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EFFECTS OF A HIGH-FREQUENCY AUGMENTED ACOUSTIC ENVIRONMENT ON PARVALBUMIN IMMUNOLABELING IN THE ANTEROVENTRAL COCHLEAR NUCLEUS OF DBA/2J AND C57BL/6J MICE

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Abstract

Neurons in the anteroventral cochlear nucleus (AVCN) of DBA/2J (D2) and C57BL/6J (B6) mice were immunohistochemically labeled for the calcium binding protein parvalbumin (PV). Prior to this, mice were treated for 12 hours nightly with a “high frequency” augmented acoustic environment (HAAE: repetitive bursts of a 70 dB sound pressure level, half-octave noise band centered at 20 kHz). This was done during the period that hearing loss occurs: pre-weaning to 55 days in D2 mice and weaning to 9 months in B6 mice. After HAAE treatment in D2 mice, high-frequency hearing loss was ameliorated and fewer PV-labeled neurons were found in the AVCN compared to untreated controls. HAAE treatment in B6 mice exacerbated high-frequency hearing loss, yet the number of PV-labeled AVCN neurons in treated mice did not differ significantly from that of control mice. The findings suggest that HAAE treatment provides relief from physiological stress caused by deprivation of auditory input from the impaired cochlea.

Keywords

mice; sensorineural hearing loss; augmented acoustic environment; cochlear nucleus; parvalbumin

1. Introduction

Mice of the inbred strains DBA/2J (D2) and C57BL/6J (B6) exhibit genetic progressive loss of cochlear hair cells and spiral ganglion cells resulting in hearing loss. In D2 mice, some elevation of auditory thresholds is evident by 3 weeks of age at high frequencies. By 2 months of age high-frequency hearing loss is severe, and thresholds for lower frequencies are elevated as well (Ralls 1967; Parham et al. 1997; Willott 1981). Hearing loss progresses more slowly in B6 mice, being near-normal in young adulthood (2 months of age) and becoming severe by 9 months of age, particularly for high frequencies (Henry, 1983; Li and Borg, 1991; Willott, 1996). Coinciding with the cochlear degeneration, losses of about 15–20% of neurons in the

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anteroventral cochlear nucleus (AVCN) are observed (Willott and Bross, 1996; Willott et al., 1987; 2008). Little additional neuron loss occurs after about 2 months of age in D2 mice or after 6 months in B6 mice.

Several hypotheses for the loss of AVCN neurons in D2 and B6 mice have been proposed (Willott, 2009), but each assumes a necessary role of auditory *deprivation*. The attenuation or partial removal of afferent neural input from the cochlea deprives AVCN neurons of activity-related trophic and/or other factors important for the health of neurons, and this causes death or pathology of neurons. It stands to reason that if a reduction of afferent input from the cochlea underlies degeneration of the AVCN, restoring or enhancing such input would have an ameliorative effect. Indeed, experiments with D2 mice using augmented acoustic environments (AAEs) have demonstrated amelioration of hearing loss, cochlear degeneration, and degenerative changes in the AVCN (Jeskey and Willott, 2000; Turner and Willott, 1998; Willott and Bross, 2004; Willott and Turner, 1999; Willott et al., 2000, 2001, 2005; 2006a,b; 2008). AAE treatments that ameliorated hearing loss and cochlear pathology have consisted of nightly 12-hr exposures to repeated bursts of either a broad-band noise or half-octave band centered at 20 kHz (“high-frequency AAE,” HAAE) from age 14 to 55 days (see Fig. 2 inset), the period when most progressive hearing loss occurs in D2 mice. Most importantly for the present study, HAAE-treated D2 mice had a less severe loss of neurons and tissue volume in the dorsal (high-frequency) half of the AVCN compared to controls (Willott et al., 2006a). Ameliorative effects of HAAE treatment on the AVCN presumably have something to do with overcoming the diminution of cochlear input associated with lower thresholds and/or increased acoustic activity from the HAAE.

B6 mice have been given AAE treatment from weaning until 9 or even 14 months of age. Although B6 mice benefit from AAE treatment using a broad-band noise (Willott and Turner, 1999; Willott et al. 2001; Willott and Bross, 2004), HAAE treatment *exacerbated* high-frequency hearing loss and hair cell damage (Willott et al., 2006b) – the opposite effect of that seen with D2 mice (see Fig. 3 inset). This was interpreted to be caused by the high vulnerability of B6 mice to noise-induced hearing loss: even though the HAAE was not intense, it caused cochlear damage over months of treatment in a vulnerable strain. Moreover, neuron loss in the B6 AVCN was minimally affected by HAAE treatment (Willott et al., 2008), presumably because the increased hearing loss did not allow the HAAE to overcome deprivation.

To gain a fuller understanding of events associated with AVCN degeneration and the effects of AAE treatment, the present study evaluated immunohistochemical labeling of the calcium binding protein parvalbumin (PV) in the AVCN. PV labeling was compared in D2 and B6 mice that had been treated with the HAAE with untreated controls. We did not obtain data from young, normal-hearing B6 mice in the present study because our interest was in HAAE effects in hearing-impaired 9-month-olds. However, it has shown that the number of PV-labeled neurons in the posteroventral CN increases by more than 80% by age 12 months in B6 mice (Idrizbegovic et al., 2004). As the following discussion suggests, this up-regulation of PV is most likely caused by the hearing loss occurring during this period. Idrizbegovic et al. (2004) did not evaluate PV in the AVCN. However, hearing loss causes up-regulation of PV in the AVCN in other preparations, so it seems likely that this would be the case in the B6 AVCN as well.

Calcium binding proteins such as PV, calbindin (CB), and calretinin are presumed to buffer cytosolic calcium transients in neurons, regulate intracellular calcium concentration, and protect cells from Ca^{2+} overload (Sloviter, 1989). Most importantly, calcium-binding proteins in central auditory neurons respond to cochlear damage. For example, Caicedo et al. (1997) used a glutamate agonist to produce temporary “functional deafferentation” of the auditory brainstem in guinea pigs. One day after the procedure, PV immunostaining was enhanced in

the cochlear nucleus. CB was also increased in the brainstem, while calretinin decreased (calretinin also decreased after cochlear ablation in ferrets; Fuentest-Santamaria et al., 2005b). They concluded that PV and CB were up-regulated as a protective mechanism after deafferentation. CB immunostaining also increased in auditory brainstem after cochlear removal in rats (Forster and Illing, 2000). PV has been used as a marker for central auditory neurons with high levels of activity in hamsters that had cochlear pathology and were prone to epilepsy (Fuentes-Santamaria et al., 2005a). The animals had decreased volume and cell size in the cochlear nucleus and other auditory nuclei which also exhibited greater densitometric immunostaining for PV. The authors interpreted this as reflecting a protective mechanism to prevent cell death in the face of reduced afferent input. Finally, older C57BL/6J and BALB/c mice with severe sensorineural hearing loss exhibited an increase in the percentage of PV and CB in the posteroventral CN (Idrizbegovic et al., 2003, 2004, 2006). Again, a protective mechanism was suggested.

The present study focuses on PV because it is the most prevalent of calcium binding proteins in the AVCN of mice and other species (Bazwinsky et al., 2008; Caicedo et al., 1996; Cruikshank et al., 2001; Lohmann and Friauf, 1996; Por et al, 2005; Vater and Braun, 1994). Given that PV tends to be up-regulated after cochlear damage, it would be predicted that HAAE-treated D2 mice, with reduced hearing loss, would have fewer PV-labeled neurons than controls, particularly in the dorsal (high-frequency) half of the AVCN, which receives input from the basal cochlea. A similar effect would not be predicted for B6 mice because the HAAE treatment did not ameliorate hearing loss, and indeed reduced sensitivity to high frequencies that comprise the HAAE.

2. Methods

2.1 Experimental animals

The D2 and B6 mice were first generation offspring of stock obtained from the Jackson Laboratory, Bar Harbor ME. They were weaned at 23 days of age and given *ad libitum* access to Purina rodent chow and tap water. D2 mice were used in an earlier study (Willott et al., 2006a) in either an HAAE-treated group (N=5) or control group (N=8). B6 mice were used in an earlier study that measured cochlear sensitivity and AVCN cell numbers in gonadectomized and gonadally intact mice (Willott et al., 2006b; 2008). Sex or gonadectomy had little effect on AVCN cell counts, so the experimental design was to pool subgroups to form two groups, HAAE-treated (N= 15) and control (N=15) mice of both sexes that would be comparable to the two D2 groups. A preliminary statistical analysis found these variables to have no significant effect on the number of PV-labeled neurons. All procedures conformed to NIH guidelines for use of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of South Florida.

2.2. Exposure to the AAE

Nightly HAAE treatment was given to D2 mice beginning around the time of birth (with dams present), whereas treatment began at weaning for B6 mice. Mice received consecutive 12-hour nights of AAE treatment until they were euthanized (age 55 days for D2 mice and 9 months for B6). Additional details can be found elsewhere (Willott et al., 2006a; 2008). Control mice (not treated with the AAE) were reared in the normal vivarium conditions.

Equipment and procedures for producing the HAAE are described elsewhere (Willott et al., 2006a; 2008). Briefly, a half-octave noise band centered at 20 kHz (rise/fall = 10 ms, duration = 200 ms, rate = 2/s; mean level within cages: 70 dB SPL re: 20 μ Pa) was synthesized with TDT equipment, and generated from Radio Shack Supertweeters placed on the filter tops above

plastic cages housing the mice. The HAAE signal was automatically timed to be on during the 12-hr dark cycle, when mice are active.

2.3. Preparation of tissue sections

Mice were anesthetized deeply with sodium pentobarbital, and intracardially perfused with physiological saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 minutes. The brain was removed and placed in 4% paraformaldehyde in 0.1M PB at 4°C, followed by 30% sucrose in 0.1M PB for cryoprotection. Serial sections were cut in the frontal plane.

For visualization of PV, a monoclonal mouse anti-PV at a dilution of 1:10,000 (No. P3088, Sigma-Aldrich) was used in the primary incubation. The monoclonal antibody against PV was produced via reaction of purified frog muscle PV with a PARV-19 hybridoma; on a Western blot this is recognized as a 12k-Da protein (data provided by manufacturer; Sigma-Aldrich Co.). After incubation in the primary antibody, the tissues were washed in PB followed by 1 hour of incubation in a 1:200 solution of 0.3% Triton X-100 and the appropriate biotinylated secondary antibody for PV (anti-mouse made in horse, No. BA-2000; Vector Laboratories, Burlingame, CA). Tissues were then washed and processed according to the avidin-biotin complex method (ABC Elite Kit, No. PK-6100; Vector) and visualized with a 0.025% solution of 3'3 diaminobenzadine (DAB) and hydrogen peroxide as described elsewhere (Husband and Shimizu, 1999). Ensuring specificity of the secondary antibody was achieved by omitting the primary antibody during incubation on alternating tissue sections. Following immunohistochemical procedures, serial tissue sections (40 µm) were cut and mounted on glass slides. Slides were then progressively dehydrated in an ethanol series. Following clearance with xylenes, slides were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

2.4. Histological analysis

The analysis of AVCN sections focused on the relationship between the HAAE treatment and counts of labeled neurons in the dorsoventral (tonotopic) dimension, so measurements were made in ventro-dorsal quartile segments in two “standard” frontal sections -- an *anterior* and *posterior* section, approximately 160 µm and 320 µm caudal to the anterior pole of the AVCN, respectively. Tonotopic frequency representation is roughly orthogonal to the dorsoventral axis in these sections (Ryugo et al. 1981; Willott et al. 1982). Sections were optically magnified to 200X using a Nikon Microphot-FXA microscope and photographed with a SPOT Insight QE camera, and SPOT Basic software. Photograph sections were then assembled into composite AVCN images in Adobe Photoshop.

An observer who was “blind” to the mouse’s experimental condition counted labeled neurons in micrographs of each AVCN quartile. A cell was counted if staining within the neuron revealed a complete soma perimeter, and it was clearly differentiated from background. All labeled cells within a section were tallied. Any potential bias was eliminated by the blind counting procedure. Each neuron was marked with a black spot using Photoshop, and the marks were counted with Image-J software (NIH). An example of the material is shown in Figure 1, which also shows how neurons were marked for counting. The counts were made in the four ventro-dorsal segments in the anterior and posterior AVCN sections.

Cell count data were statistically analyzed using 3-way (Treatment × Section × Segment) mixed analysis of variance (ANOVA), where the section and ventro-dorsal quartile segment within each section were repeated (within subject) measures and Treatment (HAAE-treated or control) was an independent variable. Significant 3-way ANOVAs ($\alpha = 0.05$) were followed up with lower-order ANOVAs and Fishers LSD (protected *t*) tests.

3. Results

3.1. Parvalbumin in D2 mice

Figure 2 shows the mean number of PV-labeled neurons for the anterior and posterior sections of HAAE and control D2 mice (the inset shows ABR thresholds of the two groups, from Willott et al., 2006a). The posterior sections are larger, which accounts for the greater number of neurons compared to the anterior sections. It is apparent that, with the exception of the ventral segments, there are more PV-labeled neurons in control mice (filled symbols) compared to HAAE-treated mice (unfilled symbols). This observation is confirmed by the ANOVAs. Of interest in the 3-way ANOVA were significant effects of Treatment [$F(1,14) = 59.8, p < 0.0001$] and interactions of Treatment $\times$ Segment [$F(3,42) = 4.29, p = 0.001$]. Follow-up 2-way mixed ANOVAs at each segment and Fisher's LSD (protected t) tests identified data points where control mice had more labeled neurons than HAAE-treated mice, and these are shown by asterisks in Figure 2.

3.2 Parvalbumin in B6 mice

The number of PV labeled neurons in B6 mice are shown in Figure 3 (the inset shows ABR thresholds derived from the data of Willott et al., 2008). The findings suggest a small effect of HAAE treatment, but the 3-way ANOVA revealed no significant effects involving HAAE treatment.

3.3. Strain comparison of PV-labeled cell densities

Because the number of neurons typically varies in direct relationship to the size of AVCN sections, the density of PV labeled neurons (number of neurons / area) was computed, as shown in Figure 4. The densities indicate an effect of HAAE treatment similar to that shown by the cell counts. In D2 mice (upper panel), control mice had a higher density of labeled cells in the dorsal half of AVCN. This observation is supported by a significant Treatment effect in the 3-way ANOVA [$F(1,11) = 14.79, p = 0.0027$] and follow-up 2-way ANOVAs for each segment showing significant Treatment effects for the two dorsal segments [F 's (1,11) = 14.79, 19.75, p 's < 0.012]. Fisher's LSD tests showed significantly higher densities in control mice in both the anterior and posterior sections (asterisks). Because dorsal AVCN segments tend to be larger in HAAE-treated D2 mice (Willott et al., 2006a), the density differences between treated and control mice are even more striking than those shown by the cell counts (Fig. 2). For example, densities of control mice are almost twice those of HAAE-treated mice in the dorsal AVCN. The comparable cell count differences were less than 50%.

PV-labeled cell densities in B6 mice, like the cell counts, suggest a small effect of HAAE treatment in the posterior section (Fig. 4, lower panel). However, the 3-way ANOVA obtained no significant effects involving HAAE treatment.

4. Discussion

PV immunolabeling of AVCN neurons provides some new insights into the central effects of chronic hearing loss and treatment with AAEs. Before proceeding, however, a word about the HAAE is warranted. Although HAAE refers to "high-frequency AAE," the 20 kHz centered half-octave band is not especially "high" within the hearing range of *Mus musculus*, which can extend two or more octaves higher in various strains. The term was originally used to differentiate it from AAEs encompassing lower frequencies (Willott et al., 2008) and has been retained here for consistency. The relatively intense HAAE is able to excite neurons in much of the dorsal half of the AVCN (segments 3 and 4) of D2 and B6 mice (Willott et al., 1982). Neurons in the more ventral AVCN of D2 mice (segment 2) also respond to the HAAE frequencies because of the less defined tonotopic organization in this strain (Willott et al.,

1982). Thus the significant effects of HAAE treatment in segments 2–4 of D2 mice and in the trend in segment 3 of B6 mice jibe with the neural responses to auditory stimuli.

4.1. PV immunolabeling in the AVCN and chronic hearing loss

As the literature reviewed earlier suggests, PV immunolabeling of AVCN neurons likely reflects a compensatory reaction to physiological stress and altered calcium regulation brought on by cochlear hearing loss. This view is supported by the inverse correlation between hearing loss and the number of PV-labeled neurons in control and HAAE-treated D2 mice, where poorer-hearing control mice had a relatively high number of labeled neurons (Fig. 2). It is certain that control and treated mice did not differ at the start of the experiments because all mice were genetically identical and born into the same environment. Thus, the difference at age 55 days must have been caused by the HAAE treatment. It is likely that this entailed a greater increase in PV up-regulation in control mice as they exhibited greater hearing loss. However, it is also possible that PV activity started at a high level in both groups and decreased in treated mice compared to controls. Both scenarios lead to the conclusion that HAAE treatment caused less PV activity in the AVCN of D2 mice.

When the two strains are compared, it appears that the degree of hearing loss alone cannot account for the number of PV-labeled neurons. Comparison of Figure 2 and Figure 3 shows that B6 mice had more PV-labeled neurons than D2 mice (note the different ordinate scales in the two figures) as do the density measurements (Fig. 4). As seen in the insets to Figure 2 and Figure 3, thresholds are generally higher in D2 mice, so the strain difference cannot be explained by ABR thresholds. The other major strain difference in hearing loss is the time course, which is much more gradual and long term in B6 mice. The 9-month-old B6 mice of the present study have had significant hearing loss for a longer duration than the 55-day-old D2 mice. The B6 mice were of course older as well. Perhaps one or both of these variables contribute to the greater PV labeling in B6 mice. Finally, unknown genetic factors cannot be ruled out.

It is important to note the obvious: the PV-labeled neurons in the present study are *surviving* neurons. In both strains, a portion of AVCN neurons had already died off by the time the experiments were terminated (Willott et al., 1987; Willott and Bross, 1996). It is also the case that little or no additional attrition of AVCN neurons occurs in either strain after the ages that the histology was performed (Willott et al., 1987; Willott and Bross, 1996). Thus, the PV-labeled neurons are presumably viable (the neurons are not in the process of dying), suggesting that chronic hearing loss is accompanied by long-term PV up-regulation.

4.2 .The deprivation hypothesis and HAAE treatment

Because of cochlear damage and threshold elevations in D2 and B6 mice, much ambient sound can no longer activate afferent neural activity to the AVCN, and this must deprive neurons of normal auditory-driven synaptic activity. HAAE treatment may overcome this deprivation in two ways. First the treatment can lessen cochlear pathology and hearing loss, as occurs for D2 mice (Fig. 2 inset; Willott et al., 2006a). This would allow ambient sound to continue stimulating AVCN neurons and maintain a more normal level of synaptic activity simply because the cochlea is healthier. Second, HAAE stimulation increases the auditory-driven afferent activity to AVCN neurons, resulting in a higher level of synaptic activity to reduce deprivation stress on the neurons. Whereas this ameliorative effect of HAAE treatment on the AVCN would be facilitated by the aforementioned beneficial effects on the cochlea, the cochlear effects might not be a necessary factor as long as the HAAE remained suprathreshold.

In D2 mice, the occurrence of fewer PV-labeled neurons following HAAE treatment indicates a reduction of deprivation stress, but the contributions of reduced cochlear pathology and

increased HAAE-evoked stimulation cannot be differentiated because both are present. In B6 mice, however, HAAE treatment made cochlear pathology and threshold elevations even worse than in controls, eliminating the first possibility. Because of lessened ambient stimulation in HAAE-treated mice, up-regulation of PV labeling would be expected, but this did not occur. The difference between HAAE-treated and control B6 mice was not statistically significant (although slightly *fewer* PV-labeled neurons were found in HAAE-treated B6 mice). This finding presents two possibilities for interpretation. First, the HAAE may have provided enough suprathreshold stimulation of AVCN neurons to prevent additional PV up-regulation that would have been caused by the exacerbated threshold elevations. The findings suggest that augmented acoustic stimulation can have some ameliorative effect even when hearing loss becomes very severe. The second possible interpretation is that the increased thresholds from HAAE treatment had the potential to cause additional up-regulation of PV, but the number of PV-positive neurons in controls was so high that further up-regulation could not occur. For example, comparing the number of PV-labeled neurons in the present study with the total number of Nissl-stained neurons from Willott et al. (2008), about 72% of the neurons in the posterior section of B6 control mice were labeled. There may have been a kind of ceiling effect for PV up-regulation where all of the neurons capable of exhibiting up-regulation had done so in both groups. The findings raise the possibility that after a certain degree of hearing loss and central reaction have been reached, additional hearing loss need not result in a greater central response.

4.3. Conclusions

The present findings support other studies in showing that hearing loss is accompanied by up-regulation of PV that is presumably associated with a perturbation of calcium regulation. Importantly, the reaction can be mitigated by augmenting the acoustic environment. This may even occur in the presence of substantial hearing loss as long as the AAE is suprathreshold. If chronic up-regulation of calcium binding proteins were to cause or be correlated with negative effects on neuronal physiology, then reversing the reaction with AAE treatment may have clinical implications for treating central responses to hearing impairment.

LIST OF ABBREVIATIONS

AAE	augmented acoustic environment
ANOVA	analysis of variance
AVCN	anteroventral cochlear nucleus
B6	C57BL/6J inbred mouse strain
CB	calbindin
CN	cochlear nucleus
D2	DBA/2J inbred mouse strain
HAAE	high frequency augmented acoustic environment
PV	parvalbumin
SPL	sound pressure level

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Figure 1. Example of histologic material. On the left is a low magnification view of the dorsal half of the AVCN of a B6 mouse. The inset box is magnified on the right. Below this are cells marked for counting with ImageJ. Faintly stained neurons were judged by a blind observer as not sufficiently labeled to be included.

Figure 2.

Mean number of PV-labeled neurons in D2 mice for the standard posterior and anterior AVCN sections. Error bars are standard errors of the mean; asterisks indicate statistically significant difference between control and corresponding HAAE-treated group. Inset shows mean ABR thresholds for control and HAAE-treated mice (from Willott et al., 2006a). Treated mice have significantly lower thresholds for tones of 16–32 kHz. As suggested by the ABR thresholds (Willott et al., 2006a), the mice would be able to hear the HAAE at the beginning of the experiment, and many neurons throughout the ventro-dorsal extent of the AVCN would have thresholds low enough to respond to the HAAE (Willott et al., 1982). At age 55 days, sensitivity to the HAAE is greatly reduced and (inset), and few neurons of control mice would be able to respond to the 70 dB HAAE (cf. Willott and Turner, 2000). Neurons of treated mice would presumably continue to respond to the HAAE.

Figure 3.

Mean number of PV-labeled neurons in B6 mice for the standard posterior and anterior AVCN sections. Error bars are standard errors of the mean. Inset shows mean ABR thresholds for control and HAAE-treated mice (from Willott et al., 2006b). Treated mice have significantly higher thresholds for tones of 12–24 kHz. At the beginning of the experiment, neurons throughout the AVCN would respond robustly to the HAAE (Willott et al., 1982; 1991). However, at 9 months of age (inset), ABR thresholds are barely below 70 dB SPL, suggesting poor responsiveness of AVCN neurons to the HAAE.

Figure 4.

Mean density (number of neurons / segment area) of PV-labeled neurons in D2 and B6 mice for the standard posterior and anterior AVCN sections. Error bars are standard errors of the mean; asterisk indicates statistically significant difference between control and corresponding HAAE-treated group.