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Degeneration in the Ventral Cochlear Nucleus after Severe Noise Damage in Mice

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Abstract

To study the mechanisms of noise-induced hearing loss and the phantom noise, or tinnitus, often associated with it, we studied a mouse model of noise damage designed for reproducible and quantitative structural analyses. We selected the posteroventral cochlear nucleus, which has shown considerable plasticity in past studies, and correlated its changes with the distribution of NT3. We used volume change, optical density analysis, and microscopic cluster analysis to measure the degeneration after noise exposure. There was a fluctuation pattern in the reorganization of nerve terminals. The data suggest that the source and size of the nerve terminals affect their capacity for regeneration. We hypothesize that the deafferentation of VCN is the structural basis of noise-induced tinnitus. In addition the immuno-fluorescent data show a possible connection between NT3 and astrocytes. There appears to be a compensatory process in the supporting glial cells during this degeneration. Glia may play a role in the mechanisms of noise-induced hearing loss.

Keywords

Hearing loss; Cochlea; mouse model; NT3; tinnitus

INTRODUCTION

Noise-induced hearing loss, a growing problem in the industrialized world (Schacht, 2008), can lead to tinnitus, a phantom sound perceived by the subject, affecting 8-13% of adults in the world (Bauer & Brozoski, 2008). The pathophysiology of tinnitus is not clear, but evidence points to a central origin (For review, see Bauer and Brozoski, 2008; also Mulders and Robertson, 2009). Noise can affect spontaneous activity in the cochlear nucleus (CN) and inferior colliculus (IC) in rats (Imig and Durham, 2005). Even moderate sound can have long-term functional effects in cat primary auditory cortex (Pienkowski and Eggermont, 2009). Noise damage can change the functional organization in chinchilla IC (Salvi et al., 2000; Wang et al., 2002), where increased spontaneous activity has been related to tinnitus (Bauer et al., 2008). The dorsal cochlear nucleus (DCN) may be the site for inducing tinnitus after noise damage (Kaltenbach et al, 2005). This conclusion is based on studies that included chinchilla, hamster and rat (for reviews see Kaltenbach, 2007; Bartels et al., 2007). Elevated activity in the fusiform cells of DCN appears to correlate with the psychophysical expression of tinnitus (Brozoski et al., 2007). Computer modeling has been developed to study hyperactivity in the DCN after hearing loss (Schaette et al., 2008, 2009).

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Studies on the effects of noise exposure on the ventral cochlear nucleus (VCN) show degenerative morphological changes (Willot et al., 1994), including apoptosis (Aarnisalo et al., 2000). Our lab has focused on the dorsal division of the posteroventral cochlear nucleus (PVCN-A). This division of the CN is a “veritable microcosm” (Morest and Potashner, 2008) of the auditory brainstem since it contains the major neuronal types of the VCN which form the ascending pathways. This region also shows considerable plasticity after cochlear damage (Bilak et al., 1997; Kim et al., 1997; Kim et al., 2004a,b,c. also see review by Morest and Potashner, 2008).

Our lab uses mouse models, including genetically mutated strains, to discover the crucial sites of the pathological changes in PVCN-A, including those occurring during perinatal development (D’Sa et al., 2007; Hossain et al., 2008). The question then arises whether the mouse model is consistent with results from other species.

In this report we quantify our results by using volume change, optic density analysis, and microscopic cluster analysis and correlate these with changes in neurotrophic factor NT3. We show that similar changes occur in mice as in the chinchilla (Morest et al., 1997; Morest et al., 1998; Muly et al., 2002; Muly et al., 2004), only faster, following a characteristic fluctuation pattern, depending on the type of ending and its source.

We suggest that the effect of cochlear damage is first of all a deafferentation of the VCN. While the functional consequences may be more obvious in the DCN and IC, the primary structural basis of tinnitus may be the damage to VCN.

Our immuno-fluorescent data show possible connections between NT3 and astrocytes. There is much current interest in the synaptic interactions with astrocytes (Fellin T, 2009). We ask whether there is a compensatory process in the supporting glial cells during this degeneration. Can we use these supporting cells to rescue the neurons? This question has implications for the future of stem cell research and the ongoing research in our lab.

MATERIALS AND METHODS

Noise exposure

Adult F1 C57bl/6J $\times$ CBA mice were used in this study. The noise delivery system consisted of a CD player connected to one channel of an integrated stereo amplifier (Harmon-Kardon, Northridge, CA) located outside a soundproof room. The amplifier was connected to a speaker (JBL Professional, Northridge, CA; driver X with horn Y) suspended from the ceiling in the soundproof room. The noise was created by passing a pseudorandom, Gaussian broadband noise through a pair of filters. The resulting signal was recorded on a CD. The cut-off of the noise delivered to the animal is around 20 kHz.

A metal cage, capable of holding up to five mice, was placed on a piece of sound absorbing foam (Sonex Illbruck, Littleton, CO) in the soundproof room under the suspended speaker. The mice were unrestrained but positioned at approximately 0.76 m below the center of the speaker. The CD containing the noise stimulus was played through the noise delivery system to expose both ears for 6 h at 115 dB sound pressure level (SPL). The SPL was monitored by a condenser microphone (Bruel and Kjaer, Denmark) connected to a measuring amplifier (Bruel and Kjaer, Marlborough, MA). The noise profile and the frequency response of the noise delivery system are shown in Fig.1.

After the noise exposure, the mice were removed from the cage and returned to the animal care facility. All procedures were in accordance with the NIH Guide for the Care and Use of

Laboratory Animals and were approved by the University of Connecticut Health Center Animal Care Committee.

Animal preparation

A total of 37 age-matched mice were used (control or 0 survival, 1 wk and 4 wk survival, 7 each; 2 wk survival, 5; 8 wk survival, 11). For numbers in each group of analysis, please see the figure legends. Mice were deeply anesthetized by 0.2 mL intraperitoneal sodium pentobarbital (45 mg/kg). Cardiovascular perfusion was performed with 2 mL of 1% sodium nitrite followed by 20 mL of 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4; the solution was kept at room temperature (Feng et al., 2010). The brain was removed and immersed in the same fixative for 1 hour at 4° C, rinsed in phosphate buffer, cryoprotected by immersion in increasing concentrations of sucrose overnight, and stored at -70° C. The cochleas were removed and treated as described below.

Assessment of cochlear damage

The temporal bones, containing the cochleas, were dissected. To remove the excess fixative, the temporal bones were washed with gentle rotation (2 h, 4° C) in 0.1 M phosphate buffer at pH 7.4 made in physiological saline (PBS). The cochleas were then stained with a mixture of 1% osmium tetroxide, 0.85% sodium chloride, 0.0165% calcium chloride, and 1% potassium dichromate, buffered at pH 7.4, followed by rinses in Tyrode's buffer. They were dehydrated in increasing concentrations of ethanol, and embedded in Araldite (Durcupan; Fluka Chemical Corp., Milwaukee, WI). The plastic and bone surrounding the organ of corti were dissected and the organ was divided into 12 to 16 segments for analysis.

Each segment was traced in the microscope using a drawing tube. The drawings were scanned into a computer, and then measured using Image J software (NIH). The length of each cochlea was determined by adding that of all of its segments.

Cochleas were examined with a differential interference contrast microscope by an observer uninformed about the identity of each specimen. When the evaluations were checked by other investigators, who knew the identity of the specimens, the results were found to be consistent throughout all of the material examined. Damage was defined as % missing inner hair cells (IHCs), outer hair cells (OHCs) and myelinated nerve fibers (MNFs) in the osseous spiral lamina. Percent missing was determined as follows. First, the numbers of healthy IHC and OHC were counted in the microscope; second, these numbers were converted to % missing by using the theoretical values from Bohne (Bohne et al., 2001) as the 100% control. Missing MNFs were estimated by comparing regions without loss (0% missing), regions of total degeneration (100% missing), and regions of partial loss by assigning a relative value to each region examined in comparison with the staining intensity of regions in the osseous spiral lamina (Bohne et al., 1985; Bohne and Harding, 2001). The averaged data were plotted in 5% increments as the % missing vs % distance from the apex of the cochlea.

Immunohistochemistry and immunofluorescence

Serial cross sections of the hindbrains were cut at 15 µm thickness in a cryostat. Sections were first treated with 0.01% H₂O₂ in PBS for 30 minutes followed by incubation with 10% normal goat serum and 0.01% tritonX in PBS for 90 minutes. Sections were treated overnight at 4° C with primary antibodies in 3% normal goat serum diluted in PBS. For immunohistochemistry the monoclonal antibody to synaptic vesicle protein SV2 (Hybridoma Bank, Dr. Kathleen Buckley) was used at 1:500, the polyclonal antibody to NT3 (Alomone Labs, Israel) at 1:750. After treatment with the primary antibody and rinsing in PBS, sections were incubated with biotinylated secondary antibody goat anti mouse for an

hour, with Vectastain Elite ABC kit (Both are from Vector Laboratories, Burlingame, CA) for another hour, and then with diaminobenzidine for the color reaction, dehydrated and coverslipped. For immunofluorescence, 1:1,000 was used for both SV2 and glutamate transporter GLT-1 (ABR, Rockford, IL). After the primary antibody, sections were treated with fluorescence labeled secondary antibody (Molecular Probes, Eugene, OR) and then coated with Vectashield and coverslipped.

CN volume determination

The image of every other SV2 immunostained section was captured at 2.5x and enlarged to fill an area of 8×10 inches. The boundaries of the PVCN-A were determined, with reference to the mouse atlas of Trettle and Morest (2001). These boundaries were traced onto transparencies, along with a scale bar, and scanned into Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). The volume of PVCN-A was obtained by multiplying the sum of the areas by the distance encompassing the sections measured.

Optical density of SV2

The intensity of SV2 staining was measured by its optical density (OD). A series of 25 images taken at 40x in a through focus mode with a water immersion lens (C-Apochromat, 40x/1.2wKerr) was obtained, using a constant light source provided by a stable power supply. A black and white video camera (cooled CCD Microimaging II) was mounted on a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY). The Z capture application of the Northern Eclipse software (Olympus Corp, Tokyo, Japan) was used for capturing images. Images were then analyzed with the Z projection application of software Image J (NIH), using the minimum intensity setting. Using Adobe Photoshop 7.0, a montage of the entire CN was constructed from overlapping images captured at the same light intensity.

OD analysis was performed in three regions, namely, PVCN-A, DCN, and a portion of cerebellum. The adjacent parts in DCN and PVCN-A were compared for changes after noise exposure, and the neighboring cerebellum served as an internal control for the staining differences between particular sections. Images of microscopic fields were acquired through an MTI Dage Series 81 black and white video camera (Dage Corp., Michigan City, IN). The OD was measured by using small blocks of 50 μm^2 from the montage. An average of the readings from 3 to 4 such blocks, each in the PVCN-A, DCN and cerebellum was used as the OD value of that region.

For normalization of the data, a calibration curve was constructed. Images of a single field were captured using 20 different neutral density filters and a stabilized DC power supply for the light source. Since the camera provided images with a depth of 8 bits, each image was rated on a 0 to 225 gray scale, using the morphometric analysis program Optimas (Version 6). In the final plot, this scale was normalized to 100% and binned in 5% increments.

Microscopic cluster analysis

The analysis (Muly et al., 2002; Kim et al., 2004c) was used to measure the decrease in the two major types of presynaptic terminals; axosomatic (perisomatic) and axodendritic (neuropil). Images were recorded as described above. A threshold gray value was determined by the investigator so that the stained objects were easily identified. Care was taken to adjust threshold values useful for detection of small changes in staining intensity by the observer. The software Optimas (Version 6.5) identifies stained objects having gray values above the pre-determined threshold. For each mouse we measured three fields, each field being 639×538 pixels. Objects with diameters over 10 μm were categorized as perisomatic, and those with diameters less than 3 μm were classified as neuropil (Muly et

al., 2002) by the investigator visually. The number of objects in each category was plotted against time after noise exposure.

Co-localization imaging

Two series of 25 through focus, black and white images were taken from the same area in fluorescence labeled sections with the 40x water immersion lens as described before. These images were then processed using the Z-projection application of Image J and corrected for background and pseudocolored with red and green. From these two series of images two composite images were generated, one in red and one in green. They were then aligned, merged, and examined for co-localization. Three sets of red-and-green merged images were made for each time point. There are three replicates for almost all time points.

Statistical analysis

Data were treated by using the analysis of variance ANOVA in SPSS (Version 6, Chicago, IL). If ANOVA shows significant differences, the significant main effects and interaction were further analyzed using Duncan's post hoc multiple comparison test. $P < 0.05$ is considered significantly different.

RESULTS

Effect of a single episode of noise exposure to the cochlea

A single exposure to noise in this study caused pronounced damage to the cochlea. This damage is specific and reproducible across all the animals and across the different time points tested after exposure. In addition, the degree of cochlear damage, as expressed by the % of missing cells and nerve fibers, did not increase significantly over the period of observation. In Figure 2 we show the averaged cochleograms from animals at one, two, four and eight weeks after noise exposure.

In the damaged cochlea, outer hair cells (OHCs) were absent from the entire length of the basilar membrane, while inner hair cells (IHCs) and myelinated nerve fibers (MNFs) were missing in different degrees from a restricted region. In the cochleograms (Fig. 2), the whole length of the basilar membrane is divided into percentiles and plotted on the X axis. This axis extends from the low frequency origin at the apex to the 100th percentile at the high frequency base. The correlation between the percentage distance from the apex and the frequency in kHz is listed in the table (right panel, bottom).

The damaged IHCs and MNFs occupied the region from around the 30th to the 85th percentile, corresponding to a frequency range from a little below 6 kHz to 50 kHz. The mouse has a hearing range from 3 – 100 kHz (Bohne BA, 2001). The data in Figure 2 supports our conclusion that the lesion is a specific response to the frequency components of the noise.

The cochlear damage appears at least as early as one week after noise exposure (Fig.2). Although the loss of both IHC and MNF increased somewhat by 8 weeks (Fig. 2, right panel), no two groups are statistically significant from each other (ANOVA: $p \leq 0.83$, $F = 0.285$, $df = 3$). Control mice (0 week), exposed only to ambient background noise, exhibited little, if any, loss of OHCs, IHCs and MNFs (data not shown).

The on-going degeneration in CN measured by volume changes

The various compartments in the CN, the first central nucleus in the auditory pathway, differ in their responses to noise damage. This difference is determined by their anatomical connections with the cochlea, specifically, with the distribution of the eighth nerve and the

intrinsic synaptic connections between those compartments. In the current study, we selected the dorsal portion of the anterior posteroventral CN (PVCN-A) for analysis. This area is one of the major targets innervated by the damaged region of the cochlea. Moreover we have learned from our previous studies that it provides a reliable site for investigating plastic changes in the CN (Kim et al., 2004c; D'Sa et al., 2007).

Figure 3 shows the region of interest. The left panel is a montage of images taken from one section; the borders of the individual images are indicated by asterisks. The right panel is from an atlas at a comparable level (Trettle and Morest, 2001). The section shown was cut through both DCN and PVCN. Only the parts used for our analysis are labeled in the montage, namely, the fusiform layer (F) and polymorphic layer (P) in DCN and (A) as PVCN-A. The DCN provides a control for the degeneration changes in PVCN-A, and the cerebellum (C) provides an internal control for staining intensity. The three small squares are examples of the 50 μm^2 sample areas chosen randomly for analysis. The grid for sampling is shown in the atlas over PVCN-A. All samples were taken from areas within the border of PVCN-A. For more details, see Methods.

Figure 4 illustrates volume change in the CN (see Methods) during the eight weeks after noise exposure. The average volume of PVCN-A did not change significantly through the fourth week after noise exposure. By the eighth week, however, the average volume decreased by 41% (ANOVA, $p = 0.04$). Thus the degeneration progressively increased up to eight weeks at least.

The on-going degeneration in CN measured by optical density

Since the decrease in volume indicates degeneration in the CN, we sought to analyze the neural components involved in this change. For this we used immunostaining against synaptic vesicle protein SV2. Previous studies (Feng and Morest, 2006) have shown that antibodies to SV2 intensely stain both axodendritic (neuropil) and axosomatic (perisomatic) nerve terminals associated with healthy synapses. A significant loss of those terminals after noise damage should induce a dramatic decrease in the labeling intensity of the affected areas.

We used optical density (OD) measurements to quantify the change in SV2 expression in several CN subdivisions following noise exposure. The result is shown in Figure 5. The data were obtained by measuring the mean gray value of 50 μm^2 blocks contained entirely within the defined borders of the PVCN-A, as well as the fusiform layer and polymorphic layer of the DCN.

The SV2 result was similar to that of the volume change. There is a significant decrease (75%) in optical density by the eighth week (ANOVA: Duncan; $p < 0.05$). The difference in PVCN-A for one, two or four weeks is much less. There were no significant changes in either layer of the DCN even by the eighth week (Fig.5, lower panel).

The images in the upper panel of Figure 5 show typical examples of anti SV2 staining in the PVCN-A at different survival times. The heavily stained perisomatic terminals, representing the normal elements (ARROWS), decreased in number as the weeks progressed from 1 to 8. At the same time, there was an increase in the number of faintly stained neural elements (ARROWHEADS), representing the on-going degeneration.

The on-going degeneration in CN measured by cluster analysis

Since the decrease in SV2 staining is prominent, we evaluated the individual elements in the affected neural structures. For this purpose we used microscopic cluster analysis (Muly et al., 2002) to sort out the decrease in two types of presynaptic terminals – the axosomatic

(perisomatic) and the axodendritic (neuropil) elements. The former reflects predominantly the synaptic endings from the eighth nerve on the CN cell bodies, while the latter reflects a more diverse source of nerve fibers, including intrinsic connections between PVCN-A and other parts of the CN. The results are summarized in Figure 6.

The upper panel in Figure 6 shows a sample area of the cluster analysis taken from a normal control. The objects above a pre-determined threshold gray value (see Methods) were selected by the software. (For details, please see Methods).

There was a large decrease by 1 week for both types of terminals. From 2 weeks to 4 weeks there was fluctuation in the changes, which were significantly different between the two categories. Both neuropil and perisomatic staining exhibited significant decreases to 30% and 25% of their respective controls by eight weeks after noise exposure.

Over all, the changes in the terminals as suggested by the cluster analysis were more dynamic than those indicated by the volume and optical density analyses.

In sum, three methods of measurement – the volume change, the optical density, and the cluster analysis – all point to a significant on-going degeneration in the CN after a single episode of noise exposure, whereas in the cochlea the same exposure causes a more rapid degeneration. The degeneration of primary nerve fibers projecting into the CN, and probably secondary intrinsic fibers, took eight weeks to reach prominence. Thus, axonal endings of primary afferents in the CN continue to degenerate long after the MNF loss.

NT3 and degeneration in CN

A previous study suggested that the eighth nerve is an important vehicle for carrying the neurotrophic factor NT3 into the CN (Feng et al., 2010). NT3 may play an important role in maintaining the stability of adult synapses in the CN. In the present study, when there was a massive degeneration and loss of synapses, we expected to see a corresponding decrease in the concentration of NT3, especially in the cochlear nerve ending. This point is addressed by Figure 7, which shows post lesion staining with antibodies against SV2, NT3 and GLT1.

By the eighth week after noise exposure, the CN continued to show signs of degeneration. During the same time the staining intensity of SV2 (Fig. 7, left column, 8WK) and NT3 (Fig. 7, middle column, 8WK) also showed dramatic decreases. This is consistent with our suggestion that the eighth nerve is a major source of NT3 supplying cochlear nerve terminals in the ventral CN. This tendency is already shown in the first week. The degeneration, as shown by the decrease in SV2 staining, coincides with decreased NT3 staining (Fig. 7). We also saw some fluctuation in the staining of SV2 and NT3 during the second to fourth weeks, in much the same way as the results with cluster analysis (Fig 6, lower panel).

While degeneration was taking place in the CN, there was a proliferation of glia and their processes. GLT1 immunostaining has proved to be a better marker for astrocytic processes than glial fibrillary acidic protein (see Walz, 2000; Josephson and Morest, 2003). GLT1 was prominent in the first week and continued to be so at the subsequent time points. This is shown in the immunostaining of astrocytic processes by anti-GLT1 (Fig. 7, right column).

Astrocytes and NT3 in degeneration

The change in NT3 after degeneration was further explored by a double-labeling study to show the cellular location of NT3 in control and degenerated CN. The result is shown in Figure 8.

The upper panels in Figure 8 show the double-labeling of NT3 (green) and SV2 (red). Yellow indicates co-localization of NT3 in nerve terminals. Prominent green and yellow staining in the control suggests that NT3 was abundant in the nerve terminals, especially in the perisomatic endings. By the eighth week, both green and yellow have diminished in their intensity. This suggests that the terminals had degenerated and lost their NT3. This loss was already prominent by the first week when there was little green or yellow staining (Fig. 8, upper panel).

The lower panel shows the double labeling of NT3 (green) and GLT1 (red), and co-localization (yellow). In the control animal, there was a certain level of NT3 present in the astrocytic processes, especially those around the neuronal cell body (Fig. 8, WHITE ARROW).

There was a prominent increase in the staining for astrocytes one week after noise exposure which is consistent with proliferation of astrocytes (see the increase in red). This coincides with a massive degeneration that occurred by the first week (Fig. 8, upper panel). This proliferation of glia can explain the small increase in the volume of PVCN-A shown in Fig. 4. The massive degeneration at week 1 is supported by the optical density decrease of PVCN-A in Fig. 5 and the significant loss of clusters in Fig. 6 at WK1. In Fig. 7, a decrease in the staining of SV2 and an increase in the staining of GLT-1 are consistent with the degeneration of nerve terminals and proliferation of astrocytes. However, there was little, if any, increase of NT3 immunostaining in the proliferating astrocytic processes (Fig. 8). Thus it appeared that the astrocytes did not respond to the noise damage by making more NT3, at least not by the first week. There is a pattern of fluctuation in the presence of NT3 in astrocytic processes (Fig. 8, lower panel), which is similar to the temporal pattern seen in the cluster analysis of the endings (Fig. 6). There is an increase by the fourth week, followed by a pronounced decrease. The possibility that these findings reflect a shift in the production or transport of NT3 in astrocytic processes will be discussed below.

DISCUSSION

We have created a mouse model for studying the effects of noise damage on the CN. We found degeneration and reorganization of nerve terminals in PVCN-A linked to the distribution of NT3. We also showed a possible connection between NT3 and astrocytes. We suggest that there is a compensatory process in the supporting glial cells during the reorganization of nerve terminals. The pathological changes in PVCN-A may be implicated in the generation of tinnitus.

The effect of noise occurs very quickly. In the cochlea all the OHCs are gone by the first week, and prominent damage occurs in both the IHCs and the MNFs; the latter presumably correspond to the loss of the spiral ganglion neurons (SGCs). The total number of IHCs did not seem to decrease significantly from one week to eight weeks, as shown by the statistical analysis (Fig. 2). The damage to IHCs, defined by a loss greater than 10%, showed a correlation with the noise spectrum used in the exposure (Fig. 2, bottom of left panel.)

Further analysis shows a correlation between the damage of IHCs and the degeneration of MNFs. In Figure 2, we use a line to indicate the high frequency edge at which 50% of IHCs were damaged. The % of the degeneration of MNFs at the same frequency and at the same time point is much lower for both one-week and two-week animals compared to the four-week and eight-week ones. This suggests that there is an ongoing deterioration in the cochlea.

In the PVCN-A, the volume change, the OD analysis, and the microscopic cluster analysis showed that the fiber degeneration continued to increase by the eighth week, the terminal

point of our measurements. This is a statistically significant change compared to the control and previous weeks, also compared to the adjacent region in DCN.

The OD analysis measures the presence of all the nerve terminals. One could ask whether degeneration and sprouting could have cancelled each other. Cluster analysis selectively measures two groups of terminals – one larger than 10 μm and another less than 3 μm . This analysis does not include all the nerve terminals, since it leaves out the intermediate group of terminals. However, it does reveal the changes in terminals from specific sources – perisomatic and neuropil. These circumstances could explain the apparent differences in the level of degeneration at one week seen in Figs. 5 and 6. At this point one could only speculate about which types of axons belong to the intermediate group. Although the % decrease of the perisomatic endings is large, their absolute numbers are much less than those of the neuropil (Fig. 6B.) Therefore this would not be reflected in the OD analysis. (Fig. 5).

Cluster analysis showed fluctuation in the number of nerve endings before the eighth week, the end point of our measurement. This fluctuation is presumably due to the interplay between degeneration and collateral sprouting. Although this fluctuation occurs for both neuropil and perisomatic endings, their pattern is different. The difference could be explained by the different sources of the two types of endings. The perisomatic cluster represents the primary nerve fibers, those coming directly from the SGCs, while the neuropil cluster also receives endings from other sources, e.g., other areas of the CN, the tegmental nuclei and commissural pathways (see Subramani et al., 2004).

What does the fluctuation represent? One possibility is that some cochlear nerve endings withdraw initially, as suggested in the chinchilla (Muly et al., 2002), and then attempt to grow back but eventually fail when the SGCs die. The other possibility is that the collaterals sprouting from surviving cochlear nerve fibers form tentative contacts with denervated somas initially; they finally die when they do not receive enough trophic support from appropriate postsynaptic sources, including the supply of NT3 or contacts with astrocytes. Cluster analysis suggests that the large and specific structure of endbulbs could arise from the sprouting collaterals, at least temporarily.

If the hyperactivity in DCN induces tinnitus after noise damage (e.g., Kaltenbach et al., 2005), this hyperactivity may be related to the degeneration in the VCN. A number of pathways interconnect the DCN with the PVCN (Morest, 1993), including collaterals of giant cells from the PVCN and projections from granule cell axons arising in the DCN. These could affect the activity levels in the DCN. Thus plastic changes in PVCN-A may provide a structural basis underlying this syndrome.

Our immunofluorescent data are consistent with our previous suggestion that the cochlear nerve is a major source of NT3 supplying the nerve terminals in the ventral CN (Feng et al., 2010). By the first week the degeneration accompanies a loss of NT3 (Fig. 8, upper panel). By the eighth week the degeneration continues to increase (Fig. 5, lower panel), and NT3 undergoes a dramatic decrease (Fig. 7, middle panel).

Figure 8 shows a connection between astrocytes and NT3 (lower panel). By the first week there was a robust growth of astrocytic processes (shown by an increase in red), and a loss of NT3 in the terminals (decrease in yellow). This suggests at least by the first week, NT3 was not transferred to, nor was it synthesized by the surrounding astrocytes in any significant amount. By the fourth week, there was an increase of NT3 in the astrocytic processes (increase in yellow and red), preceding the overall loss by the eighth week. This pattern of fluctuation of NT3 in astrocytic processes suggests that the latter may become a source of NT3 at the later stage of degeneration. This may represent a compensatory mechanism after the CN trauma.

Future research should address the use of glial cells and neurotrophic factors to rescue the traumatized neurons and endings in the noise-damaged VCN. For example, NT3 has proved to be useful in promoting axonal sprouting after spinal cord injury (Taylor et al., 2007). The present findings would also provide guidance in designing transplantation and stem cell experiments.

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Abbreviations

CN	cochlear nucleus
VCN	ventral CN
PVCN	posteroventral CN
DCN	dorsal CN
IC	inferior colliculus
IHC	inner hair cell
OHC	outer hair cell
MNF	myelinated nerve fiber
SGC	spiral ganglion cell

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Fig. 1.

The parameters of the stimulus. UPPER PANEL: Noise profile as recorded on a CD. The high frequency cut off is around 20 kHz. LOWER PANEL: The frequency response curve of the speaker.

Fig.2.

Cochleograms of noise exposed mice show loss of inner hair cells and myelinated nerve fibers along the length of the cochlea at different survival times. Graphs show the average value from each group of mice (1, 2, 8 weeks: n=3 each; 4 weeks: n=2). Percent damage vs percent distance from apex to base appears in 5% increments, from low to high frequency. The correlation between frequency and percent distance from apex is at the bottom of the right column. The calculation uses the equation, $X = 68.0 \log (F + 3550) - 239.7$. X is the percent distance from the apex, F is the corresponding frequency at that point of the basilar membrane (Ou et al, 2000). The peak of each curve overlaps with the 100% bar line. The damage distribution for inner hair cells is in the bottom panel of the left column. Any loss above 10% is one event. The curve shows the number of events vs. percent distance from apex. There are eleven mice, and the largest number of events is eleven. All inner hair cell loss occurs within a distance of 35-60% from the apex.

Fig.3. Subdivisions of CN and the sampling grid in an SV2 stained section. **LEFT PANEL:** Reconstruction in montage of CN and its neighboring cerebellum. The * show the borders between the individual images in the montage. The small squares typify the $50\ \mu\text{m}^2$ areas in the optical density and microscopic cluster analysis. **A**, PVCN-A; **F** and **P**, fusiform and polymorphic layers in DCN; **C**, cerebellum. **RIGHT PANEL:** Drawing of an atlas section (Trettle and Morest, 2001) at a comparable level. The grid covers PVCN-A. Scale: $200\ \mu\text{m}$ (left), $175\ \mu\text{m}$ (right).

Fig.4.

Volume change in PVCN-A (mean ± SEM) after noise exposure. By 8 weeks statistically significant changes occur (*, Duncan; $p < 0.05$). Mice used: control, 1 and 8 weeks, $n = 3$ each; 2 and 4 weeks, $n = 2$ each.

Fig.5. Landscape presentation of optical density analysis of SV2 staining intensity. UPPER PANEL: intensely stained perisomatic nerve terminals (SMALL ARROWS) decrease from control to 8 weeks; degenerating terminals (LARGE ARROWS) increase from 1 to 8 weeks. Scale: 20 μ m. LOWER PANEL: percent optical density (mean $\pm$ SEM) normalized to the cerebellum. At each time point the data were collected from 3-4 randomly chosen 50 μ m² blocks. There is significant change (*) in PVCN-A at 8 weeks after noise (Duncan: $p < 0.05$). No significant changes present themselves in DCN. Mice used: control, 1 and 2 weeks, $n = 3$ each; 4 and 8 weeks, $n = 4$ each.

Fig. 6. Microscopic cluster analysis of SV2 stained objects in PVCN-A. UPPER PANEL: representative field from a control. The thin white lines identify the numbered objects. LOWER PANEL: Average number of SV2 immunopositive clusters show significant fluctuations over time. Neuropil endings: A significant decrease by one week, followed by an apparent but partial recovery by two weeks, followed by a continuing decline up to eight weeks. Perisomatic endings: Significant decrease by one week, recovery by two weeks and significant increase by four weeks, followed by a decline to values significantly less than all the previous time points by eight weeks. The numbers below each bar indicate significant differences between time points. * Duncan, $p < 0.05$. Mice used: control, 2 weeks and 4 weeks, $n = 3$ each; 1 week and 8 weeks, $n = 4$ each.

Fig. 7. Immunofluorescent imaging of SV2, NT3 and GLT1 in PVCN-A of control and noise exposed mice at 1, 2, 4, and 8 weeks. By 8 weeks SV2 and NT3 reached the lowest level, as degeneration attained its peak. For other changes see Results. Scale: 20 μ m.

Fig. 8.

Landscape presentation of immunofluorescent double labeling in PVCN-A of control and noise exposed mice at 1, 2, 4, and 8 weeks. UPPER PANEL: NT3 (green) with SV2 (red); yellow is co-localization. By the eighth week the decrease in green and yellow suggests degeneration of terminals and loss of their NT3. This loss was already prominent by the first week and showed fluctuation in the second and fourth weeks. LOWER PANEL: NT3 (green), GLT1 (red), co-localization (yellow). A fluctuating pattern of NT3 in astrocytic processes appears. The arrowhead in CON indicates a site of double labeling for NT3 in astrocytic processes. For details see Results. Scale: 20 μ m.