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Small arms fire-like noise: Effects on hearing loss, gap detection and the influence of preventive treatment

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Abstract

A noise-induced loss of inner hair cell – auditory nerve synaptic connections has been suggested as a factor that can trigger the progression of maladaptive plastic changes leading to noise-induced tinnitus. The present study used a military relevant small arms fire-like noise (50 biphasic impulses over 2.5 minutes at 152 dB SPL given unilaterally to the right ear) to induce loss (~1/3) of inner hair cell synaptic ribbons (associated with synapse loss) in rat cochleae with only minor (less than 10%) loss of outer hair cells. Approximately half of the noise-exposed rats showed poorer gap detection post-noise, a behavioral indication suggesting the presence of tinnitus. There was significantly greater loss of inner hair cell ribbons in noise-exposed rats with reduced gap detection compared to noise-exposed rats retaining normal gap detection. We have previously shown systemic administration of piribedil, memantine, and/or ACEMg significantly reduced loss of inner hair cell ribbons induced by a 3 hour 4 kHz octave band 117 dB (SPL) noise. The present study examined if this treatment would also reduce ribbon loss from the small arms fire-like noise exposure and if this would prevent the reduced gap detection. As in the previous study, piribedil, memantine, and ACEMg treatment significantly reduced the noise-induced loss of ribbons, such that it was no longer significantly different from normal. However, it did not prevent development of the reduced gap detection indication of tinnitus in all treated noise exposed rats, reducing the incidence but not reaching significance.

Keywords

Noise; Cochlea; Tinnitus; Synaptopathy

INTRODUCTION

Tinnitus is the perception of a sound, in the absence of an external sound and often perceived as ringing in the ear(s). Tinnitus can lead to depression, anxiety, insomnia and

reduce quality of life. It occurs in approximately 15% of US adults with 1-2% of the population seeking professional assistance to cope with tinnitus (Shore et al., 2016 for review). Approximately 40% of all veterans report tinnitus and it is the number one co-reported service-related disability for those who served in the Gulf Wars (Theodoroff et al., 2015; Yankaskas, 2013).

A variety of noise exposure conditions can be used to induce tinnitus in animal models, including noises that induce only temporary changes in hearing thresholds (TTS) and those that induce severe permanent increases in hearing thresholds (PTS) (Bauer et al., 2008). As with people, a noise exposure generally induces tinnitus in only a sub-population of exposed animals, with the percent showing tinnitus varying depending on the noise exposure conditions and the metrics used to determine the presence of tinnitus. Our studies tested the effect of a military relevant small arms fire (SAF)-like noise that induces TTS and small PTS.

Noise exposures can induce changes in the cochlea that can then trigger a progression of further changes, including maladaptive activity dependent plasticity in both lower (auditory brain stem) and higher central auditory centers (see Auerbach et al., 2014; Eggermont and Tass, 2015; Henry et al., 2014; Knipper et al., 2013; Roberts et al., 2010; Shore et al., 2016; Wang et al., 2011 for reviews). Regions with hyper-excitability can appear and further modulations and upstream changes can continue to occur in both central auditory and non-auditory centers (Auerbach et al., 2014; Eggermont and Tass, 2015; Eggermont and Roberts, 2015; Roberts et al., 2010 for reviews). This progression of changes can be associated with an immediate tinnitus, often transient, a later occurring chronic intermittent tinnitus and ultimately to a chronic persistent tinnitus. Consequently there may be different temporally dependent targets for prevention and/or treatment of tinnitus during the different phases in this progression. A treatment might be effective during one phase in the progression of tinnitus and not during another. An initial target for intervention during the first phase would be the peripheral changes occurring in the cochlea and this is the focus of the current study. Animal studies showing tinnitus can be induced with a noise that does not cause hair cell loss or permanent changes in hearing thresholds (e.g. Bauer et al., 2008; Dehmel et al., 2012a, b), suggest that sensory hair cell loss, while perhaps sufficient, is not necessary for inducing tinnitus. Recent studies show that noise causing only a temporary threshold shift (TTS), without hair cell loss, can cause peripheral neuropathy with loss of inner hair cell (IHC) – auditory nerve (AN) synaptic connections (e.g. Kujawa and Liberman 2009). This raises the question of whether the peripheral synaptopathy could be a trigger to induce the progression of tinnitus (Bauer et al., 2007; Hickox and Liberman, 2014). Peripheral synaptopathy as an induction factor was supported by recent studies that demonstrated greater loss of IHC synaptic ribbons (marking IHC-AN synaptic connections) in noise-exposed rats that developed tinnitus compared to those that did not (Rüttiger et al., 2013; Singer et al., 2013) using a psychophysical measure of tinnitus in the rat model.

The first phase of our study examined if a SAF-like noise that generates only a small loss of outer hair cells (OHC) and small auditory brain stem response (ABR) threshold shift would produce a loss of IHC synaptic ribbons comparable to what has been shown following other types of mild noise exposures (e.g. Kujawa and Liberman, 2009; Altschuler et al., 2016).

Loss of CTBP2 immuno-labeled IHC synaptic ribbons is an indication of loss of IHC-AN synaptic connections (Kujawa and Liberman, 2009, Rüttiger et al., 2013; Singer et al., 2013). Our study also examined if the SAF-like noise would lead to reduced gap detection (GD). Reduction in GD was observed after noise exposure by Rybalko and Syka (2005) and developed by Turner et al. (2006) as a metric for the presence of tinnitus in the rat model. We then examined if there was greater loss of synaptic ribbons in the animals showing the reduced GD.

Our previous study showed that a combination of piribedil, memantine and/or ACEMg administered prior to and following a 3 hour exposure to 117dB SPL octave band noise centered around 4kHz decreased the noise-induced loss of IHC synaptic ribbons (Altschuler et al., 2016). Piribedil is a dopamine D2 receptor agonist that has previously been reported to reduce auditory nerve excitation and the noise-induced swelling and bursting of peripheral processes associated with excitotoxicity (d'Aldin et al., 1995). Memantine is a non-competitive open channel blocker that enters the NMDA receptor only when it is excessively open reducing the Ca^{++} intake that can lead to excitotoxicity (Choi et al., 1988; Gardoni and Di Luca, 2006; Lipton, 2007; Wroge et al., 2012). Local NMDA receptor blockage has been reported to reduce tinnitus (Brozoski et al., 2013). ACEMg is an anti-oxidant combination of vitamins A, C and E along with magnesium (ACEMg) that has been shown to reduce noise-induced hair cell loss (Le Prell et al., 2007). Magnesium could also reduce excitotoxicity through saturation of the Mg binding site on the NMDA receptor or by acting as a competitive Ca^{++} channel blocker to protect against damage from oxidative stress. We therefore tested if this anti-excitotoxicity combination would also reduce loss of ribbons from the SAF-like noise and if this would affect the incidence of reduced GD in the treated animals.

EXPERIMENTAL PROCEDURES

General Study Design

This study was reviewed and approved by the Institutional Animal Care & Use Committee at the University of Michigan, which is fully accredited by AAALAC International. All procedures conformed to the National Research Council's *Guidelines for the Care and Use of Laboratory Animals*: Eighth Edition. Young adult (300 – 360 g) male Sprague Dawley rats (Charles River Laboratories) were tested for baseline ABR, as well as pre-pulse inhibition (PPI) and gap inhibition of the acoustic startle reflex (ASR). Animals without normal responses were removed from study. Animals were then randomly assigned to one of three groups; Sham noise and no treatments (normal diet); Noise exposure with control (saline) injection and normal diet; Noise exposure with piribedil and memantine injections and ACEMg enhanced diet. ACEMg was provided as in LePrell et al., (2007) and Altschuler et al., (2016) beginning 3 weeks prior to noise and continuing until euthanasia at 12-15 weeks following the noise. Saline (SC) or piribedil 10mg/kg (SC) and memantine 3mg/kg (SC) was administered beginning 3 days before the noise, then 1 h prior to the noise exposure and continuing for 3 days after the noise exposure. Dosages were based on Chen et al., 1998; Kutzing et al., 2012; Wroge et al., 2012. Saline injections (SC) were administered

using an equivalent volume as in the piribedil and memantine treated animals. The SAF-like noise exposure was unilateral, to the right ear only.

Auditory Brain Stem Response (ABR)

ABR measures were taken prior to noise, immediately after the noise exposure and at 12-15 weeks after the noise (prior to euthanasia). At 12-15 weeks following the noise, animals were euthanized and processed for assessment of hair cell loss and the number of CTBP2 immuno-labeled ribbons per IHC, as a marker for IHC-AN synaptic connections. Animals were anesthetized (ketamine 65 mg/kg, xylazine 6 mg/kg). Body temperature was maintained through the use of water circulating heating pads and heat lamps. Additional anesthetic was administered if needed to maintain anesthesia depth sufficient to ensure immobilization and relaxation. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Austin, TX USA). Needle electrodes were placed at vertex (active) and the test ear (reference) and contralateral ear (ground) pinnae. Tucker Davis Technologies (TDT) System III hardware and SigGen/BioSig software (TDT, Alachua, FL USA) were used to present the stimulus and record responses. Tones were delivered through an EC1 driver (TDT, aluminum-shielded enclosure made in-house), with the speculum placed just inside the tragus. Stimulus presentation was 15 ms tone bursts, with 1 ms rise/fall times, presented 10 per second. Up to 1024 responses were averaged for each stimulus level. Responses were collected for stimulus levels in 10 dB steps at higher stimulus levels, with additional 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus level where a response was repeatedly present and 5 dB lower, where no response was present. Wave three, the peak present at the lowest intensities, was typically the response used to determine threshold. Since noise-exposed animals were not expected to develop a left (unexposed) ear hearing loss, any animal developing threshold shifts in this ear was eliminated from further analysis. Supra-threshold amplitudes and latencies for ABR peak I were analyzed in both ears (exposed and unexposed) animals pre and post exposure. The amplitude of peak I was measured from the baseline preceding the peak.

Noise Exposure (Simulated Small Arms Fire)

The SAF – like impulse noise was produced by a compression driver (JBL Selenium, model D3500Ti-Nd) powered by a linear amplifier (Parasound, model HCA-600). The output of the compression driver was coupled to a 3 mm diameter plastic speculum with a custom made aluminum coupler. The signal input to the linear amplifier was produced by a D-A converter (Tucker-Davis Technologies, model DA1). The digital input to the D-A converter was generated in software (Tucker-Davis Technologies, types SigGen32 and SigPlay32). The digital waveform was a biphasic pulse, 150 microseconds per phase, presented at 0.333 Hz. The sound output was calibrated with an impulse measuring sound level meter (Bruel & Kjaer, model 2231), and a 1/4 inch pressure response microphone (Bruel & Kjaer, type 4136) coupled to the speculum with a 0.2 cc coupler. The 50 biphasic impulses over 2.5 minutes at 152 dB SPL SAF-like noise was given unilaterally to the right ear. The normal hearing remaining in the left ear allowed for testing of GD and PPI of the ASR.

Gap Inhibition and Pre-Pulse Inhibition of the Acoustic Startle Reflex:

The Acoustic Startle Reflex (ASR) was determined using equipment and software from Kinder Scientific (<http://www.kinderscientific.com>). The animal was placed in an acoustically transparent cage, which was set atop a sensor inside a test chamber. The cage was small enough to discourage excess movement, but did not confine the animal to an abnormal posture. The ASR was assayed by playing a brief (20 ms) broad band noise pulse at 115dB SPL, a sufficient volume to elicit a “jump,” or startle reflex from the animal. This noise level and duration did not cause any hearing loss or loss of hair cells. The sensor measures the amount of movement in the jump or startle and reduced amplitude from the sensor is used to define a reduced ASR response (“reduced performance”). The ASR response can be reduced by a “pre-pulse” of another lower intensity sound just prior to the louder sound that induces the startle reflex called pre-pulse inhibition (PPI). PPI can be ineffective in inducing inhibition of the ASR if the animal cannot hear the pre-pulse, so hearing loss can influence PPI. The ASR response can also be inhibited or reduced by a brief gap in a background sound just prior to inducing the ASR called Gap Inhibition of the ASR. Gap Inhibition of the ASR can be ineffective if the animal cannot detect the Gap. If a presentation of a gap does not inhibit or reduce the subsequent ASR response then a conclusion is made that there is a reduced detection of the gap (GD). This can occur for different reasons, one of which could be tinnitus filling in the gap (Turner et al., 2006).

Both Gap Detection and PPI of the ASR were tested three times a week, first during the 2-3 weeks prior to the noise exposure to generate baseline information and then beginning 3-5 days following the noise exposure and continuing until animal euthanization at 12-15 weeks following the noise. For acclimation effects, the results from the initial two days of testing were not included in the results. To reduce habituation effects, the session (~40 min) was divided into two, with a 10-15 min break (the animal was removed from the cage) between sessions. For testing GD, a constant low-level (60 or 65 dB SPL) background noise was played, with a very short (50 ms) gap (2 ms off-on ramp) in the noise shortly (100 ms) before the startle-inducing sound. In animals with normal hearing, this gap changes their ASR and the amplitude of their response is decreased. For PPI, the startle is preceded by a brief, low-level (60 or 65 dB SPL) noise burst. This noise functions very similarly to the gap in GD, and it reduced the amplitude of the startle reflex. The pre-pulse and background noises were presented at varying frequency bands (8-10 kHz and 2-20 kHz BBN). For PPI, the relative startle ratio is the response to the “prepulse + startle” stimulus response divided by the startle response alone. For GD, the relative startle ratio is response to the gap + response to startle alone divided by the startle response alone. The values for each condition, presented in pseudo random order, with 5-9 seconds inter-trial intervals, are the average of seven trials.

Reduced Gap Detection

Reduced GD can be an indication of tinnitus (Turner et al., 2006). Reduction in GD was defined following selection criteria developed by Li et al. (2013). The amount of “normal” reduction in the ASR response from presentation of gap and normal variability was defined in sham noise exposed animals that were always assessed in parallel with noise-exposed animals. The baseline ASR response and the mean change from this baseline induced by a

gap or pre-pulse was determined for sham animals and a 95% confidence interval for variability was generated based on the distribution of performance in these sham animals. This was determined at each assessment time point. In the noise exposed group, any animals whose reduction in ASR response amplitude following the gap presentation deviated from baseline (at any frequency or broadband noise) by an amount greater than the sham group's 95% confidence interval was classified as having reduced GD and considered an indication of tinnitus. A similar comparison was done for the PPI results to determine if any noise-exposed animals had reduced PPI.

Animal Euthanasia

At 12-15 weeks following the noise (or sham), rats were anesthetized with sodium pentobarbital and then received vascular perfusion through the heart with 0.1M phosphate buffer followed by 4% paraformaldehyde fixative in 0.1M phosphate buffer. Cochleae were removed and received slow intrascalar infusion of the same fixative through the round window and an apical fenestra. Cochleae were then immersed in fixative for 12-16 hours, followed by rinse in phosphate buffered saline (PBS) and then partial decalcification in 5% EDTA in PBS for 2-3 days at 4° C. The otic capsule and tectorial membrane were carefully removed and cochleae were permeabilized and blocked in 5% normal donkey serum in 0.3% Triton X-PBS. The remaining cochlear epithelium was then incubated with mouse anti-CTBP2 antibody (BD Transduction Laboratories) diluted 1:200 in PBS for 16-20 hours at 4°C. This was followed by three ten minute rinses in PBS and then labeling with Phalloidin Alexafluor 568 (Life Technologies) diluted 1:100 and incubation with donkey anti-mouse immunoglobulin Alexafluor 488 (Life Technologies) diluted 1:500 in PBS for 2 hours at room temperature. This was followed by three rinses in PBS in the dark. The cochlear epithelium was then microdissected into three to four segments, apex, middle, base and hook, and each segment was mounted separately as a surface preparation on a glass slide with Fluoromount (EMS Inc.) and covered with a coverslip. Slides were then stored at 4°C until examination.

Assessment of Inner Hair Cell – Auditory Nerve Synaptic Ribbons

CTBP2 immunostaining of synaptic ribbons (Figure 1) was used as a marker for IHC-AN synapses as in Kujawa and Liberman (2009), Singer et al. (2013) and Altschuler et al. (2015). Up to 5% of CTBP2 immuno-labeled ribbons can be “orphans” and not opposed by peripheral processes with glutamate receptor and so the use of CTBP2 immunostaining of ribbons as a marker for IHC-AN synaptic connections has the potential for a small over-count (Sergeyenko et al., 2013).

The right ears that received the noise exposure were assessed, with the assessor blinded as to treatment condition. The length of the whole mount preparation of organ of Corti was measured using Metamorph Image Analysis software to locate the position of the regions of interest. Three regions of interest were selected along the cochlear spiral at 3.25, 5.0 and 6.5 mm from the apex for quantitative assessment. These are close to the 8, 16 and 24 kHz frequency regions (Viberg and Canlon, 2004; Müller, 1991) that were tested for ABR. Either a Zeiss LSM 510-META or a Leica SP5 Laser Scanning Confocal Microscope with a 63× objective at ×1 digital zoom were used to acquire a Z-series of 1 µm slices with a z- step

increment of 0.4 μm for each region of interest. The length of each region of interest was approximately 0.2 mm, varying because of differences in the curvature of the cochlear spiral at different positions. The digital images were analyzed using Metamorph Image Analysis software. The number of immuno-labeled CTBP2 puncta meeting size and location criteria with intensity of labeling at least five times over background was determined for each IHC base in each region of interest. The number of IHC in that 0.2 mm region was counted, and then the mean number of puncta per IHC base in the region of interest was determined.

Assessment of Hair Cell Loss

Phalloidin labeling of hair cells or of the scars replacing missing hair cells was used to identify presence or absence of IHC and OHC (Figure 1). Hair cells were counted under epifluorescence optics on a Leica fluorescent microscope using a 50 $\times$ objective and a 0.19 mm reticule in the microscope eyepiece. The number of OHC in each row and the number of IHC that were present or absent for each 0.2 mm reticule length was entered into a cytochleogram program (developed in house e.g. Lemke et al., 2009; Piu et al., 2011) starting at the apex and moving basally until the entire length of the cochlear spiral had been assessed. The program compares hair cell numbers to a normal data base. The program can generate a graph of hair cell loss by position along the cochlear spiral for each cochlea (cytochleogram) and also provide the analysis in absolute numbers or as the total percent of hair cells lost in each animal assessed.

Statistics

Significance was tested by MANOVA (Kruskal-Wallis test for nonparametric results) followed by two tailed unpaired t-test with Bonferonni correction for multiple comparisons, using Graphpad Prism or SigmaPlot. Groups further divided by differences in Gap Detection received Mann-Whitney post hoc test, using GraphPad Prism. A p-value less than 0.05 was considered necessary to infer significance.

RESULTS

Animals were removed from study if they lost ASR responsiveness, if they showed bilateral hearing loss after the unilateral noise exposure, or if they developed reduced PPI or ABR threshold shifts greater than two standard deviations from normal. Three of twenty animals in the group receiving noise exposure and no drug treatments were removed from study. Three of twelve animals in the group receiving noise exposure and the drug treatments were removed from study.

Loss of Hair Cells

The SAF-like noise produced a mean loss of 8.2% of OHC across untreated animals (saline control, normal diet). The pattern of OHC loss at 12-15 weeks following the noise is shown in a cytochleogram mapping the mean OHC loss along the cochlear spiral across animals (Figure 2). While the mean shows a primary loss between 10 and 20%, in the region 2-5 mm from the apex, there was considerable variability across animals in the OHC loss. No IHC loss was observed. The group of noise-exposed animals receiving treatment with a combination of piribedil, memantine and ACEMg had an 8.0% loss of OHCs, comparable to

the noise-exposed animals without drug treatment and there was also no major difference in the pattern of hair cell loss (Figure 2) between treated and untreated groups.

ABR

The ABR in the noise-exposed untreated animals (saline control, normal diet) at 12-15 weeks following the noise showed small but significant permanent threshold shifts of 19.0 dB at 8 kHz, and 13.1 dB at 16 kHz; the 6.7 dB increase at 24 kHz was not significant (Figure 3). The wave I Amplitude of the ABR in noise-exposed animals showed significant reductions at 8 kHz (not shown) and at 16 kHz (Figure 4) compared to Sham Noise animals but did not show a significant reduction from Sham at 24 kHz (not shown). Noise-exposed animals receiving the piribedil, memantine and ACEMg treatment showed less threshold shift than noise-exposed animals without treatment, significantly less at 16 kHz and not significantly different than sham noise animals at any of the three tested frequencies (Figure 3).

There was no drug treatment effect for combined piribedil, memantine and ACEMg, on the wave I amplitude of the ABR at 8 kHz with a SAF-like noise reduction comparable to the non-treated animals. At 16 kHz there was a treatment effect with wave I amplitude comparable to the sham noise group (Figure 4) at 70 dB ($p < 0.01$) and 80 dB ($p < 0.01$) comparable to the untreated group. The noise-induced reduction in wave I amplitude at 24 kHz did not reach significance and so while the piribedil, memantine plus ACEMg treatment resulted in a wave I amplitude that was comparable to sham, it was also not significantly different than the noise – no drug treatment group (not shown).

Loss of Synaptic Ribbons

There was a significant ($p < 0.01$) noise-induced decrease in the number of CTBP2 immunolabeled ribbons on IHCs in the region 6.5 mm from apex at 12-15 weeks following the noise. The mean number of ribbons per IHC decreased from 21.2 (± 8) in normal rats to 12.7 (± 2.4) in the noise-exposed untreated animals (saline control, normal diet) a decrease of 40% (Figures 2 and 5). There was a smaller noise-induced decrease in the number of ribbons per IHC in the regions 3.5 and 5.5 mm from the apex that did not reach significance (Figure 2). There was a significant influence of drug treatment on the noise-induced loss of IHC synaptic ribbons in the region 6.5 mm from apex. The noise-exposed group receiving combined piribedil, memantine and ACEMg treatment had ribbon counts that were significantly greater than the noise-exposed animals ($p < 0.05$) without drug treatment and not significantly different from normal animals (Figure 6).

Noise-Induced Changes – Reduced Gap Detection

None of ten sham noise animals had reduced GD. Three of twenty animals in the group receiving noise exposure but no drug treatments were removed from study. A reduction in GD without a reduction in PPI, a behavioral indication of tinnitus, was found in five of the remaining 17 animals (~30%). There were an additional three noise-exposed animals without drug treatment that showed reduced GD along with a reduction (within two standard deviations) in PPI. Reduced PPI can be an indication of hearing loss and hearing loss can influence GD. However, the ABR threshold shift and hair cell loss in these animals was no

different than those with reduced GD and normal PPI and all left cochleae were not noise exposed and retained normal hearing. If the animals with reduced PPI are included it brings the percent with reduced GD to approximately 50% (Figure 7 shows the percent having reduced GD, with and without inclusion of the animals with reduced PPI). Three of twelve animals in the noise-exposed group receiving piribedil, memantine and ACEMg treatment were removed from study. Seven of the remaining nine animals had no changes in GD, one had reduced GD with normal PPI and another had reduced GD and reduced PPI (Figure 7). This reduction in the incidence of tinnitus, however, did not reach significance, so the decrease can only be considered a “trend”.

Gap Detection and Synaptic Ribbons

The noise-exposed animals without drug treatment with reduced GD showed significantly ($p<0.05$) fewer ribbons per IHC (8.8 ± 3.3) compared to those that did not demonstrate these markers for tinnitus (18.4 ± 2.8) (Figure 6), both for those with reduced GD without reduced PPI or with reduced GD and the decrease in PPI. This difference in loss of ribbons is consistent with previous studies from the Knipper group using a different noise exposure condition and a psychophysical behavioral test for tinnitus, that also showed lower ribbon counts in noise-exposed rats that developed tinnitus compared to those that did not (Singer et al., 2013, Rüttiger et al., 2013).

DISCUSSION:

A SAF-like noise that causes only small (8%) hair cell loss and small ABR threshold shift resulted in a larger (40%) and significant loss of IHC ribbons (indicating loss of IHC-AN synaptic connections). This is consistent with previous studies with other types of mild noises (e.g. Kujawa and Liberman, 2009; Altschuler et al., 2016). The region of the cochlea with greatest loss of ribbons (6.5 mm from apex) did not coincide with the region of greatest loss of OHCs at 2-5 mm from apex. This suggests there may be differences in the sensitivity to noise related stresses between hair cells and IHC-AN synaptic connections.

There was a significant reduction in the supra-threshold amplitude of the wave I Input/Output functions in the noise-exposed non-treated animal group compared to the sham noise exposure group along with the significant loss of IHC synaptic ribbons also consistent with previous studies using a temporary threshold shift level of noise that caused loss of IHC-AN synaptic ribbons (Kujawa and Liberman, 2009) and caused greatest loss of low spontaneous rate (LSR), high threshold fibers that contribute to the amplitude of the ABR response (Furman et al., 2013).

Our previous study (Altschuler et al., 2016) found that treatment with memantine, piribedil and/or ACEMg reduced noise-induced loss of IHC ribbons from a different noise exposure condition (3 hour 4 kHz octave band 117 dB SPL noise). The present study found piribedil, memantine, ACEMg treatment also reduced loss of IHC ribbons from the SAF-like noise exposure, suggesting such “anti-excitotoxicity” treatment might have a broader application. An accompanying preservation of wave I suprathreshold amplitudes provides evidence that sparing of IHC-AN synaptic connections contributes to maintaining supra-threshold responses and dynamic range. The SAF-like noise exposure caused ABR threshold

elevations in the non-treated compared to the Control Sham group, on average, 10-15 dB above the Sham group.

The SAF-like noise resulted in significant decreases in GD in some but not all animals consistent with studies using other noise exposure conditions (see Shore et al., (2016) and other reviews). There was significantly greater loss of IHC ribbons in the noise-exposed animals showing reduced GD compared to those that did not (whether considered with or without an accompanying small reduction in PPI). Reduced GD has been suggested as an indication of tinnitus (e.g. Turner et al., 2006) and interestingly, previous studies (Singer et al., 2013; Rüttiger et al., 2013) using an operant conditioning behavioral metric for tinnitus also found greater loss of IHC ribbons in rats with this indication of tinnitus.

Limitations and future directions

If loss of IHC-AN synaptic connection is associated with induction of tinnitus then preventing their loss from noise could be a therapeutic target for noise-induced tinnitus. The present study, however, only revealed a trend with a decrease in the incidence of reduced GD in treated animals that while promising, did not reach significance. It would be useful in future studies to increase power with larger groups or use a noise exposure condition that produces a greater proportion of tinnitus in exposed animals. There is some evidence that operant conditioning tests for tinnitus identify a higher incidence of tinnitus in noise-exposed animals than seen using the reduction in GD as the metric (e.g. Jones and May, 2017; 2018). It would be useful in future studies to use both types of metrics as an assessment for noise induction of tinnitus and as a test for efficacy of treatments for tinnitus.

Wan et al., (2014) have shown that elevating NT-3 in cochlea after noise exposure induces “re-connection” of IHC-AN synapses lost from the noise and it would be interesting to test if this or other methods of reducing noise-induced loss of connections will reduce the incidence of tinnitus. It would also be interesting to see if loss of IHC-AN synaptic connections that can occur from other types of insults such as ototoxic drugs (e.g. Li et al., 2016) or aging (Sergeyenko et al., 2013; Altschuler et al., 2015) is associated with an increased incidence of tinnitus.

It is interesting that noise-exposed animals treated with piribedil, memantine and ACEMg showed less threshold shift than the non-treated noise-exposed group despite having comparable hair cell loss. A limitation of the present study is that it did not include use of distortion product otoacoustic emissions (DPOAE), a test of OHC function and it is possible that such a test could have indicated a treatment related difference in function between groups in the remaining OHCs. While the loss of IHC-AN synaptic ribbons measured following SAF-like noise was comparable to the loss reported from a TTS level noise (e.g. Kujawa and Liberman, 2009) where loss of LSR AN fibers predominated (Furman et al., 2013), it is possible that the SAF-like noise resulted in additional loss of the higher sensitivity medium and high spontaneous rate (MSR, HSR) AN fibers. Loss of MSR and HSR fibers could have contributed to the ABR threshold shift in the non-treated noise group and their sparing in the combined piribedil, memantine, ACEMg group could contribute to less threshold shift. Less threshold shift could also contribute to better supra-threshold

amplitudes in this group. It could be interesting to categorize the synaptic loss from different types of noise exposure conditions in future studies.

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Figure 1:
Surface preparations from middle of the rat cochleae (~5 mm from apex) with phalloidin staining of F-Actin (red) and immunostaining for CTBP2 (green), 2A from a normal untreated cochlea, 2B from a cochlea fifteen weeks after a SAF-like noise. Bar = ten microns.

Figure 2:

A cytochleogram of rat cochleae showing the average loss of outer hair cells (OHCs) (from all three rows) following an SAF-like noise fifteen weeks following the noise comparing groups with (red line) and without (dark blue line) treatment with memantine, piribedil and ACEMg. Below the cytochleogram the location of the three regions (3.25, 5.0 and 6.5 mm from the apex) assessed for loss of inner hair cell (IHC) ribbons (CTBP-2 immunolabeling of puncta) is shown with arrows and below the arrows the mean number of ribbons per IHC is shown for each region for normal non-treated rats (Normal) and for the rats exposed to the SAF-like noise (Noise) assessed fifteen weeks following the noise.

Figure 3:

The mean threshold shifts for the three groups of rats: a group receiving sham noise and no treatments (black circles), a group receiving SAF noise exposure and no treatments (labeled noise only – red triangles) and a group receiving SAF noise exposure and treatment with piribedil, memantine and ACEMg (labeled Noise – P&M&ACEMg - green rectangles), comparing baseline to fifteen weeks after the noise or sham. There was no significant change in sham noise animals. The SAF exposure in rats with no additional treatment resulted in significant threshold shifts ($p < 0.01$) compared to shams, with significant shifts of 19dB at 8 kHz and 13.1dB at 16 kHz at 12-15 weeks following the noise. A 6.7dB increase at 24 kHz was not significant. There was no significant difference compared to shams for the noise-exposed rats receiving piribedil, memantine and ACEMg treatment, the difference was only significant ($p < 0.1$) from the untreated noise-exposed group at 16 kHz and was not significantly different from either the sham noise group or the non-treated noise group at 8 or 24 kHz. Error bars show standard deviation.

Figure 4:

ABR wave I amplitude as a function of stimulus level is shown for three groups of rats: a group receiving sham noise and no treatments (black bars), a group receiving SAF noise exposure and no treatments (labeled noise only – red bars) and a group receiving SAF noise exposure and treatment with piribedil, memantine and ACEMg (labeled Noise – P&M&ACEMg - green bars), assessed fifteen weeks after the noise or sham. The sham wave I amplitude was significantly different from the control diet group at 80 dB ($p=0.004$). The piribedil, memantine and ACEMg group was significantly different from the control diet group at 80 dB ($p = 0.002$) and 70 dB ($p=0.007$). Error bars show standard deviation.

Figure 5:

The mean number of CTBP2 immunolabeled ribbons per hair cell in a ~0.2 mm region of interest located 6.5 mm from the apex (end of the cochlear spiral) is compared in four groups of animals: 1) rats receiving a SAF-like noise that developed reduced GD (green bar); 2) rats receiving a SAF-like noise that did not develop reduced GD (blue bar); 3) all rats that received SAF-like noise (combining both of the previous groups) (red bar) and 4) rats that received sham noise exposure (black bar). There was a significant loss of CTBP-2 immunolabeled ribbons per IHC in noise-exposed rats ($p < 0.01$). There were significantly fewer CTBP-2 immunolabeled ribbons per IHC in rats that developed GD compared to those that did not ($p < 0.05$). Asterisks show significance. Error bars show standard deviation.

Figure 6:

Treatment with piribedil, memantine and ACEMg reduces loss of inner hair cell (IHC) ribbons from a Small Arms Fire (SAF)-like noise exposure. The mean number of CTBP2 immunolabeled ribbons (puncta) per IHC for a ~0.2 mm region of interest located 6.5 mm from the apex (end of the cochlear spiral) is compared for three groups: a group receiving sham noise and no treatments (labeled No Noise), a group receiving SAF noise exposure and no treatments (labeled Noise only) and a group receiving SAF noise exposure and treatment with piribedil, memantine and ACEMg (labeled Noise-P/M & ACEMg), all assessed fifteen weeks after the noise or sham. There was a significant decrease in ribbons in the noise-exposed group with no treatments ($p < 0.01$). The noise-exposed group receiving combined piribedil, memantine and ACEMg treatment had ribbon counts that were significantly greater than the noise-exposed animals ($p < 0.05$) without drug treatment and not significantly different from normal animals. Asterisks show significance.

Figure 7:

Changes in Gap Detection are shown for three groups of rats: a group receiving sham noise and no treatment (labeled no noise), a group receiving small arms fire (SAF)-like noise exposure and no treatments (labeled noise only) and a group receiving SAF-like noise exposure and treatment with piribedil, memantine and ACEMg (labeled Noise P&M&ACEMg). Within each group the percent that had reduced Gap Detection with a reduction (less than two Standard Deviations) in Pre-Pulse Inhibition is added on with a grey bar. The treatment produced a large decrease in both measures but did not reach significance.