Concussion susceptibility is mediated by spreading depolarization-induced neurovascular dysfunction

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The mechanisms underlying the complications of mild traumatic brain injury, including post-concussion syndrome, post-impact catastrophic death, and delayed neurodegeneration remain poorly understood. This limited pathophysiological understanding has hindered the development of diagnostic and prognostic biomarkers and has prevented the advancement of treatments for the sequelae of mild traumatic brain injury.

We aimed to characterize the early electrophysiological and neurovascular alterations following repetitive mild traumatic brain injury and sought to identify new targets for the diagnosis and treatment of individuals at risk of severe post-impact complications. We combined behavioural, electrophysiological, molecular, and neuroimaging techniques in a rodent model of repetitive mild traumatic brain injury. In humans, we used dynamic contrast-enhanced MRI to quantify blood–brain barrier dysfunction after exposure to sport-related concussive mild traumatic brain injury.

Rats could clearly be classified based on their susceptibility to neurological complications, including life-threatening outcomes, following repetitive injury. Susceptible animals showed greater neurological complications and had higher levels of blood–brain barrier dysfunction, transforming growth factor β (TGFβ) signalling, and neuroinflammation compared to resilient animals. Cortical spreading depolarizations were the most common electrophysiological events immediately following mild traumatic brain injury and were associated with longer recovery from impact. Triggering cortical spreading depolarizations in mild traumatic brain injured rats (but not in controls) induced blood–brain barrier dysfunction. Treatment with a selective TGFβ receptor inhibitor prevented blood–brain barrier opening and reduced injury complications. Consistent with the rodent model, blood–brain barrier dysfunction was found in a subset of human athletes following concussive mild traumatic brain injury.

We provide evidence that cortical spreading depolarization, blood–brain barrier dysfunction, and pro-inflammatory TGFβ signalling are associated with severe, potentially life-threatening outcomes following repetitive mild traumatic brain injury. Diagnostic-coupled targeting of TGFβ signalling may be a novel strategy in treating mild traumatic brain injury.

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Introduction

Despite growing awareness of the risks associated with concussions, and specifically repetitive mild traumatic brain injury (rTBI), the mechanisms underlying the associated complications remain poorly understood and treatment does not exist. Acutely, repetitive mild TBI is associated with rare instances of catastrophic outcomes, including post-impact death.1 In the long-term, repetitive mild TBI is a risk factor for neurodegenerative brain disorders, including Alzheimer’s disease,2,3 Parkinson’s disease,4,5 and chronic traumatic encephalopathy.6–10 Additionally, cognitive and psychiatric disorders affect up to 50% of patients with mild TBI.11

The immediate effects of TBI on brain electrical activity are only partly understood. Studies using EEG reported early electrical depression12,13 as well as epileptic seizures14 after mild TBI. Previous studies using laser doppler flowmetry, intrinsic optical signalling, and electrophysiological recordings in animal models of brain injury have suggested that cortical spreading depolarizations (CSDs) are common after mild TBI.13,15,16 While epileptic seizures are characterized by excessive, synchronized neuronal firing, CSDs involve silencing of electrical activity due to abrupt, near-complete sustained depolarization of neurons.17,18 Spontaneous CSDs have been described in sedated patients after severe brain injuries, including TBI,19–21 and it has been suggested that they are associated with worse clinical outcome.20,22 The role of CSDs in neuropathology following mild TBI is not known.

Blood–brain barrier dysfunction (BBBD) has been reported following repetitive mild TBI in animal models23,24 as well as in a subset of American football and rugby players.25–27 The pathogenic role of BBBD has been studied in non-traumatic models of microvascular injury, showing that serum albumin (extravasated through the leaky BBB) binds to transforming growth factor β (TGFβ) receptors in astrocytes.28–30 Consequently, the activated TGFβ signalling pathway results in astrocytic dysfunction, neuroinflammation, alterations in the extracellular matrix, synaptogenesis, and reduced GABAergic inhibition, together leading to pathological neuropalecticy, increased excitability of the local neuronal network, and delayed neurodegeneration.25,28,31–34 Despite the detailed characterization of this molecular cascade involving BBBD, and previous reports suggesting a role of TGFβ in other models of brain injury,35,36 the role of TGFβ signalling in the outcome of repetitive mild TBI is not known.

Here we sought to evaluate the role of the immediate electrophysiological response to injury, BBBD, and TGFβ signalling in mediating susceptibility to concussion. We developed a rat model that produces clinically relevant neurological heterogeneity to study the early mechanisms underlying concussion susceptibility. We show that susceptible animals present with CSDs as the earliest electrophysiological signature after mild TBI, leading to BBBD, TGFβ signalling, and neuroinflammation. Additionally, we provide the first evidence that treatment with a TGFβ signalling blocker is BBBD-protective and may offer a novel therapeutic strategy for complications in repetitive mild TBI. Imaging data collected from contact-sport athletes in three different centres show that BBBD following delayed concussive head injury is common, heterogeneous, and cannot be readily predicted from reported concussion history or symptoms. These findings have important implications for the understanding of repetitive mild TBI pathophysiology and the diagnosis and treatment of at-risk patients.

Materials and methods

Animal care

All procedures were approved by the Institutional Animal Care and Use Committee and were performed in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals. Eight-week-old, adolescent wild-type male Sprague-Dawley rats were double-housed in standard cages and exposed to a reversed 12:12
light-dark cycle. All experimentation was performed during the dark (active) phase.

**Traumatic brain injury model and drug study**

A modified Marmarou weight-drop closed head model of TBI37–40 (500 g mass, 85 cm height) was used to induce mild TBI with substantial rotation of the animal upon impact (Fig. 1A), as described previously.28 Impacts were delivered anterior to the lambda suture line (but posterior to bregma) in the midline via alignment with the animal’s ears as an anatomical reference. Concussive mild TBI was determined using three criteria: (i) neurobehavioural performance was transiently reduced (Fig. 1B); (ii) mortality was rare (Fig. 1E); and (iii) brain MRI and gross post-mortem examination showed no intracranial bleeding or gross structural injury. Repetitive mild TBI involved one mild TBI per day for up to five consecutive days. According to institutional guidelines, animals that deteriorated significantly (i.e. sustained paralysis, or lost 20% of total body weight) were immediately euthanized. Sham controls were exposed to the same procedures, including isoflurane anaesthesia, with no injury. Pressure-sensitive film (Fujifilm PreScale, Ultra Super Low LLLW Two-Sheet Type) was used in a subset of repetitive mild TBI impacts to determine the delivered pressure.

A drug study was conducted using a selective TGF-β receptor kinase inhibitor, IPW–5371 (provided by Innovation Pathways Inc.), which is a small molecular weight drug that is BBB-penetrable. IPW was administered 10 min after the first impact by intraperitoneal injection at a dose of 20 mg/kg, consistent with previous reports of IPW’s BBB-protective effects in aged animals at this dose.30 IPW was given daily for nine consecutive days in total. TBI animals injected with vehicle solution were used as controls for all drug studies. Animals were randomized upon arrival to the facility into treatment or vehicle groups, and experimenters were unaware of intervention throughout all experimentation. Data analysis was performed only after all data were collected.

**Neurobehavioural assessments**

Neurobehavioural assessments were conducted 1 h prior to each impact and 10 min following injury. In the cohort of drug-treated animals, neurobehavioural assessment also occurred between 1 and 2 months post-injury to assess the delayed effects of treatment. Neurobehavioural assessment was performed using open field, beam walk, and inverted wire mesh tests, as described.24 All tests were filmed (Canon R700) for offline analysis by a single observer unaware of intervention. Latency in regaining the righting reflex post-impact, presence and duration of post-impact convulsive movements, and neurobehavioural task scores were analysed offline by a single observer unaware of the intervention. To assess delayed effects in drug-treated animals, neurobehavioural testing also took place between 1 and 2 months post-injury.

**Animal MRI**

T₂-weighted MRI (repetition time: 2500 ms; echo time: 64 ms; echo train length: 16; echo spacing 8 ms; 46 averages; 128 × 128 data matrix, 0.297 mm in-plane resolution, 1 mm slice thickness; acquisition time: 15.3 min) was performed using a 3 T Agilent system under isoflurane anaesthesia (1–2%, 99%, 1 l/min O₂). For BBBD assessment, dynamic contrast-enhanced (DCE) MRI was performed as described24 24 h and 1 week following the first mild TBI. Scanning protocols included 10 transverse T₁-weighted gradient-echo classic scans (repetition time: 6.03 ms; echo time: 2.98 ms; flip angle: 20°; 20 averages; 108 × 108 data matrix, 0.352 mm in-plane resolution, 1.2 mm slice thickness; acquisition time: 3 min), one immediately before and nine immediately following intravenous injection of a gadolinium-based tracer (gadobenate dimeglumine, ~211.6 mg/rat).

BBBD assessment was performed using in-house scripts (MATLAB R2018a) as reported26,41 In short, the slope of the fitted linear curve to the signal intensity change during consecutive post-contrast scans (5–20 min post-injection) was calculated. A ‘pathological’ voxel threshold was set as the slope value exceeding the 90th percentile slope values of sham controls (n = 15).

**Tissue preparation and molecular analysis**

For tissue analysis, rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg intraperitoneally) and perfused transcardially with physiological saline. Post-mortem gross brain pathology was studied by imaging a subgroup of animals (n = 17 TBI, n = 11 controls, CMOS camera, PCO Edge 5.5 model, PCO-Tech Canada).

Western blotting was performed using standard procedures.26,30 Antibodies used were: α-GAPDH (1:2000, Cell Signaling #2118), α-HMGB1 (1:1000, Abcam AB18256), α-pSmad2 (1:1000, Millipore AB3849), α-Smad2 (1:1000, Cell Signaling #5339), and α-Rabbit HRP (1:2000, Cell Signaling #7074). Membranes were visualized using chemiluminescence SuperSignal West Dura Extended Substrate (ThermoFisher Scientific #34075) on a Bio-Rad Chemidoc system with Image Lab software (4.0.1). Densitometry analysis was carried out using Image J software (National Institutes of Health) by an observer unaware of intervention. All samples were from biological replicates and were run on the same gel when comparative quantification occurred. Protein levels of interest were normalized to a GAPDH internal control for each animal.

Immunostaining was performed using standard techniques, including tissue fixation during perfusion (immediately after saline clearance) with 4% parafomaldehyde (Fisher Scientific #AC416785000). Antibodies used were: α-CFAP (1:2000, Sigma #C9205), α-iba-1 (1:2000, Wako #019-19741), and α-Rabbit Alexa Fluor488 (1:500, Invitrogen #A21206). Images were acquired (Zeiss Axiocam 503) and analysed using ImageJ software (National Institutes of Health) by an observer unaware of intervention. No-primary and no-secondary controls were used for each stain to provide background staining values that were subtracted for quantification purposes.

RNA extraction and real-time quantitative PCR (RT-qPCR) was performed using standard techniques.30 PCR products were amplified using a CFX96 Real-Time PCR System (Bio-Rad), and threshold cycles were detected using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad #172-5271). Mean threshold cycles were normalized to 18S internal control, and relative gene expression levels were quantified using the 2−ΔΔCT method by an observer unaware of intervention. See Supplementary Table 1 for primer sequences.

**Electrophysiological recordings**

Young (10–12-week-old) adult male Sprague Dawley rats (n = 35 TBI, n = 8 controls) were implanted with electrocorticography (ECOG) electrodes under deep isoflurane anaesthesia (1.5–3% isoflurane) as described.41 In short, a 2.5 cm midsagittal incision was made and two 2 mm holes were drilled for screw placement (stainless steel bone screws, Fine Science Tools, 0.86 mm × 4 mm) in the parietal bone (2 mm posterior to bregma, 2 mm anterior to lambda, and 3 mm lateral to sagittal suture). A ground electrode was inserted into neck subcutaneous tissue. Epidural ECOG electrodes were
constructed from Teflon™-insulated silver wire (280 µm diameter, A-M Systems Inc.) and miniature connectors (GS09PLG-220, Ginder Scientific). A cylindrical TBI platform (1 cm diameter, 1.5 cm height) was formed above the frontal bone using dental cement, through which the dropped weight transmitted the impact to the brain in TBI animals (Supplementary Fig. 1).

ECoG was recorded with an Octal Bioamplifier (ML138, AD Instruments). Near-direct current recordings were acquired (1kHz) with a 0.02 Hz high-pass filter, a 100 Hz low-pass filter, and a 60 Hz notch filter. A 1 h baseline recording was collected prior to impact. After TBI (or sham procedure in controls), animals were reconnected to the recording system within 10 s and brain activity was recorded for 1 h. ECoG data were analysed using LabChart software (Version 8) and MATLAB R2018a.

Intravital microscopy

A subset of rats (n = 32) underwent craniotomy under anaesthesia (1.5–3% isoflurane) as described.43 Cortical vascular imaging and BBB integrity measurements were performed under isoflurane anaesthesia using the open window technique as reported.44 Intravital microscopy (Axio Zoom V16, Zeiss GmbH and CMOS camera, PCO Edge5.5 model, PCO-Tech Canada) was performed with a parietal cranial window. CSDs were triggered at the frontal window by 3 M KCl 30 s applications via a soaked cotton ball, or by electrical stimulation (20 V and 20 Hz for 2 s) (Supplementary Fig. 1). Before and 30 min after triggering CSDs, 0.6 ml/kg Na-Fluorescein was injected in the tail vein and pial vessels were imaged at 470/525 ex/em.

Human participants and MRI protocol

Institutional ethical approval was acquired before initiation of studies on human subjects and all participants provided written informed consent. This retrospective study population included 20 amateur male American football players playing in the Israeli Football League (IFL) who reported previous concussion, and an age-matched control group (n = 61) scanned on the same MRI machine.26 In four Dalhousie University [Atlantic Football League (AFL), Halifax, Canada] American football players, repeated imaging was performed following a documented concussive event. In two mixed martial arts fighters from Dublin, Ireland, imaging was conducted before and after concussion.

In AFL players and mixed martial arts fighters, assessment took place immediately following a suspected concussion (same day as injury) as identified by the team’s medical staff or via self-referral. The Sport Concussion Assessment Tool (SCAT-5) assessment paired with clinical suspicion was used by the team medical staff to make a diagnosis of concussion, and players underwent MRI 1 week after the suspected injury.
Mechanisms of concussion susceptibility

**Results**

**Repetitive mild TBI results in neurological complications and potentially convulsions and death**

To investigate the mechanisms underlying concussion-related complications, we established a closed-head weight-drop rat model of repetitive mild TBI that induces substantial rotation of the head and body. This model was chosen due to its similarity to repetitive head injuries commonly experienced in contact-sport settings. Neurobehavioural assessment was conducted in all rats 10 min and 24 h after each impact using three tests (open field, inverted wire mesh, and beam walk—see the ‘Materials and methods’ section and Tagge et al.\(^{24}\) for details).

Impact weight and height were set to mimic a mild injury with a transient reduction in the neurobehavioural score. Ten minutes after a single impact, a decrease in the neurobehavioural score was observed, which returned to baseline 24 h post-injury. Sham controls (anaesthesia only) did not show a change in score compared to baseline (Fig. 1B). Daily repetitive impacts resulted in a progressive decrease in neurobehavioural score that became significant 24 h after three and four impacts (Fig. 1C).

In the immediate seconds following injury, convulsive movements of the hind limbs were observed in 16% of animals after a single injury and in 53% of the rats after repetitive mild TBI (Fig. 1D). Convulsive movements were never observed in sham controls. Convulsions occurred within 9.1 ± 1.5 s following impact, lasted for 22.2 ± 2.4 s (n = 32), and involved rapid onset of a tonic phase followed by rhythmic (‘tonic-clonic’) motion of the hind limbs. Death was never observed in sham controls or animals exposed to one or two impacts. After the third and fourth impacts, immediate mortality occurred in 2.7% and 8% of injured animals, respectively (Fig. 1E). In addition, 10% of animals (n = 11) were euthanized per institutional guidelines due to significant morbidity (see ‘Materials and methods’ section) following repetitive impacts (range: 3–5 impacts, median: 4).

**Consequences of repetitive mild TBI are heterogeneous**

Retrospective analysis of the distribution of neurobehavioural scores of repetitive mild TBI animals showed a divergence of score distribution with consecutive impacts. At baseline and 24 h after a single impact, no difference was found in the distribution of scores between control and repetitive mild TBI animals (Fig. 2A, left and middle). After four impacts, a non-homogenous effect of injury on outcome was indicated by a bimodal distribution of the neurobehavioural score only for repetitive mild TBI animals (Shapiro–Wilk test: \(P < 0.0001\), with a trough value of 6 (Fig. 2A, right)). The bimodal outcome of the impacts was not the result of differences in impact force, as determined via pressure sensitive films (Fig. 2C). Animals with a neurobehavioural score \(\leq 6\) (n = 39) showed a reduction in daily weight gain (\(P < 0.0001\)) compared to sham (n = 20) and animals with a neurobehavioural score \(>6\) (n = 22) who continued to gain weight during their adolescent phase of growth (Fig. 2D).
Figure 2. Heterogeneous outcomes following repetitive mild TBI. (A) The distribution of neurobehavioural scores did not differ between repetitive mild TBI and sham control animals at baseline (left) or after a single mild TBI (middle). After four impacts, neurobehavioural scores of repetitive mild TBI animals showed a bimodal (rather than a normal) distribution (Shapiro-Wilk test: P < 0.0001), with a trough value of 6 (right). (B) Left: Retrospective grouping of ‘susceptible’ and ‘resilient’ animals revealed neurobehavioural score decline in susceptible, but not resilient or sham control animals after three [72 h: one-way ANOVA: F(2,122) = 8.91, P = 0.0002; Tukey-corrected two-tailed Student’s t-test: susceptible versus control: P = 0.0006, resilient versus control: P = 0.83, susceptible versus resilient: P = 0.006; control n = 37, susceptible n = 54, resilient n = 34] and four impacts [96 h: one-way ANOVA: F(2,113) = 18.47, P < 0.0001; Tukey-corrected two-tailed Student’s t-test: susceptible versus control: P < 0.0001, resilient versus control: P = 0.21, susceptible versus resilient: P = 0.0004; control n = 37, susceptible n = 45, resilient n = 34]. Middle: Susceptible animals (but not resilient) showed a progressive increase in duration of post-impact convulsions between the first and second, and second and third impacts [susceptible: one-way ANOVA: F(3,65) = 3.64, P = 0.017, Tukey-corrected two-tailed Student’s t-tests: TBI 1 versus 2: P = 0.01, TBI 2 versus 3: P = 0.002; susceptible n = 20, resilient n = 12]. Right: Susceptible animals showed a longer latency to regain the righting reflex compared to controls (n = 14) after one [one-way ANOVA: F(2,43) = 3.70, P = 0.03; Tukey-corrected two-tailed Student’s t-test: susceptible versus control: P = 0.025; susceptible n = 20, resilient n = 12, control n = 14], two [one-way ANOVA: F(2,43) = 6.43, P = 0.004; Tukey-corrected two-tailed Student’s t-test: susceptible versus control: P = 0.003], or three impacts [one-way ANOVA: F(2,41) = 9.23, P = 0.0005; Tukey-corrected two-tailed Student’s t-test: susceptible versus control: P = 0.003]. Susceptible animals had a longer righting reflex latency than resilient animals after three impacts [one-way ANOVA: F(2,41) = 9.23, P = 0.0005; Tukey-corrected two-tailed Student’s t-test: susceptible versus resilient: P = 0.002]. (C) The mean pressure delivered during each impact (detected with pressure sensitive film) did not differ between susceptible and resilient animals (unpaired Student’s t-tests with false discovery rate correction: Impact 1: P = 0.7; susceptible n = 37, resilient n = 11; Impact 2: P = 0.7; susceptible n = 37, resilient n = 11; Impact 3: P = 0.5; susceptible n = 21, resilient n = 11; Impact 4: P = 0.2; susceptible n = 15, resilient n = 11; Impact 5: P = 0.7; susceptible n = 3, resilient n = 11). (D) Following administration of repetitive impacts, susceptible animals showed a reduction in daily weight gain [one-way ANOVA F(2,78) = 27.09, P < 0.0001, Tukey-corrected two-tailed Student’s t-tests: susceptible versus resilient: P < 0.0001, susceptible versus control: P < 0.0001, resilient versus control: P = 0.085; susceptible n = 39, resilient n = 22, control n = 20] compared to sham control and resilient animals, which continued to gain weight during their adolescent phase of growth. (E) No evidence of contusions or haemorrhage was apparent in susceptible, resilient, or control animals after a single mild TBI. (F) Post-mortem brain extraction revealed frequent (71%, n = 12 of 17) subcutaneous haematoma but no skull fractures or intracerebral haemorrhage. Susceptible (55%, n = 6 of 11), but not resilient (n = 6), animals frequently showed evidence of epidural, subdural, or subarachnoid bleeding (white arrows).

We therefore retrospectively classified rats with a combined neurobehavioural score ≤6 after four or five impacts (including those that could not be tested due to significant morbidity (see ‘Materials and methods’ section) as ‘susceptible’ to repetitive mild TBI. Animals with a neurobehavioural score ≥6 were considered ‘resilient’. Overall, 56% of repetitive mild TBI-exposed animals (n = 62 of 111) were classified as susceptible, while 44% were considered resilient (n = 49 of 111).
Susceptible (but not resilient) animals showed a gradual, consistent decrease in neurobehavioural scores during the week of repetitive mild TBI (Fig. 2B, left). In addition, susceptible rats showed a progressive increase in post-impact convulsion duration and a longer latency in regaining the righting reflex immediately post-impact compared to controls or resilient animals (Fig. 2B, middle and right). MRI anatomical sequences (T1, and T2) did not show brain pathology after a single mild TBI (Fig. 2E). Gross post-mortem brain examination in a sub-group of rats exposed to repetitive mild TBI (n = 17) showed subcutaneous haematoma in 71% of animals, with no evidence of skull fractures or intracerebral haemorrhage. Susceptible (55%, n = 6 of 11) but not resilient animals (0%, n = 6) more frequently had evidence of blood products in the epidural, subdural, or subarachnoid spaces following repetitive mild TBI (Fig. 2F). Controls (n = 11) never had cranial haematoma, skull fracture, haemorrhage, or visible blood products.

Spreading depolarizations are the first electrical signals following mild TBI and induce BBBD

To identify the immediate electrophysiological changes following mild TBI, we measured brain activity using EcoCg recordings obtained immediately following a single mild impact. We observed a large, long-duration, propagating potential in 51.6% of animals (n = 32) within 3 min following mild TBI (Fig. 3A), which was associated with depression of cortical activity, as reported for CSDs in rodents and humans. 29-31,35,36 CSDs were associated with loss of the righting reflex and, in some animals, motor convulsions were noted. Electrographical seizures were rare (n = 2 out of 62 impacts, 3.2%) and were subclinical. In one rat (1.6%), we recorded both a subclinical seizure and CSD. CSDs or seizures were never recorded in sham controls (n = 30 isoflurane exposures).

Latency to resume locomotor activity was longer in animals with recorded CSDs compared to animals with no CSDs (Fig. 3B), similar to the difference found between susceptible and resilient animals (Fig. 2B, right). TBI animals (with or without CSD) had a longer latency to locomotion than controls (Fig. 3B).

Since CSDs have been reported to induce BBBD, 37-42,49-51 we used intravital microscopy to directly measure BBB permeability before and after electrically or KCl-triggered CSDs (Fig. 3C). Triggered CSDs increased BBB permeability within 1 h in TBI-exposed animals, but not in controls (Fig. 3C and D), suggesting that repeated CSDs following TBI can directly induce microvascular changes (see ‘Discussion’ section).

Repetitive mild TBI susceptibility is associated with BBBD and neuroinflammation

To assess BBBD integrity using a clinically applicable approach, we used DCE-MRI (Fig. 4A and B). Animals were scanned 24 h (single mild TBI) and 1 week (repetitive mild TBI) post-impact. While no significant increase in BBB permeability was found 24 h after a single mild TBI, a higher percentage of brain volume with BBBD was found in susceptible, but not resilient repetitive mild TBI rats compared to controls (Fig. 4C and D).

A tight coupling between BBBD, astrocyte activation, and neuroinflammatory response has been shown in various brain pathologies. 34,54 Moreover, the hippocampus has been shown to have increased susceptibility to injury following TBI. 52-54 We therefore performed hippocampal immunofluorescent staining against GFAP and Iba-1 in brains from susceptible, resilient, and control animals collected 1 week after the first impact (Fig. 4E). We found higher GFAP and Iba-1 fluorescent intensity in the dentate gyrus of the hippocampus from susceptible compared to resilient or control animals (Fig. 4F).

RT-qPCR against the inflammatory cytokines IL-1β, IL-6, and TNFα showed elevated IL-6 expression in susceptible animals compared to resilient animals and controls, with no differences in IL-1β or TNFα expression levels 1 week after the first impact (Fig. 4G). Western blotting quantifying HMGB1, a damage-associated molecular pattern protein known to induce IL-6 expression in glial cells, revealed greater levels in susceptible, but not resilient animals compared to controls (Fig. 4H).

TGFβ signalling linked with repetitive mild TBI susceptibility and can be pharmacologically targeted

Astrocytic TGFβ signalling has been identified as a key signalling pathway linking BBBD and neuroinflammation. 26,30,31 We therefore measured the levels of phosphorylated Smad2, the major downstream TGFβ effector protein in astrocytes, in hippocampal homogenates extracted 1 week after the first impact (Fig. 5A). Susceptible animals had a higher phosphorylated fraction of hippocampal Smad2 (pSmad2:Smad2 ratio) compared to resilient or control animals.

To test the role of TGFβ signalling in the observed differences, we treated 36 animals (see the ‘Materials and methods’ section for power analysis details) with IPW-5371, a selective TGFβ receptor inhibitor shown to block brain TGFβ signalling in age-related BBBD. 30 To assess target engagement, we measured pSmad2 and Smad2 levels in a sub-group of IPW-treated rats (n = 6). One month after IPW treatment, the repetitive mild TBI-induced increase in pSmad2:Smad2 levels was blocked (Fig. 5B). DCE-MRI revealed that IPW-treated animals showed fewer voxels with BBBD compared to vehicle controls 1 week after the first impact was administered (Fig. 5C). Animals treated with IPW had shorter post-impact convulsions than vehicle controls (Fig. 5D) and regained the righting reflex earlier than vehicle controls. The righting latency for animals treated with IPW did not differ from anaesthesia-only controls (Fig. 5E). A 16% reduction in the percentage of susceptible animals was observed in the treatment group compared to vehicle controls, but this effect was not significant [vehicle: 63% versus IPW: 47%, chi-square, P = 0.19; vehicle n = 30, IPW n = 36]. However, when tested 1–2 months after injury, IPW-treated animals had improved neurobehavioural scores compared to vehicle controls (Fig. 5F).

BBBD can be detected in contact-sport athletes

Our animal data suggest that BBBD may reflect neuroinflammatory brain signalling associated with a ‘complicated’ repetitive mild TBI, and BBBD imaging can act as a biomarker for brain response to concussion. To determine whether our findings in animals can be translated to humans, we assessed BBBD integrity using DCE-MRI in 24 American football players and two mixed martial arts athletes reporting a history of concussions (Fig. 6A). Data were compared with those from 61 age-matched healthy controls with no history of concussions. 26 On average, players reported 2.6 concussions (95% CI: 1.7–3.5, min-max: 1–6; Supplementary Tables 2 and 4).

Regional analysis showed that in concussed players, the number of regions with BBBD (>2 SD of controls) deviated from a normal distribution (Shapiro-Wilk test: P = 0.004), but not in controls (Fig. 6B). While a group comparison showed no differences in the overall brain volume with BBBD in players compared with controls, up to 35% of players showed shared brain regions with BBBD, mainly in the right hemisphere (Fig. 6C and D). Importantly, BBBD was
found in a few players (n = 4) weeks and even months following the last reported concussion (see 'Discussion' section).

In a few contact sport athletes, repeated scans were available at early time points after a documented concussion: in two mixed martial arts athletes, scans were available before and 1 week following concussion, showing increased brain volume with BBBD (Fig. 6E). In four football players scanned 1 week and 1 month following a concussive mild TBI, resolution of BBB pathology was observed in three players, and increased BBBD was seen in one player (Fig. 6F and G). These results support the notion that BBBD is common in an acute concussive mild TBI, and in some individuals may last for weeks following the injury.

Discussion

We established a novel rodent mild TBI model in which a single injury results in a temporary change in neurological status, similar to a mild brain injury in which concussion-like transient symptoms are present acutely.55 Consistent with mild TBI in humans, a single mild TBI in rats revealed no gross anatomical damage, intracranial bleeding, or lesion in routine MRI.

Administration of similar repetitive impacts resulted in progressive neurological deterioration in a subset of animals, consistent with previous studies in mice and rats using closed-head models of mild TBI and repetitive mild TBI reporting substantial variation in the outcome of injury between animals.24,39 Retrospective classification of animals as susceptible or resilient to repetitive mild TBI demonstrated that while resilient rats were similar to sham controls in any tested parameter, susceptible animals showed longer acute convulsive episodes during the acute post-injury recovery period with a longer time to regain locomotion, a dramatic decrease in neurological status during the week of injury, intracranial bleeding, and in some cases (approximately 10%) death. The observed immediate mortality upon repetitive impacts may be analogous to reports of sudden death (second-impact syndrome) among individuals after consecutive mild TBIs.1 Importantly, a subset of animals died immediately post-impact...
Malignant cerebral oedema, or with a massive release of glutamate similar to that described during hypoxia.60

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CSDs in the presence of existing injury have been reported despite having neurobehavioural scores within the normal range, suggesting that acute mortality following repetitive mild TBI can occur even in the absence of overt neurological deficits or clear susceptibility to injury. Although the exact incidence of second-impact syndrome in humans is unknown,1 the mortality rate in our animal model is higher than the rate of second-impact syndrome in humans. This discrepancy may be due to the nature of the trauma, the short interval between injuries, impacting in the presence of neurological signs, or species-related differences.

While the cause of immediate post-impact death is not understood, we did observe more frequent intracranial haemorrhage, including subarachnoid and subdural bleeding in susceptible animals, compared to resilient animals (Fig. 2F). The observation that susceptible animals also show a more severe microvascular injury and BBBD (Fig. 4D, and see below) raises the hypothesis that, similar to the situation in stroke, BBBD increases the risk for secondary haemorrhage.56,57 Besides haemorrhage, immediate post-impact death could be the result of CSD spreading to the brainstem, as suggested in models of sudden unexpected death in epilepsy (SUDEP).58 CSDs are transient cortical grey matter depolarizations, resulting in suppression of brain activity and disruption of ion gradients lasting several minutes. CSD is initiated when a sufficient number of neurons are depolarized, as suggested in models of sudden unexpected death in epilepsy (SUDEP).58 CSDs are transient cortical grey matter depolarizations, resulting in suppression of brain activity and disruption of ion gradients lasting several minutes. CSD is initiated when a sufficient number of neurons are depolarized, which may occur when TBI-induced cortical shear stress surpasses a certain threshold54 or with a massive release of glutamate similar to that described during hypoxia.60

The frequent recording of CSDs in our model confirms previous studies showing increased blood flow changes within minutes after impact.61 Interestingly, CSDs are also considered as the physiologic substrate of migraine aura, which has been described in patients with complicated mild TBI and following post-traumatic headache.61,62 The extent to which repeated CSD generation with associated migraine aura persists following mild TBI is not known but is an important area for future study. In our model, while CSDs were always associated with loss of righting reflex and frequently with immobility, in some cases clear motor convulsions were noted during CSDs. Since in the sample of recorded impacts (n = 62) seizures were rare (observed in 3.2%, n = 2), we suggest that motor convulsions likely originated from spinal/subcortical activity, possibly due to the absence of normal cortical inhibition during CSD.

While the observed convulsions appear similar to concussive convulsions; Fig. 5D), or whether it is intervening in the evolutionary process of CSD, we report the presence of BBBD in susceptible (but not resilient) animals 1 week after injury, suggesting a lasting effect of injury. Consistent with previous findings, BBBD was associated with reactive gliosis, activated microglia and higher levels of HMGB1, a marker of injury-mediated neuroinflammation.54,64 This may be due to the time course of our experiments, as we assessed BBBD permeability up to 1 h following CSD induction instead of 12–24 h later. Additionally, it is challenging to directly compare the sensitivity of different methods for BBBD detection. Previous reports have assessed BBBD ex-vivo by measuring leakage of Evans blue or dextran, whereas the present experiments used intravital microscopy with sodium fluorescein injection in living animals. Whether there are potential differences in sensitivity between methods for cases of subtle leakage is unknown.

Despite the effort to control for the effects of isoflurane in our experiments, the neuroprotective, BBB protective, and anti-inflammatory effects of isoflurane41,73,74 may influence injury outcome. Our results, however, including BBBD and neuroinflammatory response, are similar to those reported for repetitive mild TBI in mice, in the absence of anaesthesia.24 In fact, if isoflurane has protective effects in our model, our findings regarding the deterioration of repetitive mild TBI animals may be conservative. Future studies may test the hypothesis of a potential selective neuroprotective effect of isoflurane in some animals and not others. An important aspect not covered in the present study is the potential difference in concussion susceptibility, underlying mechanisms, and outcome between males and female animals, given reports that female athletes may report greater concussion symptoms and longer recovery times.
Figure 4 Repetitive mild TBI leads to BBBD and neuroinflammation. (A) Slope values, reflecting a gradual change in brain signal following the injection of a non-BBB-permeable contrast agent, were calculated for each brain voxel across sequential scans. (B) A cumulative frequency histogram of slope values was constructed and showed a rightward shift for repetitive mild TBI animals 1 week (n = 19) post-impact compared to controls (n = 15) or 24 h (n = 4) post-impact. (C) Above-threshold permeability values are plotted on T2-weighted MRI scans of control, susceptible, and resilient animals 1 week after exposure to repetitive mild TBI. (D) Susceptible animals had a higher percentage of brain volume with pathological voxels than sham controls injury. Resilient animals did not differ in BBBD extent from controls [one-way ANOVA F(2,31) = 4.62, P = 0.018, Tukey-corrected two-tailed Student’s t-tests: susceptible versus control: P = 0.01, resilient versus control: P = 0.5; susceptible n = 12, resilient n = 7, control n = 15]. (E) Representative images of GFAP and Iba-1 immunofluorescent staining in post-mortem hippocampal tissue collected from control (n = 6), susceptible (n = 6), and resilient (n = 5) animals 1 week after repetitive mild TBI. Scale bar = 100 μm. (F) Quantification of total immunofluorescence intensity revealed higher (Continued)
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tected versus control: $P = 0.0006$, susceptible versus resiliant: $P < 0.0001$, resilient versus control: $P = 0.72$, susceptible $n = 9$, resilient $n = 7$, control $n = 5$. (B) pSmad2:Smad2 levels were higher in repetitive mild TBI animals compared to controls but not in animals treated with the selective TGFβ receptor inhibitor, IPW-5371, indicating successful target engagement and reduced TGFβ signalling with IPW treatment [one-way ANOVA $F(2,10) = 18.80$, $P = 0.0004$, Tukey-correction two-tailed Student's $t$-tests: vehicle versus control: $P = 0.015$, IPW versus vehicle: $P = 0.002$, IPW $n = 6$, vehicle $n = 3$, control $n = 4$. (C) IPW-treated animals have a lower percentage of brain volume with pathological voxels than vehicle controls (Mann–Whitney test: $P = 0.03$, IPW $n = 9$, control $n = 12$). (D) The duration of post-impact convulsions was shorter in animals treated with IPW following impacts two (after receiving the first dose of drug) to five compared to vehicle controls [Mann–Whitney test: $P = 0.004$, IPW $n = 14$, vehicle $n = 8$. (E) IPW-treated animals had a shorter latency to regain the righting reflex compared to vehicle controls and did not differ from sham (anaesthesia-only) controls [Kruskal–Wallis test: $F(2,106) = 21.97$, $P < 0.0001$ with Dunn post hoc: control versus vehicle $P < 0.0001$, control versus IPW $P = 0.1$, vehicle versus IPW $P = 0.01$; control $n = 56$, IPW $n = 30$, vehicle $n = 20$. (F) When tested between 1 and 2 months post-impact, vehicle TBI animals had lower neurobehavioural scores than isofluorane-only (sham) controls. Treatment with IPW resulted in higher neurobehavioural scores compared to vehicle-treated animals, and IPW animals did not differ from sham controls [one-way ANOVA $F(2,51) = 7.10$, $P = 0.002$, Tukey-correction two-tailed Student’s $t$-tests: vehicle versus control: $P = 0.002$, vehicle versus IPW: $P = 0.03$, IPW versus control: $P = 0.18$, IPW $n = 30$, vehicle $n = 13$, control $n = 11$].

Such pre-clinical models should involve careful attention to biomechanical details between animals of different ages and sizes, along with balanced psychosocial considerations of gender, to ensure accurate interpretations of sex-based differences underlying concussion susceptibility.

Finally, to test the relevance of our findings to concussions in humans, we used DCE-MRI to quantify BBB integrity in contact-sport athletes with a history of concussion. We found substantial heterogeneity in the extent of BBBD following mild TBI. Our previous work in American football players undergoing DCE-MRI suggested significant pathological variability between individual players, as approximately 40% of players had extensive BBBD.25,26 DCE-MRI studies in rugby players have also shown BBB changes in approximately 50% of players.27 Importantly, our preliminary data using repeated imaging suggests that while in most cases BBBD resolved within a few weeks after injury, in some patients it may last weeks to months after the last reported or documented trauma.26 Importantly, there was no correlation between BBBD and symptom severity, number of concussions sustained, and number of symptoms experienced by the included players (Supplementary Fig. 2). This suggests that pathologic BBBD may occur even in the absence of overt neurological symptoms and may be difficult to predict based on concussion history alone. However, these observations are limited by a small sample size and future controlled neuroimaging studies are required to further understand the genetic, environmental, and injury-related mechanisms.

Figure 4 Continued

GFAP and Iba-1 intensity in susceptible compared to resilient and control animals [GFAP: one-way ANOVA $F(2,15) = 10.76$, $P = 0.013$, Tukey-correction two-tailed Student’s $t$-tests: susceptible versus control: $P = 0.002$, susceptible versus resilient: $P = 0.004$, resilient versus control: $P = 0.97$; susceptible $n = 6$, resilient $n = 6$, control $n = 6$; Iba-1: one-way ANOVA $F(2,15) = 6.16$, $P = 0.011$, Tukey-correction two-tailed Student’s $t$-tests: susceptible versus control: $P = 0.037$, susceptible versus resilient: $P = 0.014$, susceptible versus control: $P = 0.076$, susceptible $n = 6$, resilient $n = 6$, control $n = 6$]. (G) Quantification of IL-1β, IL-6, and TNFα mRNA showed elevated expression of IL-6 mRNA in susceptible animals compared to resilient and control animals [IL-6: one-way ANOVA $F(2,20) = 5.25$, $P = 0.015$, Tukey-correction two-tailed Student’s $t$-tests: susceptible versus resilient: $P = 0.04$, susceptible versus control: $P = 0.02$, resilient versus control: $P = 0.98$; susceptible $n = 8$, resilient $n = 7$, control $n = 8$]. No differences were found between IL-1β [one-way ANOVA $F(2,20) = 0.11$, $P = 0.9$] and TNFα [one-way ANOVA $F(2,20) = 1.79$, $P = 0.2$] expression levels. (H) HMG18 protein expression was assessed by western blot, revealing greater levels in susceptible but not resilient animals compared to controls [one-way ANOVA $F(2,18) = 5.86$, $P = 0.01$, Tukey-correction two-tailed Student’s $t$-tests: susceptible versus control: $P = 0.009$, resilient versus control: $P = 0.2$, susceptible versus resilient: $P = 0.2$; susceptible $n = 9$, resilient $n = 7$, control $n = 5$].
associated with BBB injury and repair, and their role in mild TBI outcome. Additional studies should also focus on the early post-impact changes to BBB integrity, as the majority of our clinical data reflects a more chronic time course following concussive injury.

Nevertheless, DCE-MRI conducted in three different centres using two MR machine manufacturers highlights the clinical applicability of this approach as a potential quantitative diagnostic, predictive and pharmacodynamic biomarker in individuals with concussive injury. Of note, most imaging studies assess BBB permeability during the first 10 min after injection of the contrast agent (using the Tofts or modified Tofts model). Here we used the Veksler slow kinetics model that was recently found to be more sensitive in identifying BBBD after TBI. Careful attention to differences in computational approaches is therefore essential to ensure accurate comparison of barrier kinetics between imaging studies moving forward.

Our rodent data suggested that BBBD and associated pathological neuroinflammation and TGFβ signalling may have a key role in neurologic susceptibility to impacts. These results highlight the importance of further investigating the long-term consequences of persistent BBB permeability following head injury and its potential relation to the development of chronic traumatic encephalopathy and other neurodegenerative diseases.

The present study provides a framework for a better understanding of susceptibility to repetitive mild TBI. Results from our study suggest that imaging showing BBBD reflects a neuroinflammatory response and susceptibility to injury, and that diagnostic-coupled pharmacological targeting of TGFβ signalling has the

Figure 6 BBBD in contact-sport athletes. (A) Assessment of BBB integrity by DCE-MRI in 26 contact-sport athletes with a history of concussion (mean number of reported concussions: 2.6, 95% CI: 1.7–3.5, min-max: 1–6) and 61 age-matched controls with no concussion history showed diffuse areas of increased BBB leakage in contact-sport athletes. (B) Regional analysis of brain areas with greater BBBD (>2 SD of controls) showed that concussed players deviated from a normal distribution (Shapiro-Wilk test: $P = 0.004$), with a subset of concussed players having more regions with significant BBBD. (C and D) The percentage of players or controls with above-threshold BBBD in various brain areas is shown. BBBD in concussed players, but not controls, was frequently localized to the right hemisphere, with players sharing common brain regions of BBBD. (E and G) Repeated DCE-MRI scans in two mixed martial arts athletes showed increased brain volume with BBBD 1 week after mild TBI compared to baseline. (F and G) Repeated DCE-MRI scans in four American football players showed a reduction in brain volume with BBBD 1 month after concussion compared to 1 week after injury in three players, with one player showing greater BBBD 1 month post-injury.
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potential to restore microvascular integrity and prevent neurological complications in susceptible individuals.

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Competing interests

D.K. and A.F. are co-founders of Mend Neuroscience Inc., a medical therapeutics company developing pharmacologic agents for repairing BBBD. The other authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

References

1. Bey T, Ostick B. Second impact syndrome. West J Emerg Med. 2009;10(1):6–10.
2. Mortimer JA, Van Duijn CM, Chandra V, et al. Head trauma as a risk factor for Alzheimer’s disease: A collaborative re-analysis of case-control studies. Int J Epidemiol. 1991;20:528–S35.
3. Jellinger KA, Paulus W, Wrocklage C, Litvan I. Traumatic brain injury as a risk factor for Alzheimer disease. Comparison of two retrospective autopsy cohorts with evaluation of ApoE genotype. BMC Neurol. 2001;1.3.
4. Ben-Shlomo Y. The epidemiology of Parkinson’s disease. Baillieres Clin Neurol. 1997;6:55–68.
5. Goldman SM, Tanner CM, Oakes D, Bhudhikanok GS, Gupta A, Langston JW. Head injury and Parkinson’s disease in twins. Ann Neurol. 2006;60:65–72.
6. Omalu BI, DeKosky ST, Minster RL, Kamboh MI, Hamilton RL, Wecht CH. Chronic traumatic encephalopathy in a National Football League player. Neurosurgery. 2005;57:128–134.
7. Omalu BI, DeKosky ST, Hamilton RL, et al. Chronic traumatic encephalopathy in a National Football League player: Part II. Neurosurgery. 2006;59:1086–1092.
8. McKee AC, Cantu RC, Nowinski CJ, et al. Chronic traumatic encephalopathy in athletes: Progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol. 2009;68:709–735.
9. McKee AC, Gavett BE, Stern RA, et al. TDP-43 proteinopathy and motor neuron disease in chronic traumatic encephalopathy. J Neuropathol Exp Neurol. 2010;69:918–929.
10. McKee AC, Stein TD, Nowinski CJ, et al. The spectrum of disease in chronic traumatic encephalopathy. Brain. 2013;136:43–64.
11. McInnes K, Friesen CL, MacKenzie DE, Westwood DA, Boe SG. Mild Traumatic Brain Injury (mTBI) and chronic cognitive impairment: A scoping review. PLoS One. 2017;12(4):e0174847.
12. Hovda DA. The neurophysiology of concussion. Prog Neurol Surg. 2012;28:28–37.
13. Von Baumgarten L, Trabold R, Thal S, Back T, Flesnla N. Role of cortical spreading depressions for secondary brain damage after traumatic brain injury in mice. J Cereb Blood Flow Metab. 2008;28:1353–1360.
14. Bugay V, Bozdemir E, Vigil FA, et al. A mouse model of repetitive blast traumatic brain injury reveals post-trauma seizures and increased neuronal excitability. J Neurotrauma. 2020;37(2):248–261.
15. Pacheco JM, Hines-Lanham A, Stratton C, et al. Spreading depolarizations occur in mild traumatic brain injuries and are associated with postinjury behavior. eNeuro. 2019;6:ENEURO.0070-19.2019.
16. Bouley J, Chung DY, Ayata C, Brown RH, Henninger N. Cortical spreading depression denotes concussion injury. J Neurotrauma. 2019;36:1008–1017.
17. Canals S, Makarova I, López-Aguado L, Largo C, Ibarz JM, Herreras O. Longitudinal depolarization gradients along the somatodendritic axis of CA1 pyramidal cells: A novel feature of spreading depression. J Neurophysiol. 2005;94:943–951.
18. Dreier JP. The role of spreading depression, spreading depolarization and spreading ischemia in neurological disease. Nat Med. 2011;17:493–447.
19. Hartings JA, Strong AJ, Fabricius M, et al. Spreading depolarizations and late secondary insults after traumatic brain injury. J Neurotrauma. 2009;26:1857–1866.
20. Hartings JA, Watanabe T, Bullock MR, et al. Spreading depolarizations have prolonged direct current shifts and are associated with poor outcome in brain trauma. Brain. 2011;134:1529–1540.
21. Fabricius M, Fuhr S, Bhatia R, et al. Cortical spreading depression and peri-infarct depolarization in acutely injured human cerebral cortex. Brain. 2006;129:778–790.
22. Dreier JP, Major S, Pannek H-W, et al. Spreading convulsions, spreading depolarization and epileptogenesis in human cerebral cortex. Brain. 2012;135:259–275.
23. Li W, Watts L, Long J, et al. Spatiotemporal changes in blood-brain barrier permeability, cerebral blood flow, T2 and diffusion following mild traumatic brain injury. Brain Res. 2016;1646:53–61.
24. Tagge CA, Fisher AM, Minaeva OV, et al. Convulsion, microvascular injury, and early tauopathy in young athletes after impact head injury and an impact concussion mouse model. Brain. 2018;141(2):422–458.
25. Weissberg I, Veksler R, Kamintsky L, et al. Imaging blood-brain barrier dysfunction in football players. JAMA Neurol. 2014;71(11):1453–1455.
26. Veksler R, Vazana U, Serlin Y, et al. Slow blood-to-brain transport underlies enduring barrier dysfunction in American football players. Brain. 2020;143:1826–1842.
27. O’Keeffe E, Kelly E, Liu Y, et al. Dynamic blood-brain barrier regulation in mild traumatic brain injury. J Neurotrauma. 2020;37:347–356.
28. Cacheaux LP, Ivens S, David Y, et al. Transcriptome profiling reveals TGF-β signaling involvement in epileptogenesis. J Neurosci. 2009;29:8927–8935.
29. David Y, Cacheaux LP, Ivens S, et al. Astrocytic dysfunction in epileptogenesis: Consequence of altered potassium and gluta- mate homeostasis? J Neurosci. 2009;29(34):10588–10599.
30. Senatorov VV Jr, Friedman AR, Milikovsky DZ, et al. Blood-brain barrier dysfunction in aging induces hyperactivation of TGFβ
signaling and chronic yet reversible neural dysfunction. Sci Transl Med. 2019;11 eaaw8283.

31. Bar-Klein G, Cacheaux LP, Kamintsky L, et al. Losartan prevents acquired epilepsy via TGF-β1 signaling suppression. Ann Neurol. 2014;75:864–875.

32. Levy N, Milikovsky DZ, Baranaukas G, et al. Differential TGF-β1 signaling in glial subsets underlies IL-6–mediated epileptogenesis in mice. J Immunol. 2015;195(4):1713–1722.

33. Shlosberg D, Beni et al. High-resolution T1 and T2 mapping of the rat photothrombosis stroke model. J Neurotrauma. 2016;31:155–165.

34. Salas S, Lapilover E, Müller J, et al. Synaptic plasticity in area CA1 of rat hippocampal slices following intraventricular application of albumin. Neurobiol Dis. 2016;91:155–165.

35. Logan TT, Villapol S, Sykes AJ. TGF-β1 superfamily gene expression and induction of the Runx1 transcription factor in adult neurogenic regions after brain injury. PLoS One. 2013;8(3):e59250.

36. Zhang D, Hu Y, Sun Q, et al. Inhibition of transforming growth factor beta-activated kinase 1 confers neuroprotection after traumatic brain injury in rats. Neuroscience. 2013;238:209–217.

37. Marmarou A, Angoa-Perez M, Briggs DI, Viano DC, Kreipke CW, Kuhn DM. A mouse model of human repetitive mild traumatic brain injury. J Neurosci Methods. 2012;203(1):41–49.

38. Mychasiuk R, Faran A, Esser MJ. Assessment of an experimental rodent model of pediatric mild traumatic brain injury. J Neuromodulat. 2014;31:749–757.

39. Goddeyne C, Nichols J, Wu C, Anderson T. Repetitive mild traumatic brain injury induces ventriculomegaly and cortical thinning in juvenile rats. J Neurophysiol. 2015;113:3268–3280.

40. Bar-Klein G, Lubinski S, Kamintsky L, et al. Imaging blood-brain barrier dysfunction as a biomarker for epileptogenesis. Brain. 2017;140(6):1692–1705.

41. Livak KJ, Schmittgen TD. Analysis of relative gene expression using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001;25:402–408.

42. Vazana U, Veksler R, Pell GS, et al. Glutamate-mediated blood-brain barrier opening: Implications for neuroprotection and drug delivery. J Neurosci. 2016;36:7727–7739.

43. Schoknecht K, Kikhiia M, Lemale CL, et al. The role of spreading depolarizations and electrographic seizures in early injury progression of the rat photothrombosis stroke model. J Cereb Blood Flow Metab. 2021;41:413–430.

44. Serlin Y, Ofir J, Ben-Arie G, et al. Blood-brain barrier leakage: A new biomarker in transient ischemic attacks. Stroke. 2019; 50(5):1266–1269.

45. Kamintsky L, Cairns KA, Veksler R, et al. Blood-brain barrier imaging as a potential biomarker for bipolar disorder progression. NeuroImage Clin. 2020;26:102049.

46. Deoni SCL, Peters TM, Rutt BK. High-resolution T1 and T2 mapping of the brain in a clinically acceptable time with DESPOT1 and DESPOT2. Magn Reson Med. 2005;53(1):237–241.

47. Hartings JA, Bullock MR, Okonkwo DO, et al. Spreading depolarisations and outcome after traumatic brain injury: A prospective observational study. Lancet Neurol. 2011;10:1058–1064.

48. Gursoy-Ozdemir Y, Qiu J, Matsuoka N, et al. Cortical spreading depression activates and upregulates MMP-9. J Clin Invest. 2004;113:1447–1455.
neurovascular coupling and blood–brain barrier dysfunction. Epilepsia. 2019;60(2):322–336.

69. Ding M, Haglid KG, Hamberger A. Quantitative immunochemistry on neuronal loss, reactive gliosis and BBB damage in cortex/ striatum and hippocampus/amygdala after systemic kainic acid administration. Neurochem Int. 2000;36:313–318.

70. Tomkins O, Friedman O, Ivens S, et al. Blood-brain barrier disruption results in delayed functional and structural alterations in the rat neocortex. Neurobiol Dis. 2007;25:367–377.

71. Burda JE, Sofroniew MV. Reactive gliosis and the multicellular response to CNS damage and disease. Neuron. 2014;81:229–248.

72. Montagne A, Barnes SR, Sweeney MD, et al. Blood-Brain barrier breakdown in the aging human hippocampus. Neuron. 2015;85(2):296–302.

73. Statler KD, Alexander H, Vagni V, et al. Isoflurane exerts neuroprotective actions at or near the time of severe traumatic brain injury. Brain Res. 2006;1076:216–224.

74. Luh C, Gierth K, Timaru-Kast R, Engelhard K, Werner C, Thal SC. Influence of a brief episode of anesthesia during the induction of experimental brain trauma on secondary brain damage and inflammation. PLoS One. 2011;6:e19948.

75. Broshek DK, Kaushik T, Freeman JR, Erlanger D, Webbe F, Barth JT. Sex differences in outcome following sports-related concussion. J Neurosurg. 2005;102(5):856–863.

76. Berz K, Divine J, Foss KB, Heyl R, Ford KR, Myer GD. Sex-specific differences in the severity of symptoms and recovery rate following sports-related concussion in young athletes. Phys Sportsmed. 2013;41(2):58–63.

77. McGroarty NK, Brown SM, Mulcahey MK. Sport-related concussion in female athletes: A systematic review. Orthop J Sport Med. 2020;8(7):2325967120932306.

78. Sanderson K. Why sports concussions are worse for women. Nature. 2021;596(7870):26–28.