ABSTRACT

ASSESSING NEUROBEHAVIORAL DEFICITS AND NEUROINFLAMMATION FOLLOWING rLLB IN A TRANSGENIC MOUSE MODEL

by

Aakaash Gosain

As global conflicts are escalating, military personnel have long faced the constant threat of explosions, heightening the risk of blast-induced traumatic brain injury (bTBI). The recent increase in war conflicts and combat zones worldwide not only puts the military, service members, and police forces at risk but also innocent civilians who are caught in the crossfire. While moderate to severe bTBIs have been extensively studied, the pathology of repeated low-level blasts (rLLB) remains less understood. This research addresses the gap in knowledge regarding the long-term consequences of rLLB injuries in the neural anatomy. A transgenic mouse model was subjected to 70 kPa x 3 blasts at 1-minute intervals. Subsequent neurobehavioral tests, including elevated plus maze, open field test, novel object recognition, and sucrose splash test, were conducted at different post-injury time points to assess changes in behaviors of memory, anxiety, and depression. Biochemical analyses involve immunohistochemistry to assess hippocampal microglial activation. Results indicate statistically significant differences between sham and blast groups in neurobehavioral tests, suggesting increased anxiety, short-term memory loss, and depression in the blast group. Biochemical analyses reveal elevated microglial activation, indicating neuroinflammation. This study provides valuable insights into the effects of rLLB in mice, and future research could investigate chronic neuroinflammation, neurodegeneration, and blood-brain barrier (BBB) breakdown to further current findings.

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APPROVAL PAGE

ASSESSING NEUROBEHAVIORAL DEFICITS AND NEUROINFLAMMATION FOLLOWING rLLB IN A TRANSGENIC MOUSE MODEL

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“Be the change you wish to see in the world”
Gandhi

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CHAPTER 1

INTRODUCTION

1.1 Repeated Low-Level Blasts (rLLB)

Military personnel, service members, and even civilians are known to suffer from moderate to severe traumatic brain injuries (TBI) specifically caused by blast explosions resulting in blast-induced traumatic brain injuries (bTBI) (Trotter et al., 2015). Traumatic brain injury (TBI) can be caused by a direct insult to the head or body which can result in any type of neuropathological changes or injury (McKee & Daneshvar, 2015). bTBI is a particular type of TBI that is caused by pressurized shockwaves being emitted from the site of an explosion. While moderate to severe bTBIs have been extensively studied, repeated low-level blasts (rLLB) are a facet of blast injuries that require further in-depth investigation. To determine the severity of a blast a key component called blast overpressure (BOP) is measured and used to identify the level of blast. It is commonly seen in literature that any BOP value less than 90 kPa is low level or mild, 90 kPa to 180 kPa is seen as moderate to severe and any BOP exceeding 180 kPa has been deemed lethal (Mishra et al., 2016). Singular moderate to severe blasts have been rigorously studied in murine models and have undoubtedly shown biochemical and neurobehavioral changes such as oxidative stress, neuroinflammation, neural degeneration, breakdown of the blood-brain barrier (BBB), and increased signs of anxiety, depression, and memory (Ravula, Rodriguez, et al., 2022). Although there is a clear understanding of the harmful effects of high-level BOP blasts, there is a gap in knowledge regarding the pathophysiology of rLLB and the long-term effects they may cause in the neural environment. Moderate to severe blasts could be directly related to explosions from bombs or improvised explosive devices (IEDs) that

soldiers may experience while in combat. However, low-level blasts are more correlated with any artillery or firearms that soldiers use during training and war conflicts (Belding et al., 2021). During explosions, pressurized blast waves are emitted from the weapon and travel through the brain. In a repeated fashion over an elongated period, there has been anecdotal evidence to suggest that there may be chronic complications that occur from experiencing multiple explosions and suggests further investigation (Elder & Cristian, 2009). It has been shown that blasts of varying BOPs (mild to lethal) caused by explosions may cause complications in various organs such as the brain, lungs, and ears (Kocsis & Tessler, 2009). Between 2001 and 2018, the Defense and Veterans Brain Injury Center (DVBIC) reported a total of 383,947 individuals who have experienced or are currently enduring TBI, and notably over one-third of these cases resulted from exposure to various forms of blast events.

1.2 Pathophysiology of bTBI

In order to replicate and investigate bTBI injuries soldiers may face, animal models using rodents, such as rats and mice have been commonly used to assess biochemical and behavioral changes. These models are induced by explosions varying from BOPs of 27 kPa to 145 kPa (Skotak et al., 2019). Furthermore, recent investigations in the rLLB injury model have shown clear evidence of neurobehavioral deficits through behavior assessment performance and a plethora of biochemical changes such as neurodegeneration, neurotoxicity, inflammation, and curbed neural development, growth, and survival (Ravula, Das, et al., 2022). One such comprehensive study has shown that bTBI may cause the blood-brain barrier (BBB) to breakdown, resulting in an inflammatory response at the

site of injury. This rupturing of the barrier allows cells from the peripheral system to infiltrate the brain which can result in neural degeneration if these cells are present for extended periods. Neutrophils and other inflammatory markers enter the brain environment immediately following blast injury while monocytes and macrophages are present within days post-injury (Chodobski). When monocytes infiltrate the BBB, they are attracted to chemokines released by microglia and other glial cells. As glial cells are native to the neural environment, the blast causes an imbalance in the brain which results in the release of chemokines. As the monocytes are present inside the BBB, they activate to become macrophages. Simultaneously, microglia shift from a resting state to an activated state and take on a phagocytic nature. This causes the production of inflammatory cytokines, such as IL-1B, resulting in chronic neuroinflammation. Chronic neuroinflammation leads to the loss of neurons or cell death, eventually causing behavioral deficits in the organism (Murugan et al., 2020).

1.3 Gaps in Knowledge

Currently, there are limited research studies that are involved in simulating rLLB injuries in vitro or in vivo settings. Further research needs to be conducted to characterize and identify the exact effects that are caused by the rLLB injury model. Current research lacks fully comprehensive studies assessing behavioral and biological deficits that occur following rLLB. Many studies on rLLB typically employ an injury model involving one blast per day over several days. However, there exists a research gap concerning the effects of multiple blasts occurring within the same day, with intervals ranging from seconds to minutes or hours which is more in line with what a service member may face when being

exposed to multiple consecutive low-level blasts (Ravula, Das, et al., 2022). Moreover, comparisons of acute and chronic time periods post-injury need to be investigated further in order to gain a thorough understanding of the pathology of the injury and how it affects its victims following exposure (Fehily & Fitzgerald, 2017). The nuance to this type of trauma is that there are no immediate noticeable changes in behavior, however, performance decreases over time leading to long-term deficits.

1.4 Literature Search

The Center for Injury Biomechanics, Materials, and Medicine (CIBM3) have conducted a variety of research in characterizing blast waves, assessing changes in rodents, and potential therapeutic molecules to treat bTBI. A review determined that in the bTBI model assessing microglial response is imperative in determining the severity of the injury and BOP intensity used when inducing the injury (Younger et al., 2019).

In the lab, rLLB has been tested on a variety of rodent species spanning from mice, rats, chinchillas, and ferrets. Through immunoassays such as IHC, Western Blotting, and ELISA there is evidence that suggests acute and chronic microglial activation occurring in the brains of the injured rodents. Furthermore, in conjunction with biochemical testing, behavioral deficits have been also recorded through various testing protocols. Performances seen by the subjects worsen as time progresses following injury (Skotak and Ravula).

1.5 Research Objectives

This study aims to delve deeper into investigating the chronic effects of rLLB in the mouse animal model. A double-transgenic CX3CR1GFP/+:CCR2RFP/+ mouse model was used where the resident microglia were labeled with GFP and peripheral monocytes were labeled with RFP. The usage of transgenic mice allows for a higher level of accuracy in assessing microglial activation through IHC.

Repeated low-level blast injury causes neurobehavioral deficits due to chronic neuroinflammation following blast injury. We hypothesize that subjecting mice to 70 kPa x 3 blasts at 1 min intervals will result in (1) chronic neurobehavioral deficits and (2) neuroinflammation through microglial activation.

To assess chronic neurobehavioral deficits animals were subject to open field test, elevated plus maze, novel object recognition test, and sucrose splash test. These tests were implemented to verify behavioral deficits in anxiety, memory, and depression respectively. It is expected to see a decline in performance in all behavioral tests that the rodents experience.

To assess neuroinflammation mice brains were cryosectioned, mounted on glass slides, stained for microglia, and fluorescently imaged. All samples were quantified for microglia expression through skeleton analysis using ImageJ and by manual counting of the hippocampus and dentate gyrus brain regions.

CHAPTER 2

MATERIALS & METHODS

2.1 Animal Model

Double-transgenic CX3CR1GFP/+:CCR2RFP/+ mouse model was used where the resident microglia were labeled with GFP and peripheral monocytes were labeled with RFP which were used for immunohistochemistry (IHC) applications. The animals were all housed under identical conditions at a temperature of 22*C, humidity of 40%, and a 12-hour light/dark cycle. All animals were always provided with food and water and all protocols were conducted as per the Guide for the Care and Use of Laboratory Animals and were approved by Rutgers University Institutional Animal Care and Use Committee. Mice were divided into two groups: sham and blast, and 3 cohorts of animals were used for behavioral and biochemical investigation. For behavioral testing, one cohort of 10 sham and 11 blast animals were used and sacrificed PIW 4. For biochemical testing two cohorts of animals were used with 3 sham and 4 blast animals – one cohort was sacrificed PID 1 and the other cohort was sacrificed PID 7. Animals were anesthetized in an isoflurane box for ~5 min or until both mice were visibly asleep. Blast mice were blasted in the shock tube chamber while sham mice remained in the isoflurane box until all three blasts had concluded. The shock tube chamber was also supplied with isoflurane to ensure the mice did not wake up during testing. Blast animals were exposed to three 70 kPa blasts with a 1-minute interval in between each blast. Animals were sacrificed 24 hours, 7 days, and 30 days post-injury.

2.2 Injury Model

The shock tube that was used to induce the injuries is from the Center for Injury Biomechanics, Materials, and Medicine Laboratory, Newark NJ (CIBM3). The setup that was used is identical to what Ravula and their team utilized during their testing (Ravula, Rodriguez, et al., 2022). The breach length needed to be adjusted because helium gas was not available at the time of experiments and nitrogen gas was used as a substitute driver gas. A breach length of 17.75 inches was used and the driver gas was Ultra High Purity Grade Nitrogen (AirGas). Due to changes in driver gas and breach length, one 1mm thick and two 0.2mm thick mylar membranes were used to reach the desired BOP. BOPs ranged from 68 kPa to 72 kPa while testing occurred.

2.3 Neurobehavioral Studies

Sham and blast animals were subject to elevated plus maze (EPM), open field test (OFT), novel object recognition (NOR), and sucrose splash test (SST). All tests were conducted at PIW 1 and PIW 4 except for the sucrose splash test (SST) which was assessed at PIW 2. All animals were blindly handled when conducting behavioral and biochemical testing to mitigate any bias and all testing sites were sanitized with 70% ethanol in between each trial.

2.4 Elevated Plus Maze (EPM)

This test assessed anxiety levels in the animal subjects 2 days and 30 days post-injury. The testing apparatus is plus-shaped and made up of four arms with two open arms and two closed arms (Santos-Sánchez et al., 2022). The apparatus stands at 35 cm above the ground with 35 cm arms measuring from the center to the end of the arm. All animals were placed

at the center of the plus facing the open arms and were recorded for 5 mins through a camera hanging from the ceiling. Each test was recorded for time spent in open-arm vs closed-arm and analyzed through ANYMaze software. Animals that spent more time in the closed arms suggest having higher levels of anxiety than the animals that spent equal or more time in the open arms.

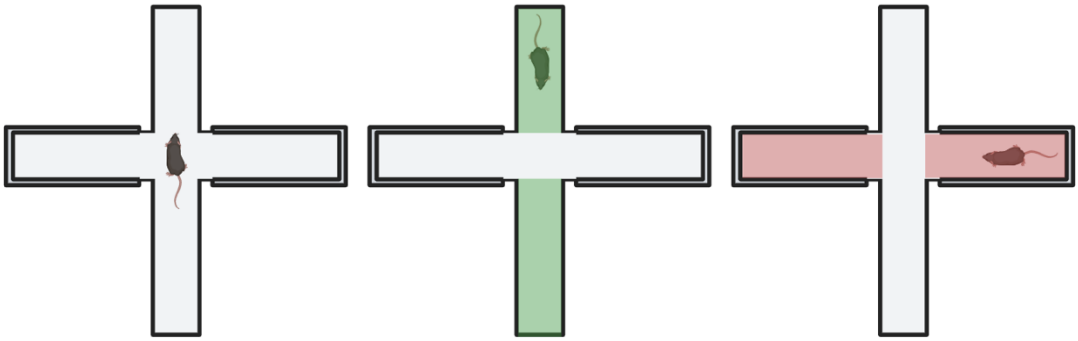

Figure 2.1 Diagram of the Elevated Plus Maze testing apparatus

Source: BioRender

2.5 Open Field Test (OFT)

This was an additional anxiety assessment given to the subjects. The testing arena was a 30cm x 30cm open square box with 30 cm walls enclosing the arena. Animals were placed directly in the center of the box and recorded for 5 min by a ceiling camera. ANYMaze recorded time spent in the center of the arena and edges of the arena. Animals that spent more time by the edges of the arena exhibited higher levels of anxiety as they were more comfortable in enclosed spaces when being placed in foreign environments and were too anxious to explore the center.

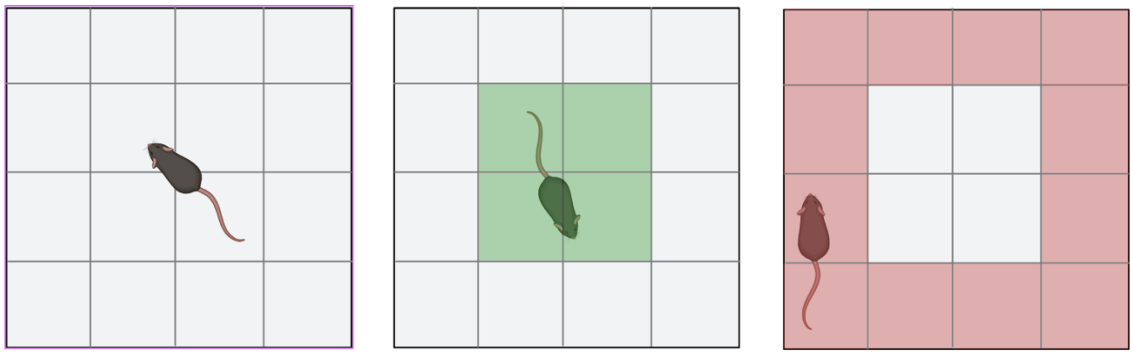

Figure 2.2 Diagram of the Open Field Test arena

Source: BioRender

2.6 Novel Object Recognition Test (NOR)

This test was implemented to assess short-term memory for the animals and was used 1 week and 4 weeks post-injury. NOR has three phases: habituation, familiarization, and testing (Kim et al., 2019). The same arena used during the OFT was used for NOR. The habituation phase occurred 24h before testing and the mouse was able to get acclimatized to the environment for 5 min. The familiarization occurred the next day with two identical objects placed in the arena and the subject was allowed to explore for 5 min. After 1h the testing phase commenced, and the animal was placed back into the arena with the same object from the familiarization phase and one object for 5 min. If the subject spent more time with the novel object than the familiar object, it can be concluded that the animal remembered the familiar object and was more interested in interacting with the novel object. If the animal's short-term memory function has been altered, then the animal would have an equal probability to interact with either object therefore not exhibiting a difference in interaction time between objects. The time spent by each object was recorded and a discrimination index was calculated. The discrimination index is a ratio of the difference in time spent interacting with each object and is calculated with the time spent with the

familiar object (tFamiliar), time spent with the novel object (tNovel), and total time spent interacting with the objects (tFamiliar + tNovel). The discrimination index calculation is as follows: DI = [(tNovel) / (tNovel + tFamiliar)]

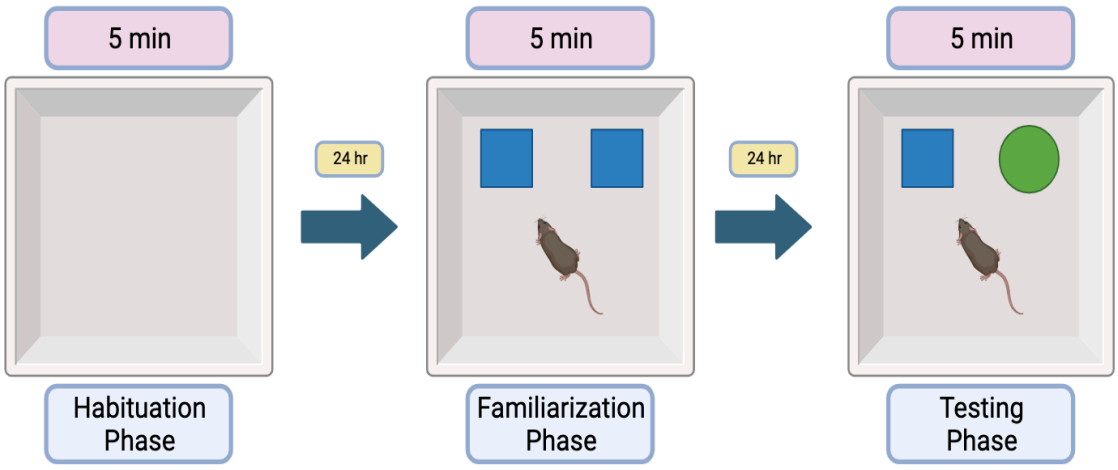

Figure 2.3 Flow chart of the Novel Object Recognition test and its phases (blue square: familiar object and green circle: novel object)

Source: BioRender

2.7 Sucrose Splash Test (SST)

This test indicates depression in animals and was conducted 14 days post-injury. Mice were splashed with a 10% sucrose solution through a spray bottle and were then placed in the testing arena for 5 min (Várkonyi et al., 2022). Their activity was recorded through the ANYMaze software, and the tester would monitor the behavior during the trial. When the animals would groom themselves by licking their fur or rubbing their head with their paws the tester recorded the time spent grooming. Lower grooming times indicate higher levels of depression as motivation for self-maintenance and cleanliness diminishes when depression is present in the animal.

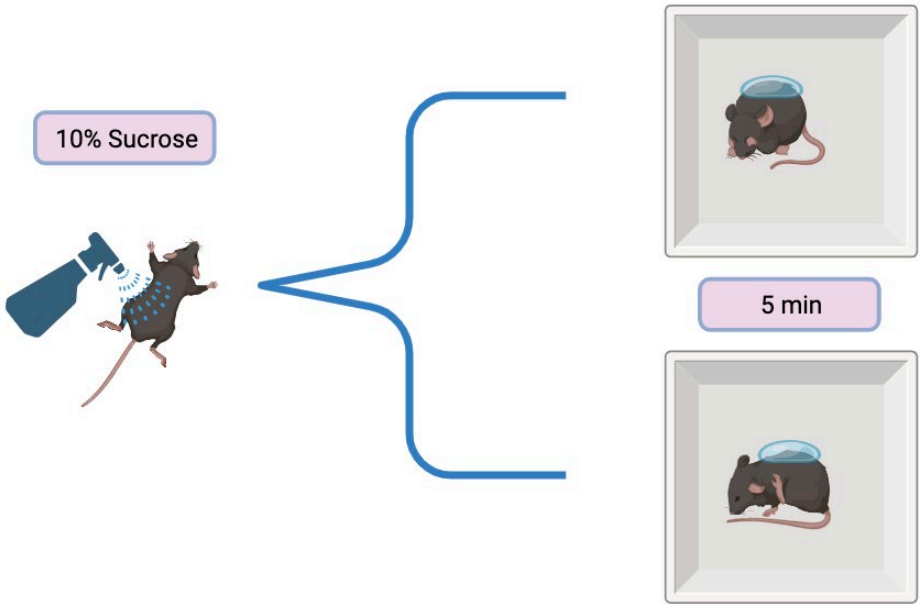

Figure 2.4 Diagram of Sucrose Splash Test

Source: BioRender

2.8 Transcardial Perfusion and Tissue Collection

Animals were anesthetized through intravenous administration of Xylamine (ketamine and xylazine) (10mg/kg) and then perfused with a saline solution followed by 4% paraformaldehyde (PFA). Brains were extracted and preserved in 4% PFA for 24h post-extraction followed by 30% sucrose solution 48h post-extraction. Brains were then frozen into OCT molds and cryosectioned for 20 um sections using Leica cryostat. Slices were preserved in 30% ethylene glycol solution -20*C and then mounted onto glass slides when ready for IHC staining.

2.9 Immunohistochemistry (IHC) and Microscopy

Immunofluorescence was conducted through Iba-1 staining for microglia. Slides were cover slipped with DAPI and then imaged using Leica Fluorescence Microscope.

Transgenic mice samples were stained with Iba-1 to enhance microglial fluorescence when imaging samples and cover slipped with DPX. Manual microglia counts and morphological skeleton analysis occurred through ImageJ.

2.10 Skeleton Analysis and Cell Counting

A skeleton analysis was employed through ImageJ to analyze the morphology of the microglia on the stained slides. The macro used with ImageJ was responsible for subtracting the background and removing particles that were not part of the microglia. Thresholds were set on average values taken from each sample within their respective groups (sham and blast, PID 1 and PID 7). Statistical analysis was conducted on the average branch length per microglia cell and the estimated cell count per unit area was obtained through the skeleton analysis.

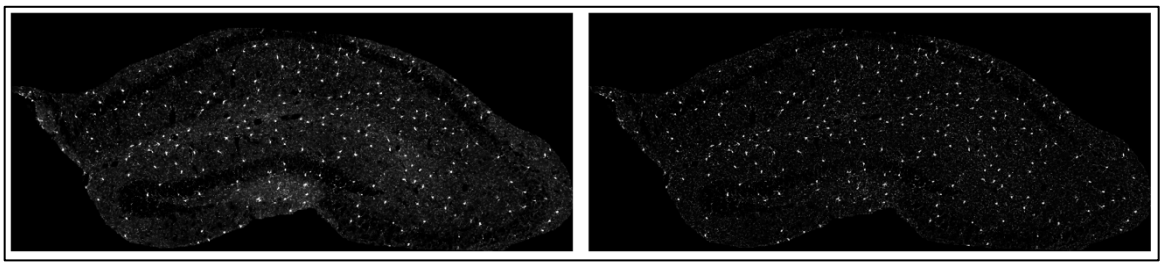

Figure 2.5 Visual comparison of a hippocampus section before (left panel) and after (right panel) of the subtract background function during the skeleton analysis.

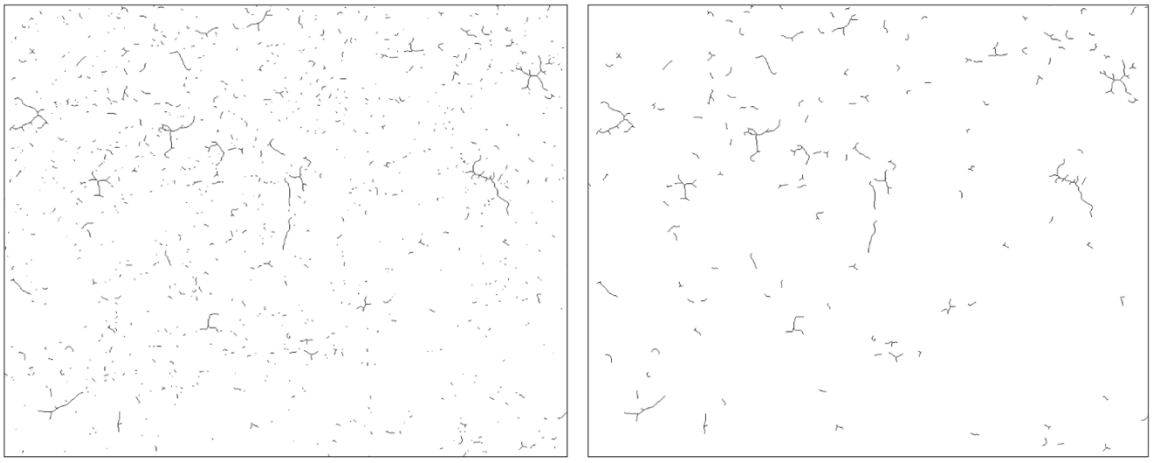

Figure 2.6 Visual comparison of a zoomed-in area of the hippocampus from the previous figure. Before (left panel) and after (right panel) of the particle remover function during the skeleton analysis.

Manual cell counting was also conducted through ImageJ. Regions of interest (ROI) surrounding the hippocampus (HC) and dentate gyrus (DG) brain regions were established and stained activated microglia cells were counted. Manual counts were all standardized by dividing the count by the area of the ROI.

2.11 Statistical Analysis

Statistical analysis on behavioral studies and IHC results were all compiled through GraphPad Prism. All groups were assessed for statistical significance through unpaired student's t-test and graphical visualizations were generated.

CHAPTER 3

RESULTS

3.1 Neurobehavioral Testing

3.1.1 Elevated Plus Maze (EPM)

The analysis of animals' time spent in the open arms during EPM testing revealed that sham animals exhibited greater exploratory behavior than blast animals. The subjects were tested at PIW 1 and PIW 4 to assess injury at acute and chronic time points. The initial comparison involved investigating time spent in the open arm at PIW 1 and PIW 4 in their respective sham and blast groups. Results showed that both groups had similar time spent in the open arms with no noticeable change from PIW 1 to PIW 4. It is important to note that the sham group overall spent more time in the open arms compared to the blast group. Further statistical analysis showed that there was statistical significance when comparing sham and blast animals and time spent in open arms during the same post injury dates. These results suggest that rLLB can induce anxiety-like behaviors immediately and chronically following blast exposure.

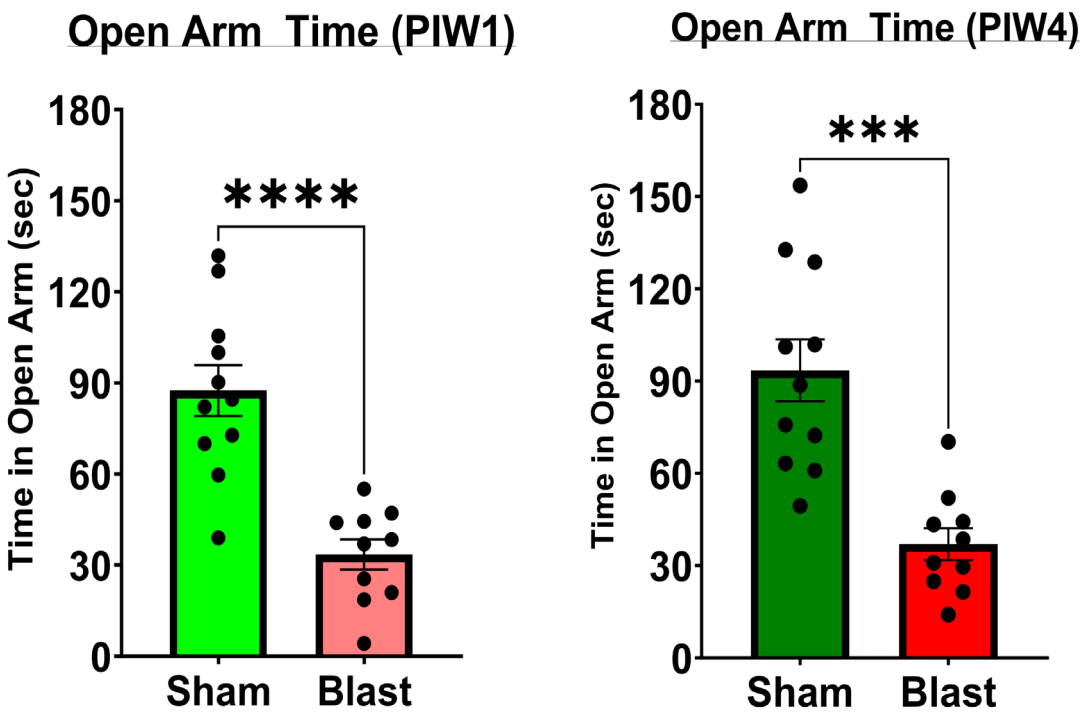

Figure 3.1 Comparison of rLLB-induced anxiety-like behavioral changes in EPM through time. Time spent in the open arms 1 week post-injury (left panel) and time spent in the open arms 4 weeks post-injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

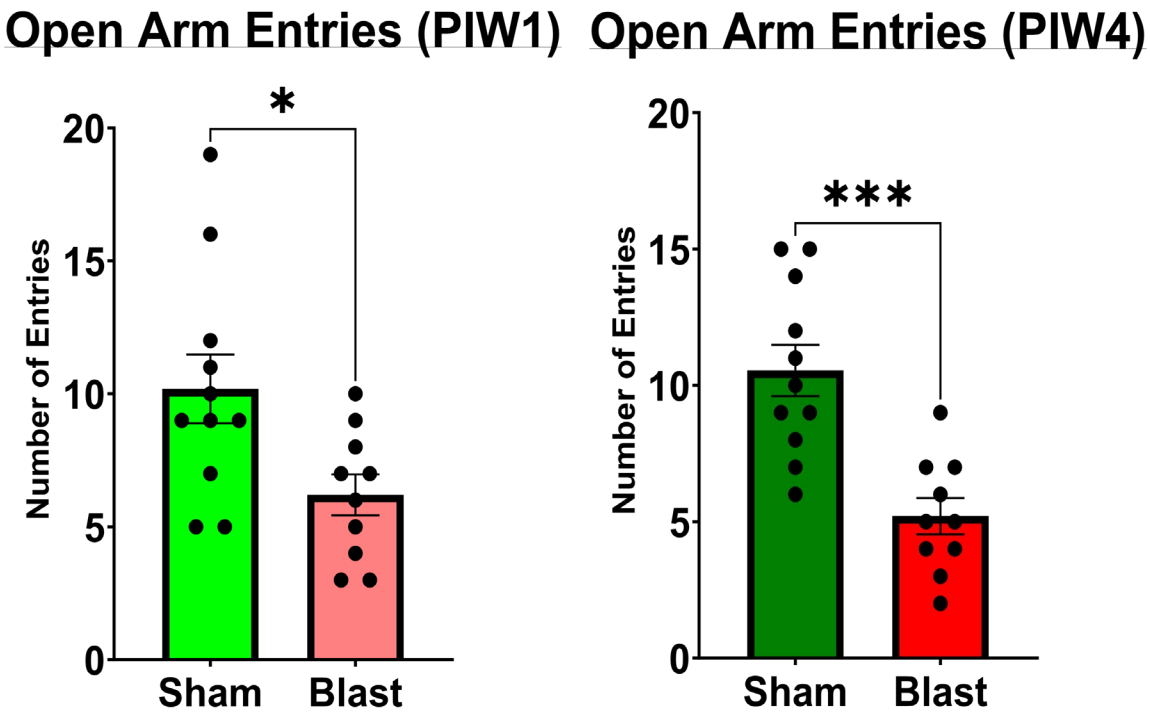

Figure 3.2 Comparison of rLLB-induced anxiety-like behavioral changes in EPM through entries. Entries to the open arm 1 week post-injury (left panel) and entries to the open arm 4 weeks post-injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

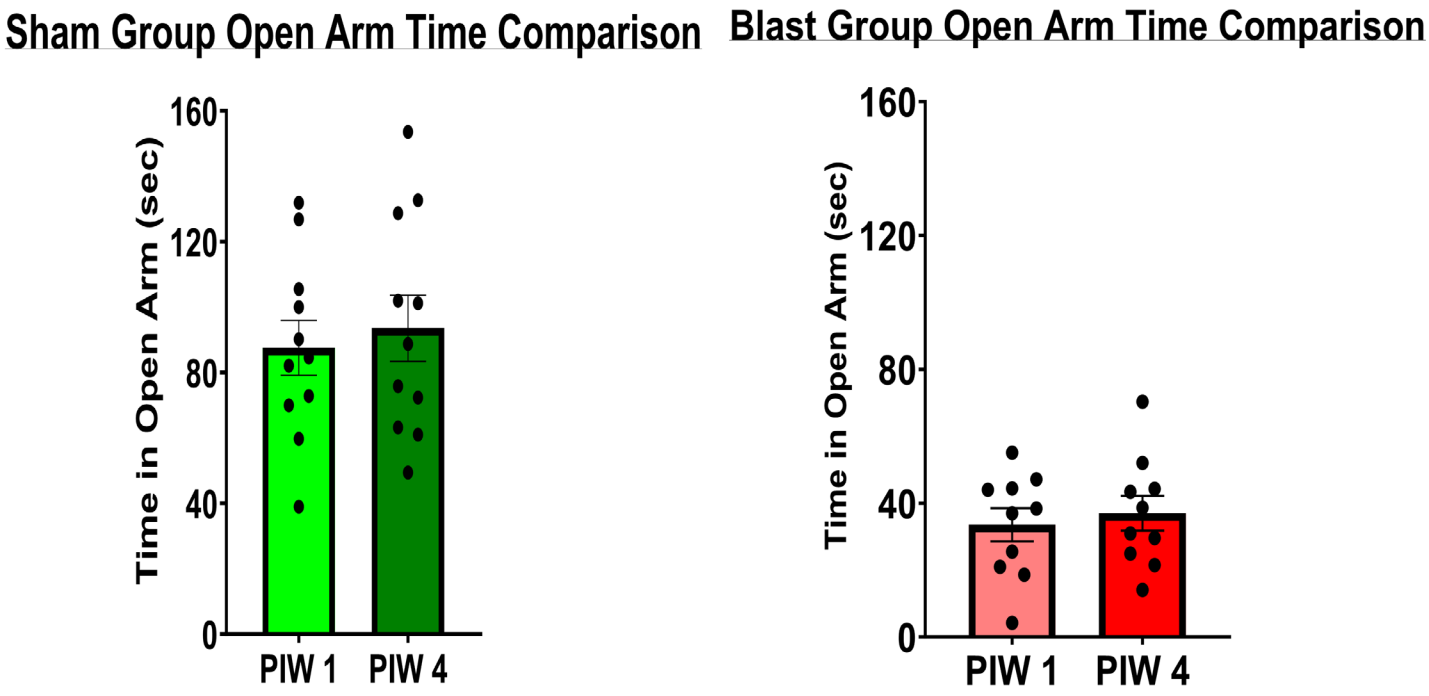

Figure 3.3 Comparison of rLLB-induced anxiety-like behavioral changes in EPM. Time spent in the open arms in the sham group between 1 week and 4 weeks post-injury (left panel) and time spent in the open arms in the blast group between 1 week and 4 weeks post-injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

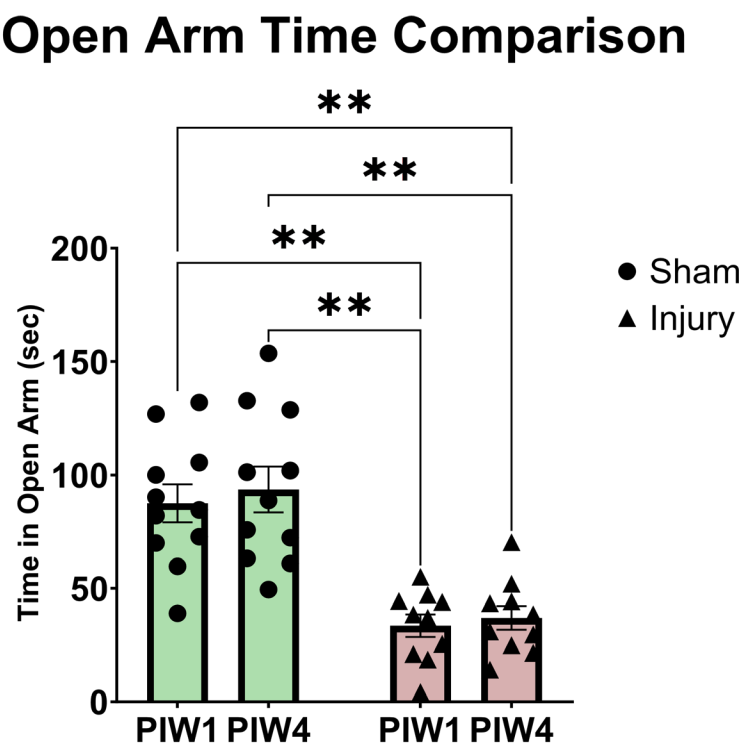

Figure 3.4 Combination of data from Figure 3.3. Time spent in the open arms in the sham group between 1 week and 4 weeks post-injury and time spent in the open arms in the blast group between 1 week and 4 weeks post-injury between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by one-way ANOVA with Tukey's post-hoc test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

The graphical comparison between the time spent and the number of entries into the open arms during the EPM testing shows that there is statistically significant evidence revealing that the sham group interacted with the open arms more than the injured blast group. This behavior could also suggest that the sham animals exhibited more signs of curiosity and willingness to explore.

3.1.2 Open Field Test (OFT)

The open-field test aims to assess an animal's levels of anxiety and willingness to explore. Statistical analysis was conducted on the number of entries into the center zone of the testing arena and the time spent in the center zone. These values were compared between sham and blast groups and were conducted for PIW 1 and PIW 4. The results are consistent with the sham group having a higher number of entries and time spent in the center zone than the blast group. All comparisons yielded statistically significant results.

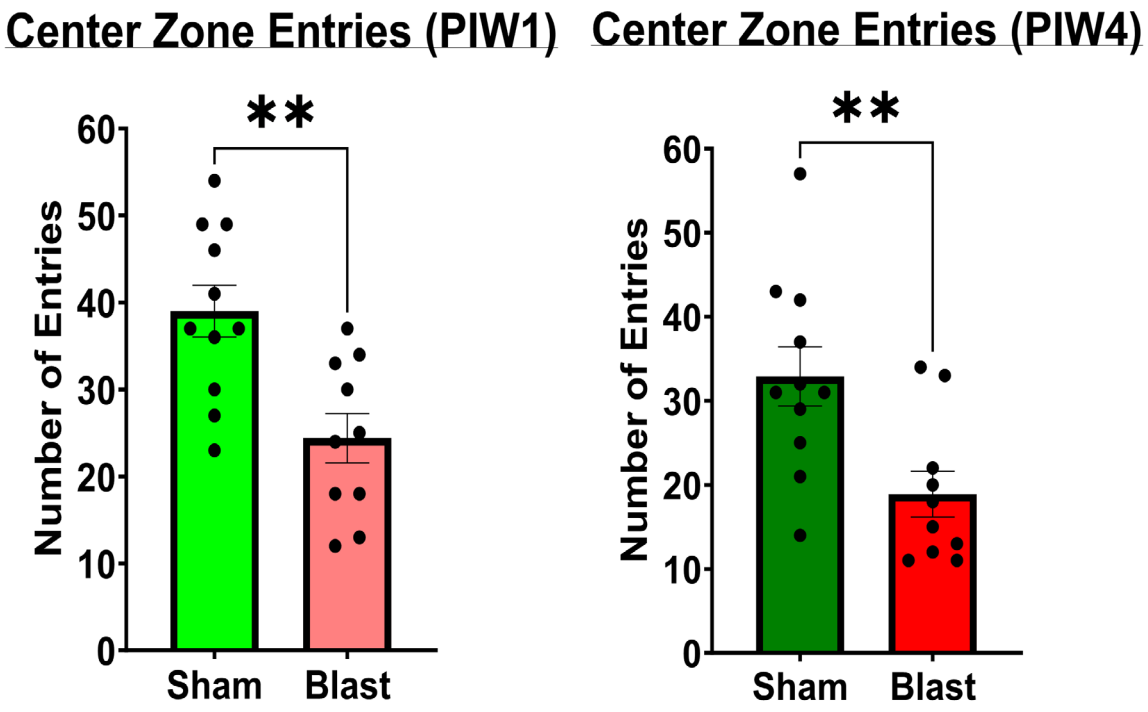

Figure 3.5 Comparison of rLLB-induced anxiety-like behavioral changes in OFT through entries. Entries to the center zone 1-week post-injury (left panel) and entries to the center zone 4 weeks post-injury (right panel) between sham and rLLB blast groups.

Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.000.1.

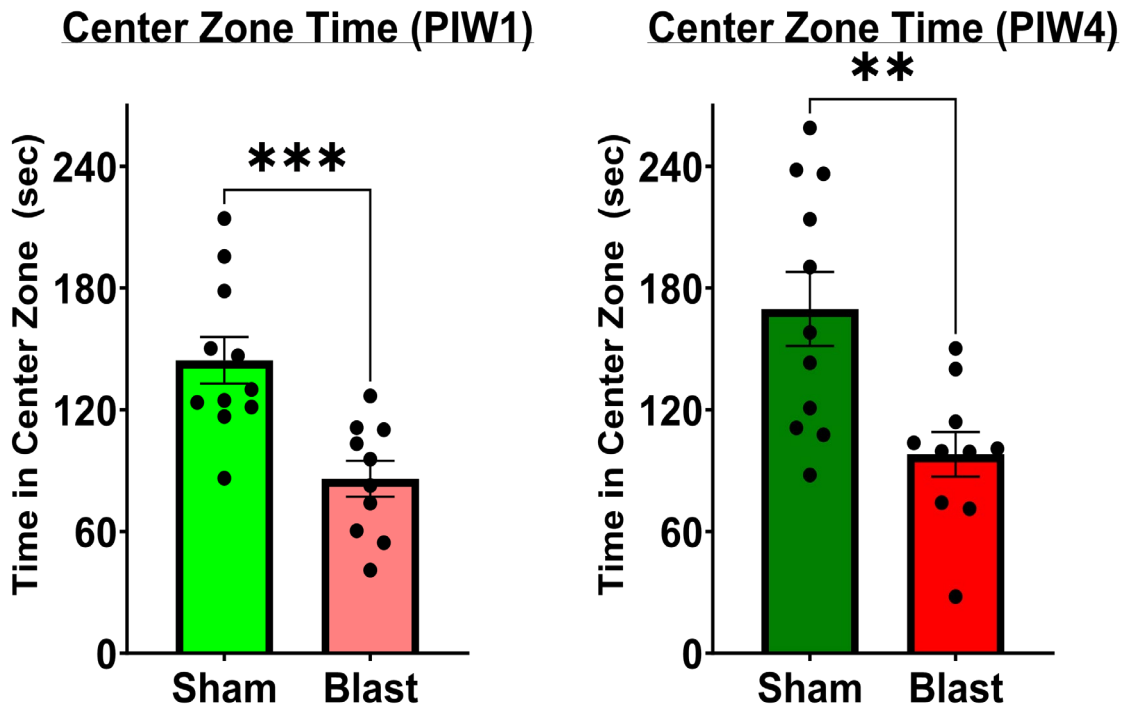

Figure 3.6 Comparison of rLLB-induced anxiety-like behavioral changes in OFT through time. Time spent in the center zone 1-week post-injury (left panel) and time spent in the center zone 4 weeks post-injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The graphical comparison between the time spent and the number of entries into the center zone during the OFT testing shows that there is statistically significant evidence revealing that the sham group interacted with the center zone more than the injured blast group. This data suggests that the sham group of animals had a higher willingness to

explore a foreign area therefore showing lower signs of anxious-like behavior in comparison to the blast group.

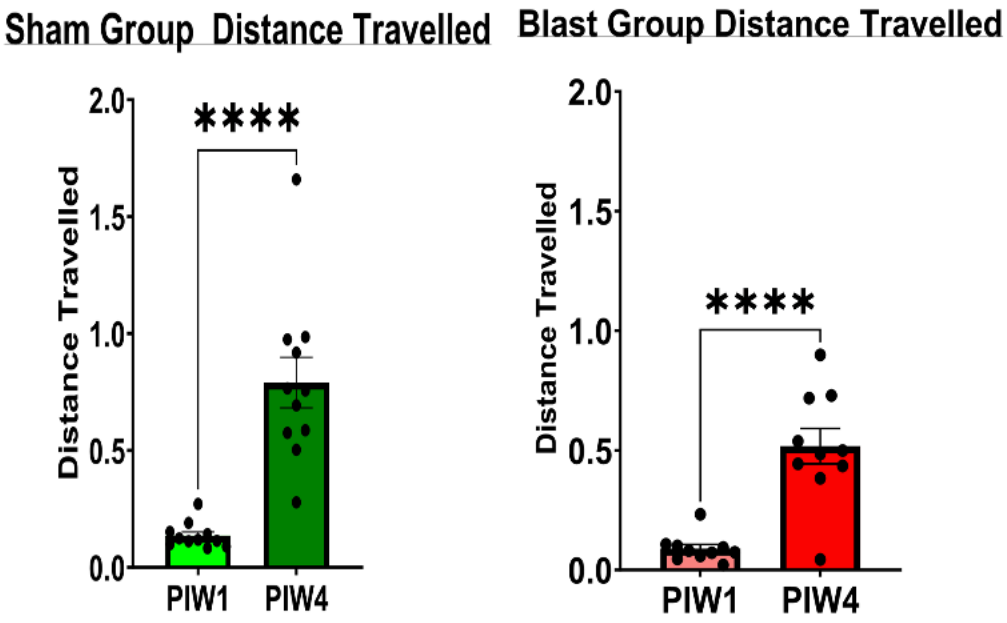

Figure 3.7 Comparison of rLLB-induced anxiety like behavioral changes in OFT through distance. Distance travelled in sham group between 1 week and 4 weeks post injury (left panel) and distance travelled in blast group between 1 week and 4 weeks post injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

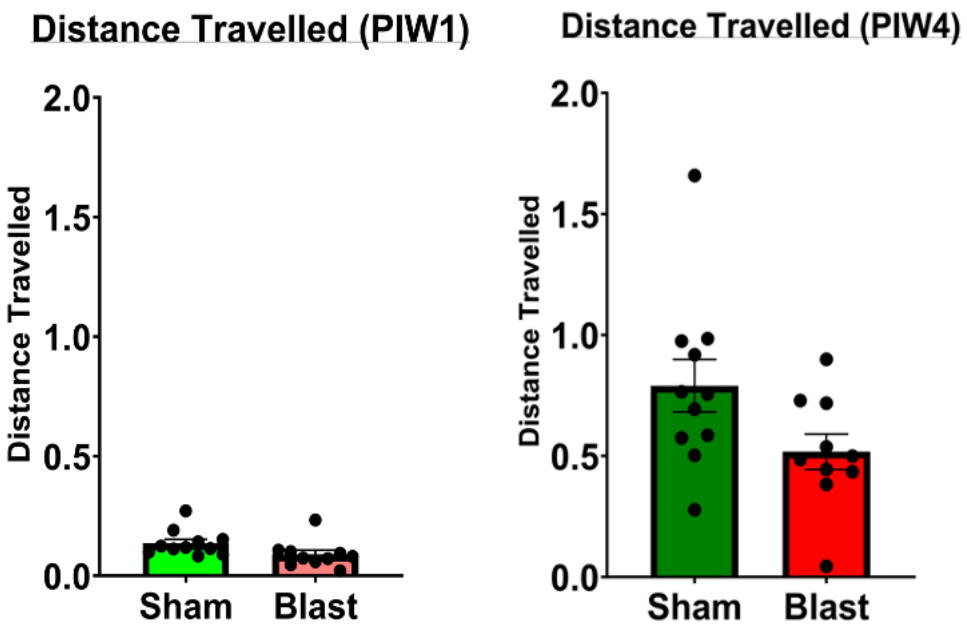

Figure 3.8 Comparison of rLLB-induced anxiety-like behavioral changes in OFT through distance and PIW. Distance traveled in sham group between 1 week and 4 weeks post-injury (left panel) and distance traveled in blast group between 1 week and 4 weeks post-injury (right panel) between sham and rLLB blast groups. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Distance traveled during OFT testing for both sham and blast animals increased from PIW 1 to PIW 4. The increase in distance traveled could be attributed to the familiarity with the arena from the two testing dates. Furthermore, when comparing the distance traveled between sham and blast during the same time point there was no significant difference between the two groups at both time points.

3.1.3 Novel Object Recognition Test (NOR)

The utilization of the NOR test was to assess the animal's memory and ability to recognize patterns and recall previous encounters. Traditionally, if a healthy animal has been exposed to a stimulus in the past, they are likely to not react as enthusiastically to the same stimulus when presented with it again. However, an animal that has undergone bTBI is susceptible to memory loss and therefore would not be able to differentiate the novel object from the standardized object. The implementation of NOR allows for further examination of short-term memory recall and pattern recognition.

When conducting statistical analysis with the NOR data first we assessed if there was a difference in the sham and blast groups and whether there was a noticeable difference in novel object identification. The sham exhibited statistical significance between the time spent interacting with the conditioned object compared to the novel object at PIW 1 and PIW 4. This result suggests that the sham animal can identify the newly introduced stimulus and their memory has not been affected. On the other hand, there was no difference in the blast group at PIW 1 or PIW 4 indicating that the blast animals were unable to identify the difference between the conditioned and novel objects.

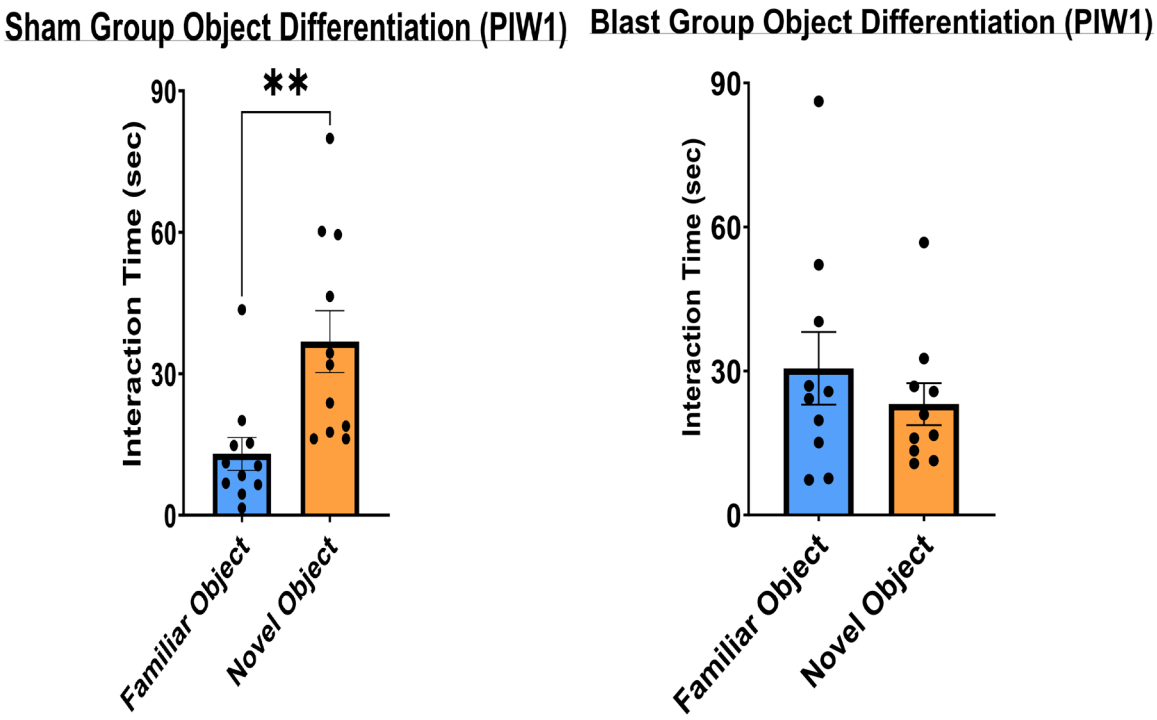

Figure 3.9 Comparison of rLLB-induced memory impairments in NOR at PIW 1. The time spent interacting with familiar and novel objects 1-week post-injury in the sham group (left panel) and blast group (right panel). Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

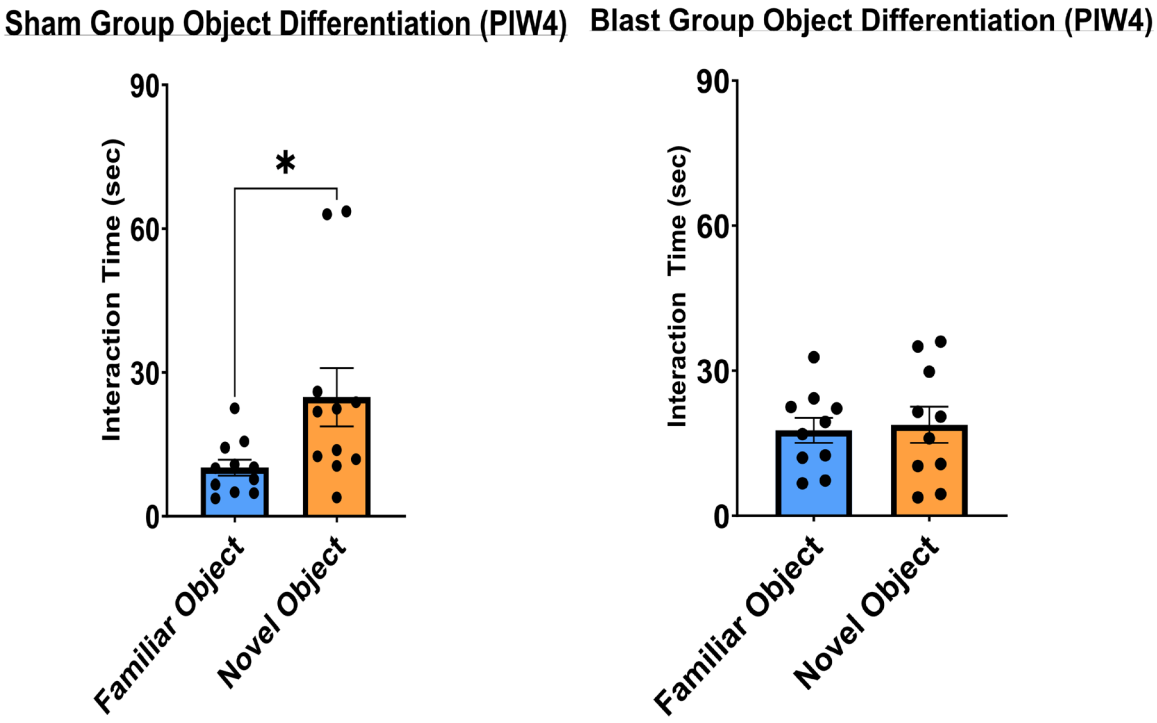

Figure 3.10 Comparison of rLLB-induced memory impairments in NOR at PIW 4. The time spent interacting with the familiar and novel objects 4 weeks post-injury in the sham group (left panel) and blast group (right panel). Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To further analyze the data, the values of time interacted with objects were taken and then calculated into a discrimination index to better visualize if there was a statistically significant difference in the performance of memory and recognition between the sham and blast groups at PIW 1 and PIW 4. The discrimination index is a ratio of the difference in time spent interacting with each object and is calculated with the time spent with the conditioned object (tConditioned), time spent with the novel object (tNovel), and total time in the arena (tTotal).

The discrimination index calculation is as follows: DI = [(tNovel + tConditioned)/(tTotal)]

The statistical analysis of the DI for the NOR test shows a statistically significant difference at PIW 1 and PIW 4 in favor of the sham group. These findings indicate that the sham group had a higher level of being able to identify the difference between the two objects and spent more time interacting with the novel object compared to the blast group.

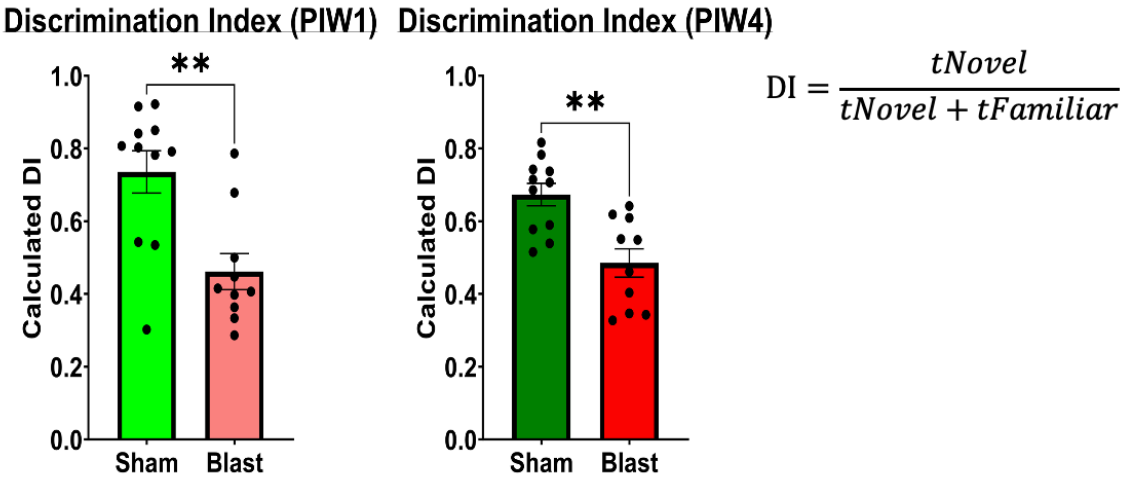

Figure 3.11 Comparison of rLLB-induced memory impairments in NOR through DI. The discrimination index in sham and blast groups 1-week post-injury (left panel) and 4 weeks post-injury (right panel). Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The graphical comparison of the calculated DI ratios during the NOR testing shows that there is statistically significant evidence revealing that the sham group interacted with the novel object more than the injured blast group. Overall, these findings suggest that following rLLB mice are susceptible to memory loss at acute and chronic time points and struggle with pattern recognition.

3.1.4 Sucrose Splash Test (SST)

The employment of the sucrose splash test was to assess depressive-like behaviors in the mice. By spraying a sugary solution onto the subjects, they are placed in a scenario where the natural response would be to groom through scratching or licking. If a mouse is experiencing depression through the repeated blasts, then they will not be as responsive to grooming as the healthy animals.

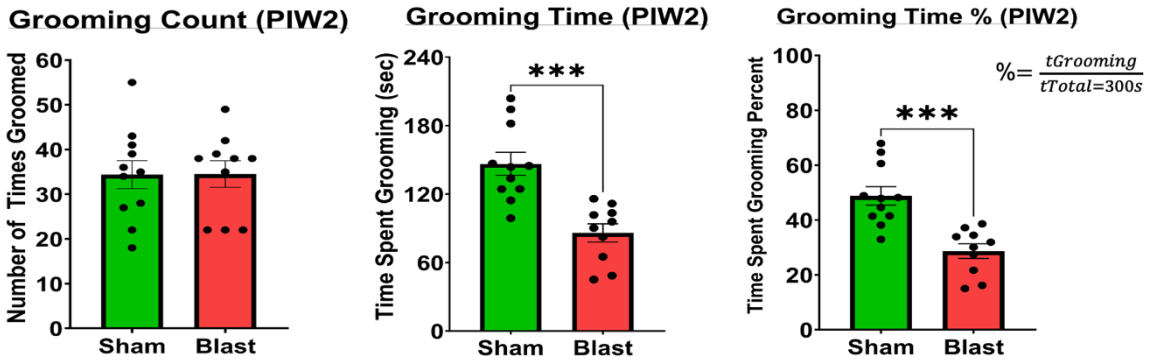

Figure 3.12 Comparison of rLLB-induced depression-like behaviors SST. The number of times groomed (left panel), time spent grooming (center panel), and percentage of time spent grooming (right panel) were all assessed 2 weeks post-injury. Data is mean ± S.E.M of 10-11 animals in each group and analyzed by student's t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

The graphical comparison of grooming time during the SST testing shows that there is statistically significant evidence revealing that the sham group groomed themselves more than the injured blast group. This behavior indicates that the blast group are showing more signs of depression compared to the sham group 14 days after injury. The data suggests that rLLB results in higher levels of depression in blasted animals than in sham animals.

3.2 Biochemical Testing

3.2.1 Skeleton Analysis

Skeleton analysis on the average branch length per microglial cell was conducted and there was statistical significance in the average branch length per microglia PID 1 between sham and blast groups. There was a trend seen at PID 7 suggesting the microglial processes were smaller in the blast group.

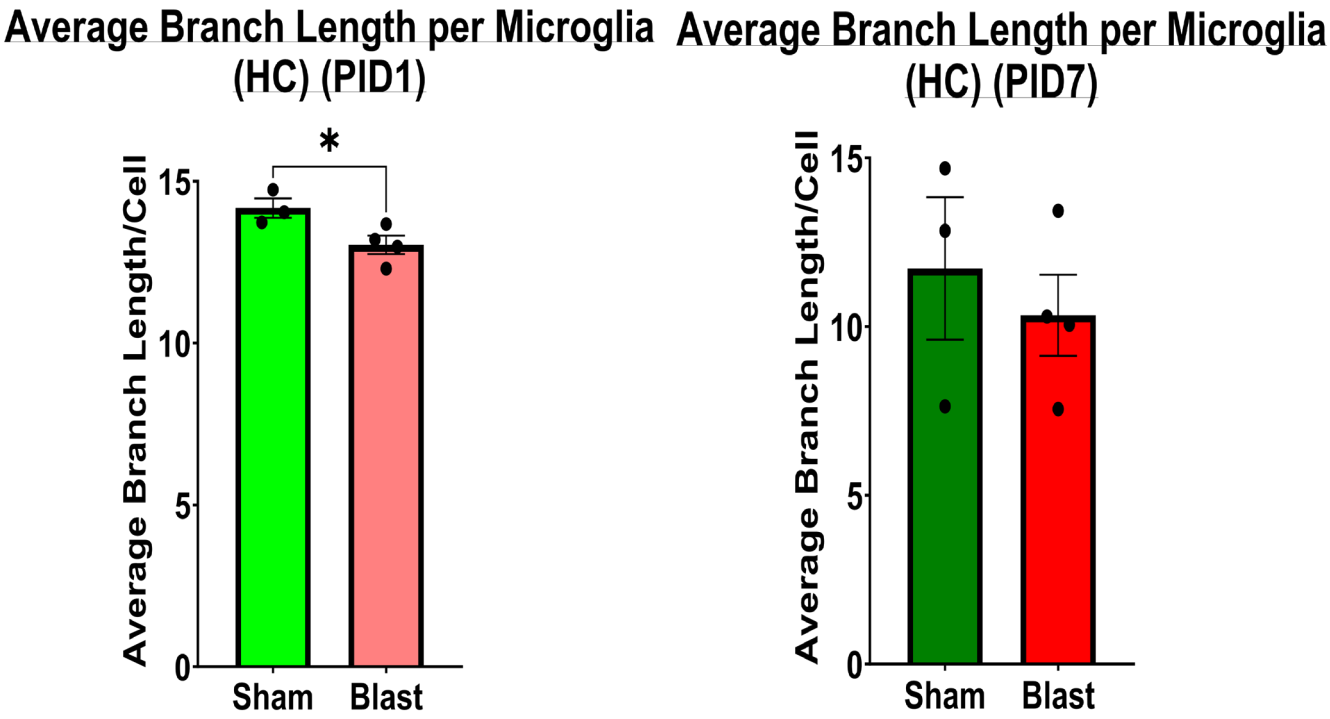

Figure 3.13 Comparison of rLLB-induced microglial process length through skeleton analysis. The average microglia branch length in sham and blast groups 1 day post injury (left panel) and 1 week post injury (right panel). Data is mean ± S.E.M of 3-4 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

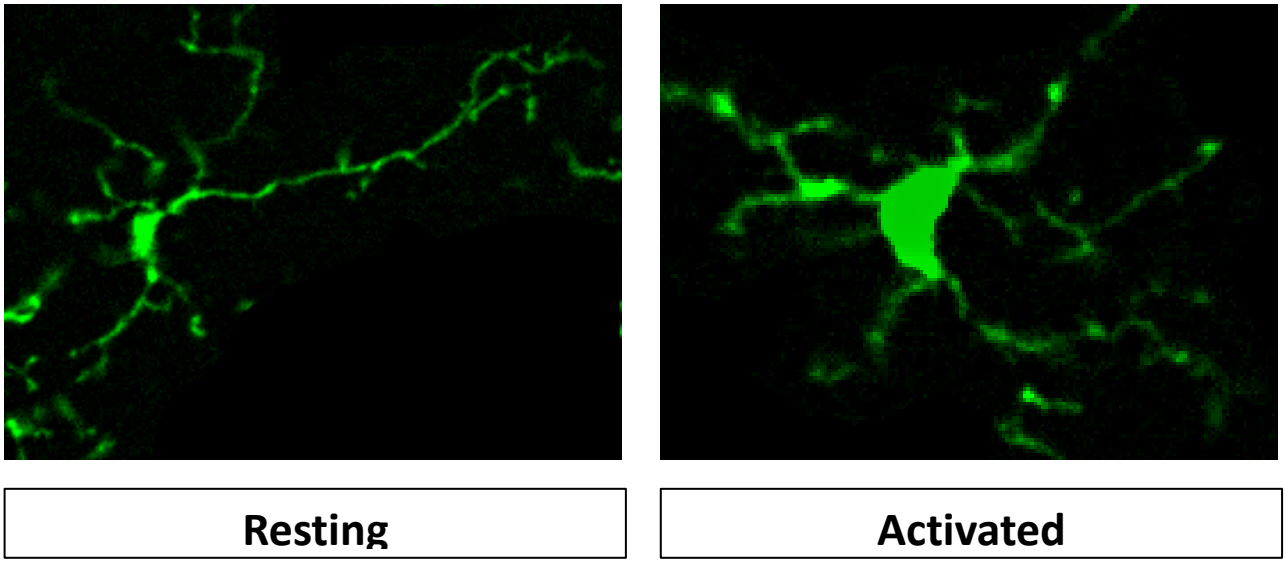

Figure 3.14 Visual aid showing the difference between resting and activated microglia in the hippocampus of a transgenic CX3CR1GFP/+ mouse model.

When microglia are in the resting state they are scanning the neural environment for any abnormalities that require attention. They present themselves with round somas with long thin processes that are spread out in all directions. Once the microglia become activated their soma begins to lose its circular shape and the processes retract towards the soma while also becoming thicker and shorter in length. The blast group shows a trend of having smaller branch lengths than the sham group indicating that the blast group had a higher level of activated microglia. Helping support the hypothesis of neuroinflammation occurring following rLLB.

Skeleton analysis of microglial counts per unit area revealed varying results. At 1 day post-injury there seemed to be a trend suggesting that the blast group presented more microglia than the sham group, however, at PID 7 they are approximately equal. It is important to note there is a data point in the sham group which is skewing the data.

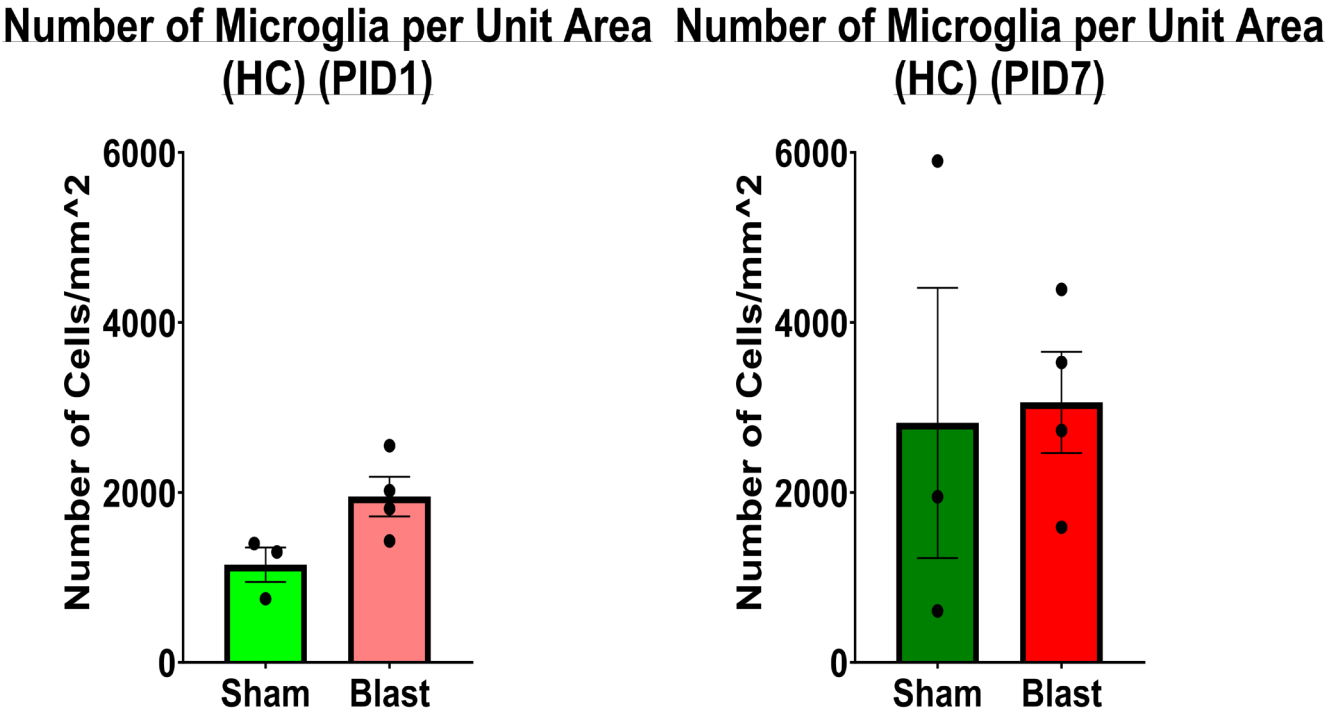

Figure 3.15 Comparison of rLLB-induced activated microglia through skeleton analysis. The number of microglia per unit area 1 day post-injury (left panel) and 1 week post-injury (right panel) between sham and rLLB blast groups in the hippocampus. Data is mean ± S.E.M of 3-4 animals in each group and analyzed by student's t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

The graphical comparison of the total number of microglia and the average number of branches per microglia shows that there is a trend suggesting that microglia and branches per microglia are higher in the injured blast group when compared to the sham group. This data suggests that there is neuroinflammation caused by rLLB, however, further studies need to be conducted to verify the results.

3.2.2 Microglia Counts

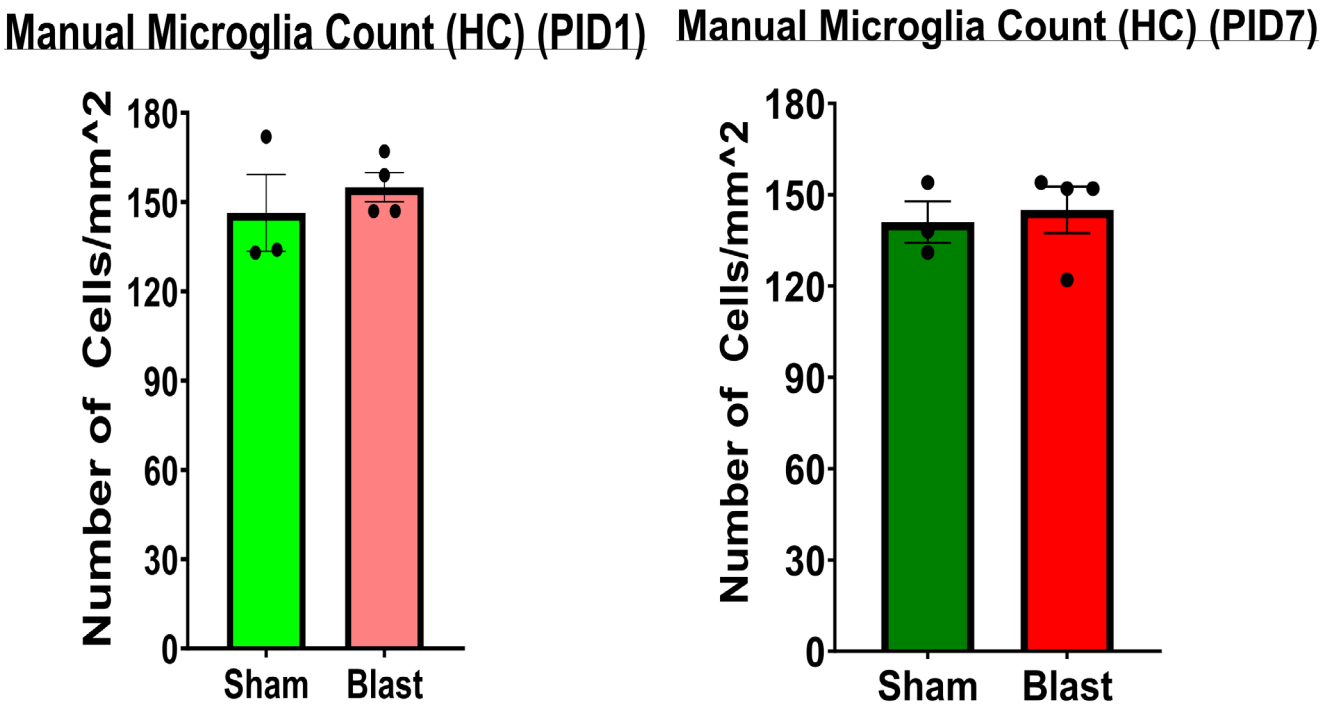

Figure 3.16 Comparison of rLLB-induced activated microglia through manual counting in HC. The number of microglia per unit area 1 day post injury (left panel) and 1 week post injury (right panel) between sham and rLLB blast groups in the hippocampus. Data is mean ± S.E.M of 3-4 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The graphical comparison of the average branch length in microglia shows that there is a trend suggesting that microglial activation in the hippocampus is higher in the injured blast group when compared to the sham group. There is no conclusive evidence to suggest there is a difference in microglia counts through the conducted manual counting. The results are inconclusive from this analysis as there is no statistical significance present

when comparing the cell counts of sham and blast animals. The absence of results prompted a more in-depth exploration of the hippocampus, focusing specifically on the region responsible for mood regulation, notably the dentate gyrus (DG), which plays a role in anxiety-like behaviors (Weeden et al., 2015).

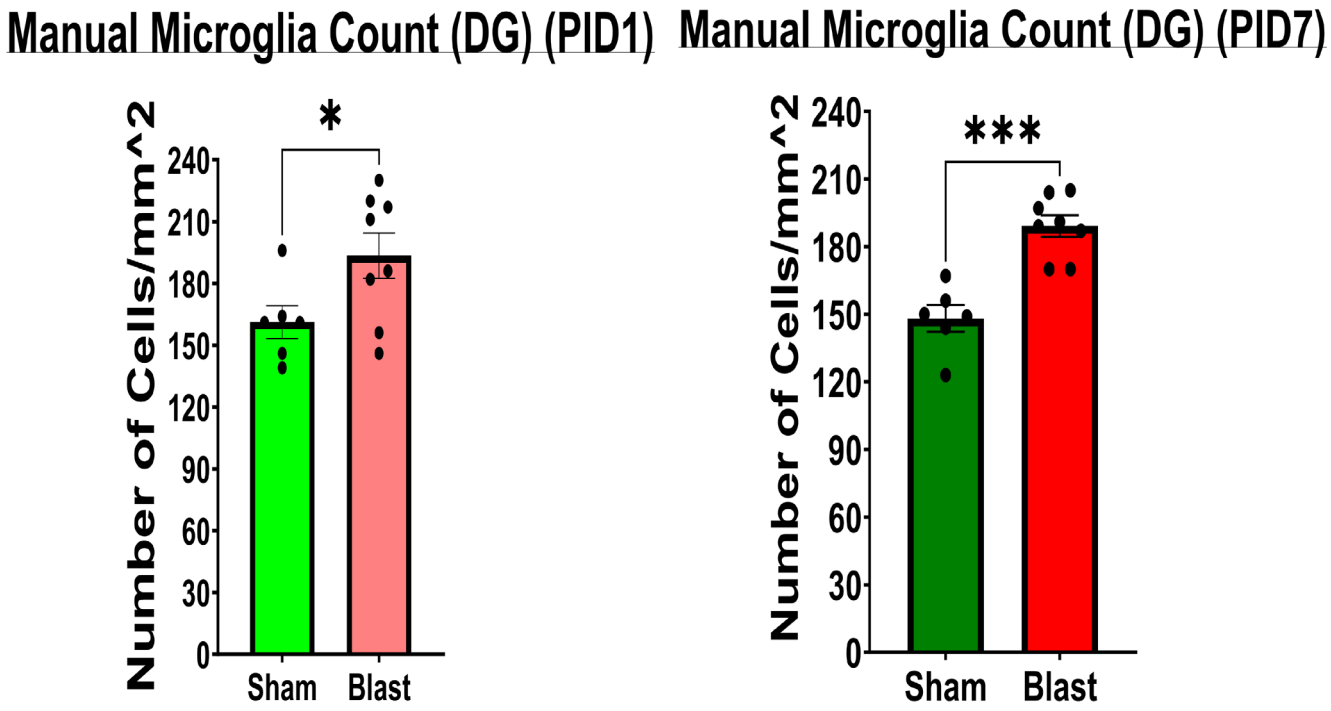

Figure 3.17 Comparison of rLLB-induced activated microglia through manual counting in DG. The number of microglia per unit area 1 day post injury (left panel) and 1 week post injury (right panel) between sham and rLLB blast groups in the dentate gyrus region. Data is mean ± S.E.M of 3-4 animals in each group and analyzed by student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Statistical analysis of the manual counts of microglia cells present in the DG region of the hippocampus shows a statistical significance in favor of the blast group having a higher number of microglia than the sham group at acute and chronic time points. As there

are higher levels of microglia presence in the blast group this may correlate with the results seen from the EPM and OFT tests.

CHAPTER 4

DISCUSSION

4.1 Neurobehavioral Deficits

Through statistical analysis of all the behavioral tests conducted, it is visible that there is a stark difference in performance between the sham and blast groups. The OFT and EPM results suggest that the blasted animals displayed higher levels of anxiety than the sham animals. By spending less time in the center zone, the subjects exhibit the behavior of feeling more comfortable near a wall rather than being open to explore. Previous work has shown that the parameters of center zone entries, time spent in the center zone, and distance traveled during testing are key measurements that should be recorded when assessing anxiety-like behaviors in a rodent model with OFT. Perez-Garcia conducted a study using rats that were blasted at 74.5 kPa for 3 consecutive days with one blast per day. When conducting OFT they found significant results of the sham group traveling more distance in the arena than the blast group and more entries and time spent in the center zone PID 2 (Perez-Garcia et al., 2018). Similarly, Blaze et al. conducted an identical injury model test on rats and found that at PID 1 the sham animals spent more time in the center zone than the blast animals. These results directly align with the results that were achieved in this study (Blaze et al., 2020). An additional measurement that could be taken when conducting the OFT testing that was not investigated during this study is the activity of where the animals are walking in the arena during testing. Although distance traveled was analyzed, where in the arena asides from the center zone were the sham and blast animals spending their time. Upon visual assessment of *Figure 4.1* it is noticeable that the blast subject had more activity concentrated in the corners of the arena and avoidance of the center zone

while the sham animal's activity was random throughout the entirety of the arena. Previous literature has shown significant results between an injured group and a control group regarding corner zone entries and time spent in the corner zones and concluded that time spent in the corner of the arena indicates decreased exploration and increased anxiety (Gillette et al., 2014). Further analysis of corner activity could be useful when assessing anxiety-like behaviors following rLLB in a rodent model.

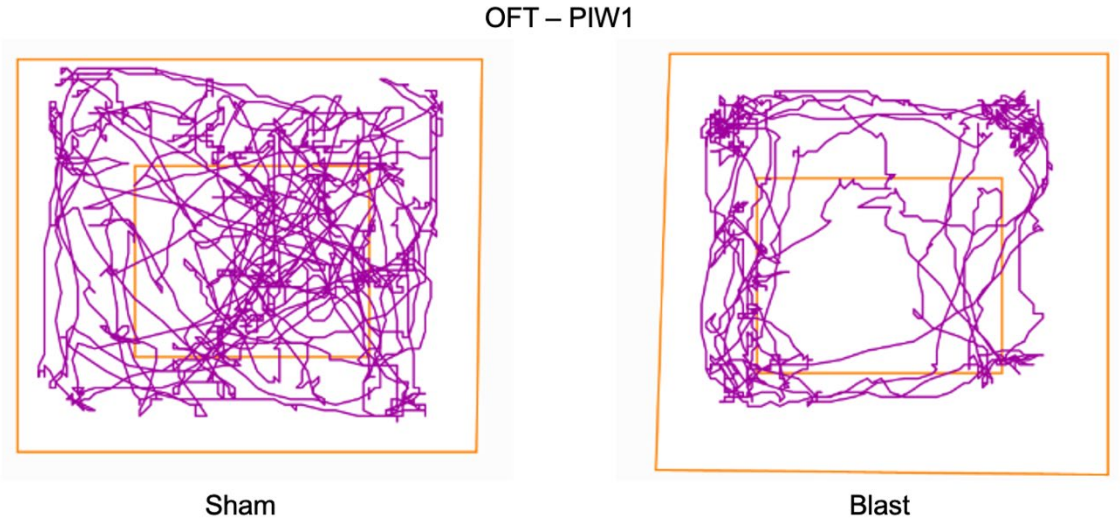

Figure 4.1 Trace map of activity during OFT testing in one random sham and blast animal at PIW 1. Visual comparison of sham and blast group activity during OFT.

The other anxiety assessment that was conducted resulted in the sham animals spending more time in the open arms during the EPM testing which suggests that the sham mice are less anxious than the injured mice. A previous study where rats were subject to 5 successive 70 kPa blasts with a 1-minute interval in between each blast showed that the blast animals preferred the closed arms during EPM testing to the open arm. The time spent in the closed arm was significantly different in favor of the blast group at PID 1 and

PID 25 suggests that the rats are exhibiting anxiety-like behaviors immediately and chronically following the blast injury (Ravula, Rodriguez, et al., 2022). Results that are closely aligned with anxiety-like behaviors when conducting the EPM test have been established as time spent in the open arms. However, by investigating entries to the open arm and distance traveled, the EPM test could also be used to assess motor function and hyperactivity of the brain following injury (Schrader et al., 2018).

Furthermore, conclusions of short-term memory loss can be seen through the results of the NOR testing. The DI ratio indicates that the sham animals were more interested in the novel object as they had already familiarized themselves with the standardized objects during the familiarization phase. The blast animals showed a lesser affinity towards investigating the novel object and spent closer to equal time exploring the standardized and novel object suggesting that they forgot that they had already interacted with the familiar object during the familiarization phase. Prior research indicates similar results when conducting NOR in a similar injury model using rodents as test subjects. Ravula found significant differences in DI ratios at PID 2 and PID 26 in favor of the sham group when blasting rats at 70 kPa with 5 repetitions (Ravula, Rodriguez, et al., 2022). Furthermore, a different study conducted by Arun et al. subjected rats to two successive blasts of 131 kPa with 2-minute intervals and they saw a decrease in the DI ratio over a span of 1 year in the repeated blast group. At PID 8 the DI was higher in the repeated blast group than the sham group but after 1-month post-injury there was a steady decline in the DI ratio for the rest of the year (Arun et al., 2019).

Lastly, the results from the sucrose splash test indicate that the blast animals are experiencing higher levels of depression than the sham animals. The less time spent

grooming from the injured animals suggests that the innate desire to keep one's health maintained seems to be absent in the blast group which is a key factor in determining depression in the murine model. A previous study induced TBI through a weight drop method on a mouse animal model and found in their splash test results that the control animals had a significantly higher time spent grooming than the TBI animals (Angoa-Pérez et al., 2014). Although, the injury model was not rLLB, the common trend of TBI causing depression can be seen through the results of the SST being conducted in both studies.

4.2 Biochemical Changes

When analyzing average branch length per microglia cell there was statistical significance at PID 1 but not at PID 7, however, a trend is present where the sham group at PID 7 visually seems to be greater than the blast group. These results suggest that the sham group had more microglial cells that were in the resting state compared to the blast group. Prior investigation by Uzunalli et al. reveals that following three 150 kPa repeated blasts, microglia in the mid brain region in the blast group had shorter and thicker processes as well as larger somata than the sham group. This result indicates that the sham group's microglia were not activated when compared to the blast group. Furthermore, the dentate gyrus region has been linked with microglial activation when assessing anxiety-like behaviors. With this research, there was a significant difference in favor of the blast group at PID 1 and PID 7 for activated microglia counts. Similarly, Bugay et al. found that after exposing mice to ~100 kPa blast over three days consecutively, there was a higher expression of activated microglia in the DG region in the blast group when compared to the sham group (Bugay et al., 2020). The results that were found in this research and prior

research align with one another suggesting that investigation of the DG region in the hippocampus may reveal insights into higher levels of anxiety in a rodent model following repeated blast injuries. These results may suggest that there is chronic neuroinflammation occurring in the brain and the sustained inflammatory response may result in the loss of neurons, confirming chronic neurodegeneration.

The rationale behind selecting the hippocampus for investigation of the rLLB model is that the HC is involved in a variety of functions related to behavioral deficits such as memory and motor control (Jimenez et al., 2018; Kerr et al., 2017). As this paper was closely assessing neurobehavioral performance and microglial activation the hippocampus allows for investigating a link between microglial activation and chronic behavioral deficits. Furthermore, half of the behavior tests assessed anxiety-like behaviors by the mice therefore it would be beneficial to analyze the HC further by exploring the microglial activation in the DG region. When rodents are exposed to stimuli that elicit an anxiety-like response, there is a recorded increase in the density of activated microglia in the dentate gyrus (DG). Moreover, stimuli that are causing anxiety in the rodent could be a reason for increased neuroinflammation in the DG, explaining the increase in activated microglia (Rooney et al., 2020).

4.3 Limitations and Future Directions

This study only investigated the novel injury model in mice. Further investigation could potentially replicate the protocol of this research and continue further testing on other rodent animals such as rats, chinchillas, and ferrets. As mentioned earlier, bTBI not only affects the brain but other vital organs such as the lungs and ears. A more comprehensive

and in-depth research might benefit by investigating other organ systems and not just the neural affects bTBI causes. A limitation seen in the analysis of the results for this research is that all results were compared to the repeated low-level blast injury model to ensure a near comparison of results. Due to the lack of literature on repeated blasts, some parameters from the behavior testing and biochemical testing may have been overlooked and the research could benefit from an in-depth investigation. Furthermore, monocyte infiltration of the BBB could be further investigated to assess BBB breakdown and the duration of the rupture by quantifying monocyte presence in the hippocampus PID 1, 7, and 30. To further confirm neuroinflammation and neurodegeneration neuron presence and counting can also be employed.

CHAPTER 5

CONCLUSION

The primary objective of this research was to investigate the chronic effects of repeated low-level blast (rLLB) injuries on neurobehavioral and biochemical changes in a mouse model. The study aimed to fill a critical gap in knowledge regarding the long-term consequences of rLLB, particularly focusing on aspects such as neurobehavioral deficits, neuroinflammation, neurodegeneration, and blood-brain barrier (BBB) integrity. The results obtained from neurobehavioral testing revealed significant differences between the sham and blast groups. The elevated plus maze (EPM) and open field test (OFT) demonstrated increased anxiety levels in the blast group, as evidenced by reduced time spent in open areas and the center zone. Novel Object Recognition (NOR) testing indicated short-term memory deficits in the blast group, as reflected in a lower discrimination index. The Sucrose Splash Test (SST) suggested higher levels of depression in the blast group, characterized by reduced grooming behavior. Biochemical analyses focused on immunohistochemistry (IHC) to assess microglial activation and neuronal loss. The results indicated a trend of increased microglial presence in the blast group, suggesting chronic neuroinflammation. Additionally, the blast group exhibited a higher mean number of branches per microglia, providing further evidence of altered microglial activation. These findings were consistent with the observed neurobehavioral deficits, indicating a link between chronic neuroinflammation and behavioral changes.

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