BRAIN
ORIGINAL ARTICLE

Concussion susceptibility is mediated by spreading depolarization-induced neurovascular dysfunction

Ellen Parker,[1,2] Refat Aboghazleh,[1,3] Griffin Mumby,[1] Ronel Veksler,[4] Jonathan Ofer,[4] Jillian Newton,[1] Rylan Smith,[1,2] Lyna Kamintsky,[1] Casey M. A. Jones,[1,2] Eoin O'Keeffe,[5] Eoin Kelly,[6,7] Klara Doelle,[1] Isabelle Roach,[1] Lynn T. Yang,[8] Pooyan Moradi,[1] Jessica M. Lin,[8] Allison J. Gleason,[8] Christina Atkinson,[9] Chris Bowen,[10,11] Kimberly D. Brewer,[10,11] Colin P. Doherty,[6,7] Matthew Campbell,[5] David B. Clarke,[1,12] Gerben van Hameren,[1] Daniela Kaufer[8,13] and Alon Friedman[1,4]

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.

1 Department of Medical Neuroscience, Dalhousie University, Faculty of Medicine, Halifax, NS, Canada
2 Faculty of Medicine, Dalhousie University, Halifax, NS, Canada
3 Department of Basic Medical Sciences, Al-Balqa Applied University, Al-Salt, Jordan
4 Departments of Physiology and Cell Biology, Brain and Cognitive Sciences, The Zlotowski Center for

Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel
5   Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Ireland
6   FutureNeuro SFI Research Centre, The Royal College of Surgeons in Ireland, Dublin, Ireland
7   Academic Unit of Neurology, Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland
8   Department of Integrative Biology, University of California, Berkeley, Berkeley, CA 94720, USA
9   Department of Family Medicine, Dalhousie University, Halifax, NS, Canada
10   Department of Diagnostic Radiology, Dalhousie University, Halifax, NS, Canada
11   Biomedical Translational Imaging Centre (BIOTIC), Queen Elizabeth ll Health Sciences Centre, Halifax, NS, Canada
12   Department of Surgery (Neurosurgery), Dalhousie University, Halifax, NS, Canada
13   Helen Wills Neuroscience Institute & Berkeley Stem Cell Center, University of California Berkeley, Berkeley, CA 94720, USA

Correspondence to: Dr. Alon Friedman
Department of Medical Neuroscience
Dalhousie University
5850 College Street PO Box 15000
Halifax, NS B3H 4R2, Canada
E-mail: alon.friedman@dal.ca

Introduction

Despite growing awareness of the risks associated with concussions, and specifically repetitive mild traumatic brain injury (TBI), 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 depression[12,13] as well as epileptic seizures[14] 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 models[23,24] 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 neuroplasticity, 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 BBB-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 TBI[37–40] (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.[26] 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 TGFβ 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

T_2-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 isofluorane anaesthesia (1–2%, 99%, 1 l/min O_2). For BBBD assessment, dynamic contrast-enhanced (DCE) MRI was performed as described[41] 24 h and 1 week following the first mild TBI. Scanning protocols included 10 transverse T_1-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 reported.[26,41] 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 Edge5.5 model, PCO-Tech Canada).

Western blotting was performed using standard procedures.[28,30] 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% paraformaldehyde (Fisher Scientific #AC416785000).[30,41] Antibodies used were: α-GFAP (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 (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 $2-\Delta\Delta CT$ method by an observer unaware of intervention.[42] 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% isofluorane) as described.[43] 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
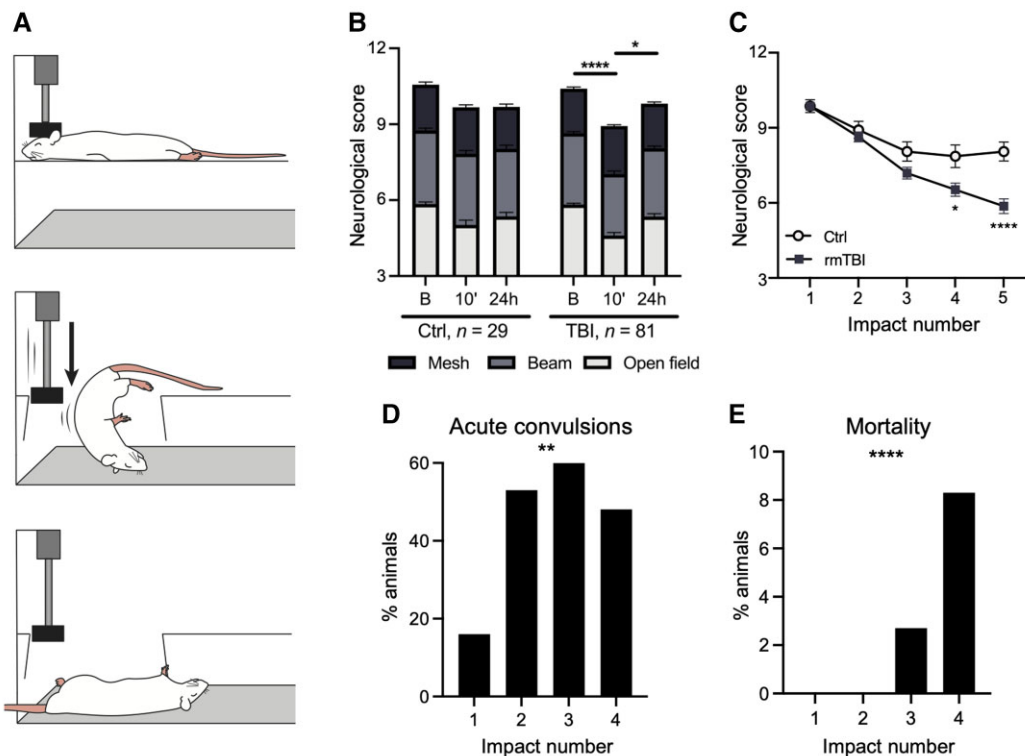
Figure 1 Modelling repetitive mild TBI. (**A**) TBI administration using a weight-drop method produced substantial rotational motion upon impact. (**B**) Ten minutes after sustaining a single mild TBI, rats had a lower composite neurobehavioural score (open field, beam walk, inverted wire mesh tests), which returned to baseline 24 h post-injury [one-way ANOVA: $F(2,262) = 11.53$, $P < 0.0001$, Tukey-corrected two-tailed Student's *t*-tests: 10 min versus baseline: $P < 0.0001$, 10 min versus 24 h: $P = 0.01$, baseline versus 24 h: $P = 0.13$; baseline $n = 92$, 10 min $n = 81$, 24 h $n = 92$]. Sham controls did not differ in neurobehavioural scoring at 10 min or 24 h post-injury compared to baseline [one-way ANOVA: $F(2,94) = 2.83$, $P = 0.07$; baseline $n = 34$, 10 min $n = 29$, 24 h $n = 34$]. (**C**) Repetitive mild TBI animals ($n = 111$) showed a marked decline in neurobehavioural score upon repetitive impacts compared to controls ($n = 37$) after three or four impacts (Mann-Whitney tests with false discovery rate correction: baseline: $P = 0.67$, 24 h: $P = 0.4$, 48 h: $P = 0.051$, 72 h $P = 0.01$, 96 h $P < 0.0001$). (**D**) Immediate post-impact convulsions were observed in 16% ($n = 5$) of animals after a single injury and increased to 53% ($n = 17$) of animals after two impacts [chi-square test for trend: $\chi^2(1,32) = 7.51$, $P = 0.006$, $n = 32$]. Sham controls did not display convulsive movements. (**E**) Post-impact mortality increased with exposure to repetitive impacts, occurring in 2.7% ($n = 3$ of 111) of animals after the third impact and in 8% ($n = 9$ of 111) of animals after the fourth impact [chi-square test for trend: $\chi^2(1,111) = 15.76$, $P < 0.0001$, $n = 111$].

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

post-injury (Supplementary Tables 3 and 4). The IFL athletes self-reported their concussion history and were assessed using the NFL sideline concussion assessment tool and underwent MRI during their football season (Supplementary Tables 3 and 4).

IFL subjects and mixed martial arts fighters were scanned on a 3 T Philips Ingenia MRI scanner in Beer-Sheva and Dublin, respectively, as described.[25–27,45] Briefly, T_1- and T_2-weighted imaging was acquired, followed by a DCE sequence. After five dynamic scans, the contrast agent gadoterate meglumine (Gd-DOTA, Dotarem®, 0.1 mmol/kg, 0.5 M, 1.5 ml/s) was administered intravenously. Images of AFL players were acquired using a 3 T MRI scanner (Discovery MR750, GE Healthcare), with a 32-channel head coil (MR Instruments) as detailed elsewhere.[46] The sequences acquired for BBB assessment included: (i) a T_1-weighted 3D sagittal anatomical scan (BRAVO, echo time/repetition time = 2/6 ms, interval time = 450 ms, field of view 22.4 cm, acquisition matrix 224 × 224 × 168, voxel size 1 × 1 × 1 mm, acceleration 2, averages 2, scan time 5 min 42 s); (ii) a T_1-weighted 3D-tilted axial sequence with variable flip angles (2°, 10°, 30°, DESPOT1, echo time/repetition time = 2/10 ms, flip angle 15°, averages 2, field of view 24 cm, acquisition matrix 192 × 192 × 34, voxel size 1.25 × 1.25 × 6 mm, scan time 6 min 39 s) for the calculation of pre-contrast T_1 map[47]; and (iii) a T_1-weighted 3D axial dynamic scan (LAVA, echo time/repetition time = 2/4 ms, filed of view 24 cm, acquisition matrix: 192 × 192 × 34, voxel size 1.25 × 1.25 × 6 mm, flip angle 15°, averages 1, Δt = 20 s) acquired between 6 and 20 min after intravenous injection of the magnetic contrast agent gadobenate dimeglumine (0.1 mmol/kg, MultiHance, Bracco Imaging Canada).

Offline analysis included BBB permeability mapping, calculated by a linear fit model.[25–27,31,46] Voxels with BBBD were defined when leakage rate was >95th percentile of controls.[26] Regional analysis included the percentage of region volume with BBBD following brain segmentation into 126 regions in accordance with the Montreal Neurological Institute brain atlas (https://github.com/neurodebian/spm12/tree/master/tpm).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 8.0 for Macintosh (GraphPad Software, La Jolla California, USA). Detailed results of statistical testing are located in the figure legends. Group means with SEM and sample size are reported. Differences between groups are reported as exact P-values and were considered statically significant at α < 0.05. Normality of data was assessed using the Shapiro-Wilk normality test. Student's t-test and the Mann–Whitney U-test were used for calculating group differences for normally or non-normally distributed data, respectively. Where multiple Student's t-tests or Mann–Whitney U-tests were used, a false discovery rate correction (Benjamini and Hochberg method) was performed to correct for multiple comparisons. When three or more groups were compared, a one-way ANOVA or Kruskal-Wallis test was performed followed by Tukey or Dunn post hoc tests to correct for multiple comparisons for normally or non-normally distributed data, respectively. A chi-square test for trend was used to assess linear trend in proportions for post-impact convulsion and mortality data.

For the rodent drug study, the sample size (n = 36) for the IPW treatment group was calculated using a power analysis with α = 0.05, a power of 0.8, and an effect size of 0.7 (based on an improvement in the neurobehavioural score from 5 to 7 with SD = 3 following treatment). An improvement in the neurobehavioural score in this range would indicate the prevention or amelioration of animals from being classified as susceptible to resilient following

treatment. The difference in susceptibility prevalence between drug-treatment groups was determined using the chi-square test.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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 <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).
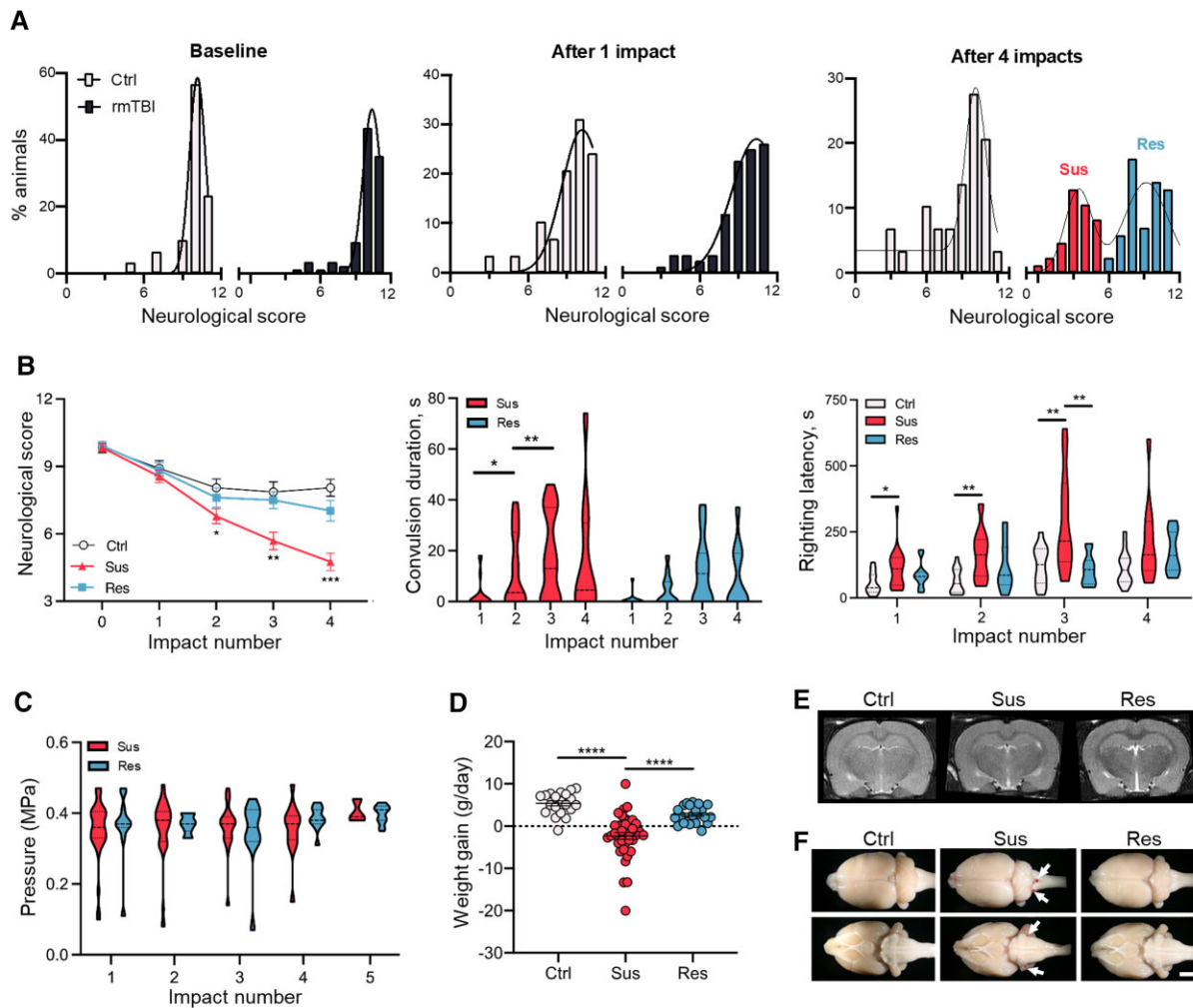
Figure 2 Heterogeneous outcomes following repetitive mild TBI. (A) The distribution of neurobehavioural scores did not differ between repetitive mild TBI and sham control animals at baseline (*left*) or after a single mild TBI (*middle*). After four impacts, neurobehavioural scores of repetitive mild 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 $n = 21$, res $n = 11$; 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 (T_1 and T_2) 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 ECoG 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.[18–20,48] 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 ($n = 30$ isofluorane 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,[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 BBB 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.[33,41] 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.[28,30,31] 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 outcomes, 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 reflex 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 BBB 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 BBB 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
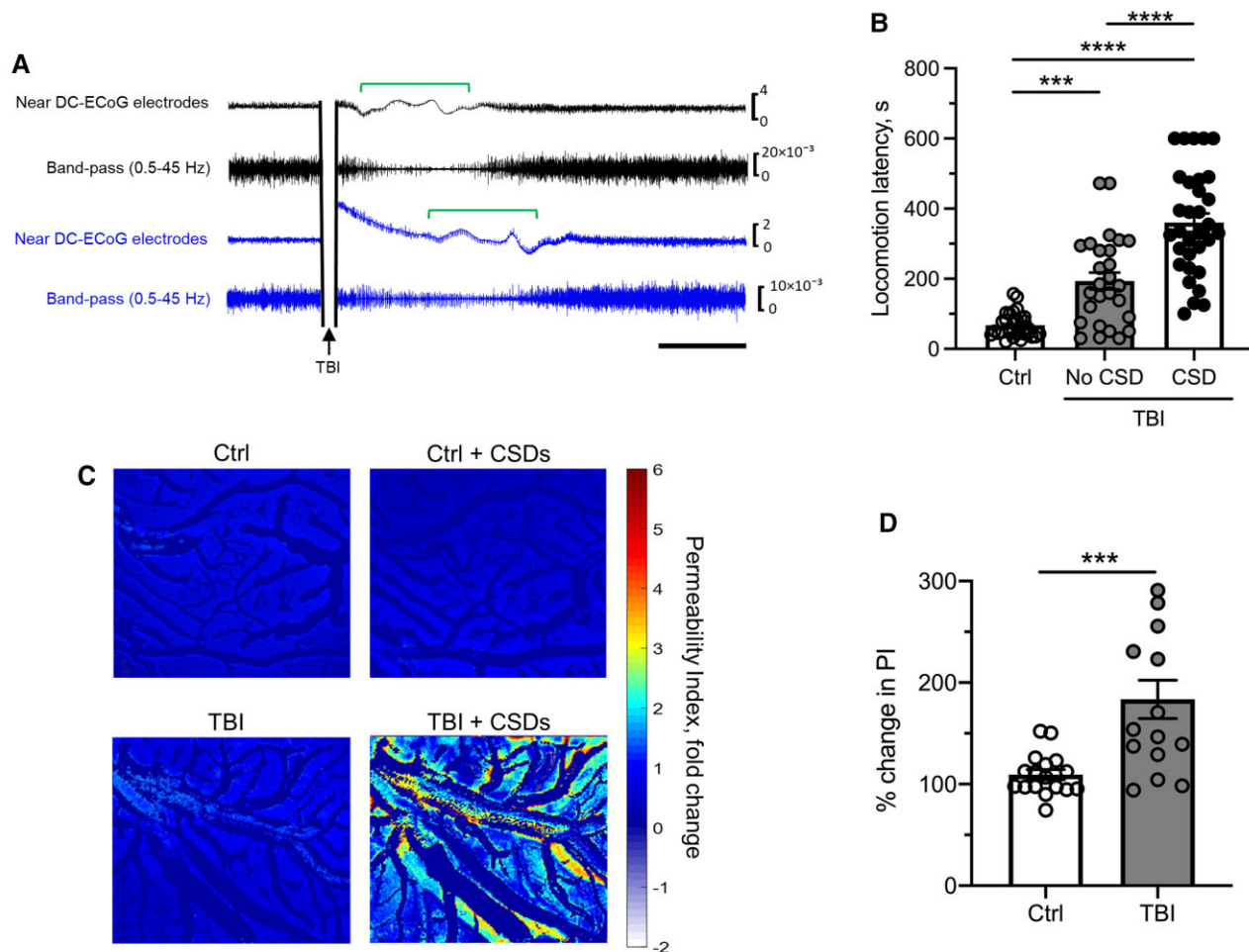
Figure 3 CSDs are the earliest electrophysiological events following mild TBI and can induce BBBD. (**A**) ECoG recordings obtained immediately following mild TBI showed a large amplitude, long-duration, propagating slow potential change associated with depression of cortical activity in 53% ($n = 37$ out of 71) impacts, consistent with the occurrence of spreading depolarization (duration indicated with green bars) recorded in both left (black trace) and right (blue trace) hemispheres. Scale bar = 60 s. Trace units = mV. (**B**) Time to regain locomotor activity was longer when CSDs were recorded following mild TBI (mean 360 ± 26 s, $n = 32$ impacts) compared to impacts without CSDs (194 ± 24 s, $n = 27$) or after isoflurane anaesthesia without impact (67 ± 6 s, $n = 30$) [one-way ANOVA, $F_{(2,86)} = 15.34$, $P < 0.0001$, Tukey-corrected two-tailed Student's t-tests CSD versus control: $P < 0.0001$; CSD versus no CSD: $P < 0.0001$; no CSD versus control: $P = 0.0002$]. (**C**) BBB permeability was assessed before and after triggered CSDs using fluorescent angiography and intravital microscopy. TBI animals showed evidence of increased BBB permeability to peripherally injected sodium fluorescein within 1 h following CSD. (**D**) BBB permeability increased (% change) in TBI animals following triggered CSDs ($n = 15$) but not in controls ($n = 17$; Mann–Whitney test, $P = 0.0007$).

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

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,[59] which may occur when TBI-induced cortical shear stress surpasses a certain threshold[24] 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.[16] 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 reported in humans,[63] there is at present no data to confirm the occurrence of CSDs nor seizures in humans in the acute setting of mild TBI. In susceptible animals, the duration of convulsive episodes increased significantly upon repetitive impacts, and was associated with post-injury complications, including worse neurological outcomes and risk of death.

We further report that triggering spreading depolarization in the injured (but not healthy) cortex leads to a rapid opening of the BBB. In previous animal studies, CSD was found to increase BBB permeability between 3 and 24 h after triggering, due to increased transcytosis,[64] likely involving the upregulation of matrix metalloprotease-9 (MMP-9).[49] Consistent with animal data are case reports in humans with complicated migraine and evidence for a leaky BBB in contrast-enhanced MRI.[65,66] The early increase in permeability observed in animals following a previous mild TBI suggests greater sensitivity of the injured brain to further endothelial injury in the presence of a recurrent injury. In both rodents and humans, CSDs in the presence of existing injury have been reported to be associated with pathological, and even inverse neurovascular coupling.[67] Such an abnormal vascular response to neuronal activity seems to be associated with injury to pericytes and higher BBB permeability,[68] and may underlie delayed ischaemia. Of note, we did not observe a significant increase in BBB permeability in control animals without a history of mild TBI, which is in contrast to previous studies that have shown increased permeability following CSD induction.[49,64] This may be due to the time course of our experiments, as we assessed BBB 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 fluoresceine injection in living animals. Whether there are potential differences in sensitivity between methods for cases of subtle leakage is unknown.

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.[24,28,41,69–71] The higher IL-6 levels we observed in susceptible animals are consistent with reports of IL-6 as the first cytokine to be upregulated and secreted by astrocytes under conditions of BBBD, and its direct role in neuronal hyperexcitability.[32]

Susceptible animals also had greater TGFβ signalling in the hippocampus, a key brain region involved in learning and memory,[72] that is particularly vulnerable to the effects of TBI.[52–54] Pharmacologic inhibition of TGFβ signalling was BBB protective, reduced the duration of post-impact convulsions, and shortened the time to righting reflex immediately post-impact. Though a partial benefit was observed, IPW treatment did not prevent the acute neurobehavioural deficits observed after mild TBI. However, animals treated with the TGFβ inhibitor showed improved neurobehavioural function when tested 1–2 months after initial injury, suggesting that the protective effect of TGFβ inhibition and BBB recovery is mainly important in preventing delayed complications due to repetitive mild TBI. Whether the effect of IPW in our studies is to inhibit CSDs (as suggested by the shorter duration of post-impact convulsions; Fig. 5D), or whether it is intervening in the CSD-induced opening of the BBB is not known and warrants a separate study. Moreover, future investigations into the time course of TGFβ signalling and BBBD following repetitive mild TBI are required to inform optimal dosing regimens. Nevertheless, our findings highlight the potential of TGFβ signalling as a novel therapeutic target to prevent complications of repetitive mild TBI and are consistent with previous studies showing the effectiveness of TGFβ inhibition in preventing epileptogenesis in young animals, as well as reducing seizure susceptibility and cognitive decline in ageing rodents.[30,31]

Despite the effort to control for the effects of isoflurane in our experiments, the neuroprotective, BBB protective, and anti-inflammatory effects of isofluorane[41,73,74] 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.[75–78]
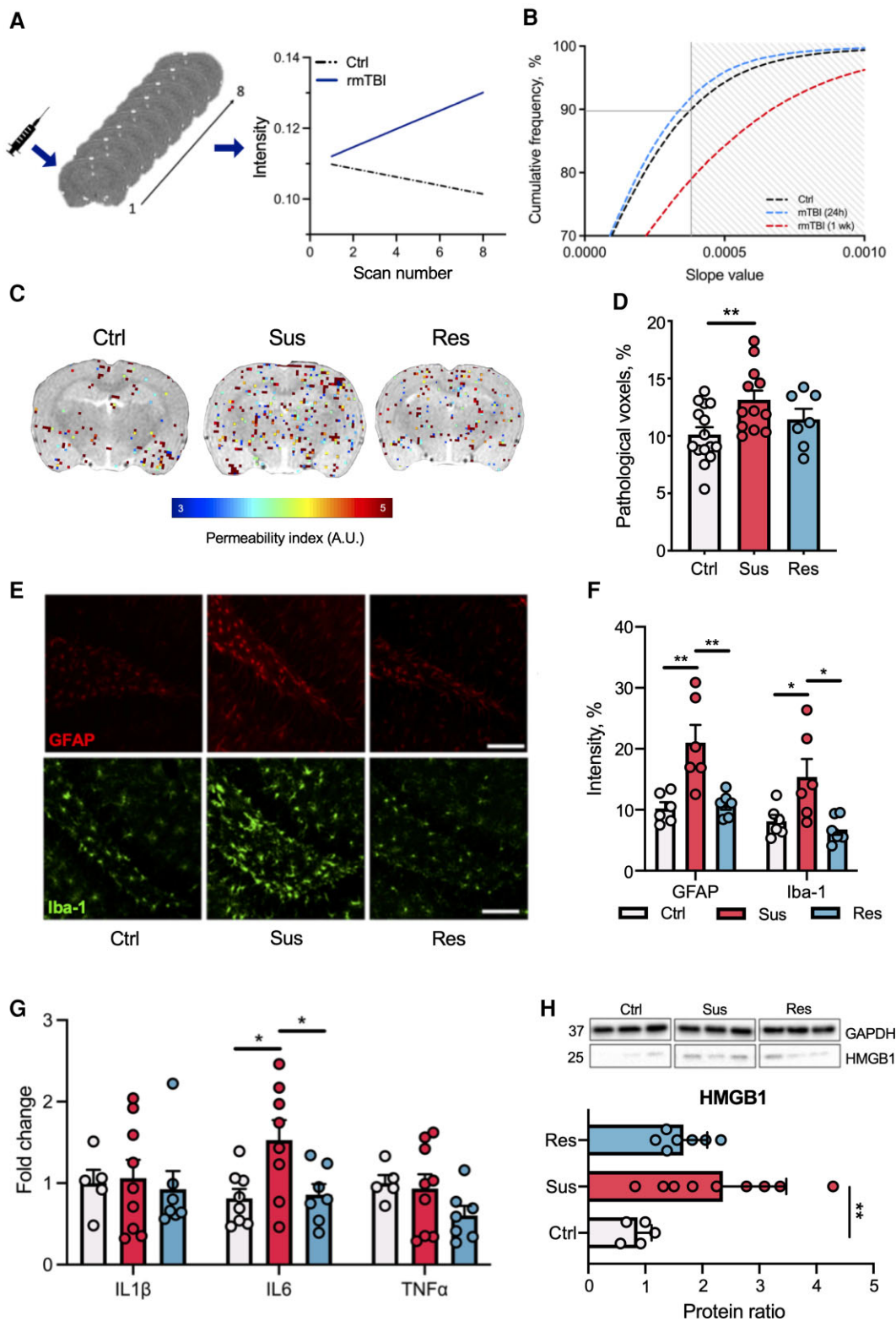
Figure 4 Repetitive mild TBI leads to BBBD and neuroinflammation. (**A**) Slope values, reflecting a gradual change in brain signal following the injection of a non-BBB-permeable contrast agent, were calculated for each brain voxel across sequential scans. (**B**) A cumulative frequency histogram of 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 T_2-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 $= 100\,\mu$m. (**F**) Quantification of total immunofluorescence intensity revealed higher
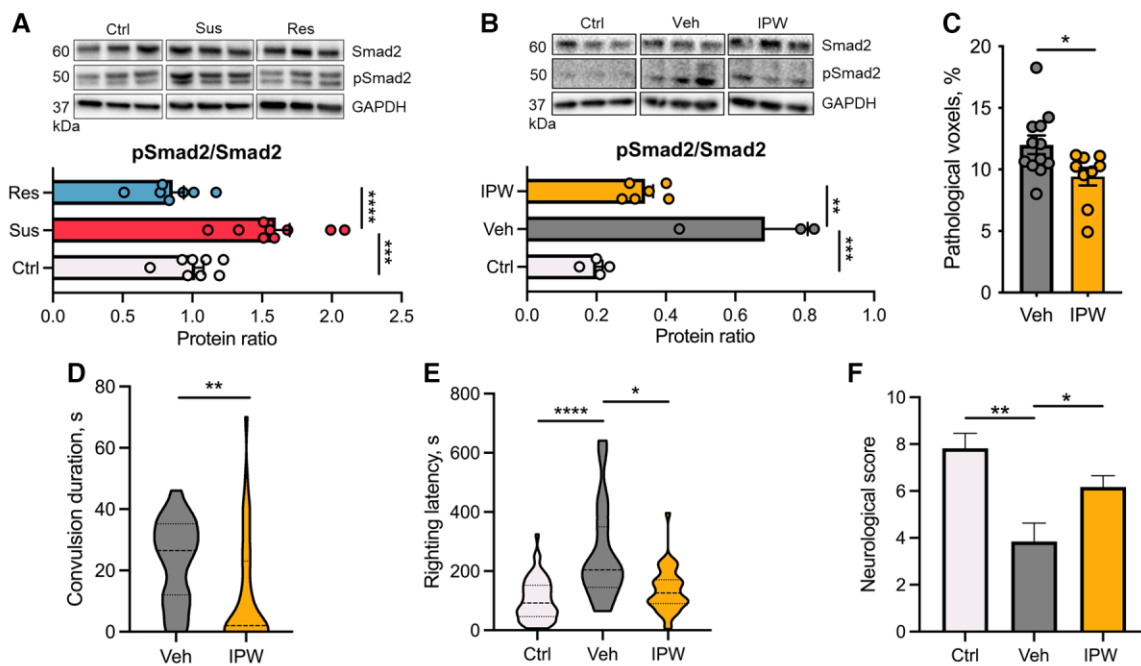
(Continued)

Figure 5 Repetitive mild TBI susceptibility is associated with hippocampal TGFβ signalling. (**A**) TGFβ signalling activity was quantified via western blot by comparing the ratio of pSmad2 and Smad2 protein expression levels. Susceptible animals showed higher TGFβ signalling activity than resilient or control animals [one-way ANOVA $F(2,18) = 20.98$, $P < 0.0001$, Tukey-corrected two-tailed Student's t-tests: susceptible versus control: $P = 0.0006$, susceptible versus resiliant: $P < 0.0001$, resiliant versus control: $P = 0.72$; susceptible $n = 9$, resiliant $n = 7$, 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-corrected two-tailed Student's t-tests: vehicle versus control: $P = 0.0003$, IPW versus control: $P = 0.15$, 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 right-ing 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-corrected 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 bio-mechanical 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-corrected 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-corrected two-tailed Student's t-tests: susceptible versus control: $P = 0.037$, susceptible versus resilient: $P = 0.014$, resilient versus control: $P = 0.87$; 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-corrected 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**) HMGB1 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-corrected 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 $n = 5$].
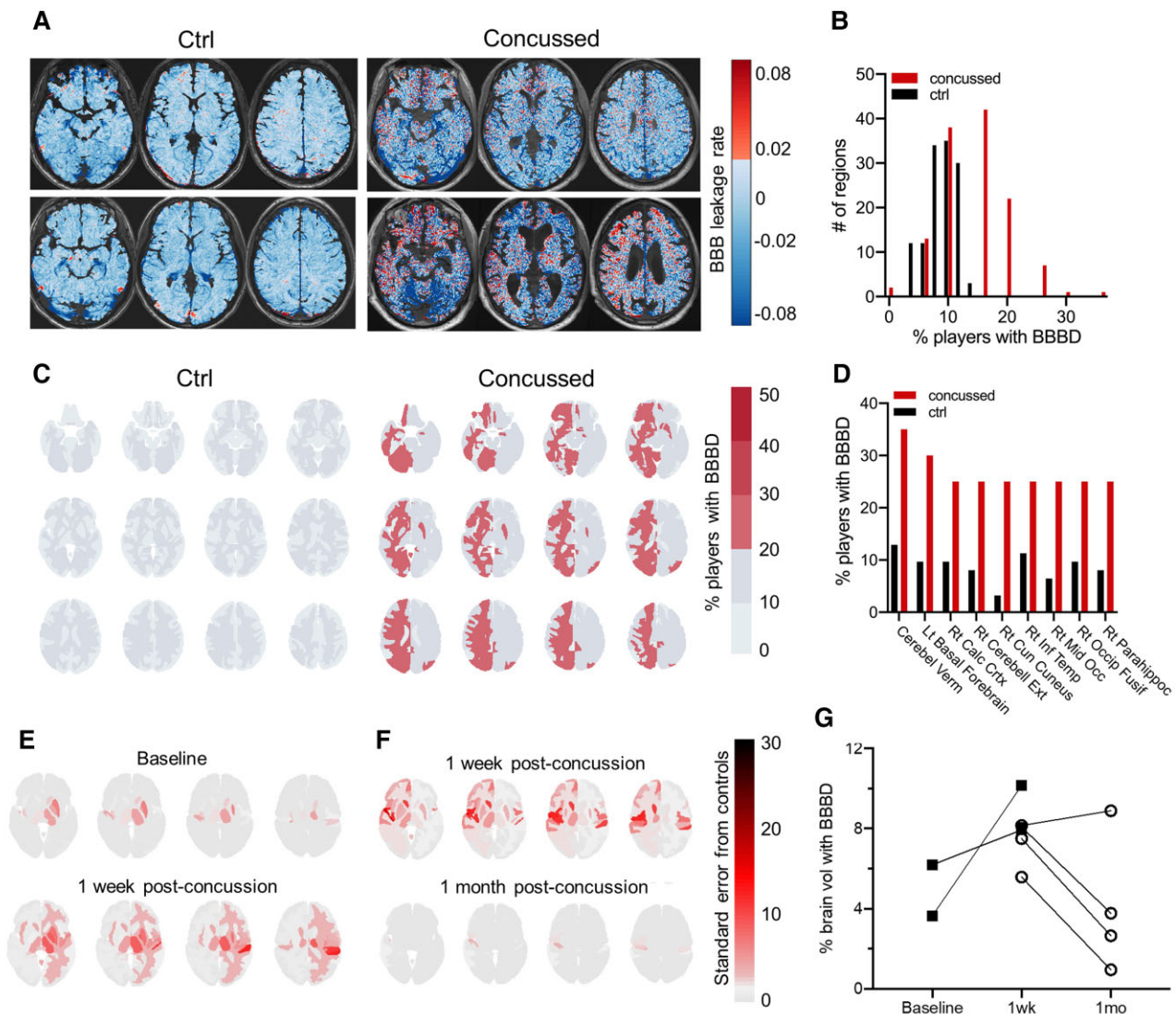
Figure 6 BBBD in contact-sport athletes. (**A**) Assessment of BBB integrity by DCE-MRI in 26 contact-sport athletes with a history of concussion (mean number of reported concussions: 2.6, 95% CI: 1.7–3.5, min-max: 1–6) and 61 age-matched controls with no concussion history showed diffuse areas of increased 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.

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.[26] 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

potential to restore microvascular integrity and prevent neurological complications in susceptible individuals.

Acknowledgements

Funding

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.

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