SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Compression injury model. An intact mouse skull bone (**a**) consists of three layers: cortical, cancellous, cortical. For standard skull thinning, a microdrill (not shown) and a microsurgical blade (**b**) 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 ~1000 μm. This preparation permits intravital imaging of the unperturbed meninges and brain parenchyma. 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 (**c**). 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 post-injury (**d**).

Figure S2. Parenchymal cell death following compression injury. Immunohistochemical analyses of coronal brain sections 12 hrs following compression injury (lower panels) 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 labeled by transcranially applying propidium iodide (red) 30 min prior to fixation. A normal uncompressed thinned skull preparation was performed on the contralateral hemisphere as a control (upper panels). Note that parenchymal cell death is not observed in any of the control images. Cell nuclei are shown in blue. Images are representative of 3 mice per group.

Figure S3. Microglia response to compression injury. Representative 5 μm maximum projections in the *xz* plane **(a, b)** or 25 μm projections in the *xy* plane **(b)** were captured from $CX3CR1^{gfp/+}$ mice at 60 min **(a)** or 6 hrs **(b)** following 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,** Following compression injury, SR101 labeled astrocytes (red) at the glial limitans are surrounded by a network of honeycomb microglia (upper panel; *xy* projection). An example is denoted with a white arrowhead. In contrast, holes in 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 following compression injury. Data are representative of 3 mice per group and at least three independent experiments.

Figure S4. Pharmacology of transcranial fluorescent compound administration. **a-c,** Steady state concentrations 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 hr following transcranial administration through a surgically thinned (uncompressed) skull. Background fluorescence was established by imaging for 30 min prior to 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 Materials & Methods for additional details about the applied fluorescent compounds. **d,** A 100 nM SR101 solution was applied to an intact mouse skull for thirty minutes, 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 $\pm$ SD) following transcranial

application to a thinned versus an intact skull. Data are representative of 3 mice per group and at least three independent experiments.

Figure S5. 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 CX3CR1$^{gfp/+}$ mice 3 hrs following compression injury. Two compression injuries were generated per mouse (over the left and right hemispheres). Prior to injury the left skull bone was treated with the denoted purinergic receptor antagonist and the right with vehicle (aCSF). When compared to the vehicle control, transcranial application of P2Y12 or P2X4 receptor antagonists impeded the ability of microglia (green) to assume both honeycomb and jellyfish morphologies, whereas P2Y6 antagonism blocked the formation of jellyfish processes only. **b,** *xy* projections were captured in LysM$^{gfp/+}$ mice 6 hrs following compression injury. Relative to the control group, transcranial administration of a P2X7 antagonist markedly reduced the number of neutrophils at the injury site. **c,** 25 µm *xy* projections were captured in CX3CR1$^{gfp/+}$ mice 3 hrs after compression injury. Transcranial administration of the connexin hemichannel inhibitor carbenoxelone (CBX), but not pannexin hemichannel inhibitor probenecid (image not shown), prevented the formation of jellyfish and honeycomb microglia (green) relative to vehicle treated mice following compression injury. **d,** 5 µm *xz* projections were captured in B6 mice 3 hrs after compression injury. SR101 (red) applied transcranially 3 hrs post-injury diffused more heavily into the brain parenchyma of CBX treated mice when compared to the vehicle control group. Data are representative of 3 mice per group and at least three independent experiments. See corresponding Figure 4.

VIDEO LEGENDS

Video 1. Dynamics of pathology following compression injury. **Part 1:** Representative B6 mice injected i.v. with Q-dots (red) were imaged 5 minutes following a normal thinned skull preparation (control; left panel) or a compression injury (compressed; right panel). Q-dot leakage into the perivascular space, visualized as a bright red accumulation around vessels (white arrowhead), is only observed after compression injury. Vascular breakdown, visualized as a diffuse red cloud erupting from a vessel (white arrow), also is only present following injury. A side view (*xz*) of the preceding compression injury video shows examples of perivascular Q-dot accumulation in blood vessels (white arrow) that descend from the meninges into the brain parenchyma. Skull bone is shown in blue. **Part 2:** Representative time lapses were captured in GFAP-GFP mice beginning 5 minutes after a normal thinned skull preparation (left panel) or a

compression injury (right panel). Astrocytes (green) normally form a continuous barrier between the meninges and parenchyma referred to as the glial limitans. Following compression injury, holes (white asterisks) appear in the glial limitans due to astrocyte death. An enlarged view from the preceding time lapse (denoted with a white box) shows an astrocyte in the glial limitans dying (white arrowhead) 45 minutes following compression injury. **Part 3:** Representative side views (*xz*) of two-photon time lapses were captured in CX3CR1$^{gfp/+}$ mice 5 minutes following a thinned skull preparation (control; top panel) or a compression injury (compression; bottom panel). Meningeal macrophages (green; white arrowhead) immediately below the skull (blue) are only visible in the control animal. Note how the sub-arachnoid space is no longer present following compression injury. The time lapses that follow show meningeal macrophages (green; long rod-like cells) undergoing rapid necrosis following compression injury (right panel). Note the green cloud generated as GFP leaks out of damaged meningeal macrophages, while microglia (green; ramified cells) beneath these macrophages remain intact. Blood vessels (red) were labeled by i.v. injection of Q-dots. **Part 4:** Representative side views (*xz*) of two-photon time lapses were captured in CX3CR1$^{gfp/+}$ mice 5 minutes following a thinned skull preparation (control; top panel) or a compression injury (compression; bottom panel). In control mice, microglia (green) never extend their processes above the glial limitans into the meningeal space. Following compression injury, however, microglial processes often extend through the glial limitans into the injured meningeal space (white arrows) as evidenced by the presence of microglial processes adjacent to the skull bone (blue).

Video 2. Compression injury induces a stable network of "honeycomb" microglia at the glial limitans. **Part 1:** A representative time lapse from a CX3CR1$^{gfp/+}$ mouse shows 3.5 hrs of continuous imaging through a normal uncompressed skull window. Afterward, a compression injury was induced and the same area was imaged for an additional 9 hrs. Prior to injury, note the presence of meningeal macrophages (green; long rod-like cells) and ramified microglia (also green) probing their surroundings. Meningeal macrophages die following compression injury (see Video 1), and microglia immediately retract their ramified processes and extend them toward the glial limitans, forming a vast network resembling a "honeycomb". This network of microglial processes is entirely formed within one hour of injury. A magnified time lapse of the area highlighted by the white box shows individual microglia converting from a ramified to honeycomb morphology between 5 min and 2 hrs post-injury. Microglia processes in the video were labeled with the Filament tool in Imaris to facilitate visualization. Note at 2 hrs how microglial processes are no longer ramified, but only extend upward towards the glial limitans,

forming a honeycomb network. Static top (*xy*) and side (*xz*) views of labeled microglial processes from the preceding section are shown at 5 min and 2 hrs following injury. Individual cells are shown in distinct colors. **Part 2.** A representative 3D projection was captured in a CX3CR1$^{gfp/+}$ mouse 3 hrs following compression injury. Astrocytes (red) were labeled by transcranial administration of SR101. Note that honeycomb microglia (green) surround individual astrocytes in the glial limitans.

Video 3. Generation and dynamics of phagocytic "jellyfish" microglia. A representative 11 hr time lapse from a CX3CR1$^{gfp/+}$ mouse was captured beginning 5 min post-compression injury. At 5 min, a microglial cell (green) in the center of the frame (white arrowhead) has already begun retracting its ramified processes. Afterward, the cell expands a single "jellyfish" process outward in all directions. Following complete retraction of processes at 2 hrs, the soma itself retracts into the jellyfish structure, and the amoeboid microglia becomes motile as other jellyfish microglia can be seen moving into the area. Note at 10 hrs post-compression that microglia in the field become significantly less motile and form a continuous barrier. Blood vessels are labeled red by i.v. injection of Q-dots.

Video 4. Three distinct microglial morphologies generated in response to compression injury. A representative time lapse showing the three distinct microglial morphologies was captured in a CX3CR1$^{gfp/+}$ mouse beginning 5 min following compression injury. The three distinct microglial (green) morphologies are highlighted with white boxes and magnified into individual time lapses. Box 1 shows an area with an immediate motile jellyfish response. Box 2 shows an area with microglial processes that extend and converge on a particular area, which is followed by the formation of a continuous barrier at the glial limitans similar to the jellyfish microglia in Box 2. Box 3 shows an area forming a network of honeycomb microglial.

Video 5. Morphological conversion of "honeycomb" into phagocytotic "jellyfish" microglia. The representative time lapse was captured in a CX3CR1$^{gfp/+}$ mouse beginning 5 hrs following compression injury. The time lapse contains examples (white arrowheads) of microglia (green) comprising honeycomb networks that collapse and immediately fill the holes in their network likely as a result of astrocyte cell death in the glial limitans. Note that large endosomes become visible in the resultant jellyfish microglia, a process taking approximately 5 minutes.

Video 6. Microglial reconstitution of the glial limitans. The representative 3D projection was captured in a CX3CR1$^{gfp/+}$ mouse 3 hrs following compression injury. Note that most microglia (green) in the center of this field have retracted their ramified processes and extended fine, thread-like processes toward the glial limitans that expand into large, circular structures with a jellyfish morphology. The jellyfish processes are juxtaposed with one another and form a continuous barrier along the glial limitans. Skull bone is shown in blue.

Video 7. Microglia form a continuous phagocytotic barrier along the glial limitans by assuming a jellyfish morphology. The representative 15 hr time lapse was captured in a CX3CR1$^{gfp/+}$ mouse beginning 5 min post-compression injury. Most microglia (green) in the field immediately retract their ramified processes, funneling all available cell mass into single, phagocytotic processes at the glial limitans. Microglial cell death was observed as early as 5 hrs post-injury (white arrowhead) and began to accelerate by 9 hrs (white arrowhead). Evidence of edema (a wave of swelling in the tissue) was observed at 11 hrs, which was followed shortly thereafter by deterioration in the overall health of the microglial barrier structure at the glial limitans.

Video 8. Myelomonocytic cell recruitment following compression injury. **Part 1:** The representative 12 hr time lapse from a LysM$^{gfp/+}$ mouse was captured beginning 5 min post-compression injury. Neutrophils (bright yellowish green) begin to swarm the area of injury at 1 hr following compression, both from adjacent areas of the meninges and by vascular extravasation (white arrowhead). Monocytes (light green) arrive later in the response (~7 hrs). Vessels (red) labeled by i.v. injection of Q-dots. **Part 2:** Representative *xy* and *xz* time lapses were captured in a LysM$^{gfp/+}$ mouse beginning 1 hr after compression injury. Thirty minutes after injury, dead cells were labeled by transcranial application of PI (red). Neutrophils localize almost exclusively to the meninges, which contains an abundance of dead cells. Skull bone is shown in blue. An enlarged view following a single neutrophil from the preceding video reveals how neutrophils interact with multiple dead cells over the course of many hours.

Video 9. Purinergic receptor and connexin hemichannel signaling mediate the innate immune response to compression injury. **Part 1:** Representative time lapses were captured in a CX3CR1$^{gfp/+}$ mice pretreated transcranially with P2Y12, P2Y6, P2X4, or P2X7 receptor antagonists and imaged beginning 5 min post-compression injury. Note that P2Y12, P2Y6, and P2X4 inhibition prevents microglia (green) from transforming into jellyfish at the glial limitans.

P2Y12 and P2X4 antagonism also reduces the formation of honeycomb networks, whereas inhibition of P2X7 has no impact on either microglial response to compression injury. In general, microglial retained more ramified processes following injury when inhibited with P2Y12, P2Y6, or P2X4 antagonists compared to P2X7. Time lapses in the next section were captured in LysM$^{gfp/+}$ mice pretreated with a P2X7 receptor antagonist or a vehicle control. P2X7 antagonism almost entirely eliminates the neutrophil (green) response to compression injury, even 12 hrs following injury. **Part 2:** Representative time lapses were captured beginning 5 min post-compression injury in CX3CR1$^{gfp/+}$ mice pretreated transcranially with a connexin hemichannel inhibitor (CBX) or a vehicle control. Inhibition of astrocytic ATP-dependent ATP release by CBX prevents microglia (green) from generating jellyfish and honeycomb responses. Microglia instead maintain ramified processes along with a single, small, ill-defined circular process at the glial limitans (white arrowhead). The structure resembles an aborted attempt to transform into the jellyfish morphology. A typical jellyfish reaction is shown in the vehicle treated control time lapse for comparative purposes.

Video 10. Glutathione reduces inflammation and glial limitans damage following compression injury. **Part 1:** Representative time lapses were captured in CX3CR1$^{gfp/+}$ mice pretreated transcranially with the ROS scavenger, glutathione, or a vehicle control. The standard compression injury was followed by physically cracking the skull (blue) with the microsurgical blade to promote a more severe inflammatory reaction. Note that meningeal macrophages (green; thin rod-like cells) and microglia near the fractured skull (green; ramified) only survive in glutathione treated animal. In addition, microglia in the glutathione-treated time lapse retain ramified processes and do not appear to sense the massive injury above them. This contrasts with the vehicle control group in which microglia immediately retract all processes and form single motile jellyfish structures. Magnified time lapses in vehicle versus glutathione treated mice following compression injury show the stark difference between microglial. Note that microglia in the vehicle control time lapse rapidly generate jellyfish structures, whereas those in the glutathione time lapse remain ramified and in a "naïve" state. **Part 2:** Representative *xz* time lapses were captured in CX3CR1$^{gfp/+}$ mice pretreated transcranially with vehicle or glutathione before compression injury. Note that within 30 minutes of injury, the subarachnoid space refills, and the compressed skull (blue) bows upward. This was never observed in the vehicle treated control group. Microglial / macrophages are shown in green. **Part 3:** Representative time lapses were captured in LysM$^{gfp/+}$ mice pretreated transcranially with vehicle or glutathione before compression injury. Glutathione pretreatment significantly

reduces the number of neutrophils (green) that migrate into the injured area relative to the vehicle control. Neutrophils occasionally become sequestered in blood vessels of glutathione treated mice, but do not extravasate and swarm the meninges.