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14. ABSTRACT Results from the second year of studies continue that a 2 ½ minute exposure to a small arms fire – like noise will induce reduced gap detection, indicating tinnitus, in approximately 2/3rds of noise-exposed rats over the two years of studies. A significant finding is that the incidence of tinnitus does not correlate with either the extent of hair cell loss / auditory brain stem response (ABR) threshold shift nor the extent of loss of inner hair cell - auditory nerve (IHC-AN) connections, in animals assessed to date. Neither increased hair cell / hearing loss nor an increased loss of connections will predict if a noise exposed animal will develop tinnitus. We are now assessing changes in auditory nerve endings in the cochlear nucleus to determine if this shows a better correlation. Studies examining the influence of anti-oxidant and/or anti-excitotoxicity treatments on hair cell loss, loss of IHC-AN connections and the generation of tinnitus are still in progress. During the third year studies will add examination of central auditory excitability and test the influence of enhancing inhibitory influences shortly after the noise exposure.					
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I. INTRODUCTION:

A high incidence of tinnitus can result as an outcome of battlefield noise and this has become a major health concern for military, reducing the ability to redeploy, reducing the quality of life of those affected and increasing health care costs. These studies have two goals. One goal is “PREVENTION”, to develop treatments that if provided prior to a noise exposure will prevent tinnitus from being induced. They test if loss of inner hair cell –auditory nerve connections plays an initiating role in generation of tinnitus and preventing this loss will prevent tinnitus from occurring. Studies first test for correlation between loss of connections and development of tinnitus and then test treatments to prevent loss of connections and progression of tinnitus. . The second goal is “TREATMENT”, to develop interventions that can halt the progression of tinnitus when given after a noise exposure. These studies are based on a hypothesis that the progression of tinnitus moves from initiation in the cochlea to changes in excitability in the central auditory pathways and therefore the focus of treatment must move from the cochlea to the central auditory system. One set of studies test enhancing the influence of glycine, a major inhibitory transmitter to prevent increased excitability from developing in central auditory pathways and to determine if this reduces the incidence of tinnitus. Additional studies will test the influence of changing ion channel properties to reduce intrinsic excitability.

II. BODY

Task 1 – Assessment of Cochlear and Cochlear Nucleus pathology

Objective: Determine if the degree of Cochlear and/or Cochlear Nucleus pathology correlates with the appearance of tinnitus (as measured in each animal).

Sub-Task 1a: Assessment of Hair Cells for Hair Cell loss

Sub-Task 1b: Assessment of Auditory Nerve connections with Inner Hair Cells (CTBP2 immunolabeling) for their loss following noise.

Sub-Task 1c: Assessment of Auditory Nerve (VGLUT1 immunolabel) terminals on neurons in Ventral and Dorsal Cochlear Nucleus (VCN, DCN) for their loss following noise.

Sub-Task 1d: Assessment of VGLUT2, VAT & VGAT immunolabeled terminals in VCN and DCN.

Sub-Task 1e: Gene Expression changes.

Sub-Task 1f: Correlation between the degree of changes (Sub-Tasks 1a-e) and the appearance of tinnitus.

Sub-Task 1a: Assessment of Hair Cells for Hair Cell loss: This is complete. Animals receiving noise exposure or sham noise exposure were terminated by vascular perfusion of 4% paraformaldehyde fixative. Cochleae were removed and received further intrascalar fixation followed by rinse. They were then dissected into surface preparations and received phalloidin staining of hair cells (for this Sub-Task 1a) as well as immunostaining for CTBP2 for Sub-Task 1b (following paragraph). The surface preparations were viewed under epifluorescent optics. A reticule in the microscope eyepiece was used to quantitate presence or absence of hair cells by position and mapped as a cytocochleogram. There was the variability in the extent of loss across individual noise-exposed animals, as is expected to be found with noise exposures (both in people and in animal models). **Figure 1** shows two representative “cytocochleograms” mapping inner and outer hair cell loss (in rows 1, 2, and 3) across the cochlear spiral, with the cytocochleogram on top showing an example of large loss found in outer hair cells in all rows and the one below showing an example of relatively minimal loss of outer hair cells that is over 10% only in the region from 2.2 to 4.2 mm from the apex. This expected variability allowed us to look for correlation between the extent of hair cell loss and loss of inner hair cell – auditory nerve connections, and between loss of hair cells and the appearance of tinnitus (did an animal showing greater hair cell loss have more loss of connections, was there more hair cell loss in animals showing noise induced tinnitus versus those that did not develop tinnitus after noise). We did not find a correlation between amount of hair cell loss and loss of connections or amount of hair cell loss or between the amount of hair cell loss and appearance of tinnitus.

Sub-Task 1b: Assessment of Auditory Nerve connections with Inner Hair Cells (CTBP2 immunolabeling) for their loss following noise. This is complete. The same cochleae that were assessed for hair cell loss (Sub-Task 1a) based on phalloidin staining of hair cells were assessed for the number of CTBP2

immunostained puncta under inner hair cells. CTBP2 is a component of the synaptic ribbon within the inner hair cells and is a marker for the connections of the inner hair cell with individual auditory nerve fibers. Laser scanning confocal microscopy was used to acquire digital image in five “regions of interest” along the cochlear spiral. A z-series with 1 μ m slices at 0.3 μ m intervals was taken of each region of interest. The digital images were imported into a Metamorph Image Analysis workstation for quantitative analysis. A line was generated in the digital image to separate each inner hair cell, based on the location of nuclei and stereocilia. The number of puncta meeting size and shape criteria and with intensity of labeling at least five times over background was determined for each inner hair cell base in the region of interest and then the mean number of puncta per inner hair cell base in the region of interest was determined. Rats receiving noise exposure were compared to rats with sham noise exposure. As a group the noise exposed animals had a significant reduction in connections compared to animals without noise exposure.

Sub-Task 1c: Assessment of Auditory Nerve (VGLUT1 immunolabel) terminals on neurons in Ventral and Dorsal Cochlear Nucleus (VCN, DCN) for their loss following noise. This is in progress. All of the animals receiving vascular perfusion with 4% paraformaldehyde also had their brain stems removed and were processed for cryostat sectioning. Cryostat sections through the ventral and dorsal cochlear nucleus were immunostained with antibody to VGLUT1, a marker for auditory nerve terminals. Digital fluorescent images of the ventral cochlea nucleus were acquired on a confocal microscope and imported into a Metamorph Image Analysis Workstation. The amount of VGLUT1 immunostaining was determined for high, medium and low frequency regions of the cochlear nucleus. This is still in progress. Data completed to date shows a reduction in the amount of VGLUT1 immunostaining in high, medium and low frequency regions of the ventral cochlear nucleus in noise exposed animals compared to sham noise controls. We have not yet reached a sufficient “n” to test for the significance for this decrease. Analysis of the dorsal cochlear nucleus is also in progress.

Sub-Task 1d: Assessment of VGLUT2, VAT & VGAT immunolabeled terminals in VCN and DCN. This is in progress. Semi-adjacent cryostat sections from the brain stems being used for Sub-Task 1c analysis were acquired and stored in a -80°C freezer for the additional analysis with these other antibodies. These sections are being assessed in year 3.

Sub-Task 1e: Gene Expression changes. Not yet initiated (in Year 2). This analysis will require qRT-PCR of cochlear nucleus and inferior colliculus from unfixed brains. We therefore could not use the same animals as for Sub-Tasks 1a-d, since these animals received vascular perfusion with fixative. Sub-tasks 1a-d were given higher priority for the animals available in year 2. Brain tissues from 30 rats have been acquired in year 3 for Sub-Task 1e and we are in the process of generating material for qRT-PCR which will be carried out later in year 3.

Sub-Task 1f: Correlation between the degree of changes (Sub-Tasks 1a-e) and the appearance of tinnitus. Some Correlations are complete and others have not yet been initiated. We have completed the determination of whether the noise-exposed animals used for Sub-Tasks 1a-d have acquired tinnitus. Tinnitus was defined as showing reduced gap detection without reduced pre-pulse inhibition (**Figures 2-5**). Over half the noise exposed animals showed acquisition of tinnitus while tinnitus did not appear in any of the sham noise exposure animals. **Figure 2** shows results at 0-2 weeks post exposure comparing animals with sham noise exposure to forty animals receiving the small arms fire – like noise exposure and **Figure 3** shows results at 4-6 weeks. Both show a shift in the histogram for Gap Inhibition (GI) of the acoustic startle reflex, indicative of tinnitus. **Figures 4 and 5** show the reduction in Gap Inhibition and in hearing (auditory brain stem response – ABR threshold shifts) for several individual animals, from the Dolan et al (2013) poster at the ARO meeting in February 2013.

We tested for correlation of acquiring tinnitus with degree of hair cell loss (Sub-Task 1a) and with degree of loss of inner hair cell – auditory nerve connections (Sub Task 1b). We find that there is no correlation between which animals acquire tinnitus after noise the amount of noise induced hair cell loss. There is a better correlation between acquisition of noise-induced tinnitus and the amount of noise-induced loss of inner hair cell – auditory nerve connections. **Figure 6** shows a comparison of the number of IHC-AN connection per inner hair in apex and base of noise exposed mice that show tinnitus (based on reduced gap inhibition of the acoustic

startle reflex) versus those that got the same noise exposure but had no reduced in gap detection thus no evidence of tinnitus occurring. There is a greater loss of connections in the mice showing tinnitus, particularly in the basal half of the cochlea. **Figure 7** compares number of connections in rats with sham noise exposure, noise exposed rats developing tinnitus and noise exposed rats that did not develop tinnitus, with a reduction in connections only seen in the group of rats that developed tinnitus. We have not completed Sub-Tasks 1c-e, so cannot yet test their correlations.

In year two we entered the “In Life Phase” for Aim 4

Objective: Test the Aim 4 hypothesis that treatments which reduce neuron excitability will reduce or eliminate noise-induced tinnitus.

Task 2 – Determine if treatment with sarcosine and retigabine following the noise exposure will reduce the number of rat that develop tinnitus.

Sub-Task 2a: Assessment of Threshold Shifts (ABRs) over the in-life period following noise or sham noise.

Sub-Task 2b: Assessment of Pre-Pulse Inhibition and Gap Detection over the in-life period following noise or sham noise (reduced Gap Detection is the metric for appearance of tinnitus).

Both Sub-Task 2a and Sub-Task 2b assessments are in progress with 30 animals completed and additional animals being tested in year 3.

III. KEY RESEARCH ACCOMPLISHMENTS:

We have shown that an exposure condition (152dB in 50 bursts over 2 ½ minutes) that mimics small arms fire will cause reduced gap detection, an indication of tinnitus, in approximately two-thirds of the rats getting this noise exposure over the first two years of studies. This was presented at the mid-winter meeting of the Association for Research in Otolaryngology (Dolan et al, 2013) and is being prepared for publication.

We have assessed several correlations. These are being presented at the upcoming (2014) meeting Association for Research in Otolaryngology (Halsey et al 2014). We find:

- **There is not a correlation between loss of hair cells and the incidence of tinnitus.**
- **There is not a correlation between ABR threshold shifts and the incidence of tinnitus (Figure 8).**
- **There is not a correlation between loss of hair cells and loss of inner hair cell – auditory nerve connections.**
- **There is a correlation between loss of inner hair cell – auditory nerve connections (Figures 6&7).**

IV. REPORTABLE OUTCOMES:

Manuscripts, Abstracts, Presentation

Dolan D, Shore S, Miller J, Halsey K, Prieskorn D, Kanicki A, Ghisleni P, Altschuler R (2013) Simulated small arms fire in rats results in reduced gap inhibition of the acoustic startle reflex suggesting induction of tinnitus, Abstracts of the 36th MidWinter Meeting of the Association for Research in Otolaryngology p108.

Halsey K, Dolan D, Eberle K, Kanicki A, DeRemer S, Prieskorn D, Shore S, Miller J, Altschuler R (2014) Title: Impulse Noise Effects on ABR, Pre-pulse Inhibition, Gap Detection, and Auditory Nerve Connections, Abstracts of the 36th MidWinter Meeting of the Association for Research in Otolaryngology, in press.

Data bases:

We have generated a normative data base of **Auditory Brainstem Response (ABR) growth functions** (Input – Output functions) at different frequencies for the Sprague Dawley rat. This normative data base is now being used as the base-line to determine changes under the experimental conditions.

We have generated a normative data base of VGlut1 immunostaining in the rat anteroventral cochlear nucleus, in high, medium and low frequency regions. This normative data base is now being used as the base-line to determine changes under the experimental conditions.

V. CONCLUSIONS:

The research completed to date shows that small arms fire – like noises will result in tinnitus in some but not all of the noise-exposed rats, with an incidence of approximately two-thirds of the noise exposed rats showing indication of tinnitus either during the first 2 weeks following the noise or over the course of two months of assessment. Rats without noise exposure (shams) do not develop tinnitus.

Most rats show some degree of hair cell loss and hearing loss from the noise exposure as well as loss of inner hair cell – auditory nerve (IHC-AN connections, however there is considerable variability in the extent of these metrics. When we look to see if the extent of hair cell loss, the extent of hearing loss or the extent of IHC-AN connection loss will predict which rats develop tinnitus and which will not we do not find any correlation with the first two (hair cell loss and ABR threshold shifts) based on animals assessed to date, but we do see a correlation with loss of IHC-AN connection.

We are continuing central auditory systems to determine if changes in auditory nerve connections in the cochlear nucleus will be a better predictor of tinnitus and these assessments are now in progress.

We have begun the in-life phase of testing therapeutics that will enhance auditory brain stem inhibition to determine if this will decrease the progression of tinnitus if given shortly after the noise exposure.

VII. SUPPORTING DATA:

FIGURE 1

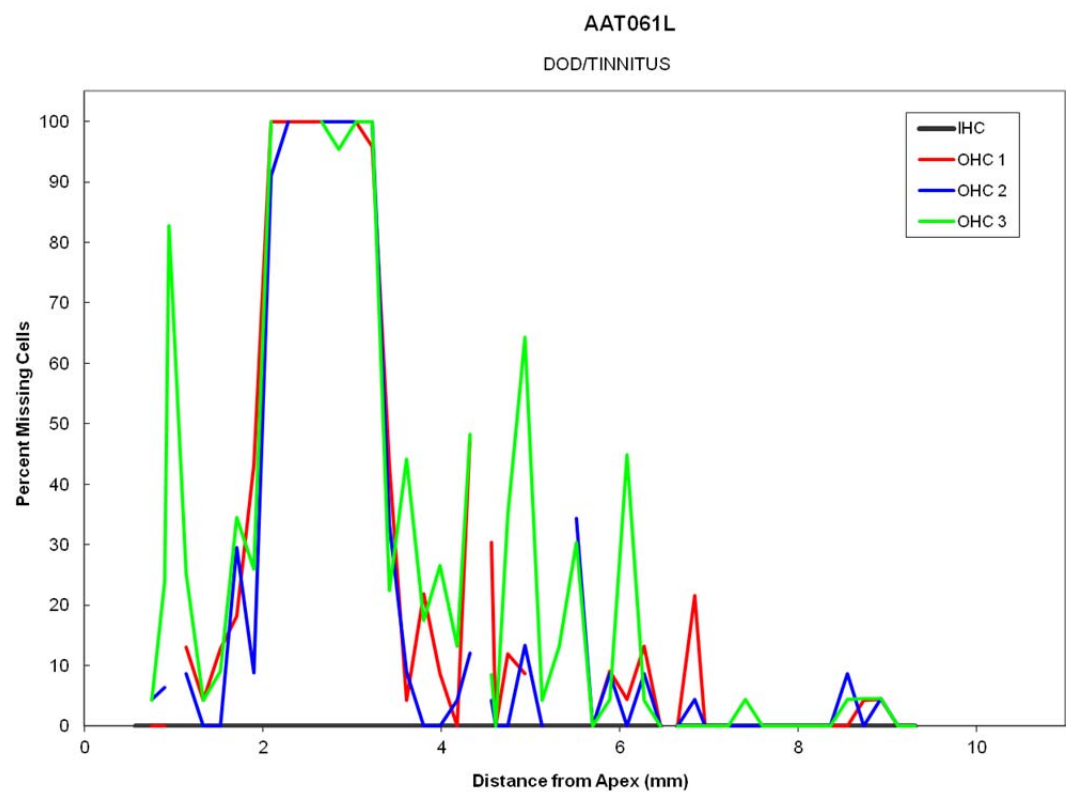


Figure 1 KHRl CytoGram - Version 3.0.7

Cytocochleograms plotting the percent of hair cell loss by position along the cochlear spiral with apex to left and base to the right. IHC= inner hair cells, OHC1, 2, 3 = outer hair cells in rows 1, 2 and 3 respectively. The plot above (AAT061) shows hair cell loss in a rat two months after the noise exposure. This rat received no preventive/protective treatments. The plot below (AAT045L) is from a rat that was on the ACEmg diet prior to and following the noise exposure and it shows less loss of hair cells at 2 months following the noise exposure.

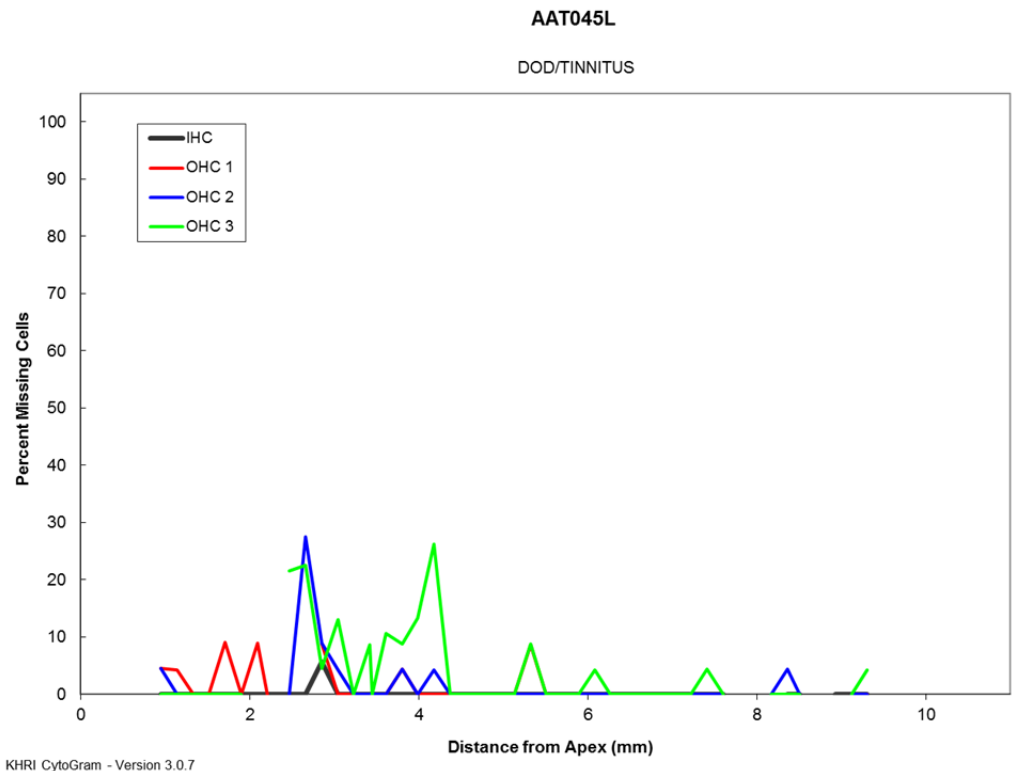


FIGURE 2
0-2 weeks post exposure

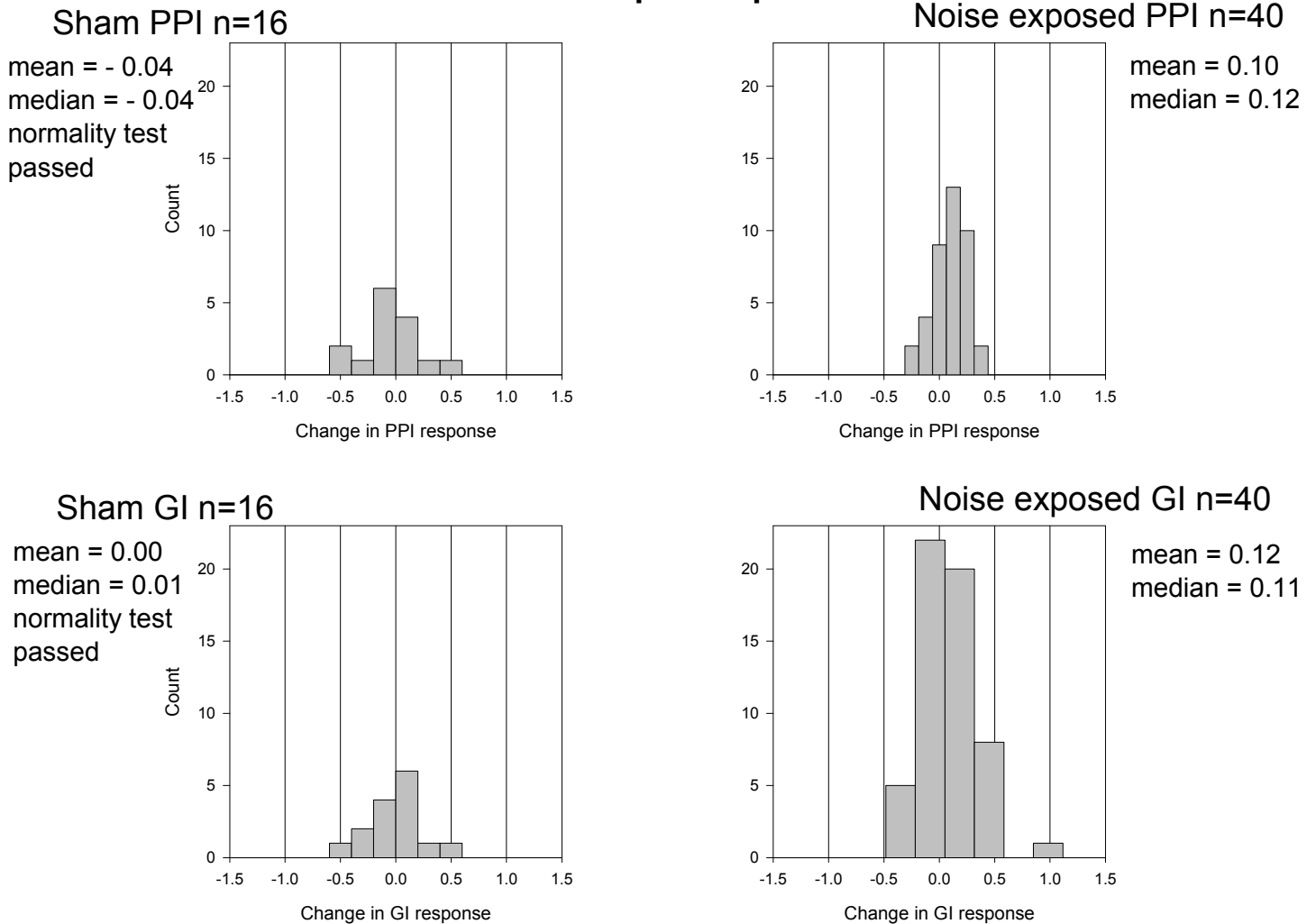


Figure 2. Probability distributions for changes in PPI (top) and gap inhibition (bottom) for the sham treated (left column) and noise treated (right column) animals over the two weeks post noise exposure. With increasing N, the data will be fitted with a normal distributions for statistical analysis to confirm normal PPI but decreased gap inhibition as an indicant of tinnitus. Initial results suggest a proportion of noise exposed animals reveal reduced gap inhibition and tinnitus.

FIGURE 3

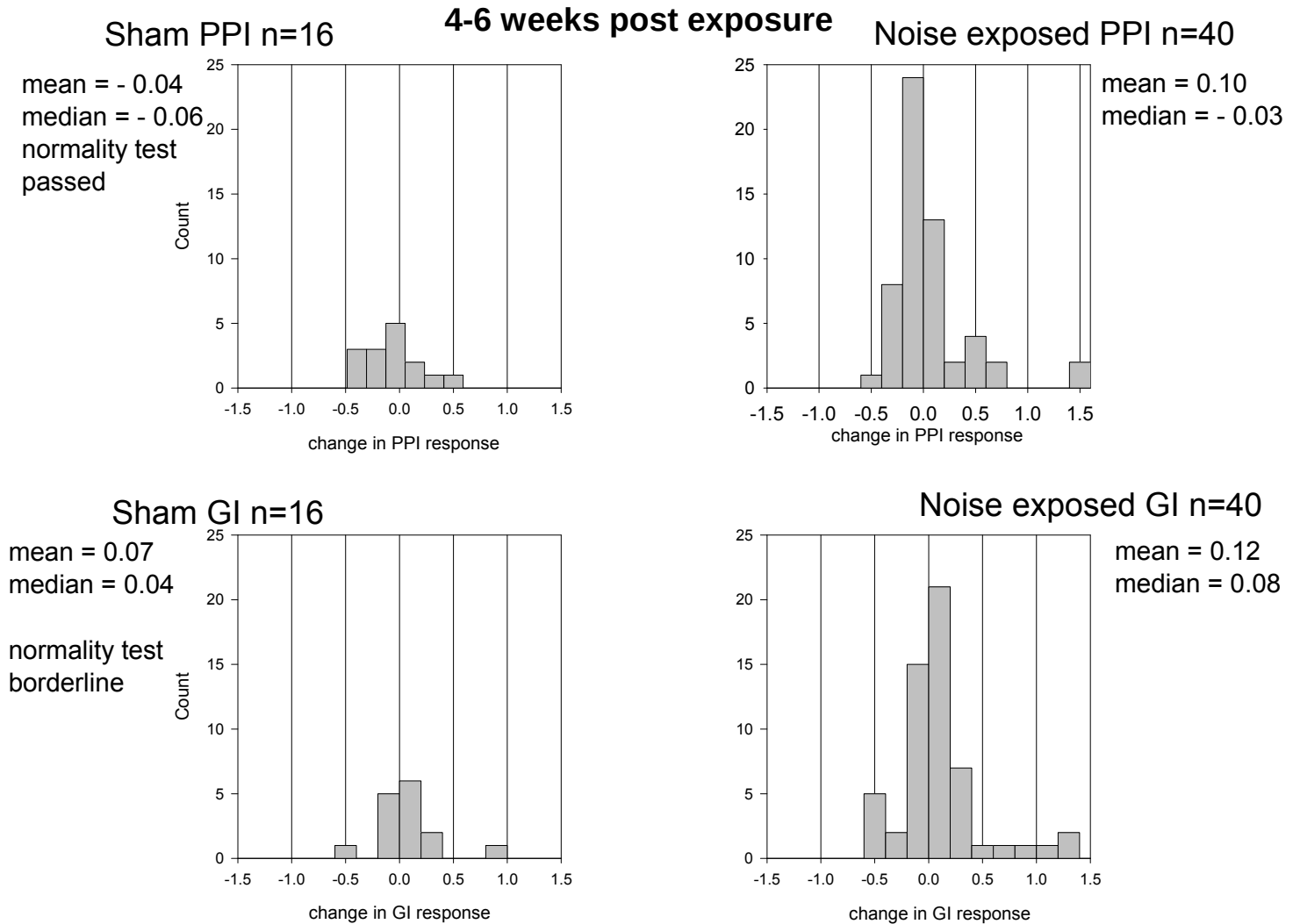


Figure 3: Probability distributions for changes in PPI (top) and gap inhibition (bottom) for the sham treated (left column) and noise treated (right column) animals over the 4-6 weeks post noise exposure period. With increasing N, the data will be fitted with a normal distribution for statistical analysis to confirm normal PPI but decreased gap inhibition as an indicant of tinnitus. Compared to the two week period (Fig.1), more animals show reduced gap inhibition at 4-6 weeks post exposure suggestive of tinnitus.

FIGURE 4

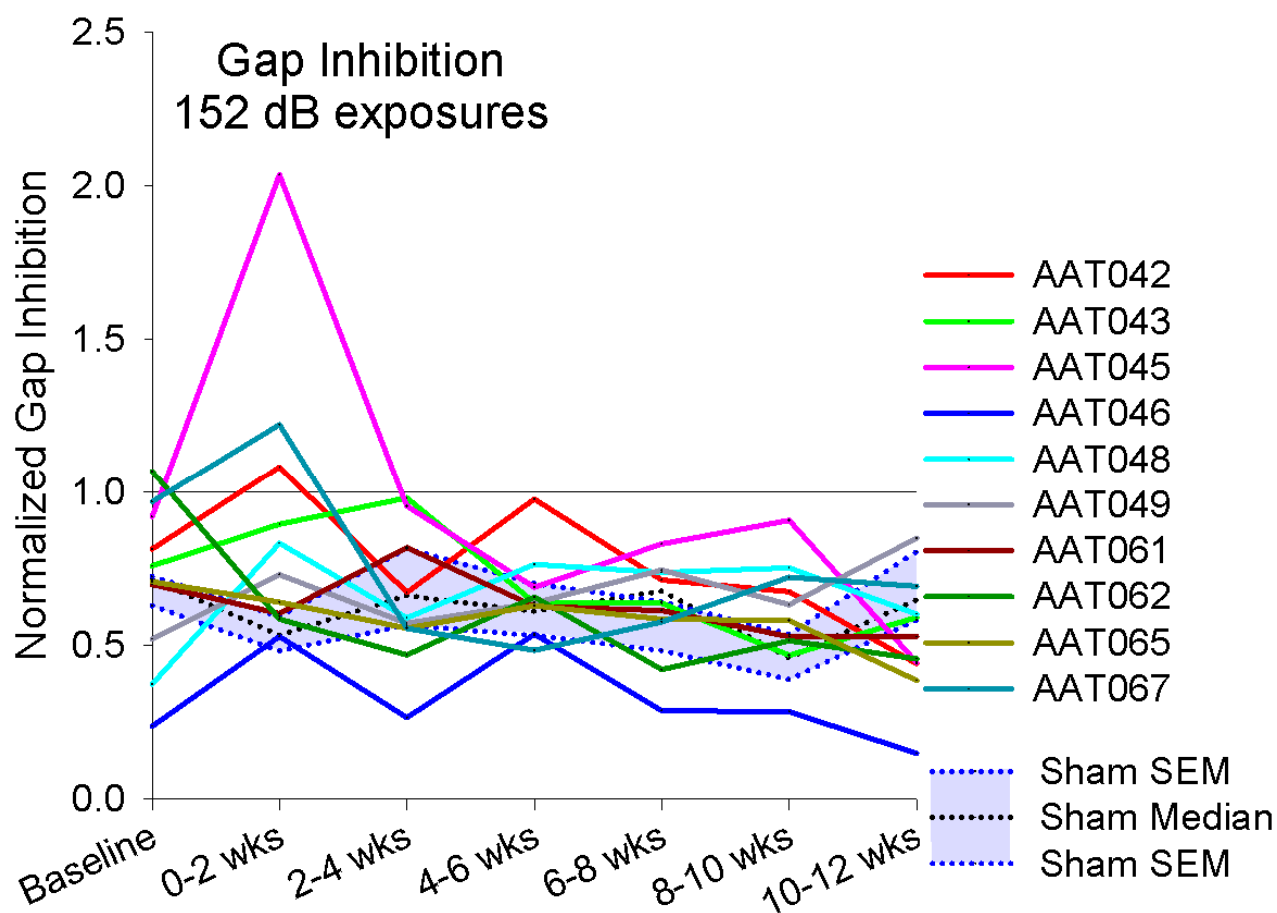


Figure 4: Individual gap inhibition results from ten noise treated animals compared to the sham treated animals (gray shaded area). Approximately half of the noise exposed animals show reduced gap inhibition compared to the sham treated animals suggesting the presence of tinnitus.

FIGURE 5

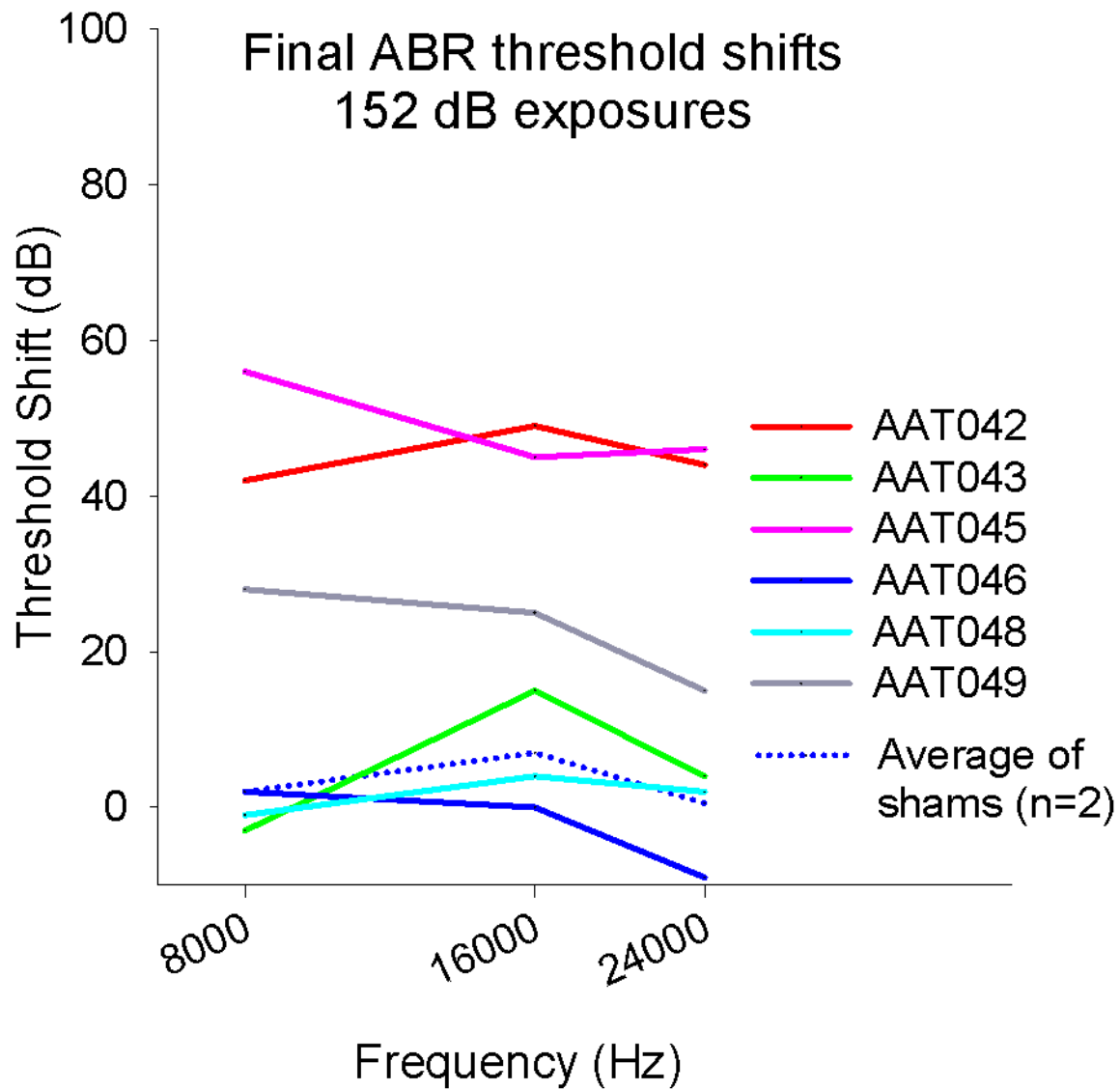


Figure 5. Terminal ABR threshold shifts for 6 noise exposed animals are compared to sham treated animals. Animals with elevated unilateral threshold shifts show reduced gap inhibition (Figure 4) indicating presence of tinnitus

FIGURE 6

	IHC-AN connections in apex	IHC-AN connection in base
Tinnitus mice	19.7	19.4
Noise but no tinnitus	17.6	12.3

Figure 6 – a comparison of the number of IHC-AN connection per inner hair in apex and base of noise exposed mice that show tinnitus (based on reduced gap inhibition of the acoustic startle reflex) versus those that got the same noise exposure but had no reduced in gap detection thus no evidence of tinnitus occurring. There is a greater loss of connections in the mice showing tinnitus, particularly in the basal half of the cochlea.

Figure 7: Comparison of the average number of CTBP2 puncta per inner hair cell (marker for the number of inner hair cell –auditory nerve terminals) in three regions of interest along the cochlear spiral. Compared among four groups of animals, rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles) , rats receiving noise exposure that did develop tinnitus (blue squares) and rats receiving noise exposure that has changes to pre-pulse inhibition (PPI). Rats receiving noise but not developing tinnitus (green triangles) had numbers that were comparable to rats receiving no noise / shams (black circles). On the other, there were large decreases in the number of connections in the noise exposed rats that did develop tinnitus (blue squares).

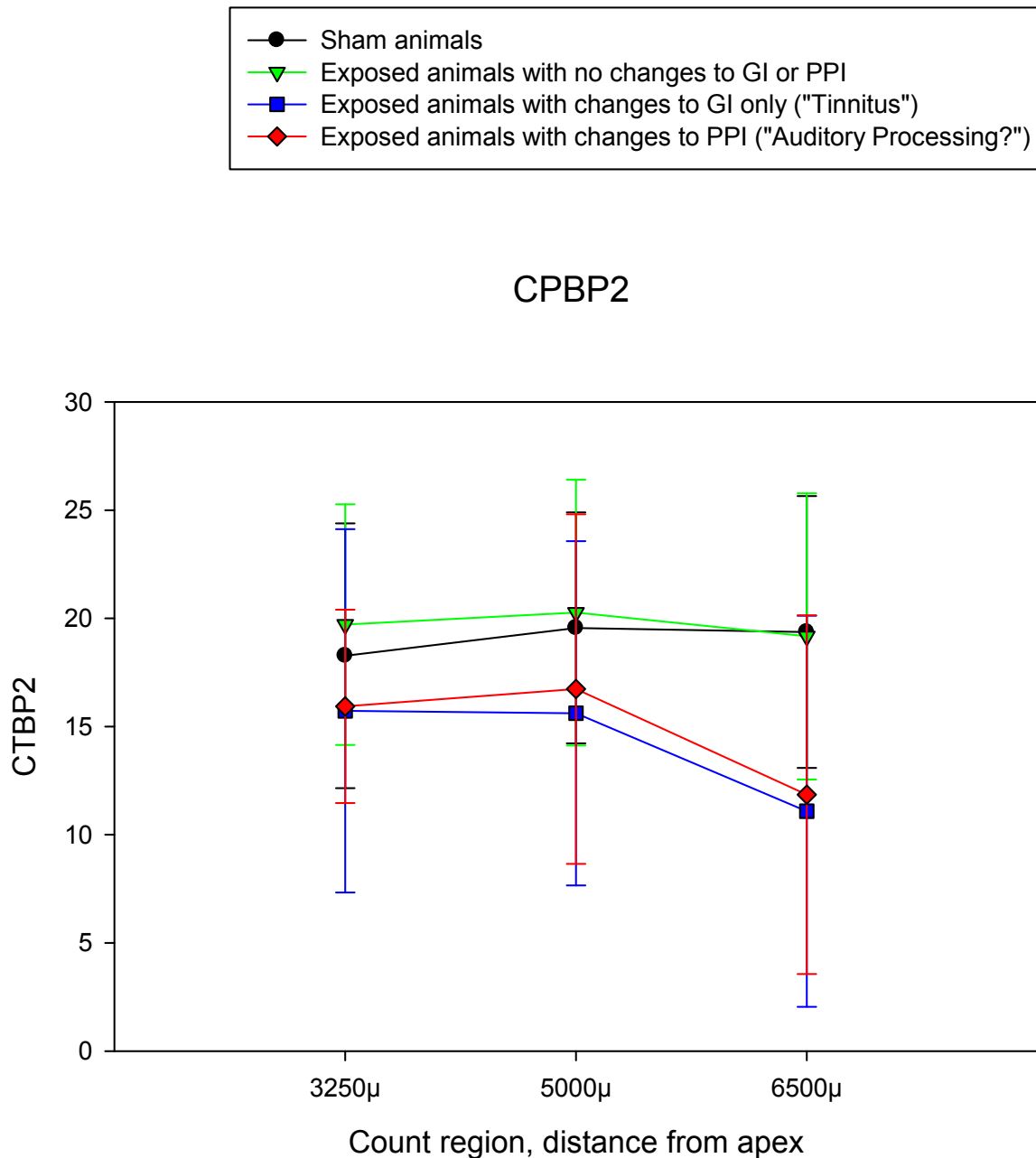


Figure 8: Comparison of threshold shift of the auditory brain stem response (ABR) in rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles) and rats receiving noise exposure that did develop tinnitus (blue squares). There was no correlation between amount of threshold shift and whether or not noise exposed animals developed tinnitus.

