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Therapeutic Function of Glucagonlike Peptide-1 (GLP-1) for Hearing Restoration After Blast Exposure or Traumatic Brain Injury (TBI)

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14. ABSTRACT
The objective of this project is to investigate the potential therapeutic function of Liraglutide (glucagon-like peptide-1 receptor (GLP-1R)) to mitigate the auditory injury after blast exposure in animal model of chinchilla. There are two aims: **Aim 1.** Identify the therapeutics of liraglutide in ameliorating auditory injuries in both pre- and post-blast treatments in relation to the blast overpressure (BOP) level or TBI severity over different time courses. **Aim 2.** Investigate the beneficial effects of liraglutide on the mitigation of the central auditory damage following repetitive exposures to the low BOP (G1) or mild TBI (G2). Accomplishments in Year 4 include: 1) hearing function tests in drug treated chinchillas at G2 BOP level of 15-25 psi for 3 blasts on Day 1 with both ears open (without protection) and in animals with additional 3 blasts on Day 4 over two time courses: 14 and 28 days; 2) summarizing of the completed experiments on therapeutic function of liraglutide for mitigation of blast-induced auditory injury in relation to the BOP level, hearing protection condition, and recovery time course; 3) immunofluorescence imaging and analysis of chinchilla brain tissues from all drug-treated groups and blast control groups (open and protected). Results demonstrate that the blast intensity and repeated blats affect the therapeutic function of liraglutide on mitigation of hearing damage in ears with and without protection and further study is needed.

15. SUBJECT TERMS
Hearing restoration, blast overpressure, auditory dysfunction, therapeutics, liraglutide, hearing function test, hearing protection devices, traumatic brain injury

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1. INTRODUCTION

Hearing damage caused by blast exposure is a frequent and common injury for Service members even at relatively low or mild blast overpressure. To date, there is no therapeutic treatment for blast-induced progressive hearing impairment. The current clinical research on military personnel indicates an increasingly strong correlation between the traumatic brain injuries (TBI) and sensorineural hearing loss, and the blast-induced hearing damage shares similar mechanisms with the TBI-induced memory deficits such as the loss of neurons but in auditory cortex and spiral ganglion. These research findings raise a crucial question: whether neurotrophic drugs offer therapeutic benefits against blast-induced auditory damage that causes cognitive deficits, decreases synaptic plasticity, and leads to neurodegeneration.

The **objective** of this project is to determine the therapeutic function of Liraglutide, the long-acting glucagon-like peptide-1 receptor (GLP-1R), to mitigate the auditory injury after blast exposure in animal model of chinchilla. Liraglutide's neurotrophic and protective activity in cellular and animal models of stroke and TBI has been reported, but the function of the GLP-1R agonist to reduce the blast-induced hearing damage and restore hearing has not been studied. Our proposed studies on Liraglutide's function to restore hearing in chinchillas will provide the first promising candidate therapeutics that promote auditory neural proliferation and reduce cell apoptosis caused by blast overpressure.

To reach the objective and long-term goal of the therapeutic treatment for blast-induced progressive hearing damage, we have a series of tasks under two specific aims to test our **general hypothesis**: hearing damage induced by repeated blast exposures at a low (below mild TBI) or mild TBI level will involve both peripheral and central auditory pathways in chinchillas, and liraglutide treatment will mitigate these abnormalities and restore hearing function.

2. KEYWORDS

Blast overpressure, auditory dysfunction, hearing restoration, therapeutics, liraglutide, hearing protection devices, traumatic brain injury

3. ACCOMPLISHMENTS

● What were the major goals of the project?

The project has two specific aims with 7 tasks.

Aim 1: Identify the therapeutics of liraglutide in ameliorating auditory function injuries in both pre- and post-treatments in relation to the blast overpressure (BOP) level or TBI severity over different time courses.

Task 1-1. To determine therapeutic efficacy of liraglutide in chinchillas repetitively exposed to low BOP (**G1)** level with hearing protection.

Task 1-2. To determine therapeutic efficacy of liraglutide in chinchillas repetitively exposed to low BOP (**G1**) level without hearing protection.

Task 1-3. To determine therapeutic efficacy of liraglutide in chinchillas repetitively exposed to high BOP (**G2**) level causing mTBI with hearing protection.

Aim 2: Investigate the beneficial effects of liraglutide on the mitigation of the central auditory damage following repetitive exposures to the low BOP or mild TBI pressure levels.
Task 2-1. To determine the effect of liraglutide on glutamate and GABA neurotransmitter receptors in the central auditory system.
Task 2-2. To determine the effect of liraglutide on synaptic plasticity changes.
Task 2-3. To examine the efficacy of liraglutide to prevent oxidative stress-induced neuronal loss.
Task 2-4. To investigate mechanisms by which liraglutide offers neuroprotection and neuro-regeneration.

- **What was accomplished under these goals?**

Key Research Accomplishments:

Aim 1:
♦ Blast tests at the BOP level: 15-25 psi or 103-172 kPa (named as G2 BOP level) for 3 consecutive blasts on Day 1 in 3 groups of chinchillas (liraglutide treated and control) with ears open without hearing protection devices (HPDs, e.g., earplugs) were completed in chinchillas over recovery time of 14 and 28 days.
♦ Chinchillas with ears protected by HPDs were exposed to 3 additional blasts at G2 BOP level on Day 4 in three experimental groups (liraglutide treated and control) over recovery time of 28 days. This new study was to establish an animal model with more severe blast-induced hearing loss to investigate the long-term effects of liraglutide treatment in chinchillas with HPDs.
♦ Hearing function tests including the auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), and middle latency response (MLR) were performed on Day 1 (pre- and post-blast) and Days 4, 7, 14, and 28, respectively, to determine the effect of liraglutide on mitigation of hearing damage over recovery time length and to identify whether the liraglutide affects the hearing restoration differently between the ears open and protected.

Aim 2:
♦ Chinchilla brain tissue samples from 6 groups of animals exposed to G1 BOP level and 6 groups of animals exposed to G2 BOP level were prepared and conducted for immunofluorescence imaging and analysis.
♦ The effect of liraglutide on blast-induced changes in cAMP-PKA and P13K-AKT pathways, excitatory (glutamate-NMDA) and inhibitory (GABAA) neurotransmitter receptor densities in vulnerable central auditory regions were identified when animals were exposed to repeated low-level blast injury with and without ear protection.

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(1) In this 4th year of the project, the major activities under Aim 1 include:

1) Performing blast tests in two liraglutide (drug) treatment groups of chinchillas (pre-blast drug treatment and post-blast drug treatment) and a blast control group with both ears open (i.e., a total of 3 experimental groups) at BOP G2 level for 3 consecutive blasts on Day 1 and conducting hearing function tests over the time course of Day 1 (pre- and post-blast) and Days 4, 7, 14, and 28.

2) Performing blast tests in two drug treatment groups of chinchillas (pre-blast drug treatment and post-blast drug treatment) and a blast control group with both ears protected by earplugs (i.e., a total of 3 experimental groups) at BOP G2 level for 3 consecutive blasts on Day 1 and 3 additional blasts at G2 level on Day 4 and conducting hearing function tests over the time course of Day 1 (pre- and post-blast) and Days 4, 7, 14, and 28.
3) Conducting statistical analysis on the data and preparing journal papers to publish the results: effects of liraglutide on mitigation of hearing damage induced by repeated high level blast exposures (G2 animals) with or without HPDs and the mitigation of hearing damage after additional repeated blast exposures on Day 4 at mild-TBI (G2 animals) with HPDs.
4) Creating new methodology for imaging and counting the hair cells in chinchilla cochlea to quantitatively determine the hair cells damage induced by blast using confocal microscope.
5) Preparing brain tissues harvested from chinchillas after the function tests on Day 14 or Day 28 and shipping to Dr. Pfister/ Dr. Chandra's lab at New Jersey Institute of Technology for histology studies (Aim 2).

The **specific objectives** are: 1) to determine the therapeutic function of liraglutide in mitigation of auditory injury in chinchillas repetitively exposed to high BOP **(G2)** level in the ears with and without hearing protection; 2) to establish the animal model with additional repeated blasts at G2 level on Day 4 or with more severe blast-induced hearing loss to investigate the long-term effects of liraglutide treatment in chinchillas with HPDs; 3) to identify if there is a different effect of liraglutide on mitigation of hearing damage in the pre-blast liraglutide (drug) treatment animals and the post-blast drug treatment animals over 14 or 28 days of recovery time; 4) to prepare chinchilla brain tissue samples from G2 animals exposed to blasts in blast control and drug-treatment groups.

(1-1) Outline of blast tests with liraglutide in chinchillas and the hearing function tests

- Animal study at high BOP (G2) level

The possible therapeutic function of glucagon-like peptide-1 (GLP-1) agonist (Liraglutide) to the acute and progressive hearing damage after repeated high-level (G2) blast exposures and recovered over 14 or 28 days was continuously investigated in animal model of chinchilla and completed in this year. Young adult chinchillas (weighing 600-800 g) were assigned to four liraglutide (drug) treatment groups: pre-blast drug treatment to blast injury with and without HPDs (e.g., earplugs) and post-blast drug treatment to blast injury with and without HPDs. For pre-blast drug treatment, liraglutide was delivered to animals with subcutaneous injection at 2 days prior to blast on Day 1 and in the consecutive 7 days; for post-blast drug treatment, liraglutide was injected to animals on Day 1 at 2 hours after the blast and in each day thereafter for 7 days. There were two blast control groups: animals without drug treatment and both ears either open or inserted with earplugs.

Figure 1 shows the time frame and experimental procedure for animals involved in pre-blast drug treatment and post-blast drug treatment groups**.** On Day 1, animals received 3 consecutive blasts with 5-minute intervals between exposures at high BOP (G2) of 15-25 psi or 103-172 kPa

as shown in **Fig. 2**. Hearing function tests, including the ABR, DPOAE, and MLR, were performed on Day 1 (pre- and post-blast) and on Days 4, 7, 14, and 28, respectively. Animals were euthanized after the function tests on Day 14 or 28. The MLR and ABR were measured to reflect the cortex and subcortical hearing function, respectively. DPOAE was measured to detect the damage of outer hair cells.

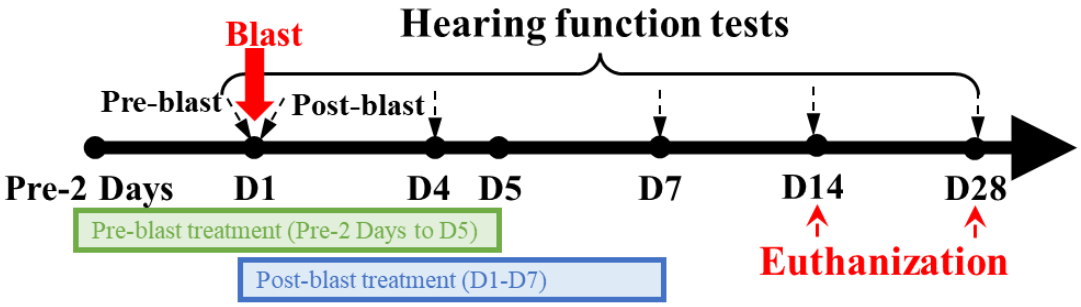

Figure 1. Diagram showing the time frame and experimental procedure for animals involved in pre-blast drug treatment, post-blast drug treatment, and blast-control groups in Aim 1. Note that each animal has pre-blast and post-blast on Day 1 (D1). The time points for function tests are denoted by dashed lines with arrows. Two subgroup animals will be euthanized for histology studies on Days 14 and 28, respectively.

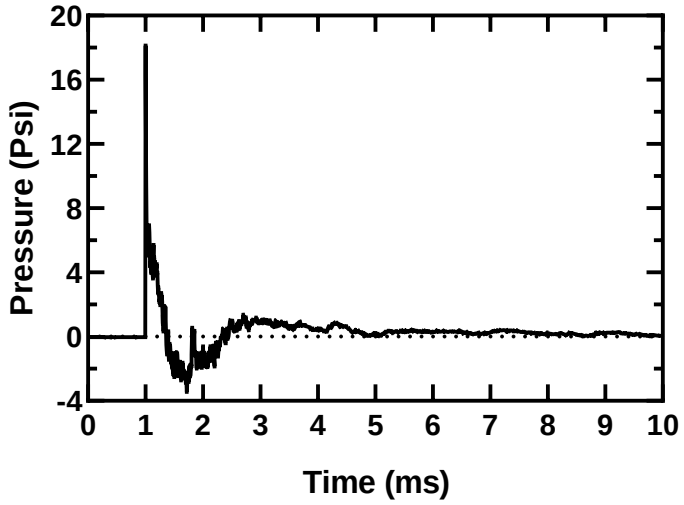

Figure 2. A recorded BOP waveform at the entrance of the ear canal from an animal test with a peak pressure of 18 psi – G2 BOP level.

(1-2) Studies on therapeutic function of liraglutide in chinchillas after repeated blast exposures at high BOP G2 level with ears open over recovery time of 14 and 28 days (To be *submitted to Hearing Research*)

● Examinations of the tympanic membrane (TM)

Three groups of chinchillas were involved in G2 BOP level study with eras open without HPDs (e. g., earplugs): pre-blast drug treatment, post-blast drug treatment, and blast control without drug treatment.

Representative images of the TM of pre-blast and post-blast on Day 1 and on Days 14 and 28 captured by a digital otoscope are shown in **Fig. 3**. In **Fig. 3A**, the TM was intact and translucent with its annulus in the middle and umbo on the right side of the picture. Due to the angle of the chinchilla ear canal, the otoscopic image could only cover approximately the inferior 50% of TM. After the blast exposures, the TM was severely damaged and no translucent membrane or the boundary of the rupture could be observed in **Fig. 3B**. The annulus was highlighted using white dashed curves in **Figs. 3B-3D**. From Post on Day 1 to Day 28, a major part of the TM remained ruptured and the recovery on the TM was limited as reflected by **Figs. 3C & 3D**. All chinchillas showed a similar trend as observed in an example shown in **Fig. 3**. Tympanometry measurements also indicated that the TMs of all chinchillas remained ruptured from post-blast to the end of the experiment.

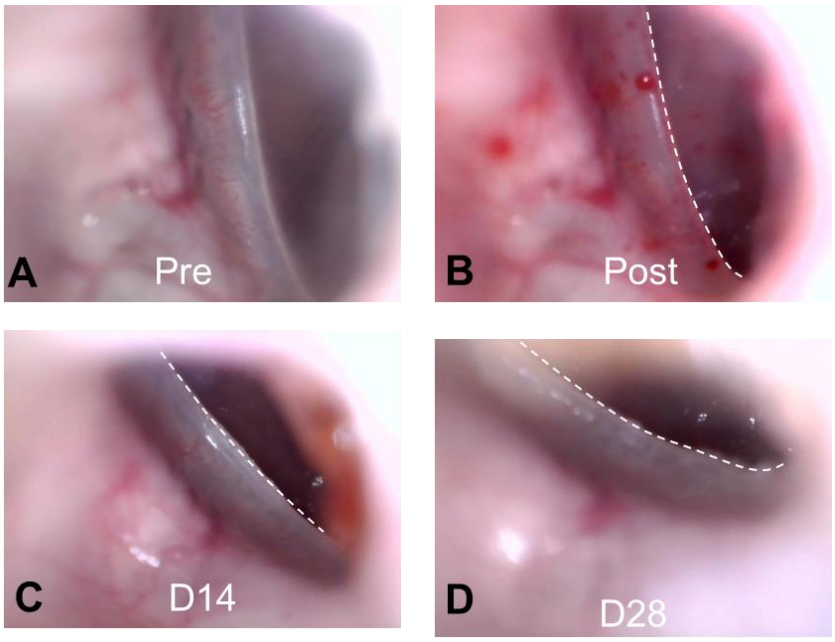

Figure 3. Representative photographs of the TM obtained from otoscopic examinations. The TM images of a chinchilla (22-1-18R) were captured (A) before blasts, (B) immediately after blasts, (C) on Day 14, and (D) on Day 28. The edge of the ruptured TM was highlighted using white dashed curves.

• <u>ABR threshold changes (shifts) observed over the time course</u>

The ABR threshold shifts (mean ± SEM) measured from pre-blast treatment, post-blast treatment, and blast control groups were plotted against frequencies in **Figs. 4A to 4C**, respectively. The sample size for results of Day 1 to Day 14 was N=14 ears in the pre-blast treatment group, N=10 ears in the post-blast group, and N=16 ears in the blast control group. The sample size for results on Day 28 was N=10 ears for all three groups. Data obtained from different time points were represented by different colors.

As shown in **Figs. 4A to 4C**, severe hearing damage was observed on Day 1 as reflected by approximately 40-65 dB mean shifts across all frequencies. The mean shifts at 4-8 kHz were higher than those at 1-2 kHz and the maximum ABR shift was observed at 4-6 kHz. The threshold shifts

decreased over time and the greatest amount of recovery was observed from Day 1 to Day 4. The recovery was almost uniform across frequencies and the shape of the curves of different colors remained unchanged from Day 1 to Day 28. By Day 28, the mean shift ranged from 10 dB at low frequencies to 37 dB at high frequencies, which indicated a significant amount of damage remained in all groups. Differences among post-blast treatment and blast control groups were difficult to observe in results obtained from all frequencies as shown in **Figs. 4B and 4C**.

The ABR threshold shifts (mean ± SEM) at 4, 6, and 8 kHz were plotted as functions of time point and presented separately in **Figs. 4D, 4E, and 4F**, respectively. Mixed-effect analysis indicated that the effect of time was significant in all experimental groups (1 kHz: F (2.54, 87.70) = 132.30, $P < 0.0001$; 2 kHz: F (2.06, 70.88) = 151.90, $P < 0.0001$; 4 kHz: F (3.08, 106.10) = 93.24, $P < 0.0001$; 6 kHz: F (2.98, 102.90) = 90.70, $P < 0.0001$; 8 kHz: F (3.08, 106.30) = 72.18, $P < 0.0001$). The significant effect of liraglutide was only observed at 4 kHz (F (2, 37) = 4.59, P = 0.017) as labeled on the title of **Fig. 4D**. Tukey posthoc tests indicated that the significant difference was detected on Day 7 between blast control and pre-blast treatment groups (P = 0.011) and on Day 14 between blast control and two treatment groups (Control Vs Pre: P = 0.004; Control Vs Post: P = 0.026), as highlighted by the brackets in **Fig. 4D**. At 4-8 kHz, the pre-blast treatment showed the lowest ABR threshold shift and such trend increased over time (see **Figs. 4A-4C**), but the statistically significant difference was only observed at 4 kHz. At 4 kHz, both liraglutide-treated groups showed lower damage than the blast control, and the difference was maximized on Day 14.

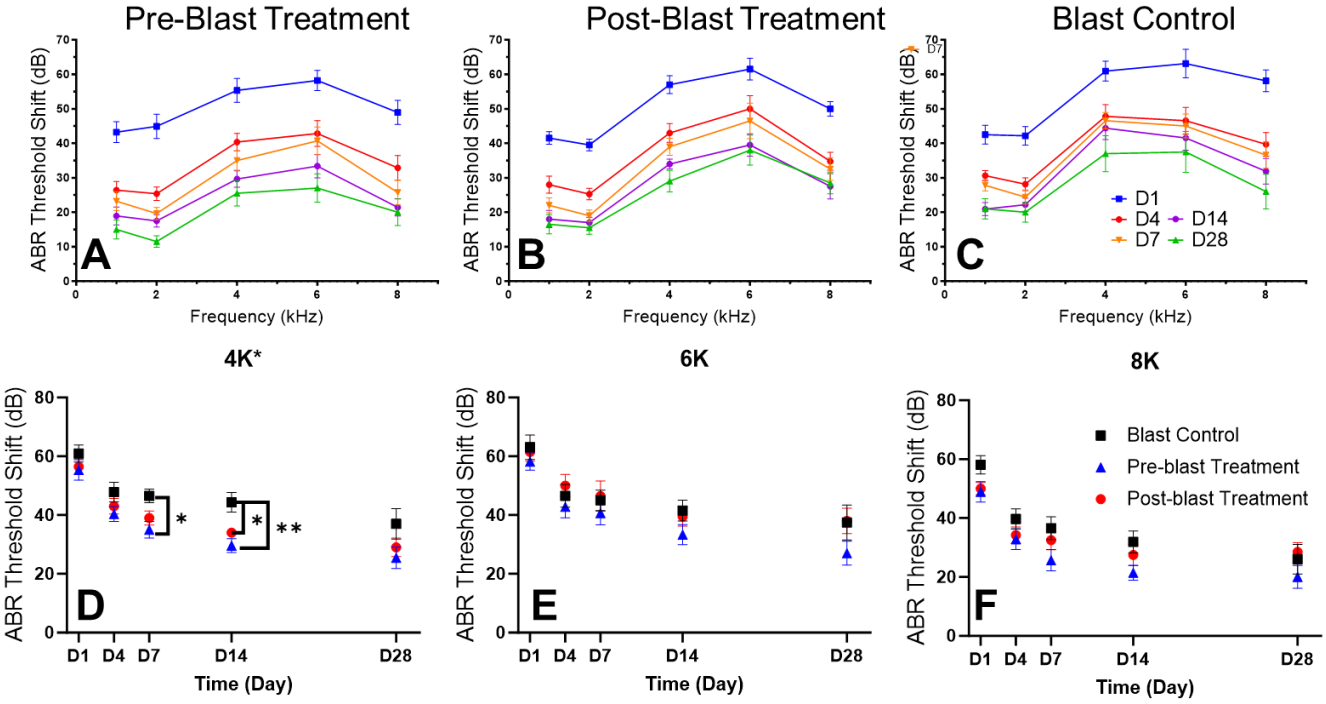

Figure 4. ABR results measured from three treatment groups at different time points. The ABR threshold shifts (mean ± SEM) measured on Days 1, 4, 7, 14, and 28 from: (A) pre-blast treatment group (D14 n=14 ears, D28 n=10 ears); (B) post-blast treatment group (D14 n=10 ears, D28 n=10 ears); and (C) blast control group (D14 n=16 ears, D28 n=10 ears). ABR threshold shifts from three chinchilla groups at 4 (D), 6 (E), and 8 (F) kHz were plotted against time with statistical results. The statistically significant effect of treatment detected by Mixed-Effect model analysis was labeled on the title and the significant difference detected by Tukey's post-hoc test was highlighted by brackets between the groups (** P < 0.05; * P < 0.10).

● ABR wave I amplitude changes observed over the time course

The peak-to-peak amplitudes of ABR wave I (mean ± SEM) were plotted as functions of stimulus level (80-100 dB SPL) in **Fig. 5**. Results from pre-blast treatment, post-blast treatment, and blast control groups were plotted in columns from left to right and results from 8 and 4 kHz were plotted in the top and bottom rows, respectively. ABR wave I amplitudes measured pre-blast, post-blast, and on Days 14 and 28 were selected as representatives and plotted in different colors as shown in the legend in **Fig. 5F**. The sample size for wave I results was the same as the ABR threshold shift results. The mean of the pre-blast amplitudes ranged between 1 to 2 μV in all groups at both frequencies.

A major reduction of the wave I amplitudes was observed after the blast, and the amplitude gradually recovered over time but did not reach the pre-blast level even on Day 28. Wave I amplitude data on Days 14 and 28 showed a certain level of slope indicating the wave I amplitude increased with the stimulus level, while the pre-blast amplitude-stimulus curve was almost flat. At 8 kHz, the mean curves measured from Day 14 and Day 28 almost overlapped with each other, while greater differences (but still within the SEM) could be observed at 4 kHz. Interestingly, the wave I amplitudes on Day 14 and Day 28 were lower in the blast control group at 4 kHz (**Fig. 5F**) compared to drug-treated groups, especially at lower stimulus levels. ABR wave I and threshold shift (**Fig. 4D**) measured at 4 kHz both indicated that the liraglutide treatment potentially improved the restoration of chinchilla peripheral auditory system function. However, the difference of the wave I results was only observed from mean values without the support of statistical significance.

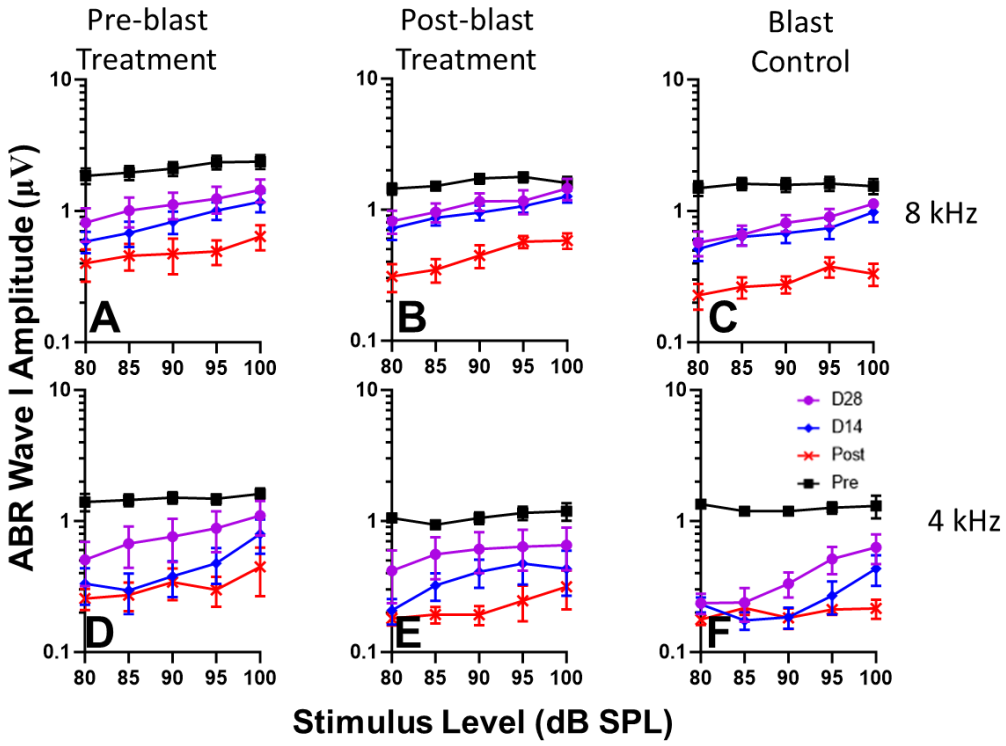

Figure 5. ABR wave I amplitudes (mean ± SEM, sample size same as ABR threshold shifts) at stimulus levels of 80 to 100 dB SPL measured from (A) pre-blast treatment group; (B) post-blast treatment group; and (C) blast control group at 8 kHz. Results measured from pre-blast treatment, post-blast treatment, and blast control groups at 4 kHz were plotted in (D), (E), and (F), respectively.

● MLR Na-Pa amplitude changes observed over the time course

An example MLR waveform measured at 500 Hz with 80 dB SPL stimuli is shown in **Fig. 6A**. The first negative peak after the ABR section (first 10 ms) was defined as the Na, and the first positive peak following the Na was defined as Pa as highlighted in **Fig. 6A**. The Na-Pa amplitude was used to represent the intensity of the MLR signal. Representative MLR signals measured from a post-blast treatment (22-1-8L) and a blast-control (22-1-16R) chinchilla ears pre- and post-blast on Day 1, and on Days 4 and 28 were presented in **Figs. 6B and 6C**, respectively. Results measured at different time points were represented by different colors. The first 40 ms of the MLR signal was selected to better display the change of the Na-Pa amplitude over time. The MLR magnitude decreased, and the latency increased immediately after the blast on Day 1. On Day 4, the Na-Pa amplitude recovered and even exceeded the pre-blast level in the post-blast treatment chinchilla (**Fig. 6B**), while the amplitude was still lower than the pre-blast level in the blast control chinchilla (**Fig. 6C**). The trend remained unchanged on Day 28.

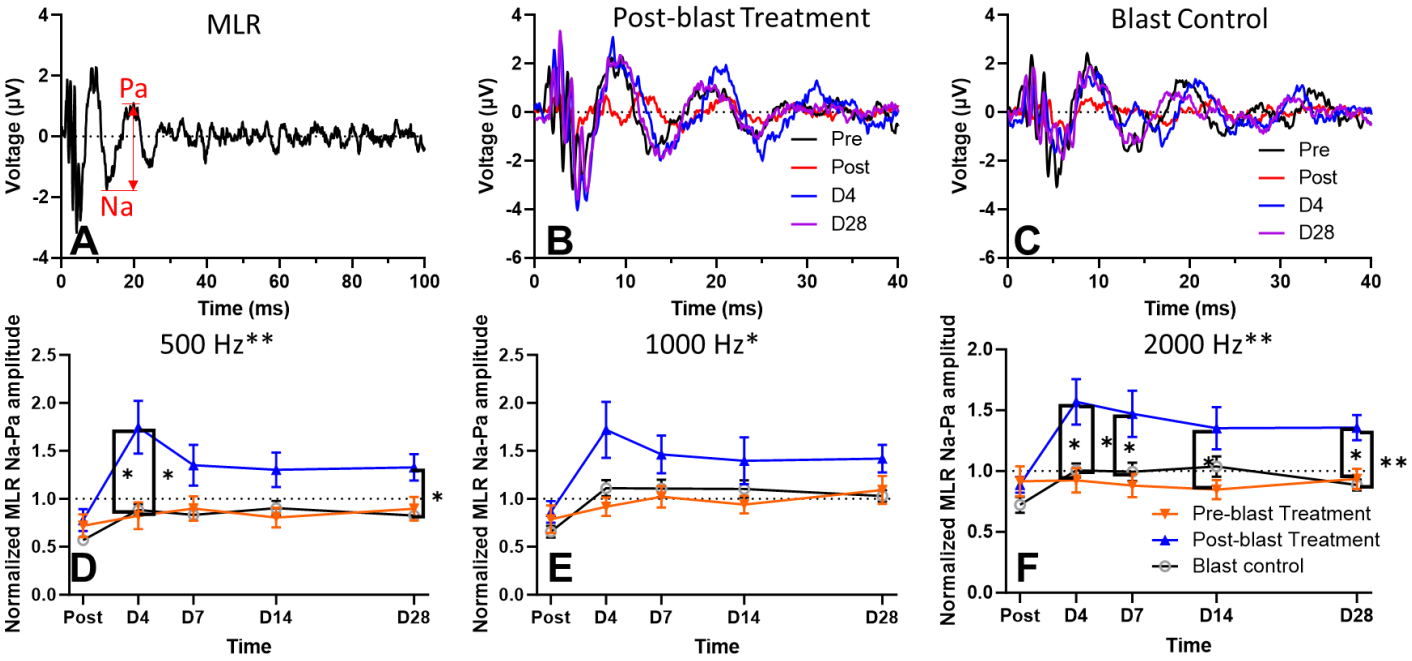

Figure 6. MLR results. An example MLR waveform measured at 80 dB, 500 Hz stimulus with a full length of 100 ms was plotted in **(A)**. Representative MLR waveforms from **(B)** post-blast treatment and **(C)** blast control ears were presented with results measured at different time points represented by different colors. Normalized MLR amplitudes from G2 open chinchillas (mean ± SEM, same sample size as ABR threshold shifts) measured at 80 dB stimuli at **(D)** 500 Hz, **(E)** 1000 Hz, and **(F)** 2000 Hz were plotted against time point. Results from different groups were represented by different colors. The statistically significant interactions between time and treatment detected by Mixed Effect analysis were labeled on the title and the significant difference detected by Tukey's post-hoc test was highlighted by brackets between the groups (** $P < 0.05$; * $P < 0.10$).

The Na-Pa amplitudes (mean ± SEM) measured at 0.5, 1, and 2 kHz were normalized by their pre-blast values and plotted as functions of time in **Figs. 6D-6F**. The sample size for results from Day 1 to Day 14 was N=14 ears in the pre-blast treatment group, N=10 ears in the post-blast group, and N=16 ears in the blast control group. The sample size for Day 28 was N=10 ears for all three

groups. As shown in **Figs. 6D-6F**, the Na-Pa amplitudes were first reduced after the blast on Day 1, increased from post-blast to Day 4, and remained almost unchanged from Day 7 to Day 28. Interestingly, the post-blast treatment group (bleu lines) showed a significantly higher Na-Pa amplitude increase from post to Day 4 which even exceeded the pre-blast level (ratio>1). The MLR amplitudes of the post-blast treatment slightly decreased from Day 4 to Day 7 but remained at a level higher than the pre-blast until Day 28. The increase from post to Day 4 was also observed in pre-blast and blast control chinchillas, but the amount of increase did not go beyond the pre-blast level, and the level remained almost unchanged from Day 4 to Day 28. The increase in the pre-blast treatment group (red lines) was relatively small, especially at 2 kHz, which indicated that the pre-blast treatment potentially altered the severity of blast-induced acute auditory injuries in the central auditory system. Results measured from three different frequencies generally showed a similar trend. Significant differences detected by Tukey's post-hoc tests were highlighted in **Figs. 6D-6F** using the brackets.

● Immunofluorescence imaging of auditory cortex (AC) and inferior colliculus (IC)

Results from the preliminary histology study are presented in **Fig. 7** from three representative animals: pre-blast drug treatment, post-blast drug treatment, and blast control. The big picture of the brain coronal section at the location of AC is shown in **Fig. 7A** and the IC is shown in **Fig. 7E**. The regions including AC and IC were highlighted using red circles. The caspase was stained in red and the cell nuclei were counterstained in blue as shown in **Figs. 7B-7D** and **7F-7H**.

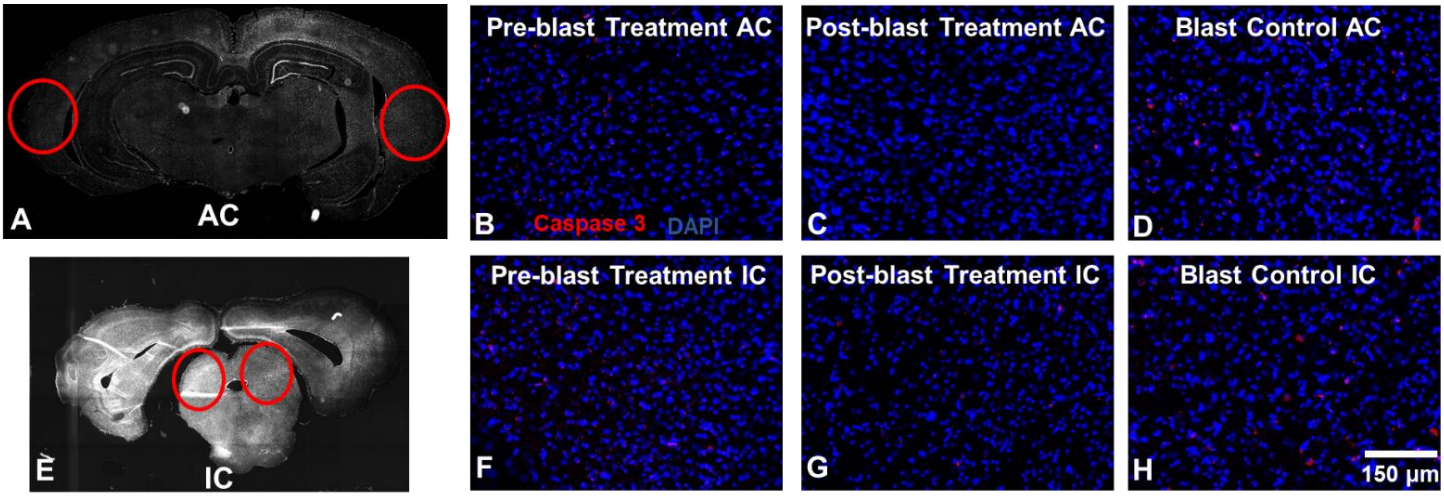

Figure 7. Results of the histology study: Low-magnification images of the (A) AC and (E) IC included the view of the entire coronal section. The regions of AC and IC were highlighted by red circles. The immunofluorescence staining of caspase-3 in chinchilla AC from (A) pre-blast treatment, (B) post-blast treatment, and (C) blast control chinchillas. The immunofluorescence staining of caspase-3 in chinchilla IC from (D) pre-blast treatment, (E) post-blast treatment, and (F) blast control chinchillas. The caspase-3 was stained in red, and the nuclei counterstain was DAPI (blue).

The expression level of caspase-3 was higher in the blast control AC than in the drug-treated ones. The integrated intensities of the caspase-3 signal for pre-blast treatment, post-blast treatment, and blast control chinchilla ACs were $1x10^{-3}$, $0.6\ x10^{-3}$, and $2\ x10^{-3}$, respectively. In IC, the expression level of caspase-3 was observed in both blast control and pre-blast treatment

chinchillas, and the post-blast treatment chinchilla showed the lowest level of caspase-3 expression. The integrated intensities of the caspase-3 signal for pre-blast treatment, post-blast treatment, and blast control chinchilla ICs were $3x10^{-3}$, $1 x10^{-3}$, and $4 x10^{-3}$, respectively. The post-blast treatment chinchilla showed the lowest level of caspase-3 expression in both AC and IC.

● Conclusions from G2 study in chinchillas with ears open and liraglutide treatment over the time course

The study on open-ear chinchillas exposed to 3 repeated G2 BOP level (15-25 psi) blasts or mild TBI aimed to assess the therapeutic function of liraglutide in animals without HPDs. The ABR and MLR were measured to monitor the change in hearing function within 28 days after the blast exposures and the pathological changes in central auditory system (CAS) were examined using histology study at the end of the experiment. Significant lower ABR threshold shifts were observed at 4 kHz in drug-treatment chinchillas, which was consistent with the higher ABR wave I amplitudes. The post-blast treatment groups showed increased MLR amplitude as well as lower levels of caspase-3 expressions in the IC and AC which indicated the possible liraglutide-induced changes in the mild TBI damaged CAS. The hearing function change and the effect of liraglutide treatment were first-time reported in the open-ear chinchillas with blast-induced mild TBI.

● Comparison of G2 study in open-ear chinchillas with the G2 study in chinchillas with ears protected on liraglutide treatment over the time course

The function of liraglutide to prevent mild TBI-induced hearing damage in chinchillas protected by earplugs during the blast exposures (15-25 psi repeated 3 times) was reported in our previous study (Jiang et al., 2023). One of the key findings from the G2 study with earplug in chinchillas was the pre-blast administration of liraglutide reduced the severity of blast-induced acute hearing damage. **Figure 8** shows the comparison of ABR threshold shifts measured from the open-ear chinchillas and those reported by Jiang et al. (2023). Results measured at 4 and 8 kHz were selected to represent the chinchilla's hearing function at middle and high frequencies, respectively. In open ears, the difference between the blast control and drug-treated chinchillas was only statistically significant on Day 7 and Day 14 at 4 kHz. This potentially suggested that the liraglutide treatment facilitated the post-blast restoration of the hearing function. At 8 kHz, a similar trend was observed but the effect of liraglutide was not statistically significant. In contrast, the difference between the pre-blast treatment group and the other two groups was most significant on Day 1 in the earplug (EP) chinchillas (**Figs. 8C and 8D**), which indicated that the pre-blast administration of liraglutide provided a certain level of protection to reduce the blast-induced acute damage. The difference between open and EP chinchillas could be a result of the protection provided by EP, which not only excluded the conductive hearing loss but also reduced the severity of the sensorineural hearing loss induced by mild TBI blast exposures.

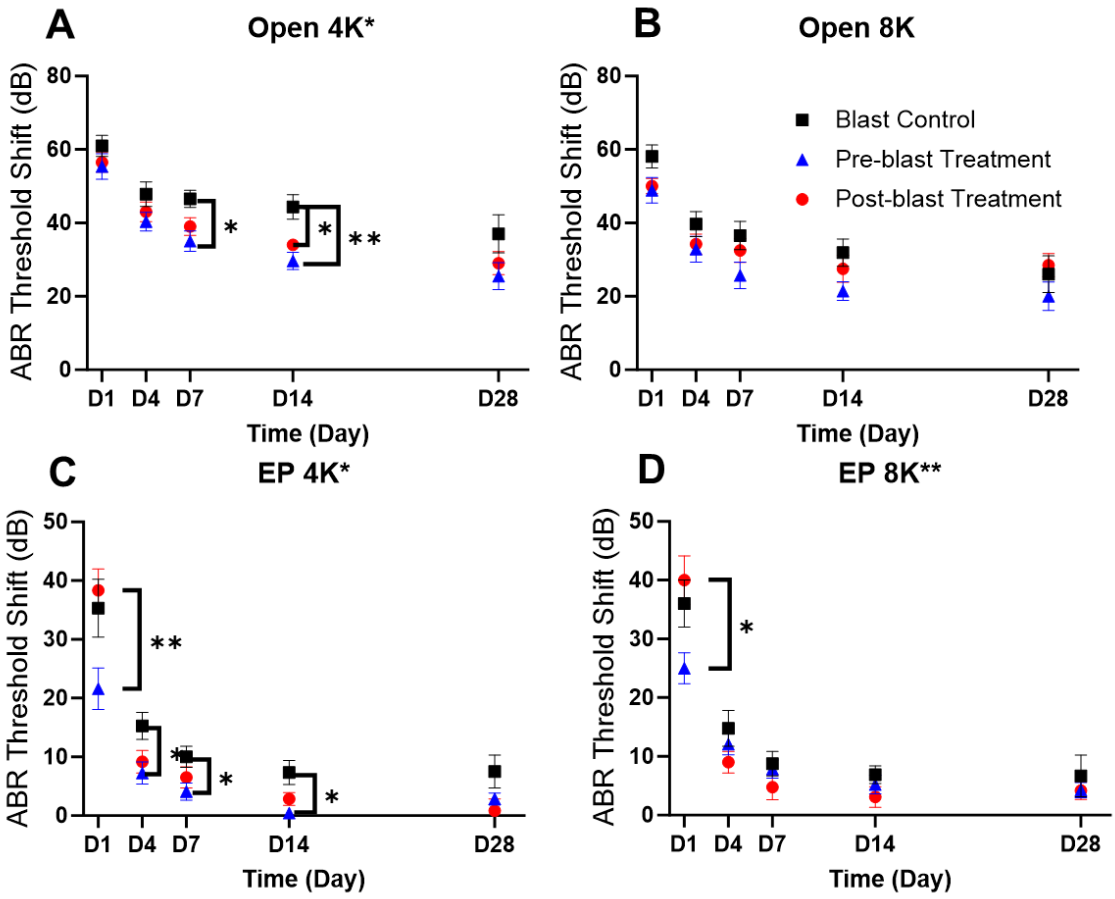

Figure 8. Comparison of ABR threshold shifts (mean ± SEM) at 4 and 8 kHz measured from open-ear and earplug (EP)-protected chinchillas experienced repeated high-intensity blasts (3x 15-25 psi). Results obtained from (A) open at 4 kHz; (B) open at 8 kHz; (C) EP at 4 kHz; and (D) EP at 8 kHz were plotted as functions of time. The statistically significant interactions between time and treatment detected by two-way ANOVA tests were labeled on the title and the significant difference detected by Tukey's post-hoc test was highlighted by brackets between the groups (** $P < 0.05$; * $P < 0.10$). (C) and (D) were obtained from Jiang et al. (accepted) and the sample sizes were: D1-14 n=18 ears & D28 n=10 ears in the pre-blast treatment group; D1-14 n=18 ears & D28 n=10 ears in the post-blast treatment group; and D1-14 n=20 ears & D28 n=10 ears in the blast control group.

Reference: Jiang S, Sanders S, Gan RZ. Mitigation of hearing damage with liraglutide treatment in chinchillas after repeated blast exposures at mild-TBI. *Military Medicine*, 2023 (In press).

(1-3) New study on therapeutic function of liraglutide in chinchillas with ears protected by HPDs - 3 additional blasts at G2 BOP level on Day 4 over recovery time of 28 days (G2 EP D4 3B study)

The results from completed G2 EP animals indicated that most of the hearing damage was spontaneously recovered after Day 4 (see **Fig. 9**). In the meantime, comparisons between the G2 EP and open animal results showed that removing the EP did not simply increase the severity of hearing damage but may change the type of blast-induced auditory injuries and the subsequent recovery process. Thus, more severe hearing damage or additional repeated blast exposures may be expected to better demonstrate the long-term effect of the liraglutide treatment. To test this idea,

we made some adjustment to the G2 EP animals by applying 3 additional blasts on Day 4 before the hearing function test. **Figure 10** shows the modified time frame and experimental procedure for animals involved in G2 EP animals with 3 additional blasts on Day 4 (e.g., G2 EP D4 3B study). There are still three experimental groups of pre-blast treatment, post-blast treatment, and blast control, following the previously established protocol. We expect to establish an animal model with more severe blast-induced sensorineural hearing loss to investigate the long-term effect of liraglutide treatment in EP chinchillas.

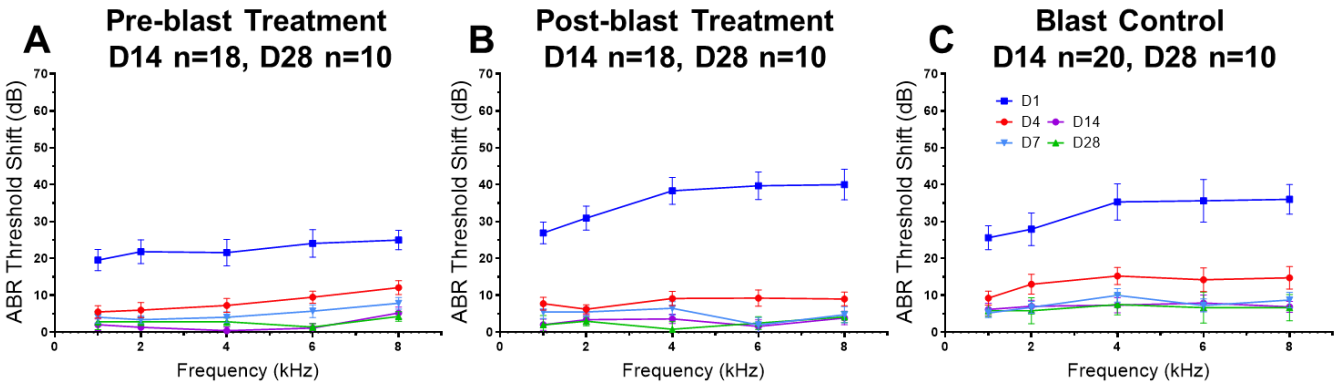

Figure 9. ABR results obtained from three experimental groups of G2 PE animals at different time points. The ABR threshold shifts (mean ± SEM) measured on Days 1, 4, 7, 14, and 28 from: (A) pre-blast treatment group (D14 n=18, D28 n=10); (B) post-blast treatment group (D14 n=18, D28 n=10); (C) blast control group (D14 n=20, D28 n=10). Note: this figure cited from reference Jiang et al. (2023).

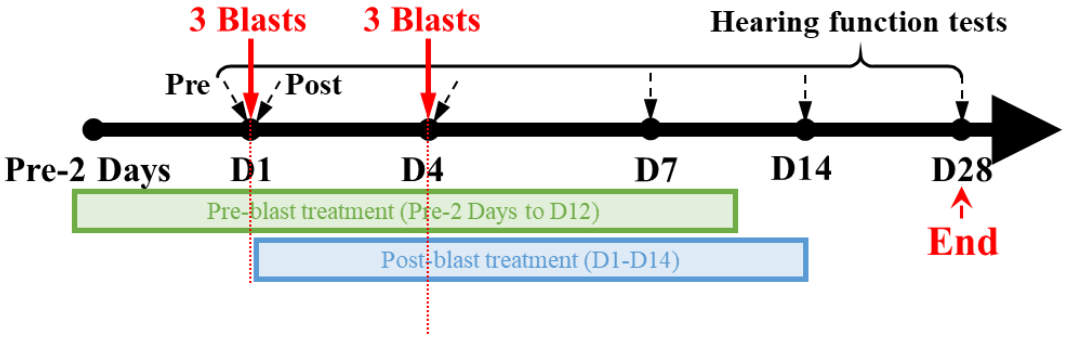

Figure 10. Diagram showing the time frame and experimental procedure for animals involved in 3 additional G2 blast on Day 4 (D4). Note that animals in all three experimental groups have 3 blasts on Day 1 (D1). The time points for function tests are denoted by dashed lines with arrows. Animals will be euthanized for histology studies on Day 28.

• <u>ABR threshold changes (shifts) observed over the time course</u>

The ABR threshold shifts (mean ± SEM) measured at frequencies of 1, 2, 4, 6, and 8 kHz were plotted in **Fig. 11** with different time points represented by different colors. Data obtained from pre-blast treatment (N = 6 ears), post-blast treatment (N = 18 ears), and blast control (N = 16 ears) groups were plotted in **Figs. 11A, 11B, and 11C**, respectively. Comparison of the ABR threshold shifts after blasts on Day 1 (all blue lines) indicated that the data from pre-blast treatment were lower than that from post-blast treatment and blast control, which showed the same result as observed from EP animals of post-blast on Day 1 in **Fig. 9**. After 3 additional blasts on Day 4, the ABR threshold shifts (all red lines) from pre- and post-blast drug treatment (**Figs. 11A and 11B**)

were much lower than that observed in blast control animals (**Fig. 11C**), which indicated the liraglutide's mitigation function to hearing damage upon the second repeated blast exposures at G2 BOP level. Without drug treatment, the ABR threshold shifts after D4 blasts almost overlapped with that of post blast on Day 1 as shown in **Fig. 11C**. From Day 4 to Day 28, the ABR shifts decreased over time and the amount of recovery between adjacent time points decreased as well in all three groups, which was also observed in the G2 EP animals with Day 1 blasts only (**Fig. 9**).

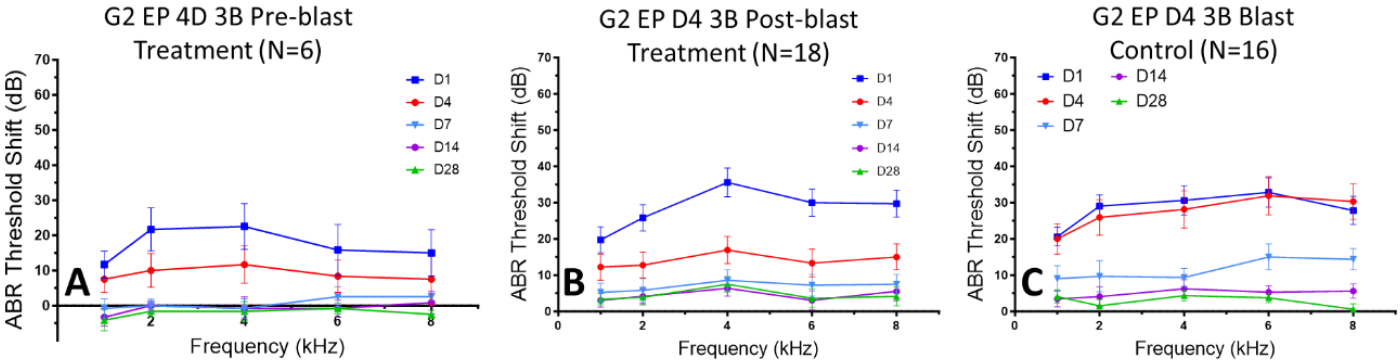

Figure 11. ABR threshold shifts (mean ± SEM) at 1 to 8 kHz measured in G2 EP D4 3B study from (A) Pre-blast treatment group (N = 6 ears), (B) Post-blast treatment group (N=18 ears), and (C) Blast control group (N=16 ears).

For further analysis, the ABR threshold shifts (mean ± SEM) were plotted with respect to time points and compared across three experimental groups at 5 individual frequencies in **Fig. 12**.

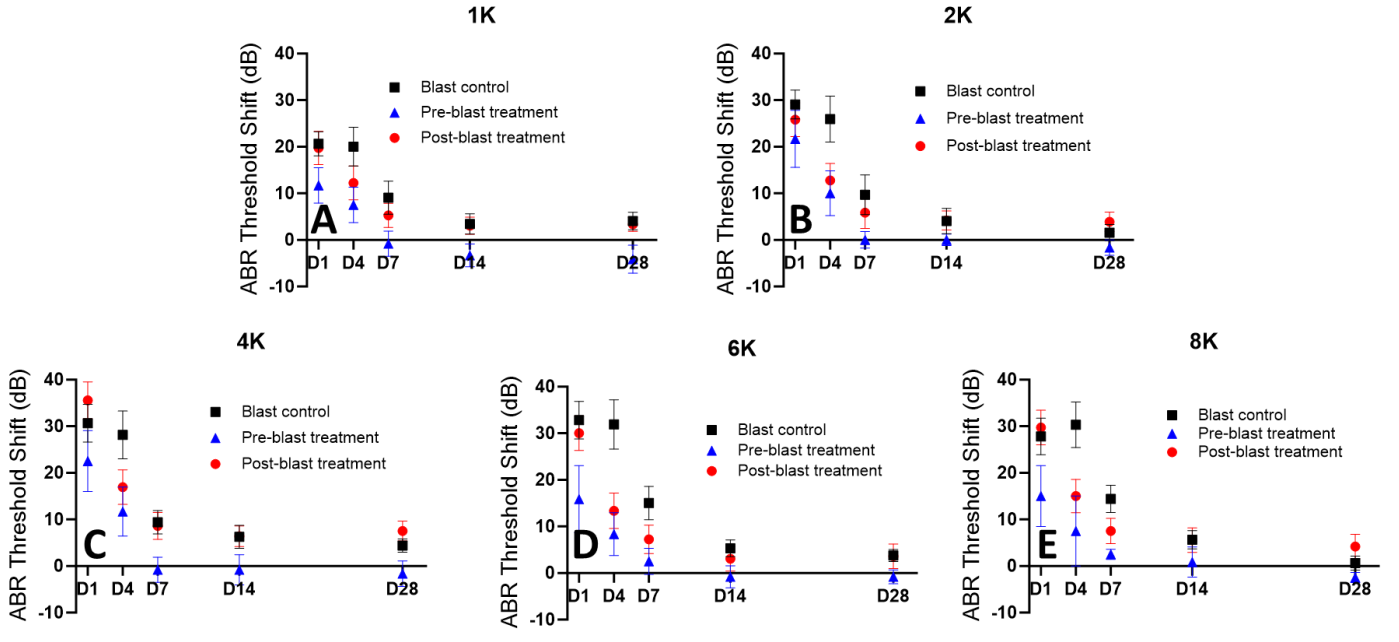

Figure 12. ABR threshold shifts (mean ± SEM) at different time points from Day 1 to Day 28 measured from three experimental groups at 5 frequencies: (A) 1 kHz, (B) 2 kHz, (C) 4 kHz, (D) 6 kHz, and (E) 8 kHz.

As shown in **Fig. 12**, the pre-blast treatment group (blue symbols) displayed the lowest threshold shifts at 4-8 kHz on Day 1 and the both drug-treated groups (blue and red symbols) displayed lower shifts than the blast control at 2-8 kHz on Day 4. On Day 7, both drug-treated groups showed lower shifts than blast control at 6-8 kHz. Thus, the additional 3 blasts on Day 4 induced more long-lasting hearing damage in the blast control group (black symbols), and the efficacy of liraglutide could be observed in drug-treated groups from Day 1 to Day 7. Note that the sample number (N) in pre-blast treatment group needs to increase for further statistical analysis. However, the qualitative results in **Figs. 11 and 12** suggested that using additional repeated blast exposures at G2 BOP level in EP animals can explore the potential therapeutic function of liraglutide on mitigation of hearing damage for ears with HPDs.

● <u>ABR wave I amplitude changes observed over the time course</u>

The ABR suprathreshold wave I results measured from the G2 EP D4 3B study and our previous study on G2 EP animals of 3 blasts on Day 1 only are shown in **Fig. 13**. The data obtained from G2 EP D4 3B are displayed in the top row and the data obtained from the previous G2 EP study are displayed in the bottom row.

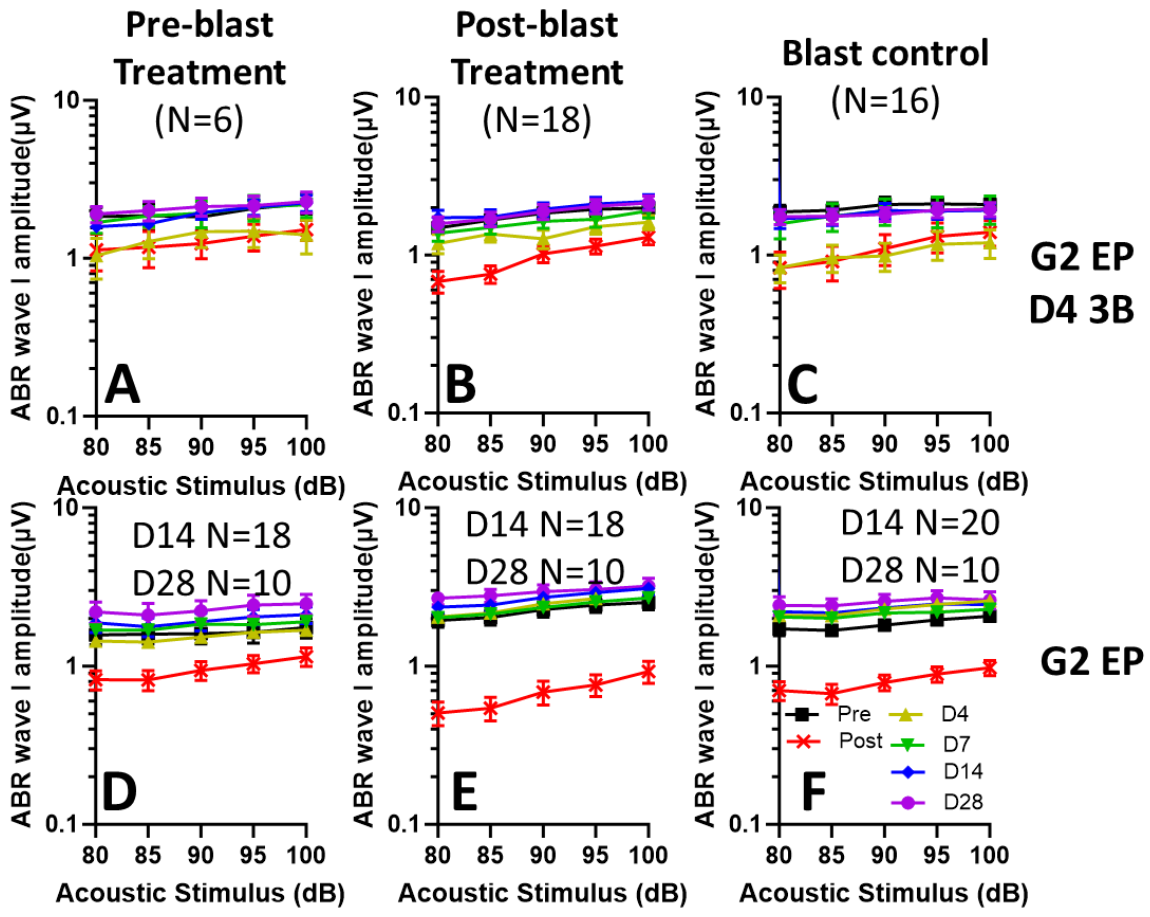

Figure 13. Comparison of ABR wave I amplitudes (mean ± SEM) in response to stimulus levels from 80 to 100 dB SPL at 8 kHz between G2 EP D4 3B and previously completed G2 EP experiments. The G2 EP D4 3B results were shown in the top row: (A) Pre-blast treatment, (B) Post-blast treatment, and (C) Blast control. The G2 EP results were shown in the bottom row: (D) Pre-blast treatment, (E) Post-blast treatment, and (F) Blast control.

Here are some observations through the comparison of the top row's results with the bottom row's: 1) the additional 3 blasts on D4 resulted in reduction of wave I amplitudes in blast control animals but not in drug treated animals, especially in post-blast treatment, which indicated the liraglutide's effect on mitigation of ABR wave I damage upon the D4 repeated blasts; 2) the wave I amplitudes returned to the pre-blast level from Day 7 to Day 28 after D4 3 blasts and in contrast, the wave I amplitude returned to pre-blast level from Day 4 to Day 28; 3) overall, the wave I amplitude results are consistent with the ABR threshold results.

● Conclusion and next step of work

The preliminary study on G2 EP D4 3B has provided evidence that repeated blast exposures at mild TBI or G2 BOP level can cause more severe sensorineural hearing loss in animal model of chinchilla. This has benefited the investigation of long-term effect of liraglutide treatment in ears with HPDs. We will continue this study in the following year with statistical analysis on more hearing function data and the histological studies on cochlear and brain tissues in chinchillas.

(1-4) New methodology for imaging and counting the hair cells in chinchilla cochlea

To better characterize the response of the peripheral auditory system to the blasts and liraglutide treatment, we developed an experimental setup to count the number of outer and inner hair cells (OHC & IHCs) in chinchilla cochlea using a confocal microscope (SP8, Leica). Here are some preliminary results obtained from this approach.

Upon the completion of the hearing function tests (on Day 14 or Day 28), cardiac perfusion of 4% paraformaldehyde saline was performed. The cochlea was then harvested, fixed in 2% paraformaldehyde and 2% glutaraldehyde in 1X PBS for over 48 hours, and decalcified in EDTA for 7 days. The cochlea was then dissected into 6 half-turns and the basilar membrane (BM) was then peeled off from the spiral ligament and osseous spiral lamina. The BM sections were blocked with 5% goat serum and immunostained using Rabbit anti-myosin VIIa (1:100, Proteus BioSciences #25–6790) as the primary antibody and Goat anti-rabbit Pacific-Blue-conjugated antibody (1:200, Thermo Fisher #P-10994) as the secondary antibody. The BM specimens were than mounted on microscopic slides covered by cover glass. The imaging process was performed on a Leica SP8 confocal microscope.

The images captured by confocal microscope are shown in **Fig. 14**. An image captured from the cochlea middle turn was shown in **Fig. 14A**. Three rows of OHCs and one row of IHCs at the base and middle turns could be clearly observed. An example image obtained from the base turn of cochlea was shown in **Fig. 14B**. The morphology of the OHC and IHC could be clearly observed. The quality of the image is sufficient for cell counting and quantifying the blast-induced damage at regions of different frequencies in the chinchilla cochlea. **Figure 14C** shows the morphology of the OHC stereocilia captured under higher magnification. Three rows of typical "V" shapes could be clearly observed under the microscope. This method will be used in the study to quantitatively assess the damage induced in the chinchilla cochlea hair cells. The morphology of hair cells and stereocilia could also be observed using this method.

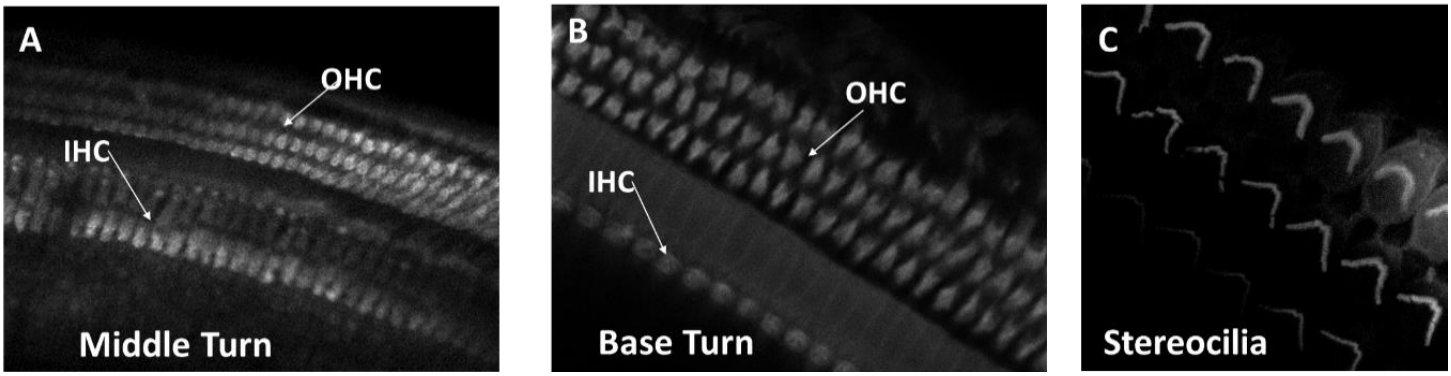

Figure 14. Images of OHC and IHC captured at the (A) middle and (B) base turns of the chinchilla cochlea under the confocal microscope. The OHC stereocilia morphology could be observed under high-magnification images as shown in (C).

(1-5) Preparing chinchilla brain tissue samples for histology study after completion of hearing function tests

Upon the completion of the hearing function tests at the time points as shown in **Fig. 1**, chinchillas were perfused transcardially with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1M PBS. The brain was excised from the cranial vault and the bullae were harvested. The brain sample for each animal was fixed in 4% paraformaldehyde in 0.1 M PBS for 48 hours. Then, the chinchilla brains were immersed in 30% sucrose solution before shipping to the laboratory of Dr. Bryan Pfister / Dr. Namas Chandra at New Jersey Institute of Technology (NJIT) – subcontractor for this project. However, there was a delay of performing all the brain tissues in the NJIT lab due to the Leica microscope malfunctioning.

(2) In this 2nd year of the project, the major activities under Aim 2 are performed in Dr. Pfister/Dr. Chandra lab at NJIT to investigate the beneficial effects of liraglutide on the mitigation of the central auditory damage following repetitive exposure to the low BOP or mild TBI pressure levels, named G1 and G2 BOP level, respectively.

Specific Aim 2: Investigate the beneficial effects of liraglutide on the mitigation of the central auditory damage following repetitive exposures to the low BOP or mild TBI pressure levels.

For Aim 2, there were two groups of injured chinchilla:
Animal group 1 (labeled as **G1 animals**) were injured at low pressure levels of 3-5 psi with 6 repeats.
Animal group 2 (labeled as **G2 animals**) were injured at moderate pressure levels of 15-25 psi with 3 repeats.

Previous findings using the originally proposed biomarkers did not lead to the identification of robust changes that could satisfactorily answer the proposed questions. Based on our result from G1 animals and the preliminary work in G2 animals, the original 8 biomarkers originally proposed did not lead to meaningful conclusions on the effects of liraglutide or ear protection (reported in the 2022 annual report).

This past year, we altered the methodology and approach to address the questions raised for Specific Aim 2. We address the following questions based on the results we have obtained thus far, organizing animal groups for analysis, presentation and interpretation of the results.

Specific objectives for Aim 2:

1) What is the effect of pre- or post-drug (liraglutide) treatment from the blast control animals?
2) What is the effect of ear protection and treatment?
3) Does the recovery time (14 vs 28 days) affect injury induced changes of central auditory damage?
4) Does low-level blast injury lead to changes in chronic inflammation in the auditory brain regions and does treatment with liraglutide reduce inflammation?

To answer these questions, we changed focus to considering changes in neuroinflammation that have been recently characterized by us and others in the rat repetitive blast model. In addition, a main action of liraglutide is reducing neuroinflammation. Accordingly, based on our experience with blast in the rodent model and the action of liraglutide, inflammation and astrogliosis are good alternative assessment instruments. Specifically, here we report findings using:

1) Iba-1 antibody to identify microglia and image analysis to quantify activation, the cell in the brain responsible for neuroinflammation. Iba 1 (Ionized calcium-binding adaptor molecule 1):
    a. Cytoplasmic protein expressed in monocyte lineage cells and in the brain and is primarily restricted to microglia
    b. Considered a marker of all microglia, rather than an activated subset
2) GFAP antibody to identify of astrocytes and image analysis to quantify activation. GFAP (glial fibrillary acidic protein):
    a. Class-III intermediate filament, is a cell-specific marker that, distinguishes astrocytes from other glial cells
    b. Cellular localization: cytoplasm (associated with intermediate filaments)

Major Activities:

A majority of the commercially available antibodies are not validated in chinchilla. Our first task this reporting period was to validate antibodies from different vendors to optimize results. Positive labeling was found in the cortex, IC and AC regions. Figures are shown below.

The accomplishments in this report include assessing neuroinflammation and therapeutic effect of liraglutide treatment between group 2 (G2) both open and protected ear 28-day survival naïve, injured, pre-treated and post-treated animals (3 repeated blasts at 15-25 psi). Accomplishments also include preparing brain slices for our regions of interest and analysis for morphological changes of microglia and astrocyte upon injury. In this report we include results and interpretations for the changes observed in terms of inflammation in the auditory cortex (AC).

Methodology:

Group 2 (G2) animals were exposed to 3 repeated blasts at 15-25 psi with and without ear protection. There were three treatment groups with 14 day and 28-day survival (Table right):

1) blast injured with no treatment
2) pre-injury treatment with liraglutide beginning two days pre-injury
3) post-injury treatment with liraglutide beginning 2-hour post-injury

Experimental Groups		
PID 14	w/o EP	No treatment (Injury)
		Pre-treatment
		Post-treatment
	with EP	No treatment (Injury)
		Pre-treatment
		Post-treatment
PID 28	w/o EP	No treatment (Injury)
		Pre-treatment
		Post-treatment
	with EP	No treatment (Injury)
		Pre-treatment
		Post-treatment

At 14 days and 28 days following blast injury, animals were sacrificed by transcardial perfusion with PBS followed by 4% paraformaldehyde and sent to the NJIT Center for Injury Biomechanics materials and medicine (CIBM3) for immunological analysis. To prepare brain slices, brains were cryoprotected and ultra-thin sections (20 µm) were cut on using a cryostat (Leica Instruments) and mounted on slides. Slices were selected to include the auditory cortex and inferior colliculus. Sections are immunostained for inflammation markers: Iba1(microglia) and GFAP (astrocytes).

Regions of interest are fluorescently imaged and digitized using Stereo Investigator (MBF Bioscience) scanner attached with fluorescent microscope (Leica Aperio Versa). Digitized images are then analyzed for morphological changes and quantified using ImageJ software. Changes in fluorescent labeled cellular structure as well as quantification of total number of microglia and astrocytes was evaluated for: Effect of liraglutide treatment in amelioration of inflammation.

Microglia Analysis: The total number of microglia was determined within the region of interest (ROI) i.e. auditory cortex in terms of Iba-1 positive cells. Further, morphological changes of microglia during their activation were identified using skeleton analysis.

Resting microglia has small spherical cell body with highly branched processes and making contacts with neighboring cells, whereas activated microglia possess larger cells body with shorter and thicker processes. We have created a plugin tool for ImageJ to measure the number of branches, number of junctions and average branch length. Once the threshold is set, the program will analyze all the images automatically. This process is able to eliminate biasedness of the manual analysis and time efficient.

Astrocyte Analysis: The total number of astrocytes was determined within the region of interest i.e. auditory cortex and inferior colliculus in terms of GFAP positive cells. Following injury, healthy astrocytes get activated into reactive astrocytes (RA). Upregulation of GFAP, the main constituent of astrocyte intermediate filaments and hypertrophic response are considered as hallmarks for reactive astrocytes. Similarly, as microglia, skeleton analysis was used to find the average branch length along with total astrocyte count.

The table below outlines samples at NJIT.

Blast level	Ear protection condition			ANIMAL ID	ANIMAL ID	ANIMAL ID	ANIMAL ID	ANIMAL ID	ANIMAL ID
G2 (15-25 psi, 3 repeats)	**w/EP**	control	PID 14	21-1-1	21-2-12	21-2-14	21-2-15		
			PID 28	21-1-8	21-1-18	21-1-19	21-2-8	21-2-9	
		pre-drug	PID 14	21-1-3	21-1-4	21-1-20	21-2-17	21-3-2	
			PID 28	21-1-9	21-1-10	21-1-11	21-1-12	21-2-13	
		post-drug	PID 14	18-4-12	21-1-5	21-1-6	21-1-21	21-2-18	21-3-1
			PID 28	21-1-14	21-1-15	21-1-16	21-1-17	21-2-11	
	w/o EP	Control	PID 14	22-1-1	22-1-2	22-1-3			
			PID 28	22-1-16	22-1-17	22-1-18			
		Pre-drug	PID 14	22-1-5	22-1-9				
			PID 28	22-1-13	22-1-14	22-1-15			
		Post-drug	PID 14						
			PID 28	21-3-20	22-1-7	22-1-8			
	Naive		17-6-3	18-1-3	19-4-12	19-4-15	22-1-19	22-1-20	23-1-20
	Analyzed								
	Sliced/ Slicing								
	Old samples								

Results and Interpretations:

We have accomplished the following tasks:

Sample preparation and analysis:

- For interpretation of results, there is a need to compare naïve animals with the injured groups to establish an expected change in neuroinflammation from injury. Accordingly, we added n= 3 naïve animals to our analysis.
- All animal brains from the Gan lab have been received, cataloged, and stored by CIBM3 at NJIT.
- Slices have been prepared from the following samples and immunolabeled outlined in the table below.

Animal Group	Ear protection condition	Treatment	PID	Animal ID	Markers analyzed	
					AC	IC
2	w EP	Control	14	21-2-12	All markers	
				21-2-14	All markers	
				21-2-15	All markers	
			28	21-1-8	All markers	
				21-2-8	All markers	
				21-2-9	All markers	
		Pre-drug	14	21-1-20	All markers	
				21-2-17	All markers	
				21-3-2	All markers	
			28	21-1-9	All markers	
				21-1-10	All markers	
				21-1-11	All markers	
		Post-drug	14	21-1-21	All markers	
				21-2-18	All markers	
				21-3-1	All markers	
			28	21-1-14	All markers	

				21-1-15	All markers	
				21-1-16	All markers	
	w/o EP	Control	28	22-1-16	All markers	
				22-1-17	All markers	
				22-1-18	All markers	
		Pre-drug	28	22-1-13	All markers	
				22-1-14	All markers	
				22-1-15	All markers	
		Post-drug	28	21-3-20	All markers	
				22-1-7	All markers	
				22-1-8	All markers	
		Naive		22-1-19	All markers	
				22-1-20	All markers	
				23-1-20	All markers	

This year, our effort has been focused on preparing, staining and imaging slices from naïve and G2 animals, protected ears 28 days survival animals. We used these samples to establish the framework for using neuroinflammation to assess the effect of liraglutide. The following results illustrate some of the final imaged samples that have been collected. Final results are analyzed for Iba1 and GFAP antibodies with n=3 per group.

Based on our results from G1 animals and the preliminary work in G2 animals, the original 8 biomarkers did not lead to meaningful conclusions on the effects of liraglutide or ear protection. As mentioned above, we are now considering neuroinflammation based on: 1) we have established neuroinflammation in the blast TBI model, and 2) liraglutide has known anti-inflammation action on the same mechanisms.

For the study on all animal groups, we first compared naïve and injured groups to establish an expected neuroinflammatory response in chinchilla to assess the efficacy of the treatment groups as shown in **Fig. 15**.

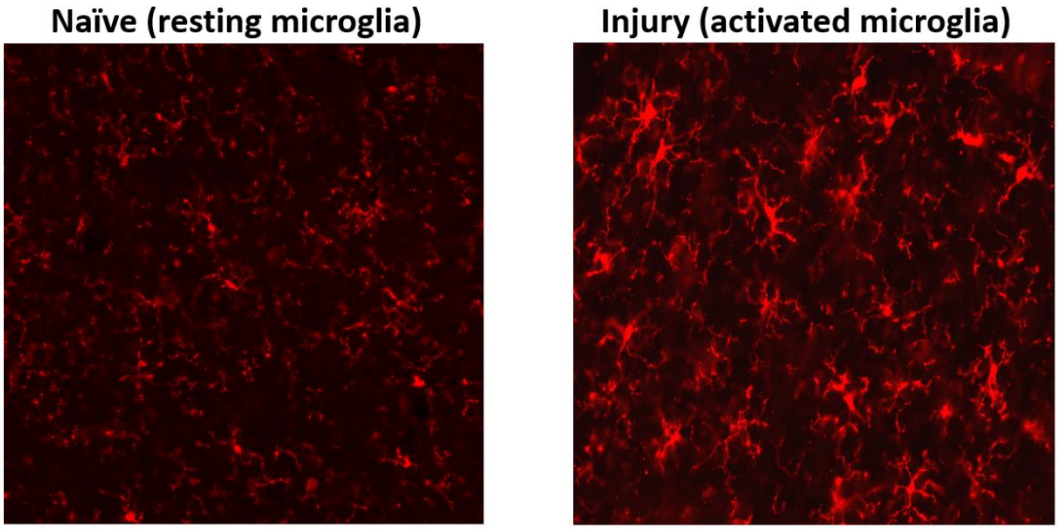

Figure 15. Microglia labeled with the Iba-1 antibody in the cortex of naïve and an injured G2 animal. This demonstrated reactivity of the Iba-1 antibody in chinchilla.

Interestingly, we have also noticed difference in microglia morphology in different region of the brain. In the image below (**Fig. 16**), in the hippocampus (HC) region (CA1) the structure of microglia is more define compared to the AC or IC region. These are representative images from G2 protected ear injured 14 days survival animals.

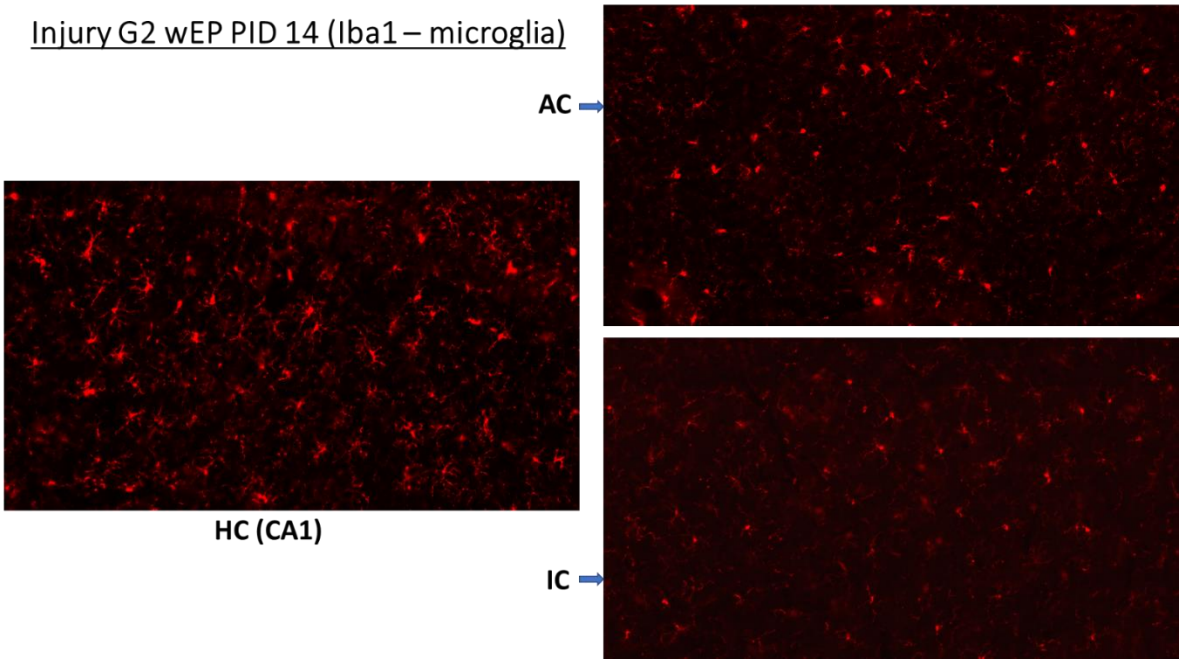

Figure 16. The microglia morphology in different region of the brain: the hippocampus (HC) region (CA1) and the AC or IC region.

The image below (**Fig. 17**) demonstrates positive staining of astrocytes in injured G2 animals, protected ear, 14-day survival. Left image showed AC of injured animal and right image is of post-treated animal.

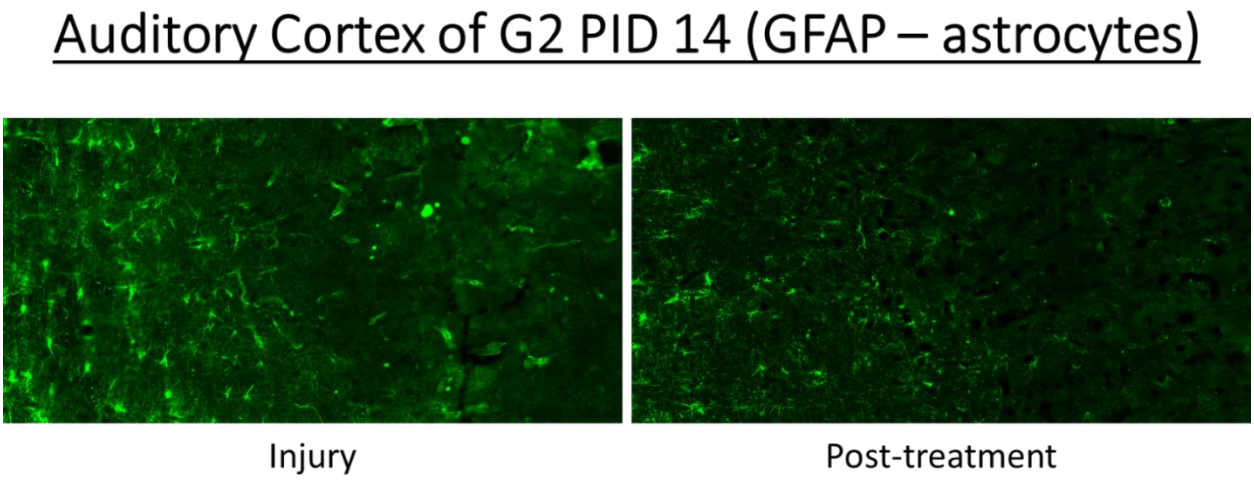

Figure 17. Positive staining of astrocytes in injured G2 animals and protected ear over 14-day survival.

Protected Ear Group Analysis:

♦ **Total number of microglia in AC**

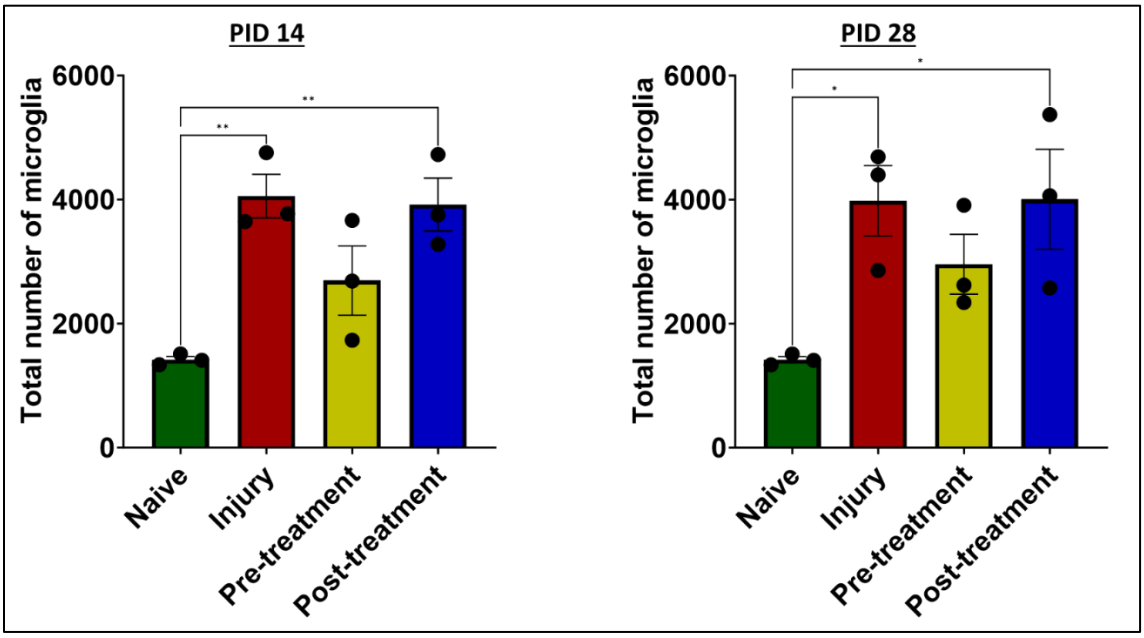

Figure 18. The total number of microglia in AC at PID 14 (left image) and PID 28 (right image).

Following injury, the total number of microglia increase. Here, we observe a significant increase in injured group when compared with naïve group. This number reduces upon pre-treatment with

liraglutide for both 14-days and 28-days survival groups. This data shows that the G2 injury paradigm induces neuroinflammation and suggests that pre-injury treatment with liraglutide can reduces inflammation below injury controls.

♦ **Mean number of branches/microglia in AC**

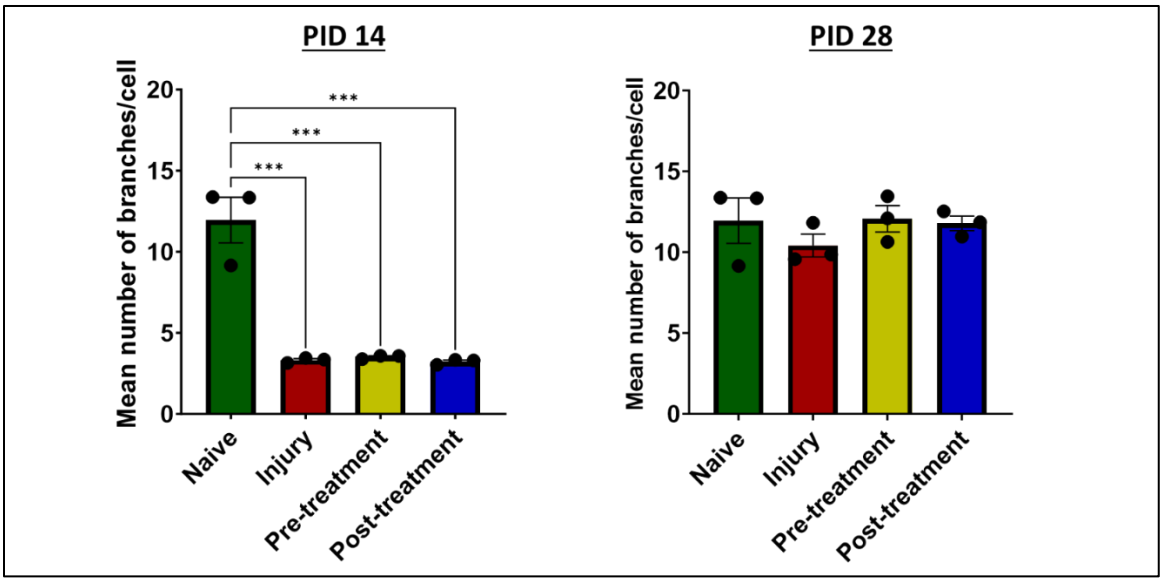

Figure 19. The mean number of branches per microglia in AC at PID14 (left image) and PID 28 (right image).

The data suggest the morphology of microglia using skeletal analysis (the number of branches per microglia) recovers to naïve controls over time with little effect of liraglutide both pre- and post-treatment when compared with injury groups in AC region.

♦ **Mean number of junctions/microglia in AC**

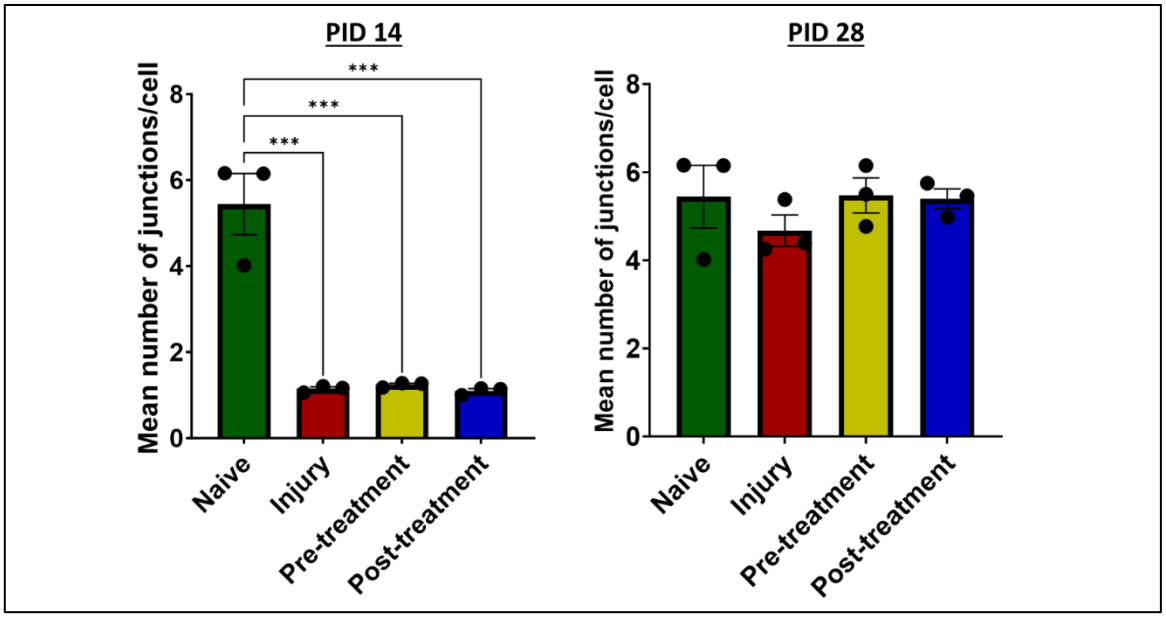

Figure 20. The mean number of junctions per microglia in AC at PID14 (left image) and PID 28 (right image).

The data suggest the morphology of microglia using skeletal analysis (mean number of junctions per microglia) recovers to naïve controls over time with little effect of liraglutide both pre- and post-treatment when compared with injury groups in AC region.

<u>Open Ear Group Analysis (PID 28 only)</u>:

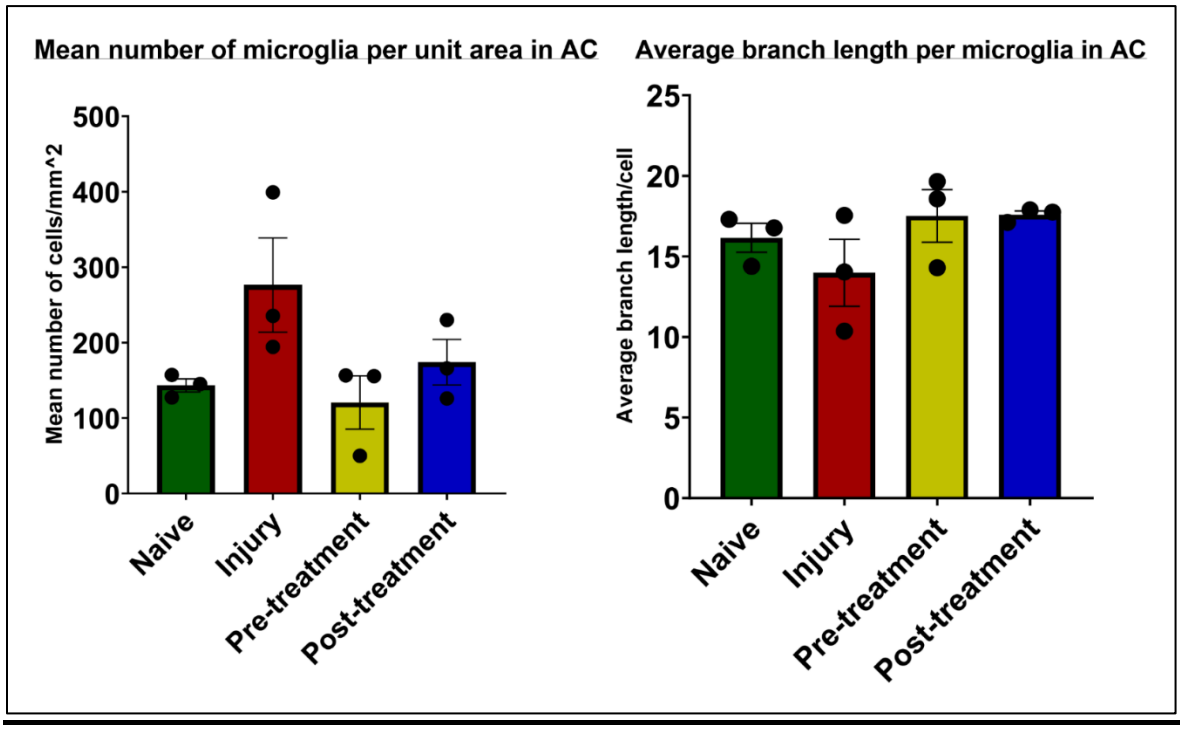

Figure 21. The total number of microglia in AC at PID 28.

Following injury, the total number of microglia (left image) increase. Here, we observe an increasing trend in injured group when compared with naïve group. This number reduces upon pre-treatment with liraglutide. Following injury, the average branch length per microglia (right image image) decrease. Here, we observe a decreasing trend in injured group when compared with naïve group which increases upon both pre- and post-treatment with liraglutide.

Protected Ear Group Analysis:

♦ **Total number of astrocytes in AC**

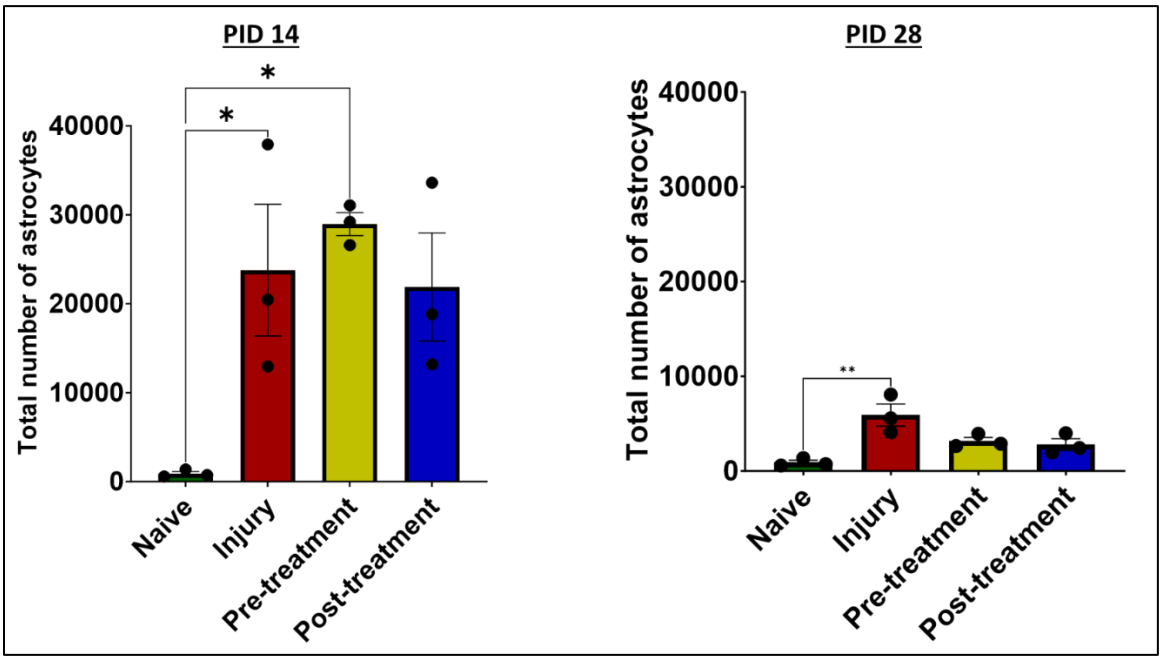

Figure 22. The total number of astrocytes in AC at PID 14 (left image) and PID 28 (right image).

Here, we observe significant increase in injured group when compared with naïve group. This number reduces upon pre- as well as post-treatment with liraglutide for 28-days survival groups suggesting a long-term effect of liraglutide treatment on astrogliosis.

♦ **Average branch length/astrocyte in AC**

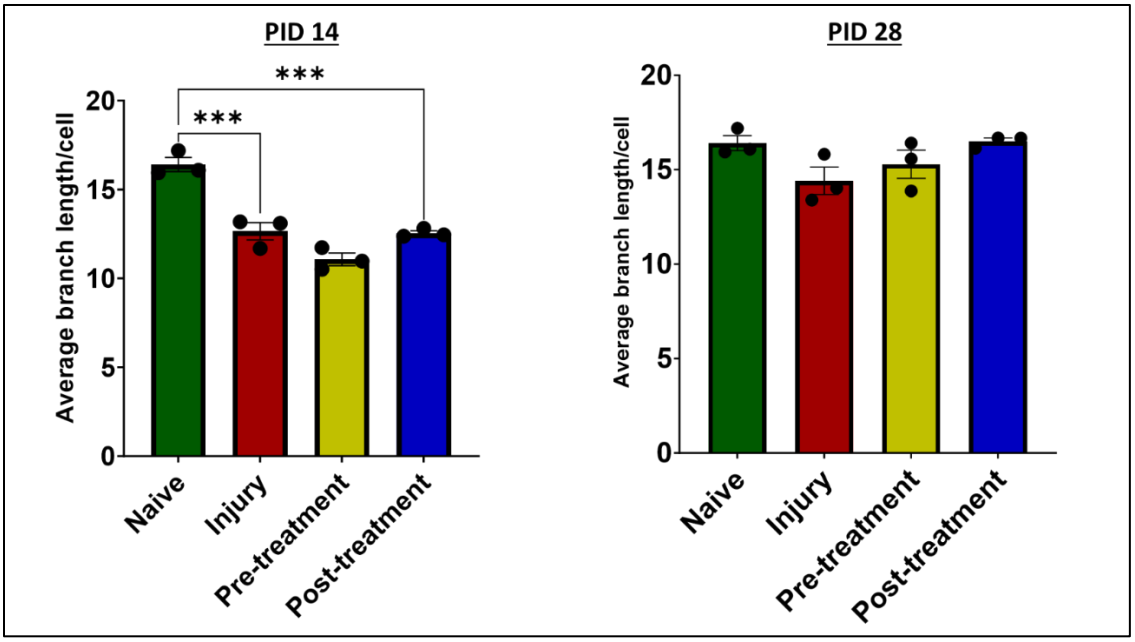

Figure 23. The average branch length per astrocyte in AC at PID 14 (left image) and PID 28 (right image).

The data suggests the branch length morphology of astrocytes using skeletal analysis recovers to naïve controls over time with little effect of liraglutide both pre- and post-treatment when compared with injury groups in AC region.

<u>Open Ear Group Analysis (PID 28 only)</u>:

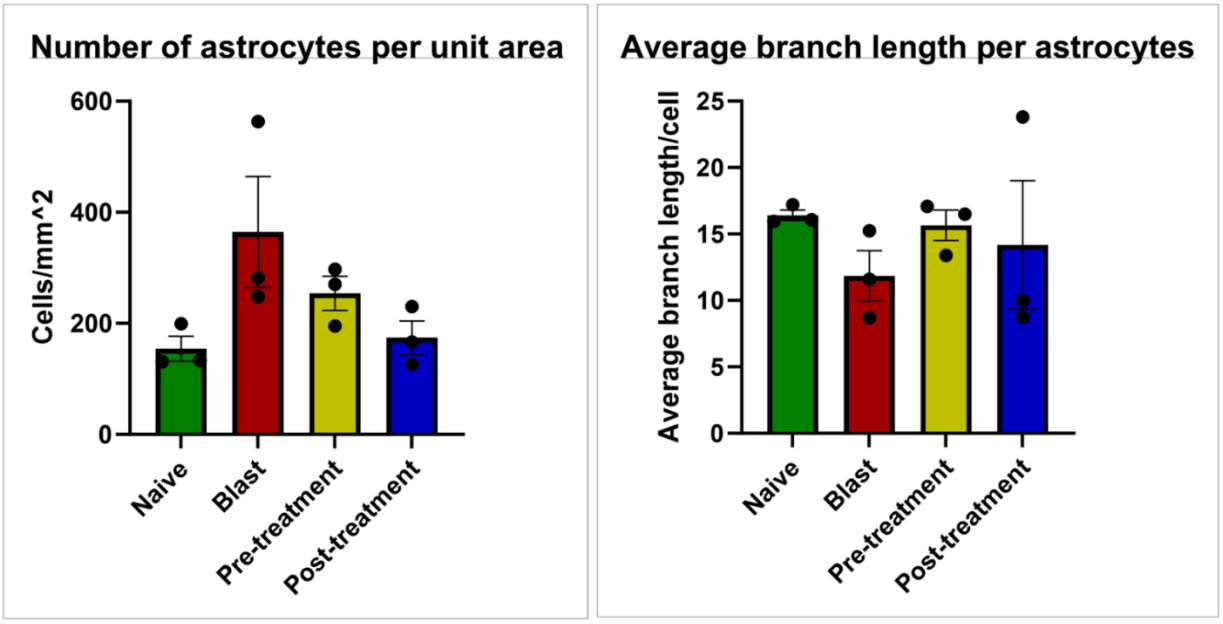

Figure 24. The number of astrocytes in AC at PID 28.

Following injury, the total number of astrocytes (left image) increase. Here, we observe an increasing trend in injured group when compared with naïve group. This number reduces upon

pre-treatment with liraglutide. Following injury, the average branch length per astrocyte (right image image) decrease. Here, we observe a decreasing trend in injured group when compared with naïve group which increases upon both pre- and post-treatment with liraglutide. Here there was an overall stronger effect of post treatment on astrogliosis.

***Interpretations of the results*:**

In this report, we were able to answer the following questions for G2 protected ear groups.

Does G2 injury cause neuroinflammation?
Data strongly shows that there is microglial activation and astrogliosis associated with G2 blasts when compared with naïve animals in AC region, thus supporting our hypothesis of inflammation post injury. In this report we have quantified this activation using ImageJ software. Total number of cells within the ROI was counted. It was observed that higher number of cells in injured group when compared the naïve group, at both PID 14 and PID 28 in AC region.

Does liraglutide reduce inflammation?
Liraglutide effect on neuroinflammation appears to be strongest with post treatment. Its effects were found to reduce the number of microglia and astrocytes in the AC region. Post treatment of liraglutide also appeared to have a long-term effect on microglia morphology and astrogliosis morphology. However, recovery time was a bigger factor in recovery of microglia and astrocyte morphology compared to liraglutide treatment.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report

- **How were the results disseminated to communities of interest?**

Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**

Under Aim 1, following the completion of animal experiments on effects of liraglutide on mitigation of hearing damage in drug treatment and blast control experimental groups under repeated blast exposures at G1 and G2 BOP levels with or without HPDs and over different recovery time course, we will complete statistical analyses of all the data collected from the experiments. One or two journal papers for summery of the animal studies under repeated blast exposures on Day 1 are expected to publish in the following year.

Under Aim 1, we will continue the study on therapeutic function of liraglutide in chinchillas with ears protected by HPDs under 3 additional blasts at G2 BOP level on Day 4 over recovery time of 28 days. We expect to establish an animal model with more severe blast-induced sensorineural hearing loss to investigate the long-term effects of liraglutide treatment in chinchillas with HPDs.

One or two journal papers for this animal studies under repeated blast exposures in addition to Day 1 are expected to publish in the following year.

Under Aim 2, we will continue working with G2 animals with new protocol to answer biochemically significant questions. Along with the mentioned markers above, we will also investigate inflammatory pathways in G2 animals. We plan to publish one or two journal papers about the outcomes from Aim 2 in the following year.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

The impact of ear protection on the beneficial effects of liraglutide in mitigating the central auditory damage following repetitive exposure to the low and high BOP levels were investigated in: 1) the hearing function recovery after blasts through ABR threshold, ABR wave 1 amplitude and latency, DPOAE, and MLR amplitude and latency measurements; 2) the biochemical difference between the drug treatment and blast injury without treatment chinchillas based on the levels of excitatory/inhibitory neurotransmitters, changes in synaptic plasticity, oxidative stress, and neuro-regeneration.

The accomplishments in this year have great impact to understanding the therapeutic efficacy of liraglutide in animal model of chinchillas repetitively exposed to high BOP (**G2)** level (15-25 psi) or mild TBI with and without hearing protection (earplugs). One of the key findings from chinchillas with earplugs was the pre-blast administration of liraglutide reduced the severity of blast-induced acute hearing damage, particularly at the middle and high frequencies (e.g., 4 and 8 kHz). In open ears, the difference between the blast control and drug-treated chinchillas was only statistically significant on Day 7 and Day 14 at 4 kHz, which potentially suggested that the liraglutide treatment facilitated the post-blast restoration of the hearing function. Our studies indicated that the efficacy of liraglutide mitigating blast-induced auditory injuries varied with blast intensity, drug administration time, peripheral auditory system condition, and hearing frequency.

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

No significant changes in approach.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

No significant problems and delays.
In NJIT lab, the new microscope is up and running. In the meantime, we are looking at additional methods for analyzing.

- **Changes that had a significant impact on expenditures**

No changes in expenditures.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

No significant changes in use and care of chinchillas. We did face the lack of qualified chinchilla suppliers in the past few years. However, with continuous support from the VPRP office of OU Norman campus and the Department of Comparative Medicine at OU Health Sciences Center, the new chinchilla supplier has been identified in 2022 and our animal study has been carried on smoothly.

6. PRODUCTS

• publications, conference papers, and presentations;
• website(s) or other Internet site(s);
• technologies or techniques;
• inventions, patent applications, and/or licenses; and
• other products.

- **Publications, conference papers, and presentations**

Publications – Journal papers:

1. Jiang, S., Sanders, S., and **Gan, R. Z.** Mitigation of hearing damage with liraglutide treatment in chinchillas after repeated blast exposures at mild-TBI. *Military Medicine* (In Press).

2. Jiang, S., Sanders, S., and **Gan, R. Z.** Hearing protection and damage mitigation in chinchillas exposed to repeated low-intensity blasts. *Hearing Research*, Vol. 429: 108703 (pp. 1-10), 2023. https://doi.org/10.1016/j.heares.2023.108703

3. Jiang, S., Welch, P., Sanders, S., and **Gan, R. Z.** Mitigation of hearing damage after repeated blast exposures in animal model of chinchilla. *J. Association for Research in Otolaryngology (JARO)*, Vol. 23: 603-616, 2022. DOI: 10.1007/s10162-022-00862-2

4. Jiang, S., Sanders, S., and **Gan, R. Z.** Potential therapeutic function of liraglutide for mitigating auditory injuries induced by high-intensity blasts in animal model of chinchilla. *Hearing Research* (Under preparation).

5. Jiang, S., Sanders, S., and **Gan, R. Z.** Therapeutic Function of Liraglutide for Mitigation of Blast-induced Hearing Damage – An Initial Investigation in Animal Model of Chinchilla. *Military Medicine* (Under preparation).

Publications – Conference papers:

1. **Gan, R. Z.**, Sanders, S., Welch, P., and Jiang, S. Therapeutic function of liraglutide for mitigation of blast-induced hearing damage – An initial investigation in animal model of chinchilla. *DoD 2023 Military Health System Research Symposium (MHSRS)*, Kissimmee, FL, August 14-17, 2023.

2. Das, T., Pfister, B., Chandra, N., Jiang, S., Sanders, S., and **Gan, R. Z.** The protective effects of Liraglutide on auditory injury after repeated mild blast TBI in chinchilla. *DoD 2023 Military Health System Research Symposium (MHSRS)*, Kissimmee, FL, August 14-17, 2023.

3. **Gan, R. Z.**, Bradshaw, J., Brown, M., and Jiang, Shangyuan. 3D computational modeling of blast wave transmission in human ear from external ear to cochlear hair cells – A preliminary study. *DoD 2023 Military Health System Research Symposium (MHSRS)*, Kissimmee, FL, 2023.

Publications – Local conference papers:

1. Jiang, S., Sanders, S., Brookes, M., **Gan, R. Z.** Potential therapeutic function of liraglutide for mitigating auditory injury after exposure to high blast overpressure in Chinchillas. *Annual Research Day of Department of Otolaryngology, Head and Neck Surgery at OUHSC,* April 15, 2023.

2. Bradshaw, J., Brown, M., Wang, X., and **Gan, R. Z.** 3D finite element model of cochlear organ of Corti for predicting hair cell response to blast overpressure. *Annual Research Day of Department of Otolaryngology, Head and Neck Surgery at OUHSC,* April 15, 2023.

3. Patel, H., Jiang, S., Brown, M., and **Gan, R. Z.** Function measurement of nanoporous aerogel earplug with acoustic testing fixture (ATF). *Annual Research Day of Department of Otolaryngology, Head and Neck Surgery at OUHSC,* April 15, 2023.

Seminar Presentations:

N/A

Books or other non-periodical, one-time publications:

N/A

- **Technologies or techniques:**

1) Staining for the chosen biomarkers
2) Digitizing the stained slices
3) Analyzing the collected data for cell counts and morphological changes

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Provide the name and identify the role the person played in the project. Indicate the nearest whole person month (Calendar, Academic, Summer) that the individual worked on the project. Show the most senior role in which the person worked on the project for any significant length of time. For example, if an undergraduate student graduated, entered graduate school, and continued to work on the project, show that person as a graduate student, preferably explaining the change in involvement.
Describe how this person contributed to the project and with what funding support. If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Rong Gan, Ph.D.
Project Role: PI
Researcher Identifier (OU ID): 112129499
Nearest person month worked: 3
Contribution to Project: Dr. Gan has involved in all research activities for the project and coordinated with NJIT on animal brain tissue immunostaining imaging.

Name: Shangyuan Jiang, Ph.D.
Project Role: Postdoc/Research Associate
Researcher Identifier (OU ID): 112979369
Nearest person month worked: 3
Contribution to Project: Dr. Jiang has been involved in all animal experiments in PI's lab at the University of Oklahoma including blast tests, hearing function tests, and chinchilla brain sample preparations.

Name: Jackson Bradshaw
Project Role: Graduate Student
Researcher Identifier (OU ID): 113474543
Nearest person month worked: 2
Contribution to Project: Jackson Bradshaw has participated in animal experiments and recording data for this project.

Name: Tulika Das
Project Role: Graduate Student
Researcher Identifier (NJIT ID): 0000-0002-9916-8502
Nearest person month worked: 3

Contribution to Project: Tulika Das has been working towards meeting the goals of the project.

Name: Aakaash Gosain
Project Role: Graduate Student
Researcher Identifier (NJIT ID): N/A
Nearest person month worked: 3
Contribution to Project: Aakaash Gosain has been working towards meeting the goals of the project.

Name: Bryan Pfister
Project Role: Professor
Researcher Identifier (NJIT ID): N/A
Nearest person month worked: 0.5
Contribution to Project: Dr. Pfister has overseen the research activities in the lab at NJIT.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

- **What other organizations were involved as partners?**

Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: The Quad Chart (available on https://www.usamraa.army.mil) shall be updated and submitted as an appendix.

A Quad Chart is submitted as an appendix.

8. APPENDICES

- **Quad Chart**
-

Therapeutic Function of Glucagon-Like Peptide-1 (GLP-1) for Hearing Restoration after Blast Exposure or Traumatic Brain Injury (TBI)

ERMS# RH180040 Award Number: W81XWH-19-1-0469

PI: Rong Z. Gan, Ph.D. **Org: University of Oklahoma** **Award Amount:** $1,290,428

Specific Aims: 1) Identify the therapeutics of GLP-1R (Liraglutide) in ameliorating auditory function injuries in pre- and post-treatments in relation to blast overpressure (BOP) level or TBI severity over the time course. **2)** Investigate the beneficial effects of liraglutide on the mitigation of the central auditory damage following repetitive exposures to the low BOP or mild TBI (mTBI) pressure levels.

Hypothesis: Liraglutide treatment will reduce hearing damage severity in blast animal model of chinchilla by modulating the excitatory and inhibitory neurotransmitter responses in the central auditory system, improving synaptic integrity, and preventing oxidative stress-induced neuronal loss.

Objectives: To investigate the potential therapeutic function of GLP-1R agonists, Liraglutide, to mitigate the auditory injury after blast exposure in animal model of chinchilla. The ability of liraglutide to offer protection, stabilization, and regeneration of neurons and synapses located along the entire auditory pathway will be investigated in chinchillas exposed to blast injury.

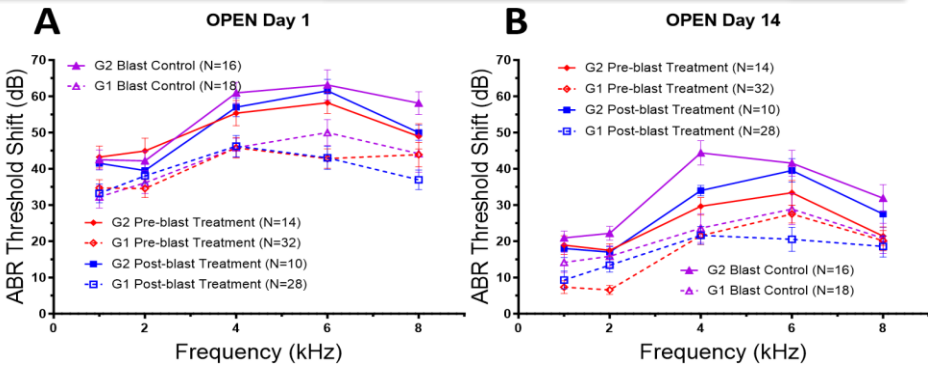

Figure. Comparison of ABR threshold shifts or elevations (mean ± SEM) measured on Day 1 **(A)** and Day 14 **(B)** in 6 groups of chinchillas receiving 3 blast exposures on Day 1 and being recovered for 14 days. There were two blast BOP levels: G1 of 3-5 psi (dashed lines) and G2 of 15-25 psi (solid lines) and animals were tested under 3 liraglutide (drug) treatment conditions: pre-blast drug treatment (**red** lines), post-blast drug treatment (**blue** lines), and blast control without drug treatment (**purple** lines).

Timeline and Cost

Activities / CY	19	20	21	22
Tasks 1-1 and 1-2 (low BOP level)				
Task 1-3 (high BOP level or mTBI)				
Tasks 2-1/2-2 (neurotransmitter)				
Tasks 2-3/2-4 (signal pathways)				
Estimated Direct Cost ($K)	**$333**	**$333**	**$334**	

Updated: October 30, 2023

Goals/Milestones

CY19 Goal – Aim 1:Task 1-1 and Aim 2: Tasks 2-1 and 2-2
☑ Hearing function tests and brain section imaging

CY20 Goal – Aim 1:Task 1-2 and Aim 2: Tasks 2-1 and 2-2
☐Investigate the effect of hearing protection on liraglutide's efficacy
☐Complete the tests at low BOP level

CY21 Goal – Aim 1: Task 1-3 and Aim 2: Tasks 2-1 and 2-2
☐ Hearing function tests and brain section imaging to demonstrate the effect of high BOP or mTBI on liraglutide's efficacy

CY22 Goal – Aim 2: Tasks 2-3 and 2-4
☐ Mechanisms of liraglutide's neuroprotection and neuro-regeneration functions

Approach: ● Conducting blast tests in chinchillas with drug treatment at two BOP levels with or without hearing protection and performing hearing function tests over the time course to determine liraglutide's therapeutics; ● Immunostaining of brain sections for fluorescence imaging to determine the effect of liraglutide on synaptic protein changes, neuroprotection, and neuro-regeneration.