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Rescue of Hearing and Protection of Auditory Structures from Noise-Induced Hearing Loss

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Rescue of Hearing and Protection of Auditory Structures from Noise-Induced Hearing Loss

by

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Dedication

This dissertation is dedicated to my Mother and my Grandmother for their endless love, support, and encouragement.

I also dedicate this dissertation to my Late Father, Morteza Amanipour, who throughout his lifetime etched in the walls of my heart the importance of education.

First, I have to thank my Mother, Dr. Mahnoush Sophia Shafiei for her love and support throughout my life, who has been a source of encouragement and inspiration to me. With her constant support I pursuit this advance degree. Thank you for giving me strength to reach and chase my dreams.

This dissertation is also dedicated to my Grandmother Monir Maleki who raised me and was there for me throughout my life and without her endless love and encouragement I would never have been able to complete my graduate studies.

It is also dedicated to my Aunt Simin, for her support and encouragement, and always being there for me. I would like to thank my Aunt and Uncle, Dr. Kathy Shafiei-Maleki and Dr. Alireza Maleki for their guidance and support.

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Table of Contents

List of Figures

Abstract

Hearing loss due to exposure to high level sound is a major health problem that causes permanent hearing impairment and has a significant impact on job productivity and quality of life. A large population suffers from noise-induced hearing loss (NIHL), a disorder for which there is currently no FDA-approved prevention, treatment or cure. The goal of my dissertation work is to provide insights into the effects of noise trauma on hearing abilities of normal hearing subjects, and determine how a new therapeutic compound (mGlu7 modulator) can protect and/or prevent loss of, or damage to sensory cells and brain neurons, including hair cells and auditory nerve fibers, due to glutamate synaptic activity hyper-excitation.

NIHL can be induced by damaging the cochlear organ of Corti; as well as hypoxia of the inner ear due to reduced blood flow; oxidative stress related to the buildup of reactive oxygen species (ROS); and neural degeneration of synaptic terminals of cochlear nerve fibers and spiral ganglion cells [1]. It is known that membrane proteins called metabotropic glutamate receptors (mGluRs) regulate synaptic neurotransmission of glutamate and helps control ionic homeostasis, and they can be activated by release of glutamate from pre-synaptic cells, such as the hair cells of the inner ear[2].

I studied the effects of a new drug that modulates mGlu7 receptors and their activity in the auditory system and investigated its effects on noise-induced hearing loss (NIHL) in a rodent animal model. We achieved this by exposing mice to a loud noise trauma, then assessing the effects of the trauma using a series of hearing tests and biomarker studies. Known physiological indicators of NIHL in rodents, such as a change in the evoked response threshold, the reduced amplitude,

increased latency of the auditory brainstem response (ABR) waves in response to noise trauma, were used to provide further insights into the overall progression of NIHL. We used ABR wave analysis to examine the responses of different levels of the auditory pathway to tone and noise burst stimuli; and distortion product otoacoustic emissions (DPOAEs) were used to examine the functionality of the outer hair cell system; and a gap-in-noise (GIN) paradigm was utilized to examine changes in auditory temporal processing due to noise damage. NIHL can also alter neurophysiological responses in the central auditory system; here we investigated effects of acoustic overstimulation on key structures in the parts of the brain used for hearing, including the cochlear nuclei (CN) and inferior colliculus (IC).

Introduction

Age-related hearing loss – ARHL, and noise-induced hearing loss- NIHL, are two major hearing and communication health problems in our society [3]. These health problems can have lasting effects, and they may result in, or be related to different types of medical co-morbidities, such as: sleeping problems [4], cardiovascular disease and diabetes [5] [6], and cognitive impairment [7]. Hearing loss effects at least 30% of people over the age of 65 [8], and nearly one in four (24%) of U.S. adults ages 20 to 69 have been exposed to loud noise that can cause permanent hearing loss [9].

NIHL has a significant impact on communication abilities, and during noise exposure a listener does not necessarily feel pain or discomfort. Even though noise trauma has a significant effect on the inner ear (cochlear hair cells and 8^{th} nerve neurons), NIHL also damages central components of the auditory pathway [10-12]. NIHL has strong impact on hair cells and they often degenerate after exposure to loud noise. It has been shown that components of the central auditory pathway will be affected by NIHL as well. For example, it was previously reported that over time, a reduction in cochlear input and peripheral damage due to loud noise in the auditory pathway decreases the dendritic spines on cells in the CN, as well as reduction in cell body and tissue size in CN and the superior olivary complex (SOC) [13] [14] [15]. Kim et al. reported that NIHL resulted in axonal degeneration in the CN as well as axonal degeneration in the trapezoid body [16] [17] [18]. NIHL also causes many difficulties for military service members during and after their duties, i.e., soldiers exposed to loud noise have difficulty identifying speech in background noise, and after deployment they may have social isolation linked to communication disorders, and

trouble seeking and retaining job employment [19, 20]. Hearing loss and concomitant hearing disorders, such as tinnitus (ringing of the ears), comprises one of the main health problems for combat veterans [21], and it costs over one billion dollars every year in our U.S. Veteran's Administration Hospital Health System [22].

The time course of NIHL varies over weeks following acoustic trauma. Immediately following acoustic over-exposure hearing loss is the most severe and are maximally affected, which is termed temporary threshold shift (TTS). There are different definitions of TTS, most studies describe TTS as the partially reversible changes during the first 24 to 48 hours after noise trauma, and others classify TTS to last up to a week after noise trauma. During this time hearing recovers to various extent depending on the duration and intensity of the trauma. In contrast, irreversible changes in hearing thresholds are called permanent threshold shifts (PTS) [23-28]. PTS is defined as a time 2 weeks after the trauma when hearing thresholds have stabilized.

It is well known that glutamate is the main excitatory neurotransmitter in the auditory system, and for the IHC auditory synapses in particular, so a worthwhile target for a NIHL drug intervention [29]. Cochlear synaptic mechanism that involves glutamate excitatory result in hearing impairment, it has been reported that large amount of glutamate are released from hair cells in response to loud noise that can be toxic. Upon synaptic release, glutamate activates metabotropic glutamate receptors (mGluRs), mGluRs are class C G-protein coupled receptors with widespread expression in inner ear. mGluRs have very low affinity for their native ligands, glutamate. Other studies have shown that glutamate synaptic activity can have a major impact on plasticity and neurotoxicity, and changes in the glutamate neurotransmitter system can play roles in ARHL and NIHL [30]. Another study looked at excitotoxicity effects of the glutamate system on neurons in the central nervous system (CNS) [31] [32]. Additionally, Puel et al. found that

application of a glutamate receptor agonist results in damage very comparable to the afferent dendrite (located below the inner hair cell- IHC) damage following noise exposure [33]. Also, it has been reported by Hakuba et al. that glutamate excitotoxicity is one of the main contributing factors in NIHL [34]. Additional studies have reported that hearing loss and cochlear damage are due to excess release of glutamate reflected in elevated perilymph concentrations of glutamate [35]. Among eight different subtypes of mGluRs, mGluR7 (type 7) is the most conserved receptor, based on previous histochemical studies, mGluR7 is expressed in hair cells and spiral ganglion cells. mGluR7 are located pre-synaptically on central synaptic sites, where it negatively regulates glutamate release. mGluR7 may remain inactive during normal neurotransmission, only becoming active during times of excessive release and acting to regulate further release, therefore this receptor might play a modulatory role in order to protect from glutamate overstimulation. Current data shows that mGluR7 is expressed at presynaptic terminals, where it is always found at the active zone of presynaptic transmitter release, emphasizing its role in glutamatergic regulation. Genome-wide association studies (GWAS) have found several single nucleotide polymorphisms (SNPs) of the glutamate metabotropic receptor 7 gene (GRM7), suggesting that it plays an important role in susceptibility to ARHL. Therefore alleles of GRM7 are associated with developing ARHL, possibly through a mechanism of altered susceptibility to glutamate excitotoxicity [36-38]. The action of novel allosteric modulators at mGluR7, that interact with GABA interneurons, and postsynaptic NMDA receptors is supporting the development of a potential breakthrough therapeutics for the treatment of patients suffering from NIHL.

Reducing the severity of NIHL using mGlu7 allosteric modulator drugs has not been previously attempted or published. So, the existing body of evidence supports our theoretical framework underlying our main hypothesis that an mGlu7 allosteric drug can provide a new

therapeutic solution to preventing or reducing key aspects of noise damage for patients potentially suffering from NIHL. This approach may result in the identification and testing of the first drug to accomplish this task for this highly prevalent sensory disorder.

In this project, we examined the effects of three pre- and post-exposure drug treatments. These conditions are representative of protection and/or treatment of hair cell/auditory nerve fiber damage due to noise-exposure. DPOAEs were used to evaluate the health and responsiveness of the cochlear outer hair cell system, GIN was used to measure auditory temporal resolution, and ABR testing showed changes in cochlear (inner ear) sensitivity and brainstem processing. We compared changes in hearing thresholds, ABR and DPOAE, and key biomarkers between the pre- and/or post-exposure drug administrations.

Chapter 1: Noise-Induced Hearing Loss in Mice

This chapter is based on a published article entitled "Noise-Induced Hearing Loss in Mice: Effects of High and Low Levels of Noise Trauma in CBA Mice" that was published in 2018 (Publisher Copyrights permission in appendix A).

The purpose of this study was to establish a NIHL paradigm that results in a level of noise damage that can be prevented or reduced utilizing the new mGlu7 drug: We examined different noise exposure paradigms using two different intensity levels to produce NIHL. Hearing function was tested after the exposure to select a suitable exposure paradigm for the future project.

1.1 Abstract

Acoustic trauma can induce temporary or permanent noise-induced hearing loss (NIHL). Noise exposed animal models allow us to study the effects of various noise trauma insults on the cochlea and auditory pathways. Here we studied the short-term and long-term functional changes occurring in the auditory system following exposure to two different noise traumas. Several measures of hearing function known to change following noise exposure were examined: Temporary (TTS) and permanent (PTS) threshold shifts were measured using auditory brainstem responses (ABR), outer hair cell function was examined using distortion product otoacoustic emissions (DPOAEs), and auditory temporal processing was assessed using a gap-in-noise (GIN) ABR paradigm. Physiological measures were made before and after the exposure (24 hours, 2 weeks, 4 weeks, and 1 year). The animals were perfused and their brain, and cochlea were collected for future biomarker studies.

Young adult un-anesthetized mice were exposed to 110 dB and 116 dB octave-band noise levels for 45 minutes, and both groups demonstrated significant threshold shifts 1 day post-noise exposure across all frequencies. However 2 weeks post-exposure, PTS within the 110 dB group was significantly reduced compared to 1 day post trauma, this improvement in thresholds was not as great in the 116 dB exposure group. At 2 weeks post-trauma, differences between the measured PTS in the two groups was significant for 4 of the 7 measured frequencies. At this 1 year time point after exposure, mice in the 110 dB group showed very minor PTS, but the 116 dB group showed a large PTS comparable to their 2 and 4 week PTS. At this time point, PTS variation between the two groups was significant across all frequencies. DPOAE amplitudes measured 2 weeks post exposure showed recovery for all frequencies within 10 dB (average) of the baseline in the 110 dB group, however for the 116 dB exposure DP amplitudes were elevated by about 30 dB. The differences in DPOAE amplitudes between the 110 dB and 116 dB groups were significant at 2 weeks, 4 weeks, and 1 year post-trauma in the mid frequency range. At 2 weeks, 4 weeks, and 1 year, DPOAE thresholds returned to within 10 dB of the baseline for the 110 dB group in the low and mid frequency range, whereas the 116 dB group still showed shifts of 30 dB for all frequency ranges. For Gap ABRs, there was a significant decrease in both noise burst 1 (NB1) and noise burst 2 (NB2) amplitudes for peaks 1 and 4 in the 116 dB group relative to the 110 dB group when measured at 1 year post trauma. These results indicate that a 6 dB increase in noise exposure intensity results in a significant increased ototrauma in both the peripheral and central auditory systems.

1.2 Introduction

Noise- induced hearing loss (NIHL) is found in over 10 million adults (6%) in the US (Center for Disease Control and Prevention, 2011-2012) [39]. Age related hearing loss

(presbycusis) affect approximately one in three people in the United States between the age of 65 and 74 (NIDCD) [8]. Men are almost twice as likely as women to have hearing loss among adults 20-69 [40].

The majority of the studies using mice, in which researchers performed comparisons between NIHL and/or age-related hearing loss [41-50] either lack a long-term comparison (up to a year) or they did not assess the exposure levels investigated in the present study (i.e., did not test noise exposures above 100 dB). Jensen and his colleagues recorded physiological measurements up to 16 months post-exposure, but the highest trauma level that they used was 100 dB [51].

Here we are focused on changes in the auditory sensitivity following noise trauma at two different sound intensities, we then measured auditory function longitudinally for up to 1 year. We report changes in physiological measures of peripheral and central hearing.

1.3 Methodology

1.3.1 Subjects

Sixteen Male CBA/CaJ mice (4-6 months of age at the time of noise exposure) with normal physiological measurements (amplitude and threshold) were used in this study. The rationale for using the CBA mouse animal model is that it loses its hearing slowly with age, like most humans. Mice were raised in our in-house colony, and were derived from breeders originally purchased from The Jackson Laboratory. Mice were housed 2-4 per cage with litter-mates, in Sealsafe Plus GM500 cages (36 x 16 x 13) connected to a Box110SS Techniplast ventilated cage rack (West Chester, PA). The housing cages were maintained at a constant temperature and humidity, using a 12 hour light cycle with water and food pellets available *ad libitum*. Animals were closely checked daily throughout the study period. Animals were tested before exposure and they showed normal

ABR amplitudes and thresholds. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of South Florida- Tampa (IACUC).

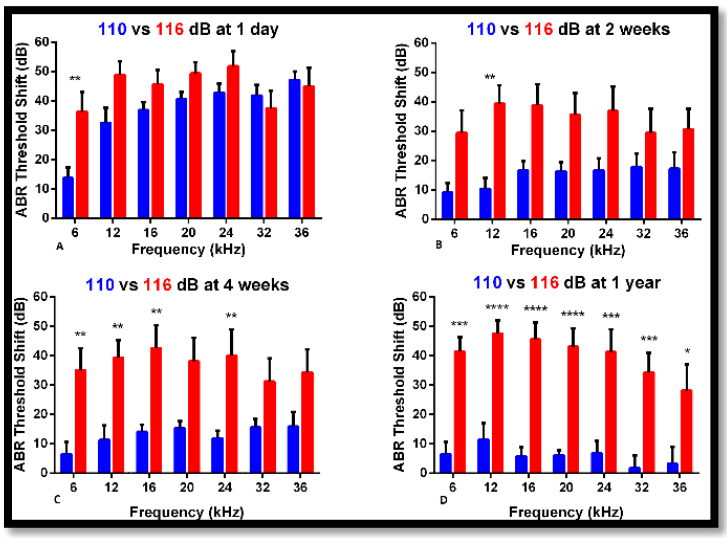

Figure 1.1 Effect of noise exposure on ABR threshold shifts (relative to pre-exposure Baseline recordings) for two groups of mice at seven frequencies. Post-hoc pair-wise comparison statistical significance levels: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

1.3.2 Acoustic Trauma

Noise exposure was performed in a double-walled soundproof room (IAC acoustics. North Aurora, IL). Each mouse was positioned individually in a 10 x 5 x 5 cm wire cage. A Technics Leaf tweeter EAS-10TH400A (Technics. Osaka, Japan) was placed on top of the mesh cage (10 cm away) and driven by an Adcom GFA-545II stereo power amplifier (ADCOM. East Brunswick, NJ). A BioSig RZ6 (Tucker-Davis Technologies (TDT) Alachua, FL) auditory processor was used to generate and control the noise. The overall noise level was measured at the center of the cage near the location of the mouse's head using a QE 7052 ½ inch microphone in combination with a Quest Model 2700 sound level meter (Quest Technologies Oconomowoc, WI). Unanesthetized mice were exposed to an octave-band noise of 8-16 kHz at 116 dB or 110 dB for 45 minutes. No food or water was provided during the duration of the exposure to noise.

1.3.3 ABR and GIN ABR

Each subject was anesthetized with a mixture of ketamine/xylazine (120/10 mg/kg body weight, respectively, IP) prior to ABR recording sessions, and respiration was monitored throughout the recordings to determine when additional supplement doses were required. Before, during, and after each recording session, body temperature was kept constant at 37°C using a warming therapy pump blanket (Stryker Medical. Portage, MI). ABR thresholds were acquired pre-exposure and at 4 different post-exposure time points, and were recorded in a sound booth (Industrial Acoustic Company Bronx, NY). Acoustic stimuli were produced by using a System III Tucker-Davis Technology system (TDT, Alachua, FL). Stimuli were attenuated and low-pass filtered (cutoff frequency 5 kHz) and averaged over 256 sweeps. Acoustic stimuli were presented to the ear via a TDT EC1 speaker. Our closed system ABR apparatus was calibrated before each recording session using a 0.6 cm type B&K microphone (Brüel & Kjær. Naerum, Denmark) in a volume that approximates the mouse ear canal [52]. ABRs were recorded using subdermal needle electrodes placed under the left pinna (ground), vertex (recording), and under the right pinna (reference). These needle electrodes were connected to an HS4 bioamp (TDT. Alachua, FL). ABR thresholds were obtained by presenting subjects with tonal stimuli at frequencies of 6, 12, 16, 20, 24, 32, and 36 kHz, attenuated in 5 dB steps from 80 dB to 5 dB SPL. Gap-in-noise ABRs were elicited by two wide band (WBN) noise bursts (NB1 and NB2) with a gap in between them, at 80 dB SPL, repetition rate was 21/s, duration of 25 ms, and 0.5 ms rise/fall time. The gap between NB1 and NB2 was varied: 0, 1, 2, 4, 8, 12, 16, 32, and 64 ms. Two replicates were acquired for each stimulus.

1.3.4 DPOAE

Outer hair cell function was assessed using distortion-product otoacoustic emissions-DPOAEs, utilizing procedures identical to those we have previously published [53, 54]. The DPOAE was recorded using Tucker Davis Technology (TDT) recording system, including stimulus generation and calibration. DP grams were acquired with frequency 1 intensity level (L1) = 65 dB, and frequency 2 intensity level (L2) = 50 dB sound pressure level (SPL). Mice were anesthetized with a mixture of ketamine and xylazine. All recording sessions were performed in a sound-proof acoustic chamber with body temperature maintained with a heating pad.

1.3.5 Data Analysis

ABR Thresholds were determined visually by three independent experimenters at the lowest intensity level by comparing two ABR wave replicates at which a defined peak was produced. ABR recordings for both groups were evaluated and compared, namely high and low intensity levels trauma. Threshold shift was computed by subtracting post-exposure level from baseline (pre-exposure). ABR GIN analysis started with identifying the location of peaks 1 and 4 (the ABR response to each noise burst consists of 4-5 peaks) for NB1 and NB2. For NB2, at short gap durations, there was no identifiable response (0 and 2 ms). Magnitudes for peaks 1 and 4 were used to formulate amplitudes statistics. The amplitudes were calculated by computing the difference between the peak and trough levels for peak 1 and peak 4. Amplitude shift values were calculated by subtracting the level at which the NB1 response occurred from the level that the NB2 response took place.

We used two-way analysis of variance (ANOVA) to perform statistical analysis for the amplitudes and amplitude shifts. When the main effects of the ANOVA were significant, in order

to assess pairwise comparison between the two groups, we used Bonferonni's multiple comparisons *post-hoc* tests (MCT).

1.4 Results

ABRs were measured before, 24 hours, 2 weeks, 4 weeks, and 1 year following exposure at seven different ABR frequencies (6, 12, 16, 20, 24, 32, and 36 kHz) and GIN ABRs were elicited by wideband noise. Amplitudes and thresholds of DPOAEs were measured before, 1 day, 2 weeks, 4 weeks, and 1 year post-exposure.

1.4.1 ABR

24 hours following noise exposure at both 110 and 116 dB significant threshold shifts were observed for all frequencies (TTS, Fig. 1.1.A), with threshold shifts for the louder exposure greater. At 2 weeks post-exposure, all mice showed elevated thresholds relative to baseline, for all 7 frequencies, the main effect was significant for the differences between the 2 groups of mice, and a significant pairwise comparison threshold shift was seen at 12 kHz (Main effect: $F(1,14) = 6.21$, $p = 0.0259$, Fig. 1.1.B). By week 4 post-exposure, the differences between ABR thresholds shifts in the two groups were significant, and at 6, 12, 16 and 24 kHz, for pairwise comparisons (Main effect: $F(1,14) = 9.51$, $p = 0.0081$, Fig. 1.1.C). Finally, at 1 year post-exposure threshold shifts for the 110 dB group had completely recovered, whereas thresholds for the 116 dB group had not, with significant differences in threshold shifts between the two groups at all frequencies (Main effect: $F(1,14) = 24.4$, $p = 0.0002$, Fig. 1.1.D).

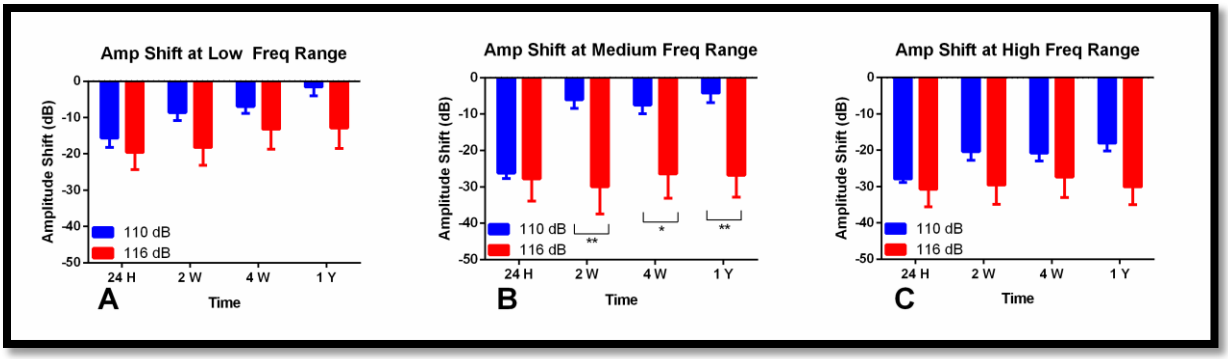

Figure 1.2. Effects of noise exposure on DPOAE amplitudes at four time points post-noise: (A) Low frequency range (5.3, 7.1, 10.7 kHz) (B) Mid-range frequency (14.3, 21.4, 28.6 kHz) (C) High frequency range (35.7, 40.2 kHz).

1.4.2 DPOAE

DPOAE frequencies were grouped into a low frequency range (5,366, 7,155, and 10,733 Hz), a mid-frequency range (14,310, 21,466, and 28,621 Hz), and a high frequency range (35,777, and 40,249 Hz). The differences in amplitude shifts at 1 year, between the two groups were affected by the two different trauma intensity levels. The amplitude shifts of the DPOAEs in the low frequency range were mostly recovered at 1 year for the 110 dB group (relatively close to 0 dB shifts), but this recovery did not occur in the 116 dB group as shown in Figure 1.2.A. For the mid frequency range the differences between the amplitude shifts were significant at 2 weeks, 4 weeks, and 1 year post-exposure (Main effect at 1 year: $F(1,14) = 6.874$, $p = 0.0201$, Fig. 1.2.B). The DPOAE amplitude shifts were more similar between the groups in the high frequency range Figure 1.2.C. The threshold shift at 24 hours post-exposure was about the same for all three frequency ranges (Fig. 1.3). However the differences were quite noticeable for 2 weeks, 4 weeks, and 1 year post-exposure; in fact threshold shift differences between the two groups were significant for the low frequency range ($F(1,14) = 13.82$, $p = 0.0023$, Fig. 1.3.A), and medium frequency range ($F(1,14) = 8.587$, $p = 0.0110$, Fig. 1.3.B).

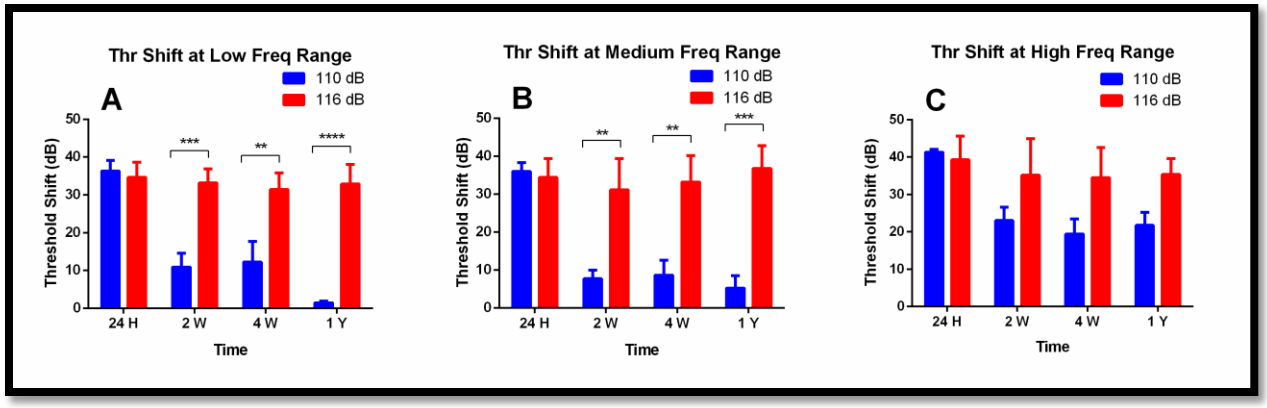

Figure 1.3. Effects of exposure on DPOAE thresholds at 4 time points post noise exposure: (A) Low frequency range (5.3,7.1,10.7 kHz) (B) Medium range frequency (14.3, 21.4, 28.6 kHz) (C) High frequency range (35.7, 40.2 kHz).

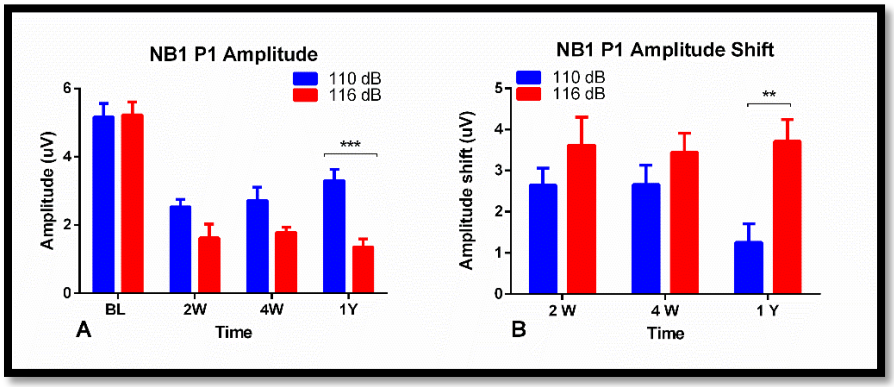

Figure 1.4. Effects of noise exposure levels on ABR GIN at three time points post noise exposure: (A) amplitude (B) amplitude shift.

1.4.3 GIN ABR

The average ABR GIN amplitude values for peak 1 are displayed in Figure 1.4. The ANOVA test revealed a significant difference between the two groups when measured 1 year post exposure ($F(1,14) = 12.17$, $p = 0.0036$, Fig. 1.4.A). The 110 dB group's amplitude showed some recovery over time, but the 116 dB group did not show any recovery in amplitude level. Similarly significant differences exist for the amplitude shifts between groups when measured 1 year post-exposure (Fig. 1.4.B). For NB2 peak 1, as gap size duration increases, the corresponding amplitude levels systematically increased in both groups. The largest amplitude shift in NB2 peak 1 was at the 1 year post-exposure time point, and the difference in NB2 peak 1 amplitude shift between 110

dB and 116 dB was significant for the 4 ms gap size ($F(1,14) = 5.610$, $p=0.0328$, Fig. 1.5.A). This difference in amplitude shift in NB2 peak 4 between the two groups was significant at most of the gap sizes (Fig. 1.5).

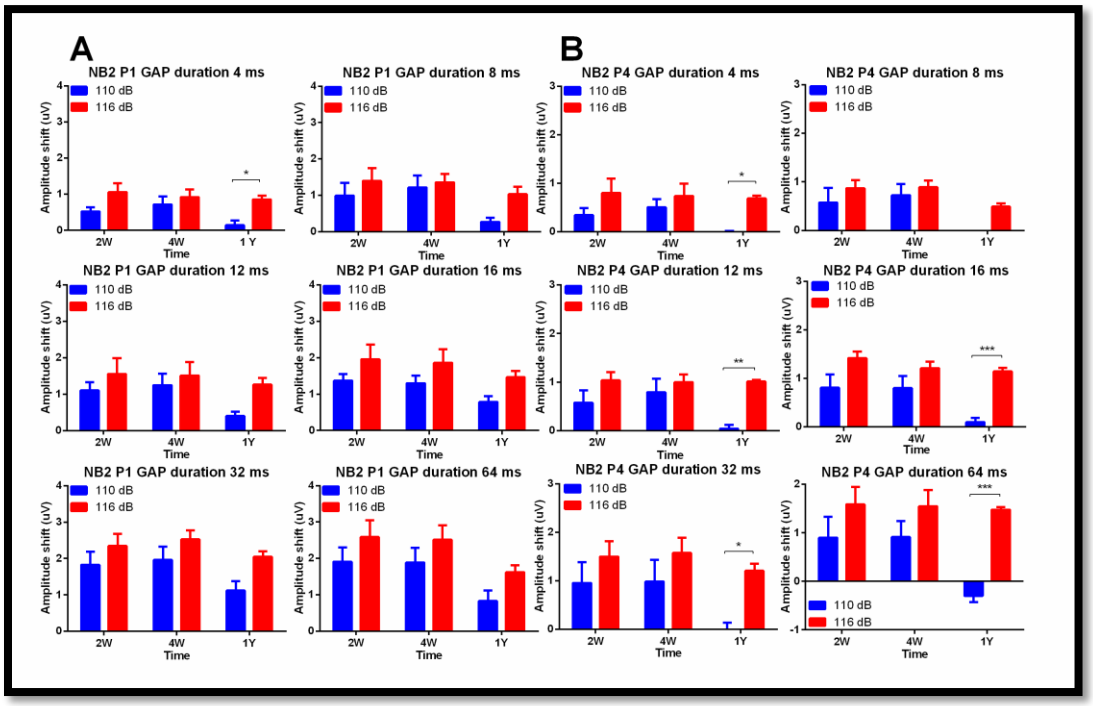

Figure 1.5. (A) NB2 Peak 1 and (B) Peak 4 amplitude shifts for three time points.

1.5 Discussion

In this study we utilized the ABR to measure hearing sensitivity, the DPOAE was used to evaluate outer hair cell functionality, and the ABR-GIN to measure auditory temporal processing. Yoshida and colleagues conducted a study in which they used CBA mice and a noise exposure setup at 100 dB for two hours. They reported maximum PTS of up to 35 dB (a range of 5 to 35 dB) 1 week post-exposure [55]. In another study Kujawa et al. used an identical exposure design, and reported TTS between 5 and 50 dB at 1 day after exposure and PTS of up to 10 dB when measured two weeks after exposure [43]. Here, we observed up to a 20 dB PTS at 4 weeks post-exposure in the 110 dB group, and up to 45 dB PTS in the 116 dB group. Our measurements 1

year post-exposure revealed that mice exposed to the 110 dB noise trauma have PTS of less than 10 dB, but subjects exposed at 116 dB still have PTS up to 50 dB.

In our present study, we found an increased PTS in normal hearing ears in unanesthetized mice due to an increase of 6 dB in noise-exposure level. Lower intensity acoustic trauma resulted in threshold shifts that typically recovered in one year, however significant threshold elevation remained in the group exposed to the 116 dB noise one year after trauma. The poorer temporal processing for the 116 dB group could be due to an age-related change in temporal processing interacting with an effect of the earlier noise trauma, which needs to be further investigated.

Cochlear amplification (outer hair cell (OHC) responses) is suppressed by axons activated in the medial olivocochlear (MOC). Excitatory and suppression effects on auditory nerve responses can also be the result of lateral olivocochlear (LOC) system activation [41]. It is known that the MOC can reduce cochlear responses in the presence of loud noise (trauma) by attenuating the gain of the OHC amplification system [56].

Our results indicate some significant ABR and DPOAE threshold and amplitude shifts, due to damage to cochlear sensory cell and neural components, including damage to OHCs [41]. It has been reported that during noise exposure glutamate excitotoxicity results in damage or loss of synaptic terminals, and/or discoupling of the synaptic ribbon from the glutamate receptors found on the inner hair cells [57, 58]. Subsequently, afferent terminal degeneration is observed which results in ABR thresholds shifts [43].

1.6 Conclusion

Our findings in the 110 dB group (of mice) indicated some significant recovery of the ABR and DPOAE thresholds, to levels close to their pre-exposure range 1 year after trauma even with

the effects of ARHL, whereas the 116 dB group showed much less recovery, consistent with findings reported in another study, where they reported up to 70 dB PTS following noise exposure [44].

Since PTS was reported in our subjects in this study, our ongoing efforts involve collecting both the cochlea and brain for anatomical and molecular biomarkers studies to elucidate the biological mechanisms underlying different levels of noise exposure damage to the auditory system. The new findings here can improve possibilities for understanding, modeling or treating noise-induced hearing loss in the future.

Chapter 2: Negative Allosteric Modulation of mGlu7 Receptors

This chapter is based on the "Negative Allosteric Modulation of mGlu7 Receptors: a Novel Approach to Prevent Noise-Induced Hearing Loss" article that is planned for submission in November 2019.

2.1 Abstract

Acoustic overstimulation causes cochlear damage, resulting in hearing loss. The main excitatory neurotransmitter in the inner ear and central auditory systems is glutamate. Excessive release of glutamate results in excitotoxicity, contributing to hearing impairment through degeneration of synaptic contacts between hair cells and spiral ganglion neurons. Here, we study the effects of PGT117, a negative allosteric modulator (NAM) of the metabotropic glutamate receptor subtype 7 (mGluR7) to prevent and reduce hearing loss following acute noise-exposure in mice. The mice were randomly placed in control and treatment groups (N=8/group). PGT117 was administered 1 hour pre-exposure and immediately following the exposure. Awake mice were exposed to an octave-band noise at 110 dB SPL for 45 minutes. Hearing threshold elevations as well as reductions in otoacoustic emission amplitudes occurred at every test frequency for the untreated group. However the treated group showed smaller threshold elevations and often no threshold shifts at 2 and 4 weeks post-exposure. Commensurate with the prevention of permanent hearing loss, preservation of spiral ganglion cells and cochlear nucleus neurons occurred in the treated mice. These results indicate that negative modulation of mGluR7 provides significant otoprotection from glutamate excitotoxicity that occurs in the cochlea and cochlear nucleus

following acoustic trauma. This novel drug is unique in that it targets the mGlu7 receptor which, until now, has not been investigated for preventing noise induced hearing loss (NIHL). In conclusion, PGT117 is a candidate on the clinical translational pathway to become the first FDA-approved drug to treat hearing loss.

2.2 Introduction

Hearing loss is a highly prevalent communication disorder affecting at least 1 in 10 persons worldwide; and the majority of people over 60 years old. Noise-induced hearing loss (NIHL) is one of the most serious and widespread forms, along with age-related hearing impairment. As industrialization progresses, so does the alarming rate of NIHL. Specifically, nearly one in four U.S. adults ages 20-69 have been exposed to loud noise that can cause permanent hearing loss [39]. This health problem can have lasting effects, that may result in, or be related to different types of medical co-morbidities, such as: sleeping problems [4], cardiovascular disease and diabetes [5, 6] and cognitive impairment [7]. In addition, recent evidence suggests that even moderate exposure to loud sounds can result in progressive cochlear synaptopathy affecting the primary synapses of cochlear inner hair cells [43]. This so-called, "hidden hearing loss" has been implicated in speech recognition deficits that can have long-lasting effects on real-world hearing abilities.

Even more surprising is the lack of any FDA-approved drug to prevent or treat NIHL; in fact, there are no drugs or medications worldwide for treating any type of sensorineural hearing loss, including NIHL. This therapeutic void calls for more research on the mechanisms of ototrauma, and utilization of novel compounds aimed at key biological mechanisms of NIHL. It is well known that glutamate is the main excitatory neurotransmitter for cochlear hair cell synapses, so a very promising target for a NIHL drug intervention [29]. Other studies have shown

that glutamate can have a major impact on synaptic plasticity, and changes in the glutamate neurotransmitter system can play roles in NIHL and age-related hearing loss [30]. Some studies have also investigated excitotoxicity effects of the glutamate system in the central nervous system (CNS) [31, 32].

The metabotropic glutamate receptor 7 (mGlu7) is one of the eight subtypes of the mGlu family (Class C GPCRs) that are expressed both centrally and in the cochlea [59]. It belongs to the Group III mGlu receptors [60], and it is localized at the pre-synaptic active zones in the CNS on both glutamatergic (auto-receptor) and GABAergic (heteroreceptor) neurons [60]. Of importance for preventing NIHL, the mGlu7 receptor has one of the lowest affinities for glutamate endogenous ligand, and is thought to act as a tonic brake of excessive glutamate release in certain pathophysiological conditions [60]. Interestingly, recent investigations showed that inhibitors of mGlu7 receptors may act primarily at GABA interneurons via a disinhibition process in the presence of high glutamate concentration, such as occurs in noise over-stimulation conditions for the inner ear [61].

Based on previous immunocytochemistry studies, the mGluR7 is expressed in cochlear IHCs, spiral ganglion neurons and cochlear nucleus. Several genome-wide association studies (GWAS) have found that several single nucleotide polymorphisms (SNPOs) of the gene for mGluR7 are linked to the susceptibility to age-related hearing loss [36-38]; NIHL and severity of tinnitus; possibly through a mechanism of altered susceptibility to glutamate excitotoxicity [36-38]. Using an mGlu7 antagonist therapeutic approach has not been previously attempted, so the main goal of the present study was to test the efficacy of a novel, highly specific, negative allosteric modulator (NAM) compound, namely PGT117 (Patent pending: Duvey, G. and Celanire, S. Preparation of novel heterocyclic compounds 1-oxo-5,6,7,8-tetrahydro-2H-phthalazine

derivatives and analogs, as mGluR7 modulators, PCT Int. Appl. (2019), WO 2019063596), via oral administration. Positive results from our experiments could put this new compound on a translational pathway to be the first drug to prevent or reduce NIHL.

2.3 Results

We have tested a newly developed, selective and specific mGlu7 NAM, PGT117, which potently reduced the mGlu7 receptor activity for both the inositol monophosphate (IP1) accumulation assay [median effective concentration (IC50) = 70,2 ± 1,2 nM; Fig. 2.1.B] and in the forskolin-induced production of cAMP assay [median effective concentration (IC50) = 22,1 ± 1,2 nM; Fig. 2.1.C], in mGlu7-expressing human cell lines. Potency of PGT117 was confirmed in mGlu7-expressing rat cell lines using the IP1 accumulation assay [median effective concentration (IC50) = 37,2 ± 1,3 nM; data not shown]. PGT117 also demonstrates a very high selectivity for the mGlu7 receptor over the other members of the mGlu receptor family, as well as a high specificity over a panel of various G protein-coupled receptors, kinase and non-kinase enzymes, transporters, nuclear receptors and ion channels (Fig 2.1.D).

PGT117 has drug-like properties, and no drug-drug interaction potential as shown by <50% inhibition of most major CYP450 isoforms at 10 µM; and *in vitro* safety profiling did not reveal either potential cardiovascular nor genotoxic risk. Finally, pharmacokinetic studies in rats showed an *in vivo* half-life of 1.27 hours, 54% oral bioavailability, and an inner ear/plasma ratio of 20% was measured 1 hour after oral dosing (Fig 2.1.E). These data were used for the design of experiments to maximize the inner ear exposure after oral administration, at the time of the noise exposure and auditory measures. In order to reach a PGT117 concentration in the inner ear, corresponding to a significant blockade of the mGlu7 receptors, a dose of 100mg/kg was

considered necessary to be 10-fold above mGlu7 IC_{50} in the inner ear, 1 hour after administration via oral gavage.

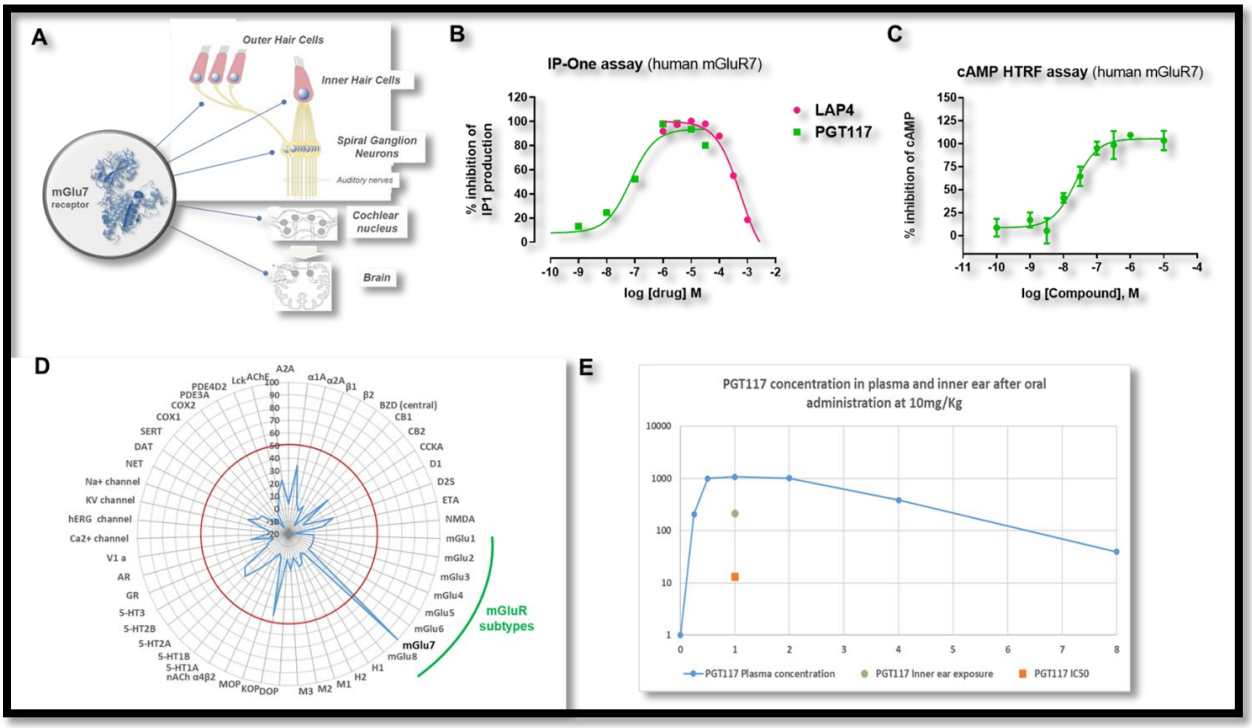

Figure 2.1. (A) Schematic representation of mGlu7 receptor expression in inner ear and brain structures. (B) Inositol Phosphate 1 (IP-one) production assay showing in vitro functional activity in recombinant cell lines expressing human mGlu7 receptors. Concentration-response curves were generated with LAP-4 in the absence or presence of PGT117. Each point represents the mean (± S.E.M.) of at least triplicate determinations from a single experiment. Antagonist activity of PGT117 is expressed as a percentage of the inhibition of reference agonist activity at its EC_{80} concentration. (C) cAMP production assay: in vitro functional activity in recombinant cell lines expressing human mGlu7 receptors. Concentration-response curves were generated with LAP-4 in the absence or presence of PGT117, as in Fig. 2.1.B. (D) Specificity profile of PGT117 on the remaining seven mGlu receptor subtypes (represented as a percentage of the inhibition of reference agonist activity at its EC_{80} concentration) and 44 additional target classes, including GPCR, enzymes, transporters, ion channels and kinases, performed at 10 µM concentration, average of n ≥ 2 independent experiments. (E) Rat pharmacokinetics of PGT117 using oral dosing, average n = 3, male Sprague-Dawley rats; dose = 10 mg/kg; Vehicle = CMC-Na 1%. Plasma and inner ear concentration measured at 1 hour time point post administration.

The Novel Compound Prevents NIHL

In young adult male CBA/CaJ mice, hearing sensitivity was measured with the auditory brainstem response (ABR), a noninvasive measurement of excitatory activity from the auditory

nerve and brainstem; a gap-in-noise (ABR GIN) test to measure temporal processing; and otoacoustic emissions (DPOAE), a measure of the health and functionality of the cochlear outer hair cell system. We assessed hearing function before and after the noise exposure and administration of the mGlu7 NAM. Mice were divided into noise-exposed experimental (treated, N=8) and noise exposed control (non-treated, N=8) groups. For these experiments, ABR, DPOAE, and ABR GIN data were acquired for each subject prior to the noise exposure, and 1 day, 2 weeks, and 4 weeks after exposure to the loud noise. The 1-day measures are an assessment of Temporary Threshold Shifts (TTS); a measure of temporary hearing loss, which typically decreases in magnitude over several days. The 2- and 4-week hearing assessments measure Permanent Threshold Shifts (PTS), considered to reflect long-term changes in hearing sensitivity. We then compared the pre- and post-exposure threshold and amplitude data for the control (noise-exposed, placebo drug) and treated groups.

PGT117 dramatically reduces auditory brainstem response (ABR) threshold shifts due to the noise exposure

The effects of noise exposure on mice that started the study with normal hearing were compared for the mice treated with PGT117, relative to non-treated controls. For the treated mice, 100 mg/kg of PGT-117 was administered 1 hour before, and immediately after the noise exposure for a total of 2 doses. We examined the differences in ABR threshold shifts (relative to the baseline pre-exposure ABRs) in treated and control groups. Figure 2.2.A plots the ABR threshold shift as a function of sound stimulus frequency measured 1 day post-exposure (TTS), and shows significant differences in the magnitude of TTS between the two groups. Note that larger, more positive threshold shifts signify greater hearing loss. TTS was much smaller, 4-20 dB in the treated group, relative to the 15- 48 dB shifts in the control group, with greater TTS at higher frequencies.

Significant main effects of drug treatment occurred for each time point (1-day, 2- and 4-weeks), and statistically significant *post-hoc* pairwise comparisons are denoted by an (*).

Figures 2.2.B-C show threshold shifts at 2, and 4 weeks post-exposure, respectively (PTS). Two weeks post-trauma, there were significant differences in threshold shifts between the two groups, with 9-18 dB PTS for the control group, and minimal shifts for the treated group. Differences between the two groups were particularly apparent for 6, 24, 32, and 36 kHz. In the 4-week post-trauma evaluation, Figure 2.2.C, noticeable PTS was still present in the control group; whereas PTS for the treated group was negligible, with the greatest differences between groups observed at 32 and 36 kHz.

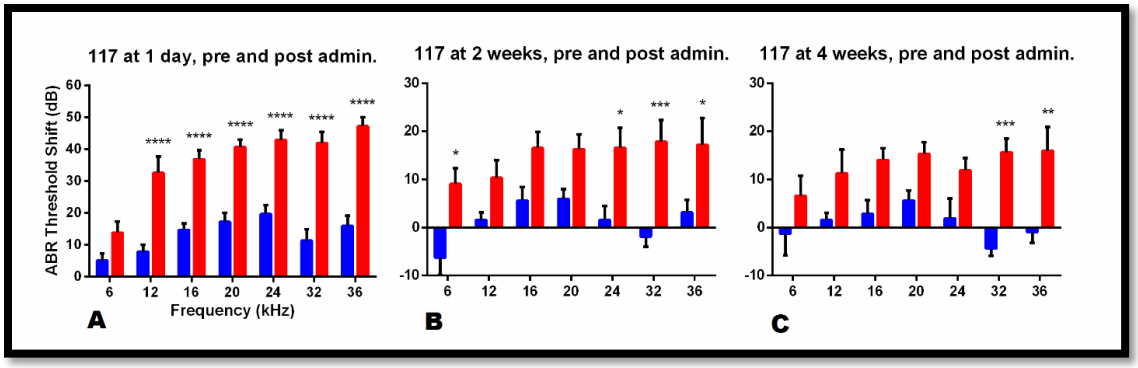

Figure 2.2. The mGlu7 NAM therapy dramatically reduces the magnitude of TTS and PTS. Effects of noise exposure on ABR threshold shifts (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies across the mouse hearing range. (A) PGT 117 was administered pre- and post-noise exposure, and the resulting TTS at 24 hours plotted as a function of ABR stimulus frequency for control (red) and treated (blue) mice. The main effect was highly significant ($F(1, 14) = 70.8$, $P < 0.0001$) at 1 day post-exposure. (B) Effects of treatment 2 weeks following noise exposure showing a significant reduction in PTS for the treated mice. The main effect was highly significant ($F(1, 14) = 15.1$, $p = 0.0016$). (C) Effects of treatment 4 weeks following acoustic trauma showing a significant reduction in PTS with the main effect highly significant ($F(1, 14) = 15.9$, $P = 0.0014$). The post-hoc pairwise comparison statistical significance levels are: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Outer hair cell system functionality is protected: Distortion product otoacoustic emission analysis

DPOAE frequencies were grouped into three frequency regions; low frequency range (5.4, 7.1, and 10.7 kHz), a medium frequency range (14.3, 21.4, and 28.6 kHz), and a high frequency

range (35.7, and 40.2 kHz). The differences in amplitude shifts at 3 different time points, between the two groups are shown in Figs. 2.3.A, B and C. Note, a decrease in the amplitude of the DPOAE, i.e., a negative amplitude shift, indicates that the functionality of the outer hair cell (OHC) system has become impaired due to the noise exposure.

Figure 2.3.A shows significant differences in DPOAE amplitude shifts for the low frequency range at 24 hours, and 2 and 4 weeks post-exposure. So, similar to the ABR threshold results presented above, the mGlu7 modulator has prevented damage to the OHC system. Within the medium frequency range at 24 hours post-exposure, DPOAE amplitude shifts, were 25 dB for the control group, but only 8 dB for the treated group (Fig. 2.3.B), which represents a significant treatment effect on protecting OHC function. Indeed, for the medium frequency range, the main effect was significant for OHC protection, along with the pairwise comparison for TTS. There were no statistically significant pairwise differences between the two groups at the PTS time points (2 and 4 weeks), however amplitude shifts were greater within the control group at both PTS evaluation points (Fig. 2.3.B); with almost no shifts in the treatment group. For the high frequency range, there was a trend for the treatment to reduce the amplitude changes somewhat, but the differences between means were not significant (Fig. 2.3.C).

DPOAE threshold shifts after noise exposure are shown in Figs. 2.3.D-F; where measurements were made before the noise exposure (baseline) and at 24 hours, and 2 and 4 weeks after exposure. Please note, as for ABR thresholds, a positive threshold shift indicates a decline in auditory sensitivity, i.e., the hearing gets worse due to the noise exposure. Within the low frequency range, the differences in threshold shifts were quite significant at all three time points (Fig. 2.3.D). Within the medium frequency range, significant differences in threshold shifts were revealed between the control and treated groups (Fig. 2.3.E). Like the low frequency data of

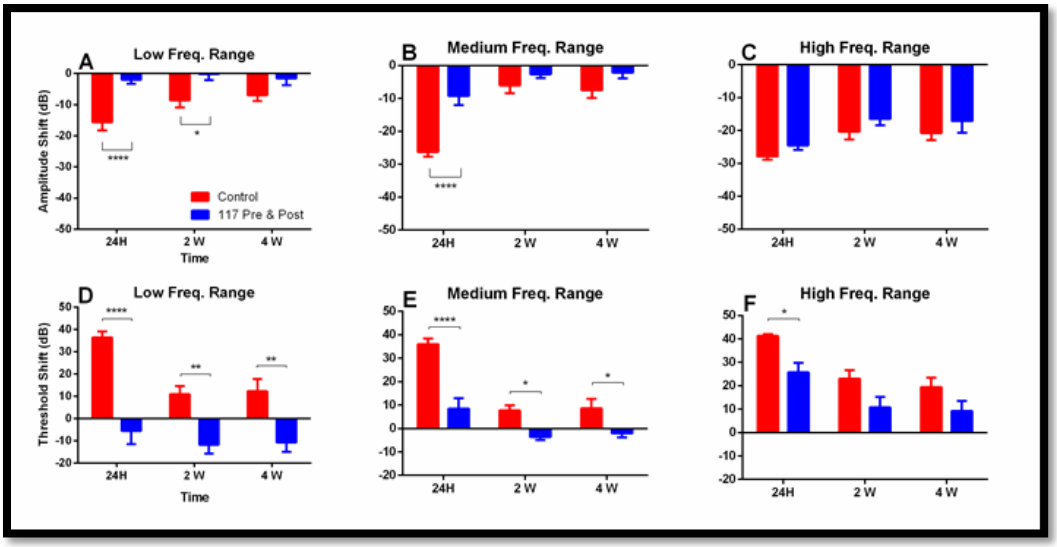

Figure 2.3. PGT 117 protects outer hair cell (OHC) function at three time points, 1 day, 2 weeks and 4 weeks; for three frequency regions: low (5.3, 7.1, 10.7 kHz), medium (14.3, 21.4, 28.6 kHz), and high (35.7, 40.2 kHz). (A) Amplitude shifts are plotted as a function of time after noise exposure. Amplitude shift is computed as: DPOAE level after noise exposure minus the baseline level; so negative shifts denote poorer OHC responsiveness. The treatment effect for low frequencies was highly significant: F (1, 14) = 16.07, p= 0.0013. (B) The ANOVA for the medium frequencies was also highly significant: F = 1, 14) = 19.53, p= 0.0006. (C) The high frequencies showed only small differences between controls and treated mice. D-F) Changes in DPOAE threshold shifts after the noise exposure. Here, threshold shift is computed as: threshold after noise exposure minus the baseline, so positive threshold shifts indicate poorer OHC responses. The main effects were all highly significant. (D) For the low frequency region, the main effect: F = (1, 14), p< 0.0001. (E) Medium frequencies main effect = F (1, 14) = 32.56, p< 0.0001. (F) High frequency range main effect: F (1, 14) = 11.71, p= 0.0041. The post-hoc pairwise comparison statistical significance levels are: *p<0.05; **p<0.01; ****p<0.0001.

Figure 2.3.D, the drug has prevented OHC hearing deficits at all three assessment time points after noise exposure. For the high frequencies the main effect for the treatment was significant (Fig. 2.3.F), and for the pairwise comparisons, the threshold shift difference between the two groups was significant at 24 hours post-exposure. The shifts were larger for the control group at 2 and 4 weeks (Figs. 2.3.E-F), so, an indication of PTS hearing protection.

PGT117 Preserves Supra-Threshold Coding: Gap-in-noise ABR analysis

The average ABR GIN amplitude differences for NB1 and NB2 for P1 and P4 are displayed in Figure 2.4.A-F. The differences in P1 and P4 NB1 amplitudes between baseline and post-

exposure measurements were smaller for the treated group, due to otoprotection by the mGlu7 modulator drug, i.e., higher P1 and P4 amplitudes after the noise exposure relative to the controls; indicating partial protection of supra-threshold coding at the level of the auditory nerve (P1) and brainstem (P4- lateral lemniscus/inferior colliculus). Please note that the main effect for P1 was statistically significant, however the P4 result was just a trend.

Peak 1 and Peak 4 NB2 GIN amplitude differences by gap duration plots (Figs. 2.4.C-F), respectively, were measured at baseline (BL), and two and four weeks post-exposure. Note the larger differences (relative to baseline) in NB2 P1 and P4 amplitude levels in the control group, when compared to the treated group at both post-exposure evaluations. Larger differences in amplitude between BL and post-exposure time points signify better gap encoding/auditory temporal processing in the treated group. Higher NB2 P1 levels indicate better suprathreshold cochlear temporal processing. Greater NB2 P4 levels reflect better brainstem auditory processing. However, these NB2 results showed a definite trend, but due to variability the main effects were not statistically significant.

PGT117 Protects Cochlear Spiral Ganglion Neurons

The spiral ganglion neurons are the cell bodies of the auditory nerve fibers of the 8th cranial nerve, which carry all of the sound coding information from the cochlea to the cochlear nucleus of the brainstem auditory system. The spiral ganglion neurons' dendrites provide the post-synaptic elements of the hair cell/auditory nerve synapses. Interactions between reduced hearing loss following noise exposure due to treatment with the mGluR7 NAM and spiral ganglion neuron density were explored 20 weeks post noise-exposure. Spiral ganglion cell counts were conducted in control (sound exposed, no drug) and treated (sound exposed, received drug) mice, with the

results shown in Figure 2.5, dramatic protection for spiral ganglion neurons was found in all three cochlear turns for the treated mice relative to the age-matched controls.

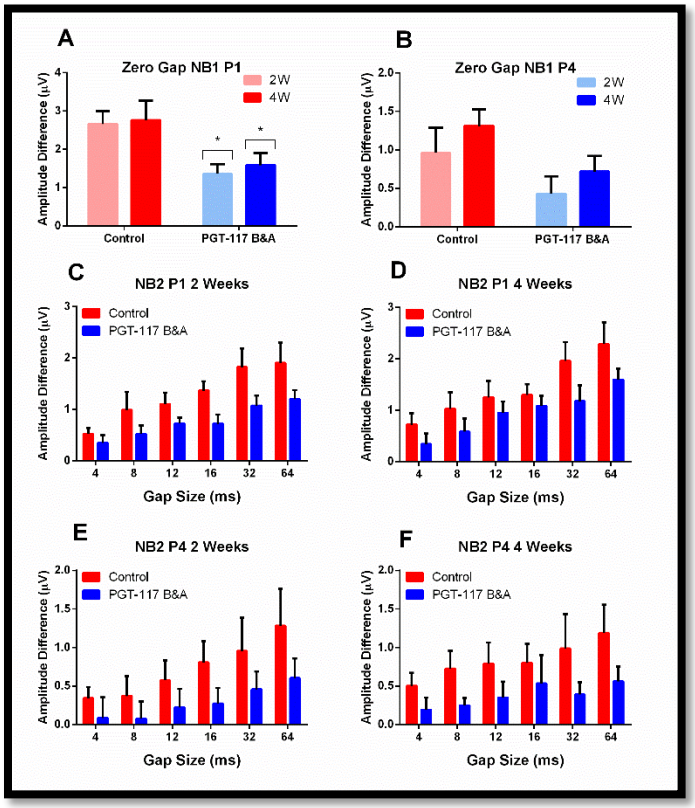

Figure 2.4. The mGlu7 drug helps preserve supra-threshold (80 dB) ABR P1 and P4 amplitudes. (A) NB1 P1 amplitude differences elicited by a wideband noise burst shown for 2, and 4 weeks post acoustic trauma for control and treated mice. Note that the differences between the amplitudes of the control baseline data, compared to 2 (2W) and 4 weeks (4W) levels are larger than that for the treated group. * indicates $P<0.05$. (B) NB1 P4 amplitude differences elicited by a wideband noise burst, shown for 2 and 4 weeks post acoustic trauma for control and treated mice. Like P1, the differences between the amplitudes of the control baseline data, compared to 2 (2W) and 4 weeks (4W) levels are larger than that for the treated group. So, there is a trend for the treatment to help preserve suprathreshold ABR coding at the level of the auditory brainstem. (C) and (D) NB2 P1 amplitude differences elicited by a wideband noise burst shown for 2 and 4 weeks post acoustic trauma for control and treated mice. Notice that the amplitudes increase with the gap duration. Like NB1, the differences between the amplitudes of the control baseline data, compared to 2 (2W) and 4 weeks (4W) levels are larger than that for the treated group. So, the treatment may help preserve suprathreshold auditory temporal processing at the level of the auditory nerve. (E) and (F) NB2 P4 amplitudes as a function of gap duration elicited by a wideband noise burst are shown for baseline, 2 and 4 weeks post acoustic trauma for control and treated mice.

PGT117 Protects Neurons in the Central Auditory System: Cochlear nucleus cell counts

The cochlear nucleus is located on the lateral side of the medulla, bounded medially by spinal trigeminal tract, and lies on the dorsal surface of the lateral recess of the fourth ventricle. It receives all of the cochlear outputs, and is the gateway for sound processing information to the central auditory system. Nissl stained anatomical sections of the anteroventral (AVCN) subdivision of the ventral cochlear nucleus are displayed in Figure 2.6.A. Note that the section from a control mouse (left) has fewer cells than that from the treated mouse (right). Nissl stained

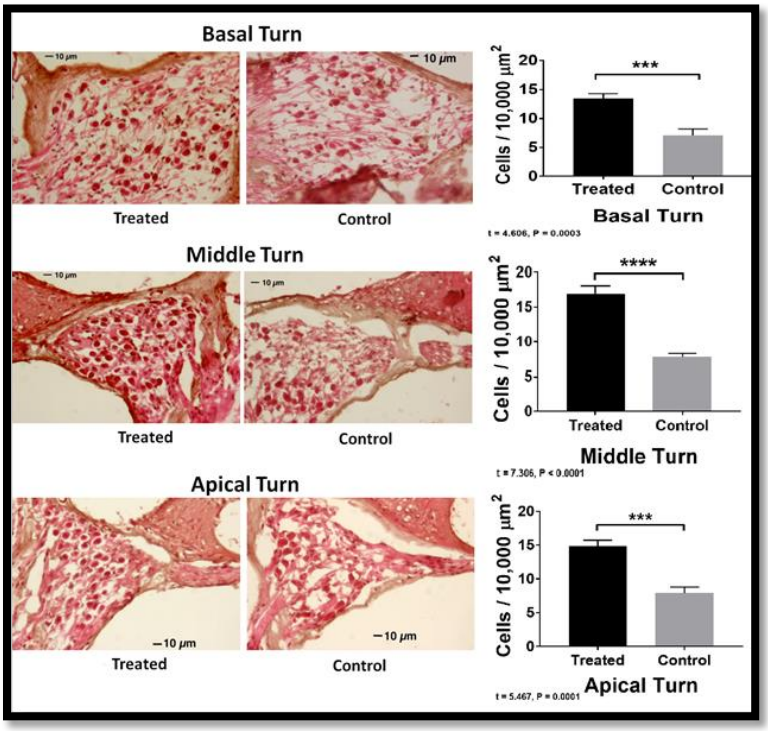

Figure 2.5. PGT 117 significantly protects spiral ganglion cells. Representative photomicrographs of radial sections through Rosenthal's canal showing spiral ganglion neuron cell body densities for treated (left panel), and control (middle panel) for the base, middle and apical cochlear turns. Right panel shows bar graphs representing spiral ganglion cell density for treated (black) and control (gray) mice. Mean ±SEM for each subject group. T test significance levels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

anatomical sections of the dorsal cochlear nucleus (DCN) are displayed in Figure 2.6.B. The mean cell density in AVCN and DCN for each group is quantified in Figure 2.6.C. Significant differences

in the cell density between control and treated groups were observed at both AVCN and DCN topographical areas of the cochlear nucleus.

Initial Biomarker Investigation: Treatment Effects on Cochlear Transcriptional Factors

Given the dramatic improvements of hearing and protection of key auditory cell types presented above for the mGlu7 NAM, investigations of cellular and molecular mechanisms are

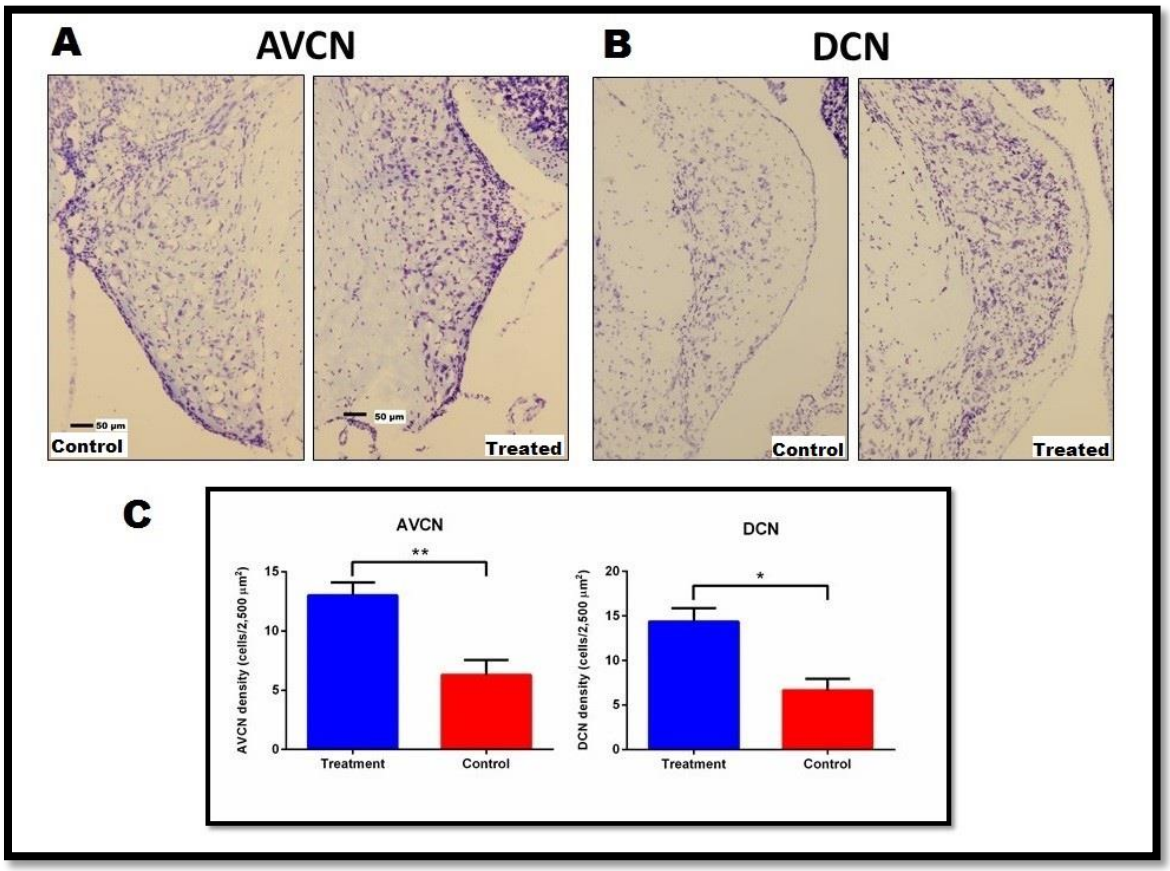

Figure 2.6. PGT 117 protects neurons of the central auditory system after noise overexposure. (A) Representative Nissl-stained sections of the anteroventral subdivision of the ventral cochlear nucleus: Left, Control; Right, Treated. Notice the greater density of cells in the treated section relative to the control section. (B) Representative Nissl-stained sections of the dorsal cochlear nucleus: Left, Control; Right, Treated. Like the anteroventral subdivision, note the greater density of cells in the treated section relative to the control section. (C) Quantitative analysis of the cell density measurements reveals statistically significant protection of cochlear nucleus cells due to the PGT 117 drug treatment. AVCN – anteroventral subdivision of the ventral cochlear nucleus; DCN – dorsal cochlear nucleus. T test statistical significance levels: *$p<0.05$, **$p<0.01$.

being initiated. Inflammatory cells are known to be activated in response to acoustic insults that induce cochlear damage and cell death. We evaluated the effects of PGT117 on two biomarkers of the cochlear inflammatory response, NF-κB and Akt. NF-κB is transcriptional nuclear factor κB involved in inflammation pathways, while the kinase Akt is a key downstream mediator of the phosphoinositide-3-kinase (PI3K) signaling pathway, involved in cellular processes, including apoptosis and inflammation. Gene expression levels for the untreated, control group were higher than the treated group, suggesting that transcriptional factors, NF-κB and Akt were down-regulated due to the treatment, as shown quantitatively in Figure 2.7; consistent with reduction in the cochlear inflammatory response.

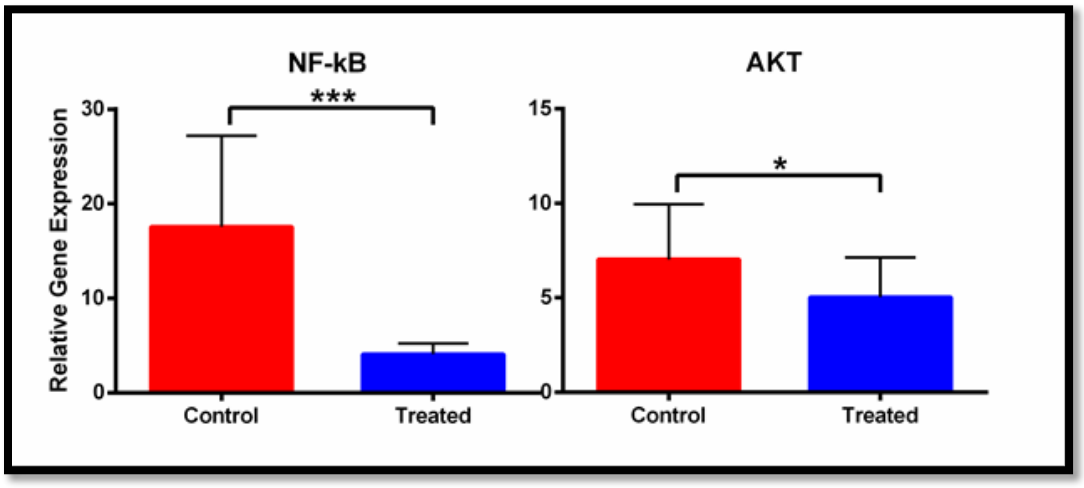

Figure 2.7. Inflammatory biomarkers are down-regulated by the mGlu7 NAM treatments for cochlear samples. Total RNA was isolated from mouse cochlea, including the organ of Corti, spiral ganglion neurons and stria vascularis, and reverse transcribed, revealing changes in the inflammatory response pathways of the cochlea due to the PGT 117 treatments. Left: RT-PCR analysis with primers representing the NF-κB NH2 terminus was performed using 10 ng of the diluted RNA. Right: RT-PCR analysis with primers for Akt NH2 terminus was performed using same amount RNA as for NF-κB. β-Actin was used as a loading control. Bar graph results are means ± SD from 3 independent experiments (N=3 mice in each group), defined as target gene expression level relative to the control (β-actin expression). T test levels of statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$.

2.4 Discussion

The pathophysiology of NIHL involves multiple etiologies, such as induction of reactive oxygen species from excitotoxicity, and the initiation of apoptotic pathways, both of which can compromise cochlear cells, including hair cells spiral ganglion neurons. In addition, sound over-stimulation results in synaptic glutamate excitotoxicity, which is also a prominent pathological component in NIHL and many neurological disorders of the central nervous system. The inner ear hair cell/auditory nerve synapses have a plethora of glutamate receptors that mediate excitatory glutamate signaling. For hair cell synapses, following the apical transduction activation cascade, glutamate is released and initiates action potentials via ionotropic AMPA receptors (GluR2/3) juxtaposed to the pre-synaptic ribbon synapse. In addition, during acoustic trauma the pre-synaptic metabotropic glutamate receptor 7 (mGluR7) is also activated by high levels of glutamate in the synaptic cleft, so we hypothesized that decreasing mGlu7 activity can act as a brake on hair cell excitotoxicity by reducing glutamate over-stimulation.

We have presented dramatic new findings on hearing protection for a novel otoprotectant (PGT117), a negative allosteric modulator of mGluR7, which displays high affinity and excellent bioavailability characteristics in both rat and mouse pharmacokinetic cochlear profiles. We administered PGT117 before and after acoustic trauma and found dramatic evidence of both functional and structural otoprotection. Previous pre-clinical studies have shown protection for NIHL in rodent models, focusing upon antioxidant approaches. Specifically, antioxidant cocktails, including vitamins A, C, E and N-acetylcysteine, given in conjunction with noise exposures have shown protective effects [62]. The mechanism of action is to attempt to neutralize or remove reactive oxygen species (ROS), free radicals, after these cellular destructive compounds are produced during and after hyper-excitability, including over-stimulation of the hair cells and spiral

ganglion neurons in response to loud noise. In contrast, a key advantage of targeting the mGluR7 receptors of the present study, is that it reduces glutamate-related excitotoxicity at the hair cell/SGN synapse, *preventing the production of the ROS and free radicals in the first place,* rather than trying to neutralize them after they are already produced and done damage. This innovative approach of our pre-clinical study should bode well for successful clinical trials in the future, whereas the antioxidant approaches have not succeeded in FDA clinical trials [63].

In addition, the results presented here suggest that the orally administered, systemic NAM may also have beneficial effects on reducing excitotoxicity effects in the central auditory system. So, even though noise trauma has a deleterious effect on the inner ear (cochlear hair cells, spiral ganglion neurons), NIHL also damages central components of the auditory pathway [10-12]. For example, it was previously reported that over time, peripheral damage, such as a reduction in cochlear inputs to the central auditory system due to loud noise decreases the dendritic spines on cochlear nucleus cells, as well as reduction in cell body numbers and size in the cochlear nucleus and superior olivary complex [13-15]. Additionally, Kim et al. reported that NIHL resulted in axonal degeneration in the cochlear nucleus and trapezoid body following noise exposure [16-18].

2.5 Materials and Methods

2.5.1 Subjects

Sixteen male CBA/CaJ mice (4-6 months of age at the time of noise exposure) with normal physiological measurements (amplitude and threshold) were used in this study. The CBA mouse animal model loses its hearing slowly with age, like most humans. Mice were raised in our in-house colony, and were derived from breeders originally purchased from The Jackson Laboratory. Mice were housed 2-4 per cage with litter-mates, in Sealsafe Plus GM500 cages (36 x 16 x 13) connected to a Box110SS Techniplast ventilated cage rack (West Chester, PA). The housing cages

were maintained at a constant temperature and humidity, using a 12 hour light cycle with water and food pellets available *ad libitum*. Animals were closely checked daily throughout the study period. Animals were tested before exposure and they showed normal ABR amplitudes and thresholds. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of South Florida- Tampa (IACUC).

2.5.2 Noise Exposure

To induce NIHL male CBA/CaJ mice (4-6 months of age at the time of noise exposure) with normal hearing were exposed to noise exposure paradigm. Noise exposure was performed in a double-walled soundproof room (IAC Acoustics, North Aurora, IL). Each mouse was positioned individually in a 10 x 5 x 5 cm wire cage. A Technics Leaf tweeter EAS-10TH400A (Technics, Osaka, Japan) was placed on top of the mesh cage (10 cm away) and driven by an Adcom GFA-545II stereo power amplifier (ADCOM, East Brunswick, NJ). A BioSig software package and RZ6 (Tucker-Davis Technologies (TDT), Alachua, FL) digital signal processor were used to generate and control the noise. The overall noise level was measured at the center of the cage near the location of the mouse's head using a QE 7052 ½ inch microphone in combination with a Quest Model 2700 sound level meter (Quest Technologies, Oconomowoc, WI). Unanesthetized mice were exposed to an octave-band noise of 8-16 kHz at 110 dB for 45 minutes.

The ABR and DPOAE testing procedures were similar to our previous reports [64, 65].

2.5.3 Auditory Brainstem Response – ABR, and GIN ABR

ABRs are relatively easily measured in rodents, without the need for surgery, and they were used in this study for longitudinal assessment of hearing, including at the level of the 8th nerve and brainstem components of the auditory system. It has been reported that peak 1 (P1) of the ABR is

produced by the auditory nerve [66], and the lateral lemniscus is believed to be the main contributor to ABR peak 4 (P4) [67]. Each subject was anesthetized with a mixture of ketamine/xylazine (120/10 mg/kg body weight, respectively, IP) prior to ABR recording sessions, and respiration was monitored throughout the recordings to determine when additional supplement doses were required. Before, during, and after each recording session, body temperature was kept constant at 37°C using a warming therapy pump blanket (Stryker Medical, Portage, MI). ABR recordings were recorded in a sound booth (Industrial Acoustic Company, Bronx, NY). Acoustic stimuli were produced by using a System III Tucker-Davis Technology system (TDT, Alachua, FL). Stimuli were attenuated and low-pass filtered (cutoff frequency 5 kHz) and averaged over 256 sweeps. Acoustic stimuli were presented to the ear via a TDT EC1 speaker coupled to a probe tube. Our closed system ABR apparatus was calibrated before each recording session using a 0.6 cm type B&K microphone (Brüel & Kjær, Naerum, Denmark) in a volume that approximates the mouse ear canal [52]. ABRs were recorded using subdermal needle electrodes placed under the left pinna (ground), vertex (recording), and under the right pinna (reference). These needle electrodes were connected to an HS4 bioamp (TDT, Alachua, FL). ABR thresholds were obtained by presenting subjects with tonal stimuli at frequencies of 6, 12, 16, 20, 24, 32, and 36 kHz, attenuated in 5 dB steps from 90 dB to 5 dB SPL. Gap-in-noise ABRs were elicited by two wide band (WBN) noise bursts (NB1 and NB2) with a silent gap in between them, at 80 dB SPL, repetition rate was 21/s, duration of 25 ms, and 0.5 ms rise/fall time. The gap between NB1 and NB2 was varied: 0, 1, 2, 4, 8, 12, 16, 32, and 64 ms. Two replicates were acquired for each stimulus.

2.5.4 Distortion Product Otoacoustic Emissions – DPOAE

Measurements of outer hair cell function with DPOAEs were accomplished using the TDT BioSig III system, utilizing procedures identical to those we have previously published [53] [54]. DPOAE measurements were completed with the constant ratio of frequency 2 (F2) to frequency 1 (F1) at 1.25, and L1 = 65 dB SPL, L2 = 50 dB SPL. The magnitude of F1, F2, as well as the 2f1 – f2 distortion product, along with noise floor components were measured and the results were exported to a Microsoft Excel sheet for further analysis. Sound pressure created by inner ear was recorded, for frequencies ranging from 5.6 to 44.8 kHz. Each measurement was repeated three times, the average of these three trials was used for the analysis. DPOAE's amplitude and thresholds were compared before and after noise exposure in both groups. DPOAE thresholds are the minimum sound level needed to evoke a threshold response of the cochlear outer hair cell system; so a measure of outer hair cell sensitivity. DPOAE amplitudes are a suprathreshold response that measures the collective strength of the outer hair cell system; i.e., an overall measure of the physiological health of outer hair cells.

2.5.5 Immunohistochemistry

After 5 months of noise exposure, Subjects were deeply anesthetized and perfused in room temperature by 0.1 M Phosphate buffer saline followed by 4% paraformaldehyde. Subjects were between 8 and 12 months old at the time of perfusion. After perfusion samples were collected by extracting the brain and both cochleae immediately after euthanasia from each animal; samples were washed and stored in our -80°C freezer until processing for molecular biology using previously-published procedures[54, 68, 69].

Samples were prepared for histology by fixing them in 4% paraformaldehyde (PFA); next samples were washed by phosphate-buffered saline (PBS) solution. After samples were washed,

cochleae were decalcified in ethylenediamine tetraacetic acid (EDTA) solution for 1 week. Brains and cochlea were dehydrated in 10% and 30% sucrose for 2 days. Brains were placed in optimal cutting temperature (OCT) compound and kept in -80 °C freezer until day of cutting. Sections of DCN, and AVCN were made with the Microm HM550; brain was sectioned at 16 µm thickness, each section was mounted on a glass slide, and air dried overnight. Glass slides were coverslipped using Mowil mounting medium.

To examine the effects of mGlu7 drugs on neural cell damage in the brain, sections were stained with cresyl violet (0.1% for 5 min); cresyl violet or Nissl staining can distinguish viable and nonviable stained cells. Nissl was selected for this study since it can label the nuclei and cell membranes of all brain cell types in a distinct manner. Cell densities for DCN and AVCN were measured from 10 samples. DCN and AVCN regions of the CN were identified, and the cross-sectional area of the selected region was measured using ImageJ software. Viable neurons with a clear round nucleus were then counted in each region and cell density was calculated.

2.5.6 Hematoxylin and Eosin (H&E) Staining

Cochlea were dissected from treated and control mice and processed for H&E staining as described in previous section. H&E protocol procedures were similar to our previous report [70]. Cochlear slides were placed in dH2O for 1 minute, stained for 5 minutes with the H&E staining kit. They were washed in dH2O for 1 minute, and then differentiated by acid wash for 1 minute, and then washed with dH2O for another minute. Cochlear slides were then inserted into blueing solution for half a minute, and washed with dH2O. In the next step cochlear slides were rinsed with 70% ETOH for 1 minute, followed by staining with Eosin II for another minute.

Finally slides were dehydrated with S2*Histo 5 X 20 seconds, xylene was also used to clear the slides; coverslips were applied using permanent mounting media. MetaMorph imaging

software in conjunction with a Leica DMR light microscope was used for semi-automated cell count, cell count method was similar to our previous publications [71-73].

2.5.7 Real-Time PCR

One of the cochleas was used to assay gene expression of NF-κB and Akt using RT-PCR, once the cochlea was dissected, total RNA was extracted from the modiolus. SV-K1 cells total RNA was extracted according to RNAeasy Mini Kit (Qiagen Inc., Valencia, CA) instructions. The RT-PCR analysis was performed as previously described [74]. In brief, total samples were vortexed for 1 min to shear genomic DNA before loading onto the RNeasy mini columns, and then eluted in a minimum volume of 30 µl and a maximum volume of 2 × 50 µl RNAse-free water. RNA obtained with this procedure was essentially free of genomic DNA. 50 ng of RNA was reverse transcribed and complementary DNA was subjected to PCR amplification. PCR reactions were performed on an AnalytikJena Tower3G apparatus with Sso Fast EvaGreen Supermix ® (BioRad, 1725201) kit. The quantitative real-time RT-PCR reaction mixture was prepared using the Ever-Green PCR master mix. The reverse transcript (RT) reaction took place at 46 °C for 20 min. Then, RT products went to PCR amplification directly. Thermal cycle protocol was 2 min at 94 °C, followed by 15 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C for 40 cycles and final holding for 5 min at 68 °C. Amplification specificity was checked using melting curves. Both negative and positive controls were included in each PCR reaction. All assays were carried out three times as independent PCR runs for each cDNA sample. Gene expression was referenced to the expression of β-actin as the housekeeping gene. Each gene expression level was normalized with respect to β-actin mRNA content. Calculations of expression were performed with relative standard curve method [75]. The primers were as follows: NF-κB-AGCTGCCAAAGAAGGACACG (F), TGGCAGGCTATTGCTCATCA (R); AKT-

AACGGACTTCGGGCTGTG (F), TTGTCCAGCACCTCAGG (R) and β-actin-GCTCTGGCTCCTAGCACCAT (F), ACATCTGCTGGAAGGTGGACAGT (R). We performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination.

2.5.8 Statistical Analysis

Results obtained in study were evaluated statistically were evaluated statistically by Student's t-test and by analysis of variance (ANOVA) using GraphPad Prism 6.01 software (GraphPad Software Inc.). If the ANOVA test shows a significant main effect, the pairwise comparisons were verified with Bonferroni tests for statistical significance ($p < 0.05$).

Chapter 3: mGluR7 Negative Allosteric Modulation, a Novel Approach for Sensorineural Hearing Loss: Preclinical Evidence from a Noise Exposure Model in CBA mice

3.1 Research Objectives

Next we administered the compound orally (as liquid), either before or after the noise exposure at a dose corresponding to 3-5 times the *in vitro* activity in the perilymph. The compound dosage of 30 mg/kg were tested.

In Aim 3.1, we tested the hypothesis that modulating mGlu7 receptor activity levels with a novel negative allosteric modulator will prevent or decrease NIHL, if given only before the noise exposure.

In Aim 3.2, we determined the optimal time for mGlu7 treatment so that eventually more patients can benefit from this therapeutic intervention. In this experiment, the drug was administered after the exposure to determine if only treatment post-noise exposure can reduce hearing loss.

3.2 Methods

3.2.1 Subjects

Twenty two male CBA/CaJ mice (4-6 months of age at the time of noise exposure) were used in this study. See Chapter 2 above for Subjects information.

3.2.2 Noise Exposure

See Chapter 2 above for noise exposure methodology.

In this project we used a compound that modulates a subtype within the group III mGluR class, this subtype mGlu7, has been implicated as important for age-related hearing loss and is expressed in hair cell/auditory nerve fiber synapses [36]. Previous studies showed that following acoustic trauma the synapse between the IHC and ANF shows evidence of elevated, presynaptic glutamate release. It is likely that degeneration of the synapses between IHCs and spiral ganglion neurons can be avoided by providing a mechanism that can prevent or reduce glutamate excitotoxicity. The Pragma group has identified negative allosteric modulators of mGlu7 for different mGluR subtypes.

3.2.3 Auditory Brainstem Response – ABR, and GIN ABR

Please see Chapter 2 above for ABR methodology.

3.2.4 Distortion Product Otoacoustic Emissions - DPOAE

Please see Chapter 2 above for DPOAE methodology.

3.2.5 Data Analysis

ABR thresholds were determined visually by three independent experimenters (two experimenters were blinded to the experimental conditions) at the lowest intensity level by comparing two ABR wave replicates at which a defined peak was produced. ABR recordings for both groups were evaluated and compared, as a function of high and low intensity levels of noise exposure. Threshold shift was computed by subtracting post-exposure level from baseline (pre-exposure). Visual detection of thresholds (VDTs) was done by three blinded judges who were not aware of what group the mice were in and the VDT were averaged across the three observers. ABR GIN analysis [52] started with identifying the location of P1 and P4 (the ABR response to each noise burst consists of 4-5 peaks) for NB1 and NB2. For NB2, at short gap durations, there was no identifiable response (0 and 2 ms). Magnitudes for P1 and P4 were used to formulate

amplitudes statistics. The amplitudes were calculated by computing the difference between the peak and trough levels for P1 and P4. Amplitude shift values were calculated by subtracting the level at which the NB1 response occurred from the level that the NB2 response took place.

We used two-way analysis of variance (ANOVA) to perform statistical analysis for the threshold and amplitude shifts. When the main effects of the ANOVA were statistically significant, in order to assess pairwise comparison between the two groups, we used Bonferonni's *post-hoc* test corrected for multiple comparisons. Unless otherwise noted, error bars in the figures are the standard error of the mean (SEM).

3.2.6 Biomarker Study

See Chapter 2 above for Biomarker study methodology.

3.2.7 Significance

PGT117, the NAM of the mGlu7 receptor modulator drug of the present investigation is not an FDA-approved drug and has not been tested in human patients yet. It is important that the efficiency of any reduction in hearing loss in animal models be evaluated so that it can be determined if further human clinical trials are worth pursuing. It was determined that the novel mGlu7 modulator appears to have the potential to be a very effective treatment for NIHL, as it can avoid or reduce hair cell and spiral ganglion neuron (SGN) loss, without systemic side effects.

3.3 Results

Electrophysiology Assays of NIHL- In the current project, we measured ABRs, a noninvasive measurement of excitatory activity from the auditory nerve and brainstem in young adult male CBA/CaJ mice before and after the administration of a NAM of mGlu7, which down-regulates activity at glutamatergic synapses, such as those at the junction of the cochlear hair cells

and auditory nerve fiber dendrites (IHC – SGN synapses). Subjects were divided into noise-exposed experimental (treated) and control (non-treated) groups. For these experiments, ABR, DPOAE, and ABR Gap data were acquired for each subject prior to the noise exposure, and 1 day, 2 weeks, and 4 weeks after exposure to noise trauma. We then compared the pre- and post-exposure amplitude and threshold data in the control and treated groups. To evaluate the effects of the mGlu7 modulatory compound in mice, we administered the drug at either before or after the exposure.

3.3.1 Study I

The effects of noise exposure on mice with normal hearing were compared for treated and untreated mice with a more potent mGlu7 compound (PGT145). Compound PGT145 is a more concentrated version of the NAM, so it could be administered at a lower dosage, which is more relevant and advantageous for future clinical applications. The groups consisted of 6 new treated mice and the same 8 control mice used in Chapter 2. The drug was administered 1 hour before the exposure to noise (but not after the noise exposure, as was done in Chapter 2). As in Chapter 2, we examined the differences in ABR threshold shifts in treated and control groups. In Chapter 3, *Study I*, the dosage of mGlu7 drug was lowered from 100 mg/kg to 30 mg/kg, to move ahead on a clinical translational pathway. Figure 3.1, shows significant differences in threshold shift between the two groups when measured at 24 hour after the noise exposure. Figures 3.2 and 3.3, show threshold shifts at 2 weeks, and 4 weeks post-exposure.

Temporary ABR threshold shifts were higher within the control group at all test frequencies (Figure 3.1), but the treatment effect, differences between the treated (blue) and untreated (red) groups, although still significant, were not as large as in Chapter 2 where the drug was administered both before and after the noise exposure (see Figure 2.2 above).

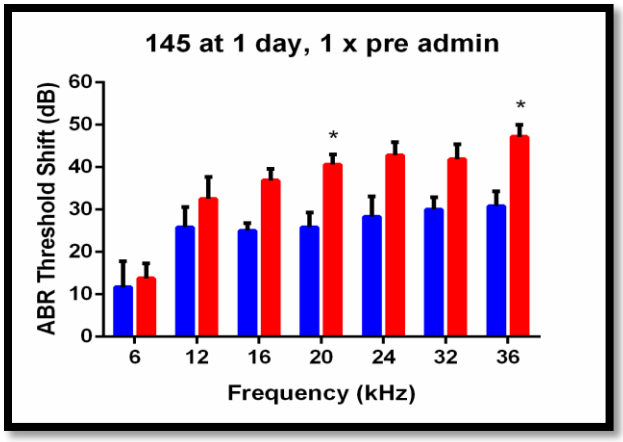

Figure 3.1. The mGlu7 modulator reduces TTS. Effect of noise exposure on ABR threshold shifts (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. The main effect was highly significant ($F(1, 12) = 8.89$, $P = 0.0111$), and the Post-hoc pairwise comparison statistical significance level: * $p<0.05$. Blue = Treatment; Red = Control.

Permanent ABR threshold shifts at two weeks post-exposure evaluation points showed treatment effects at the higher frequencies, i.e., for frequencies above 12 kHz, (Figure. 3.2).

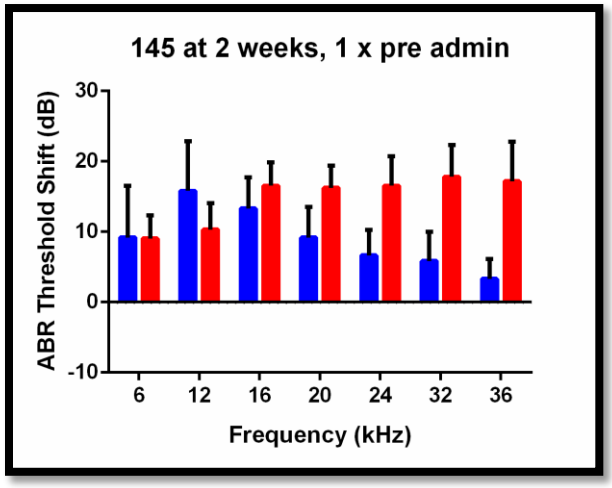

Figure 3.2. The mGlu7 modulator reduces PTS at higher frequencies. Effect of noise exposure on ABR threshold shifts (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. Blue = Treatment; Red = Control.

At the 4-week checkpoint, ABR threshold shifts were still similar at lower frequencies within the treated and control groups (6, 12 and 16 kHz), but at the remaining frequencies (20, 24, 32, and 36 kHz) dramatic decreases in PTS were achieved as a result of the pre-noise treatment of PGT117 (Figure. 3.3).

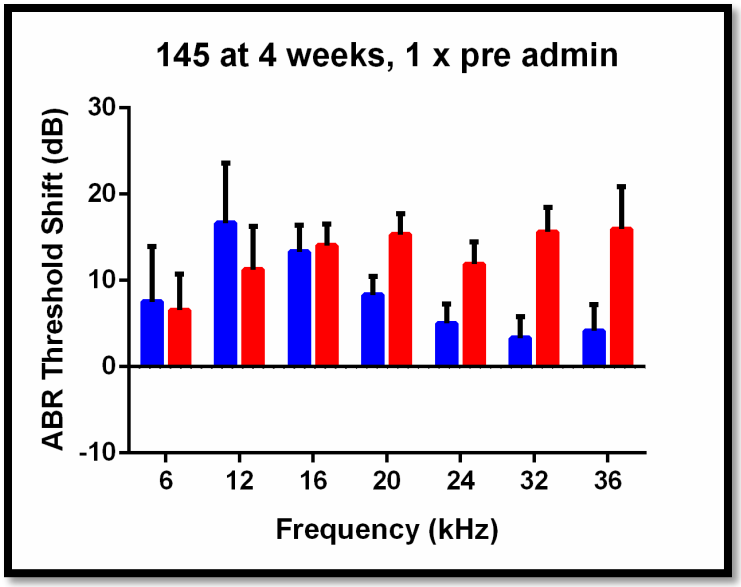

Figure 3.3. Effect of noise exposure on ABR threshold shift (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. Note that PTS of the control group was larger than that for the treated group at higher frequencies (20, 24, 32, and 36 kHz). Blue = Treatment; Red = Control.

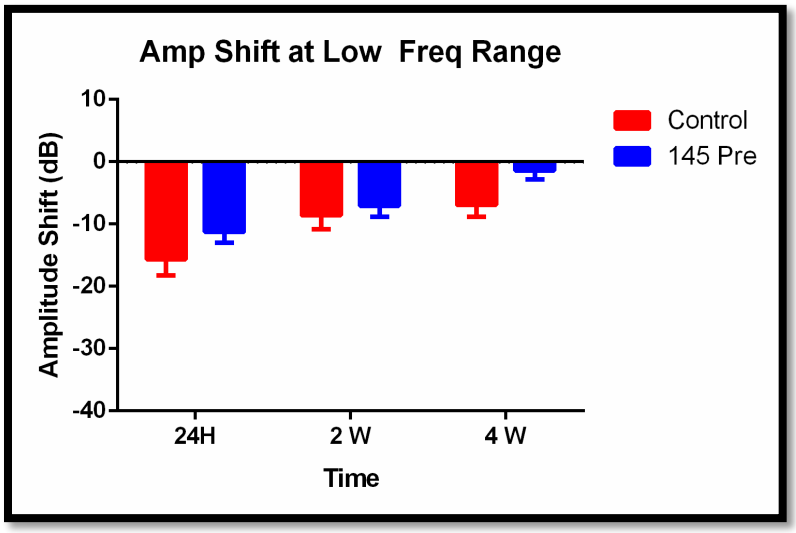

Figure 3.4. The mGlu7 modulatory drug reduces the amplitude reductions of the OHC system due to the noise at all time points. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the Low frequency range (5.3, 7.1, 10.7 kHz).

The differences in DPOAE amplitude shifts at three different evaluation time points, between the two groups in Low, Medium, and High frequency ranges are shown in Figures 3.4-6. For the Low frequency range, DPOAE amplitude shifts were higher within the control group at all three time checkpoints following the noise exposure (Figure. 3.4).

The differences in DPOAE amplitude shifts between the control and treated groups were significant during temporary hearing loss evaluation (24 hr), and there was a trend for a treatment effect at the later, PTS time points (Figure. 3.5).

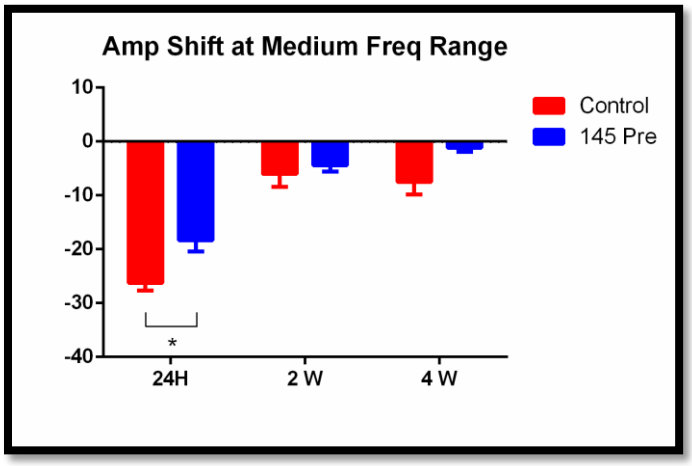

Figure 3.5 The mGlu7 modulatory drug significantly reduces the damage to the OHC system at 24 hr. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the Medium frequency range (14.3, 21.4, 28.6 kHz). The main effect was highly significant ($F(1, 12) = 6.339$, $P = 0.0270$), and the Post-hoc pairwise comparison statistical significance level: * $p<0.05$.

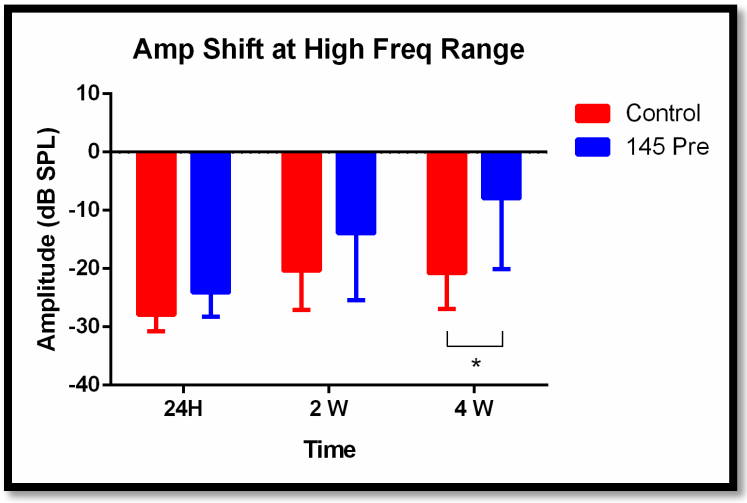

Figure 3.6. Prevention of outer hair cell damage by the mGlu7 modulator drug. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the High- range frequency (35.7, 40.2 kHz). The main effect was significant ($F(1, 12) = 4.841$, $P = 0.0481$), and the Post-hoc pairwise comparison statistical significance level: * $p<0.05$.

For the High frequency DPOAE amplitudes, there was tendency for the treatment to induce modest protection at the first two measurement points (24 hr, 2 wk), and when evaluated at four weeks post-exposure, there was a significant treatment effect (Figure. 3.6).

Otoacoustic emissions threshold shifts after noise exposure are shown in Figures 3.7-9, where measurements were made before (BL-baseline) and at 24 hours, 2 weeks, and 4 weeks after exposure. As before, DPOAE frequencies were grouped into a Low, Medium, and High frequency ranges. Permanent hearing loss sensitivity changes were completely protected for the treated group when evaluated at 2 and 4 weeks post-exposure (Figure. 3.7).

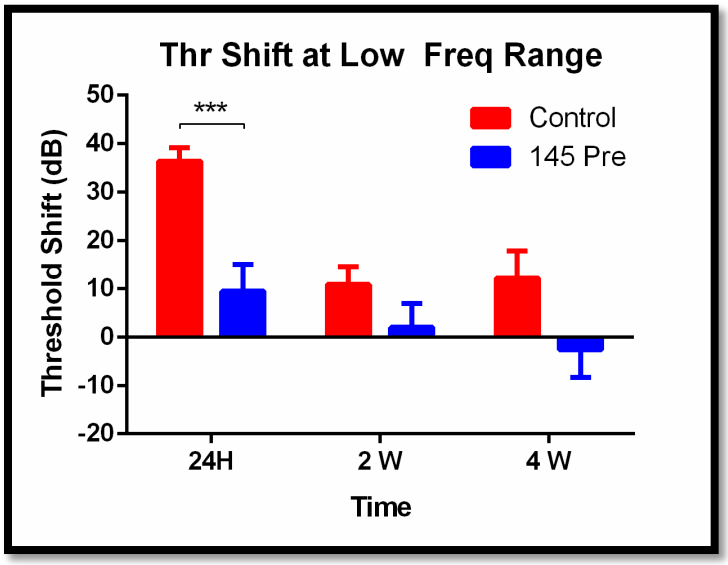

Figure 3.7. The mGlu7 modulatory drug prevents the threshold shifts of the OHC system due to the noise at all time points. Effects of exposure on DPOAE thresholds at 3 time points post-noise exposure for the Low frequency range (5.3, 7.1, 10.7 kHz) are shown. The main effect was highly significant ($F(1, 12) = 9.254$, $P = 0.0102$), and the Post-hoc pairwise comparison statistical significance level: **** $p<0.0001$.

For the Medium frequency range, the permanent hearing changes were reduced by the treatment, like the Low frequency data of Figure 3.7 Specifically, the TTS was significantly attenuated (24 hr), and the PTS was fully protected in the treated group at two and four weeks post-exposure evaluation times (Figure. 3.8).

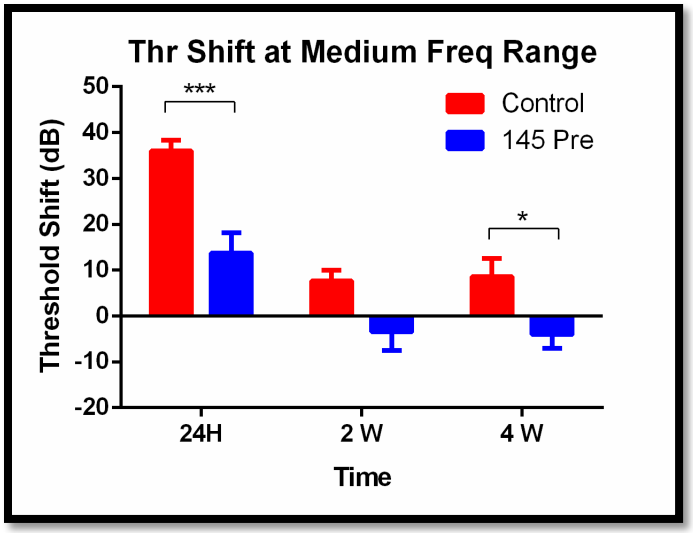

Figure 3.8 The mGlu7 modulatory drug significantly reduces the damage to the OHC system at 24 hr. Effects of exposure on DPOAE thresholds at 3 time points post-noise exposure for the Medium frequency range (14.3, 21.4, 28.6 kHz) are displayed. The main effect was highly significant ($F(1, 12) = 15.88$, $P = 0.0018$), and the Post-hoc pairwise comparison statistical significance levels: * $p<0.05$; *** $p<0.001$.

The improvement in DPOAE threshold shifts were smaller for the treated group at all evaluation time points for the higher frequencies (Figure. 3.9).

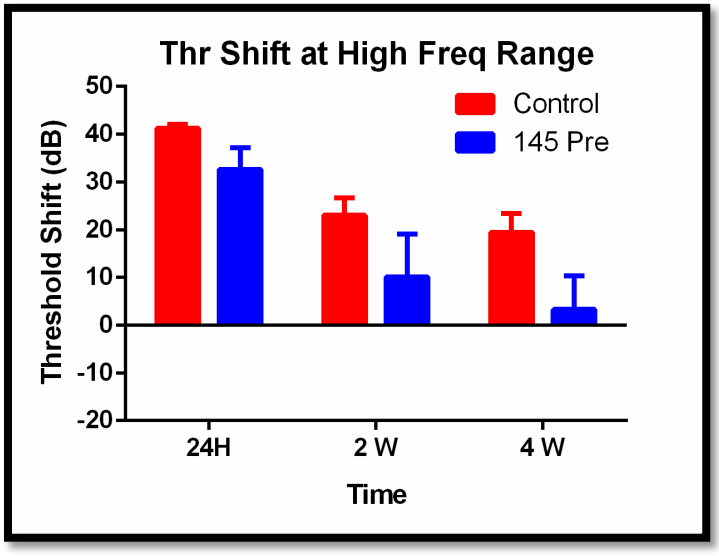

Figure 3.9 The mGlu7 modulatory drug mitigates the threshold shifts of the OHC system due to the noise at all time points. Effects of exposure on DPOAE thresholds at 3 time points post-noise exposure at High frequency range (35.7, 40.2 kHz) are displayed.

Figure 3.10, plots the amplitude differences of the auditory nerve component of the ABR (P1) in response to NB1 of the GIN test two, and four weeks post-exposure. Similar to *the Chapter 2* findings presented above, the differences in NB1 P1 amplitude levels between baseline and post-noise exposure evaluations were significantly larger in the control group, indicating positive treatment effects (Figure. 3.10). Larger differences in amplitude between BL and post-exposure values signify greater permanent effects of the noise exposure on ABR amplitudes.

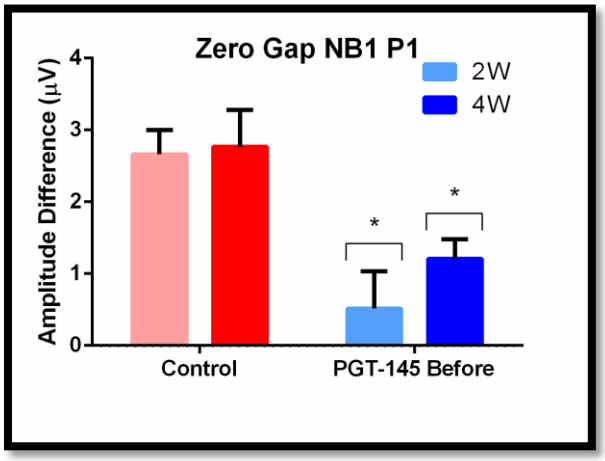

Figure 3.10 The mGlu7 drug helps preserve supra-threshold (80 dB) ABR P1 amplitude responses at the PTS assessment points (2 and 4 weeks). Note that the differences between the amplitude of the control baseline data at 2 (2W) and 4 weeks (4W) levels are significantly larger than that for the treated group.

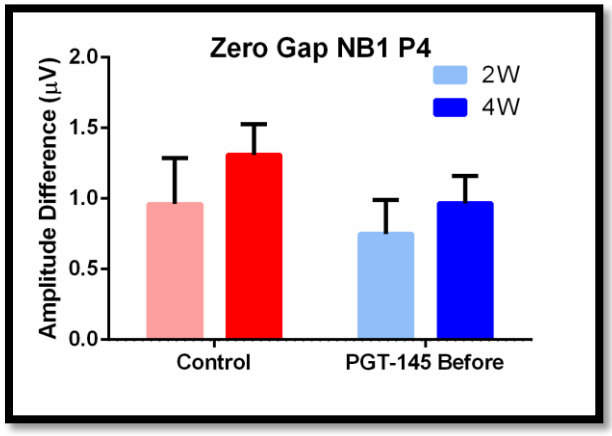

Figure 3.11. The mGlu7 drug did not have any effect on P4 for NB1 at supra-threshold (80 dB) ABR amplitude responses at the PTS assessment points (2 and 4 weeks). Note that the differences between the amplitudes of the control baseline data (BL) and 2 (2W) and 4 week (4W) levels are similar in both groups, indicating no effect of PGT117 on P4 amplitude.

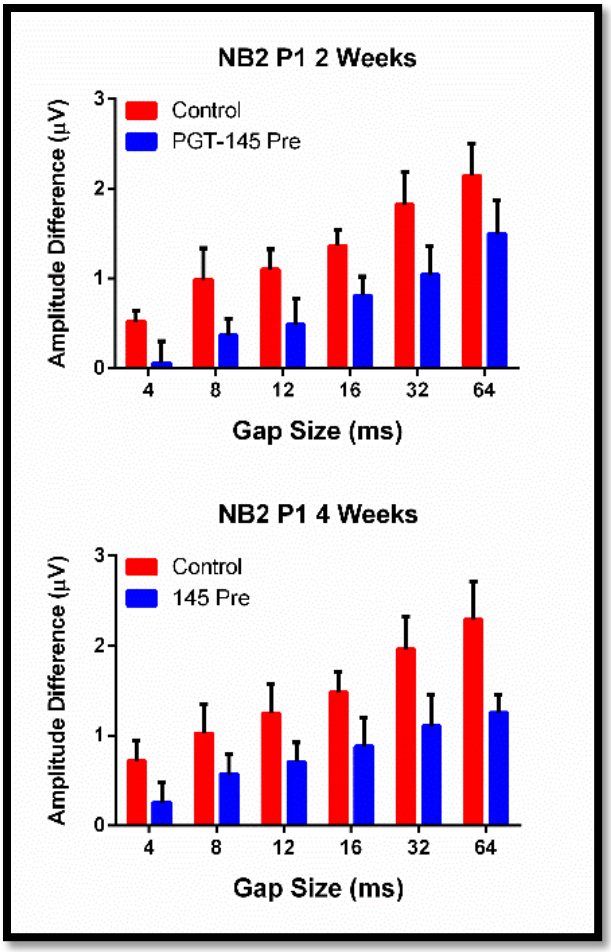

Figure 3.12. Some preservation of temporal coding is induced by the mGlu7 modulator, as 2 and 4 weeks amplitude differences for P1 of NB2 were lower for the treated group for each Gap duration.

Figure 3.11, plots the amplitude of the auditory midbrain component (P4) to NB1 of the GIN. The difference in NB1 P4 amplitude levels between baseline and post-noise exposure measurements were smaller in the mGlu7 treated group; so treatment effect was apparent here as a trend (Figure. 3.11).

Figure 3.12, plots NB2 P1 GIN amplitude as a function of gap duration. Amplitude levels of NB2 P1 showed low levels of recovery in post-exposure evaluations in the control group. Notice that the differences between baseline and post-exposure amplitude levels were smaller in the

treatment group, indicating a trend towards a beneficial treatment effect of the mGlu7 drug (Figure. 3.12).

Figure 3.13, plots NB2 P4 GIN amplitude as a function of gap duration. Post-exposure NB2 P4 amplitudes showed more recovery in the treated group at 2 and 4 weeks evaluation points when compared to the amplitude difference of the control group, indicating a suggestion of a treatment benefit at 2 and 4 weeks (Figure. 3.13).

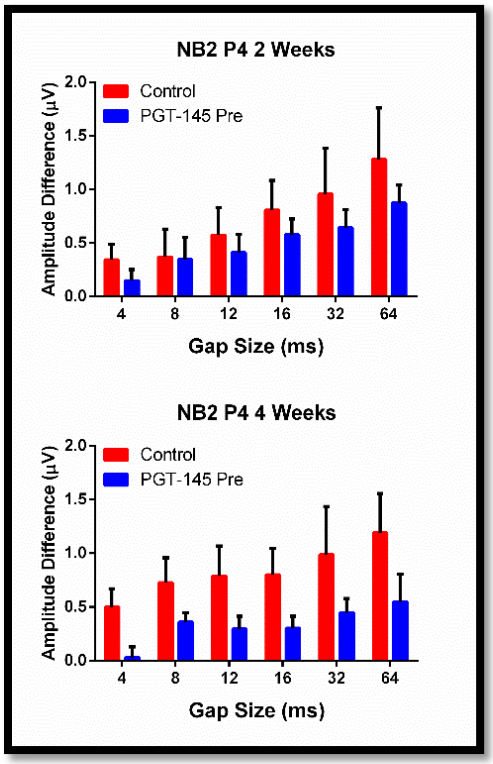

Figure 3.13. Effects of noise exposure on NB2, P4 ABR GIN amplitude at two time points post-noise exposure. Result shows larger amplitude differences in control group, i.e., treatment effect is apparent.

3.3.2 Study II

The effects of noise exposure on mice with normal hearing were compared for controls and a group of mice treated with mGlu7 compound PGT145. This group consisted of 8 new treated mice, and the 8 control mice that were utilized in Chapter 2 and Study I were also used here. In

Study II, the drug was administered three times, at 1-hour intervals *after the exposure to noise*. As before, we examined the differences in ABR threshold shifts in treated and control groups at the three post-noise exposure time points. Figure 3.14, shows significant differences in ABR threshold shifts between the two groups when measured at 24 hours after the noise exposure. Figures 3.15, and 3.16, show threshold shifts at 2 and 4 weeks post-exposure. Strong treatment effect benefits were observed consistently, particularly at the middle and higher frequencies.

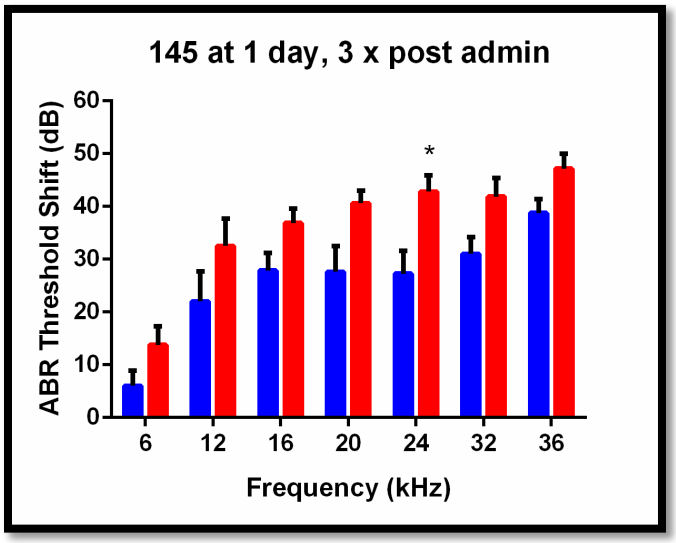

Figure 3.14. The mGlu7 modulator PGT145 reduces TTS. Effects of noise exposure on ABR threshold shifts (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. The main effect was highly significant ($F(1, 14) = 7.69$, $P = 0.0150$), and the Post-hoc pairwise comparison statistical significance level: * $p<0.05$. Blue = treatment; Red = control.

Two weeks post-exposure evaluation shows improvements at all frequencies, with statistically significant treatment benefits in ABR threshold shifts at 20 kHz and the higher frequencies (Figure. 3.15). Note that for all frequencies, except 12 and 16 kHz, PTS is eliminated by the drug intervention.

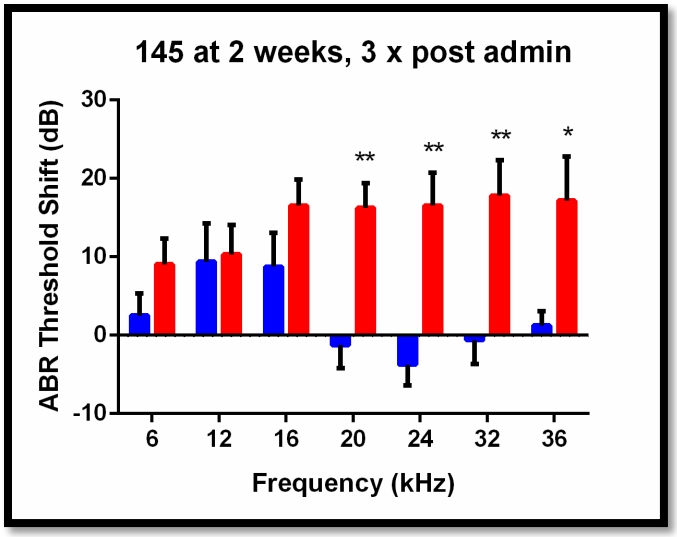

Figure 3.15. The mGlu7 modulator prevents PTS. Effect of noise exposure on ABR threshold shift (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. The main effect was highly significant ($F(1, 14) = 10.0$, $P = 0.0069$), and the Post-hoc pairwise comparison statistical significance levels: * $p<0.05$; ** $p<0.01$. Blue = treatment; Red = control.

ABR threshold shifts at four weeks post-exposure were larger for the control group, again showing that PGT145 prevented PTS at the middle and high frequencies (Figure. 3.16).

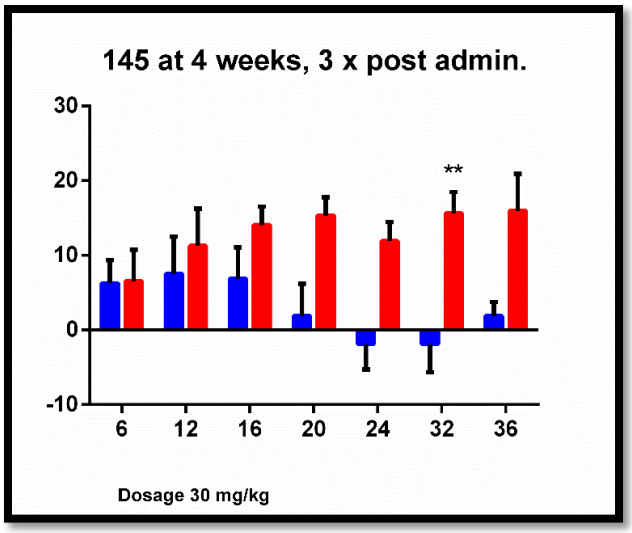

Figure 3.16. The mGlu7 modulator PGT145 reduces or prevents PTS evident in the Control group at 4 weeks post-noise exposure. Effect of noise exposure on ABR threshold shifts (relative to pre-exposure baseline recordings) for two groups of mice at seven frequencies. The main effect was highly significant ($F(1, 14) = 5.67$, $P = 0.0320$), and the Post-hoc pairwise comparison statistical significance levels: ** $p<0.01$. Blue = treatment; Red = control.

The differences in DPOAE amplitude shifts at 3 different time points, between the two groups in Low, Medium, and High frequency ranges are shown in Figures 3.17-19. For the Low frequency range, DPOAE amplitude shifts were smaller within the treated group at all evaluations, so trends for a beneficial treatment effect was observed (Figure. 3.17).

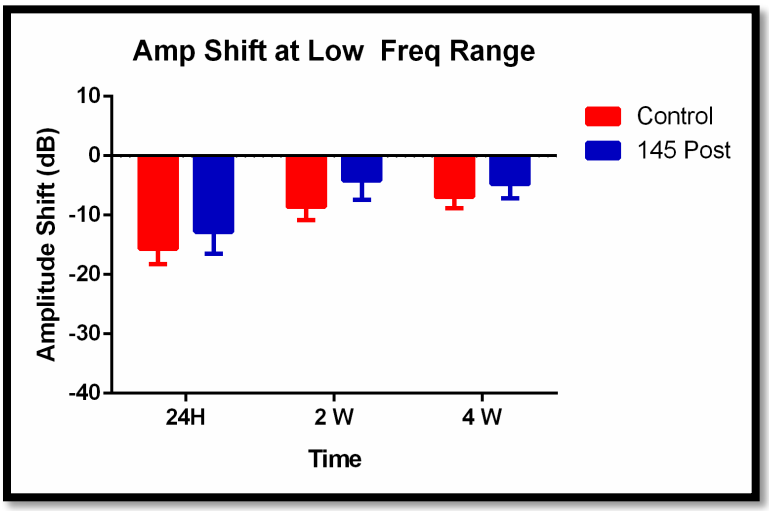

Figure 3.17. Reduction of outer hair cell damage by the mGlu7 modulator drug. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the Low frequency range (5.3, 7.1, 10.7 kHz).

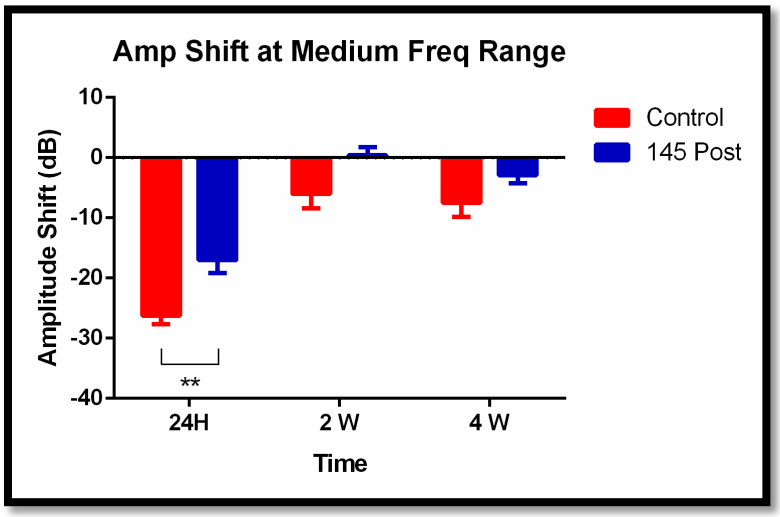

Figure 3.18. The mGlu7 modulatory drug significantly reduces the damage to the OHC system at 24 hr, with PTS damage prevention. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the Medium frequency range (14.3, 21.4, 28.6 kHz). The main effect was highly significant ($F(1, 14) = 10.11$, $P = 0.0067$), and the Post-hoc pairwise comparison statistical significance level: ** $p<0.01$.

Significant differences in DPOAE amplitude shifts exist between control and treated groups at 24 hours post-exposure within the Medium frequency range; and PTS hearing loss is prevented at the 2 and 4 week time points (Figure. 3.18).

Similar to the Medium frequency range, there is a significant difference in DPOAE amplitude shifts at 24 hours post-exposure evaluation for the High frequency range, as well as a very modest benefits at the 2 and 4 week points (Figure. 3.19).

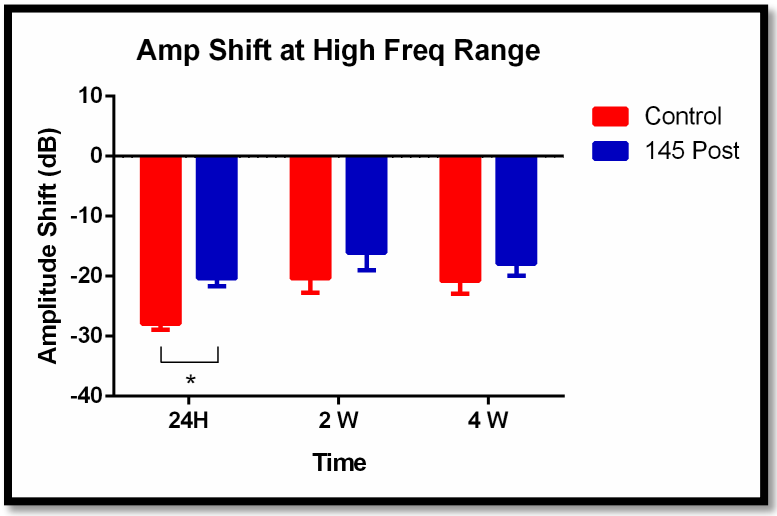

Figure 3.19. The mGlu7 modulatory drug significantly reduces the damage to the OHC system at 24 hr. Effects of noise exposure on DPOAE amplitudes at three time points post-noise for the High- frequency range (35.7, 40.2 kHz). The main effect was highly significant ($F(1, 14) = 4.762$, $P = 0.0466$), and the Post-hoc pairwise comparison statistical significance level: * $p<0.05$.

Threshold shifts after the noise exposure are shown in Figures 3.20-22, measurements are made before and at 24 hours, 2 weeks, and 4 weeks after exposure. As for the amplitudes, DPOAE frequencies were grouped into a Low, Medium, and High frequency ranges. For the Low frequency range significant differences in DPOAE TTS were observed at 24 hr, with the shift being larger in the control group (Figure. 3.20). As observed previously, the PGT145 drug treatment prevented PTS (2 and 4 weeks).

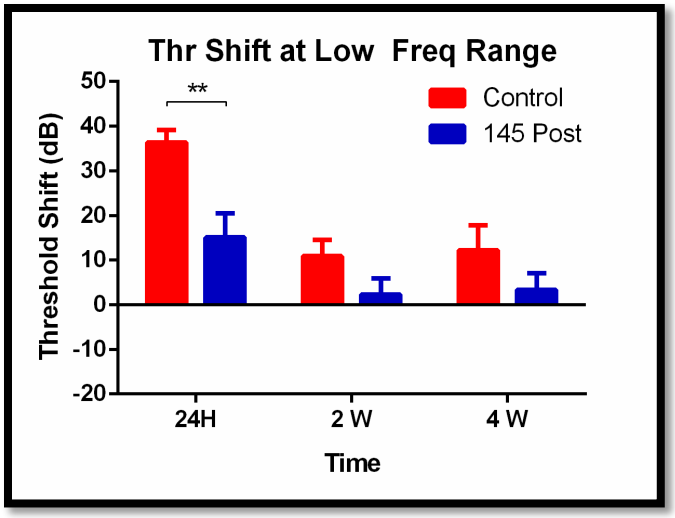

Figure 3.20. The mGlu7 modulatory drug prevents the threshold shifts of the OHC system due to the noise at 2 and 4 week time points. Effects of exposure on DPOAE thresholds at 3 time points post noise exposure for the Low frequency range (5.3, 7.1, 10.7 kHz). The main effect was highly significant (F (1, 14) = 6.473, P = 0.0234), and the Post-hoc pairwise comparison statistical significance level: ** p<0.01.

Significant differences in DPOAE threshold shifts were observed at 24 hours and two weeks post-exposure evaluations for the Medium frequency range (Figure. 3.21). Again, the drug intervention prevented permanent threshold shifts (2 and 4 weeks).

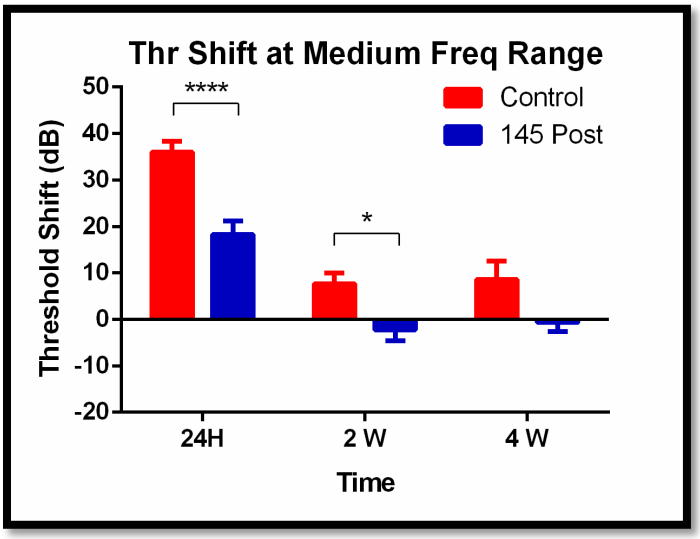

Figure 3.21. The mGlu7 modulatory drug prevents the threshold shifts of the OHC system due to the noise at 2 and 4 week time points. Effects of exposure on DPOAE thresholds at 3 time points post noise exposure for the Medium frequency range (14.3, 21.4, 28.6 kHz). The main effect was highly significant (F (1, 14) = 16.58, P = 0.0011), and the Post-hoc pairwise comparison statistical significance levels: * p<0.05; **** p<0.0001.

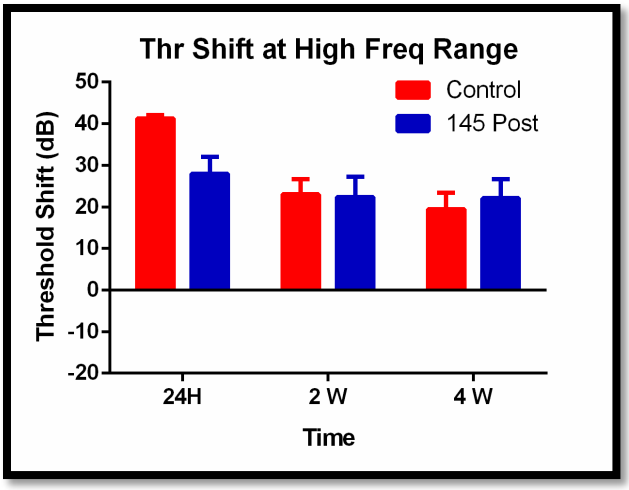

Figure 3.22. Effects of exposure on DPOAE thresholds at 3 time points post noise exposure for the High frequency range (35.7, 40.2 kHz).

For the High frequency range, TTS was greater in control group, but PTS was about the same for both subject groups when evaluated at two and four weeks post-exposure (Figure. 3.22).

The average ABR GIN amplitudes for NB1 and NB2 for P1 and P2 are displayed in Figures. 3.23-26. NB1 P1 amplitude level differences between baseline and post-exposure

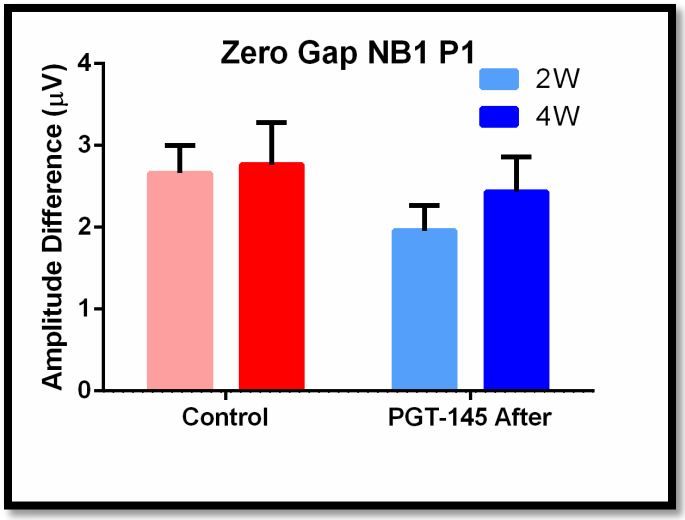

Figure 3.23. The mGlu7 PGT145 drug helps preserve supra-threshold (80 dB) ABR amplitude responses at the PTS assessment points (2 and 4 weeks). Effects of noise exposure levels on NB1, P1 ABR GIN amplitude at two time points post-noise exposure. Note that the changes between the amplitude differences of the control group at 2 (2W) and 4 weeks (4W) are larger than that for the treated group, indicating a greater deficit due to the noise exposure for the Control group.

evaluations are larger in the control group (Figure. 3.23). Larger differences in amplitude between baseline (BL) and post-exposure values signify greater effects if the noise exposure on PTS.

For the two, and four weeks post-exposure evaluations, no recovery in GIN NB1 P4 amplitude was observed, i.e., 2 and 4 week response amplitudes were similar for both the control and treated groups (Figure. 3.24).

For the longer gap durations, the GIN NB2, P1 amplitude differences between baseline and post-exposure were larger for the control group, indicating a more vigorous response for the treated group, i.e., improvements in temporal processing at the level of the auditory nerve (spiral ganglion cell responses reflected in P1 of the ABR Gap responses) (Figure. 3.25).

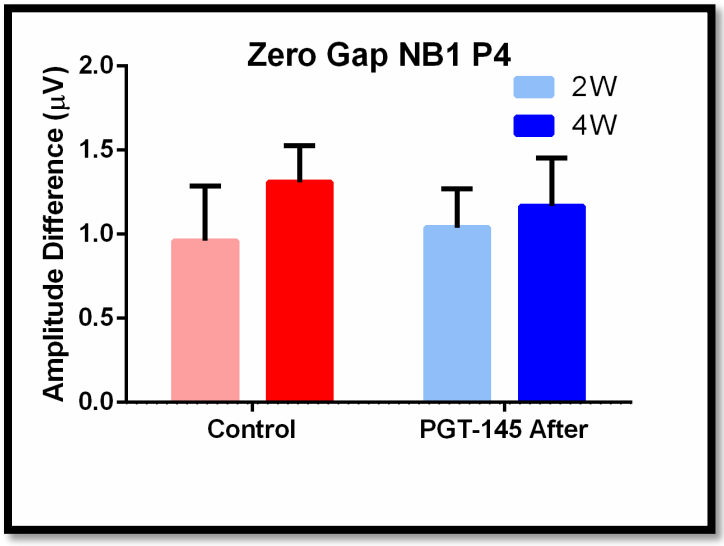

Figure 3.24. The mGlu7 drug had no noticeable effects to preserve supra-threshold (80 dB) ABR amplitude responses at the PTS assessment points (2 and 4 weeks). Note that the changes between the amplitude differences of the control data and 2 (2W) and 4 weeks (4W) levels are similar to the treated group.

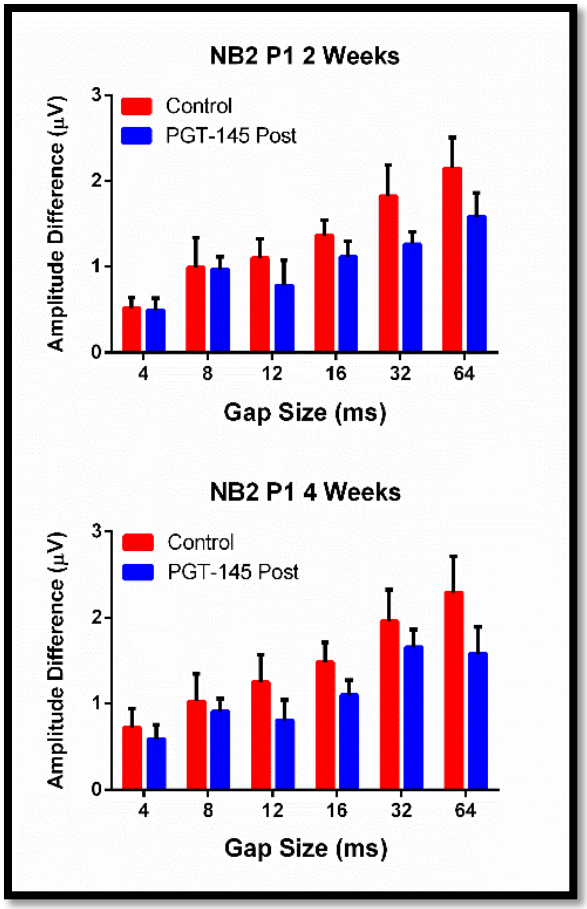

Figure 3.25. Effects of noise exposure levels on NB2, P1 ABR GIN amplitude differences at two evaluation points post-noise exposure. The Treated group showed smaller amplitude differences at the 2 and 4 week time points at the middle and high frequencies.

The post-exposure GIN NB2 P4 amplitude differences of the treated group are smaller than the control group, indicating improved temporal processing at the brainstem level due to the mGlu7 treatments (Figure. 3.26).

3.4 Summary and Discussion

In this study we measured the otoprotective effects of two novel negative allosteric modulators of the mGlur7 receptor in a mouse model of NIHL. We utilized the ABR to measure hearing sensitivity, the DPOAE was used to evaluate outer hair cell functionality, and the ABR-Gap NB2 responses were employed to measure auditory temporal processing. In *Chapter 2* of this investigation, with the initial formulation of the mGlu7 compound (formulation PGT117) we

observed up to 30 dB differences in ABR threshold shifts when measured 24 hours after exposure (TTS), where comparisons were made between the control and treated groups. Our ABR measurements 4 weeks post-exposure revealed that mice treated with the mGlu7 drug have up to 30 dB less PTS when compared to control mice, i.e., and in most cases the drug intervention prevented PTS. Within the Low frequency range, DPOAE amplitude shifts were significantly

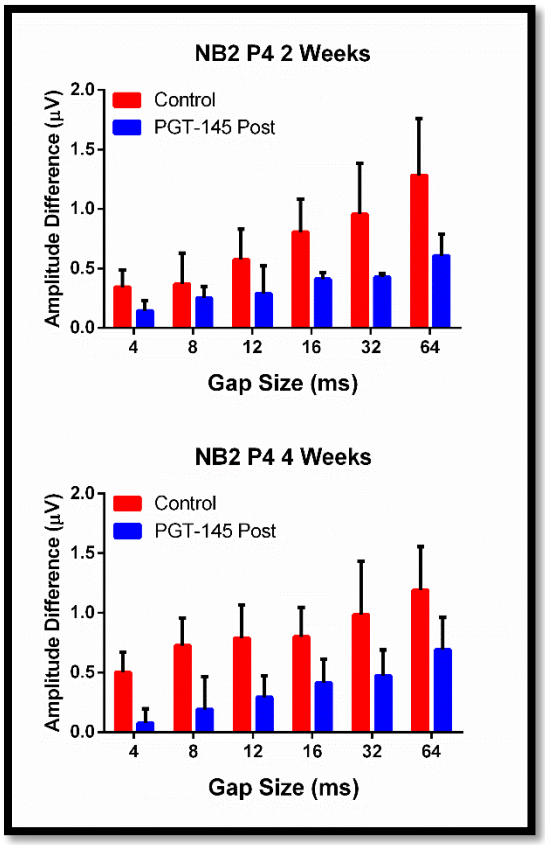

Figure 3.26 Effects of noise exposure levels on NB2, P4 ABR GIN amplitude at two time point post-noise exposure. Results differed for the two groups, i.e., treatment effect is apparent since the P4 amplitude differences of the Control group exceed those of the Treated at 2 and 4 weeks.

different between control and treated group at 24 hours, and 2 weeks post-exposure. Within the Medium frequency range amplitude shift differences between the two groups were significant only at the 24 hour time point. DPOAE threshold shifts were significant between control and treated groups within the Low and Medium frequencies ranges at 24 hours, 2 weeks, and 4 weeks post-

exposure. For the High frequency range, DPOAE threshold shifts were significant when measured 24 hours post-exposure. Cell count changes in the cochlear nucleus-CN following noise-exposure showed significant reduction in cell density for the control groups in the AVCN and DCN subdivisions.

In Chapter 3 Study I, where a more potent, concentrated form of the mGlu7 drug was employed (formulation PGT145), with dosage only before the noise exposure, we found DPOAE TTS improvements of up to 30 dB for the Low frequency range, and PTS was up to 20 dB lower in treated mice within the DPOAE Medium frequency range. Significant differences were observed in ABR threshold shifts at 20, and 36 kHz frequencies at 24 hours post-exposure.

In Chapter 3 *Study II*, we used the same drug as Study I with three administration times following the noise exposure. For the Medium frequency range we observed up to 10 dB less TTS in the treated group when measured 24 hours post-exposure. We reported for the High frequency range, up to 10 dB less PTS in mice treated with the mGlu7 modulator when DPOAE thresholds were measured 2 weeks post-exposure. Comparison between baseline and post-exposure ABR threshold shift showed significant differences between two groups. At 24 hours post-exposure significant differences in ABR threshold shift was observed at 24 kHz, 2 weeks data showed significant difference at 20, 24, 32, and 36 kHz frequencies, and this difference was most significant at 32 kHz frequency when measured 4 weeks post-exposure.

3.5 Discussion

In our study we observed significant changes in hearing thresholds post-exposure between control and those animals treated with our novel mGlu7 compound, for both potencies tested and for when the drug was administered before or after the noise exposure. Recent studies that used antioxidants to prevent or treat hearing loss following noise exposure oftentimes ended up with

good result in animal models [76-81]. Seidman reported up to 30% difference in ABR threshold difference between restricted diet group and placebo group, but in our study we showed up to 70% threshold improvement when evaluated at 1 day, and 4 weeks post noise-exposure. However, when these compounds were administered to human subjects in FDA clinical trials the results did not demonstrate effective otoprotection [82, 83]. We are the first group to evaluate temporal processing within NIHL mice after treatment, all studies mentioned in this paragraph lack GIN analysis, here we were able to show some improvement in GIN NB1 and NB2, P1 and P4 amplitudes after treating subjects with the NAM compound as we discussed above in Chapter 2, and Study I and II of Chapter 3.

Recently, Le Prell and colleagues reported that a key mechanism for hearing loss is the formation of free radicals in the inner ear [62, 84]. Nearly all of the methods using antioxidants to prevent /treat hearing loss are trying to cancel out or neutralize free radicals damage after their initial formation following exposure to the loud noise [62, 84-86]. However our innovative methodology is likely *preventing* formation of free radicals following noise-exposure instead of trying to remove them after they are already formed. There are a couple examples where patients showed lower thresholds following treatment with a glutamatergic synaptic modulator after they demonstrated signs of presbycusis [87]; in those cases patients were treated after they were diagnosed with hearing loss. In sum, we are showing a methodology where potentially patients will not lose their hearing following noise exposure, obviating the need for further treatments, since they do not lose their hearing ability initially due to the noise.

3.6 Conclusions

Considering the different potency levels and dosing paradigms employed in this Dissertation, it appears the new Pragma drugs designed to decrease glutamate synaptic activity

hold much promise for preventing or reducing levels of NIHL; at least in an animal model. Hopefully, further animal model research in a higher mammal will pave the way for FDA-clinical trials with this novel compound. It is likely that a Phase 1 FDA clinical trial would succeed, as the new drug appears to have few side effects, as studied by Pragma Therapeutics and other collaborators, at the anticipated dosage levels for humans. We certainly did not see any negative side effects in the current study, where mice survived well for 5 months after the noise exposure and drug treatments.

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