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Research Paper

Multiple mild traumatic brain injury in the rat produces persistent pathological alterations in the brain

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ABSTRACT

Multiple mild traumatic brain injury (mmTBI), in certain cases, produces persistent symptoms. However, the molecular mechanisms underlying these symptoms remain unclear. Here, we demonstrate extended pathological changes in the rat brain following mmTBI. Using the lateral fluid percussion (LFP) technique we exposed adult male Wistar rats to a mild TBI (mTBI) once a week for four weeks and compared them to surgical shams. At 90 days following the last TBI or sham procedure the animals were cognitively tested in the Morris Water Maze (MWM), euthanized, and the brains removed for immunohistochemistry. At 90 days following the last mTBI, NRF-2 staining was significantly decreased in the hilus of the hippocampus and cortex on the injured side, but did not significantly differ from shams on the un-injured side. CD68 positive microglia were significantly increased in the ipsilateral corpus callosum, cortex, and internal capsule of injured animals. Reactive astrocytosis, determined by increased GFAP staining, was also evident in the corpus callosum, cortex, internal capsule and thalamus on the injured side. Interestingly, the corpus callosum thickness at the midline was decreased in injured animals and had evident demyelination when compared to sham animals. Despite these findings, there were no significant differences in neurological assessments at 90 days following the last injury. In MWM testing there were not significant differences in the training phase, the time spent in the thigmotaxia zone, or the target quadrant during the probe trial. However, there were significant differences between shams and injured animals in platform zone crossings during the probe trial. These results demonstrate that repetitive head trauma may produce persistent, long-term pathological alterations in brain architecture that may be difficult to detect using standard cognitive and neurological assessments.

1. Introduction

There is evidence that repetitive head trauma significantly increases the severity and duration of symptoms when compared to a single injury (Aungst et al., 2014). As research into mild traumatic brain injury (mTBI) continues to grow, it has become clear that many individuals suffer from multiple head injuries over the course of their lifetime. High-risk individuals such as athletes, soldiers, construction workers and the elderly have a disproportionately high rate of repetitive head trauma (Baschera et al., 2014; Brooks et al., 2016; Goldstein et al., 2012). While these injuries may be classified as 'mild' in nature, there is evidence that when the injuries are cumulative, it leads to long-term cognitive impairment in a subset of individuals. Furthermore, there is clear evidence that a small percentage of individuals that suffer repeated head injury go on to develop a neurodegenerative disease, chronic traumatic encephalopathy (CTE) (Goldstein et al., 2012; Stein et al., 2015; Stern et al., 2011). While this condition remains relatively rare, it does demonstrate the potential long-term damage that multiple injuries may have upon the brain.

While it is clear that many individuals exposed to a mTBI recover over time, there is also evidence that certain individuals experience ongoing symptoms, beyond the expected time of recovery, such as headaches, difficulty concentrating, and impaired memory that drastically affect the quality of life (Ziino and Ponsford, 2006a,b; Stulemeijer et al., 2006; Guinto and Guinto-Nishimura, 2014). The source of these long-term symptoms has been the subject of debate among clinicians

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and researchers, with certain individuals attributing long-term neurocognitive symptoms to psychosomatic origins (Mittenberg and Strauman, 2000). However, it is possible that individuals exposed to multiple mild injuries may be experiencing specific, focal areas of damage in the brain that do not adequately repair after injury (Aungst et al., 2014; Stern et al., 2011; Guinto and Guinto-Nishimura, 2014; Bramlett et al., 1997a; Ponsford et al., 2011). This injury pattern may be leading to long-term neurocognitive issues, even in the absence of motor deficits, in certain individuals.

The study of mTBI has, in the past, been hampered by a lack of animal models that accurately reproduce repeated mild head trauma in humans. Many of the current research animal models of TBI focus on a single, moderate-to-severe injury using rodents. These models have provided important information on the molecular and pathological changes that occur after a severe injury. However, there are several reasons to study multiple mild TBI (mmTBI) in an animal model: 1) a single mild injury in rodents is typically difficult to discern as they recover much more quickly than humans; 2) the current measurements for neurological and cognitive impairment in rodents are, in many cases, not sensitive enough to detect the long-term impacts of a single mTBI in rodents; and 3) many researchers and clinicians have viewed mTBI as a linear extension of severe TBI, encompassing the same molecular processes and pathology as severe TBI, only to a lesser degree. Therefore, much of what is known about the molecular mechanisms of mTBI and mmTBI has been derived from animal models utilizing a single, severe injury. However, there is clear evidence that mmTBI and a single severe injury differ in symptom presentation, recovery, and, very likely, the mechanism(s) of long term pathology (Ponsford et al., 2011; Signoretti et al., 2011; Rosenfeld et al., 2012; Hawryluk and Bullock, 2016; Perry et al., 2016).

For the present study we utilized the lateral fluid percussion (LFP) injury device to deliver four mild TBIs over the course of 28 days, with an injury every 7 days. This interval was selected for several reasons. Winston et al. (2016) published work demonstrating that delivering thirty mild TBIs over thirty days (one injury per day) did not cause dendritic spine loss. However, when the inter-injury interval increased to 7 days there was a loss of dendritic spines, suggesting that a rest interval between injuries increased the damage pattern (Winston et al., 2016). A second reason for choosing the 7-day inter-injury interval is based on studies reported by Ruth (1935) in which each rat month equates roughly to 2.5 human years. This places 7 rat days to an equivalent of 210 days for a human. This would put the comparative human injury interval at 7 months. The authors acknowledge there is no clear median interval between concussions and it is highly variable between individuals. The authors also acknowledge this comparison may be not completely linear for brain injuries, however it does provide a recovery gap between injuries that may be more realistic with regards to human recovery between traumatic injuries.

The goal of this study was to determine if mmTBI in the rodent produced significant pathological alterations to brain structure that were not readily manifested in cognitive and neurological testing. Furthermore, we sought to determine if mmTBI produced significant alterations that were still present at 90 days following the last injury, suggesting the presence of a persistent neuropathological process associated with mmTBI.

To complete the objectives in this study we neurologically assessed the animals at 24 h following each injury and on the day of euthanization 90 days following the last injury. To assess the animals we used a modified neurological severity score (mNSS) and the Morris Water Maze (MWM), as previously published (Rau et al., 2012, 2014a,b). At 90 days after the last injury the animals were euthanized, perfused, and the brains were removed, sectioned and stained using immunohistochemistry to assess markers of inflammation, neuronal loss, and abnormal cellular structure.

2. Materials & methods

2.1. Surgical procedures

The Institutional Animal Care and Use Committee (IACUC) at The University of Montana approved all procedures in these studies. Twenty adult male, Wistar rats (350–500 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed in filtered isolator boxes with a 12-hour light/dark schedule and ad libitum access to food and water. The animals were randomly distributed into mmTBI ($n = 10$) or sham groups ($n = 10$). The LFP procedure was performed as previously described (Rau et al., 2012, 2014a,b). Briefly, animals were deeply anesthetized using 2–4% isoflurane (isoflurane anesthesia was used for all mmTBI and surgical sham procedures). A 5 mm trephination was made over the right hemisphere equidistant from the lambda and the bregma as previously described (Rau et al., 2012, 2014a,b). Animals were given a 20 ms fluid pulse to the dura at 0.98 to 1.22 atm of pressure. There was no mortality observed with these pressures. By comparison, our previous single severe TBI model (ssTBI) delivered pressures ranges of about 2.0–2.5 atm resulted in a 25–40% mortality rate (Rau et al., 2014b). All mmTBI animals had a brief apnea (mean of 1.22 s) after injury and were manually ventilated with supplemental O2 until normal breathing resumed. Every 7 days, the surgical shams underwent the same surgical procedure and were connected to the LFP device but the hammer was not released. Sham animals did not experience apnea and had an average righting time of 4 min 19 s for recovery from anesthesia. Rats receiving mmTBI were administered injuries every 7 days over a 28-day period. Righting times (the time in minutes required for an animal to bear weight on all four limbs) was recorded with an average time of 9 min 12 s. By comparison, in our previous studies of single severe TBI, ssTBI model, righting times in the TBI rats averaged 32 min with an average apnea of 126.42 s (Rau et al., 2014b).

2.2. Modified neurological severity scoring (mNSS)

The mNSS was performed as previously published (Rau et al., 2012, 2014a,b). mNSS testing was performed at 24 h following each injury and prior to euthanization at 90 days following that last injury.

2.3. Assessment of memory

Spatial memory was assessed using the Morris Water Maze (MWM) as previously published (Rau et al., 2012, 2014a,b). MWM testing began on day 84 following the last mild injury. As previously published, the training phase lasted for 5 days with a probe trial conducted on day 6 (Rau et al., 2012, 2014a,b).

2.4. Histology

Brains were harvested from mmTBI or shams at 90 days after the 4th mTBI. At the time of sacrifice rats were deeply anesthetized using isoflurane inhalation, perfused with 4% paraformaldehyde (PFA) fixative, post-fixed for 24 h in 4% PFA and divided into 2 mm coronal sections using a rat brain matrix. Slices were processed, paraffin embedded and sectioned at 7 μm. A 1 in 10 series from the slice containing hippocampus was stained with thionin to identify location relative to bregma and preliminarily assess neuronal damage. Tissue sections from sham ($n = 5$) and mmTBI ($n = 5$) rats were mounted at bregma −3.3 and −4.3, deparaffinized and rehydrated in PBS. Sections stained for either Cluster of Differentiation 68 (CD68) or NF-E2 DNA binding protein (Nrf2) and Glial Fibrillary Astrocytic Protein (GFAP) and Neurotrace (NT). CD68 sections were permeabilized in 0.3% TritonX100 for 30 min. Antigen retrieval was performed in 0.01 M citric acid. Sections were blocked in 4% normal goat serum (Vector Laboratories, Burlingam, CA) and incubated overnight at 4 °C with