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Abstract

Mild Traumatic Brain Injury (mTBI) accounts for 70-90% of TBIs recorded in the last two decades. Subjection to continued mTBIs could result in severe comorbidities appearing years later. Current research on mTBI shows significant male sex and single age skew in murine animal models. Using the Missouri Blast Model, mice were inflicted with mTBI and evaluated using standard behavior tests and a novel method not previously used for mTBI with an open blast model. With both methods, a larger clinical difference was found between age groups than sex. We were also able to show that the novel tracking method was able to identify clinical symptoms in mice consistently while traditional methods were not. These results support a need for diversification of age ranges of future murine models for mTBI studies.

Introduction

According to the Centers for Disease Control and Prevention (CDC), over 430,000 United States Armed Service members have been diagnosed with traumatic brain injury over the last twenty years, with 70-90% of those injuries being mTBI (Cassidy et al., 2004). As these soldiers have entered and returned from combat zones, they have been hospitalized for TBI in record numbers, and suffered resultant sleep disturbances, hearing loss, and long-term impairments (Hoge et al. 2008, Myers et al. 2009, Wojcik et al. 2010, Collen et al. 2012). The National Institute of Neurological Disorders and Stroke (NINDS) defines traumatic brain injury as trauma inflicted upon a brain. The severity of the injury is classified into one of three categories: mild, moderate, and severe (*Traumatic Brain Injury Information Page | National Institute of Neurological Disorders and Stroke*, n.d.). Traumatic brain injury can have long lasting and ongoing symptoms and comorbidities such as depression and Post Traumatic Stress Disorder (PTSD; Bryant, 2011). The need to identify early methods of detection of mTBI is immediate and would enable intervention and treatment for affected Armed Services personnel earlier, offering potentially improved outcomes. During a report to the United States Congress on TBI (2013) the Army Vice Chief of Staff called for development and implementation of sensors in soldiers' helmets that would enable medical personnel to identify when a soldier had been exposed to environmental conditions that could result in TBI (e.g., blasts or blunt force trauma) with the goal of expedited detection, treatment, and management of the injury.

The conditions that can create a mTBI are often less pronounced or immediately disruptive than those that result in moderate or severe TBI. A patient that experiences a mTBI may not even realize until much later when signs of neurodegenerative disorders appear. The immediate symptoms of mTBI may vary among individuals and include headache, nausea, uneven pupil dilation, confusion, convulsion, and pronounced sleepiness (CDC 2022). Upwards of 6 million individuals in the USA may experience mTBI in a given year, and of these, 15% may develop persistent or sustained disabling problems (Kushner 1998).

Current research on mTBI overwhelming skews toward single age male murine animal models. The lack of sex and age diversity in studies may have significant consequences for outcomes and results. Recent epidemiological work found that women are underrepresented in human TBI studies but are more likely to die from a severe TBI than men and more likely to experience sustained symptoms from a mTBI than men; however, these results were interactive with age, with post-menopausal women having a decreased risk of death due to TBI relative to men (Biegon 2021). Given these data, we examined the observable differences in severity of mTBI across age and sex across murine models. We anticipated differences across both age and gender, given the differences observed in human studies.

Materials and Methods

Animal Care

Thirty-two C57BL/6 mice were ordered from JAX® labs and quarantined for seven days as per Missouri University of Science and Technology IRB guidelines. They were housed in an Allentown NextGen™ Mouse 500 with 12-hour light/dark light cycle, with controlled air flow, temperature, and humidity conditions. Mice were fed a standard diet with food and water *ad libitum* for the duration of the experiment. A total of 32 individuals were used for this study (N = 16 females, N = 16 males) that were further subdivided based on litter into two groups of males (N = 8 born on 29 June 2021; N = 8 born on 10 August 2021) and two groups of females (N = 8 born on 29 June 2021; N =8 born on 10 August 2021). Mice were assigned randomly to treatments from within each litter. N = 20 mice were assigned to the blast treatment and N = 12 mice were assigned to the sham treatment (no blast exposure, but all other conditions replicated).

Blast Conditions

The day of the blast was sunny and clear with a temperature of 23 C and 47% relative humidity. All mice were transported to the blast site in their cages. The sham mice were placed into the blast cages first (n=3 sham mice per age/sex group for a total of n=12 sham mice). They were then removed from the blast site completely and returned to the vivarium before the blast was conducted. The remaining 20 mice were placed in the blast cages located 3 m from the C4 and one meter from the ground **(Fig. 1).** Mice were then subjected to an open field explosive blast, using the Missouri Blast Model, with 350g of C4. Immediately afterwards, the mice were removed from the blast cages, placed back in their cages, and returned to the vivarium. Their tails were then marked with a color-coding scheme so as to keep the observers blinded to the control/blast mice **(Fig 2).**

Mouse Monitoring

Commencing the following day, the monitoring of the mice began. The first week, monitoring was daily and then once per week for three consecutive weeks. There were two types of evaluation: video recording by an unblinded investigator and cognitive evaluations by three blinded student investigators. The mice were video recorded for a total of 5 minutes each using a GoPro video camera, by the nonblinded investigator. A specially built plexiglass chamber was built for this portion of the monitoring **(Fig. 3)** The recording cage measured as follows: outer diameter = 30 cm, wall thickness = 0.635 cm, exterior wall height = 30.5 cm, and an interior wall height = 30 cm. Masking tape was used on the outside of the recording cage to blind the mice to anything happening outside the cage to mitigate any outside influence on their behavior. The mice were weighed each day of video recording to monitor for weight changes. The recording was analyzed using MATLAB **(Fig. 4)**.

A group of blinded students individually administered a series of cognitive tests designed to detect neurological impairment in the mice and scored them. Using the SNAP (Simple Neuroassessment of Asymmetric imPairment) tests, investigators scored their performance on interactions, cage grasp, visual placing, pacing, gait and posture, head tilt, baton, and wire hang (Shelton et al. 2008). After the initial week, the mice were videoed by the non-blinded researcher weekly and the cognitive exams were repeated weekly by the blinded students. These student investigators were trained uniformly by a senior investigator on how to administer the tests, the equipment needed for them, and tests were administered on the same days**(Fig. 5).**

After the final evaluation, the mice were dispatched using CO_2 and their brains were immediately removed, then frozen with liquid nitrogen to prevent further breakdown of proteins and were retained in an $^-$80C freezer to await further study. Videos were evaluated using the MATLAB program by Dr. Yun Seong Song, an approach designed to remove human error from the equation. Data from the cognitive tests were input by a blinded student who compiled the average and standard deviations of the scores. Statistical analysis of the data was done in Excel.

Results

Analysis of the MATLAB data was conducted in Excel. The total average distance traveled, across all days of monitoring, for each test group was plotted and compared to determine any difference between controls and blast groups. There was a significant difference between the groups with young female mice traveling the least average distance and old male control mice traveling the most distance **(Fig. 6).** Travel patterns were also compared among groups by examining proportion of time spent at the center of the video chamber **(Fig. 7),** again averaging all days of monitoring together. Old male control mice spent the highest amount of time in the center, and young male blasted mice spent the least amount of time in the center.

Further analysis of the SNAP data was conducted in Excel. The first analysis shows the number of days each behavior was exhibited during the monitoring period **(Fig. 8).** Of the seven days of SNAP test administration, only Pacing, Gait/Posture, and Wire Hang had scores for each day. Head Tilt had a score of zero for all days of monitoring. The Baton test only showed scoring for two days of monitoring. Interactions, Cage Grasp, and Visual Spacing showed scores for six of the seven days. The second SNAP analysis shows the average score, across all days of monitoring, for each test group **(Fig. 9).** No score was recorded for any test group in the Head Tilt category. Old Male Blast group and Young Female Control group were the only two groups to have a score for the Baton test. Old Female Control mice exhibited no score for the Cage Grasp test. Young Female Blast mice exhibited no score for the Pacing test. All control groups exhibited scores in Interactions, Visual Spacing, Gait/Posture, and Wire Hang.

Discussion

Mice in this study experienced mTBI severity differentially based on age and sex, supporting our initial predictions. Age had a larger effect than sex, but age and sex differences were observable to the end of our tracking period. Comparing the two methods of evaluating mTBI should be analyzed. Video recording and analysis presents a standardized, analyzable and replicable method to quantify behaviors observed without relying on observer records and inference. Both the distance traveled and time that the mouse spent in the center of the chamber were informative measures for mTBI in this study. A sick or injured mouse will avoid open areas and exposure, if at all possible. We hypothesized that we would see the blasted mice travel less frequently through the inner portion of the recording chamber. As mice are prey animals, exposure to open spaces would present an excessive hazard, especially if sick or injured. Our results provided quantitative evidence that this is indeed the case – the injured mice spent considerably less time in the center of the chamber than did the control mice. The only exception for this was the Young Female Control and Young Female Blast mice, who exhibited almost identical travel through the center of the chamber. The proposed explanation for this is the more robust immune system response exhibited by younger females (Klein & Flanagan, 2016). With their ability to mount a more robust innate immune response, it is likely they were able to recover, or feel better, quicker than the other test groups.

An example of the MATLAB output showing travel patterns of young male control mice and blasted mice on Day 1 after blast is provided in **Fig. 10**. The purpose of this inclusion is for illustration purposes on how the tracking method works to evaluate the mouse's behavior in a quantifiable way. Travel by the control mouse shows high concentrations of activity at all points on the plot. Travel by the blasted mouse shows clear avoidance of the open area in the center of the recording chamber. The red lines on the outside of the circle are where the mouse stood up with front paws on the side of the chamber: There is more of this behavior by the control mouse than the blasted mouse.

The potential bias and training curve associated with SNAP assessments can be problematic and make comparing assessment data among individual investigators difficult. The SNAP assessment is currently the gold standard of evaluating mTBI severity and our data show that it is inconsistent. A score of zero across all categories would be expected for a normal, healthy mouse and this is not what we obtained from the data for our control groups. A score of 5 in any category would indicate a comatose mouse. Where we expect our control groups to be scored at zero in all SNAP tests, this is not what the data show. This suggests some problem with the test or its administration, likely the subjectivity required to make a judgement on just which behavior the mouse is exhibiting or not exhibiting. No score at all, in any category of mouse or test, averages above 0.35. With an overall average so low, there were many scores of zero. With all investigators being trained in a uniform manner at the same time, we think this is due to the individual judgement calls of each investigator. This reinforces our hypothesis that the SNAP assessment is flawed and that we need to develop a new protocol for assessing mTBI that can be applied by anyone and scored the same, removing human error, bias, and subjectivity.

Interestingly, young male and young female mice in blasted groups traveled more on the first day post-blast than older mice. We suspect this has to do with the immune response of younger groups being more robust than that of older groups (Thompson et al., 2020). The immune system ages, and the senescence of the immune system is beginning to be explored. With slower response of the innate immune system in older test groups, we submit that this is the initial reason for the increased activity of the younger groups. We recommend further study with an older group of mice in both sexes to test this theory.

Conclusion

The data show that this is a promising method to obtain more accurate data by pairing the recording method, behavioral assays, and the Missouri Open Air Blast model. Future studies should include this protocol and method of analysis. To more closely mimic the environment experienced by soldiers, the next study should also include multiple blasts where severity of mTBI could be evaluated across a range of age and sex groups. The ways in which these effects may compound and influence behavioral, physiological, and morphological features remain relatively unexplored and are an important area of future study.

Figures

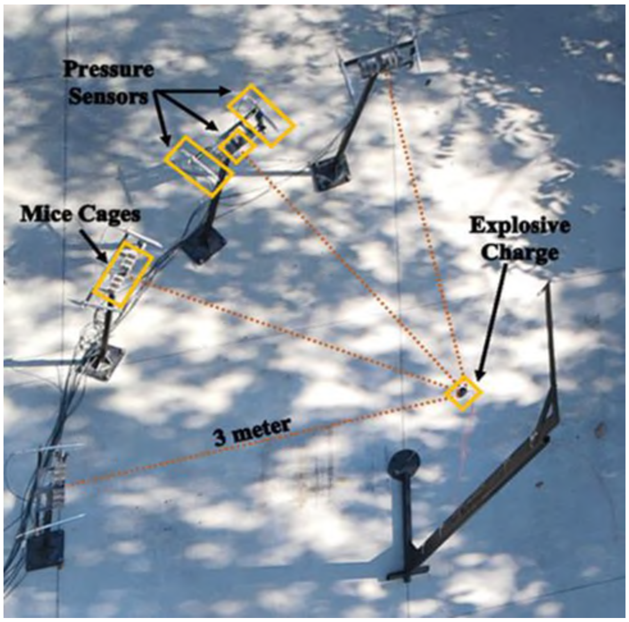


Figure 1. Aerial view of the Missouri Open Air Blast Model. The Missouri Open Air Blast Model is located in Rolla, Missouri, at the Experimental Explosives Mine, part of Missouri University of Science and Technology. It is equipped to deliver a controlled explosion, simulating what a soldier would experience in the field.

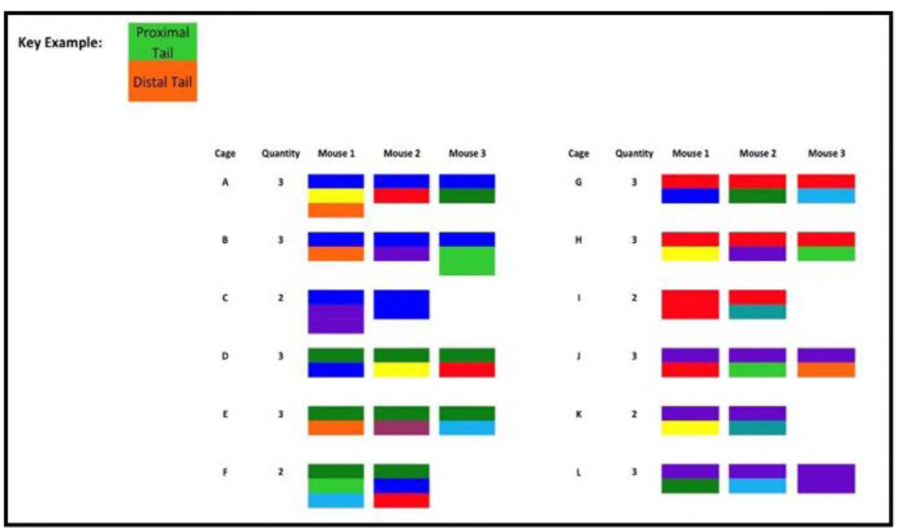


Figure 2. Color scheme for identifying mice. Color scheme used to identify each individual mouse. The scheme was used by the non-blinded investigator to identify which mouse was being recorded.

Figure 3. Specially built chambers for video recording of mice. These specially built recording chambers hold a Go Pro video camera in the mount on the top and are covered on the side to prevent any behavior alteration of the mouse due to outside activity.

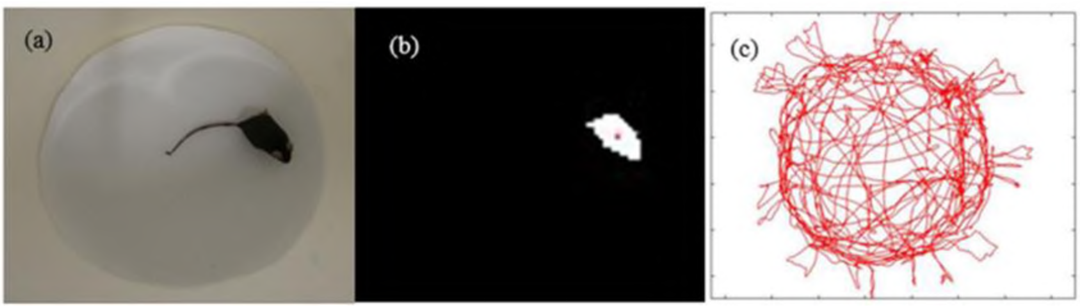


Figure 4. a) Still frame of a mouse in the chamber from our video recording. b) This is what the computer sees from that still frame. It tracks the dot on the mouse to follow the trajectory. c) Final computer output.

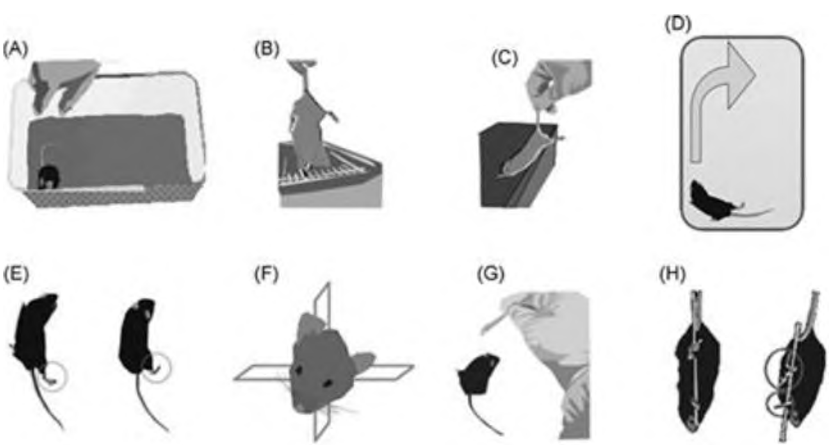


Figure 5. Visual depiction of the SNAP assessments administered to each mouse. A photo representation of each test the mouse was administered as part of the SNAP assessment. A) Interactions. B) Cage Grasp. C) Visual Spacing. D) Pacing. E) Gait/Posture. F) Head Tilt. G) Baton. H) Wire Hang.

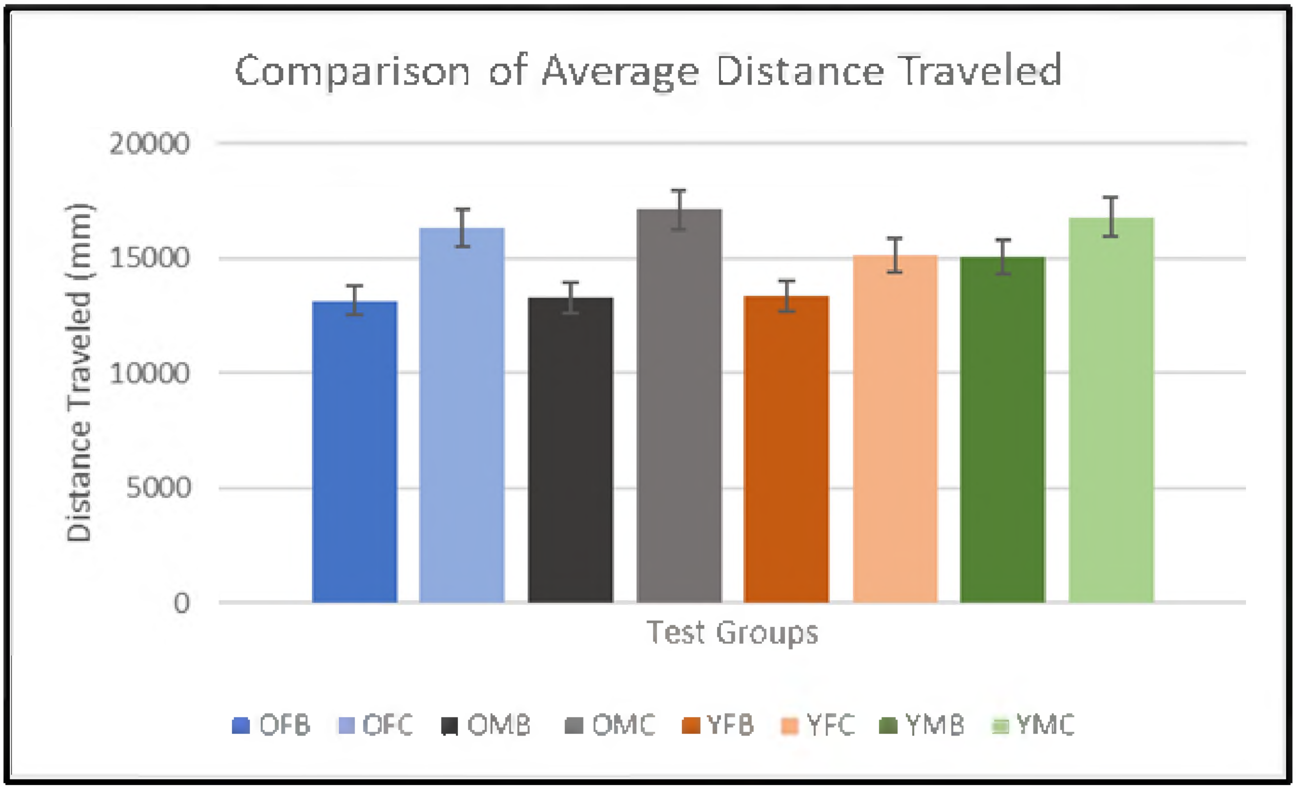


Figure 6. Average Total Distance traveled. This figure shows the average distance traveled for each group of mice, as indicated by the MATLAB evaluation. The average is each group, over all the days of evaluation. We can see here that the blast groups traveled significantly less than the control groups, on average, during the monitoring period.

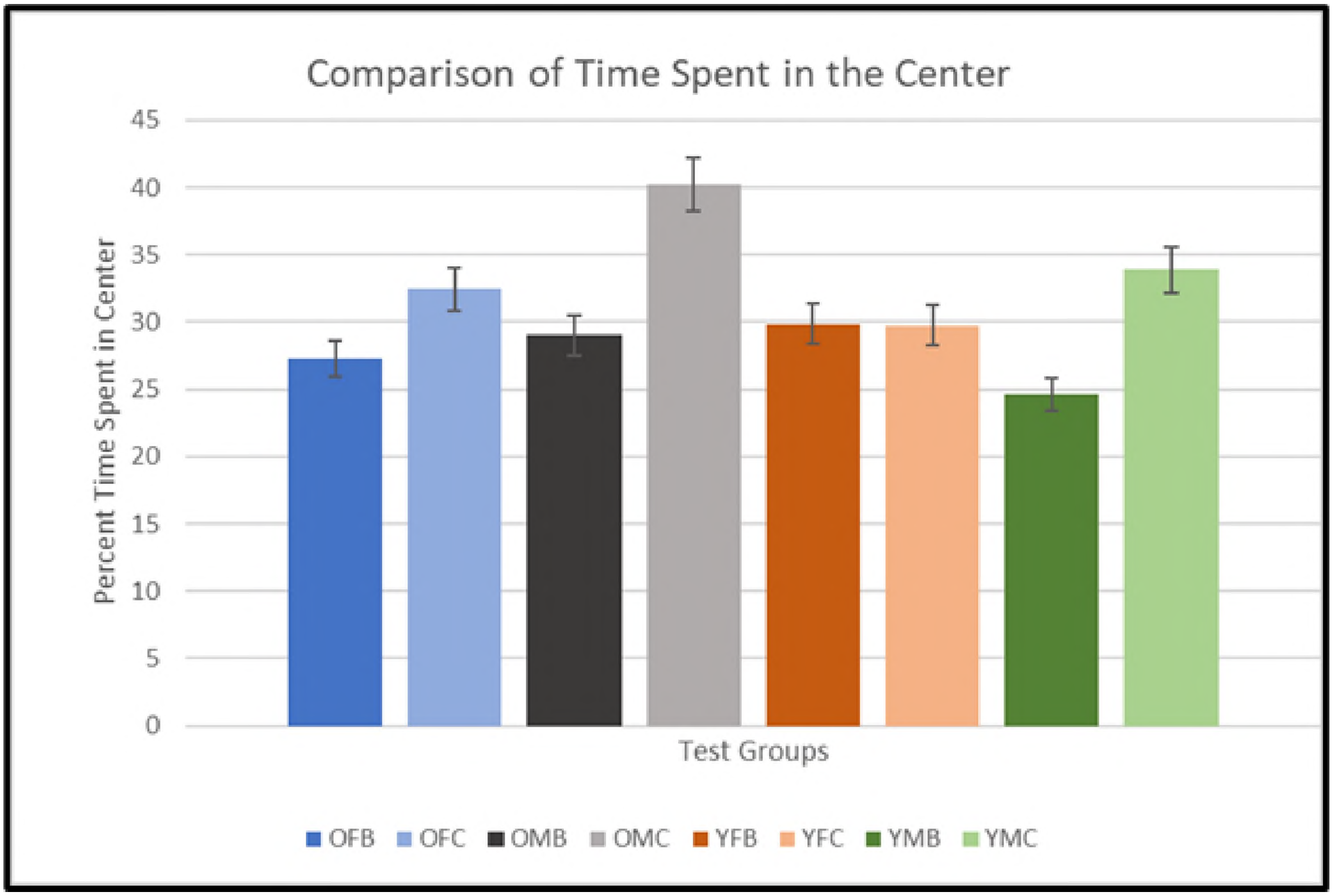


Figure 7. Average percentage of time spent in the center of the chamber, by test group. The average percentage of time the test group spent in the center of the recording chamber is useful in determining injury. A prey animal will not want to be exposed. On average, across the test period, the blasted groups were more avoidant of open areas. The sole outlier was the young female group.

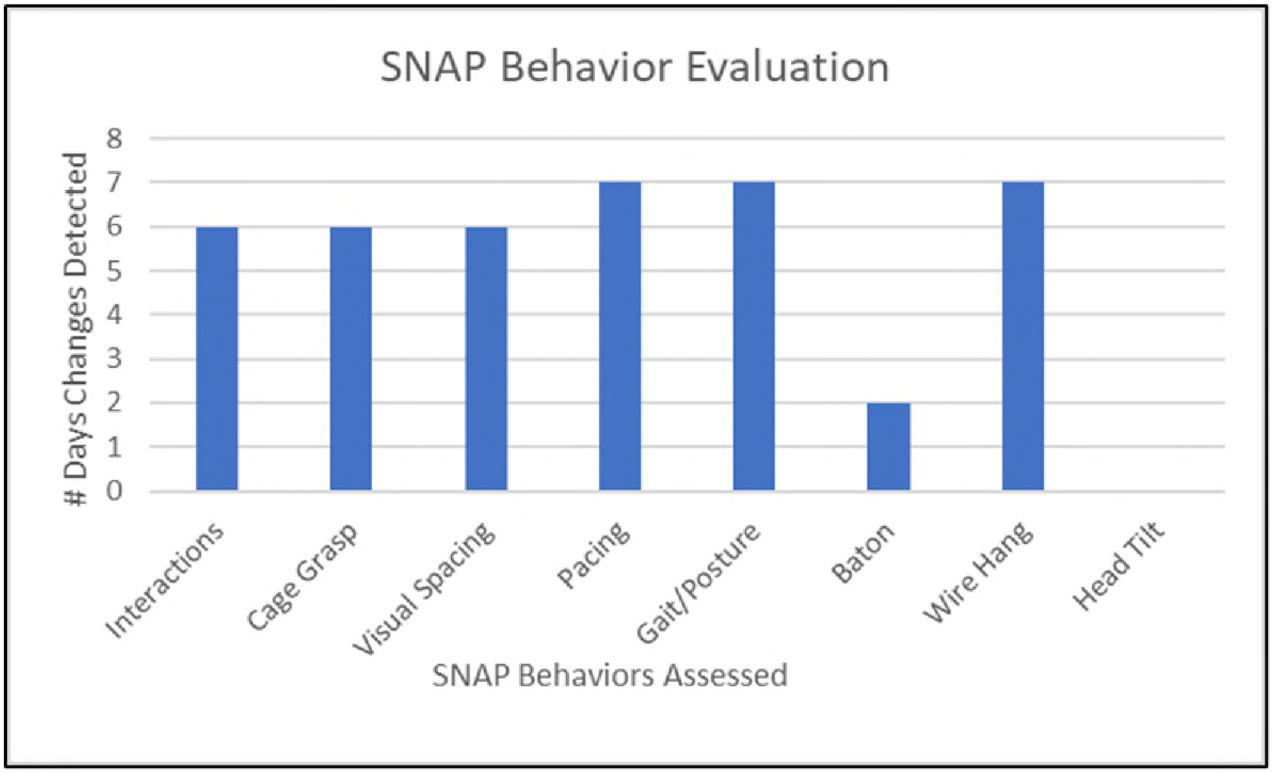


Figure 8. SNAP Behaviors Assessed. This figure shows the SNAP assessments which had scores assigned for the duration of the experiment. Not all behaviors had a score and not all behaviors were exhibited each day of monitoring.

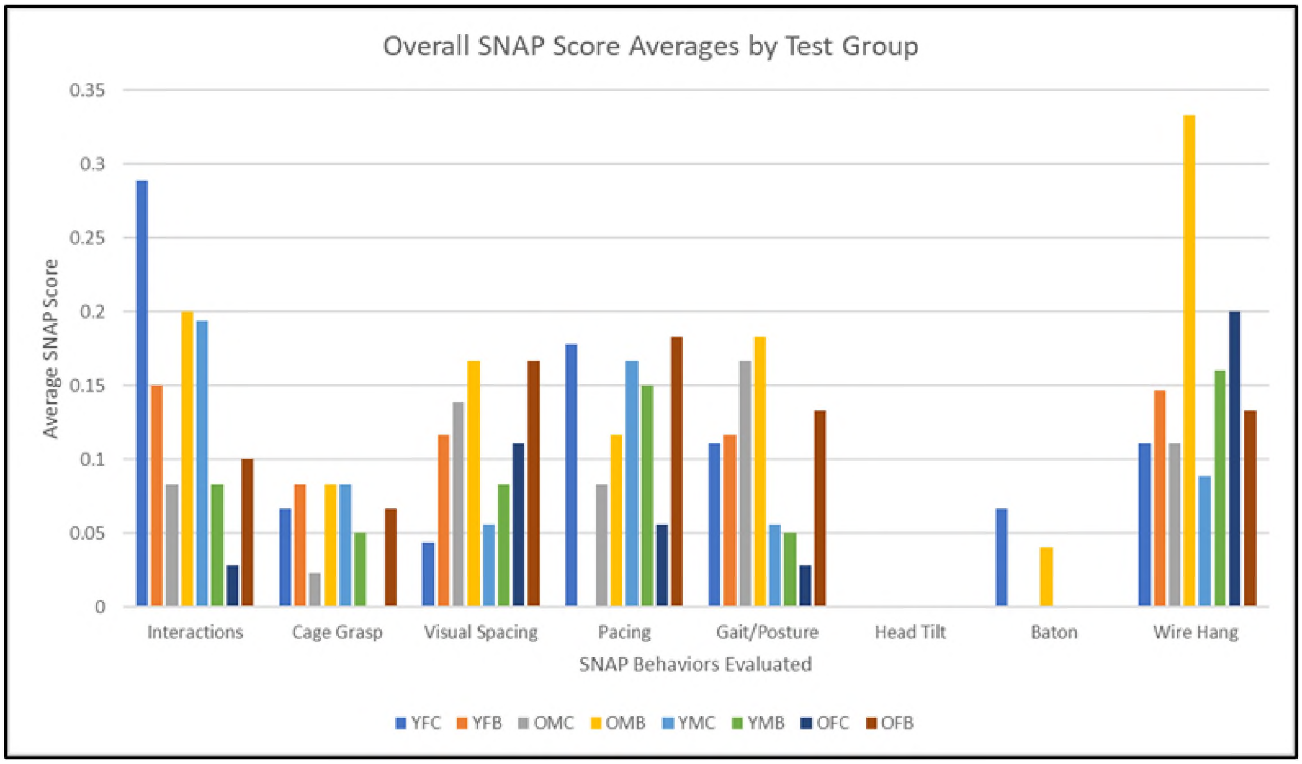
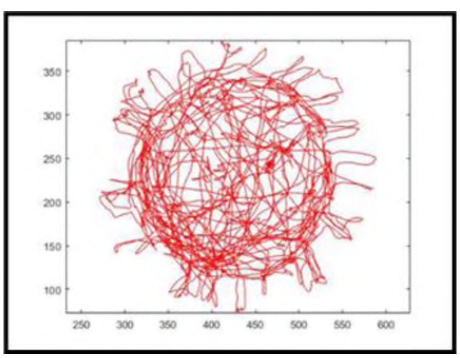


Figure 9. Overall SNAP Score Averages by Test Group. This figure shows the average overall score in each test category for each test group. We see scores exhibited for control groups on most assessments and very low averages across the board, with none higher than 0.35. A score of zero means a normal, healthy mouse. A score of five would be a comatose mouse.



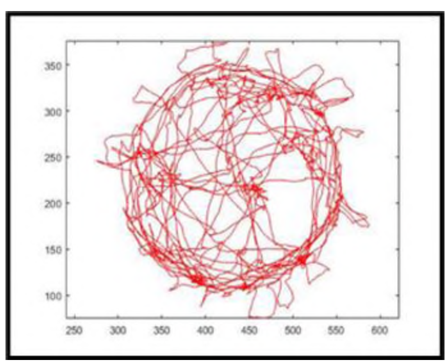


Figure 10. MATLAB output example. Depicts a juvenile SHAM mouse on Day 1 of monitoring and a Blast mouse on Day 1 of monitoring. We see what is considered normal behavior from the SHAM mouse on the left. We can clearly see a drastically reduced activity level of the Blast Mouse on the right. Avoidance of the center area and pacing along the edge is indicative of mTBI.

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