Supplementary Data

Supplementary Data

T2-weighted in vivo MRI evaluation after repetitive mild traumatic brain injury

We evaluated brain structural changes following repetitive traumatic brain injury (rTBI) by T2-weighted magnetic resonance imaging (MRI). We did not find any differences between rTBI and sham animals. Delineation of the forebrain, corpus callosum and external capsule, ventricles, cortex, and hippocampus were performed as indicated on the representative 3D reconstruction (Supplementary Fig. S2B). Quantitative analyses revealed no significant difference between rTBI and sham mice at 40 dpi for the total volume of the ventricles ($F(6,4) = 4.08$, $p = 0.55$; h2 = 0.04; two-tailed t test; Supplementary Fig. S2C), cortex ($F(6,4) = 1.06$, $p = 0.50$; h2 = 0.05; two-tailed t test; Supplementary Fig. S2D), corpus callosum, and external capsule ($F(4,6) = 1.25$, $p = 0.58$; h2 = 0.03; two-tailed t test; Supplementary Fig. S2E), hippocampus ($F(4,6) = 1.44$, $p = 0.95$; h2 = 0.0004; two-tailed t test; Supplementary Fig. S2F) and whole brain ($F(4,6) = 1.23$, $p = 0.67$; h2 = 0.02; two-tailed t test; Supplementary Fig. S2G). Next, quantitative analyses of the nT2w signal showed no significant difference between rTBI and sham groups for the cortex ($F(4,6) = 3.43$, $p = 0.12$; h2 = 0.22; two-tailed t test; Supplementary Fig. S2H), corpus callosum and external capsule ($F(4,6) = 7.60$, $p = 0.55$; h2 = 0.08; two-tailed t test with Welch's correction; Supplementary Fig. S2I), or hippocampus ($F(4,6) = 2.00$, $p = 0.60$; h2 = 0.038; two-tailed t test; Supplementary Fig. S2J).

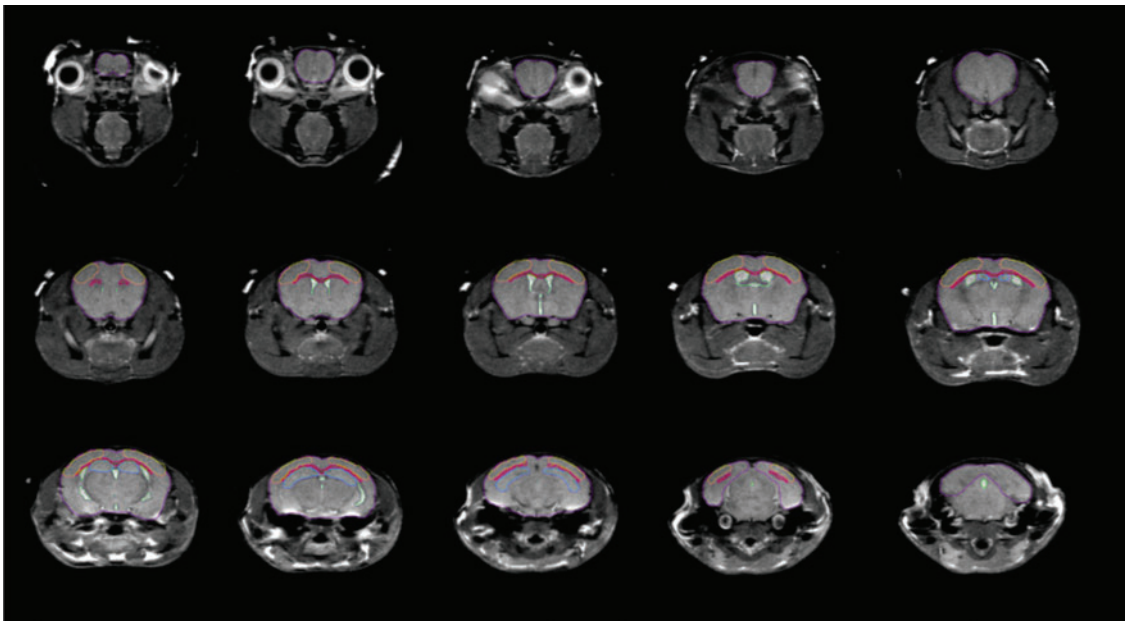
Gliosis in the hippocampus after repetitive mild traumatic brain injury

Gliosis was examined in the dorsal hippocampus at the apex of CA1 using IBA-1 to identify microglia (Supplementary Fig. S3A,B) and GFAP to label astrocytes (Supplementary Fig. S3D,E). A significant increase in the percentage area with IBA-1 labeling was identified in CA1 of the hippocampus in the rTBI sections compared with sham sections ($F(7,5) = 11.08$, $p < 0.05$; h2 = 0.42; two-tailed t test with Welch's correction, $n = 6$ sham and 8 rTBI mice; Supplementary Fig. S3C). However, no alteration in the percentage area with GFAP labeling was demonstrated ($F(6,6) = 2.48$, $p = 0.59$; h2 = 0.02; two-tailed t test, $n = 7$ sham and 7 rTBI mice; Fig. S3F).

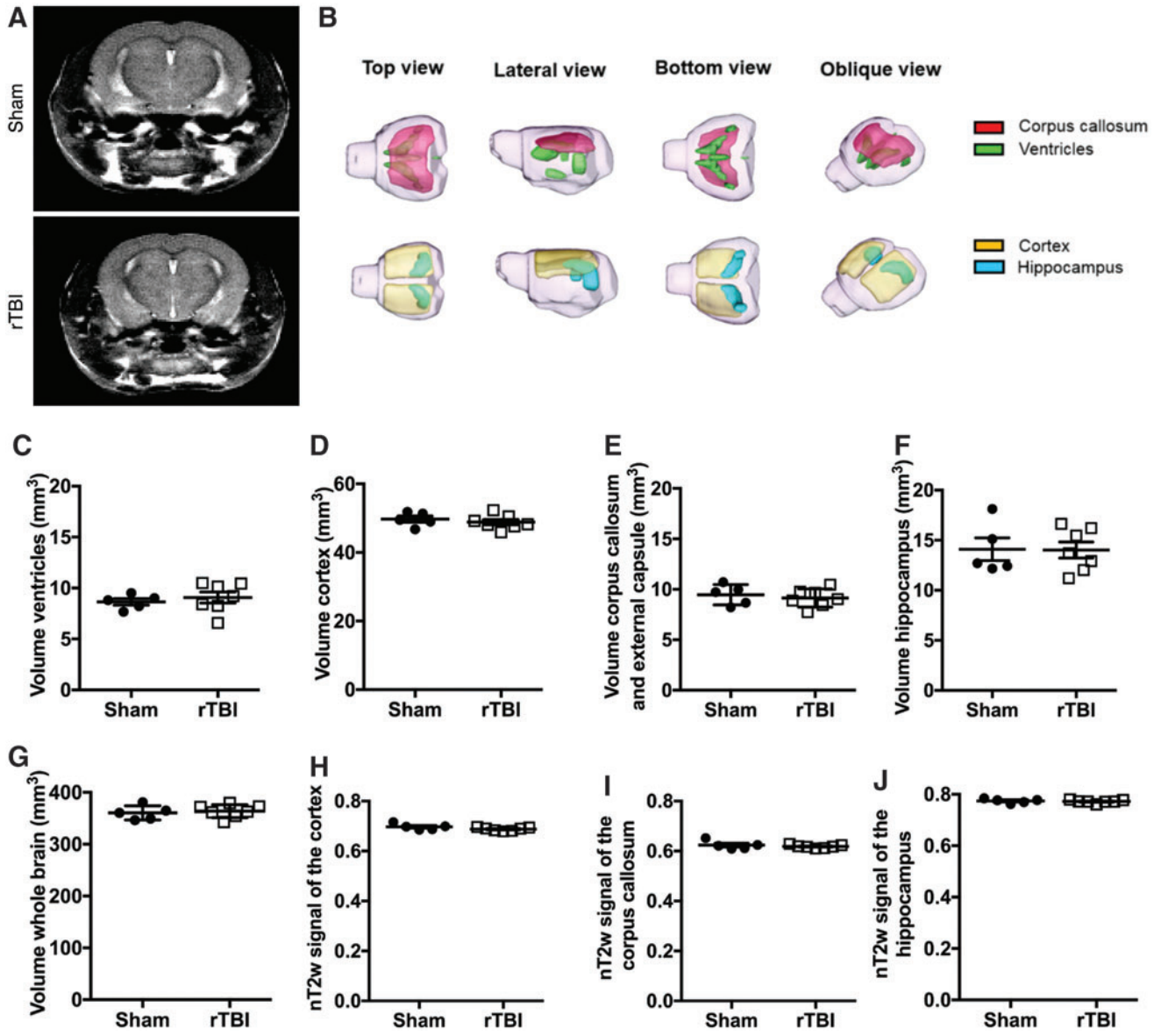
Supplementary Methods

Immunohistochemistry for hippocampal sections

Mice were terminally anaesthetized after behavioral testing using ketamine/xylazine (100 mg/kg and 10 mg/kg). They were transcardially perfused with phosphate-buffered saline (PBS) and the left hemi-brain was post-fixed in 4% paraformaldehyde for 48 h and incubated in a 30% sucrose solution before being cryoprotected in OCT and frozen at -80°C. These hemi-brains were sliced into 20 μm sections using a cryostat and air dried. Slides were frozen at -20°C until use. For staining, slides were removed from -20°C and dried in a 37°C oven for 10 min. Slides were subsequently washed in TBS-Triton for 10 min x 3, TSA-BB was used to block the sections for 30 min, and primary antibody was then applied overnight at 4°C. Primary antibodies included IBA-1 (1/500, 019-19741, Wako Chemicals USA, Richmond, VA) and GFAP (1/500, Z0334, Dako, Agilent Technology, Santa Clara, CA). The following day, slides were washed using TBS-T for 10 min x 3 and a fluorescent secondary antibody (Alexa Fluor 555 donkey anti-rabbit) was applied for 2 h at room temperature. Slides were then washed in TBS-T for 10 min x 3 prior to being

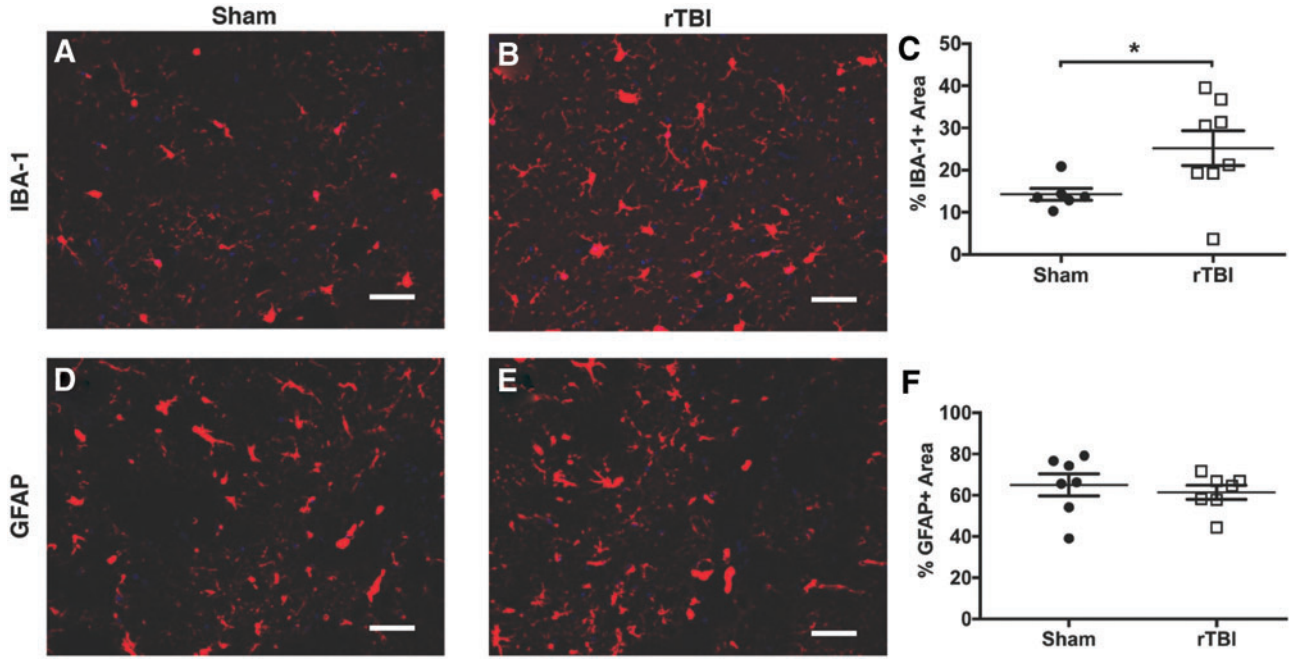


SUPPLEMENTARY FIG. S1. Anatomical delineation of brain structures. Representative delineation of the whole brain (purple), ventricles (green), corpus callosum and external capsule (red), hippocampus (blue), and cortex (orange) on T2-weighted images covering the whole brain.



SUPPLEMENTARY FIG. S2. Structural damage is not identified on *in vivo* T2-weighted magnetic resonance imaging (MRI) 40 days after repetitive traumatic brain injury (rTBI). **(A)** Representative T2-weighted images showing no anatomical difference between a sham animal and a rTBI animal at 40 dpi. **(B)** Representative 3D reconstruction of delineation of the forebrain with overlaid ventricles (green), corpus callosum and external capsule (red), cortex (yellow), and hippocampus (blue) from a rTBI animal at 40 dpi. Quantitative analyses of the total volume of the **(C)** ventricles ($F(6,4) = 4.08$, $p = 0.55$; $h2 = 0.04$; two-tailed t test), **(D)** cortex ($F(6,4) = 1.06$, $p = 0.50$; $h2 = 0.05$; two-tailed t-test), **(E)** corpus callosum and external capsule ($F(4,6) = 1.25$, $p = 0.58$; $h2 = 0.03$; two-tailed t test), **(F)** hippocampus ($F(4,6) = 1.44$, $p = 0.95$; $h2 = 0.0004$; two-tailed t test), and **(G)** whole brain ($F(4,6) = 1.23$, $p = 0.67$; $h2 = 0.02$; two-tailed t test) revealed no significant difference between sham and rTBI groups at 40 dpi. Quantitative analyses of the nT2w signal of the **(H)** cortex ($F(4,6) = 3.43$, $p = 0.12$; $h2 = 0.22$; two-tailed t test), **(I)** corpus callosum and external capsule ($F(4,6) = 7.60$, $p = 0.55$; $h2 = 0.08$; two-tailed t test with Welch's correction), and **(J)** hippocampus ($F(4,6) = 2.00$, $p = 0.60$; $h2 = 0.038$; two-tailed t test) also showed no significant difference between sham and rTBI groups at 40 dpi. All data are mean ± SEM.

cover-slipped with a vectastain aqueous mounting medium. Images were taken using a Zeiss Axioimager 1. For each mouse, 3-6 sections at the apex of CA1 in the dorsal hippocampus (between bregma, 1.58 to -2.18 mm) were imaged with a 20x objective. IBA-1 images were captured at a 400-msec exposure, whereas GFAP images were captured using a 75-msec exposure. Images were analyzed using the bioformats plugin for ImageJ to determine the percentage area covered by the appropriate fluorophore; an average percentage area was calculated for each animal.



SUPPLEMENTARY FIG. S3. Gliosis in the hippocampus, 36 dpi after repetitive traumatic brain injury (rTBI). **(A)** Representative IBA-1 immunostaining in CA1 of a sham mouse. **(B)** Representative IBA-1 immunostaining in CA1 of a rTBI mouse. **(C)** The percentage of the area of IBA-1+ staining is significantly increased in rTBI compared with sham mice. Data are mean ± SEM ($F(7,5) = 11.08$, $^*p < 0.05$; h2 = 0.42; two-tailed t test with Welch's correction, $n = 6$ sham and 8 rTBI mice). **(D)** Representative GFAP immunostaining in CA1 of a sham mouse. **(E)** Representative GFAP immunostaining in CA1 of a rTBI mouse. **(F)** The percentage of the area of GFAP+ staining is similar in rTBI and sham mice. Data are mean ± SEM ($F(6,6) = 2.48$, p 0.59; h2 = 0.02; two-tailed t test, $n = 7$ sham and 7 rTBI mice). Scale bar = 50 μm.