Published in final edited form as:

Neuroscience. 2016 February 19; 315: 114–124. doi:10.1016/j.neuroscience.2015.12.009.

Altered vesicular glutamate transporter distributions in the mouse cochlear nucleus following cochlear insult

Amarins N. Heeringa^a, Roxana A. Stefanescu^a, Yehoash Raphael^a, and Susan E. Shore^{a,b,c}

^aKresge Hearing Research Institute; Otolaryngology, University of Michigan

^bMolecular and Integrative Physiology, University of Michigan

^cBiomedical Engineering, University of Michigan

Abstract

Vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2) have distinct distributions in the cochlear nucleus that correspond to the sources of the labeled terminals. VGLUT1 is mainly associated with terminals of auditory nerve fibers, whereas VGLUT2 is mainly associated with glutamatergic terminals deriving from other sources that project to the cochlear nucleus (CN), including somatosensory and vestibular terminals. Previous studies in guinea pig have shown that cochlear damage results in a decrease of VGLUT1-labeled puncta and an increase in VGLUT2-labeled puncta. This indicates cross-modal compensation that is of potential importance in somatic tinnitus. To examine whether this effect is consistent across species and to provide a background for future studies, using transgenesis, the current study examines VGLUT expression profiles upon cochlear insult by intracochlear kanamycin injections in the mouse.

Intracochlear kanamycin injections abolished ipsilateral ABR responses in all animals and reduced ipsilateral spiral ganglion neuron densities in animals that were sacrificed after four weeks, but not in animals that were sacrificed after three weeks. In all unilaterally deafened animals, VGLUT1 density was decreased in CN regions that receive auditory nerve fiber terminals, i.e. in the deep layer of the dorsal cochlear nucleus (DCN), in the interstitial region where the auditory nerve enters the CN, and in the magnocellular region of the antero- and posteroventral CN. In contrast, density of VGLUT2 expression was upregulated in the fusiform cell layer of the DCN and in the granule cell lamina, which are known to receive somatosensory and vestibular terminals.

These results show that a cochlear insult induces cross-modal compensation in the cochlear nucleus of the mouse, confirming previous findings in guinea pig, and that these changes are not dependent on the occurrence of spiral ganglion neuron degeneration.

Correspondence to: Susan Shore, Kresge Hearing Research Inst. 1100 W. Medical Center Drive, Ann Arbor, MI 48104. Tel: 734-647-2116; Fax: 734-764-0014; sushore@umich.edu.

The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

cochlear damage; cochlear nucleus; multisensory; plasticity; tinnitus; vesicular glutamate transporters

1. Introduction

Vesicular glutamate transporters (VGLUTs) mediate the uptake of L-glutamate into synaptic vesicles. Three homologous subtypes (VGLUT1-3) have been identified in the mammalian CNS (Takamori et al., 2000, 2001, Fremeau et al., 2002), in which VGLUT1 and 2 have been studied most extensively. Occasionally, VGLUT1 and VGLUT2 co-localize, such as in the mossy fibers of the cerebellum (Hioki et al., 2003), however, generally, the spatial expression profiles of the two subtypes are distinct from each other throughout the brain (Fremeau et al., 2001, Kaneko et al., 2002, Hioki et al., 2003, Graziano et al., 2008, Balaram et al., 2015).

The cochlear nucleus is the first target of the VIIIth nerve and contains a number of different regions, which are defined by their relative locations and composition of cell types (Fig. 1). The dorsal part of the cochlear nucleus (DCN) consists of a molecular layer, a fusiform cell layer, and a deep layer (abbreviated as DCN1, DCN2, and DCN3, respectively). The ventral part of the cochlear nucleus (VCN) is subdivided in a posterior and an anterior region (PVCN and AVCN, respectively). In the mouse CN, the area where the auditory nerve enters the nucleus can be defined as a separate region, called the interstitial region (INT) or auditory nerve root (Lorente de Nó, 1933). The granule cell lamina (GCL) encapsulates the VCN on the dorsal and lateral side and primarily contains granule and small cells. The CN is the first auditory brain station where auditory input is integrated with input from other sensory modalities, such as the somatosensory and the vestibular systems (Cant and Morest, 1978, Shore and Moore, 1998, Shore, 2005, Barker et al., 2012). Both VGLUT1 and VGLUT2 are highly expressed in the CN, with distinct distributions. These distinct expression profiles correspond to the source of the glutamatergic terminals (Zhou et al., 2007, Barker et al., 2012). VGLUT1 co-labels with synaptic terminals of type I auditory nerve fibers (ANFs) and is mostly expressed in the magnocellular region of the ventral CN (VCN) and the deep layer of the dorsal CN (DCN3) (Zhou et al., 2007, Gomez-Nieto and Rubio, 2009). On the other hand, VGLUT2 co-labels with non-auditory terminals, including those from the somatosensory and the vestibular system, and is mostly expressed in granule cell lamina, where these terminals primarily innervate granule cells (Haenggeli et al., 2005, Zhou et al., 2007, Zeng et al., 2011, Barker et al., 2012). Thus, studying VGLUT1 and VGLUT2 expression provides insight into the magnitude and spatial distributions of both auditory and non-auditory innervation of the CN.

Previous studies in guinea pig have shown that the expression profiles of VGLUT1 and VGLUT2 in the CN are affected by cochlear damage. After kanamycin injections (Zeng et al., 2009, Zeng et al., 2012), VGLUT1 is down-regulated in the CN, as would be expected from the loss of afferent input from the ANFs. Interestingly, VGLUT2 is up-regulated after cochlear damage, which reflects a reactive re-innervation of the CN by inputs from the somatosensory system (Zeng et al., 2009, Zeng et al., 2012).

This cross-modal plasticity in the CN after cochlear damage is of potential importance for ‘somatic’ tinnitus, in which patients can modify their tinnitus through head and neck maneuvers, such as in jaw clenching (Levine et al., 2003), or when their tinnitus can be attributed to an insult in the head and neck region (Lockwood et al., 1998, Pinchoff et al., 1998, Levine, 1999). Indeed, animals with behaviorally confirmed tinnitus have altered functional auditory-somatosensory integration in the CN (Dehmel et al., 2012, Koehler and Shore, 2013). To further study the role of cochlear damage-induced upregulation of VGLUT2 terminals in the CN in somatic tinnitus, conditional VGLUT2 knock-out studies in mice are of high importance (Brown et al., 2008). Therefore, the current study investigates whether the changes in VGLUT1 and VGLUT2 expression in the cochlear nucleus previously shown in guinea pigs (Zeng et al., 2009, Zeng et al., 2012) are also evident in mice following cochlear insult induced by intracochlear kanamycin injection.

2. Experimental procedures

Six adult C57Bl6 mice (Charles River Inc.) of 5 weeks of age were used in this study. Mice were socially housed in a 12:12h light/dark cycle and had *ad libitum* access to food and water. Four animals were unilaterally deafened by intracochlear kanamycin injection and two age-matched animals were used as controls. VGLUT1 and VGLUT2 densities were assessed in the ipsilateral and contralateral CN of deafened animals and in both CNs of control animals. All procedures were approved by the University of Michigan School of Medicine Laboratory Animal Care and Use Committee (UCUCA protocol # PRO00005217), conformed to the NIH Guide for the Care and Use of Laboratory Animals, and followed the Society for Neuroscience’s Guidelines for the Use of Animals in Neuroscience Research.

2.2 Auditory brainstem response (ABR) measurements

ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Austin, TX USA). Animals were anesthetized with intramuscular injections of 40 mg/kg ketamine and 10 mg/kg xylazine and body temperature was maintained with a thermostatically controlled heating pad. Sub-dermal recording electrodes were placed at the vertex (posterior to bregma), and ventral to the ipsilateral and contralateral pinna for reference and ground, respectively.

Acoustic stimuli were generated and delivered, and responses were recorded with Tucker Davis Technologies (TDT, Alachua, FL USA) hardware, controlled by a Pentium PC, using TDT System II software (BioSig and SigGen). Stimuli had a sample rate of 100 kHz at 16-bit resolution and were equalized to correct for the system’s frequency response. Tone bursts (12, 16, 20, and 30 kHz; 10ms duration; 1ms rise/fall times), were presented at 10 per second and were delivered through an EC1 transducer (TDT) coupled to a speculum placed just inside the tragus. ABRs were collected for stimulus levels in 10 dB steps, starting at 80 dB SPL, with additional 5 dB steps near threshold. Up to 1024 ABRs were averaged for each stimulus level. The ABR threshold was considered the interpolation between the lowest stimulus level where a response was observed, and 5 dB lower, where no response was observed. In cases where no response was seen at 70 dB SPL, the threshold value is replaced by “no response”. ABRs were assessed before intracochlear kanamycin injections and one

day prior to euthanasia. In age-matched control mice, ABRs were measured one week before euthanasia.

2.3 Cochlear injections and brain tissue processing

Kanamycin injections were performed as described previously (Zeng et al., 2009). Briefly, animals were anesthetized with ketamine/xylazine injections (120 mg/kg and 7 mg/kg, respectively), and placed in a stereotaxic frame (David Kopf, Tujunga, CA). Rectal temperature was maintained at $38^{\circ} \pm 0.5^{\circ}\text{C}$. To expose the bulla, a small incision was made behind the left ear and the skin was retracted. A small hole was made in the bulla and 1 μl 30% kanamycin in saline was injected into the cochlea through the round window using a Hamilton syringe. Next, the skin overlying the bulla was sutured and the animal was allowed to recover.

Three to four weeks following deafening, animals were euthanized with FatalPlus (Vortech Pharmaceuticals, Dearborn, MI; 5 mg/kg, intraperitoneally). The animals were then perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in the same buffer. The brains were isolated and postfixed for 2 hours at 4°C , then transferred into a sucrose solution (20% in 0.1 M PBS) overnight at 4°C . The brainstems were sectioned on a freezing microtome at a thickness of 20 μm ; the cochleae were processed for spiral ganglion counts.

2.4 Spiral ganglion neuron assessments

Spiral ganglion neuron (SGN) densities were assessed as described previously (Zappia and Altschuler, 1989, Miller et al., 1997, Glueckert et al., 2008). Briefly, cochleae were rapidly isolated following transcardial perfusion, decalcified in 5% EDTA, and processed for embedding into JB-4 Plus. Mid-modiolar sections (5 μm) were cut in a para-midmodiolar plane such that sections had six cross-sectional profiles of Rosenthal's canal. Next, cochlear sections were mounted on slides, stained with Paragon, and coverslipped. The twelve most mid-modiolar sections were selected and every other section was used for quantitative assessment of SGN density. Digital images were acquired of the entire most basal cross sectional profile through Rosenthal's canal under bright field optics and analyzed using MetaMorph (version 6.1, Universal Imaging Corp). All SGN with a nucleus and meeting size and shape inclusion criteria were counted for each profile from each of the six slides assessed (Altschuler et al., 2015). Photomicrographs at lower magnifications were used to determine the area of the Rosenthal canal, which was used to generate a measure for SGN density within that profile. In addition, a qualitative analysis was carried out to assess the histology of the spiral limbus, spiral ligament, organ of Corti, and stria vascularis. Photomicrographs of the cochlea were adjusted for contrast enhancement in Adobe Photoshop CS6.

2.5 VGLUT immunocytochemistry

Transverse sections (20 μm) of the brainstem were mounted in four series on clean glass slides and air-dried. VGLUT1 and VGLUT2 immunocytochemistry was processed as described previously (Zhou et al., 2007, Zeng et al., 2009). All processing was done at room temperature (20–22 $^{\circ}\text{C}$). Sections were incubated for 30 minutes in a blocking solution

containing 1% normal goat serum and 0.1% Triton X-100 in 0.1M PBS (pH 7.4), followed by overnight incubation with primary antibodies for VGLUT1 (polyclonal antibody, generated in rabbit, diluted in blocking solution 1:2000, Synaptic Systems, Germany, Cat. #135 303) or VGLUT2 (polyclonal antibody, generated in rabbit, diluted in blocking solution 1:2000, Synaptic Systems, Germany, Cat. #135 403). After rinsing with 0.1M PBS, sections were incubated for 2 hours with the secondary antibody (Alexa Fluor 555-conjugated goat anti-rabbit, diluted in blocking solution 1:500; Invitrogen). After rinsing, slides were dehydrated in graded ethanol, and cover slipped using micro-cover gel (Micron Diagnostics, USA). Negative controls were conducted on sections that were not treated with either the primary or the secondary antibody, and resulted in negative immunolabeling. Positive controls for VGLUT immunolabeling were performed in the cerebellar cortex (Takamori et al., 2001, Kaneko et al., 2002, Hioki et al., 2003). To confirm antibody specificity, primary antibodies were pre-incubated with corresponding synthetic peptides (Strep-Tag® fusion protein containing amino acid residues 456 – 560 of rat VGLUT1, Cat. # 135-3P, or Strep-Tag® fusion protein containing amino acid residues 510 – 582 of rat VGLUT2, Cat. # 135-4P, Synaptic Systems, Germany), resulting in negative immunolabeling. Previously performed Western blots (Zhou et al., 2007) demonstrated anti-VGLUT1 antibody with a single band at ~ 60 kDa and anti-VGLUT2 antibody as a single band at ~65 kDa on protein extracted from both CN and cerebellum, corresponding to the molecular weights predicted for VGLUT1 and VGLUT2, respectively.

2.6 Image processing and quantification

Sections were examined using a fluorescence microscope (Leica, CM) with the appropriate filter for Alexa 555. Experimenters were blind as to whether the tissue was from normal or deafened animals. All parameters for digitizing photomicrographs were kept constant across conditions and animals. VGLUT images were taken for all CN subdivisions, as defined in the introduction, including the molecular layer, the fusiform cell layer, and the deep layer of the dorsal cochlear nucleus (DCN1, DCN2, and DCN3, respectively), GCL, INT, AVCN, and PVCN (Fig. 1D). For each side of each animal, three photomicrographs (63X) were taken at equal intervals from caudal to rostral for each of the selected regions (i.e. one picture from the 25th percentile, one from the 50th percentile and one from the 75th percentile), in order to create an average pixel value density for each region.

Quantification was performed as previously described (Zhou et al., 2007). Photomicrographs were imported to Adobe Photoshop for contrast adjustment and imported to Metamorph for automatic quantification. Pixel intensity, determined by VGLUT-immunoreactivity was determined in each of the above mentioned CN regions using MetaMorph. The total pixel value was divided by the chosen area in each photomicrograph to yield the VGLUT density (in pixel value/ μm^2).

2.7 Statistical analyses

To investigate changes in ABR thresholds, SGN density, and VGLUT1 and VGLUT2 pixel values as a result of intracochlear kanamycin injections, the ipsilateral side of the deafened animals was compared to the contralateral side of the deafened animals and to both sides of the control animals pooled together. One-way ANOVA was used to assess statistical

differences in SGN density following kanamycin injection. Two-way ANOVA and three-way ANOVAs were followed by one-way ANOVA post-hoc tests with Holm-Bonferroni correction to evaluate significant changes in ABR thresholds and VGLUT expression, respectively. All statistical tests were evaluated in MatLab (R2015a, Mathworks) using functions from the Statistical Toolbox.

3. Results

3.1 Effects of intracochlear kanamycin injections on ABR thresholds and SGN densities

Three to four weeks after unilateral kanamycin injections, ipsilateral ABR responses were completely abolished at 70 dB SPL (Fig. 2). Contralateral ABR hearing thresholds were slightly elevated (14 dB on average across frequencies) by kanamycin injection (two-way ANOVA with factors f1) before/after and f2) frequency: f1) $F(1) = 10.37$, $p = 0.0037$). However, post-hoc tests were not significant for any of the tested frequencies (one-way ANOVA: $p = 0.278$, $p = 0.128$, $p = 0.236$, $p = 0.241$ for 12, 16, 20, and 30 kHz, respectively, corrected for multiple comparisons). ABR thresholds of age-matched control animals were similar to ABR thresholds of normal-hearing C57Bl6 mice (Fujita et al., 2015, Shi et al., 2015).

Assessment of SGN density revealed that ipsilateral SGN density was not significantly different from contralateral (One-way ANOVA: $F(1) = 3.52$; $p = 0.1097$) or control ears (One-way ANOVA: $F(1) = 1.27$; $p = 0.3028$), however, a downward trend could be observed (Fig. 3A). This downward trend could be attributed to two out of four mice, which showed a prominent decrease in SGN density ipsilateral to the kanamycin injections (M46, M49; Fig. 3B). These two animals were sacrificed four weeks after surgery, whereas M23 and M30 were sacrificed three weeks after surgery.

Representative photomicrographs show the spiral ganglion and organ of Corti of the ipsilateral and contralateral cochleae of two animals injected with kanamycin: one that was sacrificed after three weeks (Fig. 4A and 4B for contralateral and ipsilateral, respectively) and one that was sacrificed after four weeks (Fig. 4C and 4D for contralateral and ipsilateral, respectively). Three weeks following intracochlear kanamycin injection, inner and outer hair cells were unaffected in the contralateral ear (solid and open arrows, respectively, Fig. 4A), whereas inner and outer hair cells degenerated in the ipsilateral ear (Fig. 4B). The tunnel of Corti was collapsed, but supporting cells, including pillar cells and Deiters cells, remained present. The spiral limbus, spiral ligament, and stria vascularis were not affected three weeks following kanamycin injections in either the contralateral or the ipsilateral ear. Furthermore, SGN density appeared unaffected, supporting the quantification (Fig. 3B).

Four weeks following intracochlear kanamycin injection, the ipsilateral organ of Corti degenerated, leaving no sensory epithelium (Fig. 4C), whereas the contralateral organ of Corti was unaffected (Fig. 4D). Furthermore, the stria vascularis appeared thinner ipsilateral to the injection and the spiral ligament showed vacuolization. These changes were not apparent in the contralateral ear. The spiral limbus appeared unaffected (Fig. 4D). As quantified above, SGN density was greatly reduced four weeks after kanamycin injection (Fig. 3D).

Even though effects of kanamycin injection on SGN densities and other cochlear structures were variable, changes in VGLUT1 and VGLUT2 density were similar across all four deafened animals.

3.2 VGLUT1 immunolabeling in the cochlear nuclei after unilateral cochlear insult

Immunolabeling of VGLUT1 in the cochlear nucleus revealed that VGLUT1 expression was significantly decreased in CN regions that receive ANF inputs, including the VCN and the deep layer of the DCN, ipsilateral to the kanamycin injection. Representative photomicrographs of all examined CN regions are depicted in Figure 5 and demonstrate a decreased VGLUT1-immunoreactivity in the INT (Fig. 5A), PVCN (Fig. 5B), AVCN (Fig. 5C), and the deep layer of the DCN (Fig. 5G) in the ipsilateral CN in an animal with cochlear insult. These changes were seen in all animals that were unilaterally injected with kanamycin. Three-way ANOVA, with factors f1) control/deafened, f2) ipsilateral/contralateral, and f3) region, revealed significant effects of deafening (f1) and side of deafening (f2) and two-way interactions between deafening and side of deafening (f1xf2) and between side of deafening and region (f2xf3) (f1) $F(1) = 21.31$, $p < 0.001$, f2) $F(1) = 8.32$, $p = 0.0043$, f3) $F(6) = 228.81$, $p < 0.001$; f1)xf2) $F(1) = 8.32$, $p = 0.0043$, f2)xf3) $F(6) = 3.37$, $p = 0.0033$). Post-hoc tests revealed that VGLUT1 expression was significantly decreased in the ipsilateral DCN3, AVCN, PVCN and INT as compared to the contralateral side and to the control ($p < 0.01$, Holm-Bonferroni corrected), and in the ipsilateral DCN2 as compared to the control DCN2 ($p < 0.05$, Holm-Bonferroni corrected, Fig. 5H).

Because VGLUT1 is associated with terminals of the auditory nerve, we investigated whether the extent of VGLUT1 decrease was related with the extent of SGN degeneration. CN regions innervated by the ascending branch of the auditory nerve (AVCN and INT) showed a 8.2- and 3.8-fold decrease in the two animals without major SGN densities changes (M23 and M30, respectively, Fig. 3B), and an average 4.5- and 3.8-fold decrease in animals with evidence of marked SGN degeneration (M46 and M49, respectively, Fig. 3B). CN regions innervated by the descending branch (PVCN and DCN3) showed an average of 4.7- and 2.3-fold decrease in M23 and M30, respectively, and an average of 6.1- and 2.6-fold decrease in M46 and M49, respectively. Furthermore, the pattern of VGLUT1 decrements seen in the CN was similar when animals that were sacrificed either after three or four weeks post-injection. This suggests that SGN degeneration is not necessary for downregulation of VGLUT1 in CN regions innervated by the ascending or the descending branch of the auditory nerve.

3.3 VGLUT2 immunolabeling in the cochlear nuclei after cochlear insult

VGLUT2-immunoreactivity was significantly increased in the ipsilateral GCL and DCN2, the primary recipients of non-auditory inputs in rodents (Zhou and Shore, 2004, Haenggeli et al., 2005, Zeng et al., 2011). Figure 6 shows photomicrographs of all examined CN regions and demonstrates increased VGLUT2-immunoreactivity in the ipsilateral GCL (Fig. 6D) and DCN2 (Fig. 6F) as compared to the contralateral side (Fig. 6D' and 6F', respectively) of an animal with cochlear insult. These are typical changes seen in all four animals with cochlear insult. Threeway ANOVA, with factors f1) control/deafened, f2) ipsilateral/contralateral, and f3) region, revealed significant effects and two- and three-way

interaction effects (f2) $F(1) = 13.08$, $p < 0.001$; f3) $F(6) = 336.76$, $p < 0.001$, f1)xf2) $F(1) = 13.08$, $p < 0.001$, 18 f2)xf3) $F(6) = 5.6$, $p < 0.001$; , f1)xf2)xf3) $F(6) = 6.15$, $p < 0.001$). Post-hoc tests revealed significant increases of VGLUT2 pixel value density in DCN2 and GCL ($p < 0.01$, Holm-Bonferroni corrected). VGLUT2 density was decreased in AVCN and INT ($p < 0.01$ and $p < 0.05$, respectively, Holm-Bonferroni corrected; Fig. 6B). No significant changes in VGLUT2 were seen in the molecular layer of the DCN and the deep layer of the DCN or PVCN.

Because cochlear insult-induced VGLUT2 increases reflect the time course of SGN degeneration in guinea pig (Zeng et al., 2009), we investigated here whether the extent of VGLUT2 increase was related with SGN degeneration. Mice without major SGN densities changes (M23 and M30) had a 1.7- and 1.6-fold increase of VGLUT2-, respectively, in DCN2 and GCL, the two major recipients of non-auditory input (Zhou and Shore, 2004, Zhou et al., 2007). Animals with evidence of SGN degeneration (M46 and M49, Fig. 3B) had a 2.1- and 1.4-fold increase of VGLUT2. Moreover, similar to the changes seen in VGLUT1, VGLUT2 increases in the CN were similar when animals that were sacrificed after three weeks or four weeks. This suggests that, similar to decreases in VGLUT1, SGN degeneration is not necessary for upregulation of VGLUT2 density in the fusiform cell layer of the DCN and the GCL.

4. Discussion

The discovery and identification of three members of the solute carrier family, Slc17a6-8, that act as VGLUTs, has enabled the correct identification of glutamate signaling neurons and their role in glutamatergic neurotransmission. VGLUT1 and VGLUT2 are considered the most reliable markers of glutamatergic neurons and have mostly complementary distributions (Takamori et al., 2000, Fremeau et al., 2001, Takamori et al., 2001). In the CN, ANF synaptic terminals express VGLUT1, while terminal endings from other sensory systems express VGLUT2 (Zhou et al., 2007). Furthermore, after unilateral cochlear damage by kanamycin (Zeng et al., 2009) VGLUT1 is diminished while VGLUT2 is increased. This coincides with a reduction in ANF input, but enhancement of somatosensory input to the CN of the guinea pig (Zeng et al., 2012).

In the present study, the distribution of VGLUT1 and VGLUT2 in the CN, previously described for guinea pig, was replicated in mouse and showed similar changes in VGLUT1 and 2 distributions after unilateral cochlear insult. VGLUT2 was significantly increased in regions of the CN that are the major recipients of somatosensory and vestibular inputs (fusiform cell layer of the DCN and GCL) (Ryugo et al., 2003, Zhou et al., 2007, Barker et al., 2012). VGLUT1, in contrast, was significantly decreased in regions of the CN that receive ANF input (DCN3, INT, PVCN and AVCN) (Ryugo and Parks, 2003, Zhou et al., 2007, Gomez-Nieto and Rubio, 2009). However, not all glutamatergic terminals are necessarily derived from outside of the cochlear nucleus. Two types of multipolar cells have been defined, 20 which reside in the VCN and project to the neurons in the DCN (Doucet and Ryugo, 1997). Since D-multipolar cells are glycinergic (Doucet et al., 1999), they are not contributing to VGLUT immunoreactivity. On the other hand, it has been suggested that T-multipolar cells are glutamatergic (Ferragamo et al., 1998), but it is not known whether

these neurons are positive for VGLUT1 or for VGLUT2. Since the vast majority of glutamatergic neurons of the VCN co-express genes for VGLUT1 and VGLUT2 (Ito et al., 2011), it is likely that T-multipolar cells are positive for both VGLUT1 and VGLUT2. Therefore, if intracochlear kanamycin injections would affect glutamatergic terminals of T-multipolar cells, VGLUT1 and VGLUT2 densities in the DCN would likely to be altered in the same direction, which was not observed in our results. Granule cells are also glutamatergic and are positive for VGLUT1 (Rubio et al., 2008). These cells have long parallel fibers that extend along the molecular layer of the DCN where they innervate apical dendrites of fusiform cells and cartwheel cells. This is reflected by the high VGLUT1 immunoreactivity in DCN1. Our results suggest that glutamatergic neurotransmission of granule cells is not affected by unilateral cochlear insult. Other cells that make connections within the cochlear nucleus are cartwheel cells and vertical cells, but these are both glycinergic (Kuo et al., 2012). Therefore, our study suggests that unilateral cochlear insult induced by kanamycin injections reduced glutamatergic terminals from the auditory nerve, which was compensated by an upregulation of non-auditory glutamatergic terminals.

Because VGLUT1 is associated with terminals of the auditory nerve, it was hypothesized that decreases in VGLUT1 were related to the extent of spiral ganglion cell survival. Interestingly, this study showed that degeneration of SGNs is not necessary for decreases in VGLUT1 to occur, neither in regions innervated by the ascending branch (AVCN and INT), nor in regions innervated by the descending branch of the auditory nerve (PVCN and DCN3). This is in contrast to the results in guinea pig that showed decreases both in SGN densities and VGLUT1-labeled puncta following intracochlear kanamycin injections (Zeng et al., 2009, Zeng et al., 2012). Our results indicate that the threshold for initiating VGLUT changes in the CN does not require degeneration of the SGN. Other possible signals for triggers for VGLUT1 decrease and VGLUT2 increase in the CN include loss of cochlear neurotrophin secretion, which is also reflected in ABR and hair cell changes (Wan et al., 2014). Further research is needed to elucidate these signals.

In guinea pig, increases in VGLUT2-labeled puncta in the small cell cap, fusiform cell layer, and deep layer of the DCN seem to reflect the time course of SGN degeneration (Zeng et al., 2009). Yet, this study showed that, in mouse, increases in VGLUT2 densities were of similar magnification in animals without major SGN degeneration as compared to animals with SGN degeneration. Possibly, VGLUT2-labeled terminals are upregulated in response to a local signal related to downregulation of VGLUT1 terminals, rather than in response to degeneration of SGNs. This signal might derive from local microglia activation that regulates reinnervation of the cochlear nucleus after cochlear ablation (Janz and Illing, 2014). Future research on the time course of SGN degeneration, downregulation of VGLUT1-labeled terminals, and upregulation of VGLUT2-labeled terminals is needed to further elucidate these signals and relationships.

C57Bl6 mice are known to develop age-related hearing loss, which is typically thought to affect ABR thresholds after approximately 8 months of age (Hunter and Willott, 1987, Johnson et al., 1997, Zheng et al., 1999). Since the animals used in this study were 5 weeks of age upon surgery and 8 to 9 weeks of age upon euthanasia, the presence of sensorineural hearing loss is unlikely. Indeed, ABR thresholds of the mice in the current study are similar

to thresholds of normal hearing C57Bl6 mice (Fujita et al., 2015, Shi et al., 2015). Moreover, VGLUT changes occurred only in the CN ipsilateral to the kanamycin injection, whereas sensorineural hearing loss would presumably affect both sides equally. Therefore, it is unlikely that the mouse strain was a confound in this particular study.

To introduce unilateral cochlear insult, kanamycin was injected through the round window of the cochlea. It is well known that systemically applied aminoglycosides, such as kanamycin, damage the cochlea, inducing sensorineural hearing loss (Forge and Schacht, 2000). Outer hair cells degenerate first after kanamycin, followed by inner hair cells. Hair cell degeneration shows a gradient of sensitivity, beginning at the base and progressing to the apex of the cochlea. This range of sensitivity is determined by intrinsic susceptibility to free radicals that are formed by the aminoglycosides (Forge and Richardson, 1993, Wu et al., 2002). Degeneration of the SGNs is traditionally thought to be a secondary effect, caused by a loss of trophic support from the hair cells, but direct injury due to the aminoglycosides cannot be ruled out. These effects can be observed *in vivo* by assessing ABR thresholds, which are elevated following kanamycin-induced cochlear damage. Mice are less susceptible to aminoglycoside ototoxicity than guinea pigs (Poirrier et al., 2010). This species difference is thought to be caused by a difference in pharmacokinetics of aminoglycosides, rather than a resilience of the hair cells to degenerate when aminoglycosides are present (Wu et al., 2002). Indeed, when mice are injected with a combination of kanamycin and furosemide, which opens the blood-brain barrier and reduces renal clearance, aminoglycoside ototoxicity is more severe (Jansen et al., 2013). This suggests that kanamycin applied directly in the cochlea, as carried out in the current study, would be as effective in mice as in guinea pig (Zeng et al., 2009, Zeng et al., 2012).

Physiological studies show that the VGLUT2-labeled terminals from somatosensory ganglia and brainstem nuclei (Zeng et al., 2011, Zeng et al., 2012), have significant impact on tinnitus development in guinea pigs. This is demonstrated by increased responsiveness of CN principal neurons to somatosensory electrical stimulation after noise damage (Shore et al., 2008). Furthermore, the somatosensory influence is hyper-excitatory in fusiform cells in animals with behavioral and physiological evidence of tinnitus (Dehmel et al., 2012, Koehler and Shore, 2013). Thus, if VGLUT2 was absent or reduced, there would be less excitatory influence from somatosensory fibers on CN neural responses, and less probability of developing fusiform cell hyperactivity and tinnitus.

Acknowledgments

Supported by the Tinnitus Research Initiative, NIH P30 DC-05188 (SES) and NIH R01 DC004825 (SES). The authors thank C. Zeng, A. Kanicki, and L. Shreve for providing technical assistance. We thank Sanford Bledsoe for insightful comments on the manuscript and the anonymous reviewers, whose critical reviews have substantially improved the current manuscript.

Abbreviations

ABR	auditory brainstem response
ANF	auditory nerve fiber

ANOVA	analysis of variance
AVCN	anteroventral cochlear nucleus
CN	cochlear nucleus
DCN	dorsal cochlear nucleus
DCN1	molecular layer of the dorsal cochlear nucleus
DCN2	fusiform cell layer of the dorsal cochlear nucleus
DCN3	deep layer of the dorsal cochlear nucleus
GCL	granule cell lamina
INT	interstitial region
PVCN	posteroventral cochlear nucleus
SGN	spiral ganglion neuron
VCN	ventral cochlear nucleus
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2

References

- Altschuler RA, Dolan DF, Halsey K, Kanicki A, Deng N, Martin C, Eberle J, Kohrman DC, Miller RA, Schacht J. Age-related changes in auditory nerve-inner hair cell connections, hair cell numbers, auditory brain stem response and gap detection in UM-HET4 mice. *Neuroscience*. 2015; 292:22–33. [PubMed: 25665752]
- Balaram P, Isaamullah M, Petry HM, Bickford ME, Kaas JH. Distributions of vesicular glutamate transporters 1 and 2 in the visual system of tree shrews (*Tupaia belangeri*). *J Comp Neurol*. 2015; 523:1792–1808. [PubMed: 25521420]
- Barker M, Solinski HJ, Hashimoto H, Tagoe T, Pilati N, Hamann M. Acoustic overexposure increases the expression of VGLUT-2 mediated projections from the lateral vestibular nucleus to the dorsal cochlear nucleus. *PLoS One*. 2012; 7:e35955. [PubMed: 22570693]
- Brown SD, Hardisty-Hughes RE, Mburu P. Quiet as a mouse: dissecting the molecular and genetic basis of hearing. *Nature reviews Genetics*. 2008; 9:277–290.
- Cant NB, Morest DK. Axons from non-cochlear sources in the anteroventral cochlear nucleus of the cat. A study with the rapid Golgi method. *Neuroscience*. 1978; 3:1003–1029. [PubMed: 85282]
- Dehmel S, Pradhan S, Koehler S, Bledsoe S, Shore S. Noise overexposure alters long-term somatosensory-auditory processing in the dorsal cochlear nucleus--possible basis for tinnitus-related hyperactivity? *J Neurosci*. 2012; 32:1660–1671. [PubMed: 22302808]
- Doucet JR, Ross AT, Gillespie MB, Ryugo DK. Glycine immunoreactivity of multipolar neurons in the ventral cochlear nucleus which project to the dorsal cochlear nucleus. *J Comp Neurol*. 1999; 408:515–531. [PubMed: 10340502]
- Doucet JR, Ryugo DK. Projections from the ventral cochlear nucleus to the dorsal cochlear nucleus in rats. *J Comp Neurol*. 1997; 385:245–264. [PubMed: 9268126]
- Ferragamo MJ, Golding NL, Oertel D. Synaptic inputs to stellate cells in the ventral cochlear nucleus. *J Neurophysiol*. 1998; 79:51–63. [PubMed: 9425176]
- Forge A, Richardson G. Freeze fracture analysis of apical membranes in cochlear cultures: differences between basal and apical-coil outer hair cells and effects of neomycin. *Journal of neurocytology*. 1993; 22:854–867. [PubMed: 8270950]

- Forge A, Schacht J. Aminoglycoside antibiotics. *Audiology & neuro-otology*. 2000; 5:3–22. [PubMed: 10686428]
- Freneau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:14488–14493. [PubMed: 12388773]
- Freneau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH. The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron*. 2001; 31:247–260. [PubMed: 11502256]
- Fujita T, Yamashita D, Uehara N, Inokuchi G, Hasegawa S, Otsuki N, Nibu K. A high-fat diet delays age-related hearing loss progression in C57BL/6J mice. *PLoS One*. 2015; 10:e0117547. [PubMed: 25625852]
- Glueckert R, Bitsche M, Miller JM, Zhu Y, Prieskorn DM, Altschuler RA, Schrott-Fischer A. Deafferentation-associated changes in afferent and efferent processes in the guinea pig cochlea and afferent regeneration with chronic intrascalar brain-derived neurotrophic factor and acidic fibroblast growth factor. *J Comp Neurol*. 2008; 507:1602–1621. [PubMed: 18220258]
- Gomez-Nieto R, Rubio ME. A bushy cell network in the rat ventral cochlear nucleus. *J Comp Neurol*. 2009; 516:241–263. [PubMed: 19634178]
- Graziano A, Liu XB, Murray KD, Jones EG. Vesicular glutamate transporters define two sets of glutamatergic afferents to the somatosensory thalamus and two thalamocortical projections in the mouse. *J Comp Neurol*. 2008; 507:1258–1276. [PubMed: 18181146]
- Haeggeli CA, Pongstaporn T, Doucet JR, Ryugo DK. Projections from the spinal trigeminal nucleus to the cochlear nucleus in the rat. *J Comp Neurol*. 2005; 484:191–205. [PubMed: 15736230]
- Hioki H, Fujiyama F, Taki K, Tomioka R, Furuta T, Tamamaki N, Kaneko T. Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. *Neuroscience*. 2003; 117:1–6. [PubMed: 12605886]
- Hunter KP, Willott JF. Aging and the auditory brainstem response in mice with severe or minimal presbycusis. *Hear Res*. 1987; 30:207–218. [PubMed: 3680066]
- Ito T, Bishop DC, Oliver DL. Expression of glutamate and inhibitory amino acid vesicular transporters in the rodent auditory brainstem. *J Comp Neurol*. 2011; 519:316–340. [PubMed: 21165977]
- Jansen TT, Bremer HG, Topsakal V, Hendriksen FG, Klis SF, Grolman W. Deafness induction in mice. *Otology & neurotology: official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology*. 2013; 34:1496–1502.
- Janz P, Illing RB. A role for microglial cells in reshaping neuronal circuitry of the adult rat auditory brainstem after its sensory deafferentation. *J Neurosci Res*. 2014; 92:432–445. [PubMed: 24446187]
- Johnson KR, Erway LC, Cook SA, Willott JF, Zheng QY. A major gene affecting age-related hearing loss in C57BL/6J mice. *Hear Res*. 1997; 114:83–92. [PubMed: 9447922]
- Kaneko T, Fujiyama F, Hioki H. Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain. *J Comp Neurol*. 2002; 444:39–62. [PubMed: 11835181]
- Koehler SD, Shore SE. Stimulus timing-dependent plasticity in dorsal cochlear nucleus is altered in tinnitus. *J Neurosci*. 2013; 33:19647–19656. [PubMed: 24336728]
- Kuo SP, Lu HW, Trussell LO. Intrinsic and synaptic properties of vertical cells of the mouse dorsal cochlear nucleus. *J Neurophysiol*. 2012; 108:1186–1198. [PubMed: 22572947]
- Levine, R. Somatic modulation of Tinnitus appears to be a fundamental attribute of tinnitus. *Proceedings of the 6th International Tinnitus Seminar; Cambridge UK*. 1999. p. 93-197.
- Levine RA, Abel M, Cheng H. CNS somatosensory-auditory interactions elicit or modulate tinnitus. *Exp Brain Res*. 2003; 153:643–648. [PubMed: 14600798]
- Lockwood A, Salvi R, Ciad B, Towsley M, Wack M, Murphy M. The functional neuroanatomy of tinnitus: Evidence for limbic system links and neural plasticity. *Neurology*. 1998; 50:114–120. [PubMed: 9443467]
- Lorente de Nó R. Anatomy of the eighth nerve: III.-General plan of structure of the primary cochlear nuclei. *The Laryngoscope*. 1933; 43:327–350.

- Martin MR. Morphology of the cochlear nucleus of the normal and reeler mutant mouse. *J Comp Neurol*. 1981; 197:141–152. [PubMed: 6164700]
- Mikula S, Trotts I, Stone JM, Jones EG. Internet-enabled high-resolution brain mapping and virtual microscopy. *NeuroImage*. 2007; 35:9–15. [PubMed: 17229579]
- Miller JM, Chi DH, O’Keeffe LJ, Kruszka P, Raphael Y, Altschuler RA. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. *Int J Dev Neurosci*. 1997; 15:631–643. [PubMed: 9263039]
- Pinchoff R, Burkard R, Salvi R, Coad M, Lockwood A. Modulation of tinnitus by voluntary jaw movements. *American J Otology*. 1998; 19:785–789.
- Poirrier AL, Van den Ackerveken P, Kim TS, Vandenbosch R, Nguyen L, Lefebvre PP, Malgrange B. Ototoxic drugs: difference in sensitivity between mice and guinea pigs. *Toxicology letters*. 2010; 193:41–49. [PubMed: 20015469]
- Rubio ME, Gudsruk KA, Smith Y, Ryugo DK. Revealing the molecular layer of the primate dorsal cochlear nucleus. *Neuroscience*. 2008; 154:99–113. [PubMed: 18222048]
- Ryugo DK, Haenggeli CA, Doucet JR. Multimodal inputs to the granule cell domain of the cochlear nucleus. *Exp Brain Res*. 2003; 153:477–485. [PubMed: 13680048]
- Ryugo DK, Parks TN. Primary innervation of the avian and mammalian cochlear nucleus. *Brain research bulletin*. 2003; 60:435–456. [PubMed: 12787866]
- Shi L, Liu K, Wang H, Zhang Y, Hong Z, Wang M, Wang X, Jiang X, Yang S. Noise induced reversible changes of cochlear ribbon synapses contribute to temporary hearing loss in mice. *Acta oto-laryngologica*. 2015; 135:1093–1102. [PubMed: 26139555]
- Shore SE. Multisensory integration in the dorsal cochlear nucleus: unit responses to acoustic and trigeminal ganglion stimulation. *Eur J Neurosci*. 2005; 21:3334–3348. [PubMed: 16026471]
- Shore SE, Koehler S, Oldakowski M, Hughes LF, Syed S. Dorsal cochlear nucleus responses to somatosensory stimulation are enhanced after noise-induced hearing loss. *Eur J Neurosci*. 2008; 27:155–168. [PubMed: 18184319]
- Shore SE, Moore JK. Sources of input to the cochlear granule cell region in the guinea pig. *Hear Res*. 1998; 116:33–42. [PubMed: 9508026]
- Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature*. 2000; 407:189–194. [PubMed: 11001057]
- Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J Neurosci*. 2001; 21:RC182. [PubMed: 11698620]
- Wan G, Gomez-Casati ME, Gigliello AR, Liberman MC, Corfas G. Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma. *eLife*. 2014; 3
- Wu WJ, Sha SH, Schacht J. Recent advances in understanding aminoglycoside ototoxicity and its prevention. *Audiology & neuro-otology*. 2002; 7:171–174. [PubMed: 12053140]
- Zappia JJ, Altschuler RA. Evaluation of the effect of ototopical neomycin on spiral ganglion cell density in the guinea pig. *Hear Res*. 1989; 40:29–37. [PubMed: 2768082]
- Zeng C, Nannapaneni N, Zhou J, Hughes LF, Shore S. Cochlear damage changes the distribution of vesicular glutamate transporters associated with auditory and nonauditory inputs to the cochlear nucleus. *J Neurosci*. 2009; 29:4210–4217. [PubMed: 19339615]
- Zeng C, Shroff H, Shore SE. Cuneate and spinal trigeminal nucleus projections to the cochlear nucleus are differentially associated with vesicular glutamate transporter-2. *Neuroscience*. 2011; 176:142–151. [PubMed: 21167260]
- Zeng C, Yang Z, Shreve L, Bledsoe S, Shore S. Somatosensory projections to cochlear nucleus are upregulated after unilateral deafness. *J Neurosci*. 2012; 32:15791–15801. [PubMed: 23136418]
- Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hear Res*. 1999; 130:94–107. [PubMed: 10320101]
- Zhou J, Nannapaneni N, Shore S. Vesicular glutamate transporters 1 and 2 are differentially associated with auditory nerve and spinal trigeminal inputs to the cochlear nucleus. *J Comp Neurol*. 2007; 500:777–787. [PubMed: 17154258]

Zhou J, Shore S. Projections from the trigeminal nuclear complex to the cochlear nuclei: a retrograde and anterograde tracing study in the guinea pig. *J Neurosci Res.* 2004; 78:901–907. [PubMed: 15495211]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

VGLUT1 decreases in magnocellular CN ipsilateral to cochlear insult

VGLUT2 increases in ipsilateral CN regions receiving non-auditory nerve input

VGLUT1 and VGLUT2 changes suggest cross-modal compensation after cochlear insult

VGLUT changes in mice provide impetus for using genetic tools in elucidating mechanisms of tinnitus

Figure 1. The mouse cochlear nucleus

A. Nissl staining of a coronal section of the mouse brain (Mikula et al., 2007), showing the location of the cochlear nucleus in the brainstem. This section was located at approximately 5.88 mm posterior to bregma and 2.08 mm posterior to the interaural axis. **B.** Coronal sections along the rostrocaudal axis of the mouse CN, stained with cresyl violet and luxol fast blue, modified from (Martin, 1981). **C.** Schematic drawing of the section shown in panel **A**, presenting the location of the dorsal and ventral cochlear nucleus in the mouse brainstem. **D.** Schematic drawings of the sections shown in panel **C**, modified from (Martin, 1981). Regions where photomicrographs were taken for VGLUT analysis are illustrated with arrows and include the molecular layer, fusiform cell layer, deep layer of the DCN (DCN1, DCN2, and DCN3, respectively), GCL, INT, AVCN, and PVCN.

Figure 2. ABR responses are absent at 70 dB SPL in the ipsilateral ear three to four weeks after intracochlear kanamycin injections

Mean ABR thresholds $\pm$ SEM before and three to four weeks after kanamycin injection (solid and dashed lines, respectively). Thresholds of the ipsilateral ear are shown in red; thresholds of the contralateral ear are shown in blue. Two normal-hearing age-matched control mice are shown for comparison in black. Kanamycin injection resulted in absence of ABR responses at 70 dB SPL in the ipsilateral ear three to four weeks following surgery. Contralateral thresholds were not affected by kanamycin injection.

Figure 3. SGN densities are not significantly changed three to four weeks after unilateral deafness

A. Mean SGN densities (cells/10,000 μm^2) $\pm$ SEM are shown for the contralateral and ipsilateral ears of the deafened mice and for both ears pooled together of the normal-hearing control animals. **B.** Individual SGN densities of the deafened (M23, M30, M46, M49) and the control (M35, M36) animals. Ipsilateral and contralateral SGN densities are shown in red and blue, respectively; control left and right ears are shown in black and grey.

Figure 4. Damage to the organ of Corti and spiral ganglion

Photomicrographs (20X) of the contralateral (**A**) and ipsilateral (**B**) spiral ganglion neurons and the organ of Corti of an animal that was sacrificed three weeks following kanamycin injection (M23) and of an animal that was sacrificed four weeks following kanamycin injection (M46) (panels **C** and **D**, for the contralateral and ipsilateral side, respectively). Closed arrows mark the location of inner hair cells; open arrows mark the location of outer hair cells. The calibration shown in panel D is the same for all panels.

Figure 5. VGLUT1 decreases in the CN ipsilateral to cochlear insult

Representative photomicrographs (63x) of VGLUT1-immunoreactivity in the ipsilateral and contralateral INT (A), PVCN (B), AVCN (C), GCL (D), DCN1 (E), DCN2 (F), and DCN3 (G), three weeks after intracochlear kanamycin injection. Images were adjusted for contrast enhancement in Photoshop. Scale bar indicates 30 μm (A–C) or 15 μm (panel D–G). **H.** Mean VGLUT1 density (pixel value/μm²) ± SEM across CN regions for controls, ipsilateral, and contralateral CNs. * indicate p < 0.05, ** indicate p < 0.01 (Holm-Bonferroni corrected).

Figure 6. VGLUT2 increases in the CN ipsilateral to cochlear insult

Representative photomicrographs (63x) of VGLUT2-labeled puncta in the ipsilateral and contralateral INT (A), PVCN (B), AVCN (C), GCL (D), DCN1 (E), DCN2 (F), and DCN3 (G), three weeks after kanamycin injection. Images were adjusted for contrast enhancement in Photoshop. Scale bar indicates 30 μm (A–C) or 15 μm (D–G). **H.** Mean VGLUT2 density (pixel value/μm²) ± SEM across CN regions for controls, ipsilateral and contralateral CNs. * indicate $p < 0.05$, ** indicate $p < 0.01$ (Holm-Bonferroni corrected).