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EFFECTS OF EXPOSING C57BL/6J MICE TO HIGH- AND LOW-FREQUENCY AUGMENTED ACOUSTIC ENVIRONMENTS: AUDITORY BRAINSTEM RESPONSE THRESHOLDS, CYTOCOCHLEOGRAMS, ANTERIOR COCHLEAR NUCLEUS MORPHOLOGY AND THE ROLE OF GONADAL HORMONES

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Abstract

Gonadectomized and intact adult C57BL/6J (B6) mice of both sexes were exposed for 12 hours nightly to an augmented acoustic environment (AAE): repetitive bursts of a 70 dB SPL noise band. The high-frequency AAE (HAAE) was a half-octave band centered at 20 kHz; the low-frequency AAE (LAAE) was a 2-8 kHz band. The effects of sex, gonadectomy, and AAE treatment on genetic progressive hearing loss (a trait of B6 mice) were evaluated by obtaining auditory brainstem response (ABR) thresholds at ages 3-, 6-, and 9-months. At 9-months of age, hair cell counts (cytococholeograms) were obtained, and morphometric measures of the anteroventral cochlear nucleus (AVCN) were obtained. LAAE treatment caused elevation in ABR thresholds (8-24kHz), with the highest thresholds occurring in intact females. LAAE treatment caused some loss of outer hair cells in the basal half of the cochlea (in addition to losses normally occurring in B6 mice), with intact females losing more cells than intact males. The loss of AVCN neurons and shrinkage of tissue volume that typically occur in 9-month-old B6 mice was lessened by LAAE treatment in intact (but not gonadectomized) male mice, whereas the degenerative changes were exacerbated in intact (but not gonadectomized) females. These LAAE effects were prominent in, but not restricted to, the tonotopic low-frequency (ventral) AVCN. HAAE treatment resulted in some loss of neurons in the high-frequency (dorsal) AVCN. In general, LAAE treatment plus male gonadal hormones (intact males) had an ameliorative effect whereas HAAE or LAAE treatment plus ovarian hormones (intact females) had a negative effect on age-related changes in the B6 auditory system.

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1. Introduction

The present study is the latest in a series investigating the effects of treatment with augmented acoustic environments (AAEs) on progressive hearing loss and accompanying anatomical changes in mice of the C57BL/6J (B6), DBA/2J (D2), and other inbred strains of mice that exhibit genetic age-related hearing loss (Turner and Willott, 1998; Willott and Turner, 1999; Willott and Bross, 2004; Willott et al., 2000; 2005; 2006a,b). Treatment using a middle-frequency noise band AAE (MAAE; peak levels from 8-20 kHz) of moderate intensity (70 dB SPL re: 20 μ Pa) has generally ameliorated age-related hearing loss in several strains. MAAE treatment has also lessened degenerative changes in portions of the cochlea and anteroventral cochlear nucleus (AVCN) of B6 and D2 mice. However, AAE treatment has also had some negative effects. In B6 mice evaluated at age 12-14 months, MAAE treatment exacerbated neuronal loss in the ventral and dorsal extremes of the AVCN (Willott and Bross, 2004). Treatment of D2 mice with a high-frequency AAE (HAAE, centered at 20 kHz) increased the loss of inner hair cells (IHCs) in the extreme basal (high-frequency) cochlear region, while having otherwise beneficial effects on the survival of hair cells and AVCN neurons (Willott et al., 2006a). The same HAAE worsened high-frequency hearing loss and hair cell loss in 9-month-old B6 mice (Willott et al., 2006b). The present study evaluates the central correlates of the negative HAAE effects in B6 mice, as well as the peripheral and central effects of treatment with a low-frequency AAE (LAAE) that has not been employed previously.

The present study first compares the effects of LAAE treatment in B6 mice on ABR thresholds and cytochleograms with those previously reported for HAAE treatment. The 2-8 kHz LAAE noise band covers a range of frequencies for which threshold elevations are relatively mild (about 10 dB) in B6 mice by 9 months of age (the end point in this study). Thresholds for frequencies within the HAAE noise band around (20 kHz) exhibit more severe elevations by age 9 months. Thus, the two AAEs stimulate regions of the cochlea with different vulnerabilities to genetic damage in B6 mice.

A second aspect of the present study was to evaluate the central effects of both HAAE and LAAE treatment on morphological measures in the AVCN. The AVCN is tonotopically organized, with a general representation of low frequencies ventrally to high frequencies dorsally. Thus, it is important to determine the effects of increased auditory-evoked neural activity from LAAE or HAAE stimulation on tonotopic regions of the AVCN.

A third aspect of the study is part and parcel of the other two: to further examine sex differences in hearing loss in B6 mice and effects of AAE treatment thereon. Our strategy is to employ gonadectomized mice (gonadal hormone levels are greatly reduced) and intact mice. Several studies have shown that high-frequency hearing loss becomes more severe in female B6 mice by 6-9 months of age (Henry, 2002, 2004; Willott and Bross, 2004; Willott et al., 2006b). Moreover ovarian hormones may contribute to this deficit in females (Willott et al., 2006b). Thus, all analyses use both gonadectomized and intact mice of both sexes.

The present study used a new cohort of mice exposed to the LAAE for ABR thresholds, cytochleogram, and AVCN analysis plus AVCN tissue from the mice used in the previous study of HAAE effects on ABR thresholds and cytochleograms. Both cohorts consisted of intact and gonadectomized male and female mice. Control mice (not treated with an AAE) were included in both cohorts.

2. Methods

2.1 Experimental animals

C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, ME at age 4–6 weeks. Half of the mice received gonadectomies at age 2–3 weeks by highly experienced surgeons at the Jackson Laboratory before being shipped. Mice served in one of 12 subject groups based on sex (M, F), gonadal status (gonadally intact [int], ovariectomized [OVX], orchidectomized [ORX]), and AAE treatment (high AAE [HAAE], low AAE [LAAE], control [con]). The 12 groups were as follows: intFcon (n = 9), intMcon (n = 9), OVXcon (n = 7), ORXcon (n = 7), intFHAAE (n = 4), intMHAAE (n = 5), OVXHAAE (n = 5), ORXHAAE (n = 5), intFLAAE (n = 8), intMLAAE (n = 4), OVXLAAE (n = 7), ORXLAAE (n = 6). Young control B6 mice (5 males; 1 female) 6 weeks old, (Ycon) were also tested but not used in analysis of variance. ABR thresholds and body weights do not differ significantly between males and females at this age (e.g., Turner and Willott, 1998). Body weights at 9 months of age were largest in the three intact male groups (36.5g, 36.5g, 37.3g), followed by the three OVX groups (30.9g, 31.3g, 33.4g), ORX male groups (28.1g, 29g, 32.6g), intact female groups (22.5g, 23.4g, 24.8g), and young controls (17 g).

The room temperature in the vivarium was maintained between 23°C and 24°C and a 12-hour light/dark schedule was used. Mice were given *ad libitum* access to Purina rodent chow and tap water.

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

Upon receipt from The Jackson Laboratory (age 4–6 weeks), mice were placed in a plastic cage (12 × 13 × 30 cm), 4 mice per cage. They received 12-h per night of AAE treatment every night through 9 months of age in the vivarium.

The HAAE was a half-octave noise band centered at 20 kHz; the LAAE was a 2–8kHz noise band (both: rise/fall = 10 ms, duration = 200 ms, rate = 2/s) synthesized with Tucker-Davis Technologies (TDT) equipment and downloaded to an Apple Mini-ipod. This signal was amplified with a Radio Shack MPA-200 audio amplifier and sent to Radio Shack Supertweeters placed on the filter tops above the plastic cages. The amplifier gain was set so that the mean SPL within the cages was 70 dB. The level was calibrated by inserting the microphone of a Brüel and Kjær Type 2203 Precision Sound Level Meter coupled to a Type 1613 octave band filter into holes drilled in various locations of a calibration cage. The signal was automatically timed to be on during the 12-hr dark cycle. Mean ambient noise levels in the vivarium were less than 55 dB SPL at octave bands centered at 2 kHz to 31.5 kHz.

2.3. ABR thresholds

ABR thresholds were obtained at ages 3 months, 6 months, and 9 months with a range of ± 2 weeks due to the large number of mice to be tested. Testing was performed at midday (noon ± 1.5 hours). The order of testing was balanced among the different subject groups for all three test sessions.

The procedures have been described elsewhere (Willott et al., 2006a,b). Stimuli were synthesized and produced using TDT System 3 hardware and software and an ESL electrostatic speaker (Tucker-Davis Technologies), which has a relatively flat frequency response between 2 kHz and 100 kHz. The speaker was mounted 20 cm in front of the mouse's head in the free field in a foam-padded, shielded acoustic chamber. The output was calibrated by placing the

Brüel & Kjær microphone in the space to be occupied by the mouse's head and determining the appropriate setting for the desired acoustic output. A maximum SPL (re: 20 μ Pa) of 80 dB was employed for all stimuli.

Mice were anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (20 mg/kg), and acepromazine (3 mg/kg) and kept warm with a heating pad during ABR recordings. A subdermal (active) needle electrode was inserted at the vertex, and ground and reference electrodes were inserted subdermally in the loose skin beneath the pinnae of opposite ears. TDT System 3 hardware and software were used to obtain ABRs, with 500 stimulus repetitions per record. Preamplifier filters were set at 100 Hz high pass and 3000 Hz low pass.

Mice were tested with tone pips (1 ms rise/fall; 3 ms duration; 4, 8, 12, 16, 24, and 32 kHz). ABRs were obtained at each frequency by reducing the SPL in 5 dB steps beginning with a maximum of 80 dB SPL. Threshold was defined as the lowest 5 dB step at which an ABR could be recognized. Judgment of threshold was made from ABR records off-line by two independent, experimentally blind observers. The two observers obtained the same thresholds in the vast majority of cases and any discrepancies were rarely more than 5 dB. Minor between-rater discrepancies in thresholds were resolved, subject to agreement by both observers. If ABRs were not evoked by the maximum SPL of 80 dB, a nominal threshold of 85 dB was assigned.

2.4. Preparation of AVCN tissue sections

At 9 months of age, 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 at least 12 hours for cryoprotection. Cochleas were removed after perfusion, the same time as the brains, placed in 4% paraformaldehyde in 0.1M PB, and shipped for processing and analysis.

2.5. Cytocochleograms

Cytocochleograms were prepared at the University at Buffalo under subcontract to Dr. Richard Salvi. The procedures for preparing and analyzing mouse inner ears have been described in detail in previous reports (McFadden et al., 1999a; McFadden et al., 1999b; Willott et al., 2006b). Briefly, the organ of Corti was carefully microdissected out from base to apex, and specimens stained with Ehrlich's hematoxylin solution (Ding et al., 2001). Specimens were mounted in glycerin on glass slides and examined with a light microscope with differential interference contrast optics (Zeiss Standard, 400X magnification). Hair cells were counted as present if the cell body and cuticular plate were intact. Cytocochleograms were constructed by plotting the percentage of missing OHCs or IHCs as a function of percent distance from the apex of the cochlea. Percentages were computed relative to laboratory norms for young CBA mice (Spongr et al., 1997).

2.5. Analysis of AVCN sections

After several days of fixation, the brain was bisected midsagittally, and the right hemisphere was frozen and cut in frontal sections at 40 μ m on a sliding microtome. Serial sections were mounted on gelatin-coated glass slides, stained with cresyl violet (Sigma), and coverslipped using Permount. The area and shape of AVCN sections and size of neurons were measured using Nissl-stained AVCN sections. All measurements were made by observers who were "blind" to the experimental condition.

Serial sections from the rostral pole of the AVCN to its caudal border (entrance of the eighth nerve) were visualized with a Nikon SMZ-1500 stereoscope, and photographed at 40X

magnification. Photographed images were loaded into graphics software (Canvas 9.0; ACD Systems, Miami, FL). These were used to identify the two standard sections for detailed analysis. With the rostral pole of the AVCN as a reference, the fourth section was designated as the *anterior section* and the 8th section as the *posterior section*. (i.e., approximately 160µm and 320µm posterior to the rostral pole, respectively). Measurements were made on these two standard frontal sections of each mouse, as in our previous studies (Willott and Bross, 2004; Willott et al., 2005). These sections lie, respectively, within the anterior and posterior divisions of the AVCN in the atlas of Trettel and Morest (2001), where additional cytoarchitectonic details may be found. Tonotopic frequency representation is roughly orthogonal to the ventro-dorsal axis (Berglund and Brown 1994; Ramon y Cajal, 1909; Ryugo et al. 1981; Willott et al. 1982).

2.5.1. Segmentation of AVCN sections—Because this study was designed to determine the frequency specificity of AAE effects, the detailed analysis of AVCN sections focused on the relationship between the high- or low-frequency AAE treatment and morphological properties in the ventro-dorsal (tonotopic) dimension. It was expected that the high- and low-frequency AAEs would have different effects on tissue along this dimension. 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 Elements. The 200X optical magnification provided sufficient detail to clearly identify neurons while providing a depth of field allowing visualization of all neurons in a section on a single focal plane. To reflect AVCN tonotopicity, the imaging software was used to separate each posterior section into 8 segments of equal ventro-dorsal distance. The smaller anterior sections were divided into 4 ventro-dorsal segments. Thus, the ventro-dorsal segments roughly paralleled tonotopicity. In both sections, segments were typically about 100 µm in height. All neurons within the anterior and posterior AVCN sections were outlined and filled with black using Photoshop Elements. No attempt was made to categorize neuron type (e.g., spherical, multipolar cells). The cell types are similar in size and often difficult to distinguish in the mouse AVCN (see Trettel and Morest 2001) and, more importantly, it might be the case that degenerative changes would confound differentiation of type in B6 mice. ImageJ software (NIH) was then used to filter background, isolate neuron masks, and compute the area of each neuron, the number of neurons in each segment, and the area of each segment.

3. Results

3.1 ABR thresholds

A summary of ABR thresholds for all groups (age 9 months) used in the present study on LAEE treatment are shown in Figure 1. Data for tests at 3 and 6 months are not shown because effects occurring at 9 months were not yet present (except for an emerging sex effect at 6 months). Females are indicated by black symbols, males by green; Young B6 (Ycon;6 weeks old) are in red (these are for comparison with 9-month-olds but were not used in ANOVAs). The frequency spectrum of the LAEE is shown by the shaded bar. It is obvious that all 9-month-old groups exhibited threshold elevations at 24 and 32 kHz compared to young B6 controls. Groups also differed from one another. The group differences were confirmed by a 2-way mixed ANOVA (8 groups × 6 test frequencies, with the latter as a repeated measure). There was a main effect of Group [$F(7,60) = 6.64, p < 0.0001$] and Group × Frequency interaction [$F(35,300) = 3.00, p < 0.0001$]. Additional analyses of sex, LAEE treatment, and gonadectomy effects are presented below.

3.1.1. Sex effect—A significant sex effect occurred, with female mice exhibiting more severe ABR threshold elevations than males at age 9 months. For example a 2-way ANOVA

comparing intact males and females at 9 months showed a main effect of Sex [$F(1,15) = 28.13$, $p < 0.001$] and Sex $\times$ Frequency interaction [$F(5,75) = 8.93$, $p < 0.0001$], with Fisher's LSD tests showing superior male thresholds at 8-32Kz. This is especially clear graphically at 24 kHz, where all four male groups (green symbols) are clearly separated from the four female groups (black symbols).

3.1.2. Effects of LAEE treatment and gonadectomy

3.1.2.1. Males: Treatment with the LAEE in males resulted in more severe threshold elevations at some frequencies compared to control (untreated) males. This can be seen in the middle panel of Figure 1, where intMcon and ORXcon (green filled and unfilled circles) had lower ABR thresholds than their LAEE-treated counterparts, intMLAAE and ORXLAAE. A 3-way mixed ANOVA for male groups (2 treatment conditions $\times$ 2 gonadal conditions $\times$ 6 test frequencies) showed a significant effect of LAEE Treatment [$F(1,31) = 11.34$, $p = 0.0046$] and LAEE Treatment $\times$ Frequency interaction [$F(5,155) = 3.14$, $p = 0.0098$]. Fisher's LSD tests showed greater threshold elevations in LAEE treated males from 8-24 kHz.

In males, orchidectomy had little effect on ABR thresholds, as there were no significant differences between intMcon and ORXcon, or between intMLAAE and ORXLAAE groups (see middle panel of Fig. 1).

3.1.2.2. Females: Ovariectomy was a significant variable, having a beneficial effect on LAEE-treated females. The 3-way ANOVA on females showed a main effect of Gonadal status [$F(1,29) = 14.52$, $p = 0.0007$] and Gonadal status $\times$ Frequency interaction [$F(5,145) = 2.3$, $p = 0.036$]. The effects of LAEE treatment were detrimental in intact females (intFLAAE), as was the case for males. However, this was not the case for ovariectomized mice treated with the LAEE (OVXLAAE). Indeed, ABR thresholds of OVXLAAE mice were lower than those of intFLAAE mice between 4-16 kHz (Fisher's LSD tests). At 16 kHz, thresholds of OVXLAAE were the lowest of any group (lower panel of Fig. 1, black diamonds), with Fisher's LSD tests significant against intFLAAE and intFcon mice. In other words, ovariectomy ameliorated hearing loss induced by the LAEE.

3.2. Cytocochleograms

Cytocochleograms for OHCs and IHCs are presented in Figures 2 and 3, respectively. In each figure, cytocochleograms for the LAEE-treated mice are shown in the upper panel. The lower panel presents both LAEE data and HAAE data from the earlier paper, with intact and gonadectomized groups combined to make the AAE comparisons easier. The abscissa represents the percent distance from the apex (i.e., 100% = extreme base)

3.2.1. Outer hair cells—For mice treated with the LAEE (upper panel of Figure 2) only one comparison of interest was significant: intFLAAE females (black squares) had more severe hair cell loss than intMLAAE males (green squares). The 2X10 ANOVA obtained a significant Group $\times$ Segment interaction [$F(9,99) = 2.29$, $p = 0.023$], with Fisher's LSD tests showing the intact males to have more OHCs in segments 70% and 80% (distance from the apex).

Comparing the effects of LAEE and HAAE treatment on OHCs (Fig. 2, lower panel), substantial group differences are evident for the basal half of the cochlea, with HAAE-treated mice faring worse than LAEE mice. The differences are confirmed by the 2-way mixed ANOVA (6 groups $\times$ 10 cochlear segments as a repeated measure) with a main effect of Group [$F(5,52) = 6.23$, $p = 0.0001$] and Group $\times$ Segment interaction [$F(45,468) = 5.09$, $p < 0.0001$]. Among males, HAAE-treated mice had a more severe loss of OHCs than controls or LAEE-treated mice for the 60%, 70%, and 80% segments. The least severe OHC loss is seen in male control mice (Mcon, filled green circles). For female groups FHAAE mice (black diamonds)

had more severe OHC loss at the 70% and 80% segments than the other groups (follow-up ANOVAs and Fisher's LSD tests).

[illegible]

Mean IHC cytochrome c oxidase (COX) immunohistochemical (IHC) staining comparing LAE- and HAE-treated mice are shown in the lower panel of Figure 3. Cochleas of HAE-treated mice had more severe hair cell loss than LAE-treated mice. The 2-way ANOVA found a main effect of Group [$F(5,53) = 4.72, p = 0.0012$] and Group X Segment interaction [$F(45,477) = 6.46, p < 0.0001$]. Follow-up ANOVAs and Fisher's LSD tests confirmed that HAE groups had more severe loss of IHCs than LAE groups in basal segments (70% to 90% segments in FHAE; 80% to 100% segments in MHAE).

3.3. AVCN analysis

The effects of AAE treatment on AVCN morphology were evaluated for mice used in the LAEE experiments together with those used in the HAAE experiments (whose ABR thresholds and cytochleograms were first reported in the Willott et al., 2006b). The overall size of AVCNs (summing the serial sections from which the standard posterior and anterior sections were chosen) did not differ significantly among the groups. The strategy was to first obtain an overall ANOVA for the 8 segments of the posterior section or the 4 segments of the anterior section using all 12 subject groups, then follow up with additional ANOVAs for comparisons of interest. Young control B6 mice (Ycon) were not used in the ANOVAs but were available for comparisons with the 9-month-olds as needed.

The analyses focus on two measures: the number of neurons per ventro-dorsal AVCN segment and the area of each segment. Measures that did not differ significantly among groups were the size (filled area) of neurons and the packing density (number of neurons per mm² of each section, and these are not analyzed here.

3.3.1. Posterior section—The standard posterior sections were divided into 8 ventro-dorsal segments paralleling the low-to-high tonotopic organization of the AVCN. As a reference to this, the upper left and right portions of each panel indicate the segments for which thresholds in young B6 mice are less than 50 and 60 dB SPL for frequencies within the LAEE and HAAE bands (based on a multiple-unit, frequency-threshold mapping study of Willott et al., 1982).

3.3.1.1. Number of AVCN neurons: For clarity, the numbers of AVCN neurons for each ventro-dorsal segment are separated with respect to HAAE and LAEE in Figure 4 (control groups appear in each panel). However, ANOVAs were performed on the entire set of 12 groups. The overall ANOVA obtained main effects of subject Group [$F(11, 64) = 2.98, p = 0.003$] and AVCN Segment [$F(7,77) = 62.9, p < 0.0001$]. To further investigate the differences among segments and groups, 1-way ANOVAs were performed for each segment, followed by Fisher's LSD tests.

The neuron counts varied most among groups in the low-frequency (ventral) and high-frequency (dorsal) regions of the AVCN (Fig. 4). In the low-frequency, ventral region ANOVAs were significant for segments 1 [$F(11,64) = 2.36, p = 0.016$] and 2 [$F(11,64) = 2.41, p = 0.014$]. Fisher's LSD tests showed the intMLAAE males (lower panel, green squares) to have the most neurons, and the intFLAAE females (lower panel, black squares) and ORXHAAE males (upper panel, green diamonds) to have the least neurons. In the high-frequency, dorsal region, 1-way ANOVAs were significant for segments 7 and 8 [$F(11,64) = 1.92, p = 0.05$ for each]. The most neurons were found in the intMLAAE males (lower panel,

green squares) and the least in the intFHAAE (upper panel, black squares) and OVXHAAE females (upper panel, black diamonds). Fisher's LSD test showed each of these group to differ significantly from at least 4 other groups.

Several observations from Figure 4 are supported by the statistics. First, the number of surviving neurons in the intMLAAE males (lower panel, green squares) was similar to the number of neurons in young controls (Ycon, red diamonds). Indeed, the group of intact males treated with the LAEE was the only group that did not have significantly fewer neurons than Ycon (2-way mixed ANOVAs comparing Ycon with other groups had p 's < 0.05).

LAEE-treated, intact females (intFLAAE) had the fewest neurons in the ventral, low-frequency AVCN, especially in segment 2 (Fig. 4, lower panel, black squares). In the dorsal, high-frequency AVCN, females treated with the HAAE had relatively few neurons irrespective of their gonadal status (upper panel, black squares and black diamonds).

To highlight these influences of sex, AAE treatment, and gonadal status, Figure 5 shows the neuron counts in segments 2 and 7, respectively. Tonotopicity of segment 2 corresponds to the LAEE, and segment 7 corresponds to the HAAE. Among the intact mice (symbols to left), the intMLAAE males (filled green squares) had the most neurons in both the ventral and dorsal segments. The intFLAAE females had the fewest neurons in the segment 2 (upper panel, filled black squares), whereas intact females treated with the HAAE had relatively few neurons in segment 7 (lower panel, unfilled black squares). In general, orchidectomy in males was associated with fewer neurons, whereas ovariectomy in females was associated with more neurons or was neutral.

3.3.1.1.1. Sex effect: Evidence for a general sex difference was not strong. Sex differences were not significant comparing intact groups intMcon versus intFcon or intMHAAE versus intFHAAE. The ANOVA for intMLAAE versus intFLAAE showed a main effect of Group [$F(1,10) = 6.18$, $p = 0.03$], but the intMLAAE groups had more neurons than other groups irrespective of sex.

3.3.1.2. Area of ventro-dorsal segments: The mean area of each ventro-dorsal segment for the posterior section are shown in Figure 6. Again, the intMLAAE group stands out (lower panel, green squares). The 2-way ANOVA for all groups found a significant main effect of Segment [$F(7,441) = 199.5$, $p < 0.0001$], again primarily due to the intMLAAE males. The 1-way ANOVA for segment 2 was significant [$F(11,63) = 2.07$, $p = 0.036$], and Fisher's LSD test showed the MLAAE mice to have a larger segment 2 than all other groups except intMcon (filled green circles). Other groups did not differ significantly from one another.

As was the case for the number of neurons, the only significant sex differences among intact mice was between intMLAAE and intFLAAE. There was a Group X Segment interaction [$F(7,70) = 3.26$, $p = 0.005$], with segments 1-3 and 7-8 being larger in males.

3.3.2. Anterior section—The anterior section is somewhat smaller than the posterior section. Although low frequency (ventral) to high frequency (dorsal) tonotopicity is present at this rostro-caudal level, we do not have sufficient physiological data to indicate the segments specifically representing the LAEE and HAAE, and have not done so in the figures.

3.3.2.1 Number of AVCN neurons: The number of neurons in the 4 ventro-dorsal segments of the standard anterior section is shown in Figure 7, and intMLAAE males and intFLAAE females again stand out with the most and least ventral AVCN neurons, respectively. The 2-way ANOVA obtained a main effect of Segment [$F(3,33) = 29.12$, $p < 0.0001$], but not of Group. Examination of Figure 7 (lower panel) suggests that this is because of LAEE treatment in these

two groups, especially in segment 2 (compare green and black squares). This is supported by a significant 1-way ANOVA for segment 2 [$F(11,61) = 1.99, p = 0.045$] and Fisher's LSD tests. The latter showed that, in segment 2, intMLAAE mice (lower panel, green squares) had significantly more neurons than 8 of the other groups, whereas intFLAAE mice (lower panel, black squares) had significantly fewer neurons than 7 of the other groups. The intMLAAE group did not differ significantly from the Ycon group.

As was the case for the posterior segment, the only significant sex difference for intact mice (Sex X Segment mixed ANOVAs) was for intMLAAE and intFLAAE [$F(1,10) = 11.04, p = 0.008$].

3.3.2.2. Area of ventro-dorsal segments: The area of the 4 ventro-dorsal segments of the anterior section is shown in Figure 8. As was the case for neuron counts, intMLAAE males had the largest ventral segments, and intFLAAE mice had the smallest (compare lower panel green and black squares). The 2-way (12 groups X 4 segments) ANOVA obtained a main effect of Group [$F(11,61) = 1.2, p = 0.044$] and Group X Segment interaction [$F(33,183) = 1.86, p = 0.006$]. The 1-way ANOVA for segment 1 (ventral) was significant [$F(11,61) = 2.25, p = 0.022$], and Fisher's LSD tests showed intFLAAE females to have smaller segments than 9 groups. For segment 2 the ANOVA was significant [$F(11,61) = 2.15, p = 0.029$], with Fisher's LSD tests showing segments of intMLAAE males (lower panel, green squares) to be larger than all other groups, and intFLAAE females (lower panel, black squares) to be smaller than 7 groups.

As was the case for other measures (above) the only significant sex effect was intMLAAE versus intFLAAE [$F(1,9) = 12.20, p = 0.007$]. The intMLAAE group did not differ significantly from young controls.

4. Discussion

The present study found that ABR thresholds, hair cell loss, and AVCN morphology were affected by the type of AAE (LAAE, HAAE), sex and/or gonadal status, and tonotopicity of tissue regions. However, all these factors must be considered together.

4.1. Hearing loss / cochlear pathology

4.1.1. Sex effect on hearing loss—The sex effect found in earlier studies (Henry, 2002; Willott and Bross, 2004; Willott et al., 2006b) was again replicated. Intact female B6 mice that were not treated with an AAE exhibited higher thresholds than males at age 9 months (Fig. 1), whereas no sex differences were observed when they were tested at age 3 months. Cytocochleograms of untreated control mice showed greater hair cell loss in females compared to males (Figs. 2, 3), but these differences were not statistically significant. Nonetheless, the greater ABR threshold elevations in females are likely related to cochlear damage. It may be that the dysfunction or damage fell short of hair cell loss, and that other metabolic markers of hair cell function might reveal these sex differences (e.g., Ding et al., 1999). Also, using a greater number of cytochleograms in the analysis might results in a statistically significant effect (the sex difference barely missed a $p < .05$). More will be said about the sex effect below.

4.1.2. Effects of AAE treatment on hearing—Treatment with the LAAE exacerbated ABR threshold elevations in the intMLAAE and ORXLAAE males and the intFLAAE females, which had the highest thresholds of all. The increased threshold elevations in these groups occurred for 8-24 kHz tones (Fig. 1) -- frequencies extending upwards from the high cutoff of the LAAE spectrum. Willott et al. (2006b) showed that HAAE treatment caused ABR threshold elevations at 24 and 32 kHz -- frequencies that were also at the upper cutoff and above the HAAE spectrum. OHC cytochleograms of the intFLAAE females (Fig. 2, upper panel, black

squares) indicate more severe loss than intMLAAE males (green squares) in cochlear regions serving frequencies higher than the LAEE. In the study by Willott et al. (2006b), the HAAE also caused hair cell loss in the basal cochlear regions serving frequencies higher than the HAAE.

The findings of “upward spreading” of ABR threshold elevations and hair cell loss from both LAEE and HAAE noise bands suggest that noise-induced hearing loss may be responsible. It is well known that noise-induced threshold elevations generally affect frequencies above the exposure noise spectrum, typically 0.5 to 1 octave above the center frequency of the noise and that the basal (high-frequency) cochlea is most vulnerable (see Colvin and Luxon, 2007 for a recent review). Although the AAEs were only 70 dB SPL, it is known that B6 mice are especially vulnerable to noise-induced hearing loss (Davis, 2001; Davis et al., 2003; Harding et al., 2005; Holme and Steel, 2003; Vasquez et al., 2004). Taken together, the findings suggest that prolonged exposure to the LAEE or HAAE plus the genetic vulnerability of B6 mice caused some damage to the cochlea.

4.1.3. Interaction of hormones and AAE treatment—Hormonal status appears to interact with AAE treatment in affecting auditory sensitivity in B6 mice. The most deleterious effects of either LAEE treatment (present study) or HAAE treatment (Willott et al., 2006b) on ABR thresholds and OHCs occurred in intact females. This was also the case for AVCN effects discussed below. These findings, while preliminary, suggest that ovarian hormones in some way facilitated noise-induced damage. Supporting this notion is a study by Shen et al. (2007) that found protective effects of calcium channel blockers on noise-induced hearing loss to be greater in male B6 mice.

If female B6 mice are exceptionally vulnerable to noise-induced hearing loss, it is conceivable that the sex effect typically observed in B6 mice (Henry, 2002; Willott and Bross, 2004; Willott et al., 2006b) might be due to noise-induced hearing loss. That is, a relatively low level of ambient sound in the vivarium might cause females to have greater cochlear damage and hearing loss than males by middle age. Note that intact female controls (intFcon) mice had slightly higher thresholds than OVXcon females (Fig. 1, lower panel), albeit not statistically significant. Thus, the sex difference in B6 mice may indeed be modulated by hormones, but only in the context of noise-induced hearing loss.

4.2. The AVCN

The findings for both the posterior and anterior AVCN sections present a consistent picture with respect to the number of neurons and size of segments in the ventral, low-frequency region of 9-month-old B6 mice when LAEE treatment is used. Intact males treated with the low-frequency AAE (intMLAAE) exhibited the most surviving neurons among 9-month-olds in both the anterior and posterior AVCN sections (lower panels of Figs. 4 and 7, and Fig. 5, green squares). In contrast, intact females treated with the same low-frequency AAE (intFLAAE) exhibited the greatest loss of neurons (black squares). The relatively high number of neurons in the intact male mice (intMLAAE) and low number in the intact females (intFLAAE) were paralleled by larger and smaller AVCN segments respectively (lower panels of Figs. 6 and 8). Thus, it appears that the combination of LAEE treatment and male gonadal hormone(s) ameliorates the progressive degenerative changes in the ventral AVCN, whereas the combination of LAEE treatment plus ovarian hormone(s) exacerbates these changes.

Although the intact males had larger body weights than intact females (see Methods), this does not appear to be a factor with respect to segment size or neuron number. The segment size effect was observed for only some segments. Moreover, the number of neurons is presumably not determined by body weight.

With respect to high-frequency AAE effects, several groups of HAAE-treated mice wound up with relatively few neurons in the dorsal, high-frequency segments (posterior AVCN section), and there were no ameliorative effects in any segments. A preponderance of negative effects was also the case for ABR thresholds and cytochrome c oxidase of HAAE-treated mice (Willott et al., 2006b). It would appear that stimulation of the vulnerable high-frequency regions of the B6 auditory system generally exacerbates the genetically mediated damage.

4.3. Role of hormones

Gonadal hormones have the potential to affect the auditory system both peripherally and centrally (reviewed in detail by Willott and Bross, 2004; 2006b). An earlier hypothesis (Willott and Bross, 2004) proposed that ovarian hormones were beneficial to cochlear health in young adult B6 mice. As fertility and estrogen levels declined toward the end of the first year of life, the protective effects waned, so that progressive hearing loss accelerated in female B6 mice. The hypothesis was not supported by the Willott et al. (2006b) study because increased hearing loss in females could be seen as early as age 6 month, when ovarian functioning was still high. The present study takes this one step further, providing evidence that ovarian hormones actually exacerbate damage to the peripheral and central B6 auditory system when moderately increased environmental noise (the AAE) is present. The increased hearing loss may, in turn, lessen auditory-evoked neural activity in high- and low-frequency regions of the AVCN, affecting central degeneration. It is not clear if ovarian hormones also have a direct effect on the AVCN, independent of evoked neural activity, and this cannot be ruled out. The situation in B6 males is quite different as there appears to be an additional, beneficial androgenic effect. *The combination of male hormones and low-frequency stimulation lessens degenerative changes in the AVCN.*

It is important to emphasize that this hypothesis applies only to the interaction of hormones and responses to particular AAEs in B6 mice. When noise-induced hearing loss is not an obvious factor, ovarian hormones may have a protective effect on hearing in humans (Hultcrantz et al, 2006) and rodents (e.g., Coleman et al., 1994; Guimaraes et al., 2004) as well as having other neuroprotective or neurotoxic effects (e.g., Garcia-Segura et al., 2001; Picazo et al., 2003). Moreover, while sex differences in noise-induced hearing loss occur in species other than the B6 mouse, the relative vulnerability of basal and apical cochlea may differ from that reported here (e.g., McFadden et al., 1999, 2000).

4.4. Previous AAE studies

Several findings of our previous studies of AAE effects need to be addressed in order to clarify some issues raised by the present results. First, treatment with a middle-frequency (MAAE, peak SPL from 8-20 kHz) had ameliorative effects on progressive hearing loss in B6 and other inbred strains (Turner and Willott, 1998; Willott and Turner, 1999; Willott and Bross, 2004; Willott et al., 2001; 2005; 2006a,b). Vazquez et al. (2004) also found evidence of OHC preservation after moderate noise exposure. The middle portion of the cochlea is the most resistant to age-related changes in B6 mice, so it may be that MAAE stimulation of “healthy” cochlear tissue does not induce damage, but is beneficial instead.

Second, treatment of D2 mice with the same HAAE used here resulted in amelioration of hearing loss and degenerative changes in the dorsal, high-frequency region of the AVCN (Willott et al., 2006a). The hypothesis proposed to account for this brought into account the fact that D2 mice have very early, high-frequency hearing loss even at 3 weeks of age (Ralls, 1967; Willott et al., 2006a). D2 mice hear well enough to respond to the HAAE, but the noise band is barely above threshold. Thus, the effective intensity of the HAAE was diminished, and this presumably prevented noise-induced hearing loss. A second factor is that tonotopic organization in the D2 AVCN is abnormal (Willott et al., 1982). The “high-frequency” dorsal

AVCN never responds well to high frequencies because of the early cochlear pathology. The dorsal AVCN region develops relatively low thresholds for the frequencies the mice can hear (<20 kHz) which encompass a portion of the HAAE spectrum. Thus, the HAAE evokes neural responses throughout the AVCN, as is the case for the MAAE, which has beneficial effects.

Third, the study of MAAE treatment by Willott and Bross (2004) had the mice survive until 12-14 months of age. Male mice had fewer neurons than untreated controls in the extreme dorsal and ventral AVCN segments, but treated females differed little from controls. These mice were 3-5 months older than those used in the present study and had significantly more hearing loss, so the findings are not especially comparable. Nonetheless, the apparent advantage in females (the opposite of the present study) can be explained by a negative impact of ovarian hormones and/or positive impact of androgens. By 12-14 months of age, fertility and ovarian hormone activity have declined greatly in female B6 mice (Nelson et al., 1981, 1982, 1992). Thus, any negative effect of these hormones must have diminished substantially. Male hormones do not decline as precipitously in B6 mice (Nelson et al., 1975), but it is feasible that the ameliorative impact has lessened. The sex effect on progressive hearing loss in B6 mice may also have been a factor. If stimulation by the MAAE were having negative effects on the AVCN of the older mice, the older females would be partially protected because of their elevated thresholds. Taken together, these factors could reverse the sex differences in the AVCN in older mice.

4.5. Implications

The present study has shown that several factors determine whether AAE treatment has positive, negative, or neutral effects in the cochlea or AVCN. These factors include sex, gonadal hormones, site of the effect (cochlea or AVCN), frequency spectrum of the AAE, tonotopic organization, hearing sensitivity in responding to the AAE, and age. Moreover, the variables may interact with one another, and some of these are “moving targets.” For example, as B6 mice approach middle age (9 months), ovarian hormonal function begins to decline in females, implying that their influence on the auditory system changes. During the same period, genetic cochlear pathology and hearing loss ensue, altering the way the auditory system responds to an AAE; in turn, this alters the ventro-dorsal pattern of auditory-evoked neural activity and tonotopic organization in the AVCN. At the same time, a percentage of AVCN neurons disappear in B6 mice irrespective of AAE treatment (Willott and Bross, 1996; Willott et al., 1987). A 1-month-old B6 mouse morphs gradually into a rather different 9-month-old during the course of AAE treatment. Thus, it is very difficult to determine how and when the various factors might interact with AAE treatment to produce the findings reported here.

An understanding of the parameters that determine whether moderate noise exposure is beneficial or detrimental has obvious implications with respect to hearing aid use or controlling environmental noise in people who are at risk for hearing loss. The findings reported here suggest that the relationships among several variables, including hormones, must be determined in order to gain this understanding. Importantly, over a long period of time both positive and negative effects may be brought about by moderate levels of sound. These sorts of effects might have implications for observations that audiograms obtained from older women may show higher thresholds for low-frequency tones than men, even though men have worse high-frequency hearing (Glorig, 1957; Hinchcliffe and Jones, 1968; Moscicki et al., 1985).

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Figure 1.

Mean ABR thresholds for mice used in the LAAE experiments at age 9 months. Most standard errors of the mean were between 2-3 dB, but these are not shown for graphic clarity. The frequency band of the LAAE noise is shown by the shaded bar. Symbol code: intM = intact males; intF = intact females; ORX = orchidectomized males; OVX = ovariectomized females; LAAE = treated with the low-frequency AAE. The groups were: intact and gonadectomized controls (intMcon, ORXcon, intFcon, OVXcon), mice treated with the LAAE (intMLAAE, ORXLAAE, intFLAAE, OVXLAAE) and young B6 control mice (Ycon). Upper panel: all groups; middle panel: males; lower panel: females.

Figure 2.

Mean cytochromeochleograms (% missing hair cells) for outer hair cells. The abscissa refers to the percent distance from the apex (i.e., 100% = extreme base). Upper panel: mice used in the present LAEE experiments. Lower panel: Intact and gonadectomized mice in the LAEE groups and HAAE groups (from Willott et al., 2006b) were combined for graphic clarity. LAEE = treated with the low-frequency AAE; HAAE = treated with the high-frequency AAE.

Figure 3.

Mean cytochleograms (% missing hair cells) for inner hair cells. The abscissa refers to the percent distance from the apex (i.e., 100% = extreme base). Upper panel: mice used in the present LAEE experiments. Lower panel: Intact and gonadectomized mice in the LAEE groups and HAAE groups (from Willott et al., 2006b) were combined for graphic clarity.

Figure 4. Mean number of neurons per ventro-dorsal segment in the posterior AVCN section. Standard error of the mean (SEM) bars on some curves are typical of others (not shown for graphic clarity). The upper panel includes only HAAE-treated groups; the lower panel includes only LAEE groups; the same control groups appear in each panel.

Figure 5. Mean number of neurons in segment 2 (upper panel) and segment 7 (lower panel) of the posterior AVCN section. Error bars = SEMs.

Figure 6.

Mean area of each ventro-dorsal segment in the posterior AVCN section. Standard error of the mean (SEM) bars on some curves are typical of others (not shown for graphic clarity). The upper panel includes only HAAE-treated groups; the lower panel includes only LAEE groups; the same control groups appear in each panel.

Figure 7.

Mean number of neurons per ventro-dorsal segment in the anterior AVCN section. Standard error of the mean (SEM) bars on some curves are typical of others (not shown for graphic clarity). The upper panel includes only HAAE-treated groups; the lower panel includes only LAEE groups; the same control groups appear in each panel.

Figure 8.

Mean area of each ventro-dorsal segment in the anterior AVCN section. Standard error of the mean (SEM) bars on some curves are typical of others (not shown for graphic clarity). The upper panel includes only HAAE-treated groups; the lower panel includes only LAEE groups; the same control groups appear in each panel.