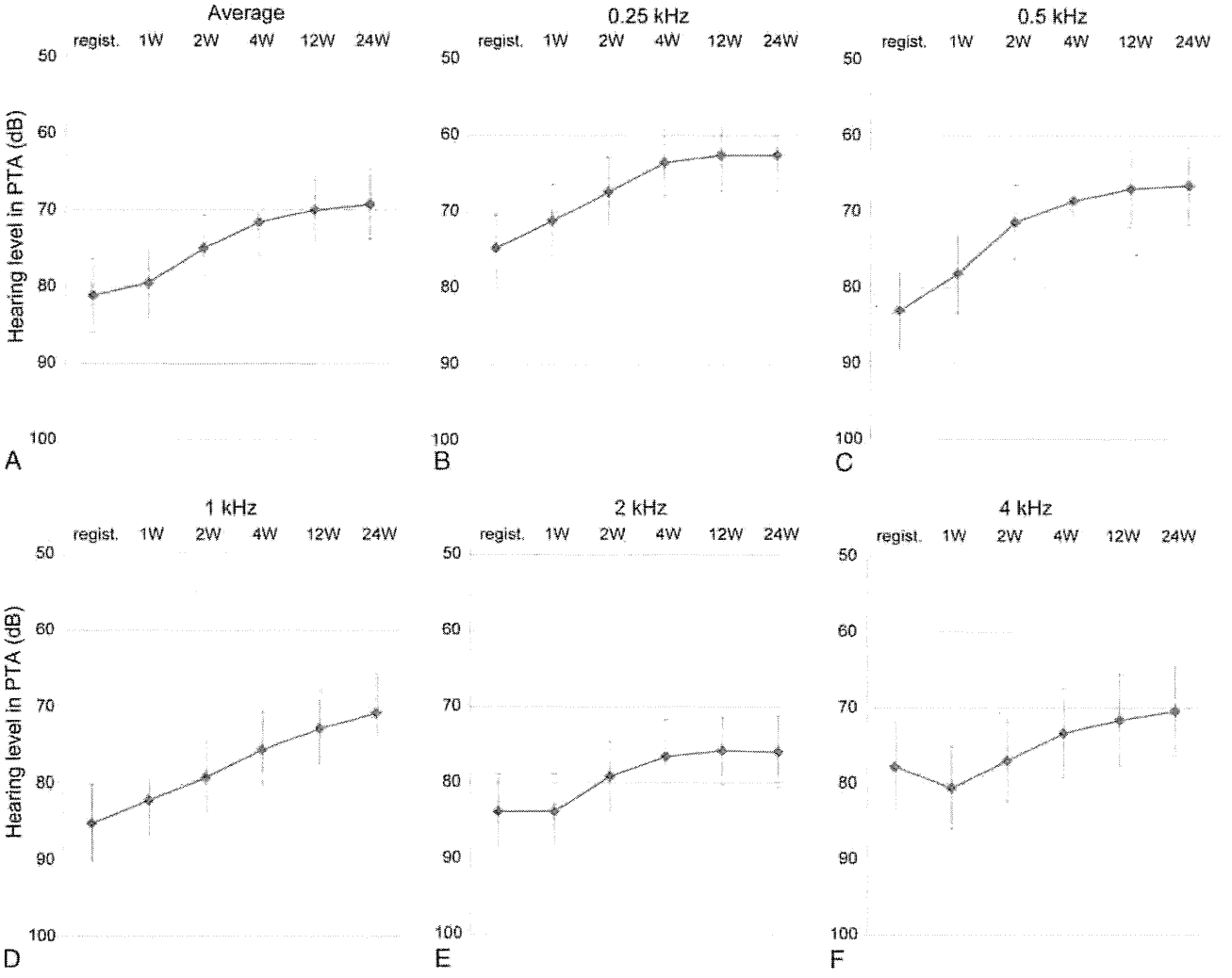

FIG. 2. Hearing levels in PTA at registration (regist.) and after topical IGF1 treatment, in the average of 5 frequencies tested (*A*), at 0.25 (*B*), 0.5 kHz (*C*), 1 kHz (*D*), 2 kHz (*E*), and 4 kHz (*F*). *Bars* represent standard errors.

frequencies tested were examined by the repeated measures analysis of variance. The final recovery in PTA thresholds after topical IGF1 treatment was compared with that in a historical control of hyperbaric oxygen therapy for 199 patients with glucocorticoids-resistant SSHL in Kyoto University Hospital between October 2000 and September 2006 (15) by unpaired *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Alterations in PTA Thresholds

Figure 1 shows alterations in mean PTA hearing levels between at registration and 24 weeks after treatment for each patient. The mean PTA hearing level at registration was 81.2 dB (95% confidence interval [CI], 71.2–91.1), and that of 24 weeks after treatment was 69.3 dB (95% CI, 59.8–78.7). The time course of alterations in averages of PTA thresholds is shown in Figure 2A and was statistically significant ($p < 0.001$). Figure 2, B to F, shows the time courses of alterations in PTA thresholds at frequencies of 0.25, 0.5, 1, 2, and 4 kHz, which were all found to be statistically significant ($p < 0.001$). These findings indicate that topical IGF1 treatment via gelatin hydrogels has significant effects on the recovery of PTA thresholds in patients with SSHL refractory to systemic steroids.

Difference in PTA Recovery Among Frequencies

The mean recovery for the average PTA threshold of 5 frequencies tested 24 weeks after treatment was 11.9 dB (95% CI, 5.8–17.9), and means of recoveries in PTA thresholds for each frequency are shown in Figure 3. Differences in the recovery of PTA thresholds among frequencies were statistically significant ($p = 0.0003$), and we observed a trend that hearing recovery at 2 or 4 kHz was smaller than that at lower frequencies. These findings indicate that PTA hearing recovery after topical IGF1 treatment predominantly occurred at low frequencies.

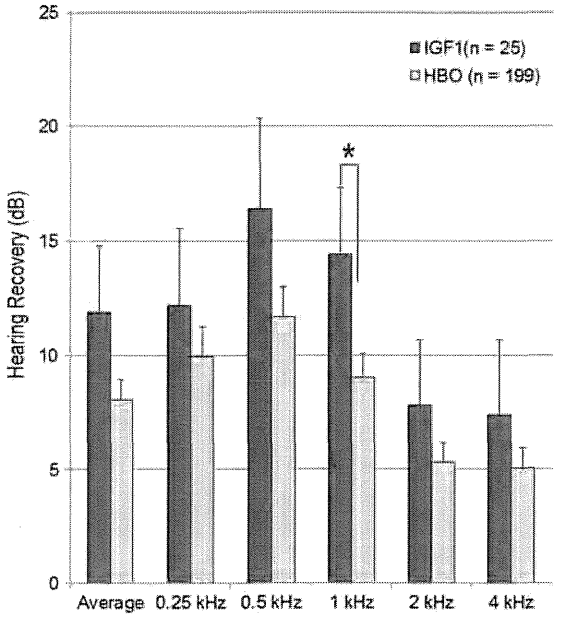

FIG. 3. Final hearing recovery in the average of 5 frequencies tested and at each frequency tested after topical IGF1 treatment or in a historical control of hyperbaric oxygen therapy. An *asterisk* indicates a significant difference in hearing recovery between topical IGF1 treatment and hyperbaric oxygen therapy. *Bars* represent standard errors.

Time Points for Hearing Recovery

Cumulative numbers of patients showing 10- or 20-dB recovery in PTA during the observation period are shown in Figure 4. For the average PTA threshold of 5 frequencies tested, 14 patients showed 10-dB recovery, and 8 showed 20-dB recovery at the end of the observation period. Four weeks after treatment, 10-dB recovery was found in 11 patients (79%) and 20-dB recovery in 4 patients (50%) (Fig. 4). For each frequency, 10-dB recovery at 4 weeks was found in 89% (17/19) of patients at 0.25 kHz, 89% (17/19) at 0.5 kHz, 68% (13/19) at 1 kHz, 86% (12/14) at 2 kHz, and 67% (8/12) at 4 kHz (Fig. 3, B–F). At 4 weeks, 20-dB recovery appeared in 64% (7/11) of patients at 0.25 kHz, 85% (11/13) at 0.5 kHz, 70% (7/10) at 1 kHz, 50% (3/6) at 2 kHz, and 83% (5/6) at 4 kHz. These findings demonstrate that PTA hearing recovery after topical IGF1 treatment occurred during the initial 4 weeks in the majority of patients but that further recovery was evident in some patients at a later stage.

Comparison With a Historical Control

The recoveries of PTA thresholds for each frequency tested and for the average of 5 frequencies 24 weeks after topical IGF1 treatment were compared with those in a historical control of hyperbaric oxygen therapy (Fig. 3). The recovery of the average PTA thresholds after topical IGF1 treatment was larger than that of historical controls (11.9 ± 2.9 [mean ± standard error] for IGF1 versus 8.0 ± 0.9 for historical control), but the difference was not statistically significant ($p = 0.08$). For each frequency, there is a trend that topical IGF1 treatment shows better PTA threshold recoveries than a historical control (Fig. 3). The difference in PTA threshold recoveries at 1 kHz between the 2 groups was statistically significant at $p = 0.04$, whereas at other frequencies, no significant differences in PTA threshold recoveries were observed.

DISCUSSION

The present study demonstrates that topical IGF1 treatment has significant effects on the PTA thresholds of patients with SSHL refractory to systemic steroids. Topical IGF1 application using gelatin hydrogels was performed at 23 days after SSHL onset and led to an average recovery of 11.9 dB in PTA thresholds over 5 frequencies. In comparison with a historical control of hyperbaric oxygen therapy, there is a trend that topical IGF1 treatment shows better hearing recovery than hyperbaric oxygen therapy, and a significant difference in hearing recovery is found at 1 kHz. After topical IGF1 treatment, recovery at or above 10 dB was found at 0.25, 0.5, and 1 kHz frequencies. Maximum recovery of 16.0 dB occurred at 0.5 kHz, whereas hearing recovery at 2 or 4 kHz was less than 10 dB, suggesting that irreversible degeneration might occur in the high-frequency region of the cochleae. On the other hand, significant hearing recovery also was observed at low frequencies, suggesting that damage in the low-frequency region may be reversible and respond to IGF1 delivered by gelatin hydrogels from the round window.

Regarding the time course of alterations in PTA thresholds for the average of 5 frequencies, hearing recovery was initiated 2 weeks after treatment and had stabilized by 4 weeks, which corresponds to recovery starting approximately 5 weeks after the onset of SSHL symptoms and

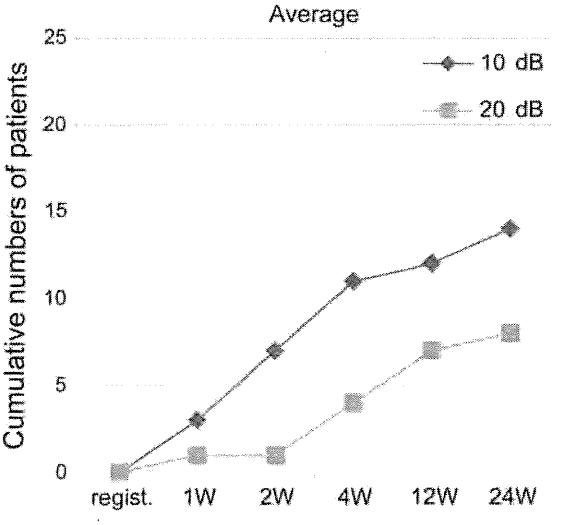

FIG. 4. Cumulative numbers of patients showing 10- or 20-dB recovery in the average of 5 frequencies during the observation period. *Bars* represent standard errors.

TABLE 1. *Audiometric outcomes in studies of the intratympanic injection of steroids for sudden sensorineural hearing loss refractory to systemic steroids*

First author	Sample size	Steroid, dose (mg/ml)	Treatment protocol	PTA baseline (dB)	Hearing improvement (dB)
Plontke, 2005 (23)	23	Dex (4) or Mp (40)	RW microcatheter for 4 wk	103	16
Choung, 2006 (24)	34	Dex (5)	2 injections/wk for 2 wk	72	9
Roebuck and Chang, 2006 (25)	31	Dex (24)	1 injection	69	12
Kilic, 2007 (26)	20	Mp (62.5)	5 injections at 3-d intervals	65	22
Haynes, 2007 (27)	40	Dex (24)	1 injection	NA	5
Kakehata, 2011 (28)	24	Dex (4)	1 injection per day for 8 d	75	17

Dex indicates dexamethasone; Mp, methylprednisolone; NA, not available; PTA, pure tone audiometry; RW, round window.

continuing until 7 weeks after the onset. In general, spontaneous SSHL recovery has been shown to occur between Days 3 and 14 after its onset (1,2), so the PTA recovery observed in the present study may not be spontaneous. PTA thresholds at 1 kHz exhibited a trend of linear recovery during the observation period, whereas those at other frequencies and alterations in cumulative numbers of patients with a 10 dB recovery showed a similar trend to the average of all 5 frequencies. An increase in cumulative numbers of patients showing 20-dB recovery was slightly delayed in comparison with that showing 10 dB recovery, which initiated at 4 weeks after treatment and continued to 12 weeks. In short, hearing recovery after topical IGF1 treatment seemed to take approximately 4 weeks, and in cases with good recovery, further delayed recovery occurred.

Previous animal experiments have demonstrated crucial roles of IGF1 in the physiopathology of hearing (16,17). We also have revealed that IGF1 has otoprotective effects against noise- and ischemia-induced cochlear damage using in vivo animal models (11–13). As for mechanisms of hearing recovery by IGF1, two possible explanations are aroused. One possible mechanism is the rescue of cochlear cells from cell death via IGF1 receptor-mediated actions, which has been indicated by animal experiments. IGF1 binding to IGF1 receptors activates several intracellular signaling pathways associated with the promotion of cell survival (18). Another is the regulation of glucose transport in cochlear cells. IGF1 also has insulin-like actions including promoting glucose transport (19), which can be associated with the survival of cochlear hair cells (20,21). Both mechanisms might be involved in hearing recovery by topical application of IGF1. Further studies are required for elucidation of detailed mechanisms.

Recently, the intratympanic injection of steroids has gained considerable attention as an alternative for systemic steroids that may reduce the risk of developing systemic side effects and enables the delivery of more steroids into the cochlea. Rauch et al. (22) reported that the intratympanic injection of steroid effects as an initial treatment for SSHL are not inferior to those of systemic steroids, and there is an increasing number of case series evaluating intratympanic steroids as a salvage therapy for SSHL refractory to systemic steroids (23–28). Table 1 is a summary of 6 studies, of which, evidence levels are superior to IV and, of which, sample sizes are larger than 20 subjects. The median PTA recovery obtained in these studies is 14.0 dB (range, 5–22 dB), which is in agreement with that obtained in the present study.

Of previous studies on intratympanic steroids as salvage treatment, 2 describe detailed time courses of PTA thresholds. Ho et al. (29) reported a time course of alterations in PTA thresholds after the intratympanic injection of dexamethasone. Four weeks after the initiation of treatment (approximately 6 wk after the onset), an average recovery of 24.3 dB was reported, with little further improvement. Similarly, Kilic et al. (26) examined PTA thresholds for an average of 4 frequencies after intratympanic injections of methylprednisolone every 3 days for a total of 5 applications. At 4 weeks after the initiation of treatment (approximately 8 wk after the onset), an improvement of 19.8 dB was found, with no further improvements occurring during the observation period. The time course of hearing recovery after topical IGF1 treatment therefore seems to be similar to that which occurs after intratympanic steroid application.

CONCLUSION

The present results indicate that topical IGF1 application via gelatin hydrogels may be efficacious for SSHL refractory to systemic steroids. Although the level and time course of hearing recovery after topical IGF1 treatment is similar to that after IT steroids, the baselines of PTA thresholds and methods for evaluation vary among studies. In addition, pharmacologic actions of IGF1 differ from those of steroids. Therefore, the difference in therapeutic potential for SSHL refractory to systemic steroids between topical IGF1 and intratympanic steroid treatment is unclear. To clarify this, we are currently performing a randomized trial to compare the efficacy of topical IGF1 treatment with that of intratympanic steroids for SSHL refractory to systemic steroids, which may provide critical information on salvage treatments for refractory SSHL.

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Trauma-Specific Insults to the Cochlear Nucleus in the Rat

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The effect of acoustic overstimulation on the neuronal number of the cochlear nucleus (CN) was investigated by using unbiased stereological methods in rats. We found that, after 9 weeks of recovery, neurons in the anteroventral cochlear nucleus (AVCN) degenerated, whereas those in the posteroventral and dorsal cochlear nuclei (PVCN and DCN) were preserved. The noise trauma induced near complete loss of the outer hair cells throughout the cochlea, and the inner hair cells were preserved only in the more apical regions. This pattern of selective loss of AVCN neurons in this study was different from trauma induced by auditory deafferentation by mechanical compression of auditory neurons. In contrast to noise trauma, mechanical compression caused loss of neurons in the PVCN and DCN. After 5 weeks of recovery from mechanical compression, there was no loss of inner or outer hair cells. These findings indicate that auditory deprivation, induced by different experimental manipulations, can have strikingly different consequences for the central auditory system. We hypothesized that AVCN neuronal death was induced by excitotoxic mechanisms via AMPA-type glutamate receptors and that excitatory neuronal circuits developed after acoustic overstimulation protected the PVCN and DCN against neuronal death. The results of the present study demonstrate that hearing loss from different etiologies will cause different patterns of neuronal degeneration in the CN. These findings are important for enhancing the performance of cochlear implants and auditory brainstem implants, because diverse types of hearing loss can selectively affect neuronal degeneration of the CN.

Key words: auditory nerve; cochlear nucleus; compression injury; neurodegeneration; noise exposure

The morphological and physiological effect of acoustic overstimulation to the auditory periphery is relatively well characterized, but less is known about the effects on the central auditory system. The hair cells in the cochlea transduce acoustic information to auditory nerve fibers, and these signals are then transmitted to the cochlear nucleus (CN). The information that is conveyed to the CN is therefore dependent on functionally intact hair cells and nerve fibers. Any temporary or permanent disruption in the peripheral organ will have physiological consequences for the processing and perception of acoustic information throughout the central auditory pathway (CAP). Understanding how different peripheral pathologies affect the subregions of the CN is important for characterizing functional deficiencies and for developing strategies to restore function.

We demonstrated in a previous study that the number of neurons in the posteroventral cochlear nucleus (PVCN) and dorsal cochlear nucleus (DCN) decreased, while the neurons in the anteroventral cochlear nucleus (AVCN) were preserved after auditory deprivation induced by mechanical compression of auditory neurons (Sekiya et al., 2009). Mechanical compression did not affect the hair cells but resulted in complete loss of the ABR when stimulated at high intensities.

As an extension of this finding, we questioned how noise trauma, designed to cause hair cell loss, would affect neuronal numbers in the subregions of the CN and if the changes would differ from the changes found after mechanical compression. To address this question, we quantified hair cells in the cochlea and neurons in the CN. Central auditory pathology was assessed by using unbiased stereological methods.

In contrast to mechanical compression to the auditory nerve, in which auditory neurons become primarily hypoactive, it is feasible that noise trauma could have complex consequences for the CN, because the neurons initially become hyperactive, followed by a

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hypoactive state, as revealed by the degree of hair cell injury after trauma. Although they have not been verified quantitatively, such differences in the insults may cause different effects on the CN. The findings of the present study clearly indicate that auditory deprivation, induced by different experimental manipulations, can have strikingly different consequences for central auditory morphology and neuronal number.

MATERIALS AND METHODS

Trauma Models and Auditory Brainstem Responses

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments at Kyoto University. Male adult Sprague-Dawley rats (P12–14 weeks) were exposed to a short-duration/high-intensity sound, as reported elsewhere (Kojima et al., 2007). Briefly, each animal was deeply anesthetized by an intraperitoneal injection of pentobarbital sodium (Nembutal; 40 mg/kg; Abbott, North Chicago, IL) and fixed by clamping the incisors and tails to a handmade apparatus before exposure to acoustic overstimulation. An octave-band noise centered at 4 kHz of 137 dB SPL was generated by a combination of a noise generator (NP-203; JR Sound, Tokyo, Japan), an amplifier (SRP-P150, Sony, Tokyo, Japan), and speakers (horn super tweeter T925A; Fostex, Tokyo, Japan). The experimental rats were exposed to the noise for 1 min in a soundproof room used for human examination. Sound levels were monitored at the animal's head and calibrated with a sound level meter (LA2560; Ono Sokki, Yokohama, Japan).

The procedure for mechanical compression has been described previously (Sekiya et al., 2000). Briefly, the axons of the auditory neurons were quantitatively compressed in the cerebellopontine angle, without permanent compromise of the blood supply to the cochlea. Each animal was anesthetized by an intraperitoneal injection of ketamine (100 mg/ml; Sankyo, Tokyo, Japan) and xylazine (9 mg/ml; Bayer, Tokyo, Japan). After exposure of the seventh and eighth cranial nerve trunks through right retromastoid craniectomy, an L-shaped stainless-steel wire was placed on the auditory nerve to compress the nerve and simultaneously record the CAP.

Auditory brainstem responses (ABRs) were recorded between the base of the pinna and the vertex, with the ground electrode at the base of the right forelimb. Click stimuli (100 dB SPL) were presented to the ear at a rate of 9.5 pulses/sec through a tube earphone driven by a 100-μsec rectangular pulse wave fed by a stimulator. Evoked potentials were amplified with a bandpass of 50 Hz to 3 kHz and averaged using a processor (Synax 1100; NEC Medical Systems, Tokyo, Japan) with a sampling interval of 20 μsec and 500 data points in each recording. The responses to 100 successive clicks were averaged for ABR recordings and stored in a computer. Alternating clicks were used to stimulate the ABR. For the recordings of cochlear microphonics (CM), condensation and rarefaction clicks were given separately in each recording session using the same electrodes, and the responses to 50 successive clicks were averaged. ABR recordings were performed in both sides of noise-exposed and control rats.

Quantification of Hair Cells

The cochleae from the control (n = 3), nerve compression (n = 3), and noise trauma groups (n = 3) were stained with phalloidin (TRITC; Molecular Probes, Eugene, OR; 1:80; 60 min) and dissected into half-turns, and the pieces were placed separately on an eight-well microscope slide (Histolab Products, Gothenburg, Sweden) in a drop of Citifluor. The entire basilar membrane was dissected, yielding a total averaged length of approximately 6.0 mm. The criterion for cell loss was the identification of a scar formation. The number of scar formations was counted using a Zeiss Axiovert light microscope with epifluorescence and a ×40 oil objective, as previously described (Viberg and Canlon, 2004).

Quantification of CN Neurons

Nine weeks after noise exposure, the rats were sacrificed by cardiac perfusion using freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) under deep anesthesia with pentobarbital, and the brains were removed en bloc. The region of the brainstem containing the CN was sectioned away from the brain and cryoprotected in 20% sucrose in PBS for 24 hr in a refrigerator (4°C). The specimens were frozen in isopentan with dry ice and stored at −20°C. With a Zeiss cryostat (HM 560), section thickness was set at 50 μm. The frozen sections were collected and stored in Eppendorf tubes with cryoprotection solution (40% PBS, 30% glycerol, 30% ethylenglycol) at −20°C.

Seventy-five sections were evenly dispersed in 15 Eppendorf tubes so that every tube contained five sections that were 750 μm apart (Fig. 1).The staining procedure began by thawing the sections and then placing them in cell strainers into six-well plates with PBS on a rotating table. The protocol for immunostaining was obtained from the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA), with some modifications. The tissue was permeabilized using 0.3% Triton in PBS for 30 min, followed by a PBS rinse (3 × 10 min), then exposed to 3% H_2O_2 in PBS, 30 min to quench endogenous peroxidase. The sections were blocked for 1 hr using 1.5% normal horse serum for NeuN in 0.15% Triton in PBS, followed by a 2 × 5 min rinse in 0.15% Triton in PBS. The sections were incubated with the primary antibody NeuN (1 mg/ml; MAB377; Chemicon/Millipore, Temecula, CA; anti-mouse 1:600 in 0.3% Triton in PBS) overnight in a refrigerator (4°C). The secondary antibody was incubated for 30 min (0.5% anti-rabbit IgG and 1.5% normal goat serum for NeuN; Vector Laboratories). The Vectastain ABC Elite kit reagent was added for 1 hr, followed by a 3 × 10 min 0.15% Triton in PBS rinse and then 7 min of exposure to DAB-nickel. The sections were rinsed in distilled water, mounted on Superfrost slides, and allowed to dry overnight. The sections were then counterstained with cresyl violet (0.25%) and dehydrated in graded ethanol and finally cleared in xylol and coverslipped with Mountex (Histolab Products).

Stereology

The quantification of the total number of neurons in the entire, anatomically delineated area of the CN was made by using stereological methods (Gundersen et al., 1988; Idriz-

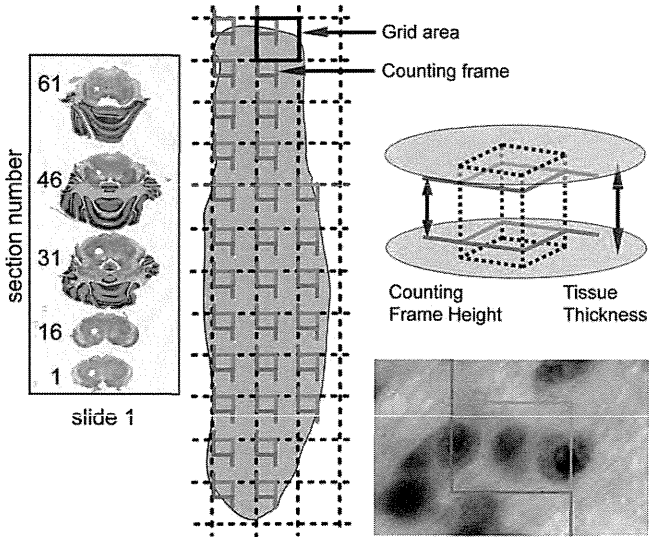

Fig. 1. Arrangement of CN sections used for quantifying the total neuronal number. The series of sections is distributed over 15 slides, and every third slide is used for analysis. Each slide has five sections; for example, slide 1 has sections 1, 16, 31, 48, and 61. All sections are collected from the region of interest. The quantification of the total number of neurons is made by first anatomically delineating the area of interest. Grid areas are systematically placed over the area. A computer-generated counting frame is superimposed on the screen, and the counting frame height is set within the midregion of the section and discards the rough outer edges. By using the optical fractionator with optical sectioning, approximately 150 neurons are counted (×100 oil-immersion objective). An example is shown of a counting frame in which red lines indicate rejection regions (neurons in contact with the red line are not counted). The neurons within the frame and touching the green line are counted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

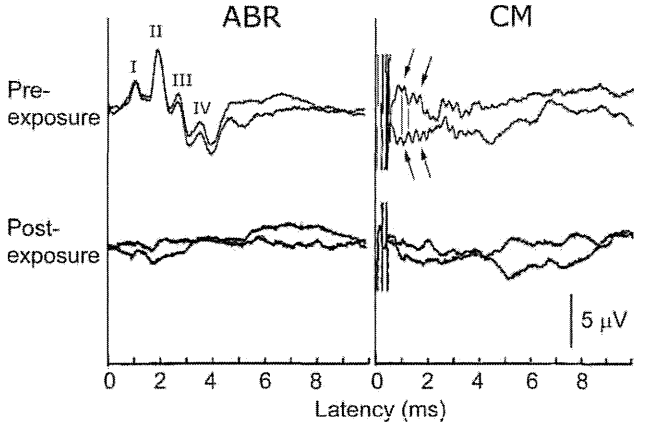

Fig. 2. Auditory brainstem response (ABR) recordings of a rat before and 9 weeks after noise exposure. The ABR and cochlear microphonics (CM; small arrows) were lost 9 weeks after noise exposure. Vertical bars indicate phase reversal of CM in response to condensation (upper trace) or rarefaction clicks (lower trace). I–IV, wave I to IV of ABR.

begovic et al., 2006; Sekiya et al., 2009) in n = 11 (AVCN), n = 12 (PVCN), and n = 10 (DCN) bilateral samples from the noise-exposed rats. The mechanical compression analyses consisted of n = 4 AVCN and PVCN and n = 5 DCN unilateral samples. The controls consisted of AVNC (n = 10) and PVCN (n = 10) and DCN (n = 11) unilateral samples. The delineation of the PVCN and DCN was performed at low magnification (×5) using an Axio Imager M2 Zeiss microscope connected to an Olympus DP72 digital camera, which transmits the image to the monitor. A computer-generated counting frame was superimposed on the screen in CAST-GRID 3.6.2.0 software (Visiopharm, Hørsholm, Denmark; Fig. 1). Two sets of motors are connected to the microscope to move the section at a known distance in the x and y directions. The optical fractionator design was used together with optical sectioning so that approximately 150 neurons would be counted under a ×100 oil-immersion objective with a numerical aperture of 1.40. All neurons positively stained for NeuN were counted, and the equation used to calculate the total number of neurons in each subdivision of the CN was $N = 1/ssf \times 1/asf \times 1/hsf \times Q^{-}$, where ssf is section sampling fraction, asf is area sampling fraction, and hsf is height sampling fraction (Dorph-Petersen et al., 2001). The area of the counting frame was dimensioned so that two or three neuronal somas would typically be sampled (Fig. 1). The dimensions of the counting frame were 33.7 × 25.4 μm^2 (856 μm^2), and the height of the optical dissector was 10 μm (Fig. 1). The step size used for AVCN was 4% (step length 146.3 μm) and for DCN and PVCN 12% (84.4 μm). The section thickness was measured at each step in the given areas in the section, and a weighted thickness was calculated (Dorph-Petersen et al., 2001). Every third section was analyzed. The changes in the total neuronal number were evaluated by a one-way ANOVA in SigmaStat 3.5.

RESULTS

Nine weeks after noise trauma, there was a total loss of all ABR peaks and CM in all animals (Fig. 2). This was a consistent finding in all the animals measured. The quantification of inner and outer hair cell loss is illustrated for the control, nerve compression, and noise trauma groups (Fig. 3). Hair cell loss was not found for the control group or for the mechanical compression group. Quantification of hair cell loss 9 weeks after noise trauma demonstrated total loss of all outer hair cells. The inner hair cells were partially preserved in the more apical cochlear regions, whereas the middle to basal regions showed complete loss (Fig. 3). Thus, the noise trauma used in this study caused severe peripheral damage.

Under microscopic observation, reduction of neuronal numbers after the noise exposure was evident in the AVCN (Fig. 4). To determine quantitatively how peripheral damage induced by noise trauma affects the neuronal count in the CN, we performed unbiased stereology to count the number of neurons in the AVCN, PVCN, and DCN (Fig. 5). In the AVCN from the noise trauma animals, there were 16,243 ± 5,752 neurons (n = 11) and in the unexposed control animals 23,513 ± 4,886 neurons (n = 10). The noise trauma

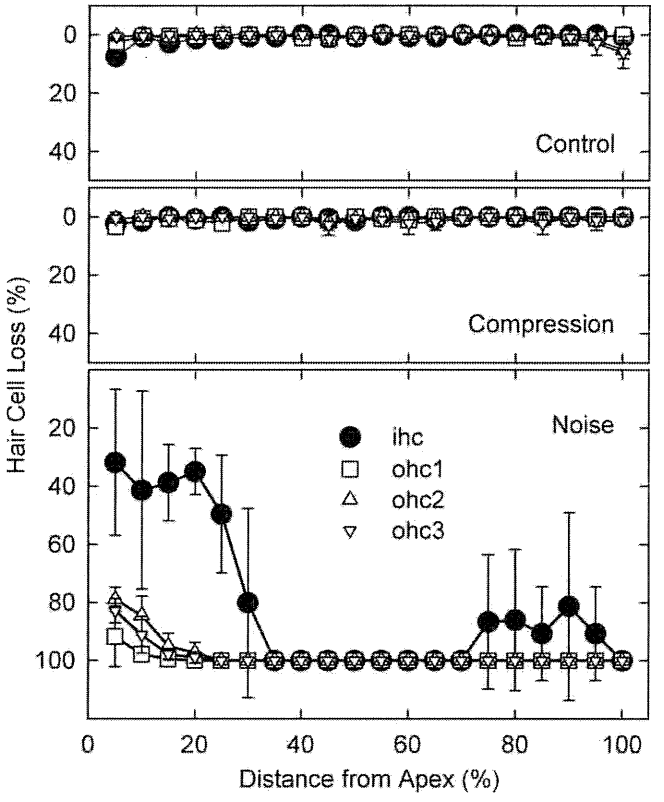

Fig. 3. Cochleogram illustrating the percentage loss of inner and outer hair cells for the controls, noise exposed, and mechanical compression groups. Hair cell loss was not detected in the control or mechanical compression groups, but severe hair cell loss was noted 9 weeks after noise exposure.

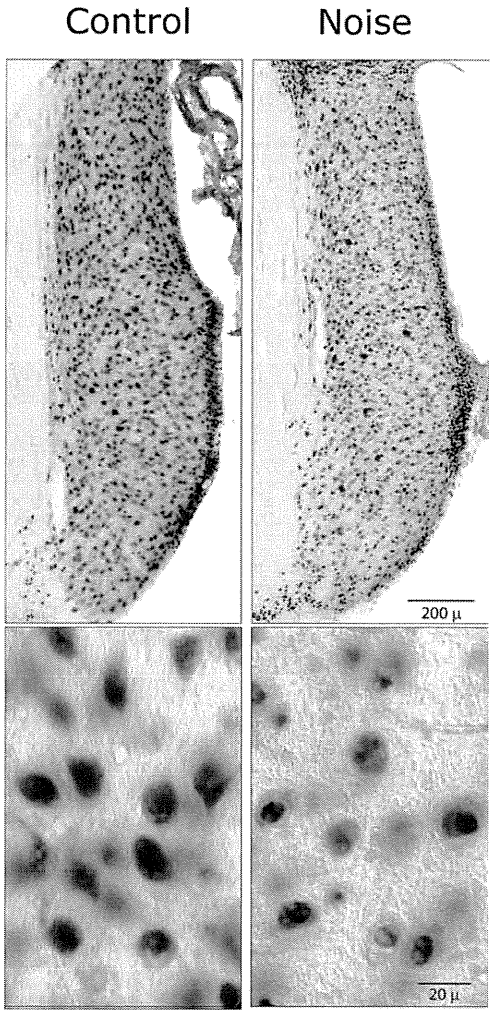

Fig. 4. Low-magnification micrographs of the control (**left**) and acoustic overstimulation (**right**) groups. The AVCN shows a slight reduction in size and a reduction in the neuronal population (**top**). Enlarged views demonstrate the paucity of neurons and shrinkage of nuclei of residual neurons in the acoustic overstimulation group in comparison with the control group (**bottom**).

PVCN had 9,796 ± 5,157 neurons (n = 12), and the control PVCN had 13,545 ± 2,837 (n = 10). The DCN in the noise group had 9,347 ± 1,873 neurons (n = 10), and the control DCN had 10,069 ± 1,957 neurons (n = 11). The AVCN demonstrated a statistically significant difference between the noise-exposed and the unexposed groups ($P < 0.006$, F = 9.641, df = 1, ANOVA; Fig. 5). In contrast, neither the PVCN nor the DCN showed a statistically significant difference between the noise-exposed and the control groups (PVCN, $P < 0.054$, F = 4.2, df = 1; DCN, $P < 0.399$, F = 0.743, df = 1, ANOVA).

As reported previously, mechanical compression did not change the mean number of neurons in the AVCN compared with the controls (mean ± SD, 23,513 ± 4,886 on the control side, n = 10; and 27,935 ± 3,131 on the compressed side, n = 4). In the PVCN and DCN, however, the total number of neurons significantly decreased on the compressed sides compared with controls (Fig. 5). In the PVCN, the mean number neurons was 13,545 ± 2,837 (n = 10) on the control side and 5,849 ± 1,363 on the compressed side ($P < 0.001$, F = 26.03, df = 1, n = 4). In the DCN, the mean number of neurons was 10,069 ± 1,957 (n = 11) on the control sides and 5,514 ± 938 on the compressed sides ($P < 0.001$, F = 23.88; df = 1, n = 5; Sekiya et al., 2009). Collectively, quantification of the total number of neurons in each structure demonstrates the different effects that noise trauma and mechanical compression have on the CN (Fig. 5).

DISCUSSION

CN degeneration is shown to be dependent on the trauma used for insult. Here we compare the long-term effects of noise trauma and mechanical compression in untreated animals and show different patterns of degeneration. In this report, the total number of neurons was counted in the entire CN, and it was found that the AVCN showed a statistically significant decrease after exposure to broadband acoustic trauma, whereas the

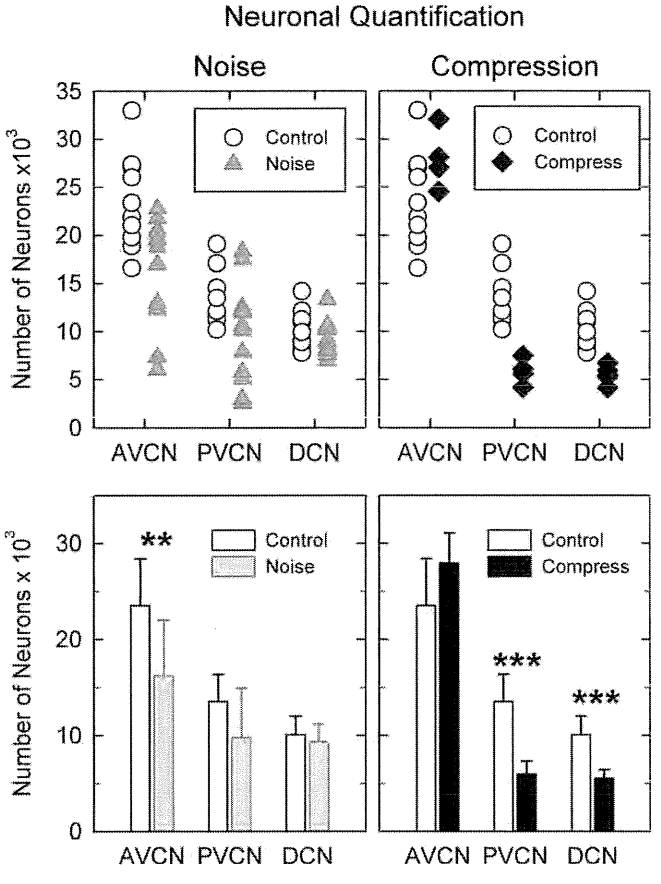

Fig. 5. Quantification of the total number of neurons in the AVCN, PVCN, and DCN. Individual data for the noise exposure and mechanical compression groups compared with the control group. Neuronal quantification for the individual animals (**top**) and mean ± standard deviation for the noise exposed, mechanical compression, and control groups (**bottom**). There is a statistically significant reduction in the number of neurons in the noise-exposed AVCN compared with the control group. In the mechanical compression group, the PVCN and DCN showed statistically significant reductions in the number of neurons compared with the control group. $^{**}P < 0.01$, $^{***}P < 0.001$.

PVCN and DCN did not show any statistically significant change in neuronal number. In contrast, mechanical compression resulted in a loss of neurons in the PVCN and DCN. To the best of our knowledge, this is the first experimental study to quantify neuronal numbers in the entire CN after trauma using unbiased stereological methods. A previous paper reported neuronal numbers for all subdivisions of the CN after acoustic overstimulation, but the entire area in each subdivision was not evaluated, so total neuronal estimates are not known (Willott et al., 1994). When determining the change in neuronal number after insult, only the total counting of neurons is a reliable measure (Oorschot, 1994; West, 1999). Density measurements cannot be used for this purpose because they are indirect values and are not accurate (Oorschot, 1994). In counting cells, we used the optical dissector and fractionators, and this technique never requires tissue volume estimates (Charleston, 2000). Moreover, with this unbiased method, the traditional problems of "split nucleus" issues and unknown effects on tissue volume resulting from tissue processing, including fixation, dehydration, embedding, sectioning, and staining, can be avoided (Charleston, 2000).

Excitatory Neuronal Death in the AVCN Neurons After Noise Trauma

Intracochlear mechanical damage is known to induce axonal loss in the CN and upper auditory nuclei in a manner that corresponds topographically to the damaged cochlea (Morest et al., 1997). However, noise trauma produces zones of axonal loss that are larger than damaged areas in the cochlea, suggesting an expansion of axonal loss resulting from excitotoxic processes (Kim et al., 1997, 2004a,b; Morest et al., 1998; Muly et al., 2004). In the present study, only AVCN neurons were statistically significantly affected by noise trauma. Calcium loading of the cytoplasm by the calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor because of synaptic overactivity constitutes severe metabolic challenges to the nervous system (Petralia et al., 2000). Excitotoxic processes lead to toxic neurodegeneration, such as necrosis and apoptosis (Canlon et al., 1998; Mattson, 2007; Rainey-Smith et al., 2010). In the acute stage of acoustic overstimulation, glutamatergic synaptic release in the CN increases, consistent with hyperactivity of glutamatergic transmission (Willott et al., 1994; Muly et al., 2004). The calcium permeability of AMPA receptors is inversely correlated with the relative abundance of GluR2 subunit, so the GluR2-lacking AMPA type is calcium permeable, whereas the GluR4 subunit promotes assembly of more rapidly gated receptors (Hollmann et al., 1991; Geiger et al., 1995). Studies of mRNA expression and immunolabeling indicate that most AMPA receptor complexes in AVCN neurons contain mainly GluR4 and GluR3 but lack GluR2 subunit. In contrast, GluR2 is widespread in the DCN (Hunter et al., 1993; Petralia et al., 1997, 2000; Wang et al., 1998; Caicedo and Eybalin, 1999). The VCN neurons receive stronger direct afferent input from the auditory nerve than DCN neurons (Groschel et al., 2010), and their neuronal responses are shaped by their intrinsic conductances rather than synaptic interactions (Forsythe, 2001). In contrast, the DCN contains a substantial number of interneurons that mold the response to sound through synaptic interactions between neurons (Young and Oertel, 2004; Fredrich et al., 2009). Overall, it is assumed that the VCN neurons are more vulnerable to calcium overloading than the DCN neurons following acoustic overstimulation, which could be one explanation for the differences found in the present study. In contrast to our results, an earlier paper reported that the effect of intense noise exposure on neuron number was minimal in AVCN, a part in PVCN and DCN (Willott et al., 1994). In that study, however, a traditional dye staining method was used to

identify pale-staining nucleus and nucleolus the cells (Willott et al., 1994). In our study, however, the cells were immunostained with an antibody that could specifically indicate neuronal nuclei. The differences among the staining methods could be one of the main causes of the inconsistency.

Spherical Bushy Cells and Other Principal Cells in the AVCN

The AVCN includes several principal cell types, including spherical bushy, globular bushy, multipolar, and small cells, that are present throughout the VCN (Malmierca, 2003; Young and Oertel, 2004). Numerical data from the cat indicate that the total numbers of spherical bushy cells are 36,600, globular busy cells 6,300, and multipolar cells 9,400 (Young and Oertel, 2004). Moreover, the spherical bushy cells are present exclusively in the AVCN and make up more than 70% of total neurons in the CN (50,000 cells; Young and Oertel, 2004). Spherical bushy cells receive an auditory nerve ending, one of the largest endings in the brain, the endbulb of Held, which contains an enormous number of synaptic release sites (a mean of 155, ranging from 85 to 217; Nicol and Walmsley, 2002) to secure the precise transmission of timing information from auditory stimuli to spherical bushy cell (Pfeiffer, 1966; Baker et al., 2010). On the other hand, however, this abundance of glutamate release sites at the endbulb of Held may expose spherical bushy cells to the threat of excitotoxic cell death after noise trauma.

Here we counted neurons by using NeuN antibodies that could stain neuronal nuclei exclusively. We did not use Golgi staining, which allows the identification of individual cell types in the CN. Nevertheless, we obtained data on the specific numerical differences among the principal cells in the AVCN. We conclude that the decrease by more than one-third of the total neuronal number in the AVCN mainly reflects the loss of spherical bushy cells.

Trauma-Specific Changes in the Rat CN

During acoustic overstimulation, an excessive flow of action potentials enters the CN through auditory neurons until the peripheral organ becomes incapacitated (Muly et al., 2004). As previously demonstrated, the AVCN was resistant to auditory deprivation by mechanical compression of auditory neurons, although the PVCN and DCN were vulnerable in these rats (Sekiya et al., 2009). In contrast, the present experiment demonstrated the reduction of neurons was observed only in the AVCN, whereas the PVCN and DCN were preserved. Collectively, the degeneration patterns of CN neurons between these two models are complementary to each other (Fig. 6). In both of these insult models, the CN neurons experience auditory deprivation. Nevertheless, the PVCN and DCN neurons were not sensitive to auditory deprivation once they had experienced acoustic overstimulation.

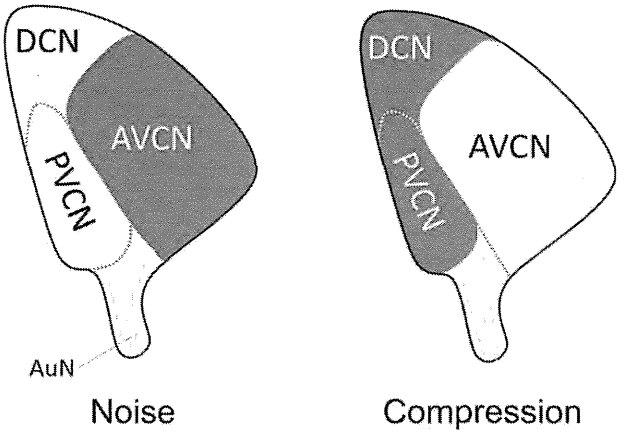

Fig. 6. Summary illustration of the effects of noise trauma (**left**) and mechanical compression (**right**) in the CN. The shaded area represents the CN region with loss of neurons. Noise exposure reduced the neurons in the AVCN, whereas mechanical compression reduced neurons in the PVCN and DCN. Taken together, the degeneration patterns of CN neurons were different between these two insults that commonly cause a reduction of peripheral output to the CN eventually.

After acoustic overstimulation, the net number of excitatory endings can recover, but the inhibitory endings are only partially recovered. This could then favor excitation and result in hyperexcitability and increased rates of neuronal discharge in the PVCN (Kim et al., 2004a) and in the DCN, where inhibitory neurons are far more predominant than excitaroy neurons compared with the VCN (Fredrich et al., 2009). Chronic increase in spontaneous activity was recorded broadly across the DCN after intense noise exposure (Zhang and Kaltenbach, 1998; Kaltenbach, 2007). It is likely that the fusiform cells in the DCN become hyperactivated with the loss of glycinergic inhibition after acoustic overstimulation (Brozoski et al., 2002). Predominantly excitatory neural transmissions in the PVCN and DCN might have prevented the neurons in these nuclei from dying because of the loss of auditory input following acoustic overstimulation, although it should be further elucidated how excitatory and inhibitory endings recover after acoustic overstimulation in the DCN, as has already been verified in PVCN (Kim et al., 2004a).

Clinical Implications

The VCN is a primary auditory structure that executes phase-locked temporal processing of auditory information (Oertel, 1997) and has been regarded as the main structure in relaying electrically coded information from auditory processors to higher level auditory structures (McCreery, 2008). Our present results suggest that CN neurons degenerate differently depending on whether auditory input to the CN is lost with or without the precedent stage of auditory hyperactivation. Clinically, cochlear implant and auditory brainstem implant are useful for patients with hearing loss from

various causes (Toh and Luxford, 2008). This variety of causative factors for hearing loss implies that the pattern of neuronal degeneration in the CN may vary among the patients. Although the VCN has been regarded as the main neural pathway for artificial hearing, the DCN might also play vital roles in obtaining hearing sensation with these devices (Zhang and Zhang, 2010).

We found that the total number of neurons degenerated only in the AVCN after acoustic overstimulation, whereas the neurons in the PVCN and DCN remained unchanged. This pattern of selective loss of CN neurons was complementary to that in our previous study with auditory deafferentation by mechanical compression of auditory neurons (Fig. 6). Taken together, these findings indicate that the pattern of CN neuronal death depends on the causative factors. It was likely that the loss of AVCN neurons was caused through glutamatergic excitotoxic mechanism and that plastic changes induced in the CN following acoustic overstimulation prevented the death of PVCN and DCN neurons. Because cochlear implants and auditory brainstem implants are useful for patients with various underlying causes, it is important to understand the relationship between the type of hearing loss and the various patterns of neuronal degeneration in the CN.

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