Transcranial amelioration of inflammation and cell death after brain injury

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Traumatic brain injury (TBI) is increasingly appreciated to be highly prevalent and deleterious to neurological function1–3. At present, no effective treatment options are available, and little is known about the complex cellular response to TBI during its acute phase. To gain insights into TBI pathogenesis, we developed a novel murine closed-skull brain injury model that mirrors some pathological features associated with mild TBI in humans and used long-term intravital microscopy to study the dynamics of the injury response from its inception. Here we demonstrate that acute brain injury induces vascular damage, meningeal cell death, and the generation of reactive oxygen species (ROS) that ultimately breach the glial limitans and promote spread of the injury into the parenchyma. In response, the brain elicits a neuroprotective, purinergic-receptor-dependent inflammatory response characterized by meningeal neutrophil swarming and microglial reconstitution of the damaged glial limitans. We also show that the skull bone is permeable to small-molecular-weight compounds, and use this delivery route to modulate inflammation and therapeutically ameliorate brain injury through transcranial administration of the ROS scavenger, glutathione. Our results shed light on the acute cellular response to TBI and provide a means to locally deliver therapeutic compounds to the site of injury.

TBI encompasses injuries that range from mild to severe1–3, and occurs when the brain is exposed to external forces that induce focal and/or diffuse pathologies, including vascular damage, oedema, axonal shearing and neuronal cell death4–10. TBI is usually divided into two phases: the primary insult and the ensuing secondary reaction. It is postulated that primary cell death cannot be prevented without avoiding the injury itself, but that secondary damage is amenable to therapeutic intervention because it is driven by pathogenic parameters such as ROS11,12, calcium release13, glutamate toxicity14,15, mitochondrial dysfunction15, inflammation15, and so on. Animal models of TBI have been developed that reflect mild, moderate and severe forms of injury5, but therapeutic research in these models has not yet translated successfully into the clinic6,13. Thus, there is an increasing need to develop additional TBI models, temporally map the dynamics of brain injury responses, and devise therapeutic interventions.

In humans, primary injury to the meninges and vasculature can be observed in the absence of conspicuous brain damage after minor head trauma. As part of an ongoing study of mild TBI, we evaluated research magnetic resonance imaging (MRI) with contrast from patients presenting to the emergency room within 48 h of a minor head injury. Over a period of 30 months, 142 patients were enrolled with a baseline Glasgow Coma Scale of 15, reporting loss of consciousness or post-traumatic amnesia, and a clinical computed tomography (CT) scan without evidence of injury to the parenchyma. Meningeal haemorrhage was seen on CT in 18 patients (12.7%), including subarachnoid blood in 13 (9.1%) and subdural blood in 7 (4.9%). Focal enhancement of the meninges was observed on post-contrast fluid attenuated inversion recovery (FLAIR) MRI imaging (Fig. 1a) in 69 (48.6%) patients, and without concomitant meningeal haemorrhage in 53 (36.9%) patients. Enhancement is the result of extravasation of gadolinium contrast into space containing free fluid with a T1 relaxation time constant equivalent to that of cerebrospinal fluid (CSF)4. To understand better the immunopathogenesis of focal brain injury, we developed a novel closed-skull model of mild TBI amenable to intravital imaging studies. Thinning the murine skull bone to ~30 μm allows the underlying meninges and brain parenchyma to be imaged by two-photon laser scanning microscopy (TPM) without overt brain injury or inflammation12. Thinning the skull bone beyond 30 μm causes increased pliability and concavity, which compresses the meningeal space (referred to as a compression injury) (Extended Data Fig. 1). Sequential thinning of the skull bone from 50 to 10 μm induced increasing amounts of meningeal cell death (Fig. 1b). Cell death and inflammation associated with over-thinning was reproducibly generated by quickly thinning the skull bone to ~20–30 μm and then manually promoting concavity with minimal downward pressure (Extended Data Fig. 1). We used this model to define the dynamics of inflammation and the mechanisms that cause cell death after focal TBI.

Using TPM we first mapped the kinetics and severity of brain pathology, starting 5 min after compression injury. Immediately after injury quantum dots injected intravenously leaked from vessels into the subarachnoid and perivascular spaces (Fig. 1c and Supplementary Video 1). Within 30 min, ROS were detected in the meninges (Fig. 1d) and holes appeared in the glial limitans due to astrocyte cell death (Fig. 1e and Supplementary Video 1). Transcranially administered SR101, a 600 molecular weight (MW) dye, leaked through the glial limitans into the parenchyma after compression injury, but remained largely within the meningeal space after standard skull thinning (Fig. 1f). Compression also induced cell death in the meninges that increased steadily over time, but was not observed in the parenchyma until 9–12 h after injury (Fig. 1g, h). Parenchymal cell death at 12 h was indiscriminate, as neurons, astrocytes, oligodendrocytes and microglia were all lost in the lesion site (Extended Data Fig. 2).

We next sought insights into the dynamics of the innate inflammatory response. Meningeal macrophages (long, rod-like cells) died within 30 min of compression injury (Fig. 2a and Supplementary Video 1). In response to meningeal cell death, microglia extended processes through the compromised glial limitans into the meninges (Extended Data Fig. 3a and Supplementary Video 1). We also observed a coordinated microglial response to compression injury. Most microglia within 50 μm of the meninges retracted all processes except for ~2–3 that extended towards the glial limitans, forming a stable contiguous network resembling a ‘honeycomb’ structure (Fig. 2b and Supplementary Video 2). Long-term TPM revealed that the honeycomb network formed within an hour of injury and could be maintained for up to 12 h (Supplementary Video 2). Honeycomb microglia surrounded surviving astrocytes in the glial limitans and aligned with the junctions between individual cells (Extended Data Fig. 3b and Supplementary Video 2).

In response to astrocyte death (Fig. 1d and Supplementary Video 1), a morphologically distinct microglial reaction was observed; microglia retracted their ramified processes and extended a single, non-branching process towards the glial limitans that resembled a jellyfish (Fig. 2c, Extended Data Fig. 3b and Supplementary Video 3). 'Jellyfish' microglia

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started to form almost immediately after compression injury (Supplementary Videos 3, 4), and some were motile, whereas others remained stationary (Supplementary Videos 3–5). We commonly observed honeycomb networks of microglia interspersed with clusters of jellyfish microglia (Supplementary Videos 4, 5), which probably reflects variation in lesion severity along the glial limitans. Honeycomb microglia could even transform within 5 min into jellyfish microglia, presumably after astrocyte cell death (Supplementary Video 5), whereas naive microglia required ~30 min to acquire a jellyfish morphology. Jellyfish projections were usually linked to cell bodies via thin processes (Supplementary Video 6) and often formed a continuous phagocytic layer at the glial limitans (Fig. 2c, Extended Data Fig. 3b and Supplementary Videos 3, 6, 7). Over time, microglia residing in the glial limitans died, particularly after tissue swelling (or oedema) was observed (Supplementary Video 7). Peripherally derived myelomonocytic cells (neutrophils and monocytes) also responded to brain damage. Within an hour of compression injury, required ~30 min to acquire a jellyfish morphology. Jellyfish projections were usually linked to cell bodies via thin processes (Supplementary Video 6) and often formed a continuous phagocytic layer at the glial limitans (Fig. 2c, Extended Data Fig. 3b and Supplementary Videos 3, 6, 7). Over time, microglia residing in the glial limitans died, particularly after tissue swelling (or oedema) was observed (Supplementary Video 7). Peripherally derived myelomonocytic cells (neutrophils and monocytes) also responded to brain damage. Within an hour of compression injury,
Dextran of 40,000 MW and below were able to pass through the intact skull into the meninges (Fig. 3b), although larger dextrans required longer diffusion times (Fig. 3c). A 70,000 MW dextran was unable to pass transcranially in 30 min. In addition, a variety of fluorescent small molecules and macromolecules passed through the murine skull bone and achieved measurable steady-state concentrations in the meninges dependent on molecular weight (Fig. 3d and Extended Data Fig. 4). Passage through an intact skull yielded a meningeal concentration approximately one half that achieved by thinned skull application (Extended Data Fig. 4d, e). We assessed the feasibility of passing compounds through thicker skulls by applying the contrast agent manganese chloride to an

Figure 2 | Innate immune response to a compression injury. a–d. Twenty-five micrometre xy maximum projections from CX3CR1gfp/+ mice (a–c) or LysMgfp/+ mice (d) captured at 30 min (a), 1 h (b), 2 h (c) or 6 h (d) in a normal thinned skull preparation (uncompressed) or after a compression injury. a. Meningeal macrophages (green) visualized in CX3CR1gfp/+ mice were highly motile, and interacted with dead cells during the 12 h observation period (Fig. 2d and Supplementary Video 8).

To modulate TBI lesions locally, we applied compounds to the intact skull bone. We discovered that SR101, when applied to an intact (non-thinned) skull bone passed directly into the meninges within 10 min (referred to as a ‘transcranial application’) (Fig. 3a). We next tested a range of differently sized fluorescent dextrans (3,000–70,000 MW),

Figure 3 | Metrics of transcranial diffusion through the skull bone. a. A 600 MW fluorescent dye, SR101 (red), was applied to an intact mouse skull for the indicated time and then the skull (blue) was quickly thinned and imaged. Five micrometre xy maximum projections show that SR101 is detectable in the meninges beginning 10 min after application and fully saturates the space within 15 min. White dotted line indicates glial limitans. Scale bar, 50 μm. b. The size dependence of diffusion through an intact skull bone was evaluated 30 min after continuous transcranial application of the indicated molecular weight dextrans (red). Dextrans that passed successfully through the intact skull generated fluorescence in the meninges. The skull bone is shown in blue, and the glial limitans is denoted with a white dotted line. Scale bar, 50 μm. c. A colour-coded table summarizing the imaging results shown in panels a and b denotes the presence (green) or absence (red) of fluorescent dye in the meninges at the indicated molecular weight and time. Grey, not tested. d. Fluorescent compounds of increasing molecular weights were passed transcranially through a thinned skull window during imaging. Steady-state concentrations (% applied) of the fluorescent compounds in the meninges and parenchyma were quantified from normalized fluorescence intensities. See also Extended Data Fig. 4. e. Manganese chloride (Mn; 500 mM solution) applied transcranially to an intact rat skull (~1 mm thick) is visible by MRI in the brain parenchyma 2 h after application (white arrow). The mean parenchymal manganese concentration ± s.d. is provided. Scale bar, 1 mm. All data in the figure are representative of three mice (or rats) per group and at least three independent experiments.

myelomonocytic cells (probably neutrophils) localized exclusively to the meninges, were highly motile, and interacted with dead cells during the 12 h observation period (Fig. 2d and Supplementary Video 8).

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intact rat skull bone (~1 mm thick) and imaging transcranial passage by MRI (Fig. 3e). Transcranially applied manganese chloride was clearly visible in the rat brain parenchyma 2 h after application.

We next defined the mechanisms underlying compression injury–induced inflammation. Transcranial application of purinergic receptor antagonists (P2RY12 or P2RX4) inhibitors before compression injury prevented both honeycomb and jellyfish morphologies, whereas P2RY6 antagonism only blocked the jellyfish response (Fig. 4a–c, Extended Data Fig. 5a and Supplementary Video 9). In contrast, P2RX7 antagonism had no effect on microglia, but almost entirely eliminated neutrophil recruitment (Fig. 4a–c, Extended Data Fig. 5b and Supplementary Video 9). Astrocytes are known to amplify purinergic receptor signalling through ATP-induced ATP release via connexin hemichannels, which can be blocked with carbenoxolone (CBX)17. Transcranial application of CBX, but not a specific pannexin inhibitor (probenecid), before compression injury caused microglia to remain ramified, extending only small, ill-defined circular processes at the glial limitans, similar to what was observed after P2RY6 antagonism (Fig. 4d, e, Extended Data Fig. 5c and Supplementary Video 9), CBX inhibited the formation of honeycomb and jellyfish microglia, whereas pannexin inhibition slowed the onset and magnitude of neutrophil recruitment (Fig. 4f).

Pre-treatment with CBX also significantly increased SR101 leakage through the glial limitans, suggesting that ATP release by astrocytes and purinergic signalling in microglia help maintain barrier integrity between the meninges and parenchyma (Fig. 4g and Extended Data Fig. 5d).

To identify the primary mediator of cell death after compression injury, we focused on the role of ROS, which appeared in the meninges shortly after compression injury (Fig. 1d). Transcranial administration of the ROS scavenger glutathione (GSH) resulted in near complete preservation of meningeal macrophages after a more severe injury (that is, skull fracture) (Fig. 5a and Supplementary Video 10) as well as glial limitans preservation (Fig. 5b, f). In addition, microglia beneath this layer remained in a non-reactive, ramified state (Fig. 5a, e and Supplementary Video 10). Preservation of the glial limitans after GSH treatment resulted in refilling of the subarachnoid space beneath the compression injury, which pushed the thinned skull bone upward (Supplementary Video 10). GSH administration also eliminated the recruitment of myelomonocytic cells (Fig. 5c, g and Supplementary Video 10).

Cell death was first observed in the meninges and later spread to the parenchyma after compression injury (Fig. 1h). Transcranial pretreatment with GSH resulted in a 50% reduction in meningeal death, but administration after injury had no effect (Fig. 5h), indicating that half the initial meningeal cell death following compression injury is due to ROS. GSH, when applied continuously starting at 15 min or 3 h after injury, reduced parenchymal cell death at 12 h by 67% and 51%, respectively (Fig. 5d, i). These data indicate that ROS are a mediator of cell death after compression injury. The contribution of inflammation to cell death was assessed by transcranially inhibiting neutrophils and microglia with P2RX7 or P2RY6 antagonism, respectively. Inhibition of neutrophil recruitment through P2RX7 antagonism increased cell death in the meninges 12 h later, but had no impact on parenchymal cell death (Fig. 5h, i). Conversely, inhibition of microglia through P2RY6 antagonism increased parenchymal cell death at 12 h, but did not affect meningeal cell death (Fig. 5h, i). These data suggest that inflammation is neuroprotective within the first 12 h of compression injury.

TBI induces a complex reaction that can result in permanent damage and neurological dysfunction. In this study, we observed evidence of meningeal damage in ~50% of patients with mild head injury, indicating that this is a common pathology in humans. We sought mechanistic insights into this process by developing a novel closed-skull model of brain injury and imaging the acute cellular injury response from its inception. Importantly, we discovered that the skull bone is porous and permits the passage of small molecules (~40,000 MW) and contrast agents by passive diffusion, which should facilitate local delivery.
of therapeutics and other molecules into the central nervous system. Pathologically, compression injury initially caused meningeal cell death, vascular damage, ROS generation, and disruption of the glial limitans, which ultimately gave rise to indiscriminate parenchymal cell death. ROS are commonly observed in TBI lesions\(^a\), and transcranial delivery of GSH preserved the glial limitans, reduced cell death, and eliminated the sterile injury response. GSH significantly reduced parenchymal cell death even when administered 3 h after injury, providing a therapeutic window for treatment of focal brain injury.

In the absence of GSH, the brain responded to damage by eliciting an anatomically partitioned sterile immune reaction\(^a\). Microglia first fortified the glial limitans through the generation of honeycomb and an anatomically partitioned sterile immune reaction\(^a\). Microglia first window for treatment of focal brain injury. Parenchymal, myelomonocytic cells invaded the damaged meninges in a P2RX7- and pannexin-dependent manner, consistent with a recent study showing P2RX7-dependent neutrophil recruitment into the injured liver\(^d\). Collectively, our data suggest that the acute inflammatory reaction to brain injury is beneficial\(^d\). Moreover, ROS and purines represent major drivers of the injury response and are amenable to transcranial therapeutic manipulation.

**METHODS SUMMARY**

Human patients presenting to the emergency room within 48 h of mild head injury were evaluated as part of an ongoing Traumatic Head Injury Neuroimaging Classification (THINC) study. MRI scans were obtained and evaluated after injection of a gadolinium-based contrast agent. To model the pathology of mild head injury, we surgically thinned the murine skull bone over the barrel cortex to a thickness of ~20–30 μm and then applied minimal downward pressure to promote concavity above the glial limitans (indicated by a white dotted line) in the GSH-treated group. Scale bar, 50 μm. c, In GSH pre-treated mice, no neutrophil response (green) to compression injury was observed at 6 h. Scale bar, 100 μm. d, GSH administered 15 min or 3 h after a compression injury significantly reduced parenchymal cell death observed at 12 h. PT\(^+\) dead cells (red) reside primarily in the meninges of GSH-treated mice. White dotted line indicates glial limits. Scale bar, 50 μm. e–g, Bar graphs (mean ± s.d.) show quantification of honeycomb and jellyfish microglia (e), glial limitans permeability (f) and neutrophil recruitment (g) in vehicle- versus GSH-treated mice after compression injury at the time points denoted above. Uncomp., uncompressed skulls. h, The number of PT\(^+\) dead cells per mm\(^3\) (mean ± s.d.) was quantified in the meninges at 30 min or 12 h after compression injury. GSH significantly reduced meningeal cell death if applied before (pre-treatment (pre-treat.)), but not after (0 min, 15 min, 3 h) compression injury. P2RX7 blockade increased meningeal cell death when administered 15 min after compression injury. i, Parenchymal cell death was quantified 12 h after compression injury in the denoted groups. All data are representative of three independent experiments, and asterisks denote a statistically significant difference (\(P < 0.05\)) from the vehicle control group.

**Figure 5 | Transcranial administration of glutathione reduces inflammation and cell death after compression injury.** a–i, Twenty-five micrometre × yz maximum projections (a, c) and 5 μm xz projections (b, d) were captured in CX3CR1gfp\(^i\) (a), B6 (b, d) or LysMgfp\(^–\)– (c) mice after compression injury (\(n = 3\), a–c, e–g: \(n = 4\), d, h–i). a, GSH pre-treatment prevented jellyfish and honeycomb microglia formation (green) 1 h after a compression injury that resulted in a cracked skull (blue). GSH administration also promoted survival of meningeal macrophages (macs: green; white arrows). Scale bar, 100 μm. b, Relative to the vehicle control group, GSH pre-treatment prevented glial limitans breakdown observed 1 h after injury. SR101 (red) localizes above the glial limitans (indicated by a white dotted line) in the GSH-treated group. Scale bar, 50 μm. c, In GSH pre-treated mice, no neutrophil response (green) to compression injury was observed at 6 h. Scale bar, 100 μm. d, GSH administered 15 min or 3 h after a compression injury significantly reduced parenchymal cell death observed at 12 h. PT\(^+\) dead cells (red) reside primarily in the meninges of GSH-treated mice. White dotted line indicates glial limits. Scale bar, 50 μm. e–g, Bar graphs (mean ± s.d.) show quantification of honeycomb and jellyfish microglia (e), glial limitans permeability (f) and neutrophil recruitment (g) in vehicle- versus GSH-treated mice after compression injury at the time points denoted above. Uncomp., uncompressed skulls. h, The number of PT\(^+\) dead cells per mm\(^3\) (mean ± s.d.) was quantified in the meninges at 30 min or 12 h after compression injury. GSH significantly reduced meningeal cell death if applied before (pre-treatment (pre-treat.)), but not after (0 min, 15 min, 3 h) compression injury. P2RX7 blockade increased meningeal cell death when administered 15 min after compression injury. i, Parenchymal cell death was quantified 12 h after compression injury in the denoted groups. All data are representative of three independent experiments, and asterisks denote a statistically significant difference (\(P < 0.05\)) from the vehicle control group.
Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions T.L.R., D.N. and D.B.M. designed all murine experiments and interpreted the data. L.L.L. provided and interpreted the human TBI data. T.A. and A.P.K. contributed the transcranial manganese data. T.L.R. and D.B.M. wrote the paper.

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METHODS

Human subjects and MRI protocol. As part of the ongoing Traumatic Head Injury Neuroimaging Classification (THINC) study, patients presenting to the emergency room or trauma service at Suburban Hospital (SH; Bethesda, Maryland) and MedStar Washington Hospital Center (WHC; Washington, DC) within 48 h of head injury are screened and enrolled (NCT01132937). In most patients, a conventional non-contrast CT scan was obtained for clinical purposes. Following consent, a research MRI was obtained that included a single dose of a gadolinium-based contrast agent: 0.1 mmol kg⁻¹ gadopentetate dimeglumine (Bayer Healthcare) or 0.2 mmol kg⁻¹ gadobenate dimeglumine (Bracco Diagnostics) according to local site policy. The imaging was performed on either a 1.5T (SH) or 3T (WHC) scanner using a protocol of ~25 min duration, which included T2-FLAIR (fluid attenuated inversion recovery) of ~2:30 duration, obtained before and again 5 min after intravenous (i.v.) contrast administration. An additional three-dimensional T2-FLAIR sequence was acquired post-contrast on the 3T at WHC to permit better visualization of the morphology of enhancement on a surface rendering of the head. This study was approved by the NINDS Institutional Review Board.

Rodents. C57BL/6J (B6), B6.129P2-Cx3cr1< disruptions/> (CX3CR1< disruptions/>; ref. 22) and FVB/N-Tg(FAPGFPE)14Mels (ref. 23) were obtained from The Jackson Laboratory. C57BL/6J mice were generated by crossing B6 mice with CX3CR1< disruptions/> mice in a closed breeding facility at the NIH. B6 LysM<sup>−/−</sup> mice (LysM<sup>−/−</sup> mice) were provided by T. Graf and maintained at the NIH. All mice were 8–16 weeks of age. Adult male Sprague–Dawley rats (body weight ~200 g) were obtained from Harlan Laboratories. All rodents were housed under specific pathogen-free conditions and treated in accordance with the Institutional Animal Care and Use Committee at the NIH.

Skull thinning and compression injury. For imaging experiments, mice were anaesthetized with ketamine (85 mg kg⁻¹), xylazine (13 mg kg⁻¹) and acepromazine (2 mg kg⁻¹) in PBS and maintained at a core temperature of 37 °C. The skull bone over the barrel cortex was then thinned to a thickness of ~30–40 μm as described. For this procedure, the bone was manually thinned over a 30 min period, and the amount of downward pressure was kept to a minimum. To induce a compression injury, the skull was thinned in ~1–2 min to a thickness of ~20–30 μm. Once thinned, the blunt end of a surgical instrument was used to gently press the pliable skull bone downward to promote concavity in the bone. The downward pressure applied was minimal and only performed until a concave bone was observed. This resulted in the skull bone collapsing (without breaking) inward towards the surface of the brain. In one set of experiments, the skull bone was intentionally cracked to induce a severe injury. Imaging was performed beginning 5 min after injury.

Intravital two-photon laser scanning microscopy. Mice with a thinned or compressed skull bone were imaged using a Leica SP5 two-photon imaging system (Leica Microsystems) equipped with an 8,000 Hz resonant scanner, a ×20/1.0 NA dipping objective, and two Mai Tai HP DeepSee Lasers (SpectraPhysics) tuned to 905, 920 or 970 nm. Fluorescence emission was separated by high efficiency dichroic mirrors (Semrock) and collected with a NDD4 external detector (Leica). Stacks of images were obtained using a step size of 1.0 μm, and two Mai Tai HP DeepSee Lasers (SpectraPhysics) were tuned to 905, 920 or 970 nm. Fluorescence microscopy was used to examine the identity of dead cells in the brain parenchyma, a compression injury and normal skull thinning procedure were performed on opposite hemispheres of the same skull. Dead cells were labelled by transcranial propidium iodide application, and the mice were perfused with 4% paraformaldehyde (PFA) at 12 h after injury. Whole mouse heads were post-fixed in 4% PFA for 8 h and then stored in 30% sucrose for another 24 h. After decalcification of the skull bone, 6 μm serial coronal brain sections through the injury and control sites were produced by Histovar. Before antibody staining, sections were blocked with 10% fetal bovine serum (FBS) (GEAP and Iba1 stains), 10% BSA plus 35 μg mL⁻¹ donkey anti-rabbit (NeuN stain), 10% BSA plus 35 μg mL⁻¹ donkey anti-mouse F(ab) plus 0.1% Triton X-100 (APC stain). The following cell types were labelled: neurons (anti-NeuN; 1:800; Chemicon), astrocytes (anti-GFAP; 1:800; DakoCytomation), microglia (anti-Iba1; 1:400; Wako) and oligodendrocytes (anti-APC; 1:50; Oncogene Research). Cell-marker-specific antibodies were detected with secondary antibodies conjugated to Alexa Fluor 488 (1:200; Jackson ImmunoResearch Laboratories). Working stocks of primary and secondary reagents were diluted in PBS containing 2% FBS (NeuN, GFAP, Iba1) or 10% FBS with 0.1% Triton X-100 (APC). All sections were incubated with 1 μg mL⁻¹ 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 3 min at room temperature (24 °C) to stain cell nuclei.

Confocal microscopy. Two-dimensional images shown in Extended Data Fig. 2 were captured from stained 6-μm frozen sections using an Olympus Fluoview confocal microscope equipped with a ×20 objective. Images were collected using sequential scanning with the 405, 488 and 559 nm laser lines to produce three separate overlays.

Purinergic receptor, hemichannel and ROS antagonism. Before skull thinning, antagonists diluted in aCSF were applied directly to the skull bone, and vehicle was simultaneously applied to the opposite hemisphere to serve as a control. The following antagonists from Sigma were used: TNP-ATP hydrase (PR2X4; 25 mM), oxidized ATP (PR2X7; 10 mM), MRS2578 (PR2Y6; diluted in a 50 mM DMSO stock 1:100 in aCSF to a final concentration of 500 μM), MeSAMP (PR2Y12; 10 mM), CBX (connexin hemichannels; 100 mM), provenecid (pannexin hemichannels; 100 mM) and glutathione (ROS; 100 mM). Vehicle and purinergic receptor antagonists were applied as a 3 mm diameter bubble on the skull surface and replenished as needed over a 30 min incubation period to prevent drying. This ensured compounds were replenished as needed to prevent drying. After the 30 min incubation, the skull was dried and then thinned to induce a compression injury over both hemispheres. Imaging was initiated immediately after injury. For most studies, the skull bone pre-incubated with antagonists was imaged continuously for 3–10 h, after which a three-dimensional stack was captured from both the vehicle- and antagonist-treated areas for quantitative purposes. Long-term continuous intravital TPM alone did not induce an injury response (data not shown). Glutathione was also applied at 15 min and 3 h after compression injury to determine the impact on cell death. For these studies, glutathione was added directly to the aCSF submerging solution (100 mM) while imaging. The glutathione was maintained in the submerging solution for the entire imaging experiment. Similar studies were conducted using 10 mM oxidated ATP and 500 μM MRS2578.

Gliolim 2-leakage assay. For permeability studies, areas of skull bone were pre-incubated with vehicle, CBX (100 mM) or glutathione (100 mM) for 30 min as described earlier. Afterwards, a meningeal compression injury was induced. While imaging, antagonists (10 mM CBX or 100 mM glutathione) were added directly to the aCSF submerging solution. After 3 h of imaging, SR101 (1 mM) was applied for 15 min, followed by a 5 min aCSF wash. A three-dimensional stack was then captured to quantify the degree of SR101 leakage through the glial limits.

Skull bone permeability analyses. For skull bone permeability studies, the following rhodamine-labelled dextrans were placed directly on the intact skull bone for up to 30 min: 3,000 MW (25 mM), 10,000 MW (5 mM), 40,000 MW (1 mM) and 70,000 MW (0.5 mM). SR101 (1 mM) was used as a representative 600 MW compound. Compounds were replenished as needed to prevent drying. After the incubation period, the skull bone was quickly thinned and imaged. A three-dimensional stack was captured to determine whether the fluorescence could be found beneath the skull bone. For quantitative analyses of skull bone permeability through a thinned skull window, the following fluorescent compounds were used: Nile Red (318 mM; 100 mM), fluorescein (376 MW; 100 mM), SR101 (606 mM; 100 mM), rhodamine B (1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (DHEP) (phospholipid; 1,334 mM; 1 mM), 5-tetramethylrhodamine (TMR)-RRADESDDDDDDD (TMR-tagged peptide; 1,706 MW; 100 mM), 5′-Rhodamine Red-X-NA-ATGAT-3′ (Rhodamine-Red-tagged oligonucleotide; 2,335 MW; 1 μM), 3,000 MW dextran (100 μM), 10,000 MW dextran (10 mM) and 40,000 MW dextran (100 mM). Dextran 190,000 MW was used as this skew is moderately pass through the skull bone. After the B6 mouse followed by 30 min of imaging in aCSF to establish a baseline. Without interrupting the imaging sequence, the aCSF was replaced after 30 min with a solution containing the fluorescent compounds at the denoted concentrations and then imaged continually for another 60 min. After acquisition, 100 × 100 × 5 μm (x,y,z) boxes were created using the ‘surfaces’ tool in Imaris and placed 25 μm above the skull, in the meninges (0.5 μm below the skull), and in the parenchyma (15–20 μm below the skull). The background fluorescence intensity for each anatomical region was calculated using the first 30 min of imaging. Instantaneous concentrations of fluorescent compounds were then determined from 30 to 90 min (at 1 min intervals) for the skull, meninges and parenchyma by calculating the average fluorescent intensities in the 100 × 100 × 5 μm boxes. After subtracting the background fluorescence, the resultant number was divided by the fluorescent intensity of the applied compound in solution and multiplied by 100 to generate a percentage. To determine the concentrations in the meninges achieved by transcranial loading through an intact murine skull bone, 100 mM SR101 was applied transcranially through the intact skull for 20 min followed by a quick (~2 min) thin skull preparation and...
Transcranial manganese delivery. Rats were anaesthetized with 5% isoflurane and then switched to 1–2% isoflurane for maintenance. Their body temperature was maintained by a heated water bath. The animals were placed in a stereotaxic apparatus, a single midline incision with a sterile scalpel was made through the skin of the skull, and the skull bone was exposed by scraping away the periosteum. The following sterile saline solutions were placed directly on the skull bone above the S1 area (left/right, +3; rostral/caudal, −1.4; from Bregma): 500 mM MnCl₂, 250 mM MnCl₂ (not shown) and pure saline as a control. The solutions were pipetted over a 2 h period and replenished as needed. After 2 h, the skull was rinsed three times with saline, the skin sutured, and the animal promptly placed in an MRI scanner to detect manganese diffusion into the brain. Images were acquired on an 11.7 T/31 cm horizontal magnet (Agilent) interfaced to a Bruker Avance III console (Bruker BioSpin) equipped with a 12 cm gradient set (Resonance Research). A 9 cm laboratory-built birdcage coil was used for signal transmission and a 2 cm surface coil placed on the rat head was used for signal acquisition. During imaging, anaesthesia was maintained at 1.5% isoflurane, and the body temperature was maintained at 37 °C using a heated water bath. Contrast enhancement by manganese was detected using a T₁-weighted spin echo pulse sequence (TE 7.6 ms, TR 500 ms, Nav = 8, 100 μm in-plane resolution, 30 1-mm-thick axial slices). The T₁ relaxation times were measured using a saturation recovery spin–echo sequence (TE = 7.6 ms, TRs 0.4, 0.97, 1.77, 3.124 and 10 s; 200 μm in-plane resolution).

Two-photon image analysis. All quantitative analyses and processing of three-dimensional/four-dimensional imaging data were performed using Imaris 7.0 software (Bitplane). Supplementary Videos were constructed and annotated using Adobe Premiere Pro CS4. Microglia honeycomb reactions were quantified from 434 × 434 × 150 μm (xzy) three-dimensional image stacks obtained at selected time points in CX3CR1<sup>Gr<sup>1</sup></sup> mice. This was accomplished by measuring the total length of microglial processes in contact with the glial limitans. Microglial cell bodies were first selected using the Imaris ‘spots’ tool. Afterwards, ten microglia per mouse were randomly selected for quantification of process length. Only cells with all of their processes in the field of view and no more than 50 μm beneath the skull bone were quantified. Microglial processes that were touching (flat against) the glia limitans were labelled and measured using the Imaris ‘filaments’ tool. Data were then represented on a per cell basis as the length of microglial processes in contact with the glial limitans. Microglia with a jellyfish morphology were identified as those having processes greater than 20 μm in diameter. The number of jellyfish microglia was then divided by the total number of microglia within 50 μm of the skull bone and multiplied by 100 to generate a percentage. To quantify cell death, propidium-iodide-positive cells were labelled in three-dimensional stacks using the Imaris ‘spots’ tool. Dead cells from 0 to 5 μm below the compressed skull were considered meningeal, whereas cells from 5 to 100 μm were considered parenchymal. The number of dead cells was divided by the volume analysed and represented as cells per mm³. Neutrophils were quantified in LysM<sup>Gr<sup>1</sup></sup> mice using the Imaris ‘spots’ tool. After compression injury, neutrophils were never observed in the brain parenchyma and, therefore, were quantified only in the meningeal space (0 to 5 μm below the compressed skull). The number of neutrophils was divided by the volume analysed and represented as cells per mm³. To quantify leakage of SR101 through the gial limitans, a 50 × 50 × 100 μm (xzy) solid box was generated using the Imaris ‘surfaces’ tool. The box was placed 25 μm beneath the epicentre of the compression injury (that is, the lowest point of the compressed skull bone). The mean fluorescent intensity of the SR101 signal inside of this box was then calculated. The value obtained beneath the antagonist-treated skull (glutathione or CBX) was divided by the vehicle control area (from the opposite hemisphere) to generate a fluorescence ratio.

MRI image analysis. MRI images from rats receiving transcranial manganese were analysed using ImageJ (http://rsb.info.nih.gov/ij/) and MIPAV (http://mipav.cit.nih.gov/) software developed at NIH. T₁ relaxation maps for brain slices were calculated using the MRI Analysis Calculator plugin. Background tissue T₁ values and standard deviations for every slice were obtained by placing a region of interest (ROI) over an area of the cortex opposite from the one of manganese administration. T₁ relaxation maps were thresholded using the value of the background T₁ minus two standard deviations in order to obtain ROIs in which manganese had significantly shortened the T₁ of the cortex tissue. The average T₁ relaxation times as well as the total number of voxels in those ROIs were used to determine the local concentration and total amount of manganese in each slice, using the equation: 1/(T₁<sub>observed</sub>) = 1/(T₁<sub>background</sub>) + r<sub>1</sub> × c. The value of 4.7 s⁻¹ mM⁻¹ was used for cortex T₁ relaxivity as previously reported. The total amount of manganese was integrated through the relevant slices.

Group sizes and statistical analysis. Group sizes of 3–4 rodents were used for all experiments to ensure reproducibility and that statistical differences could be detected. All experiments were independently replicated at least three times. Only animals that succumbed to anaesthesia were excluded from further analysis. For experiments with experimental and control groups, littermates were randomized before assignment to a particular condition. Statistical significance (P < 0.05) was determined using a Student’s t-test (two groups) or a one-way analysis of variance (ANOVA) (more than two groups). A rank sum test or ANOVA on ranks was used for data sets with non-parametric data. All graphs and statistical analyses were performed using SigmaPlot version 11.

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Extended Data Figure 1 | Compression injury model. a, An intact mouse skull bone consists of three layers: cortical, cancellous, cortical. b, For standard skull thinning, a microdrill (not shown) and a microsurgical blade are used to manually thin all three layers of bone until only 30 μm of lower cortical bone remains. The diameter of the thinned area is ~1,000 μm. This preparation permits intravital imaging of the unperturbed meninges and brain parenchyma. c, Compression injury is induced by thinning the bone to ~20–30 μm and then using the flat blunt edge of a microsurgical blade to apply minimal downward pressure, which promotes concavity in the thinned skull region. d, This entire procedure is performed in 1–2 min, after which the cellular response to the compression injury can be continuously imaged by TPM as soon as 5 min after injury.
Extended Data Figure 2 | Parenchymal cell death following compression injury. Immunohistochemical analyses of coronal brain sections 12 h after compression injury (bottom) shows that cell death (red) extends several layers into the neocortex. Cell death at this time point was indiscriminate, as the lesion site was largely devoid of neuronal (NeuN\(^+\)), astrocytic (GFAP\(^+\)), oligodendrocyte (APC\(^+\)), and microglia (Iba1\(^+\)) staining (all in green). Dead cells were labelled by transcranially applying propidium iodide (red) 30 min before fixation. A normal uncompressed thinned skull preparation was performed on the contralateral hemisphere as a control (top). Note that parenchymal cell death is not observed in any of the control images. Cell nuclei are shown in blue. Images are representative of three mice per group.
Extended Data Figure 3 | Microglia response to compression injury.

a, b, Representative 5 μm maximum projections in the xz plane (a, b) or 25 μm projections in the xy plane (b) were captured from CX3CR1<sup>gfp<sup> mice at 60 min (a) or 6 h (b) after a normal thinned skull preparation (uncompressed) or compression injury. a, Microglia (green) extend processes into the meningeal space (white arrow) only after compression injury. The glial limitans is denoted with white dotted lines. b, After compression injury, SR101-labelled astrocytes (red) at the glial limitans are surrounded by a network of honeycomb microglia (top, xy projection). An example is indicated with a white arrowhead. In contrast, holes in the astrocytic coverage at the glial limitans are filled by jellyfish microglia (white arrow). A side view (xz projection) reveals microglia (green, white arrows) to be an integral part of the glial limitans after compression injury. Data are representative of three mice per group and at least three independent experiments.
Extended Data Figure 4 | Pharmacology of transcranial fluorescent compound administration. a–c, Steady-state concentrations (Conc) of fluorescent compounds in aCSF were calculated by two-photon microscopy above the skull (blue) as well as in the meninges (orange) and brain parenchyma (green) for 1 h after transcranial administration through a surgically thinned (uncompressed) skull. Background fluorescence was established by imaging for 30 min before adding the fluorescent compounds. Low molecular weight compounds established higher steady-state concentrations in the meninges than larger compounds. The hydrophobic, low molecular weight compound Nile Red was also able to pass through the glial limitans into the brain parenchyma.

See Methods for additional details about the applied fluorescent compounds. Oligonuc., oligonucleotide. d, A 100 nM SR101 solution was applied to an intact mouse skull for 30 min, then immediately thinned and imaged while the skull was submerged in aCSF to determine the decay in the meningeal SR101 concentration over time. e, The bar graph shows a comparison of the meningeal SR101 concentration (mean ± s.d.) after transcranial application to a thinned versus an intact skull. Data are representative of three mice per group and at least three independent experiments.
Extended Data Figure 5 | Role of purinergic receptors and connexin hemichannels in the compression injury response. 

a, Representative 25 μm maximum projections in the xy plane were captured in CX3CR1gfp/+ mice 3 h after compression injury. Two compression injuries were generated per mouse (over the left and right hemispheres). Before injury the left skull bone was treated with the indicated purinergic receptor antagonist and the right with vehicle (aCSF). When compared to the vehicle control, transcranial application of P2RY12 or P2RX4 receptor antagonists impeded the ability of microglia (green) to assume both honeycomb and jellyfish morphologies, whereas P2RY6 antagonism blocked the formation of jellyfish processes only.

b, xy projections were captured in LysMgfp/+ mice 6 h after compression injury. Relative to the control group, transcranial administration of a P2RX7 antagonist markedly reduced the number of neutrophils at the injury site. c, Twenty-five micrometre xy projections were captured in CX3CR1gfp/+ mice 3 h after compression injury. Transcranial administration of the connexin hemichannel inhibitor CBX, but not the pannexin hemichannel inhibitor probenecid (image not shown), prevented the formation of jellyfish and honeycomb microglia (green) relative to vehicle-treated mice after compression injury.

d, Five micrometre xz projections were captured in B6 mice 3 h after compression injury. SR101 (red) applied transcranially 3 h after injury diffused more heavily into the brain parenchyma of CBX-treated mice when compared to the vehicle control group. Data are representative of three mice per group and at least three independent experiments. See also Fig. 4.