ABSTRACT

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There is limited information about sex differences in mRHI, despite some studies suggesting females participating in contact sports experience more head injuries than males. This study will test the hypothesis that mRHI will lead to more severe neurological deficits in female mice than in male mice. C57BL/6 female mice were assigned to sham and mRHI groups (n=30/group). Lightly anesthetized mice received 25 mild head injuries, once a day (M-F) over 5 weeks using a weight drop model that included a free fall with rotational injury. Acutely, mRHI female mice performed worse than sham injured mice on the balance beam ($F(1,28) = 4.309$, $P=0.0472$) whereas there was no difference in males. 5 weeks and 15 weeks after injury mice underwent a 3-week series of behavioral tests. Both male and female mice in the mRHI groups performed significantly (T-test $P< 0.01$) worse on the Rotarod than uninjured controls. Only males in MWM showed significant impairment on memory for 5-week and significant impairment on spatial learning and memory for 15-week (Probe T-test $P< 0.05$). Only 15-week male mice showed deficits in elevated plus maze (EPM) (T-test $P< 0.05$). Acutely, female mice showed balance deficits that were not apparent in males. Fifteen weeks after mRHI, males no longer displayed deficits in the rotarod, but female mice continued to have a decrease in performance compared to controls (T-test, $P<0.05$). Unlike the males, female mice did not display any significant deficits in the MWM and EPM.

ASSESSMENT OF SEX DIFFERENCES FOLLOWING

REPEATED MILD HEAD INJURY

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THESIS

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CHAPTER I

BACKGROUND AND LITERATURE

In people younger than 45 years, accidental injury has become the major causes of death in the developed countries (8, 43). Traumatic brain injury (TBI) is a major cause of disability, morbidity, and mortality among injuries and causes a significant proportion of all traumatic deaths in the U.S. The general incidence of TBI of developed countries is said to be around 200 per 100,000 population at risk per year (8). According to other studies from multiple cities and countries, children, adolescents, and young adults have a higher incidence rate compared to other groups **(Figure 1)** (8). The increase in incidence among the adolescent and elderly shown in **Figure 1** may be impacted by activities from the outdoors and falling, respectively. Many factors, like increasing awareness of the acute and chronic effects from sports related TBI and U.S. military service members sustaining head injury during deployment, have increased interest in TBI. However, both sporting and military cohorts now show higher risks of mild TBI (mTBI) when compared to the general population (6). In fact, up to 20% of all TBIs, including concussion, are believed to be sports related TBI (SR-TBI) (7). Among the people that had SR-TBI, half of them were children and adolescents (7). Depending on the strength of impact, TBIs can be classified as mild, moderate, or severe. There have been studies that show a relationship with head trauma and multiple degenerative diseases, like Alzheimer's disease (AD), Parkinson's Disease (PD), Chronic Traumatic Encephalopathy (CTE) (4). When looking at the sequelae of cognitive deficits in TBI, **Table 1** shows the acute and chronic sequalae of TBI in

all severity, looking at loss of consciousness, posttraumatic amnesia, memory, attention, processing speed and executive (39). Although most cases in mild TBI and moderate-severe TBI are resolved rapidly after injury, there are still chronic effects that can persist for weeks, months or years (39). Especially among mTBI patients, the long-term effects may not be immediately noticeable since they may not experience any acute symptoms.

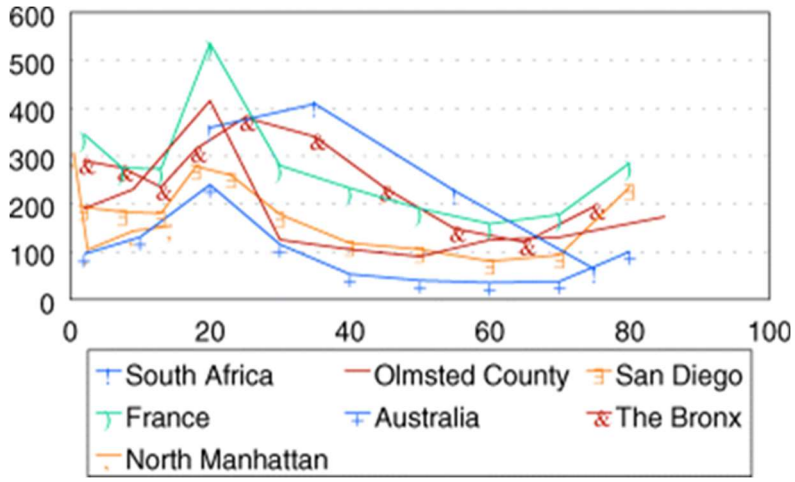

Figure 1: Age-specific incidence of TBI (8). The x-axis is age and y-axis is incidence rate of 100,000 people

	Mild TBI	Moderate-Severe TBI
Acute		
Loss of consciousness	0–30 min	> 30 min
Posttraumatic Amnesia	0–24 hrs	>24 hrs
Subacute and Long-term		
Memory	- Resolves rapidly within 80–85% patients (7, 11–13) May persist in ~15% of patients (14)	- Persists in ~65% of patients (9) - Can include deficits of awareness, reasoning, language, visuospatial processing, and general intelligence (15–17)
Attention		
Processing Speed		
Executive Functions		

Table 1: Acute and Long-term Cognitive Sequelae of TBI by Levels of Severity (39)

Even in sports that have less contact, like soccer, studies show that players that head a soccer ball repetitively or collide with a player show poor neurocognitive performances (5). Having those mild TBIs occur multiple times puts athletes at risk of potential neurological deficits.

Although there has been an increased awareness about mild TBI, there is a lack of information about repetitive mild TBI (mRHI). Not to mention, most studies used male subjects for their research. The lack of female representation limits findings about sex differences in mTBI and mRHI, despite some studies suggesting females participating in contact sports experience more head injuries than males (8).

Severities of a TBI

Depending on the strength of impact, TBIs can be classified as mild, moderate, or severe. In terms of health complications and symptoms from experiencing a TBI of any severity, some TBI patients may experience transient neurological effects, while other patients will experience chronic or life-threatening symptoms that could affect their cognitive and motor function. TBIs can be described as: focal (specific) or diffuse (widespread), primary (direct) or secondary (cascade of events following primary injury), and acute (short/transient) or chronic (long-lasting) (10). Some common acute symptoms from TBIs are headache, dizziness, imbalance, impaired cognition, diffuse vascular and axonal injury, and hemorrhage if severe (10). Repeated mild hits in the head, that usually occur in contact sports, have shown an increased risk in CTE, a progressive condition that occurs when repeated blows in the head happen (13). CTE can result into a decline in memory and cognition, and behavioral changes, like aggressiveness, depression, poor impulse control, and dementia (13).

There are multiple methods that were used to detect a TBI and its severity. One of the major methods used by clinicians to determine the severity of a TBI is the Glasgow Coma Scale

(GCS) (9). There are three parameters used: best eye response, best verbal response, and best motor response. Each parameter is measured from 1 to 5 and the total score is from 3 to 15, 3 being the worst and 15 being the best. Although it has helped access the severity of TBI when triaging and prioritizing patients, this method seems to be effective with patients experiencing acute symptoms and if the victim/patient is effectively communicating with clinicians. Language barriers, intellectual or neurological deficits, and inability/impairment to communicate can affect the objectiveness of the GCS (9). Along with that, the GCS will not be effective for long-term symptoms since it is mainly used after impact, and the symptoms used to describe the scale are acute. Not to mention, some hits, like mild/repetitive hits, could show a high score but have persisting symptoms in the future. According to the CDC, 75% to 85% assessments have been categorized as mTBI with a score of 13 to 15 (10, 11). Impacts that are less severe than a concussion and do not result in overt symptoms, but have subtle neuropsychiatric deficits in functional MRI, are known as sub-concussive (13). Sub concussive injuries are diagnosed by exclusion as a blow to the head that does not result in concussive symptoms. Thus, patients that have a high GSC score will not necessarily be treated since patients that experience little to no symptoms could be cleared to go. Although having a high GCS score may appear good for the patient, there have been studies showing chronic symptoms and impairments after a mild TBI. Fifteen to thirty percent of patients that experience a mild TBI developed prolonged neurocognitive and behavioral changes (10,12). Some of those changes are amnesia, irritability, and slowed reaction time (13). However, a majority, if not all, changes/symptoms are usually resolved and overlooked by patients. Although most symptoms resolve themselves in a few days, some symptoms last longer. If those symptoms have not been resolved within a few months, those persisting symptoms become Post-Concussive Syndrome

(PCS) (12).

Another method used for detecting TBI is diagnostic imaging of the brain. Damage from a TBI can result in both primary and secondary injuries. Primary injuries, like hematomas and traumatic axonal injury (TAI), occur as a direct result from the impact (14). Secondary injuries occur after primary injury and as a biochemical cascade of events, which result into cerebral swelling and herniation (14). As a result, secondary injuries can cause an increase in intracranial pressure (ICP) leading to a reduction in cerebral perfusion pressure (the pressure gradient driving oxygen delivery and nutrition to the brain (CPP, 14). The ICP rise and CPP drop causes the brain to become ischemic, which will cause the cerebral autoregulation induces cerebrovascular vasodilation to maintain blood flow to the brain (14). These effects, however, can be managed and monitored through clinical imaging, like a computed tomography (CT) scan or magnetic resonance imaging (MRI).

Today, traditional CT and MRI scans are used to identify TBI (15). For severe/urgent TBIs, imaging can be critical for diagnosis and management. A non-contrast CT helps identify any intracranial hemorrhage and intra-axial hemorrhage (19). Both CT and MRI scans are proved useful during the hours of injury (first 24 hours to 72 hours), MRI being more superior than CT scan 48 to 72 hours after injury (33). CT scans are proved to be clinically valuable when evaluating for moderate and severe TBI; however, CT scans show limited usefulness when evaluating for mTBI (32). There have been multiple new imaging techniques used to explore evaluating an mTBI and obtaining more information for all levels of severity in TBI. Diffusion tensor imaging (helps detect white matter pathology through the movement of water in the cerebral white matter), perfusion imaging (looks at increased permeability of the brain-brain barrier, vascular injuries, and altered cerebral blood flow), positron emission tomography (PET,

focuses using perfusion imaging and has potential to look at tau/amyloid plaques), and functional MRI (looks at blood flow to depict brain activity and blood oxygen level-dependent signal to demonstrate cerebral function are good examples for detecting mTBI (15,16,41). However, studies also show that standard MRI techniques are not helpful in identifying mTBI in patients who are likely to have delayed recovery (33). A fMRI study, however, found changes in mTBI patients in activation pattern of working memory when compared to a control group (34). Although this information would be useful to detect mTBI with fMRI, the cost/benefit of performing fMRI for mTBI makes it difficult to do more studies and for clinical use since mTBI usually resolves on its own.

Biochemical markers

With the limited usefulness of imaging modalities, using potential biomarkers and biochemical markers showed promise to detect TBI, especially mTBI, in patients even if a CT or MRI scan detects no abnormalities. TBI biomarkers have been looked at for research in helping detect TBIs in an effective and quick manner. There are multiple biomarkers in many locations, like brain tissue, blood, plasma, and cerebrospinal fluid (CSF), that have been used. However, there are limitations to using the right biomarkers and what severity they can be detected from. Currently, there is only one TBI biomarker, glial fibrillary acidic protein (GFAP), that is FDA cleared and rapidly detects injury (18). GFAP is an astroglial marker that usually shows elevated levels within 3 to 34 hours in CSF and serum/plasma after severe TBI and in serum and plasma samples after a moderate and mild TBI (19). GFAP is an important intermediate filament protein in astrocytes that helps maintain homeostasis in the brain (61). Astrocytes function in axon guidance, synaptic support, and controlling the blood brain barrier (BBB) (34). GFAP is mostly released from injured brain tissue into biofluids, like CSF and plasma, after a

TBI. GFAP has the potential to be used as a disease prognosticator and a diagnostic tool since it allows reduces the usage of a CT scan and determine the likelihood of mid- and long-term prognosis (19). Multiple studies have shown GFAP levels correlate with outcome after TBI. Increased levels of GFAP in patients with TBI corelated with clinical outcomes (20). Serum GFAP were also significantly higher in patients who died or experienced an unfavorable outcome and predicted the neurological outcome at six months (21). Studies also have shown high levels of GFAP were predictive with increased mortality (52). Although the FDA approved diagnostic tool can be used to rule out the need for a CT scan, it cannot clear a patient for having an mTBI or any TBI in this case. Not to mention, GFAP is known to peak withing a couple of hours of injury and remain elevate for only a few days (**Figure 2**) (19).

There have been multiple protein markers, however, that can be used for long-term detection, like phosphorylated tau (P-Tau), and ionized calcium binding adaptor molecule 1 (Iba-1). These markers have been shown to elevate in months or years after the impact, which can help detect chronic/subacute TBI that can be left untreated if not diagnosed (**Figure 2**) (19). There has been increasing evidence that TBI may be a risk factor for the development of age-associated neurodegenerative disorders, like Alzheimer's Disease, Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Multiple Sclerosis (MS). A TBI can initiate the protein aggregation-related seeding event leads to protein aggregation accumulation, which can result to a neurodegenerative disorder. In autopsy studies, moderate to severe TBI have resulted in increased amyloid deposition in the brain (19). Another study showed that a deposition of P-Tau occurred after a TBI (22). Elevated levels of P-Tau have been detected in the brain after mRHI had occurred (23). Not to mention, elevated levels of P-Tau not only stay in acute and subacute periods, but in chronic periods. P-Tau has the potential to be used as a monitor for

development of CTE, AD or PD. P-Tau can also be a predictor of poor outcome when levels are elevated (19).

Iba-1 is a microglial marker that can persist in elevated levels after a TBI. When a TBI occurs, microglia proliferate and cause neuroinflammation from a secondary injury mechanism (45). Studies have shown that microglial activation found to last for many years after a TBI, particularly a moderate/severe injury, and microglial activation occurred in the injured cortex (4). Iba-1 would be a good injury indicator of microglial inflammation and neurodegeneration.

Tight junctions (TJ) play a critical role in maintaining the BBB. The BBB functions to keep cerebral homeostasis functioning. Dysfunctions in the BBB, like alteration of TJs at the BBB, can affect proper neural function and lead to progression of neurodegenerative diseases (57). When a TBI occurs, the BBB can leak and cause increased expression of tight junctions. Tight junctions that are important to study their effects following a mRHI would be: occludin and claudin (claudin 5). Claudins are the principal proteins that establish the backbone of TJ, and they are critical determinants of paracellular tightness between the adjacent BBB endothelial cells (58). Claudin is also known to be the dominant TJ in the BBB. Studies have shown that deletion of claudin-5 in mice have normal TJs in the BBB but can lead to detrimental brain defects and prenatal death (59). Another study showed that there was an overexpression of claudin in cultured brain micro vessel endothelial cells that caused increased paracellular tightness, which suggested that expression levels of claudin correlate with TJ function (53). Another TJ that would be important to study is occludin. Occludin is a transmembrane TJ that works with claudin to maintain the BBB (58). Occludin plays an important role as a regulator of TJ assembly and functions (56). A study that mice with occludin knocked out have normal TJ function but display histological abnormalities (61).

It is important to assess these TJ to determine whether the proteins increase or decrease expression and how it affects the BBB following an mRHI TBI. A study showed decreased expression levels of TJ-associated protein, like claudin and occludin, after a TBI (54), which could cause BBB leakage and exacerbate neurological deficits in the brain. However, another study showed increased levels of occludin following a mTBI (first 8 hours after injury) (62). A rise in occludin levels can help maintain the BBB from leaking after an mTBI, which could decrease the effects that an mTBI could have if hit.

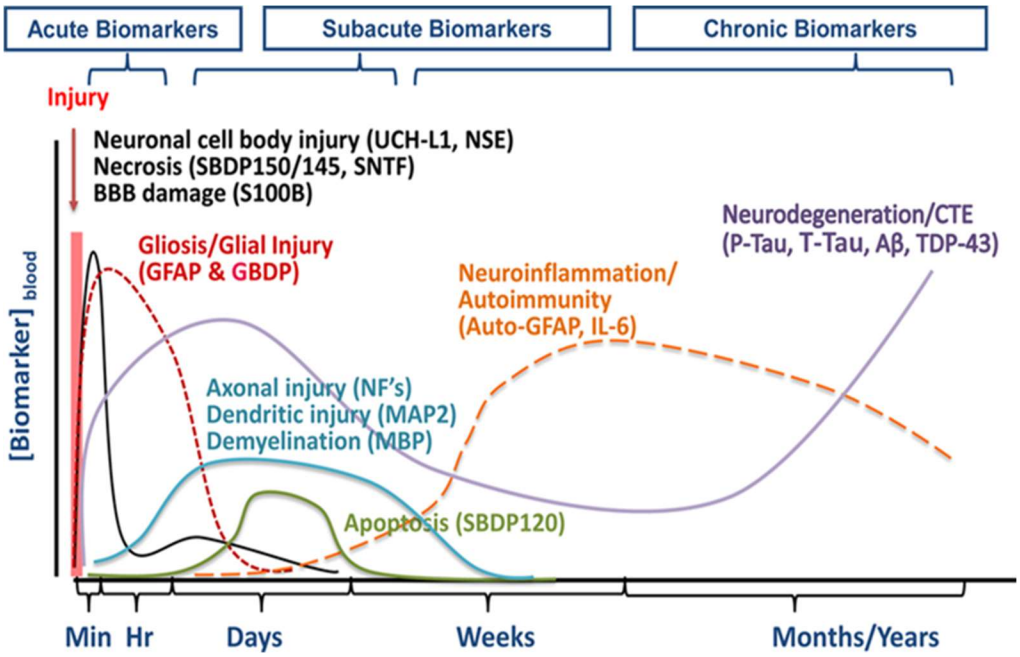

Figure 2: A continuum of protein biomarkers in tracking different phases of TBI (19)

Mouse Models for Mild TBI

There have been multiple models used for TBI studies. These include fixed-head and free-movement models, open skull and closed skull models, focal impacts and diffuse impacts, and a variety of tools for providing impact (pneumatic pistons, weight drops, pendulums, etc.) (42, 43). Injuries may also differ in location (front, top, lateral aspect of the head) or protection of the head as with the use of disks or helmets to protect the rodent's head from impact (42, 43, 44). **Figure 3** summarizes models used in different studies along with procedures done to

rodents, type of impact tip and how they fall after impact (42).

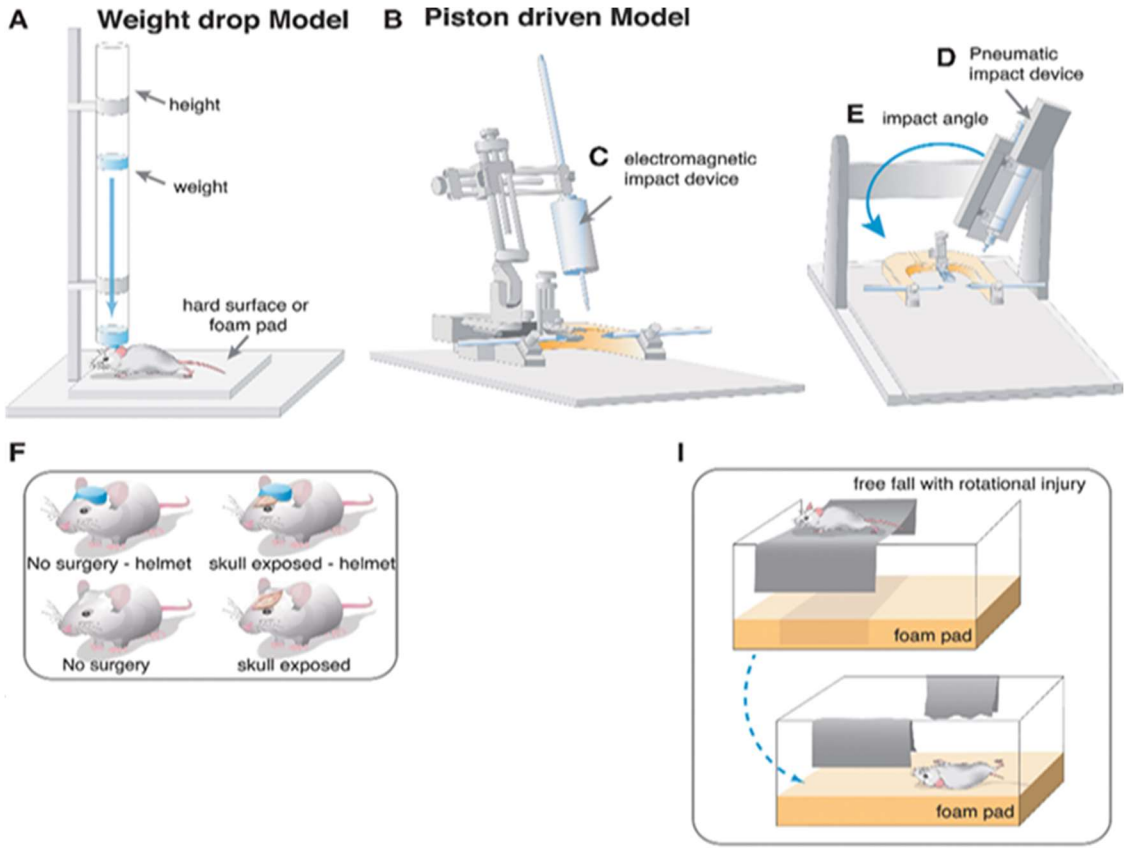

Figure 3: Example experimental set-up for weight drop and piston mTBI models. In the weight drop model **(A)**, a variable weight is dropped from a variable height onto the head of the animal and the animal can be on either a hard surface or a foam pad. Piston driven models **(B)**, use either an electromagnetic **(C)** or a pneumatic **(D)** driven piston that is set to a specified velocity and impact depth and strikes the head of the animal. In piston driven models, the impact angle **(E)** can vary between study designs. In both the weight drop and the piston driven models the impact surface can vary with either a helmet or no helmet on the intact scalp or the exposed skull **(F)**. An emerging model utilizes rotation following impact by placing the animal on a thin sheet following impact the animal falls through the sheet onto a foam pad **(I)**. (42).

Although most models have been shown effective in modeling effects of mild, moderate, and severe TBI, they do not all reflect in realistic in terms how someone receives a sport-related mRHI. A patient that receives a mild TBI will not have an open skull and usually has less of an effect on them. Along with helmets, this does not best represent sports that use no helmets, like soccer or basketball. A most sound representation from a mild TBI in sports would be using free fall with rotational injury since most injuries will involve a form of rotation motion upon

impact as shown in **Figure 3I**. Therefore, this is the model chosen for my studies.

Sex differences

In sex difference studies, there has been a lack of female representation for TBI when related to sports or mild and repetitive hits. As a result, there is not much information looking at how differently both sexes will experience a TBI, whether it is a mild or severe hit.

When looking at different outcomes, there can be multiple ways to interpret outcomes for sex differences. One may do a retrospective observational study and look at incidence in TBI complications and mortality, while other studies could use a Glasgow coma scale. An observational study in Japan looked at 51,000 TBI patients between 2004 and 2018 (25). They used odd ratios in mortality and post-injury complications between males and females with age groups. Results show males experience higher mortality than females, specifically among younger (10-19) and older (60+) generations (25). Elderly males experience more TBI complications than elderly females. However, the causes of trauma in the study were from vehicle accidents and falls, which can typically have moderate to severe TBI. The study does not indicate much about mRHI or mTBI, especially among contact sports. According to other studies, females sustain more concussion compared to males, especially in sports (23,24). The mortality rate in TBI for males and females was reported as 1.6% and 3.4%, respectively (27). When compared to the Extended Glasgow Outcome Scale (GOSE) and Functional Status Examination (FSE), females had worse outcomes than males in both assessments (28). When compared to post concussive syndrome (PCS), female sex had a significantly higher odds of poor outcome after a mTBI, especially for women who are bearing a child (29). However, there are multiple studies having mixed outcomes in terms of symptoms and behavioral tests. According to a study by Gupte, among all human studies, 47% of the studies reported worse

outcome in women than men, 26% of men reported better outcomes in women, and 18% found no sex differences (4). Multiple factors may impact why there are mixed outcomes like selecting the type of subjects of TBI, what kind of TBI they got, and what kind of tests were done to determine the outcome. In animal studies, most of the studies are male mice or rodents only, which impairs how different types of TBI models and severity affect female mice as well. Some studies have shown that female rodents outperformed male rodents in rotarod performance and performance recovery; however, there were multiple models that different studies used with different behavioral outcomes, like female mice performing better in controlled cortical impact model but male mice performing better in enriched environment (CCI) (30,31). Given how most TBI occurs in sports and in younger ages, it would be ideal to use a repeated mild TBI model by using a weight drop model with free fall since it best represents that population at risk (3,8,23).

In a sports study that reviews sex differences from sports related TBI, it looked at multiple results showing no sex differences and sex differences. Multiple studies used an imPACT testing battery, which tests for neurocognition, with a graded symptom checklist (26). These studies, including one study that showed females sustained significantly more concussions than males, show that there were no significant sex differences in concussion symptoms and neurocognition. One study that used imPACT found that sex differences in symptom, reporting, symptom severity, and time to become asymptomatic, while other imPACT studies showed females experienced poorer performance in visual memory tasks and neurocognitive testing (26). The summary of those studies shows that females will also be vulnerable to the development of post-concussion migraines. One area that the review used to measure output was cognitive function, which showed that females experience worse cognitive functioning

(visual memory, reaction time, and emotion cognition) in females compared to males after a sports-related concussion. The trajectory of recovery was also reviewed, in terms of recovery time and symptom severity, where studies indicated that females are at risk for increased severity and prolonged recovery after a sports-related concussion (26). Studies also used vestibular-oculomotor function and gait performance to test concussive effects. It showed that females recovered more slowly in both dizziness and vestibular-oculomotor function along with higher vestibular ocular reflex scores than males (26). In gait performance, the authors of that study suggested that executive functioning may be affected between sexes, where concussed females showed greater change between single-task (walking down a hallway) and dual-task (walking down a hallway while concurrently completing a cognitive task) conditions for step rate variable when compared concussed males (26). Fluid biomarkers were done in studies to see potential biomarkers to be used. A study showed that males exhibited significantly higher baseline concentrations of UCH-L1 and S100B than females, while females experienced higher levels of CNPase than males (27). Females also experienced higher levels of t-tau and worse outcomes after a sports-concussions. Although these studies showed most outcomes demonstrate that females experience worse outcomes in multiple areas, these studies examined these outcomes within a few weeks after a concussion, which lacks information about the long-term effects of TBI. Not to mention, most sport-related hits won't be classified as concussive but can be detrimental to neurocognitive functioning.

CHAPTER 2

SPECIFIC AIMS

Because studies in football and soccer players reveal that players can receive hundreds of non-concussive head impacts each season without concussive symptoms, the potential role for such injuries in long-term neurological outcomes is an important public health concern. Understanding the progression of injury, including neuropathology and peripheral biomarkers could enhance the ability to monitor participants and reduce the risk of developing chronic neurological injury. Importantly, most preclinical studies have been performed in males since the participation of male humans in contact sports (i.e., American football and ice hockey) is much greater than that of females (23). Nevertheless, an increasing number of girls and young women are now participating in contact sports and studies suggest that they experience a proportionally greater likelihood of concussion.

Previous studies in our laboratory have shown that male mice experience early motor and delayed cognitive deficits after experiencing repeated mild traumatic brain injuries (mRHI). Along with motor and cognitive deficits, we observed an increase in the expression of phosphorylated tau (Phospho-Tau) and glial fibrillary acidic protein (GFAP) in the cerebral cortex (CTX), a biochemical marker for neurological disease. The role of sex differences in TBI outcome in animal models is mixed, with some studies showing worse outcomes in males and some showing worse outcomes in females (4). However, published studies have not focused on the kind of repetitive mild injuries that are prevalent in sport. The objective of my research is to

use the mRHI model used in previous studies to assess whether there are sex differences between male and female mice. A secondary goal is to also determine if female mice experience any motor and cognitive deficits from the mRHI model.

Specific Aim 1: We will test the hypothesis that mRHI will lead to more severe neurological deficit in female mice than in male mice.

Specific Aim 2: We will test the hypothesis that sex differences in neurological function will be reflected in biochemical differences in male and female mice.

SIGNIFICANCE & INNOVATION

Significance

This proposal is **significant** because it will provide insight into whether there is a sex difference in the mRHI model between male and female. From what the background has stated about sex differences, females are also likely to have worse outcomes, like worse cognitive function, vestibular-oculomotor function, and recovery, after an mTBI than males would. There have been multiple studies that show that females sustain more concussions than male (23,24). A study by Gupte showed that 47% of studies reported worse outcomes than men, compared to 26% of men reported better outcomes in women, and 18% found no sex differences (4). However, these studies are not accounting for effects of repetitive hits in the head. Injuries like an mTBI can happen more than once, especially in contact sports and military combat. When looking at sports studies, women also perform worse in neurocognitive functioning (visual memory, reaction time, and emotion cognition) (26). Although sport-related TBI (SR-TBI) are usually mild and repetitive, most sport studies assess the acute effects, rather than the chronic effect. Although, acute symptoms may be important for moderate/severe TBI, mTBI usually little to no acute symptoms. However, fifteen percent of those mild cases can have chronic/subacute symptoms that can persist for months or years. Injuries like an mTBI can happen more than once, especially in contact sports and military combat. With an increasing rise in women playing in contact sport and participating military combat, it is important to assess the acute and chronic effects of mRHI in females and assess the sex differences between them.

Innovation

This study is **innovative** for a few key reasons: 1) We used a mRHI model that includes a trap door for rotational injury (42). When looking at most rodent models, some would use a piston driven model, or a weight drop without rotational injury. However, these models don't best represent what a mild TBI should feel like. A mild TBI, especially from contact sports, will have rotational injury to the head upon impact. It's very important to use a model that represents what an mTBI would feel like. 2) We are measuring the effects of chronic symptoms five weeks/fifteen weeks after repetitive hits in the head have been performed. Most mTBI show little to no acute symptoms, so it is essential to look at the effects of chronic symptoms since they can persist after an injury. Assessing these chronic symptoms helps us assess whether the mRHI model leads to motor and cognitive deficits following a rest from the hits. 3) We are looking at protein markers, like GFAP, P-Tau, and Iba-1, to see whether there is a difference between the mRHI and sham groups. The GSC scale is mainly based on observations and symptoms when assessing a patient with a TBI. However, if a patient gets a score of 13 or higher or if the patient is unable to communicate well, it would be very difficult to diagnose someone with a TBI. Biomarkers have the potential to assess whether someone has a TBI or not, and these biomarkers can help indicate any chronic symptoms or neurodegenerative disease.

MATERIALS AND METHODS

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of University of North Texas Health Science Center in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Two cohorts of C57BL/6 female mice were used for this study. Cohort 1: Thirty female mice were housed in groups of five. Two groups of fifteen were randomly assigned into a sham control group or mRHI group. This cohort will have a 5-week rest period after hits, shown in figure. Cohort 2: Thirty female mice were housed in groups of five. Two groups of fifteen were randomly assigned into a sham control group or mRHI group. This cohort will have a 15-week rest period

after hits, shown in **Figure 4**.

Experimental Design

Weight drop model: For five times a week until five weeks, Monday to Friday, the mice are taken to a weight drop model including a free fall with rotational injury to test the effects of repetitive mild TBI on female mice **(Figure 5)**. The weight drop model is used to impact the dorsal side of

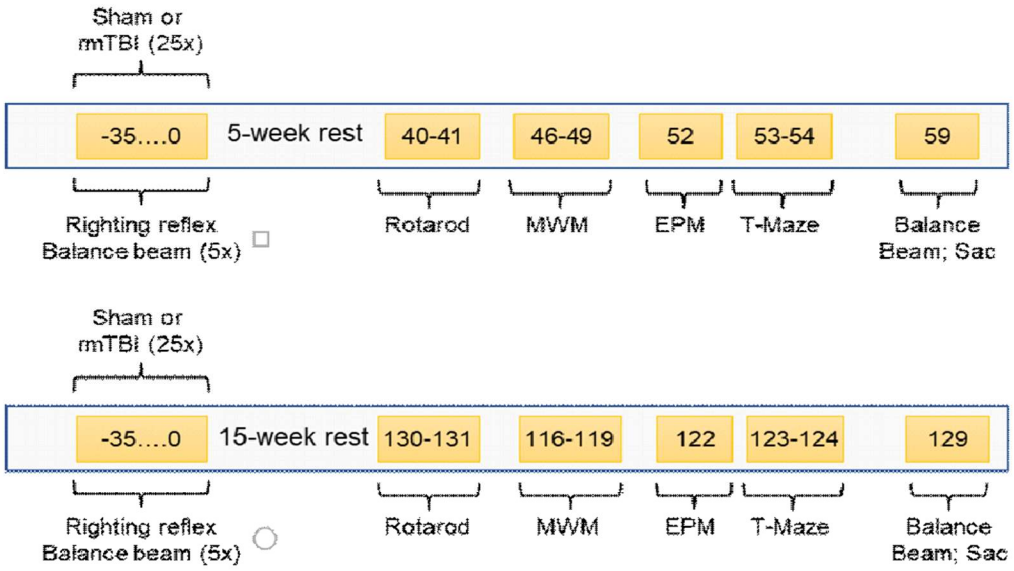

Figure 4: A timetable for how the female mice will be experimented on in the order of what experiment will be done. Numbers stand for **days**. MWM (Morris Water Maze), EPM (Elevated Plus Maze), and Sac (Sacrifice). **(A)** Cohort 1 with female mice having a five-week rest. **(B)** Cohort with female mice having a fifteen-week rest.

the head while anesthetized. Upon the impact on the head, a trap door opens below them to demonstrate the impact of angular acceleration from an impact in the head. People who experience mRHI will feel a sudden change in rotational velocity upon impact. These types of hits are common in contact sports and military combat zones. The trap door used for free fall replicates the rotational injury that occur in mRHI. Two groups are evaluated, sham and TBI group, while the sham groups are not hit with a weight and only dropped through a trapdoor. Once fallen, both groups were taken to a cage on their backs to time how long the mice wake up due to their righting reflex. People that experience a TBI usually have loss of consciousness upon

impact. The righting reflex allows us to see if female mice experience loss of consciousness from mRHI.

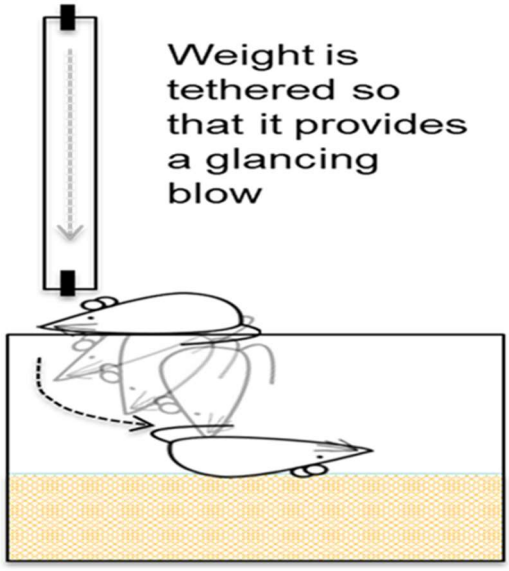

Figure 5: mRHI model. A **65-gram** steel weight is dropped through a tube to strike the head of an anesthetized mouse. The force will open the trap door open under the mouse and allows head acceleration. The mouse flips 180° and lands on a soft foam bed.

Experimental Procedures

Behavioral tests: Multiple tests were performed to observe the acute and chronic effects following an mRHI hit from the model. The righting reflex and balance beam are both tests that observe acute effects that can occur from an mRHI. The righting reflex is a measure for loss of consciousness. When laid on their backs, mice usually right themselves back to their front. However, the anesthesia and impact cause the female mice to not perform the reflex immediately, which allows us to observe the effects from loss of consciousness. In all severities of TBI, patients experience loss of consciousness after a TBI (<30 minutes for mild and >30 minutes for moderate and severe). The balance beam is a measure of vestibular function and sensory motor integration (30). It involves the brain vestibular system and motor control areas, such as the cerebellum. A study showed that 87% of 111 patients experienced vestibular symptoms, like feeling unbalanced, gait ataxia, etc. (31). At the end of the week, we performed the balance beam 5 minutes after the

mice have completed the righting reflex. The balance beam has two platforms and a circular rod that is connected to them. The mice were placed on the rod to see how long they stay on the rod or reach the platform. We collected the time at which the mice fell or reached the platform. Once the impacts were made and the balance beam was conducted for the female mice, we waited five weeks to test the cognitive and motor skills of each mouse to test the chronic effects of mRHI. The following behavioral tests used were rotarod (RT), Morris water maze (MWM), elevated plus maze (EPM), and T-maze, respectfully in that order.

In rotarod, the female mice stood on a spinning rod that accelerated to 4 rpm in 5 minutes **(Figure 6)**. The mice did their best to stay on the rod and were timed for how long they stay on as the rod spins. Once the mice fell off, their times were recorded. Rotarod tests for motor coordination in female mice (32). The cerebellum is responsible for motor coordination and “fine-tuning" movements. Studies have shown that mice following a mild TBI had deficits in motor coordination (38). The mice performed these tests for four sessions. Data was analyzed over a period of four sessions and average of all four sessions when compared with sham and mRHI groups.

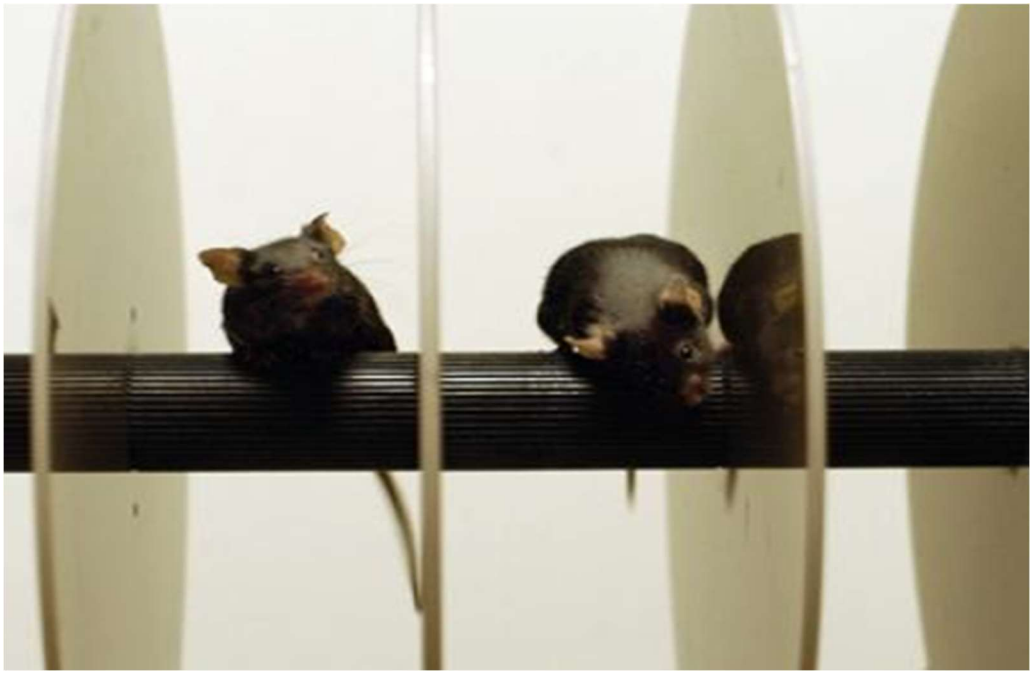

Figure 6: Rotarod. Mice stand on the spinning rod (**accelerating to 4 rpm in 5 minutes**) as time was recorded for how long the mice stayed on the rod.

In MWM, the female mice were placed in an oblique pool of water where a hidden platform was also used **(Figure 7)**. Mice can typically recognize water depths and tend to avoid swimming in deep water (1), so the female mice would want to find that hidden platform. Once placed in the water, the video camera, placed on top of the pool, tracked motion of the female mice in the water until they found the hidden platform or two minutes passed. The video camera analyzed the distance it took the female mice to reach the platform. The camera followed the motion of the female mice and analyzed the motion into a path length Four sessions were performed, and in each session, the female mice and hidden platform were placed in different quadrants to allow the mice to learn from a different location. MWM tests for spatial learning (2). The hippocampus is one of the key brain areas for spatial learning (3). A study showed that some male mice that experienced mild and severe TBI spent less time in a target quadrant compared to the sham group (35). However, the model used in this study will be different since our MWM will include using a hidden platform. Three parameters were measured: path length, learning index, and time spent in

annulus **(**40cm**)**. Path length measures the distance it took to reach the target platform. The lower the path length, the less amount of distance it took for the female mice to reach the target. The learning index was calculated using the average of the last three sessions. The learning index measures how well the female mice remembered where the platform was. The lower the learning index, the better the mice performed in understanding where they were and where to go. Last, time spent in annulus was measured for how the female mice stayed in a 40 cm region, where the hidden platform was. Time spent in annulus allowed us to see if female mice learned to go to that target area due to the hidden platform used to being there.

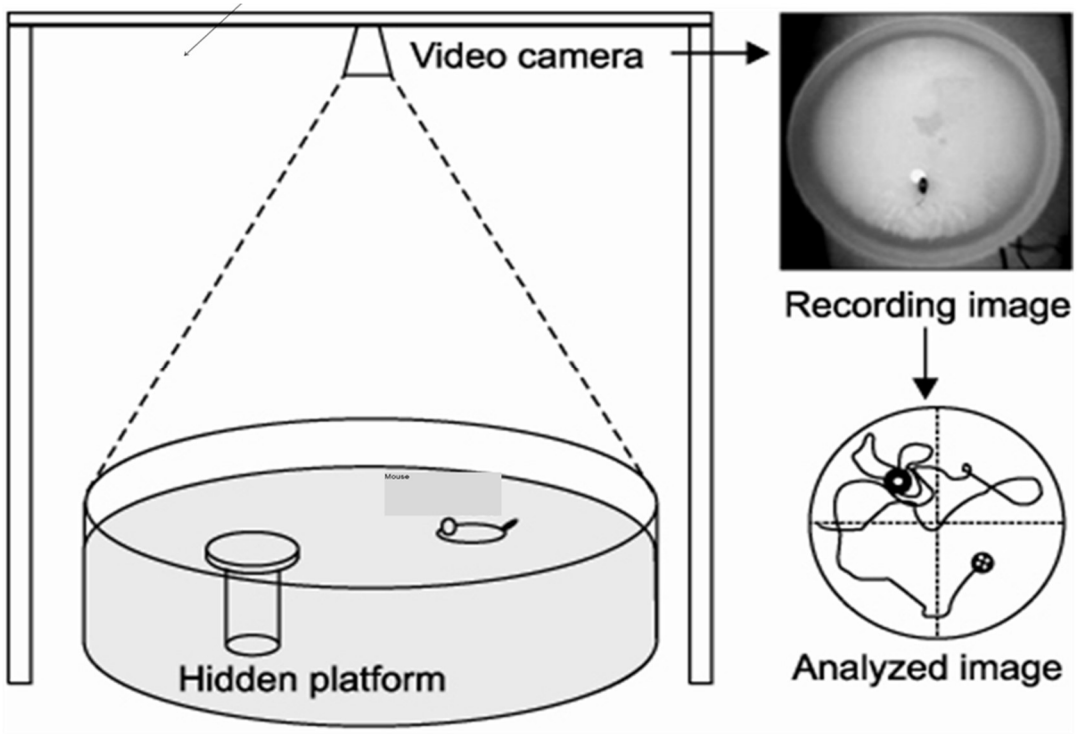

Figure 7: Morris Water Maze. Mice must learn to swim to a platform hidden below the surface of an opaque pool. A video camera is placed on top of the pool to observe the motion of the mice in the water. The recorded imaged will be analyzed to sketch path lengths of where the mice are going.

In EPM, the female mice were placed in a plus-sign shaped platform with 2 sides being open while the other 2 sides are closed with walls **(Figure 8)**. Time spent in the open arms was measured and calculated into a percentage out of the total time the female mice spent in the apparatus. EPM tests for anxiety-like behaviors from mice that are based on mice's natural

aversion for open and elevated areas (36). When mice are no longer having a natural aversion to open arms, the female mice have an increase in disinhibition. The more the female mice spent on the open arms, the more disinhibition that the female mice will show. The frontal and temporal lobes affect the disinhibition of such behaviors. A study showed that a patient with a TBI showed severe disinhibition including violence (37). He attacked the nurses without provocation and started kicking and shouting when being asked questions in bed (37). The patient was impulsive and couldn't control his behavior. Using EPM would allow us to test if mice exhibit disinhibition following an mRHI.

Figure 8: Elevated Plus Maze. The apparatus has **2 open arms and 2 closed arms**. Mice typically avoid open arms due to their natural aversion.

In T-maze tests, female mice were placed in a T-shaped apparatus and were lightly shocked until they move the right or left arms **(Figure 9)**. An arm was assigned for where the female mice were supposed to go. If they did not go to the target arm after a couple seconds, a shock was administered to the female mice until they reached the desired location. The objective for the

female mice was to find the understand that the target arm was desirable and anywhere else was not (active avoidance). In previous studies from our lab, T-maze showed male mice progressively took more trials to meet criteria as they received more hits. However, behavioral tests were performed immediately after the injury, giving no resting period for the mice. T-maze was measured by trails to criterion, meaning the number of trials it took for the female mice to get that criterion. The female mice had to attempt four out of the five trials, by turning to the correct arm and avoiding a shock, to reach a criterion.

Figure 9: T-Maze. Mice are trained to make a **preemptive response** involving **a simple discrimination** (turning to the correct arm of a T-maze), to avoid a **punishing stimulus** (shock to the feet).

Brain tissue collection: Upon finishing behavioral data for the female mice, a final weighing and balance beam were performed. After the tests were complete, thirty-two female mice, sixteen each from the sham and mRHI groups, were randomly chose to be sacrificed and collected the brain tissue. The brain tissue was cut in pieces to retrieve the female mice's cortex (CTX) and

hippocampus (HP). The CTX and HP of the female mice brain were used to collect protein from the brain tissue for western blot data. The remainder of the female mice were also sacrificed and collected their brains intact. Those brains will be cut to small pieces for immunohistochemistry and stained to observe any increased levels of expression.

Western blotting: Protein was extracted from brain tissue in a solution of tissue protein extraction reagent (TPER, Pierce) and HALT protease inhibitor (Pierce). Protein concentrations were determined using a commercial kit (BCA, Pierce). Equal amounts of protein were separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad 4-20% gradient gels. Proteins were transferred to a nitrocellulose membrane for western blotting. The distribution of samples for the gel was three sham/three mRHI. Chameleon Duo pre-stained protein ladder was the marker used to help visualize gel migration. Proteins were transferred to a nitrocellulose membrane for western blotting. The total protein was then quantified using a total protein stain kit (LI-COR) and scanned using the LI-COR Odyssey Imaging System. The membrane was also blocked with Interpret TBS blocking buffer (LI-COR). GFAP polyclonal antibody (Thermal Fisher, 1:1000), P-Tau polyclonal antibody (Thermal Fisher, 1:1000), Iba-1 (Wako, Fuji Film 1:1000), Occludin polyclonal antibody (Invitrogen, 1:1000), and Claudin 5 polyclonal antibody (Invitrogen, 1:1000) were used as primary antibodies to detect the target proteins in the membrane. Goat anti-rabbit (H+L) cross-absorbed secondary antibody (Invitrogen, Dylight 800, 1:20,000) was used to bind to the primary antibody and emit a fluorescence. Washes were performed using a 1X TBS buffer for 5 minutes. Once the protocol finished, the membrane was scanned using the LI-COR Odyssey Imaging System. The scanned images were analyzed using Image Studio and Empiria Studio. Using the total protein stain images, normalization of each sample was performed.

Immunohistochemistry: Deeply anesthetized mice were transcardially perfused with saline and 4% formaldehyde. Brains were removed and post-fixed in 4% formaldehyde for 48 hours before being moved to sodium phosphate buffer for storage. Coronal 30 μm serial brain sections were made with a vibratome and stored in cryoprotectant solution at -20C in 12 well plates.

Free-floating serial sections (1:6) were processed for immunohistochemistry for Iba-1 (Wako, Fuji Film 1:1000). Staining was revealed with biotinylated donkey anti-rabbit secondary antibodies (Jackson Immunochemicals) and Vector Labs ABC Elite avidin biotin kit (#6100) followed by Vector Labs IMMPact DAB kit. All sections were run simultaneously and mounted on gelatin coated slides. Slides were dried overnight and coverslipped with DPX mounting medium (Sigma). A treatment-blinded observer identified sections between -1.34 and -1.58 mm Bregma for each animal. The optic tracts and corpus callosum were identified with phase-contrast microscopy and outlined using Neurolucida software (Microbrightfield) on an Olympus BX60 upright microscope using a 10x objective. Individual Iba-1 positive cells were identified and marked within these areas using a 20x objective under brightfield illumination with Neurolucida.

Cell counts and area size were calculated using Neuroexplorer (Microbrightfield). Cell density (cells/μm2) was averaged across the sections counted for each animal.

Statistical Analysis

Group differences (mRHI vs sham groups) in single endpoint measures, like mean latency to fall, learning index, trials to criterion, and percent time spent in open arms, were determined by student's T-Test. Group differences that occurred over time, like latency to fall and path length, were determined by one-way analysis of variance (ANOVA) with repeated measures followed by, if needed, a Sidak's post-hoc test (factor 1: group membership). For sex differences and time spent in annulus, a two-way ANOVA with repeated measures followed by Tukey post-hoc test

was used (factor 1: group membership) (factor 2: sex for sex differences/sessions for time spent in annulus). Values are presented as mean± SEM. A one-tail statistical analysis was done for t-test, one-way ANOVA, and two-way ANOVA (for MWM). Multiple studies have shown that people and animals that experience a TBI or head injury (HI) will show neurological deficits. If compared to the sham group, studies support that mRHI group would perform worse and show more severe deficits after a head injury.

Power analysis: The number of animals was estimated to calculate the necessary number of female mice need in each experiment to achieve a power of at least 0.80 with a probability of a Type I error of 0.05.

RESULTS

The primary objective of these experiments was to investigate the effects of mRHI in female mice when compared to male mice. The secondary objective was to determine whether female mice experienced any motor or cognitive deficits in an mRHI model compared using the mRHI and sham groups.

5-week cohort

Acute symptoms from mRHI for 5-week cohort.

Behavior tests that assessed acute symptoms from mRHI were waking time and balance beam. **Figure 10** shows average wake time data for both sham and mRHI groups and sexes over a span of 5 weeks. A one-way ANOVA with repeated measures was performed between the mRHI and sham groups followed by Sidak post-hoc test. For female mice, not only did the data show a significant difference overall **($p < 0.0001$)**, but there was significant difference between both groups in each week **($p < 0.05$)**. For male mice, it showed a significant difference overall **($p < 0.01$)**, but there was one week that had a significant difference between both groups in each week **($p < 0.01$)**. A two-way ANOVA with repeated measures was performed between both sexes and with sham and mRHI groups followed by Tukey post-hoc test. There was a significant difference between the groups and sexes overall **($p < 0.0001$)**, and multiple comparisons showed that female mRHI group and male mRHI group have a significant difference when compared

with their single data points to each week.

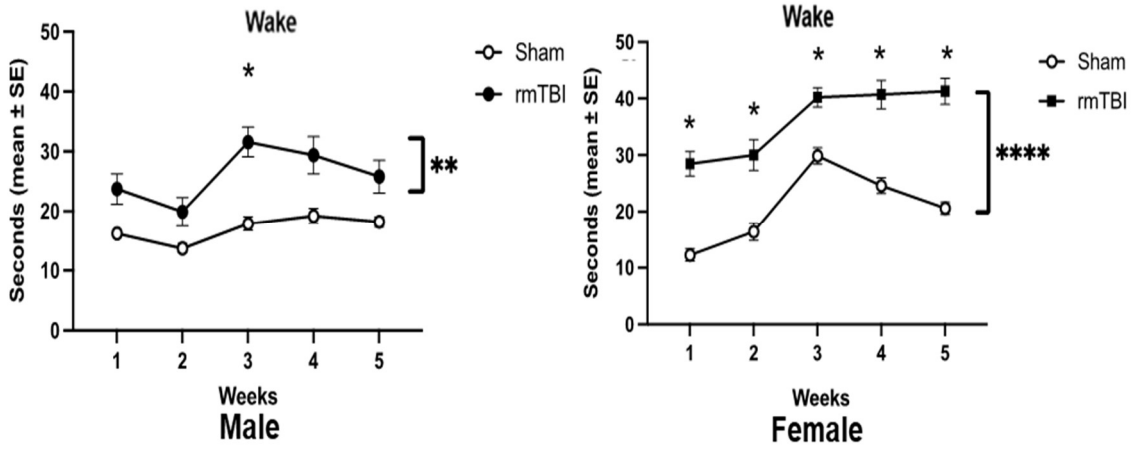

Figure 10: Wake time/righting reflex for 5-week cohort. A one-way ANOVA with weeks as repeated measures followed by Sidak post-hoc test yielded significant differences **(*= *p*< 0.05) (**= *p*< 0.01) (****= *p*< 0.0001)** between sham and mRHI groups for both male and female mice. A two-way ANOVA with weeks as repeated measures followed by Tukey post-hoc test yielded a significant difference between male and female mice when compared with sham and mRHI groups.

Balance beam for the 5-week male mice cohort cannot be compared with the 5-week female mice cohort since the rod used in the male mice cohort was square, while the female mice cohort used a round rod instead since it would cause the mice to fall off more likely. Due to male mice staying still and not moving on the rod, the square rod showed a male standing on the rod for longer periods of time than female mice did. A one-way ANOVA with repeated measures, followed by Sidak post-hoc test, showed a significant difference overall in the balance beam test when comparing the mRHI and sham groups **(Figure 11, *p*< .05)**, but no single data points

showed any significant differences each week.

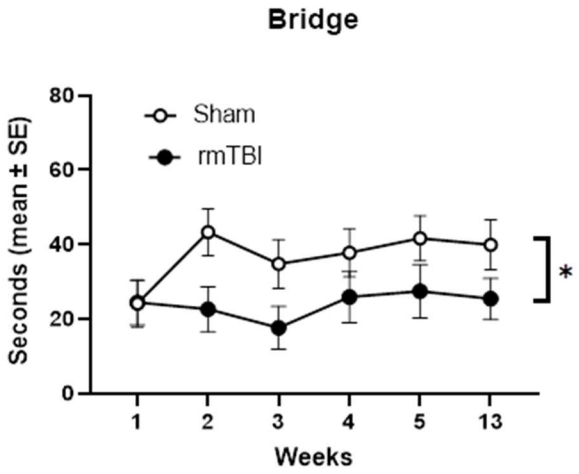

Figure 11: Balance beam/bridge for 5-week cohort. A one-way ANOVA with weeks as repeated measure followed by Sidak post-hoc test **(*= $p < 0.05$)** between sham and mRHI groups for female mice.

Chronic symptoms from mRHI for 5-week cohort

Behavioral test that assessed chronic symptoms from mRHI were rotarod, MWM, EPM, and T- maze. Using a one-way ANOVA with repeated measures, followed by Sidak post-hoc test, both male and female mice showed a significant difference overall when comparing both mRHI and sham groups in rotarod **(Figure 12A, $p < 0.05$)**. Only female mice show significant differences in single data points between both groups for the first two sessions. When the mean latency (separating them by sex and groups) was calculated, the data showed the means of sham and mRHI groups for male mice was **70.21± 3.78 and 54.50± 3.47**, and for female mice was **73.30± 3.71 and 54.17± 4.25**, respectively. For both male and female mice, the student's t-test showed a significant difference between mRHI and sham groups' mean latency **(Figure 12B, $p < 0.05$)**.

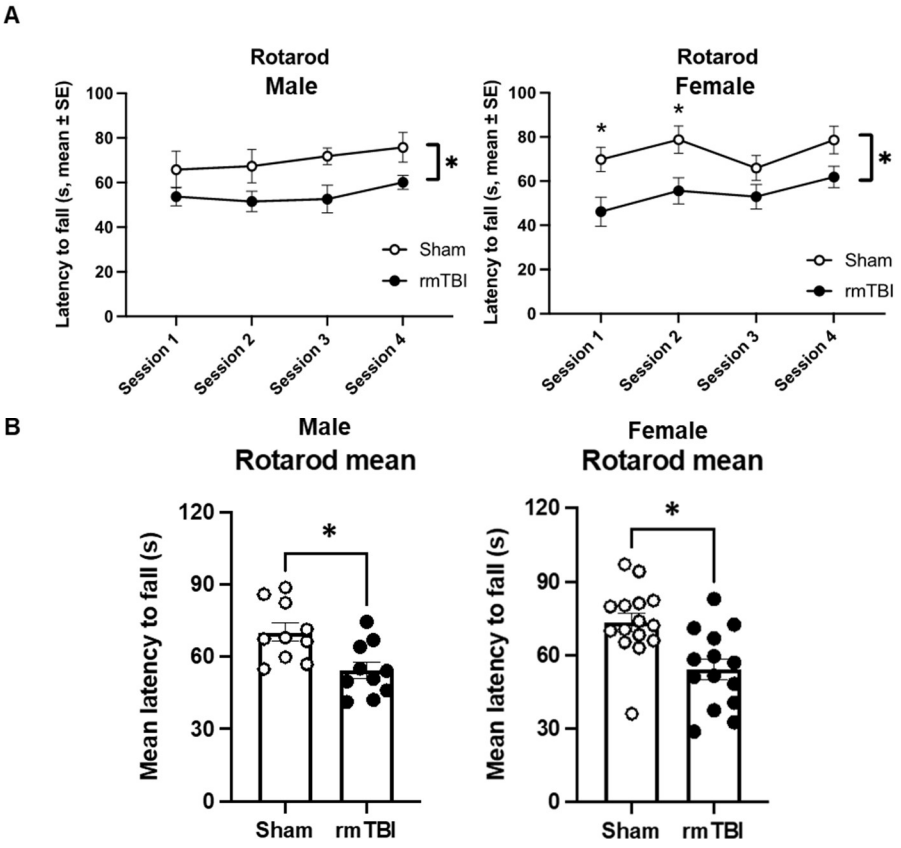

Figure 12: Rotarod for 5-week cohort. **(A)** Latency to fall from a rotating rod was grouped by sham and mRHI over 4 sessions (Rod accelerated to 4rpm in 5 min). A one-way ANOVA with sessions as repeated measure yielded significant difference **(*p*< .05)** between sham and mRHI groups for both genders. **(B)** Mean latency to fall from a rotating rod was grouped by sham and mRHI and averaged over 4 sessions. A Student's T-Test yielded significant differences **(*p*< .05)** between sham and mRHI groups for both genders.

In MWM, three parameters were assessed: path length, learning index, and time spent in annulus. For path length, a one-way ANOVA with repeated measures showed no significant differences between the mRHI and sham group **(Figure 13)**. For learning index, the means of sham and mRHI groups for male mice was **516.95± 47.63 and 503.40± 54.65**, and for female mice was **405.86± 47.78 and 463.25± 60.35**, respectively. For both male and female mice, a student's t-test showed no significant difference between the mRHI and sham groups **(Figure 13)**. For time spent in annulus session 2, the means of sham and mRHI groups for male mice was **19.41± 3.91** and **21.87± 4.35**, and for female mice was **19.52± 2.74** and **12.98± 2.17**, respectively. For time spent in annulus session 4, the means of sham and mRHI groups for

male mice was **13.26± 3.33** and **4.94± 1.97**, and for female mice was **24.94± 4.08** and **17.22± 2.81**, respectively. A two-way ANOVA only showed a significant difference between the mRHI and sham groups in session 4 **(Figure 13, $p< 0.05$)**.

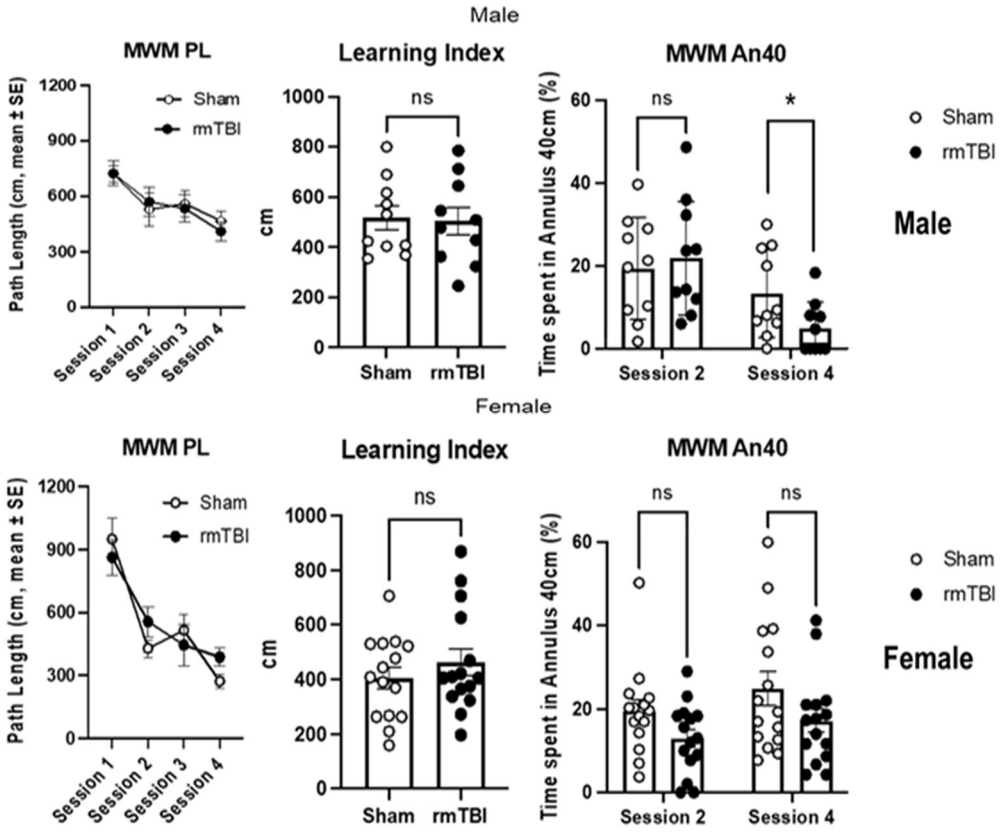

Figure 13: Morris water maze for 5-week cohort. Three parameters were measured: path length, learning index, and time spent in annulus. A two-way ANOVA yielded a significant difference for session 4 of time spent in annulus for male mice **(*=p<0.05)**.

In EPM, the time spent in open arm (%) was measured and averaged with all subjects and grouped by mRHI and sham groups. The means of sham and mRHI groups for male mice was **19.24± 2.02 and 23.07± 4.85**, and for female mice was **9.95± 1.75** and **10.06± 1.80**, respectively **(Figure 14)**. The student's t-test did not show any significant difference for both sexes. However, female mice showed a lower mean for time spent in open arms (%) when compared to male mice.

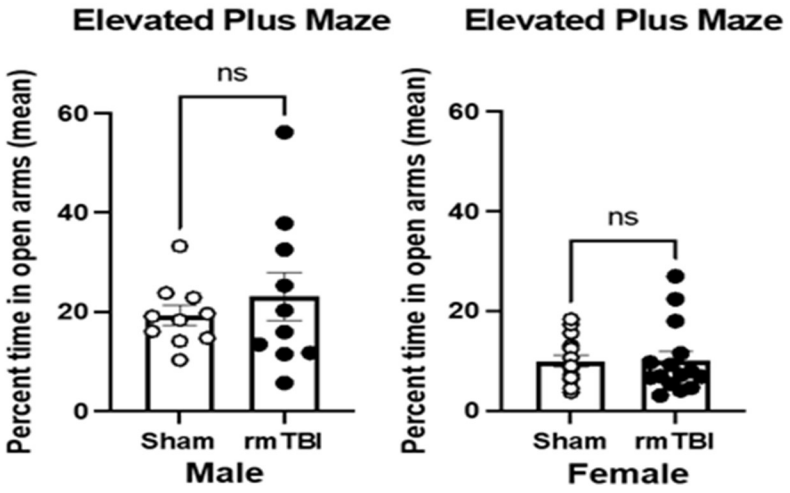

Figure 14: Elevated plus maze for 5-week cohort. The student's t-test showed no significant differences between the mRHI and sham groups for both male and female mice.

In T-maze, the trials performed until criteria met was measured and averaged with all subjects and grouped by mRHI and sham groups. The means of sham and mRHI groups for male mice was **13± 0.91 and 14.2± 1.38**, and for female mice was **9.93± 0.87** and **12.73± 1.58**, respectively (**Figure 15)**. The student's t-test didn't show any significant difference for both sexes.

Figure 15: T-maze for 5-week cohort. The student's t-test showed no significant differences between the mRHI and sham groups for both male and female mice.

Biochemical markers in the 5-week cohort

Five protein markers were assessed: GFAP, P-Tau, Iba-1, occludin, and claudin. Two areas of the brain were used to assess these protein markers: the cerebral cortex (CTX) and hippocampus (HP). However, claudin was not assessed for the female's CTX. In male mice, a student's t-test yielded there was no significant difference between mRHI and sham groups for all protein markers that were observed in both the CTX and HP. **(Figure 16A)**. However, in female mice, a student's t-test yielded significant differences **($p<0.05$)** in P-Tau, Iba-1, and occludin (Iba-1 and occludin had small but significant increases) between the sham and mRHI groups in the CTX. In female mice, a student's t-test also yielded significant differences in Iba-1 between the sham and mRHI groups in the HP **(Figure 16B)**.

Although mRHI male mice showed no significant increases in Iba-1, Iba-1 was also assessed through immunohistochemistry of the male mice brain **(Figure 17)**. Immunohistochemistry for the female mice was not assessed since the project is still ongoing. In the optic tract (OT), a student's t-test yielded a significant difference **($p<0.05$)** between the male mice sham and mRHI groups in cell density for Iba-1. In the corpus callosum, a student's t-test yielded a significant difference **($p<0.05$)** between the male mice sham and mRHI groups for cell density in Iba-1.

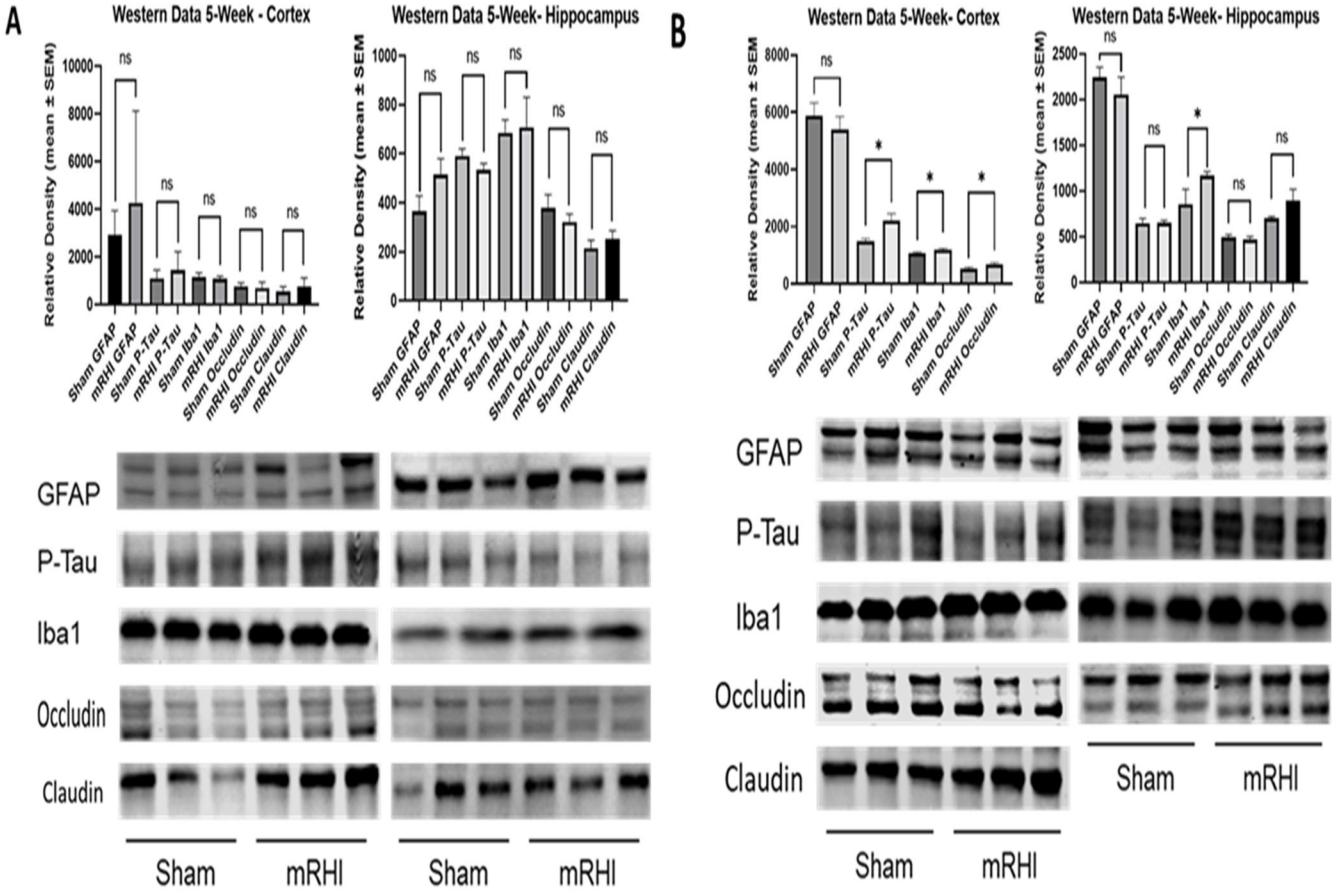

Figure 16: Western blot data of the CTX and HP for 5-week cohort. (**A)** Male mice from 5-week cohort. The student's t-test showed no significant differences in all protein marker in CTX and HP between the mRHI and sham groups for male mice. **(B)** Female mice from 5-week cohort. The student's t-test showed significant differences **(*=p<0.05)**. In P-Tau, Iba-1, and occludin in CTX and Iba-1 in HP between the mRHI and sham groups for female mice.

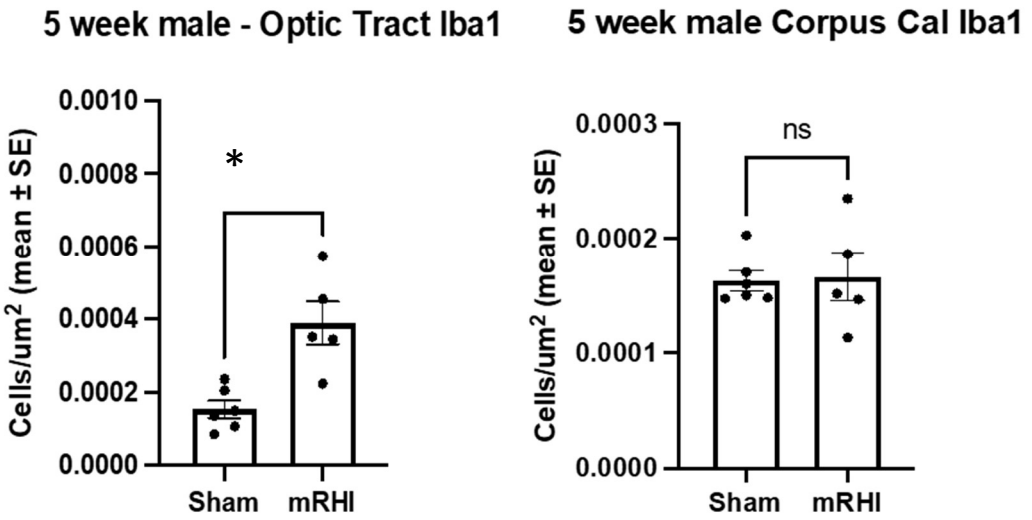

Figure 17: Cell densities for Iba-1 in optic tract (OT) and corpus callosum (corpus cal) for 5-week cohort male mice. In the OT The student's t-test showed a significant difference **(*=p<0.05)** for cells densities in Iba-1 between the mRHI and sham groups in male mice. In the corpus callosum, the student's t-test showed no significant difference for cells densities in Iba-1 between the mRHI and sham groups in male mice.

15-week cohort

Acute symptoms from mRHI for 15-week cohort.

Like the 5-week cohort, behavior tests that assessed acute symptoms from mRHI were waking time and balance beam. **Figure 18** shows average wake time data for both sham and mRHI groups over a span of 5 weeks. A one-way ANOVA with repeated measures followed by Sidak post-hoc test showed a significant difference overall **(p< 0.01)**, and all weeks, except week 2, had a significant difference between both groups **(p< 0.05)**.

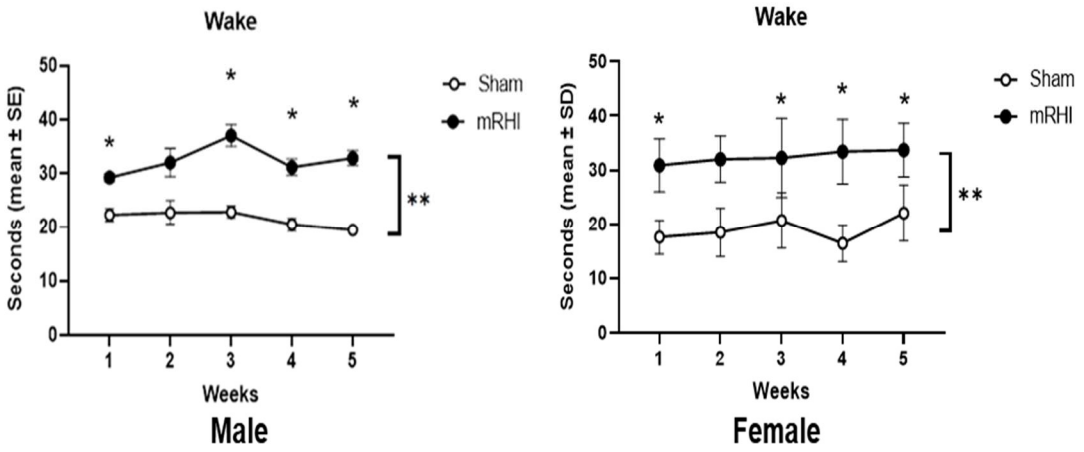

Figure 18: Wake time/righting reflex for 15-week cohort. A one-way ANOVA with weeks as repeated measures followed by Sidak post-hoc test yielded significant differences **(*= *p*< 0.05) (**= *p*< 0.01)** between sham and mRHI groups for male mice.

Balance beam for the 15-week male mice cohort swapped with a round rod. A one-way ANOVA with repeated measures showed no significant difference overall in the balance beam test when comparing the mRHI and sham groups in male mice. However, a one-way ANOVA with repeated measures, followed by Sidak post-hoc test, showed a significant difference overall in the balance beam test when comparing the mRHI and sham groups (***p*< .05)**, and the weeks 2, 3, and 4 data points showed significance differences. **(Figure 19)**.

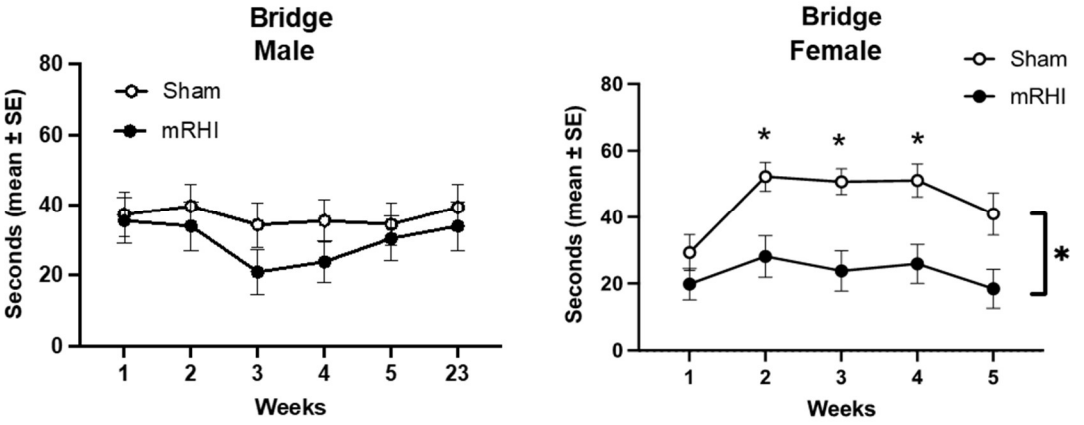

Figure 19: Balance beam/bridge for 15-week cohort. A one-way ANOVA with weeks as repeated measure followed by Sidak post-hoc yielded a significant difference **(*p*<0.05)** between sham and mRHI groups for only female mice.

Chronic symptoms from mRHI for 15-week cohort

Like the 5-week cohort, behavioral test that assessed chronic symptoms from mRHI were rotarod, MWM, EPM, and T- maze. Using a one-way ANOVA with repeated measures followed by Sidak's post-hoc test, only female mice showed a significant difference overall when comparing both mRHI and sham groups in rotarod **(Figure 20A, $p<0.05$)**. When the mean latency (separating them by groups) was calculated, the data showed the means of sham and mRHI groups for mice males was **59.17± 4.81** and **48.61± 3.64**, and for female mice was **70.49± 3.67** and **58.32± 4.55**, respectively. The student's t-test showed a significant difference between the mRHI and sham groups' mean latency for female mice **(Figure 20B, $p<0.05$))**.

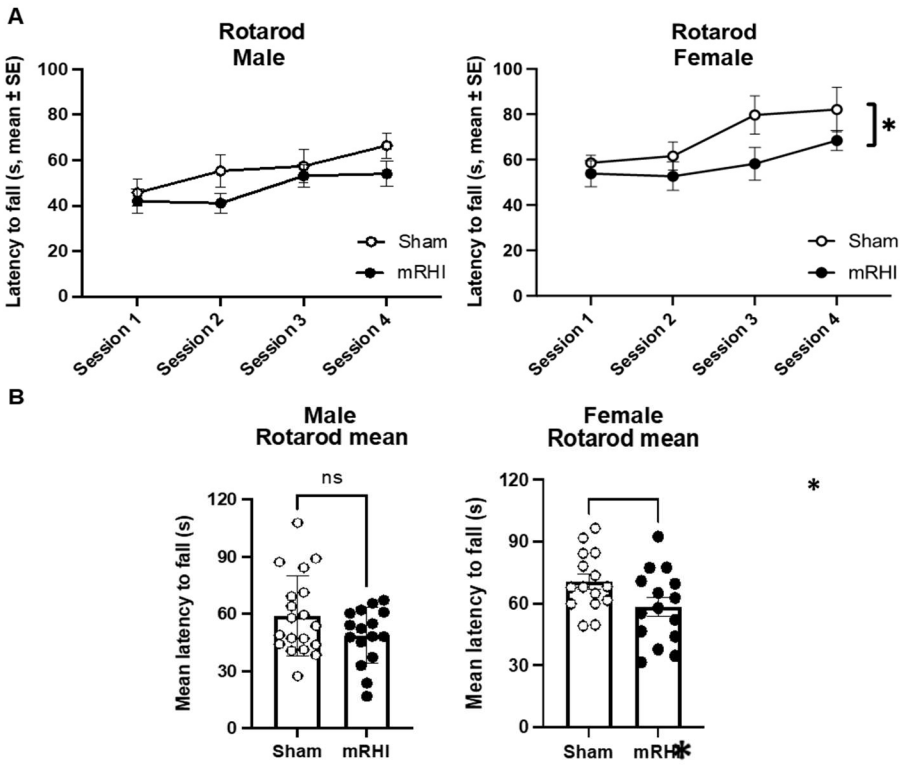

Figure 20: Rotarod for 15-week cohort. **(A)** Latency to fall from a rotating rod was grouped by sham and mRHI over 4 sessions (Rod accelerated to 4rpm in 5 min). A one-way ANOVA with sessions as repeated measure followed by Sidak post-hoc test yielded a significant difference **($p<0.05$)** between sham and mRHI groups for only female mice. **(B)** Mean latency to fall from a rotating rod was grouped by sham and mRHI and averaged over 4 sessions. A Student's T-Test yielded a significant difference **($p<0.05$)** between sham and mRHI groups for only female mice **($p<0.05$)**.

In MWM, three parameters were assessed: path length, learning index, and time spent in annulus. For path length, a one-way ANOVA with repeated measures followed by Sidak post-hoc test showed a significant difference overall between the mRHI and sham group for male mice, while female mice didn't **(Figure 21, $p<0.05$)**. For learning index, the means of sham and mRHI groups for male mice was **343.28± 32.43** and **479.61± 41.82**, and for female mice was **347.40± 50.40** and **304.73± 23.84,** respectively. In learning index, a student's t-test showed a significant difference between the mRHI and sham groups for male mice, while female mice didn't **(Figure 21, $p<0.05$)**. For time spent in annulus session 2, the means of sham and mRHI groups for male mice was **17.06± 2.01** and **13.67± 2.10**, and for female mice was **20.32± 2.84 and 22.11± 3.87**, respectively. For time spent in annulus session 4, the means of sham and mRHI groups for male mice was **26.96± 2.11** and **23.31± 3.11,** and for female mice was **24.26± 3.53 and 18.30± 2.36**, respectively. Looking at time spent in annulus, a two-way ANOVA only showed a significant difference between the mRHI and sham groups in session 2 for male mice, while female mice showed no significant difference in both sessions **(Figure 21)**.

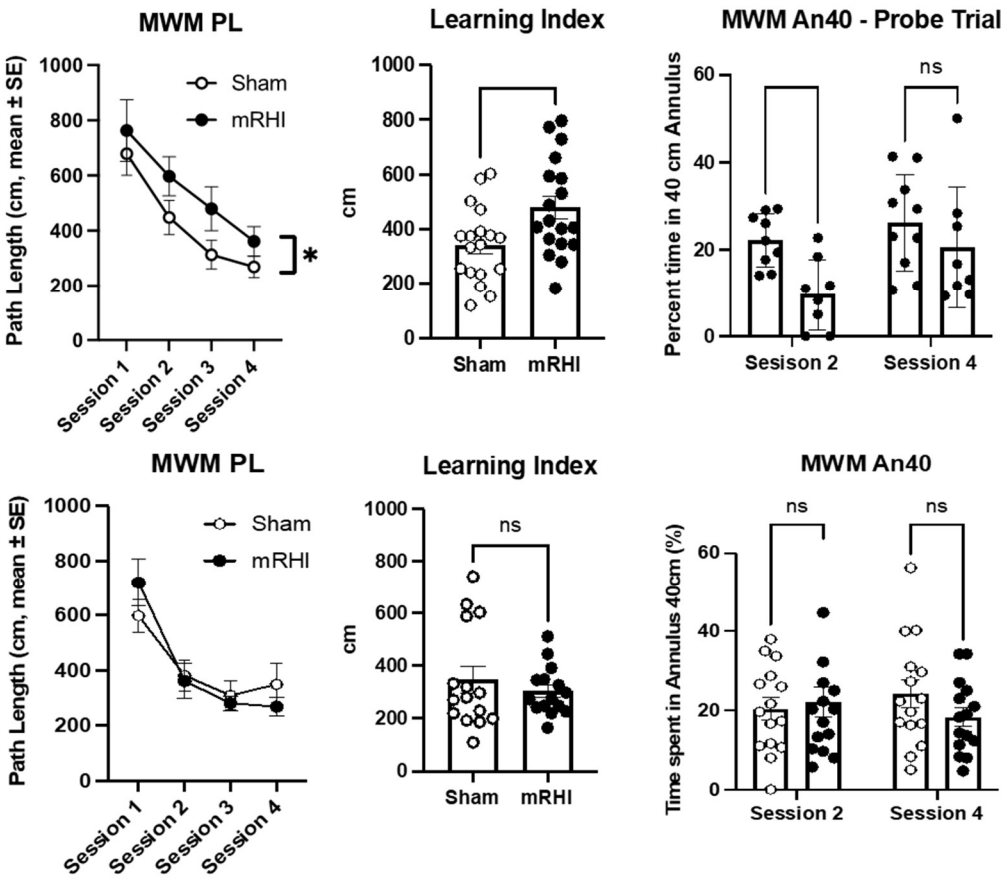

Figure 21: Morris water maze for 15-week cohort. Three parameters were measured: path length, learning index, and time spent in annulus. In path length, a one-way ANOVA with repeated measures followed by Sidak post-hoc test yielded a significant difference **(p<0.05)** between sham and mRHI groups for only male mice. In learning index, a student's t-test yielded a significant difference **(p<0.05)** between sham and mRHI groups for only male mice**.** In session 2 of time spent in annulus, a two-way ANOVA yielded a significant difference between the sham and mRHI groups in male mice only.

In EPM, the time spent in open arm (%) was measured and averaged with all subjects and grouped by mRHI and sham groups. The means of sham and mRHI groups for male mice was **19.16± 3.40** and **28.24± 1.65**, and for female mice was **23.12± 1.35** and **19.65± 1.81**, respectively. **(Figure 22)**. The student's t-test showed a significant difference for male mice but not for female mice **(p<0.05)**.

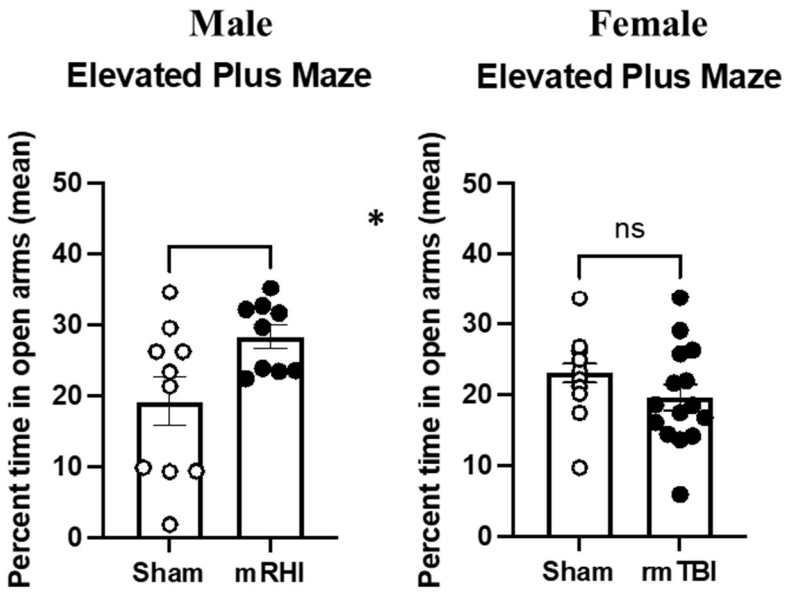

Figure 22: Elevated plus maze for 15-week cohort. The student's t-test showed a significant difference between the mRHI and sham groups for male mice **(p<0.05)** but not female mice.

In T-maze, the trials performed until criteria met was measured and averaged with all subjects and grouped by mRHI and sham groups. The means of sham and mRHI groups for male mice was **15.31± 1.15** and **16.28± 1.44,** and for female mice was **16.52± 2.74** and **15.78± 2.17**, respectively. **(Figure 22)**. The student's t-test didn't show any significant difference for both male and female.

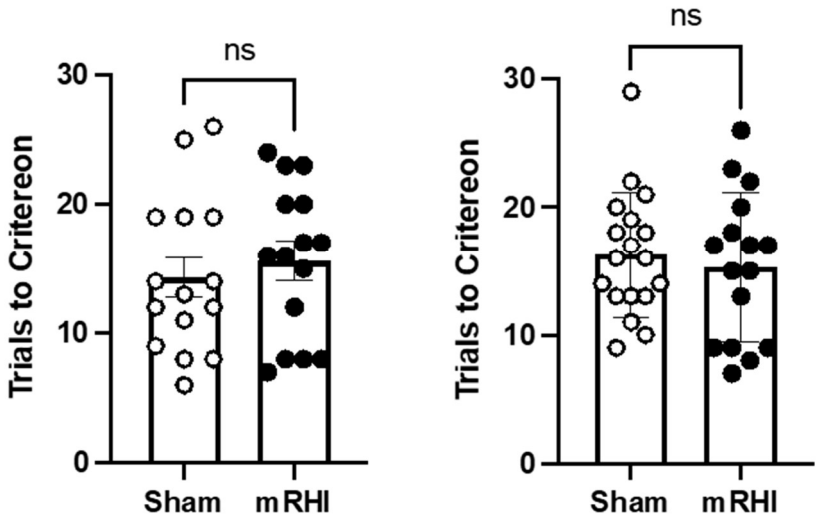

Figure 23: T-maze for 15-week cohort. The student's t-test showed no significant differences between the mRHI and sham groups for male mice.

DISCUSSION

In this study, we evaluated whether there was sex difference between effects of mRHI in male and female mice, and if female mice exhibited motor or cognitive deficits following injury from a mRHI model. Furthermore, we sought to determine whether there was a different developmental time-course of acute and delayed behavioral deficits between the sexes. The key findings of this study are: 1) For both the 5-week and 15-week cohorts, female mice in the mRHI group showed acute motor deficits (balance beam), acute loss of consciousness (wake time), and chronic motor deficits compared to the sham group. 2) Female mice experienced more severe motor deficits following an mRHI but didn't experience no cognitive deficits and changes in affective behavior following an mRHI compared to male mice.

In the 5-week cohort, a two-way ANOVA with repeated measures showed that female mice took a longer time to perform a righting reflex after impact when compared to male mice. However, the loss of consciousness only measures acute symptoms after injury. Looking at preliminary data from a previous study, both the female and male mice after injury showed motor deficits from the rotarod motor test. This result is similar to a human study that evaluated the motor function in patients that experienced a severe TBI along with the improvements in motor function (46). Their initial data showed that about 30% of the patients have arm and leg paresis (46), which shows that motor deficits have occurred from a severe TBI. Even with a milder and more repetitive model, the result shows motor deficits similar to the human study. However, only male mice showed cognitive deficits in MWM for time spent in annulus. This data supports that the mice with mRHI have persistent deficits that could last after recovery. As

noted by Yang et al., there were significant deficits motor coordination and cognition that appeared following an mTBI (40). However, the results for female mice in MWM, EPM, and T-maze and for male mice in EPM and T-maze differ with a study that looks at chronic cognitive deficits in TBI (51). A study showed that humans that have cognitive problems like deficits in impulse control, problem solving, memory, attention, and learning were observed after a TBI. The female mice fail to show deficits in learning, memory, and impulse control, while male mice only experience deficits in memory.

In the 15-week cohort, the rotarod showed female mice exhibited motor deficits after an mRHI while male mice didn't. This result shows that male mice seem to resolve their motor deficits over time while female mice's motor deficits continue to persist. The male mice's improvement in motor function is similar to a study about patients with severe TBI shows improvements after six months from paresis, ataxia, and postural instability (55). Over time, the patients began to improve their motor skills, which was similar to male mice with their motor skills. However, this study had severe TBI, while our study is milder and more repetitive, so the effects of how well the subject improved would differ. In cognitive behavioral tests like MWM and T-maze, male mice showing cognitive deficits in spatial learning and memory; however, female mice failed to show those deficits. Like the 5-week cohort female mice, the results for female mice in MWM, EPM, and T-maze are disagreed by a study that looks at chronic cognitive deficits in TBI (51). In the EPM, male mice only showed changes in affective behavior and stayed on the open arms more than the sham group, while female mice didn't. As change in affective behavior in male mice is like disinhibition in humans, this result for male is similar to a human study about a male patient experiencing a change in behavior/disinhibition after a car accident (37). The patient was attacking nurses without

provocation and kept shouting them when the nurses asked questions. The patient failed to control his behavior when needed to, like the male mice when they were trying to suppress their anxiety by staying in closed arms. The injury was most likely a moderate/severe TBI since the patient also experienced a subdermal hemorrhage (37). The results showed changes in affective behavior, even with a milder and repetitive model form of TBI. However, effects of severity could differ since our model is milder. The results are also supported by another study about the pathophysiology of mTBI. The study found that some patients that experience personality changes, like aggression, impulsivity, irritability, emotional liability, and apathy (47). Although the male mice didn't show aggression, irritability, emotional liability, and apathy, they were more impulsive or had a change of behavior that led them to go to the open arms more than the sham group, which is similar to a study that uses another animal model for impulsivity. However, the result for female mice differs with this study since mRHI female didn't significantly travel to open arms more than the sham group (37, 47). The results show female mice were able to suppress their anxiety of by staying in the closed arms, which showed their behavior is normal.

Our mRHI model followed another study's TBI model that also performs repetitive and mild head injuries (63). However, we didn't follow the helmet procedure to put on mice. The Briggs model showed about 10% of the mice sustained severe symptoms, like fractures, bleed, and death, while also showing neurological deficits (63). The study's results show that the model can provide mild head injuries with less risk of causing a moderate/severe head injury. According to the Briggs study, the mice that took about 100 seconds to perform a righting reflex (63), while the mice in our model performed the reflex less than the Briggs. Since the Briggs model demonstrates deficits that can occur with less risk of more severe symptoms, this

study provides support that our model can provide mild and repetitive hits since the mice from our result performed a righting reflex sooner, and most mice did not sustain severe injuries as well.

There could be many reasons why female mice failed to show more severe cognitive deficits and changes of affective behavior than male mice. Effects from an mRHI could be reduced female hormones like estrogen since there are studies that show that show female steroid hormones exert neuroprotective effects using anti-inflammatory and antioxidant mechanism (50). The study shows that high levels of 17β-estradiol lower brain damage, and progesterone initiates its neuroprotective effects when anti-inflammatory cytokines, like TNF-α and IL-6, have decreased levels (50). Female mice may have neuroprotective effects from female hormones that could reduce the effects of cognitive deficits.

In the 5-week cohort, male mice showed no elevated levels of any protein marker in CTX or HP, while female mice had elevated levels in P-Tau, Iba1, and occludin in CTX when compared to their respective sham groups. However, Iba-1 and occludin had a small increase that was significantly different. Although the protein markers are significantly different from their sham groups, the levels of both mRHI and sham group should physiologically similar, which means that occludin and Iba-1 have would have the same effect to the body. Additionally, the male and female mice results follow the how long GFAP typically stays in the body after a TBI (19). GFAP lasts around 3 hours to 2 days with multiple studies that show increased levels of protein markers that we were assessing. Although female mice showed significant differences in proteins that weren't significant in male mice, female mice's deficits were similar to male mice's deficits. The similarity of the results could mean that female mice could have more severe deficits over time since elevated P-Tau has been shown to be a

predictor of poor outcome. For Iba-1 in male mice, the western results showed no increased expression of Iba-1. However, the immunohistochemistry of the male brain tissue showed increased expression of Iba-1 in the optic tract, which shows that male mice have increased expression of Iba-1 following an mRHI.

Limitations: There are few limitations to this study that could affect our results. Outcomes for female and male mice may vary when compared with behavioral and biochemical marker data. Certain mice may experience far more severe outcomes from a TBI, while other mice may experience little to no outcomes from a TBI. Multiple results in thus have shown a wide spread of datapoints in western blot data and behavioral data. When looking at protein markers like GFAP, P-Tau, and Iba-1, the markers were collected by dissecting a mouse brain by splitting the cortex and hippocampus. Although these markers showed increases compared to the sham group, it would be unpractical to use a patient's brain tissue to measure the markers' levels. The most appropriate way to measure for patients would be from drawing blood, but the levels shown from extracting brain tissue may differ from the levels in drawing blood. Lastly, when collecting brain tissue for western blot data, we collected the entire cortex and hippocampus rather than specifically extracting a specific portion, which could affect the true levels of the markers. The immunohistochemistry result showed a significant difference while the western blot results did not, which supports the dissecting of the brain may impact the true results.

Future work: Immunohistochemistry and western blot for the 15-week cohort female mice. Performing those procedures will allow us to compare the data for sex differences. These protein markers need to be evaluated whether they can be indicators of the deficits we found. If the protein markers were assessed before behavioral tests were performed, we can evaluate whether the results from the western blot can reflect the behavioral data. However, extracting

the mice brain can no longer work if we were to approach the following procedure. Drawing blood of the mice instead of the brain could be a viable alternative. Another alternative that can be done would be looking at other biomarkers, like miRNA or exosome. miRNA are RNA molecules that regulate gene expression at a post-transcriptional level (19). They have known to circulate around multiple human diseases and disorders. There have been miRNAs have been known in rodent studies to elevate TBIs of multiple severities. These miRNAs that have potential in rodents are miRNA-Let-7i (seen in acute CSF), MiR-376a, MiR-214, and MiR-199a-3p (seen in acute serum samples in mild close head injuries) (19). There is also human data that detected elevations in 3 miRNA (MiR-16, MiR-92a, MiR-765) in acute plasma samples after a severe TBI (19). miR-151–5p, miR-195, miR-20a, miR328, miR-362–3p, miR30d, miR-451, miR-486, miR-505, and miR-92a in acute CSF and/or serum samples from human TBI, ranging from mild to severe TBI have been able to distinguish an mTBI from healthy volunteers and orthopedic injury control samples (19). miR-142–3p and miR-423–3p in another study have been used to identify mTBI patients who are likely to have the post-concussive syndrome (19). Exosomes are lipid-bilayer encapsulated particles that are released from cells (healthy or injured) into biofluids, like extracellular fluid, CSF, and blood. They may contain contents like miRNA or a specific protein when the human is unwell from a disease or disorder. A study showed that the CSF of TBI patients has elevated levels of several protein markers (19). Exosomes with tau-like p-tau would be a good diagnostic tool for chronic TBI patients at risk for CTE.

SUMMARY AND CONCLUSION

In summary, female mice in both cohorts showed motor deficits following an mRHI but showed no cognitive deficits, while male mice in both cohorts showed motor (only 5-week cohort) and cognitive deficits following an mRHI. Although female mice continue to show motor deficits for a longer period while male mice seemed to resolve their motor deficits after 15 weeks, female mice do not have more severe outcomes when compared to male mice 5 weeks and 15 weeks after injury. The mRHI model does, however, lead to acute deficits and chronic motor deficits following an injury.

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