Cochlear Damage Changes the Distribution of Vesicular Glutamate Transporters Associated with Auditory and Nonauditory Inputs to the Cochlear Nucleus

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Integration of multimodal information is essential for understanding complex environments. In the auditory system, multisensory integration first occurs in the cochlear nucleus (CN), where auditory nerve and somatosensory pathways converge (Shore, 2005). A unique feature of multisensory neurons is their propensity to receive cross-modal compensation after deafening. Based on our findings that the vesicular glutamate transporters, VGLUT1 and VGLUT2, are differentially associated with auditory nerve and somatosensory inputs to the CN, respectively (Zhou et al., 2007), we examined their relative distributions after unilateral deafening. After unilateral intracochlear injections of kanamycin (1 and 2 weeks), VGLUT1 immunoreactivity (ir) in the magnocellular CN ipsilateral to the cochlear damage was significantly decreased, whereas VGLUT2-ir in regions that receive nonauditory input was significantly increased 2 weeks after deafening. The pathway-specific amplification of VGLUT2 expression in the CN suggests that, in compensatory response to deafening, the nonauditory influence on CN is significantly enhanced. One undesirable consequence of enhanced glutamatergic inputs could be the increased spontaneous rates in CN neurons that occur after hearing loss and that have been proposed as correlates of the phantom auditory sensations commonly called tinnitus.

Introduction

Vesicular glutamate transporters (VGLUTs) package glutamate into synaptic vesicles and serve as excellent markers of glutamatergic projections. Although the homologous subtypes, VGLUT1 and VGLUT2, accumulate glutamate with similar transport kinetics, their distributions in the brain are distinct, with VGLUT1 more prominent in the cerebral and cerebellar cortices and hippocampus and VGLUT2 predominantly expressed in the thalamus, deep cerebellar nuclei, and brainstem regions including the cochlear nucleus (CN) (Freneau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Kaneko et al., 2002).

In the CN, VGLUT1 and VGLUT2 are distinctly associated with synaptic terminals of auditory nerve fibers (ANF) and brainstem somatosensory nuclei, respectively. VGLUT1 expression is intense in the core region of ventral cochlear nucleus (VCN) and deep dorsal cochlear nucleus (DCN), where ANFs terminate on principal projection neurons, whereas VGLUT2 expression is stronger in the granule cell domain (GCD), which receives most of the nonauditory inputs to the CN (Wright and Ryugo, 1996; Ohlrogge et al., 2001; Zhou and Shore, 2004, 2006b; Haengeli et

al., 2005; Zhan et al., 2006; Zhan and Ryugo, 2007; Zhou et al., 2007). Furthermore, terminal endings originating in the spinal trigeminal nucleus (Sp5), cuneate nucleus, and lateral reticular nucleus colabel with VGLUT2 and not VGLUT1 (Zhou et al., 2007; Cui and Shore, 2008). In contrast, type I ANF terminals colabel with VGLUT1 but not VGLUT2. Type II ANFs (Benson and Brown, 2004) do not label with either VGLUT1 or VGLUT2 (Zhou et al., 2007). The VGLUT2-positive nonauditory endings activate granule cells, which project to the principal cells in the DCN, where they are combined with VGLUT1-positive ANF inputs, resulting in multisensory integration (Shore, 2005).

One feature unique to multisensory neurons is their propensity for receiving cross-modal compensation after sensory deprivation: In visually deprived mammals, auditory input from the inferior colliculus and auditory thalamus is redirected to the visual thalamus and cortex (Batzri-Izraeli et al., 1990; Izraeli et al., 2002; Piche et al., 2007). Similarly, the anterior ectosylvian region of the cat's parietal association cortex, which receives visual, auditory, and somatosensory inputs (Dehner et al., 2004; Meredith et al., 2006), becomes dominated by auditory and somatosensory inputs after visual deprivation and results in superior sound-localizing abilities (Rauschecker, 1995). Deafness can also result in an infiltration of primary auditory cortex by neurons responding to somatosensory and visual stimuli (Hunt et al., 2006). Thus far, such cross-modal reorganization after deafness has not been demonstrated at peripheral levels of the auditory system. Here, we study cross-modal compensation in the CN by examining changes in the differential distributions of VGLUT isoforms associated with nonauditory (VGLUT2) and ANF (VGLUT1) pro-

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jections to the CN. Because VGLUT immunoreactivity (ir) is correlated with quantal release of glutamate (Wilson et al., 2005; Moechars et al., 2006), the degree of change in VGLUT-ir should be an indication of changes in the amount of glutamatergic transmission from auditory and nonauditory inputs.

Materials and Methods

Thirteen pigmented guinea pigs (300–400 g) with normal Preyer's reflexes were used in this study. Ten were unilaterally deafened and three were untreated controls. Five of the ten unilaterally deafened were allowed to survive for 1 week, and five were allowed to survive for 2 weeks after the deafening. For the unilaterally deafened animals, the contralateral and the ipsilateral CNs were compared with each other and to the normal controls ($N = 6$, using both sides). All procedures were performed in accordance with the National Institutes of Health (NIH) *Guidelines for the Use and Care of Laboratory Animals* (NIH publication No. 80–23) and guidelines provided by the University Committee on Use and Care of Animals of the University of Michigan.

Deafening. The 10 animals to be unilaterally deafened were anesthetized with intramuscular injections of ketamine hydrochloride (Ketaset; 40 mg/kg) and xylazine (Rompun; 10 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments). Rectal temperature was maintained at $38 \pm 0.5^\circ\text{C}$ with a thermostatically controlled heating pad. Using a post-auditory approach, a small incision was made behind the left ear and the skin retracted to expose the bulla. A small hole in the bulla was made and kanamycin (20 μl , 30% in normal saline) was injected into the cochlea using a Hamilton syringe, through the round window. After the injection, the skin overlying the bulla was sutured, and the animal was allowed to recover. After 1 week (five animals) or 2 weeks (five animals), auditory thresholds were tested using auditory brainstem responses (ABRs), and the animals were killed.

ABR measurements. ABRs were performed in deafened animals before deafening, and 1 or 2 weeks after deafening on the day of killing. Normal animals received ABRs for both ears. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems). Animals were anesthetized with 58.8 mg/kg ketamine, 2.4 mg/kg xylazine, and 1.2 mg/kg acepromazine (Unit for Animal Laboratory Medicine, University of Michigan), and body temperature was maintained with thermostatically controlled heating pads. Subdermal recording electrodes were placed at vertex (1 cm posterior to bregma), reference (ventral to the pinna on the tested ear), and ground (ventral to the pinna on the contralateral ear) sites.

Digital signals were generated and delivered to the Tucker Davis Technologies (TDT) hardware by a Pentium PC, using the TDT software package SigPlay32. Stimuli were generated using a sample rate of 100 kHz at 16-bit resolution. Equalization to correct for the system response was performed on the digital waveforms in the frequency domain. Acoustic stimuli (15 ms tone bursts, with 1.5 ms rise/fall times), were presented at 10 per second. TDT System II hardware and SigGen/Biosig software (TDT) were used to present the stimulus and record responses. Tones (4, 10, and 20 kHz) were delivered through a Beyer driver (Beyer Dynamic; aluminum-shielded enclosure made in house), using a speculum placed just inside the tragus. Up to 1024 responses were averaged for each stimulus level. Responses were collected for stimulus levels in 10 dB steps at higher stimulus levels, with additional 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus level where a response was observed and 5 dB lower where no response was observed.

After final ABR measurements, the animals were killed with Nembutal (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. After perfusion fixation, the brain and cochleae were extracted and placed in the same fixative for 2 h at 4°C . The brain was then transferred to 20% sucrose in 0.1 M PBS overnight at 4°C . The brainstem was sectioned on a freezing microtome at a thickness of 40 μm . The cochleae were processed for hair cell counts (see below).

Cochlear hair cell and spiral ganglion assessments. Two animals (one at 1 week and one at 2 weeks after deafening) were processed for cochlear hair cell counts, and eight animals (four 1 week and four 2 week deaf-

ened) were processed for spiral ganglion counts. Left (deafened) and right sides were compared.

Cochlear hair cell assessments. After the perfusions, the temporal bones were immediately removed, the round and oval windows exposed, and the cochleae fixed with intrascalar infusion of 4% paraformaldehyde in PBS through the round window. After fixation, cochleae were microdissected and stained with 1% rhodamine phalloidin. Surface preparations of the cochlear spiral were prepared, and individual turns of the organ of Corti were mounted on glass slides. Surface preparation assessment was performed under epifluorescent illumination on a Leitz photomicroscope. Surviving hair cells were counted in 0.19 mm reticules and plotted as cytochleograms [percentage of outer hair cell (OHC) loss, relative to normal hearing ears] using a Microsoft Excel program kindly provided by Dr. David Moody (University of Michigan, Ann Arbor, MI). Raw data for each row of OHC were collected and assessed individually. In addition, data for the three rows of OHC were collapsed together and analyzed as a group.

Cell density of spiral ganglion cells. Every third midmodiolar section was mounted on a glass slide until 15 sections had been mounted and a random start point generated; the six sections from that point were used to generate an "analysis length" of 72 μm . Six Rosenthal's canal profiles, visible on each section (two from the basal cochlea, two from the middle turn, and two from the apical turn), were assessed for each toluidine blue/basic fuchsin-stained section by an observer blind to treatment condition. Spiral ganglion cells were counted as normal if they met the following criteria: (1) had a diameter of 14–22 μm and an area of 153–380 μm^2 ; (2) and had a nucleus, with a diameter of 5–10 μm . The outline of each profile of Rosenthal's canal was traced using a computer mouse. Cell count and cell area were then used to calculate cell density. All measurements were calculated using the MetaMorph Image Acquisition, Enhancement, and Analysis System (Molecular Devices).

Brain tissue and immunocytochemistry processing. Transverse sections of the brainstem were mounted in serial order on clean glass slides and air dried. Alternate serial sections were used to label VGLUT1 and VGLUT2.

All tissue processing was done at room temperature (20 – 22°C) unless otherwise stated. The anti-VGLUT1 polyclonal antibody was generated in rabbits against Strep-Tag fusion protein containing amino acid residues 456–560 of rat VGLUT1 (Synaptic Systems; Cat. #135 303), and the anti-VGLUT2 polyclonal antibody was generated in rabbits against Strep-Tag fusion protein containing amino acid residues 510–582 of rat VGLUT2 (Synaptic Systems; Cat. #135 403). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen) and Cy3-conjugated goat anti-mouse antibody (Jackson Immunological Research Laboratories; code 115-165-146). All primary and secondary antibodies were diluted in the same blocking solution containing 1% normal goat serum (Jackson ImmunoResearch Laboratories; 005-000-121) in 0.1 M phosphate buffer with 0.1% Triton X-100 (Sigma; 9002-93-1), pH 7.4. The sections were incubated in the blocking solution for 30 min, followed by overnight incubation with primary antibodies (1:1000 for both VGLUT1 and VGLUT2). After thoroughly rinsing in PBS, sections were reacted for 2 h with the secondary antibody. Sections were then rinsed, dehydrated in graded ethanol, and coverslipped with Microcover (Micron). Negative controls were conducted on sections that were not treated with either primary or secondary antibodies, resulting in no immunolabeling. VGLUT antibodies were preincubated with corresponding synthetic peptides, resulting in negative immunolabeling. (Strep-Tag fusion protein containing amino acid residues 456–560 of rat VGLUT1, Cat. #135-3P, and Strep-Tag fusion protein containing amino acid residues 510–582 of rat VGLUT2, Cat. #135-4P; Synaptic Systems). Western blots performed previously (Zhou et al., 2007) demonstrated anti-VGLUT1 antibody with single band at ~ 60 kDa on protein extracted from both CN and cerebellum and anti-VGLUT2 antibody as a single band at ~ 65 kDa, corresponding to the molecular weights predicted for VGLUT1 and VGLUT2, respectively. Positive controls for VGLUT-ir were performed in the cerebellar cortex (Takamori et al., 2001; Kaneko et al., 2002; Hioki et al., 2003).

Image processing. Sections were examined using a fluorescent microscope equipped with the appropriate filters for rhodamine and Alexa Fluor 488 (Leica). Photomicrographs of immunolabeling were digitized

and imported to MetaMorph for quantification of immunolabeling. The parameters for digitizing photomicrographs were determined in a pilot study with optimal contrast between labeling and background and were then kept constant across conditions and animals. Photomicrographs were imported to Adobe Photoshop for contrast adjustment that was consistent for all sections.

Analysis of VGLUT-ir was conducted for all subdivisions of the CN, with emphasis on the following regions: regions receiving primarily non-auditory inputs, i.e., the GCD, which includes the Shell region and the second layer of DCN, the fusiform cell layer (DCN2), and contains numerous small cells including granule cells (Weedman et al., 1996; Wright and Ryugo, 1996; Alibardi, 2004), and the deep DCN–DCN3, which contains some nonauditory terminals.

Regions that receive primarily type 1 ANF input include the antero-ventral cochlear nucleus (AVCN) and posteroventral cochlear nucleus (PVCN) magnocellular areas and layer three of the DCN (AVCNm and PVCNm and DCN3). DCN1 (also called the molecular layer) is comprised mostly of the parallel fiber axons of granule cells but also contains some terminals from nonauditory regions (Zhou and Shore, 2004; Haenggeli et al., 2005).

Quantification and statistical analysis. Quantification was performed blind as to whether the tissue was from normal or deafened animals. The VGLUT-ir density was quantified in each of the following CN regions: DCN1, DCN2, and DCN3, the Shell region in the vicinity of the small cell cap, AVCNm, and PVCNm. The sections for AVCNm and PVCNm were taken from the midfrequency regions (i.e., halfway between the most dorsal aspect and the most ventral aspect of the VCN). This would, thereby, encompass only regions affected by the hearing loss (Fig. 1, high and midfrequency regions). For each side of each animal, three pictures (40 $\times$) were taken at equal intervals from caudal to rostral for each of the above selected regions except DCN1 (i.e., one picture from the 25th percentile, one from the 50th percentile, and one from the 75th percentile). For DCN1, a higher magnification (63 $\times$) was used to take three pictures evenly from caudal to rostral, leaving a total of 18 pictures per side in each animal. The photomicrographs were then transferred to MetaMorph for automatic quantification. To ensure that puncta were counted reliably, visual inspections and manual corrections were always conducted after each automated (thresholded) counting. This was to ensure that puncta were not merged by the thresholding procedure. The number of VGLUT-labeled puncta was divided by the chosen area to yield the puncta density. The chosen areas were consistent in size and position across animals. No distinction was made between puncta on cell bodies and puncta on dendrites or in the neuropil. Means and SEs of the mean were calculated for the puncta density of VGLUT1 and VGLUT2-ir. The deafened and contralateral sides ($N = 5$, 1 week; $N = 5$, 2 weeks) were compared with the normal controls ($N = 6$). A mixed effects, repeated-measures ANOVA was conducted with groups (normal, 1 week, 2 weeks) as a between-subjects factor and side (ipsilateral, contralateral), using the SPSS statistical package, region of the cochlear nucleus (DCN1, DCN2, DCN3, Shell, AVCN, PVCN), and section (caudal, medial, rostral) as within-subject factors.

Results

Unilateral injections of kanamycin resulted in profound, unilateral hearing loss

All 10 animals with unilateral intracochlear injections of kanamycin had profound, unilateral hearing losses as demonstrated with ABR measurements performed before killing (Fig. 2A). No ABR responses were measurable at 90 dB sound pressure level (the limits of our sound system) in the deafened ears. Figure 2A shows the mean ABR thresholds for these animals at three test frequencies (4, 10, and 20 kHz) for the left (ipsilateral, deafened) and right (contralateral) ears, as well as six normal control ears in three normal hearing animals.

Cytocochleograms show major inner and outer hair cell loss

Figure 2B shows an example of a typical cytocochleogram obtained for the ipsilateral and contralateral ear in one animal taken

Figure 1. Schematic of the locations within the CN that were used for measurements and micrographs. Each region of interest is indicated with square. **A–C**, Drawings of transverse sections from the rostral to caudal ends of CN. icp, Inferior cerebellar peduncle; tz, trapezoid body; 7n, facial nerve.

at 1 week after kanamycin injection. The cytocochleogram shows the percentage of missing inner and outer hair cells along the length of the cochlea. The deafened animal's ipsilateral cochlea (1 week) showed complete hair cell loss (inner and outer) for the basal half of the cochlea and severe inner and outer hair cell loss for the remaining 20% of the cochlea, excluding the apex. There was hair cell preservation in the apical 10% of the cochlea. This pattern of loss was also evident at 2 weeks after the injection. Such severe hair cell loss would be expected to result in the profound hearing losses exhibited using ABRs (Fig. 2A).

Spiral ganglion density is significantly reduced 2 weeks after deafening

Figure 2C shows the mean spiral ganglion densities for the whole cochlea as well as the basal turn at 1 and 2 weeks after deafness. The left side for the whole cochlea ($F = 221.24$; $df = 1, 28$; $p < 0.001$) and the basal turn ($F = 140.36$; $df = 1, 40$; $p < 0.001$) shows decreased spiral ganglion density when compared with the right at both 1 and 2 weeks, additionally the decrease at 2 weeks is greater than at 1 week for the whole cochlea ($F = 37.59$; $df = 1, 28$; $p < 0.001$) and the basal turn ($F = 20.01$; $df = 1, 40$; $p < 0.001$). Spiral ganglion density for the undeafened ear (right) was not significantly different between the 1 and 2 week groups ($p > 0.719$ for both whole and basal turn only) and is similar to values reported for normal animals (Miller et al., 2007).

Figure 2. *A*, Mean ABR thresholds for five animals 1 ($N = 5$) and 2 ($N = 4$) weeks after deafening by an injection of kanamycin into the left cochlea. ABRs are shown before (left and right) and after (left and right) deafening. Three normal hearing, control animals (left and right ears) are also shown for comparison. ABRs indicate profound deafness at both 1 and 2 weeks after kanamycin injection. No effect of the kanamycin injections was observed in any of the right ears. Closed square symbols at 90 dB for 4 and 20 kHz indicate no response at the limits of the speaker (indicated by upward arrows). *B*, A representative cytochrome C histogram is shown from one guinea pig 1 week after a kanamycin injection into the left ear. The percentage of missing inner and outer hair cells is indicated along the length of the cochlea. The deafened cochlea showed complete hair cell loss (inner and outer) in the basal half of the cochlea and severe inner and outer hair cell loss in the remaining 20% of the cochlea, excluding the apex. There was hair cell preservation only in the apical 10% of the cochlea. The yellow line shows the extent of hair cell loss in the normal (contralateral) ear. *C*, Spiral ganglion counts (SGC) are shown for eight animals, 1 ($N = 4$) and 2 ($N = 4$) weeks after kanamycin injections into the cochlea. Mean spiral ganglion densities for the whole cochlea and the basal turn at 1 and 2 weeks after cochlear damage are shown. Filled asterisks indicate significant differences of $p < 0.01$.

VGLUT expression is significantly altered in the cochlear nuclei ipsilateral to unilateral deafening

VGLUT1-ir density, primarily representing VIIIth nerve synaptic terminals in CN, is decreased after unilateral deafness

Figure 3, *A* and *C*, shows photomicrographs of decreased VGLUT1-ir density in the ipsilateral AVCNm and PVCNm in one animal (2 weeks after deafening) compared with the con-

tralateral side (Fig. 3*B,D*). The regions where the photomicrographs were taken are shown by arrows in Figure 1, *A* and *B*. The photomicrographs in Figure 3, *B* and *D*, show strong VGLUT1 labeling in the contralateral AVCNm and PVCNm, especially around cell bodies in this region, reflecting VIIIth nerve input. This pattern is similar in the normal control animals (Zhou et al., 2007). In contrast, sparse labeling is observed on the side ipsilateral to the deafening (Fig. 3*A,C*). For the deafened animals, VGLUT1-ir density was significantly decreased in magnocellular cell areas of VCN (VCNm; AVCNm and PVCNm) that receive VIIIth nerve input on the ipsilateral side compared with both the contralateral side and the normal CNs at both 1 and 2 weeks after deafness (Fig. 4). A mixed effects, repeated-measures ANOVA was conducted with groups (normal, 1 week, 2 weeks) as a between-subjects factor and side (ipsilateral, contralateral), region of the cochlear nucleus (DCN1, DCN2, DCN3, Shell, AVCN, PVCN), and section (caudal, medial, rostral) as within-subject factors. The main effects of side ($F = 68.30$; $df = 1, 13$; $p < 0.001$) and region ($F = 3605.28$; $df = 5, 65$; $p < 0.001$) as well as the interactions of side with region ($F = 64.47$; $df = 5, 65$; $p < 0.001$) and side with group ($F = 18.34$; $df = 2, 13$; $p < 0.001$) and side $\times$ region $\times$ group ($F = 17.63$; $df = 10, 65$; $p < 0.001$) were significant. The main effect of section was not significant, nor did it interact with any of the other factors indicating that the changes were relatively uniform across the rostral–caudal axis. The interaction of side with group and region was broken down into its simple effects, and Bonferroni adjusted comparisons were made as depicted in Figure 4. On the side, contralateral to the cochlear damage, there was no change in regulation of VGLUT1. On the side ipsilateral to the cochlear damage, no significant increase or decrease in VGLUT1 levels relative to normal is seen in the DCN2, DCN3, and Shell regions. However, the levels in the AVCN, PVCN, and DCN1 regions were significantly down for both 1 and 2 week groups after correction ($p < 0.003$) for those comparisons.

Note that DCN3 is also a recipient of nonauditory inputs (see below, “VGLUT2-ir, representing nonauditory synaptic terminals in CN (Zhou and Shore, 2006a), is increased after unilateral deafness”).

VGLUT2-ir, representing nonauditory synaptic terminals in CN (Zhou and Shore, 2006a), is increased after unilateral deafness

Figure 3 shows photomicrographs of increased VGLUT2-ir density in the ipsilateral Shell region of one animal (2 weeks after deafening) compared with the contralateral side (Fig. 3*E,F*). The region where the photomicrographs were taken is shown by an arrow in Figure 1*B*. Although VGLUT2 is intensely expressed in the Shell regions of all the animals, even on the contralateral side (Fig. 3*F*) as shown previously (Zhou et al., 2007), it is significantly increased above both contralateral and normal control levels at 2 weeks after deafening (Fig. 5). A mixed effects, repeated-measures ANOVA was conducted with groups (normal, 1 week, 2 weeks) as a between-subjects factor and side (ipsilateral, contralateral), region of the cochlear nucleus (DCN1, DCN2, DCN3, Shell, AVCN, PVCN), and section (caudal, medial, rostral) as within-subject factors. The main effects of side ($F = 21.37$; $df = 1, 13$; $p < 0.001$) and region ($F = 151.18$; $df = 5, 65$; $p < 0.001$) as well as the interactions of side with region ($F = 5.82$; $df = 5, 65$; $p < 0.001$) and side with group ($F = 6.56$; $df = 2, 13$; $p = 0.01$) were significant. Again, the main effect of section was not significant, nor did it interact with any of the other factors. The interaction of side with group and region was broken down into its simple effects, and Bonferroni adjusted compar-

sons were made as depicted in Figure 5. On the side contralateral to the cochlear damage, there appears to be a temporary downregulation of VGLUT2 in the DCN2, DCN3, and Shell regions [after Bonferroni adjustment only DCN3 ($p < 0.001$) retained significance], which recovers to normal levels by 2 weeks. On the side ipsilateral to the cochlear damage, no significant increase or decrease in VGLUT2 levels is seen at 1 week; however, by week 2, levels are significantly increased in the DCN2, DCN3, and Shell regions (all $p < 0.001$ after correction). The increase in the AVCN region failed to retain significance after correction.

Thus, VGLUT2-ir density, primarily reflecting nonauditory inputs to the CN, was thus significantly increased in regions previously shown to receive inputs from the Sp5, trigeminal ganglion, and other nonauditory nuclei (Weinberg and Rustioni, 1987; Shore et al., 2000; Zhou and Shore, 2004; Haenggeli et al., 2005; Shore and Zhou, 2006; Zhan et al., 2006). VGLUT2 puncta densities are significantly increased also in DCN3 ($p < 0.001$), which receives both VIIIth nerve and nonauditory inputs. Interestingly, VGLUT2 is increased in ipsilateral AVCNm in the same region where VGLUT1 is decreased at 1 and 2 weeks after deafness, although this was not significant after Bonferroni correction.

Changes in the relative distributions of VGLUT1 and VGLUT2 across the CN

To highlight the changes in the relative distributions of the two glutamate transporters, we replotted the data from Figures 4 and 5 for the deafened and normal ears as ratios. Figure 6 shows that, 2 weeks after deafening, the distributions of glutamatergic inputs become dominated by VGLUT2, signifying a major reorganization of projections from auditory nerve and nonauditory regions, including the spinal trigeminal nucleus, cuneate nucleus, and lateral reticular nucleus. Two weeks after deafness, there are decreases in VGLUT1 in all regions, but especially AVCNm and PVCNm, which receive major ANF inputs. In contrast, there are increases in VGLUT2 in DCN1, DCN2, the Shell, and DCN3 (predominant recipients of nonauditory inputs), as well as AVCNm and PVCNm, suggesting a broadening of areas receiving nonauditory inputs.

Discussion

Glutamatergic projections from nonauditory nuclei to the CN terminate in CN regions that are distinct from those that receive auditory nerve synapses, i.e., primarily the Shell regions of CN and deep DCN (i.e., Shell, DCN2, DCN3) (Zhou and Shore, 2004; Haenggeli et al., 2005; Zhan et al., 2006; Zhou et al., 2007).

Figure 3. VGLUT1-ir is decreased, whereas VGLUT2 is increased in the CN ipsilateral to the cochlear damage. **A–D**, Photomicrographs of VGLUT1-ir from the VCNm of one animal (2 weeks after deafening). **A**, **B**, VGLUT1-ir in the AVCNm. VGLUT1 is strongly expressed in AVCNm on the control side (**B**) as previously shown (Zhou et al., 2007) but weakly expressed after deafening (**A**). **C**, **D**, VGLUT1-ir in the PVCNm. VGLUT1 is sparsely expressed in ipsilateral PVCNm compared with contralateral side. The decrease in VGLUT1-ir reflects primarily decreased VIIIth nerve synaptic inputs after kanamycin injections into the cochlea. **E**, **F**, Photomicrographs of VGLUT2-ir from SHELL regions of CN. VGLUT2-ir in the ipsilateral SHELL region is increased compared with the contralateral side. Scale bars: **A–D**, 50 μ m; **E**, **F**, 20 μ m.

In contrast, type I VIIIth nerve fibers synapse on the cell bodies and dendrites of principal cells in the magnocellular regions of CN and some small cells in the cell cap (VCNm, DCNm) (Hackney et al., 1990; Snyder and Leake, 1997; Tsuji and Liberman, 1997). Type II ANFs project primarily to the dendrites of small neurons in the region of the small cell cap, but it is not yet clear whether they make synaptic contacts with granule cells (Benson and Brown, 2004), nor do they label with VGLUT1 or VGLUT2 (Zhou et al., 2007). Type II ANFs also project to cell bodies of multipolar or bushy cells, the targets of type I ANFs.

Figure 4. Mean VGLUT1 puncta densities in each CN subdivision in the ipsilateral, contralateral, and normal control CNs ($N = 6$), 1 ($N = 5$) and 2 weeks ($N = 5$) after deafness. VGLUT1 puncta densities (expressed as thousands per square millimeters) are significantly decreased in the ipsilateral (compared with the contralateral and normal CNs) AVCNm, PVCNm, and DCN1 compared with both the contralateral and normal control CNs ($p < 0.0003$, open stars). The magnocellular VCN receives major VIIIth nerve synaptic terminals.

Figure 5. Mean VGLUT2 puncta densities in each CN subdivision, ipsilateral, contralateral, and normal control CNs ($N = 6$), 1 ($N = 5$) and 2 weeks ($N = 5$) after deafness. VGLUT2 puncta densities at 2 weeks are significantly increased in the ipsilateral (compared with the contralateral and normal CNs) Shell and DCN2 that receive nonauditory synaptic terminals. Filled stars indicate $p < 0.001$. VGLUT2 puncta densities are also significantly increased but to a lesser extent in DCN3, which receives both VIIIth nerve and nonauditory inputs. Error bars represent SEM.

Our observation that VGLUT1 is primarily (but not exclusively) associated with type I VIIIth nerve synapses, whereas VGLUT2 is associated almost exclusively with nonauditory inputs to the CN (Zhou et al., 2007), has enabled us to study changes in these inputs after cochlear damage. Unilateral cochlear kanamycin injections, which produced significant cochlear hair cell and spiral ganglion cell loss, accompanied by profound deafness, resulted in major, significant reductions in VGLUT1-ir in those regions receiving major VIIth nerve input (AVCNm and PVCNm, with a similar trend in DCN3) 1 week and 2 weeks after deafness. Interestingly, there was no

VGLUT1-ir reduction in the Shell region, suggesting that the type I VIIIth nerve input to the Shell region (Tsuji and Liberman, 1997) is less affected by the cochlear damage. In agreement with our hypothesis that loss of VIIIth nerve fibers through deafening would cause cross-modal reinnervation of the cochlear nucleus, VGLUT2-ir was significantly increased in regions that receive nonauditory inputs (DCN2, DCN3, and Shell regions). These increases were increasingly evident at 2 weeks after deafness, suggesting the compensation is not immediate and may be the result of axonal sprouting (Boulland et al., 2007). This time line also correlates with the greater loss of spiral ganglion cells at 2 weeks after deafness.

Although it is well documented that cochlear deafferentation causes a decrease in the synaptic input from the VIIIth nerve to CN neurons (Muly et al., 2002; D'Sa et al., 2007), this is the first study to report potential increased synaptic inputs to the cochlear nucleus from nonauditory regions after cochlear damage. The increased VGLUT2-ir shown here could indicate an increase in the number of axonal inputs from nonauditory regions or simply an increase in the amount of glutamate transporter. The possibility that axonal sprouting may occur after cochlear damage has been suggested by reports of the re-emergence of GAP-43 and synaptophysin after acoustic trauma (Illing and Horváth, 1995; Bilak et al., 1997; Fuentes-Santamaria et al., 2007). The present study provides evidence that some of these new axons are glutamatergic and originate in somatosensory nuclei, reticular formation, pontine nucleus, and other nonauditory areas (Shore et al., 2000; Zhou and Shore, 2004; Babalian, 2005; Haenggeli et al., 2005; Zhan and Ryugo, 2007; Zhou et al., 2007) to compensate for the loss of excitatory input from the cochlea. The signaling pathways needed to initiate these changes would likely be the parallel fibers, the axons of granule cells that carry the nonauditory information to the principal cells of the dorsal cochlear nucleus (Mugnaini et al., 1980; Manis, 1989). Indeed, the VGLUT1-ir parallel fibers in the present study (DCN1) showed less immunoreactivity after cochlear damage, possibly providing the needed signal for increasing axonal projections from nonauditory regions to their cell bodies, the granule cells. This region also showed an increase in VGLUT2-ir, probably reflecting an increase in the amount of nonauditory inputs that are occasionally seen in this region in the normal animal (Zhou and Shore, 2004; Haenggeli et al., 2005). Communication between the Shell region and VCN neurons has been suggested by the branching of the dendrites of D stellate cells into the granule cell domain (Oertel et al., 1990).

Over the past decade, the cochlear nucleus has been estab-

Figure 6. VGLUT immunoreactivity in the CN ipsilateral ($N = 10$) to the deafening is replotted as ratios over the normal ears ($N = 6$), where 1 = no difference between deafened and normal ears. **A**, The distributions of VGLUT1 and VGLUT2-ir across different regions of the CN are shown in the same figure to highlight the changes in the relative distributions of the two VGLUT transporters after deafness. At both 1 and 2 weeks after deafness, ipsilateral VGLUT1 is decreased in VCNm (AVCN and PVCN) and DCN3, reflecting the large reduction of VIIIth nerve terminals after hair cell loss after the kanamycin injections. By 2 weeks, VGLUT1 is decreased more dramatically in all regions. In contrast, VGLUT2-ir is elevated above normal in DCN1, DCN2, and the Shell regions at 1 week after deafness and is elevated in all regions by 2 weeks. The areas receiving primarily auditory nerve inputs or nonauditory inputs are designated by the curly brackets above the graphs.

lished as a multisensory center that integrates auditory information provided by the VIIIth nerve with multimodal information from somatosensory and other nonauditory modalities to aid in orientation to sounds and suppression of body generated sounds (Davis et al., 1996; Kanold and Young, 2001; Shore et al., 2003, 2008; Shore, 2005). It is well known that cross-modal compensation can occur after deafferentation or sensory deprivation in one sense (Goel et al., 2006; Oh et al., 2007). Until now, this compensation has only been shown in higher structures, such as the inferior colliculus, superior colliculus, or cortex (Batzri-Izraeli et al., 1990; Izraeli et al., 2002; Piche et al., 2007). The present study demonstrates that cross-modal reinnervation can occur also in a second order structure, the CN. The changes demonstrated here are consistent with recent reports that, after cochlear damage, responses of CN neurons to trigeminal ganglion stimulation are enhanced as evidenced by shorter latencies and reduced thresholds, as well as a redistribution of excitatory and inhibitory responses (Shore et al., 2008). These changes could be adaptive, by enhancing the presumed functions of these pathways such as suppression of self-generated sounds, thus enabling the animal to attend more easily to external, environmental stimuli. Recent studies in primates indicate that the DCN circuitry documented for rodents and other mammals such as cats is also fully present in primates (Rubio et al., 2008), suggesting that these findings would be generalizable, even to humans.

One undesirable consequence of these increased glutamatergic inputs could be increased spontaneous rates in CN neurons that have been observed after hearing loss (Kaltenbach and Afman, 2000; Brozoski et al., 2002; Sumner et al., 2005; Shore et al., 2008), and that have been proposed as correlates of the phantom auditory sensations commonly called tinnitus (Zhang and Kaltenbach, 1998; Brozoski et al., 2002; Kaltenbach et al., 2004; Kaltenbach, 2007). In this context, the correlation of spiral ganglion loss with increases in VGLUT2 in the present study is consistent with the results of a recent study showing that loss of large-diameter auditory nerve fibers can be a reliable predictor of tinnitus after acoustic exposure (Bauer et al., 2007).

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