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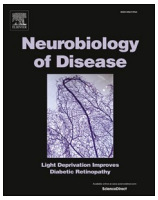

Behavioral, axonal, and proteomic alterations following repeated mild traumatic brain injury: Novel insights using a clinically relevant rat model

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ABSTRACT

A history of mild traumatic brain injury (mTBI) is linked to a number of chronic neurological conditions, however there is still much unknown about the underlying mechanisms. To provide new insights, this study used a clinically relevant model of repeated mTBI in rats to characterize the acute and chronic neuropathological and neurobehavioral consequences of these injuries. Rats were given four sham-injuries or four mTBIs and allocated to 7-day or 3.5-months post-injury recovery groups. Behavioral analysis assessed sensorimotor function, locomotion, anxiety, and spatial memory. Neuropathological analysis included serum quantification of neurofilament light (NfL), mass spectrometry of the hippocampal proteome, and ex vivo magnetic resonance imaging (MRI). Repeated mTBI rats had evidence of acute cognitive deficits and prolonged sensorimotor impairments. Serum NfL was elevated at 7 days post injury, with levels correlating with sensorimotor deficits; however, no NfL differences were observed at 3.5 months. Several hippocampal proteins were altered by repeated mTBI, including those associated with energy metabolism, neuroinflammation, and impaired neurogenic capacity. Diffusion MRI analysis at 3.5 months found widespread reductions in white matter integrity. Taken together, these findings provide novel insights into the nature and progression of repeated mTBI neuropathology that may underlie lingering or chronic neurobehavioral deficits.

1. Introduction

Mild traumatic brain injuries (mTBIs), such as concussions, account for >80% of TBI cases (Dewan et al., 2018). mTBI is particularly common in sports with the highest participation rates for adolescents and young adults (Daneshvar et al., 2011; Finch et al., 2013). It is also increasingly prevalent in military personnel (e.g. warzone blast exposure (Escolas et al., 2020; Lindquist et al., 2017)), and is a leading cause

Abbreviations: mTBI, mild traumatic brain injury; NfL, neurofilament light; MRI, magnetic resonance imaging; PPCS, persistent post-concussion symptoms; CTE, chronic traumatic encephalopathy; ACHI, awake closed head injury; DTI, diffusion tensor imaging; PND, post-natal day; EPM, elevated plus maze; NE, north-east; NW, north-west; SE, south-east; SW, south-west; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TCEP, tris(2-carboxyethyl)-phosphine-hydrochloride; CAA, 2-Chloroacetamide; FA, fractional anisotropy; AD, axial diffusivity; RD, radial diffusivity; ADC, apparent diffusion coefficient; TBSS, tract-based spatial statistics; TFCE, threshold free cluster enhancement; ROI, region of interest; ANOVA, analysis of variance; CC, corpus callosum; EC, external capsule; IC, internal capsule; Fim, fimbria of hippocampus.

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of emergency department visits (Centers for Disease Control, 2019; Gaw and Zonfrillo, 2016). Although the neurobehavioral (e.g. cognitive and emotional impairments) and physical (e.g., headache, motor deficits) effects of mTBI most often resolve within 7–10 days, approximately 20% of individuals are plagued by symptoms that persist for several weeks, months or longer (i.e. persistent post-concussion symptoms; PPCS) (Quinn et al., 2018). There is mounting evidence that a history of mTBI may be the strongest risk factor for suffering a future mTBI, and also for experiencing more symptoms for a longer duration should another mTBI occur (Dretsch et al., 2015; Iverson et al., 2017; Miller et al., 2013). Risk of exacerbated symptoms is thought to be particularly high if mTBIs are experienced in short succession (Eisenberg et al., 2013; Silverberg et al., 2013). Moreover, several recent retrospective studies on individuals with a history of mTBI or repetitive head trauma have provided evidence of an increased risk for long-term neurological conditions, such as depression and cognitive impairment (Manley et al., 2017), or progressive neurodegenerative diseases such as Alzheimer's and chronic traumatic encephalopathy (CTE) (Lehman et al., 2012; Mez et al., 2017). However, there is still a great deal of controversy and debate surrounding the associations between repeated mTBI and persistent or progressive neurological conditions (Iverson et al., 2019; Smith et al., 2019), and this topic requires further investigation.

The last two decades have seen a significant increase in understanding of the neuropathology that may underlie the effects of repeated mTBIs. Post-mortem analyses of individuals with an extensive history of mTBI have found evidence of microstructural pathology, including widespread axonal disruption and proteopathies, and in some cases, significant atrophy of certain brain regions (McKee et al., 2013; Smith et al., 2013). Clinical in vivo neuroimaging studies have also provided evidence of reduced white matter integrity (Koerte et al., 2015; Wright et al., 2020), tau pathology (Barrio et al., 2015; Cherry et al., 2016) and microglial activation in people with a history of repeated mTBI (Cherry et al., 2016; Coughlin et al., 2015). Nonetheless, the retrospective nature and presence of confounding variables in many of these studies make it difficult to decipher the true prevalence and magnitude of these neuropathological changes. Moreover, with most studies conducted at single time-points several years or decades following the final injury, the temporal progression of neuropathology is poorly understood.

Animal models of repeated mTBI have allowed for important neuropathological insights not possible in clinical studies. Multiple rodent studies have found that repeated mTBIs can trigger significant and potentially lasting changes in energy metabolism, blood brain barrier damage, oxidative stress, neuroinflammation, axonal damage, tau pathology, and neurodegeneration (Cheng et al., 2019; Eyolfson et al., 2020; Fehily and Fitzgerald, 2017; Lyons et al., 2018; Mouzon et al., 2018; Shultz et al., 2011; Webster et al., 2015; Wright et al., 2019). Nevertheless, the clinical relevance of some animal models used to create this understanding has been questioned, with anaesthetics or craniotomy both shown to alter TBI pathophysiology and outcomes, and some models producing structural changes (e.g. cavitation) not typically seen in clinical mTBI (Archer et al., 2018; Shultz et al., 2017). Moreover, although clinical and pre-clinical studies have increased understanding of the potential cellular and molecular consequences of repeated mTBI, a number of important questions remain, particularly regarding the progression of these changes through the acute and chronic stages of injury. To provide further insights into the pathophysiological and neurobehavioral effects of mTBIs, we have recently developed and characterized an awake, closed-head injury (ACHI) model in rats. A single injury with this anaesthetic- and surgery-free model results in transient neurobehavioral symptoms that typically resolve within 24–48 h post-injury, and transient increases in glial reactivity that appear to resolve within two weeks (Pham et al., 2019). We have also recently shown that repeated ACHIs result in acute changes in white matter that resemble those seen in clinical mTBI (Wortman et al., 2018). In the current study, we aimed to determine the acute and chronic effects of repeated ACHIs separated by 48 h. To do so, we implemented a detailed neurobehavioral battery to characterize the extent and evolution of neurobehavioral changes, blood (neurofilament light; NfL) and neuroimaging (diffusion tensor imaging; DTI) biomarkers to assess axonal injury, and high-resolution mass spectrometry to investigate the hippocampal proteome at both acute and chronic stages after repeated mTBI.

2. Materials and methods

2.1. Animals

Sixty-four male Long-Evans rats were obtained from Animal Resource Centre (WA, Australia) at post-natal day (PND) 28. Rats were housed in pairs at the La Trobe University Central Animal House, with a 12 h:12 h light/dark cycle and food and water available ad libitum. All procedures were performed in accordance with the Animal Ethics Committee at La Trobe University (AEC 17–06) and were within the guidelines of the Australia code of practice for the use of animals for scientific purposes by the Australian National Health and Medical Research Council. All animals were allowed 48 h to acclimatize to their enclosures upon arrival before experimenter contact. After this period animals were handled 4 days per week until the first sham or injury procedure on PND 40–42.

2.2. Mild TBI

Rats were randomly allocated into groups that received four sham injuries or four ACHI procedures, with each procedure separated by 48 h. This injury interval was chosen as we previously found behavioral deficits at 48 h post-ACHI (Pham et al., 2019). Sham and ACHI procedures were completed similar to those described previously (Pham et al., 2019). Briefly, a steel helmet was placed over the Decapicone™-restrained rat (Braintree Scientific, MA, United States) and centered over the left parietal bone, with the rat placed on a foam bed and lightly held in position by the lower body. Once positioned, a 5 mm tip attached to a controlled cortical impactor (Leica Biosystems, IL, United States) was triggered and impacted the helmet (velocity: 6.5 m/s, depth: 8 mm, dwell time: 100 ms). The total duration of the procedure was less than 1 min. Sham procedures were performed identically to ACHIs, with the impact instead triggered adjacent to the animal's head. No skull fractures were observed during post-mortem analysis. The lack of anesthesia allowed for inspection of visual signs that may be associated with loss of consciousness (e.g. apnea, absence of hindlimb withdrawal reflex, lying motionless). Incidence of apparent transient loss of consciousness increased after each ACHI (1st ACHI, 25.8%; 2nd ACHI, 35.5%; 3rd ACHI, 45.2%) before slightly decreasing at the 4th ACHI (29.0%). Although these measures were conducted by an experimenter blinded to the experimental condition, it should be acknowledged that assessment of loss of consciousness is inevitably a subjective measure in this context. Once rats had self-righted and were mobile (typically within 1 min post-injury), the beam task was used to assess sensorimotor coordination for all rats for each procedure (as described below). One rat was excluded prior to injury due to a skin wound sustained in its home cage. Rats were assigned into either acute 7-day or chronic 3.5-month recovery groups.

2.3. Neurobehavioral testing

All rats performed beam task immediately post-each injury. Rats assigned to seven-day recovery (i.e. acute) performed open field (24 h following final sham/ACHI), Y-maze (two days), elevated plus maze (EPM; three days) and beam task (three days). To avoid repeated testing, rats assigned to chronic recovery were exposed to the same tasks beginning at three months, with the addition of water maze. All behavioral assessments, excluding beam task (recorded with a stationary camera), were recorded by an overhead camera and analyzed using tracking software Ethovision (Ethovision XT 10; Noldus, the Netherlands). With the exception of the beam task conducted

immediately post-injury, experimenters were blinded to the rat group at all stages during testing. All video/data analysis was conducted by a researcher blinded to the experimental conditions.

Sensorimotor function was assessed via the beam task at baseline one hour prior to the first injury, and one minute post each procedure for all recovery groups similar to that described previously (Pham et al., 2019; Wright et al., 2016). The acute recovery rats repeated the task at three days following the final sham/ACHI, and chronic rats 3.5 months. The task consisted of a 2 cm wide, 1.5 m long wooden beam, elevated 75 cm above the ground, with a protective mattress placed below to soften any falls. At the start-end of the beam there was a bright light (i.e. an aversive stimulus), as well as a camera to record the task. At the finish-end, a dark "home box" was placed with home cage bedding to encourage completion of the task. Prior to testing, beam training took place as previously described (Pham et al., 2019). Rats were required to traverse 1.0 m on the beam to the home box to complete a trial, with five trials used for each session. In a small number of cases, trials were re-performed if rats were immobile for longer than five seconds. Average latency to cross the beam and hindlimb slips were later determined through captured video analysis by a researcher blinded to the experimental conditions. Rats that failed to complete a trial within 20 s were assigned this value. Rats that fell were automatically given this maximum time as well as 2 hindlimb slips.

Locomotion/activity was assessed in an open field similar to that described previously (Brady et al., 2015; Pham et al., 2019). This task consisted of a 100 × 100 cm sawdust-covered arena, with 40 cm high walls to prevent escape. Rats began in the center of the field and were allowed to explore for five minutes before they were returned to their home cage. Locomotion was assessed by total distance travelled. To measure anxiety, the arena was divided into an inner zone (66 × 66 cm^2) and outer zone and the time spent in the inner zone was determined.

We previously found that a single ACHI induced spatial memory deficits in the Y-maze at 24 h, with resolution apparent at 48 h (Pham et al., 2019). Therefore, the current study used Y-maze to assess spatial memory, similar to that described previously (Johnstone et al., 2018; Pham et al., 2019), 48 h after the final injury in the acute recovery groups, as well as at 3.5 months in the chronic recovery groups. Rats were introduced into a Y-maze for a 10 min exploration phase, with the novel arm closed, then returned to their home cage. One hour later, rats were reintroduced to the Y-maze for a 5 min testing phase with all arms open. Entries and time spent in the novel arm and familiar arm were recorded and used to generate a discrimination preference index for each measure. The discrimination index was calculated as follows: (novel arm - familiar arm) / (novel arm + familiar arm) (Rachmany et al., 2013).

Anxiety-like behavior was measured using an EPM similar to that described previously (Pham et al., 2019; Sun et al., 2019). The plus-shaped maze platform was elevated 50 cm above ground and consisted of two open and two closed arms (50 × 10 cm/arm) and a square (10 × 10 cm) central platform. Rats were placed into the center of the EPM and allowed to explore the arms of the maze for 5 min before returning to their home cage. Duration spent in the open arms was quantified.

After the completion of other behavioral tasks, spatial cognition of chronic recovery rats was assessed using water maze similar to as previously described (Webster et al., 2015). A 163 cm diameter circular pool was filled with tap water (28 ± 1 °C). Non-toxic white paint (150 ml) was used to opacify the water and create contrast for rat tracking. The arena was divided into four quadrants, north-east (NE), north-west (NW), south-east (SE) and south-west (SW). A circular 10 cm diameter acrylic escape platform was submerged 2 cm below the water surface in the center of a randomized quadrant. Testing consisted of two days; acquisition (day 1) and reversal (day 2) consisting of 10 trials per day with a maximum time of 60 s per trial. Trials began when rats were placed into the pool facing the wall at one of eight pseudorandomized locations (N, S, E, W, NE, NW, SE, SW) and required to swim to locate and stand on the hidden platform to be 'rescued' by the experimenter. If the maximum time elapsed, rats were led to the hidden platform by the experimenter and left for 15 s before being 'rescued'. Settings for reversal were the same as for acquisition, with the exception of the hidden platform, which was placed in the opposite quadrant before trials commenced. Average latency to platform, percentage of time in target quadrants and percentage of direct and circle swims were calculated.

2.4. Tissue and blood collection

Rats were euthanized for collection of fresh or fixed tissue at either seven days post- or 3.5 months post-last injury. To obtain fresh tissue, rats were anesthetized with Lethobarb and culled for collection. The ipsilateral cortex and hippocampus were flash frozen in liquid nitrogen and stored at −80 °C before analysis. Blood was collected by cardiac puncture and transferred to 3.5 ml Becton Dickinson Vacutainer® SST™ II Advance blood collection tubes (Franklin Lakes, NJ, United States), and allowed to clot at room temperature for 30 min. Following centrifugation at 1500*g* for 10 min, serum was snap frozen in liquid nitrogen and stored at −80 °C until analysis. Chronic recovery rats were anesthetized with Lethobarb, and following cardiac blood collection, were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS before brains were removed and post fixed with PFA at 4 °C overnight. Samples were then transferred to 1× PBS and washed twice daily for 3 days on a rocker at 4 °C and stored in 1× PBS at 4 °C before they were prepared for magnetic resonance imaging (MRI).

2.5. Serum NfL quantification

Quantification of serum NfL was performed using a 'Simoa® NF-light Advantage Kit' run on the Simoa HD-X Analyzer (Quanterix, Billerica, MA, USA). A single assay was performed on eight randomly selected samples per group, and was run in a temperature-controlled laboratory by an experimenter blinded to the experimental conditions. Samples were tested in duplicate, with a total serum volume for each sample of 106 µl. All samples measured above the lower limit of quantification for NfL (0.174 pg/ml).

2.6. High-resolution, data-independent acquisition mass spectroscopy

The ipsilateral hippocampus was chosen for proteomic analysis, as our previous study using this ACHI model found evidence of hippocampal gliosis (Pham et al., 2019). Flash frozen ipsilateral hippocampus samples were homogenized in liquid nitrogen and lysed in 4% SDS, 100 mM HEPES, pH 8.5, which was then heated at 95 °C for 5 min and sonicated three times for 10 s each at an amplitude of 10 µm. The lysates were clarified by centrifugation at 16,000*g* for 10 min and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo). Equal protein amounts were denatured and alkylated using Tris(2-carboxyethyl)-phosphine-hydrochloride (TCEP) and 2-Chloroacetamide (CAA) at a final concentration of 10 mM and 40 mM, respectively, and incubated at 95 °C for 5 min. SDS was subsequently removed by chloroform/methanol precipitation. Sequencing grade trypsin was added at an enzyme to protein ratio of 1:100 and the reaction was incubated overnight at 37 °C. The digestion reaction was stopped by adding formic acid to a concentration of 1%. The samples were cleaned up with BondElut Omix Tips (Agilent) and concentrated in a vacuum concentrator prior to analysis by mass spectrometry.

Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap column (100 µm × 2 cm, nanoViper, C18, 5 µm, 100 Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 µm × 50 cm, nanoViper, C18, 2 µm, 100 Å; Thermo Scientific). The peptides were separated by increasing concentrations of 80% ACN / 0.1% FA at a flow of 250 nl/min for 158 min and analyzed with a

QExactive HF mass spectrometer (Thermo Scientific) operated in data-independent acquisition (DIA) mode. Sixty sequential DIA windows with an isolation width of 10 *m/z* have been acquired between 375 and 975 m/z (resolution: 15.000; AGC target: 2e5; maximum IT: 9 ms; HCD Collision energy: 27%) following a full ms1 scan (resolution: 60.000; AGC target: 3e6; maximum IT: 54 ms; scan range: 375–1575 m/z).

2.7. Mass spectrometric data analysis

The acquired DIA data have been evaluated in Spectronaut 13 Laika (Biognosys) using an in-house generated spectral library derived from rat brain samples. Of note, potential plasma protein contaminants have been removed from this library using an in-house generated spectral library derived from rat plasma samples in combination with homologs identified in the Human Blood Atlas (Uhlen et al., 2019). Missing values have been imputed using background signals, protein intensities were $\log_2$ transformed and median normalized, and two pairwise comparisons (7 day ACHI vs. 7 day sham and 3.5 months ACHI vs. 3.5 months sham) were carried out. Protein fold changes and FDR adjusted *p*-values (q-values) were calculated. GO terms associated with differentially regulated proteins (i.e. those with q-value <0.05 were extracted from String database (Szklarczyk et al., 2019).

2.8. DTI

2.8.1. Ex vivo magnetic resonance imaging acquisition

Ex vivo MRI was performed using a 9.4 T Bruker (Bruker™ BioSpin®, USA) and actively decoupled volume transmit and four-channel cryogenically-cooled surface receive coils. Whole fixated brains were embedded with 3% agar in 50 ml Falcon tubes for scanning. DTI was performed in 81 directions with diffusion duration (δ) = 4.5 ms, diffusion separation (Δ) = 13.5 ms and b-value = 4000 s/mm^2. We also obtained one non-diffusion (b_0) volume. Image parameters also included repetition time (4500 ms), echo time (34 ms), field of view (32 × 24 mm^2), 48 slices of 250 μm thickness and matrix size (128 × 96) giving an isotropic spatial resolution of 250 × 250 × 250 μm^3.

2.8.2. MRI processing and analysis

MRtrix3 (www.mrtrix.org) was used to process DTI (Tournier et al., 2019). DTI metrics including fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD) and the apparent diffusion coefficient (ADC) were calculated and registered to a study-specific template as described previously (Wright et al., 2018). Whole-brain voxel-wise analysis was performed using tract-based spatial statistics (TBSS) with threshold free cluster enhancement (TFCE).

ITK-SNAP (Yushkevich et al., 2006) was used to manually trace regions of interest (ROI) on seven consecutive coronal MRI T_2-weighted structural images. ROIs included the cortex, corpus callosum and hippocampus and the total volume of each ROI was calculated using ITK-SNAP.

2.8.3. Statistical analysis

All outcomes were analyzed using GraphPad Prism version 8.3.1 for Windows (GraphPad Software, CA). Water maze percentage time in target quadrant, direct and circle swims were analyzed using unpaired *t*-test and presented as mean + standard error of the mean. For correlation analysis of serum NfL levels and beam performance, Pearson r was used to assess normally distributed data, while Spearman r was used to assess data not normally distributed. Permutation testing of DTI metrics was performed using FSL's *randomize* with 5000 permutations and fully corrected for multiple comparisons with TFCE. Two-way repeated measures analysis of variance (ANOVA) were used for beam task analysis immediately after injury. All other data was analyzed with two-way ANOVA, with variables of injury and time. Sidak's multiple comparisons performed where appropriate. Statistical significance was accepted if $p \leq 0.05$.

3. Results

3.1. ACHI resulted in sensorimotor deficits immediately post-injury

Beam task was used to assess sensorimotor function at baseline and one minute after each sham/injury procedure (n = 31/group; Fig. 1). The average time taken to traverse the beam one minute after each ACHI (Fig. 1a) was analyzed using repeated measures two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)} = 60.27$; $p < 0.0001$) and time ($F_{(4, 240)} = 8.543$; $p < 0.0001$), and a significant interaction of injury by time ($F_{(4, 240)} = 13.88$; $p < 0.0001$). Post hoc analysis found repeated ACHI rats took significantly longer to traverse the beam compared to sham rats after each ACHI procedure (Fig. 1a; $p < 0.0001$ for all time-points). Total number of hindlimb slips 1 min after each ACHI (Fig. 1b) was analyzed using two-way ANOVA, revealing a main effect of injury ($F_{(1, 60)} = 114.0$; $p < 0.0001$) and time ($F_{(4, 240)} = 18.74$; $p < 0.0001$), and a significant interaction of injury by time ($F_{(4, 240)} = 18.51$; $p < 0.0001$). Post hoc comparisons found that injured rats displayed significantly more hindlimb slips at 1 min after each ACHI compared to sham rats (Fig. 1a; $p < 0.0001$ for all time-points).

3.2. Repeated ACHI resulted in persistent sensorimotor deficits

Beam task was also used to assess sensorimotor deficits at three days (n = 14-15/group) and 3.5 months (n = 17/group) after the final injury (Fig. 2). One rat, allocated to the long-term recovery group, completed beam task at 3 days and therefore was included for this analysis only. For time taken to traverse the beam three days and 3.5 months after final-sham/ACHI (Fig. 2a), a two-way ANOVA was performed after excluding one rat with incomplete data (video recording error), and revealed a main effect of injury ($F_{(1, 59)} = 6.44$; $p = 0.014$), and time ($F_{(1, 59)} = 12.50$; $p < 0.001$), with no interaction of injury and time ($F_{(1, 59)} = 0.86$; $p = 0.356$). For hindlimb slips (Fig. 2b) there was a main effect of injury ($F_{(1, 59)} = 4.04$; $p = 0.049$), but not for time ($F_{(1, 59)} = 0.14$; $p = 0.709$), or an injury by time interaction ($F_{(1, 59)} = 1.82$; $p = 0.182$).

3.3. No differences in locomotion and anxiety-related behavior

Locomotor activity and anxiety-like behavior were assessed using open field at one day (n = 14/group) and 3.5 months (n = 17/group) following repeated sham/injury, with no differences found in total distance travelled (Supplementary Fig. 1a) and time spent in center zone (Supplementary Fig. 1b).

EPM was used to assess anxiety-like behavior at three days (n = 14/group) and 3.5 months (n = 17/group) after repeated sham/ACHI (Supplementary Fig. 2). For the duration of time spent in the open arms there was a main effect of time ($F_{(1, 58)} = 30.57$; $p < 0.0001$), however there was no main effect of injury ($F_{(1, 58)} = 0.00$; $p = 0.980$) and no injury by time interaction ($F_{(1, 58)} = 0.03$; $p = 0.853$).

3.4. Impaired spatial memory at acute, but not chronic, stages post-ACHI

The effect of repeated ACHI on spatial memory of the Y-maze was assessed at two days (n = 13/group) and 3.5 months (n = 16-17/group) post-final sham/ACHI (Fig. 3). Rats were excluded if they spent less than 10 s in each arm (n = 2) or escaped the maze (n = 1). For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury ($F_{(1, 55)} = 5.04$; $p = 0.029$), with no main effect of injury ($F_{(1, 55)} = 1.26$; $p = 0.267$) or time ($F_{(1, 55)} = 0.72$; $p = 0.399$). Post hoc analysis found a trend for repeatedly injured rats to spend less time in the novel when compared to the familiar arm at 2 days ($p = 0.056$), but not at 3.5 months post-final injury compared to sham rats ($p = 0.641$). Entries into the novel/familiar arms between sham and repeat ACHI rats was also assessed, revealing no main effect of injury ($F_{(1, 55)} = 0.60$; $p = 0.442$), time ($F_{(1, 55)} = 3.02$; $p = 0.088$), or injury by time interaction ($F_{(1, 55)} = 2.34$; $p = 0.132$).

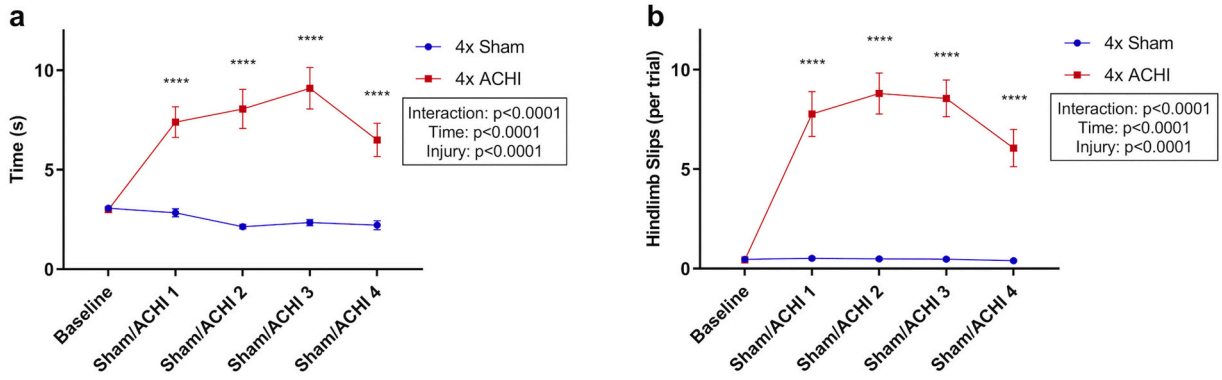

Fig. 1. Effect of repeat ACHI on sensorimotor function immediately after each injury. a) At 1 min after each ACHI, rats took significantly longer to traverse the beam compared to sham rats (ACHI 1–4, each ****$p < 0.0001$). b) Repeated ACHI rats also displayed more hind-limb slips compared to repeated sham rats (ACHI 1–4, each ****$p < 0.0001$). $n = 31$/group at each time-point. Data presented as mean ± SEM.

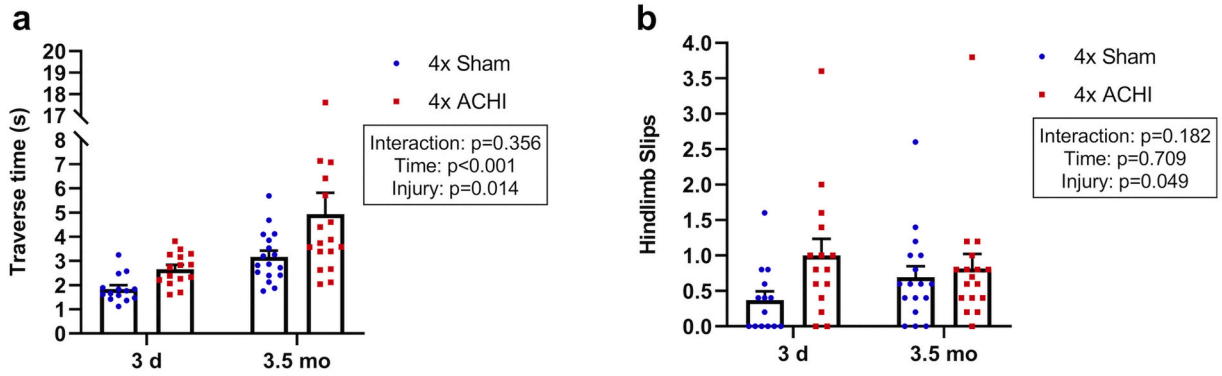

Fig. 2. Effect of repeat ACHI on sensorimotor function at 3 days and 3.5 months after final ACHI. a) A main effect was found in injury ($p = 0.018$) for beam traverse time at 3 days and 3.5 months after final-sham/ACHI b) For hindlimb slips per trial at 3 days and 3.5 months post-final ACHI, there was a main effect of injury ($p = 0.048$). n = 30-31/group at baseline, n = 14-15/group at 3 days, n = 16-17/group at 3.5 months. Data presented as mean + SEM.

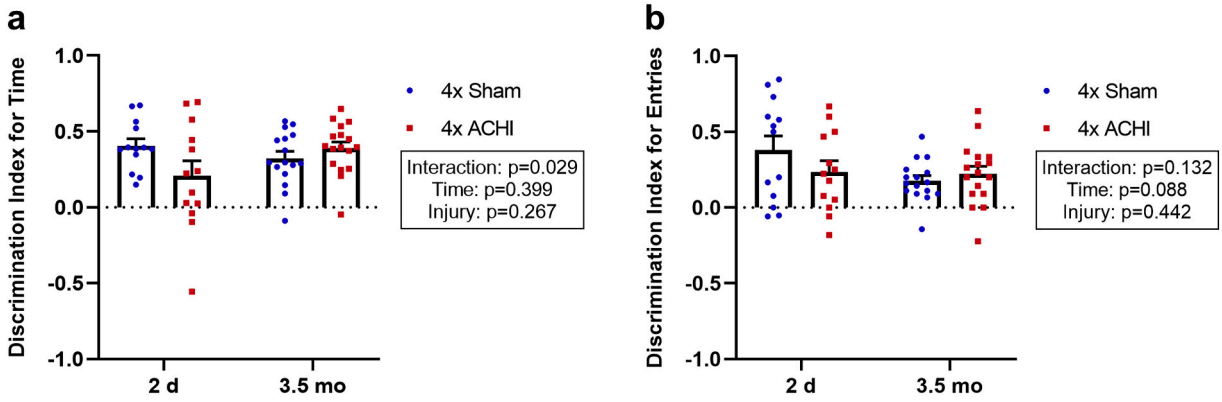

Fig. 3. Effect of repeat ACHI on spatial memory. a) For time in novel arm relative to the familiar arm, there was a significant interaction of time and injury b) No significant differences were observed in novel arm entries between repeat ACHI and sham rats. n = 13-17/group. Data presented as mean + SEM.

Spatial memory at the chronic timepoint was also assessed using water maze (n = 17/group). In both acquisition and reversal testing, no differences were seen for latency to reach the platform, time in target quadrant, time in previous target quadrant (reversal), and direct and circle swims (Supplementary Fig. 3).

3.5. Acute but not chronic elevation in serum NfL levels

A Simoa HD-X Analyzer was used to quantify serum NfL levels at 7 days (n = 8) and 3.5 months (n = 8) after repeated sham or repeated ACHI (Fig. 4; Supplementary Fig. 4). Two-way ANOVA found a significant main effect of injury ($F_{(1, 27)} = 16.57$; $p < 0.001$), time ($F_{(1, 27)} = 6.40$; $p = 0.018$), and interaction of injury and time ($F_{(1, 27)} = 15.48$; $p < 0.001$) (Fig. 4a). Post hoc analysis revealed a significant difference in serum NfL between sham and ACHI rats at 7 days ($p < 0.0001$) but not 3.5 months ($p = 0.994$). A positive correlation (Pearson $r = 0.72$; $p = 0.044$) was found between 7-day serum NfL and the average number of beam slips immediately after each ACHI procedure (Fig. 4b; Supplementary Fig. 4a). A positive correlation was also revealed between 7-day serum NfL and beam traverse time at three days post-injury (Spearman r

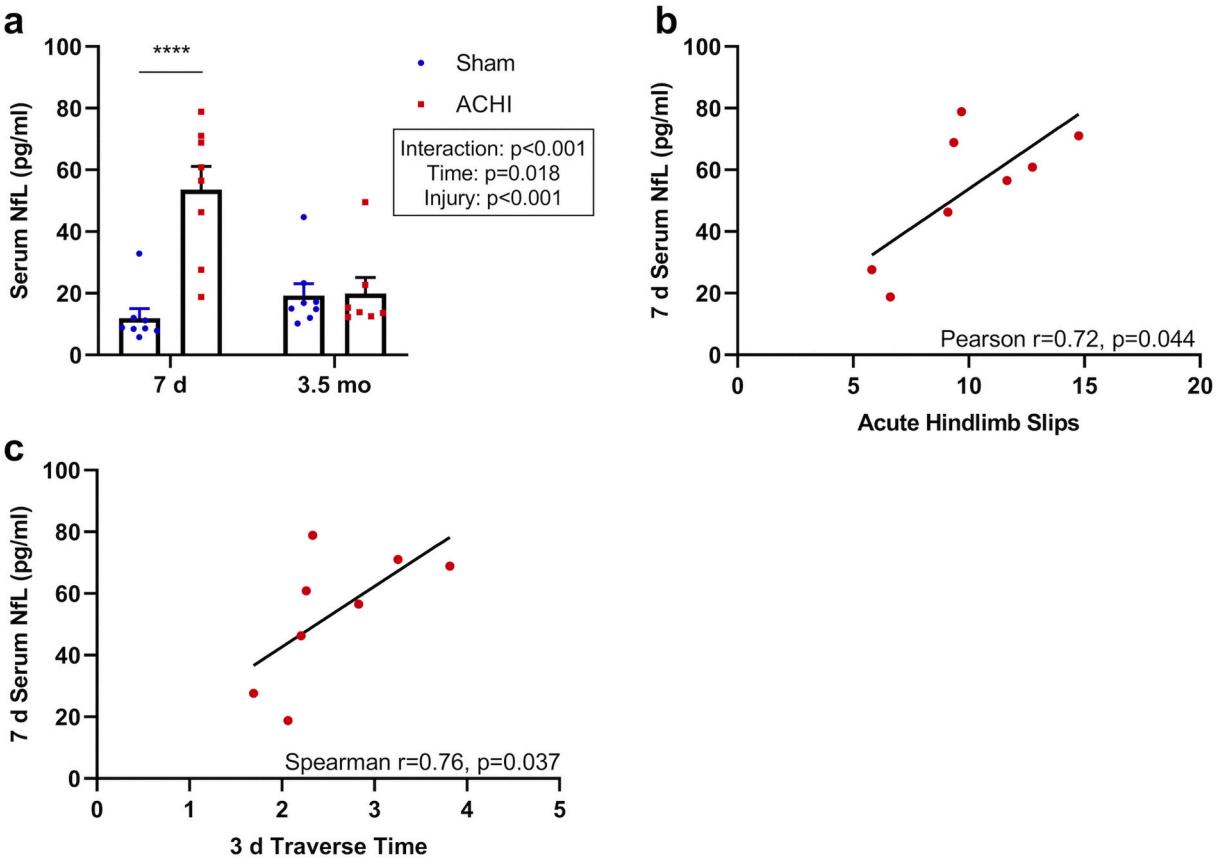

Fig. 4. Serum NfL levels after repeat ACHI. a) Serum NfL was significantly elevated at 7 days post-final ACHI (****$p < 0.0001$) but not at 3.5 months when compared to repeat sham rats. $n = 8$/group. Data presented as mean + SEM. b) A significant relationship between serum NfL levels at 7 days and average beam hindlimb slips immediately post ACHI at each timepoint (Pearson $r = 0.72$, $p = 0.044$). c) A significant correlation between serum NfL levels at 7 days and average beam traverse time at 3 days post ACHI (Spearman $r = 0.76$, $p = 0.037$). Linear regression line of best fit is plotted for visualisation purposes. n = 8/group.

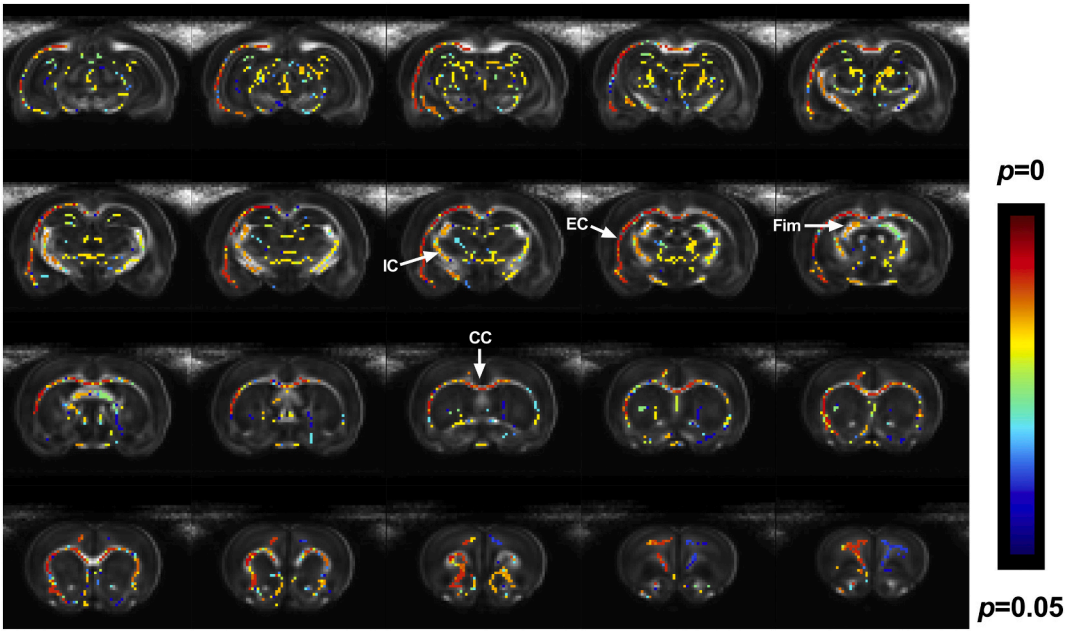

Fig. 5. Whole-brain TBSS revealed chronic FA changes in response to repeat ACHI. This figure depicts the FA study template (i.e. a representative FA image reconstructed from sham and mTBI rats in grayscale), with colored voxels representing significant ($p < 0.05$) group differences in FA between sham rats and mTBI rats at 3.5 months post-injury. Color bar indicates threshold-free cluster enhanced p-value. When compared to sham rats, repeated ACHI rats had reduced FA in voxels of various matter tracts. In particular, reduced FA appeared most evident in the corpus callosum, external capsule, internal capsule and fimbria of the hippocampus (indicated by white arrows). n = 8/group. CC = corpus callosum, EC = external capsule, IC = internal capsule, Fim = fimbria of the hippocampus.

= 0.76; $p = 0.037$) (Supplementary Fig. 4d). No other correlations were observed for beam performance and serum NfL at seven days or 3.5 months (Supplementary Fig. 4).

3.6. Widespread reductions in white matter FA

TBSS was used to assess FA of white matter tracts of ex vivo sham and repeat ACHI rat brains at 3.5 months (n = 8/group). Widespread, significant reductions in white matter FA were observed in both contralateral and ipsilateral hemispheres (Fig. 5). No volumetric differences were seen in the ipsilateral and contralateral cortex, corpus callosum and hippocampus between ex vivo scans of 3.5 month repeat sham and mTBI brains (results not shown).

3.7. Acute and chronic alterations in the hippocampal proteome

High-resolution, data-independent acquisition mass spectrometry was used to analyze the proteomic differences between fresh ipsilateral hippocampal tissue (n = 4-6/group) isolated from sham and repeated mTBI rats, and a total of 5053 proteins has been quantified across all samples. At seven days, 26 proteins were found to be significantly altered between sham and repeated mTBI rats (Supplementary Fig. 5a), and 72 proteins were significantly different in expression at 3.5 months (Supplementary Fig. 5b) when considering a false discovery threshold of 0.05. String database analysis of GO terms revealed 78 biological functions altered at seven days, and 182 altered at 3.5 months (data not shown). After reviewing GO terms, the Uniprot database (UniProt Consortium, 2018), and the literature, a selection of proteins deemed to have evidence for potential involvements in neurobiology and neuropathology were selected and presented in Table 1 (acute) and Table 2 (chronic).

4. Discussion

The neurobehavioral and pathophysiological alterations induced by repeated mTBI are still poorly understood. To provide new insights into the nature and progression of these changes, we implemented our clinically relevant rodent model of mTBI, along with detailed behavioral testing, blood and DTI biomarkers of axonal injury, and mass spectrometry of the hippocampal proteome. Our findings revealed that repeated ACHI was associated acutely with sensorimotor and subtle cognitive deficits, robust increases in serum NfL levels that were correlated with motor deficits, and increased levels of hippocampal proteins linked with changes in energy metabolism and glial activation. At the chronic stages there were subtle neurobehavioral deficits, widespread reductions in white matter integrity, and alterations in several hippocampal proteins including those associated with neurogenesis.

4.1. Behavioral deficits

Clinically, balance and gait are commonly assessed in acutely in patients suspected of suffering a mTBI (McCrory et al., 2017), with deficits usually resolving within days (Guskiewicz, 2001); however, there is some evidence that those with a history of mTBI may display prolonged subtle balance and gait deficits (Lynall et al., 2019; Sosnoff et al., 2011). Here, repeated mTBI resulted in significant sensorimotor deficits on the beam immediately after each impact, providing an indication of injury consistency. Analysis of beam performance at three days and 3.5 months revealed that repeated mTBI rats had sensorimotor impairments. Previously, we found that a single ACHI induced beam deficits that resolved within 24 h (Pham et al., 2019), thus indicating that repeat ACHI appears to have prolonged the duration of sensorimotor deficits. A main effect of time was found for time to traverse the beam, likely reflective of the increased age or size of the rats at the chronic time-point (Sun et al., 2019).

Impairments to memory are also common in clinical mTBI (McCrory et al., 2017), and repeated mTBI may cause cumulative and prolonged cognitive deficits (Broussard et al., 2018; Guskiewicz et al., 2005; Luo et al., 2014; Schatz et al., 2011). Y-maze testing revealed an injury by time interaction for relative time spent in the novel arm, providing some evidence of acutely impaired spatial memory in repeat mTBI rats. There were no deficits Y-maze and water maze test results at the chronic time-point. It is possible that tests conducted over multiple sessions (e.g. Morris water maze) may be required to detect more robust visuospatial memory deficits at acute stages and chronic time-points post-injury. Nonetheless, our findings indicate that in contrast to some other preclinical studies repeated mTBI did not result in overt and persisting spatial memory deficits (Shultz et al., 2019); however, differences in injury model, severity and schedule are likely to be contributing factors.

Table 1
Proteins of interest with altered ipsilateral hippocampal expression at 7-days following repeated mTBI compared to sham.

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
3-ketoacyl-CoA thiolase, peroxisomal	↑	Acetate CoA-transferase activity	Mitochondrial fatty acid beta-oxidation	(Chater-Diehl et al., 2016)
Annexin A2	↑↑	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	(Li et al., 2018; Zhao and Lu, 2007)
Calretinin	↑↑↑	Ca^{2+} binding	Ca^{2+} buffering. Regulation of presynaptic cytosolic Ca^{2+} concentration and long-term potentiation	(Buritica et al., 2009; Schmidt et al., 2013)
Collagen alpha-1(I) chain	↑↑↑	Fibrillar forming collagen	Component of glial scar	(Neo and Tang, 2017; Schwaller, 2014)
Dynein light chain 1, cytoplasmic	↓	Microtubule transport	Enables retrograde motility of vesicles and organelles along microtubules	(Gillardon et al., 1998)
ELAV-like protein 2	↑	RNA binding	Neurodevelopment and synaptic function.	(Berto et al., 2016; Zybura-Broda et al., 2018)
Electron transfer flavoprotein subunit beta	↑↑	Electron transfer activity	Mitochondrial fatty acid beta-oxidation and amino acid catabolism	(Shimazu et al., 2018)
60 kDa heat shock protein, mitochondrial	↑	Chaperonin involved in mitochondrial protein import	Mitochondrial protein import and macromolecular assembly. Promotes cytokine release and immune cell activation	(Dukay et al., 2019; Lehnardt et al., 2008)
Isocitrate dehydrogenase [NADP], mitochondrial	↑↑	Isocitrate dehydrogenase ($NADP^+$) activity	Mitochondrial enzyme that plays an essential role in intermediary metabolism and energy production	(Han et al., 2017; Kim et al., 2016)
Myosin-11	↑↑	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	(Rex et al., 2010)
Propionyl-CoA carboxylase beta chain, mitochondrial	↑	Propionyl-CoA carboxylase activity	Mitochondrial catabolism of odd chain fatty acids, branched-chain amino acids and other metabolites	(Tan et al., 2018)
Vimentin	↑↑	Class-III intermediate filaments of non-epithelial cells	Marker of reactive astrocytes. Promotes elongation of astrocytic processes and glial scar formation	(Ribotta et al., 2004; Wilhelmsson et al., 2004)

Table 2
Proteins of interest with altered ipsilateral hippocampal expression at 3.5 months following repeated mTBI relative to sham.

Protein	Injury Effect	Molecular Function	Potential Biological Function	Ref
Alpha-actinin-4	↓	F-actin crosslinking protein	Important for cell motility and structural plasticity of neurons	(Kalinowska et al., 2015; Tentler et al., 2019)
Alpha-2-HS-glycoprotein	↓↓↓	Kinase inhibitor activity	Anti-inflammatory marker	(Shi et al., 2019; Wang et al., 2009)
Arachidonate 15-lipoxygenase	↓↓↓	Non-heme iron-containing dioxygenase	Generates anti-inflammatory molecules in hippocampal-prefrontal cortex. Depletion may compromise cognition	(Shalini et al., 2018)
Ankyrin-2	↑	Ion channel binding	Axonal domain organization and establishment. Essential for synaptic stability. Adaptor and scaffold for various neuronal ion channels	(Choi et al., 2019; Weber et al., 2019)
Annexin A2	↓↓↓	Ca^{2+}/phospholipid-binding	Anxa2-KO mice have exacerbated neuroinflammation post-TBI. May assist in maintaining endothelial integrity post-cerebrovascular injury	(Li et al., 2018; Zhao and Lu, 2007)
Sodium/potassium-transporting ATPase subunit beta-1	↑	Regulatory hydrolysis of ATP	Preservation of Na^{+}/K^{+} ATPases	(Wang et al., 2018; Wen et al., 2018)
Non-muscle caldesmon	↓↓↓	Actin binding protein	Enhances axon extension in hippocampal neurons	(Morita et al., 2012)
Contactin-1	↑	Nervous system development	Present at junctions of axons, myelin, and glial cells. Role in myelin sheath development	(Çolakoğlu et al., 2014)
Isocitrate dehydrogenase [NADP], mitochondrial	↓↓	Intermediary metabolism and energy production	Deficiency can cause oxidative stress and intensify mitochondrial dysfunction leading to neuronal death	(Han et al., 2017; Kim et al., 2016)
Integrin beta-1	↓↓↓	Collagen receptor	Itgb1 signalling promotes axon assembly. Removal causes axon formation deficits	(Huang et al., 2006; Lei et al., 2012)
Galectin-1	↓↓↓	Lectin, binding carbohydrates	Neuroprotection in ischemic brain injury	(Horie and Kadoya, 2002; Wang et al., 2015)
Lamin-B1	↓↓↓	DNA binding	Neuronal migration. Deficiency leads to abnormal neuronal development	(Mahajani et al., 2017)
Microtubule-associated protein 1B	↑	Microtubule binding	Present in axons and neurons. Essential for regulation of microtubule and synaptic interaction and stability	(Bodaleo et al., 2016; Gödel et al., 2015)
Myosin-11	↓↓	Actin and ATP binding	Necessary for synaptic plasticity and memory formation	(Rex et al., 2010)
2-oxoglutarate dehydrogenase, mitochondrial	↓	Oxoglutarate dehydrogenase (NAD+) Activity	Impairment of Ogdh complex has key role in glutamate mediated neurotoxicity during TBI, leading to neuronal death	(Weidinger et al., 2017)
Tubulin beta-4B chain	↑↑	Structural component of cytoskeleton	Major structural constituent of microtubules. Important to neurodevelopment and plasticity	(Wu et al., 2009)

4.2. Axonal injury

4.2.1. TBSS

At 3.5 months, ex vivo MRI revealed no overt structural changes in all repeated mTBI rats, with volumes of the ipsilateral and contralateral cortex, corpus callosum and hippocampus all comparable to sham rats. Analyses of white matter integrity with TBSS did however reveal significant diffusion differences in repeated mTBI and sham rats, with reductions in FA observed in hemispheres ipsilateral and contralateral to the site of impact.

Reduced FA is commonly reported following mTBI in humans in both acute and chronic stages of injury (Dean et al., 2015; Rutgers et al., 2008; Wada et al., 2012; Wright et al., 2020). Although FA can also be increased by factors such as gliosis after TBI (Budde et al., 2011), reduced FA is widely thought to reflect white matter pathology such as axonal damage, demyelination, and reduced fibre density (Budde et al., 2011; Hutchinson et al., 2018). Although the extent of FA reductions appeared to be greater in the contralateral hemisphere, widespread changes in white matter structures were observed throughout the brain, with the corpus callosum, external capsule, internal capsule, and the fimbria of the hippocampus and corticofugal pathways particularly affected. Notably, these regions are commonly affected in clinical mTBI (Dean et al., 2015; Girgis et al., 2016; Messé et al., 2011; San Martín Molina et al., 2020), and we have previously found DTI changes in many of these regions in the acute stages after repeated ACHIs (Wortman et al., 2018). As such, damage to these regions may have been a key component of the cognitive deficits observed acutely, and the prolonged sensorimotor deficits. For example, the corpus callosum and corticofugal pathways are both thought to be critical to sensorimotor function, therefore damage to these regions may have contributed to the beam deficits in repeated mTBI rats (Li et al., 2015; Lindenberg et al., 2010). Nonetheless, further studies are required to understand the contribution of white matter changes to symptoms, as well as the temporal progression of these changes throughout acute, sub-acute stages after repeated mTBI.

4.2.2. Serum NfL

Quantification of serum NfL at seven days and 3.5 months after repeated mTBI allowed additional insights into the nature and progression of axonal damage. A cytoskeletal protein that is abundant in large-calibre myelinated axons, NfL is thought to be released into body fluids such as the cerebrospinal fluid (Shahim et al., 2016; Zetterberg et al., 2006) and blood (Shahim et al., 2018) following axonal injury or degeneration. Numerous studies have found serum NfL to be a dynamic biomarker of axonal pathology neurodegenerative diseases (Gaetani et al., 2019), with diffuse axonal injury hypothesised to be a key component of symptom outcomes following TBI (McKee and Daneshvar, 2015; Smith and Stewart, 2020).

In the present study, we found a robust increase of NfL concentrations in serum at seven days after repeat ACHI compared to repeat sham rats. This result is similar to findings in studies of boxers exposed to multiple head traumas, with increased serum NfL found at 7–10 days after competition (Shahim et al., 2017). Despite excellent rodent cross-reactivity of the Simoa NfL assay, relatively few pre-clinical studies have utilised this assay in the context of TBI (Cheng et al., 2019; Cheng et al., 2018). Our novel finding of robustly elevated NfL in serum at seven days after repeated mTBI indicates significant potential of this assay in pre-clinical research, and further indicates that the extent of axonal damage was likely substantial in the ACHI model. Moreover, increases in serum NfL correlated with acute sensorimotor deficits provides evidence of the potential of this biomarker as an indicator of mTBI severity.

A plethora of recent studies have found that serum NfL is highly sensitive to axonal damage in neurodegenerative diseases (Gaetani et al., 2019). Indeed, there is also emerging evidence that white matter damage may be a key contributing factor to some cases of PPCS (Khong et al., 2016; Miller et al., 2016). Given the associations with these conditions and repeated mTBI exposure, we hypothesised that serum NfL would remain elevated at 3.5 months post-injury; however, despite extensive white matter changes apparent at this stage, the resolution of this biomarker to sham levels is likely to indicate a lack of ongoing axonal damage. Future repeated mTBI studies are required to

understand if neurodegeneration is triggered at later points (e.g. 6–12 months post-injury) that may correspond to the typical age of onset of neurodegenerative conditions in humans (i.e. >60 years of age), and whether serum NfL levels can predict and monitor the rate and extent of potential neurodegeneration.

4.3. Hippocampal proteome

A variety of notable differences were found in the hippocampal proteome at seven days or 3.5 months post-injury. Although requiring further investigation, these findings shed new light into the short- and long-term effects of repeated mTBI.

4.3.1. Acute alterations

Unbiased mass spectroscopy revealed up-regulation of several mitochondrial proteins, particularly those associated with β-oxidation of fatty acids. Although the energy demands of the brain are primarily met by glucose (Mergenthaler et al., 2013), recent studies have indicated that capacity of the brain to oxidise fatty acids is greater than previously thought (White et al., 2020). As such, given that significant reductions in cerebral glucose metabolism have been reported in the days after single and repeated mTBI (Prins et al., 2013; Yoshino et al., 1991), it is possible that increased fatty acid catabolism represents a compensatory mechanism in order to meet the metabolic requirements of the injured brain. Furthermore, given the high lipid concentrations in the brain, increased β-oxidation may also represent a protective mechanism to minimize a potentially toxic build-up of fatty acids released by cellular damage. Future studies are required to test these hypotheses, and to understand whether β-oxidation of fatty acids may represent a novel target for neuroprotective therapies and biomarkers of injury neurobiology.

In addition to the proteins associated with adaptations to hippocampal energy metabolism, we also observed increases in proteins associated with gliosis and the formation of a glial scar, buffering of calcium, and maintenance of endothelial cell integrity. These findings provide further evidence that neuroinflammation, disrupted calcium homeostasis, and cerebrovascular damage, are all likely prominent in the acute and sub-acute stages after repeated mTBI.

4.3.2. Chronic alterations

We observed some increases in protein expression at 3.5 months after repeated mTBI that may be reflective of neurorestorative functions activated by repeated mTBI. For example, there is evidence that ankyrin 2 and contactin-1 are critical to formation and organization axons and synapses (Chatterjee et al., 2019; Koch et al., 2008; Weber et al., 2019). Contactin-1 also has a central role in oligodendrocyte myelin formation. Moreover, increases in microtubule-associated protein 1B and tubulin beta-4b, two key components of the axonal cytoskeleton, provide further evidence of potential activation of repair mechanisms after repeated mTBI.

In contrast, we also found a reduction in several proteins in animals subjected to repeated mTBIs that may be associated with impairments in neurodevelopment or neuroremodelling, or a reduced neurogenic capacity. For example, we observed reductions in caldesmon and myosin II, with the interaction of these two proteins previously shown to be critical to axonal extension in hippocampal neurons (Morita et al., 2012). In addition, integrin beta-1 is a cell surface receptor thought to have diverse and important roles in the central nervous system (Panov et al., 2014), including outgrowth of hippocampal progenitor cells (Harper et al., 2010) and axon assembly during development (Lei et al., 2012), with multiple studies demonstrating that deficiency of integrin beta-1 results in impaired long-term potentiation (Huang et al., 2006), synaptic plasticity and working memory (Chan et al., 2006). Although further research is required to confirm and understand the significance of these decreases in protein expression, when considering the significant literature on their neurobiological importance, we speculate that these proteomic changes may contribute to chronic neuropathology and behavioral impairments after repeated mTBI.

Although this study has provided some important new insights into the potential effects of repeated mTBIs, there are some limitations to the study that should be acknowledged. Firstly, it is important to note that given this is a single sex study, there may be limitations in generalizing the current findings to females. Second, although we chose not to run acute behavioral analysis on the chronic recovery rats to avoid the potential confound of repeated task exposure, serial testing may have provided greater insights into the temporal progression of behavioral phenotypes after repeated mTBI. In addition, TBSS analysis was chosen to enable unbiased assessment of white matter throughout the brain structures; however, future studies could apply ROI analysis to examine changes in defined brain structures. Using this approach, one could also examine how DTI changes in specific brain structures relate to other functional (e.g. sensorimotor deficits) and molecular (e.g. NfL) changes. However, serum NfL levels appear to normalize by the chronic stages of injury, and as such, we hypothesize that correlation with FA values appears unlikely. Although we observed robust changes in white matter with TBSS, further studies are required to validate these changes with complementary techniques such as histology. Finally, proteomic analysis in this study was limited to the ipsilateral hippocampus and analysis with mass spectrometry, therefore further studies are required to validate these changes and investigate potential alterations in other brain regions.

5. Conclusions

With increasing concern that exposure to repeated mTBIs can lead to lingering or progressive neurological impairments, it is imperative that pre-clinical research is conducted to better understand these associations and their pathophysiological underpinnings. Using a clinically relevant rat model of repeated mTBI, our acute recovery studies revealed that while still in the symptomatic period, rats had changes in several hippocampal proteins suggestive of adaptations in energy metabolism and increased glial reactivity, as well as a profound increases in serum axonal injury biomarker NfL that correlated with sensorimotor deficits. At a chronic stage of injury, despite displaying minimal neurobehavioral deficits and a return of NfL to sham levels, we found significant disruptions to the hippocampal proteome, and widespread reductions in white matter integrity. Given the significant promise of serum NfL as an early indicator of neurodegenerative disease onset, we speculate that resolution of this biomarker indicates that changes in white matter were not progressive at 3.5 months post-injury, with future studies required to determine if progressive neurodegenerative changes are apparent at a more chronic time-points.

Author contributions

LP conducted all behavioral experiments, performed tissue collection and preparation, generated the data and wrote the draft manuscript. SRS and SJM conceptualized and supervised the project. WTO assisted with individual experiments. DKW generated and analyzed MRI data and wrote the text of the final manuscript. RBS, CH and ADS performed mass spectrometry, analyzed the data and wrote the text of the final manuscript. PMC, MS and JB critically analyzed data. CGS supervised the project. LP, SRS, SJM, DKW, WTO, MS, PMC, JB, RBS, CH, ADS, CGS, RDB, TJO and RM reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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References

Archer, D.P., et al., 2018. Neuroprotection by anaesthetics in rodent models of traumatic brain injury: a systematic review and network meta-analysis. Br. J. Anaesth. 121, 1272–1281.

Barrio, J.R., et al., 2015. In vivo characterization of chronic traumatic encephalopathy using [F-18]FDDNP PET brain imaging. Proc. Natl. Acad. Sci. U. S. A. 112, E2039–E2047.

Berto, S., et al., 2016. ELAVL2-regulated transcriptional and splicing networks in human neurons link neurodevelopment and autism. Hum. Mol. Genet. 25, 2451–2464.

Bodaleo, F.J., et al., 2016. Microtubule-associated protein 1B (MAP1B)-deficient neurons show structural presynaptic deficiencies in vitro and altered presynaptic physiology. Sci. Rep. 6, 30069.

Brady, R.D., et al., 2015. Experimental traumatic brain injury induces bone loss in rats. J. Neurotrauma 33, 2154–2160.

Broussard, J.I., et al., 2018. Repeated mild traumatic brain injury produces neuroinflammation, anxiety-like behaviour and impaired spatial memory in mice. Brain Inj. 32, 113–122.

Budde, M.D., et al., 2011. The contribution of gliosis to diffusion tensor anisotropy and tractography following traumatic brain injury: validation in the rat using Fourier analysis of stained tissue sections. Brain 134, 2248–2260.

Buritica, E., et al., 2009. Changes in calcium-binding protein expression in human cortical contusion tissue. J. Neurotrauma 26, 2145–2155.

Centers for Disease Control, 2019. Traumatic Brain Injury & Concussion Emergency Department (ED) Visits. https://www.cdc.gov/traumaticbraininjury/data/tbi-ed-visits.html (accessed 03.17.2020).

Chan, C.-S., et al., 2006. Beta 1-integrins are required for hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory. J. Neurosci. 26, 223–232.

Chater-Diehl, E.J., et al., 2016. Alteration of gene expression, DNA methylation, and histone methylation in free radical scavenging networks in adult mouse Hippocampus following Fetal alcohol exposure. PLoS One 11, e0154836.

Chatterjee, M., et al., 2019. Contactins in the central nervous system: role in health and disease. Neural Regen. Res. 14, 206–216.

Cheng, W.H., et al., 2018. Age at injury and genotype modify acute inflammatory and neurofilament-light responses to mild CHIMERA traumatic brain injury in wild-type and APP/PS1 mice. Exp. Neurol. 301, 26–38.

Cheng, W.H., et al., 2019. CHIMERA repetitive mild traumatic brain injury induces chronic behavioural and neuropathological phenotypes in wild-type and APP/PS1 mice. Alzheimers Res. Ther. 11, 6.

Cherry, J.D., et al., 2016. Microglial neuroinflammation contributes to tau accumulation in chronic traumatic encephalopathy. Acta Neuropathol. Commun. 4, 112.

Choi, C.S.W., et al., 2019. Ankyrin B and Ankyrin B variants differentially modulate intracellular and surface Cav2.1 levels. Mol. Brain 12, 75.

Çolakoğlu, G., et al., 2014. Contactin-1 regulates myelination and nodal/paranodal domain organization in the central nervous system. Proc. Natl. Acad. Sci. U. S. A. 111, E394–E403.

Coughlin, J.M., et al., 2015. Neuroinflammation and brain atrophy in former NFL players: an in vivo multimodal imaging pilot study. Neurobiol. Dis. 74, 58–65.

Daneshvar, D.H., et al., 2011. The epidemiology of sport-related concussion. Clin. Sports Med. 30, 1–vii.

Dean, P.J.A., et al., 2015. Long-term structural changes after mTBI and their relation to post-concussion symptoms. Brain Inj. 29, 1211–1218.

Dewan, M.C., et al., 2018. Estimating the global incidence of traumatic brain injury. J. Neurosurg. 1-18.

Dretsch, M.N., et al., 2015. Multiple past concussions are associated with ongoing post-concussive symptoms but not cognitive impairment in active-duty army soldiers. J. Neurotrauma 32, 1301–1306.

Dukay, B., et al., 2019. Heat-shock proteins in neuroinflammation. Frontiers 10.

Eisenberg, M.A., et al., 2013. Time interval between concussions and symptom duration. Pediatrics 132, 8–17.

Escolas, S.M., et al., 2020. Traumatic brain injuries: unreported and untreated in an Army population. Mil. Med. 185, 154–160.

Eyolfson, E., et al., 2020. Examining the progressive behavior and neuropathological outcomes associated with chronic repetitive mild traumatic brain injury in rats. Cereb. Cortex Commun. https://doi.org/10.1093/texcom/tgaa002.

Fehily, B., Fitzgerald, M., 2017. Repeated mild traumatic brain injury: potential mechanisms of damage. Cell Transplant. 26, 1131–1155.

Finch, C.F., et al., 2013. Increasing incidence of hospitalisation for sport-related concussion in Victoria, Australia. Med. J. Aust. 198, 427–430.

Gaetani, L., et al., 2019. Neurofilament light chain as a biomarker in neurological disorders. J. Neurol. Neurosur. Ps. 90, 870–881.

Gaw, C.E., Zonfrillo, M.R., 2016. Emergency department visits for head trauma in the United States. Emerg. Med. J. 16, 5.

Gillardon, F., et al., 1998. Induction of protein inhibitor of neuronal nitric oxide synthase/cytoplasmic dynein light chain following cerebral ischemia. Neuroscience 84, 81–88.

Girgis, F., et al., 2016. Hippocampal neurophysiologic changes after mild traumatic brain injury and potential neuromodulation treatment approaches. Front. Syst. Neurosci. 10, 8.

Gödel, M., et al., 2015. Microtubule associated protein 1b (MAP1B) is a marker of the microtubular cytoskeleton in podocytes but is not essential for the function of the kidney filtration barrier in mice. PLoS One 10, e0140116.

Guskiewicz, K.M., 2001. Postural stability assessment following concussion: one piece of the puzzle. Clin. J. Sport Med. 11, 182–189.

Guskiewicz, K.M., et al., 2005. Association between recurrent concussion and late-life cognitive impairment in retired professional football players. Neurosurgery 57, 719–726.

Han, S.J., et al., 2017. Mitochondrial NADP(+)-dependent isocitrate dehydrogenase deficiency exacerbates mitochondrial and cell damage after kidney ischemia-reperfusion injury. J. Am. Soc. Nephrol. 28, 1200–1215.

Harper, M.M., et al., 2010. Integrins contribute to initial morphological development and process outgrowth in rat adult hippocampal progenitor cells. J. Mo. Neurosci. 40, 269–283.

Horie, H., Kadoya, T., 2002. Galectin-1 plays essential roles in adult mammalian nervous tissues. Roles of oxidized galectin-1. Glycoconj. J. 19, 479–489.

Huang, Z., et al., 2006. Distinct roles of the beta 1-class integrins at the developing and the mature hippocampal excitatory synapse. J. Neurosci. 26, 11208–11219.

Hutchinson, E.B., et al., 2018. Diffusion MRI and the detection of alterations following traumatic brain injury. J. Neurosci. Res. 96, 612–625.

Iverson, G.L., et al., 2017. Predictors of clinical recovery from concussion: a systematic review. Br. J. Sports Med. 51, 941–948.

Iverson, G.L., et al., 2019. Chronic traumatic encephalopathy neuropathology might not be inexorably progressive or unique to repetitive neurotrauma. Brain. 142, 3672–3693.

Johnstone, M.R., et al., 2018. Gambogic amide, a selective TrkA agonist, does not improve outcomes from traumatic brain injury in mice. Brain Inj. 32, 257–268.

Kalinowska, M., et al., 2015. Actinin-4 governs dendritic spine dynamics and promotes their remodeling by metabotropic glutamate receptors. J. Biol. Chem. 290, 15909–15920.

Khong, E., et al., 2016. Diffusion tensor imaging findings in post-concussion syndrome patients after mild traumatic brain injury: a systematic review. Front. Neurol. 7, 156.

Kim, H., et al., 2016. IDH2 deficiency promotes mitochondrial dysfunction and dopaminergic neurotoxicity: implications for Parkinson's disease. Free Radic. Res. 50, 853–860.

Koch, I., et al., 2008. Drosophila Ankyrin 2 is required for synaptic stability. Neuron 58, 210–222.

Koerte, I.K., et al., 2015. A review of neuroimaging findings in repetitive brain trauma. Brain Pathol. 25, 318–349.

Lehman, E.J., et al., 2012. Neurodegenerative causes of death among retired National Football League players. Neurology 79, 1970–1974.

Lehnardt, S., et al., 2008. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J. Neurosci. 28, 2320–2331.

Lei, W.-L., et al., 2012. Laminin/β1 integrin signal triggers axon formation by promoting microtubule assembly and stabilization. Cell Res. 22, 954–972.

Li, Y., et al., 2015. The microstructural status of the corpus callosum is associated with the degree of motor function and neurological deficit in stroke patients. PLoS One 10, e0122615.

Li, W., et al., 2018. Annexin A2 is a Robo4 ligand that modulates ARF6 activation-associated cerebral trans-endothelial permeability. J. Cerebr. Blood F Met. 39, 2048–2060.

Lindenberg, R., et al., 2010. Structural integrity of corticospinal motor fibers predicts motor impairment in chronic stroke. Neurology 74, 280–287.

Lindquist, L.K., et al., 2017. Traumatic brain injury in Iraq and Afghanistan veterans: new results from a National Random Sample Study. J. Neuropsychiatr. Clin. Neurosci. 29, 254–259.

Luo, J., et al., 2014. Long-term cognitive impairments and pathological alterations in a mouse model of repetitive mild traumatic brain injury. Front. Neurol. 5, 12.

Lynall, R.C., et al., 2019. Functional balance assessment in recreational college-aged individuals with a concussion history. J. Sci. Med. Sport 22, 503–508.

Lyons, D.N., et al., 2018. A mild traumatic brain injury in mice produces lasting deficits in brain metabolism. J. Neurotrauma 35, 2435–2447.

Mahajani, S., et al., 2017. Lamin B1 levels modulate differentiation into neurons during embryonic corticogenesis. Sci. Rep. 7, 4897.

Manley, G., et al., 2017. A systematic review of potential long-term effects of sport-related concussion. Br. J. Sports Med. 51, 969–977.

McCrory, P., et al., 2017. Consensus statement on concussion in sport—the 5th international conference on concussion in sport held in Berlin, October 2016. Br. J. Sports Med. 51, 838–847.

McKee, A.C., Daneshvar, D.H., 2015. The neuropathology of traumatic brain injury. Handb. Clin. Neurol. 127, 45–66.

McKee, A.C., et al., 2013. The spectrum of disease in chronic traumatic encephalopathy. Brain 136, 43–64.

Mergenthaler, P., et al., 2013. Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends Neurosci. 36, 587–597.

Messé, A., et al., 2011. Diffusion tensor imaging and white matter lesions at the subacute stage in mild traumatic brain injury with persistent neurobehavioral impairment. Hum. Brain Mapp. 32, 999–1011.

Mez, J., et al., 2017. Clinicopathological evaluation of chronic traumatic encephalopathy in players of American football. JAMA J. Am. Med. Assoc. 318, 360–370.
Miller, K.J., et al., 2013. Self-reported mild TBI and postconcussive symptoms in a peacetime active duty military population: effect of multiple TBI history versus single mild TBI. J. Head Trauma Rehabil. 28, 31–38.
Miller, D.R., et al., 2016. White matter abnormalities are associated with chronic postconcussion symptoms in blast-related mild traumatic brain injury. Hum. Brain Mapp. 37, 220–229.
Morita, T., et al., 2012. Caldesmon regulates axon extension through interaction with myosin II. J. Biol. Chem. 287, 3349–3356.
Mouzon, B.C., et al., 2018. Lifelong behavioral and neuropathological consequences of repetitive mild traumatic brain injury. Ann. Clin. Transl. Neur. 5, 64–80.
Neo, S.H., Tang, B.L., 2017. Collagen 1 signaling at the central nervous system injury site and astrogliosis. Neural Regen. Res. 12, 1600–1601.
Panov, A., et al., 2014. Fatty acids in energy metabolism of the central nervous system. Biomed. Res. Int. 2014, 472459.
Pham, L., et al., 2019. Mild closed-head injury in conscious rats causes transient neurobehavioral and glial disturbances: a novel experimental model of concussion. J. Neurotrauma 36, 2260–2271.
Prins, M.L., et al., 2013. Repeated mild traumatic brain injury: mechanisms of cerebral vulnerability. J. Neurotrauma 30, 30–38.
Quinn, D.K., et al., 2018. Prolonged postconcussive symptoms. Am. J. Psychiatry 175, 103–111.
Rachmany, L., et al., 2013. Cognitive impairments accompanying rodent mild traumatic brain injury involve p53-dependent neuronal cell death and are ameliorated by the tetrahydrobenzothiazole PFT-α. PLoS One 8, e79837.
Rex, C.S., et al., 2010. Myosin IIb regulates actin dynamics during synaptic plasticity and memory formation. Neuron 67, 603–617.
Ribotta, M., et al., 2004. Glial scar and axonal regeneration in the CNS: Lessons from GFAP and vimentin transgenic mice. In: Mechanisms of Secondary Brain Damage from Trauma and Ischemia. Springer, pp. 87–92.
Rutgers, D.R., et al., 2008. White matter abnormalities in mild traumatic brain injury: a diffusion tensor imaging study. AJNR Am. J. Neuroradiol. 29, 514.
San Martín Molina, I., et al., 2020. In vivo diffusion tensor imaging in acute and subacute phases of mild traumatic Brain injury in rats. eNeuro 7 (ENEURO.0476–19.2020).
Schatz, P., et al., 2011. Early indicators of enduring symptoms in high school athletes with multiple previous concussions. Neurosurgery 68, 1562–1567 (discussion 1567).
Schmidt, H., et al., 2013. Nanodomain coupling at an excitatory cortical synapse. Curr. Biol. 23, 244–249.
Schwaller, B., 2014. Calretinin: from a "simple" ca(2+) buffer to a multifunctional protein implicated in many biological processes. Front. Neuroanat. 8, 3.
Shahim, P., et al., 2016. Neurochemical aftermath of repetitive mild traumatic brain injury. JAMA Neurol. 73, 1308–1315.
Shahim, P., et al., 2017. Serum neurofilament light as a biomarker for mild traumatic brain injury in contact sports. Neurology 88, 1788–1794.
Shahim, P., et al., 2018. Neurofilament light and tau as blood biomarkers for sports-related concussion. Neurology 90, e1780–e1788.
Shalini, S.-M., et al., 2018. Distribution of Alox15 in the rat brain and its role in prefrontal cortical Resolvin D1 formation and spatial working memory. Mol. Neurobiol. 55, 1537–1550.
Shi, X., et al., 2019. Acute anti-inflammatory markers ITIH4 and AHSG in mice brain of a novel Alzheimer's disease model. J. Alzheimers Dis. 68, 1667–1675.
Shimazu, T., et al., 2018. Role of METTL20 in regulating β-oxidation and heat production in mice under fasting or ketogenic conditions. Sci. Rep. 8, 1179.
Shultz, S.R., et al., 2011. Repeated mild lateral fluid percussion brain injury in the rat causes cumulative long-term behavioral impairments, neuroinflammation, and cortical loss in an animal model of repeated concussion. J. Neurotrauma 29, 281–294.
Shultz, S.R., et al., 2017. The potential for animal models to provide insight into mild traumatic brain injury: translational challenges and strategies. Neurosci. Biobehav. Rev. 76, 396–414.
Shultz, S.R., et al., 2019. The clinical relevance of behavior testing in animal models of traumatic brain injury. J. Neurotrauma. https://doi.org/10.1089/neu.2018.6149.
Silverberg, N.D., et al., 2013. Post-concussion symptom reporting after multiple mild traumatic brain injuries. J. Neurotrauma 30, 1398–1404.
Smith, D.H., Stewart, W., 2020. 'Concussion' is not a true diagnosis. Nat. Rev. Neurol. 16, 457–458.
Smith, D.H., et al., 2013. Chronic neuropathologies of single and repetitive TBI: substrates of dementia? Nat. Rev. Neurol. 9, 211–221.
Smith, D.H., et al., 2019. Chronic traumatic encephalopathy — confusion and controversies. Nat. Rev. Neurol. 15, 179–183.
Sosnoff, J.J., et al., 2011. Previous mild traumatic brain injury and postural-control dynamics. J. Athl. Train. 46, 85–91.
Sun, M., et al., 2019. Aged rats have an altered immune response and worse outcomes after traumatic brain injury. Brain Behav. Immun. 80, 536–550.
Szklarczyk, D., et al., 2019. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47, D607–d613.
Tan, K.N., et al., 2018. Triheptanoin protects against status epilepticus-induced hippocampal mitochondrial dysfunctions, oxidative stress and neuronal degeneration. J. Neurochem. 144, 431–442.
Tentler, D., et al., 2019. Role of ACTN4 in tumorigenesis, metastasis, and EMT. Cells 8, 1427.
Tournier, J.D., et al., 2019. MRtrix3: a fast, flexible and open software framework for medical image processing and visualisation. NeuroImage 202, 116137.
Uhlen, M., et al., 2019. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. Science. 366.
UniProt Consortium, T, 2018. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 46, 2699.
Wada, T., et al., 2012. Decreased fractional anisotropy evaluated using tract-based spatial statistics and correlated with cognitive dysfunction in patients with mild traumatic brain injury in the chronic stage. AJNR Am. J. Neuroradiol. 33, 2117–2122.
Wang, H., et al., 2009. Peripheral administration of fetuin-A attenuates early cerebral ischemic injury in rats. J. Cerebr. Blood F Met. 30, 493–504.
Wang, J., et al., 2015. Galectin-1-secreting neural stem cells elicit long-term neuroprotection against ischemic brain injury. Sci. Rep. 5, 9621.
Wang, J., et al., 2018. Interaction of DCF1 with ATP1B1 induces impairment in astrocyte structural plasticity via the P38 signaling pathway. Exp. Neurol. 302, 214–229.
Weber, T., et al., 2019. The Ankyrin repeat domain controls presynaptic localization of Drosophila Ankyrin2 and is essential for synaptic stability. Front. Cell Dev. Biol. 7.
Webster, K.M., et al., 2015. Progesterone treatment reduces neuroinflammation, oxidative stress and brain damage and improves long-term outcomes in a rat model of repeated mild traumatic brain injury. J. Neuroinflammation 12, 238.
Weidinger, A., et al., 2017. 94 - glutamate excitotoxicity induced by nitric oxide mediated dysfunction of the mitochondrial 2-Oxoglutarate dehydrogenase complex. Free Radic. Biol. Med. 112, 74.
Wen, H., et al., 2018. Neuroglobin mediates neuroprotection of hypoxic postconditioning against transient global cerebral ischemia in rats through preserving the activity of Na+/K+ ATPases. Cell Death Dis. 9, 635.
White, C.J., et al., 2020. Determining the bioenergetic capacity for fatty acid oxidation in the mammalian nervous system. Mol. Cell. Biol. 40 (e00037-20).
Wilhelmsson, U., et al., 2004. Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. J. Neurosci. 24, 5016–5021.
Wortman, R.C., et al., 2018. Diffusion MRI abnormalities in adolescent rats given repeated mild traumatic brain injury. Ann. Clin. Transl. Neur. 5, 1588–1598.
Wright, D.K., et al., 2016. Behavioral, blood, and magnetic resonance imaging biomarkers of experimental mild traumatic brain injury. Sci. Rep. 6, 28713.
Wright, D.K., et al., 2018. Telomere length and advanced diffusion MRI as biomarkers for repetitive mild traumatic brain injury in adolescent rats. Neuroimage Clin. 18, 315–324.
Wright, D.K., et al., 2019. Repeated mild traumatic brain injuries induce persistent changes in plasma protein and magnetic resonance imaging biomarkers in the rat. Sci. Rep. 9, 14626.
Wright, D.K., et al., 2020. White matter abnormalities in retired professional Rugby league players with a history of concussion. J. Neurotrauma. https://doi.org/10.1089/neu.2019.6886.
Wu, C., et al., 2009. Beta4 tubulin identifies a primitive cell source for oligodendrocytes in the mammalian brain. J. Neurosci. 29, 7649–7657.
Yoshino, A., et al., 1991. Dynamic changes in local cerebral glucose utilization following cerebral concussion in rats: evidence of a hyper- and subsequent hypometabolic state. Brain Res. 561, 106–119.
Yushkevich, P.A., et al., 2006. User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. NeuroImage 31, 1116–1128.
Zetterberg, H., et al., 2006. Neurochemical aftermath of amateur boxing. Arch. Neurol. 63, 1277–1280.
Zhao, W.Q., Lu, B., 2007. Expression of annexin A2 in GABAergic interneurons in the normal rat brain. J. Neurochem. 100, 1211–1223.
Zybura-Broda, K., et al., 2018. HuR (Elavl1) and HuB (Elavl2) stabilize matrix Metalloproteinase-9 mRNA during seizure-induced Mmp-9 expression in neurons. Front. Neurosci. 12.