

RESEARCH DESIGN:

Significance: *Microhemorrhages are linked to cognitive decline in humans.* Clinical studies (two just published in 2012) have correlated the increased incidence of microhemorrhages, caused by the bleeding of the smallest blood vessels in the brain, with cognitive decline and dementia risk¹⁻⁵. The impact of bleeding from a brain blood vessel on neural health and function depends critically on the size of the hematoma. Large intracerebral hemorrhages lead to widespread cell death and are associated with high rates of mortality and morbidity²³. In contrast, small MRI-detectable microbleeds do not produce acute stroke symptoms, but have been correlated with cognitive dysfunction^{24, 25}, and with more severe cognitive impacts after head trauma²⁶. For the smallest hemorrhages (diameter < 200 µm), clinical studies are more challenging because small hematomas can only be detected in postmortem histology. These microhemorrhages are common in multiple types of dementias, such as Alzheimer's disease and vascular dementia²⁷, and in patients with CADASIL²⁸. Several studies have independently linked increased numbers of microhemorrhages to poorer cognitive performance¹⁻⁵. Conversely, there have been no reports suggesting cell death near these lesions (although there are only limited studies in humans). New experimental studies of the impact of microhemorrhages on the health and function of nearby brain cells could help to shed light on the importance of their contributions to cognitive decline.

Because animal models are lacking, mechanisms linking microhemorrhage and cognitive dysfunction remain unclear. It has been difficult to study the pathological consequences of a microhemorrhage because good animal models of microvessel bleeds were not available. Current hemorrhage models involve injection of either blood directly into the brain or of enzymes that degrade the extracellular matrix, causing bleeding from many vessels²⁹. Neural death and inflammation was found in the vicinity of these hemorrhages, however the hematomas created are greater than 1 mm in diameter and the mechanisms that cause cell injury after these lesions may not play the same role in microhemorrhages. Transgenic mice, such as hypertensive or CADASIL (Notch 3 mutation) animals, develop microhemorrhages with diameters of several hundred micrometers throughout the brain, which have been shown to trigger inflammation³⁰. However it is difficult to follow the effects of microhemorrhages in these animals because of the unpredictable timing and location of the lesions.

In recent work, we have shown that our model of laser-induced microhemorrhage recapitulates the human findings of increased inflammation, including activation of astrocytes and microglia⁷. Interestingly, neurons near the microhemorrhage and their dendrites showed no sign of death or alteration in dendrite arborization⁷, consistent with the limited human autopsy data available³⁰. In contrast, occlusion of the same size vessel leads to widespread and nearly complete dendrite degeneration near the ischemic core^{7, 31}. This suggests that while occlusions of microvessels could impact cognitive function through cell death and neurite destruction, microhemorrhages likely affect the brain through more subtle mechanisms. While other experimental models of microscopic-scale injury, such as direct laser lesioning of the parenchyma, can also cause increased inflammation and have been used to study microglia dynamics³²⁻³⁴, our microhemorrhage model produces bleeds that mimic the human lesions identified at autopsy in both size and gross pathology (Fig. 1), making them more relevant for understanding human disease.

Elevated dendritic spine turnover could indicate a mild pathological state without cell death. In the most severe injuries, neurons die and their processes irreversibly disappear. In less severe damage, such as in the penumbra of an ischemic stroke, dendrites can "bleb," while spines disappear³¹. This process can be reversed if blood flow is rapidly restored, but extended ischemia leads to infarction³¹. Because it happens within minutes of the onset of ischemia and can be replicated in cultured neurons under hypoxia/hypoglycemia, this dendrite blebbing is likely an entirely neuronal process and does not involve microglia³¹. In milder insults, such as the trauma that results from cranial window surgeries¹⁸, dendritic processes remain unblebbed, and the total number of synaptic spines remains unchanged, but the turnover rate of spines is elevated. Such elevated spine turnover has been linked to plasticity that contributes to functional recovery after neural damage³⁵. After the mild injury from a craniotomy, which does not cause cell death, the increased spine turnover more likely leads to the formation of spurious connections and the dismantling of necessary synapses. Similarly, we suggest that, because neurons are not significantly injured, elevated synaptic rewiring after a microhemorrhage is likely not linked to regenerative plasticity and might, instead, contribute to abnormal function.

Inflammatory cells interact with synapses and may drive increases in synaptic turnover in pathological states. Recent work on microglia has shown that these multifaceted cells can serve many functions and show many phenotypes depending on the situation and the particular stimulus. In the normal state, microglia rapidly explore their local region by the extension and retraction of fine processes. These have been shown to make brief (minutes) contact with synapses, suggesting that microglia are "monitoring" the state of the synapse¹⁵. The duration of contact between microglia processes and synapses appears to be related to the amount of

neural activity¹⁷. In a disease state, the microglial response is graded by the severity of the damage to the tissue and also by the type of the pathogenic stimulus¹⁵. For focal injuries, work from our lab and others has shown that microglia rapidly extend processes toward the injury site. Within minutes to hours, the microglia processes change to a thicker shape and surround the lesion. Studies with knockout animals and pharmacological manipulations have shown that this process relies on P2Y-mediated detection of extracellular ATP gradients that are likely sustained by activated astrocytes²¹. In models of ischemic stroke, the dynamics of microglia processes change in the penumbral region. Microglia processes remain in contact with synapses for about an hour, and this often results in the disappearance of the synaptic spine¹⁷. Under inflammatory stimuli such as LPS and amyloid-beta application, microglia have been observed to phagocytose neurons that have surface-expressed phosphatidylserine^{36, 37}. In both injured and healthy brain, especially during development and times of high plasticity, microglia have also been shown to phagocytose spines³⁸. Finally, microglia have been shown to internalize both pre- and post-synaptic elements¹⁰. The recent recognition that signals formerly thought to be reserved for the immune system, such as MHC1¹⁹, are involved in synapse regulation suggests that alterations in microglia triggered by a microhemorrhage could result in neuronal functional changes. Taken together, this data suggests that activated microglia (and perhaps invading macrophages) may accelerate spine loss in otherwise healthy neurons. The loss of synaptic input due to such spine loss may drive homeostatic mechanisms that trigger these same neurons to actively seek new synaptic connections. In principle, this process could lead to a stable synaptic density, but with aberrant elevations in the rate of synapse turnover.

Innovation: *Inflammation-mediated increases in spine turnover may represent a novel mechanism for brain dysfunction.* In this proposal, we test the hypothesis that microhemorrhages lead to brain dysfunction not by killing neurons or other brain cells, but rather through elevation of synaptic turnover, mediated by increased activity of brain-resident and/or blood-derived inflammatory cells. If true, inflammation-triggered aberrant synapse turnover could be a novel mechanism for cognitive dysfunction following a microhemorrhage or, potentially, as a result of other triggers of neuroinflammation, such as in neurodegenerative disease.

Distinguishing microglia and macrophage inflammatory responses after microhemorrhage may reveal differential role for these cell types. We will utilize an experimental strategy for *in vivo* imaging of the response of inflammatory cells to injury that enables us to distinguishing the role of activated brain-resident microglia from that of invading blood-derived macrophages. This is done by generating chimeric animals through bone marrow transplants between different strains of fluorescent protein expressing transgenic mice and wild type mice, thereby creating animals with only labeled microglia or macrophages. Increasing evidence suggests that brain inflammation can have both neuroprotective and neurodegenerative effects, and it is likely that brain-resident microglia and invading macrophages play different roles in both the positive and negative aspects of the inflammatory response¹⁵. Because macrophages could be more easily modulated through systemically-administered drugs than microglia, distinguishing the neuroprotective and neurodegenerative roles of microglia and macrophages could help guide the development of therapeutic approaches that could mitigate the ill effects or, possibly, enhance the protective effects of the inflammatory response.

Unique technical approaches to producing microhemorrhages and studying their impact enable these studies. We use a pioneering methodological approach⁶ that is well-matched for investigating our hypothesis and for which our lab is uniquely capable. Highly nonlinear absorption of individual, tightly-focused, high-energy, femtosecond-duration laser pulses is used to rupture of the wall of a targeted cortical arteriole. The vessel bleeds out into the surrounding tissue until clotting at the vessel wall stops the bleeding⁴. The lumen of the ruptured vessel does not clot, so no ischemia is produced. In addition, the laser energy that is deposited leads to direct cellular injury only in a few cubic micrometers of tissue right at the location of the laser focus, ensuring that the subsequent effects we observe are caused by the presence of red blood cells and blood plasma in the brain, not the technique used to induce the bleeding. The mechanism of laser-based cellular injury we use to rupture the vessel wall is distinct from and more controlled than the use of long exposures to high repetition-rate trains of femtosecond laser pulses (i.e. the laser systems used for 2PEF imaging), where accumulation of heat at and around the laser focus plays an important role and leads to more widespread direct injury of tissue by the laser^{32, 39, 40}. Though requiring complex laser technology, our amplified femtosecond laser-based approach to producing a microhemorrhage uniquely enables these studies, as it allows us to target both the location and time of creation of the lesion. This method produces microhemorrhages that resemble the lesions that have been found in the brain of humans with dementia (Fig. 1). When paired with *in vivo* imaging using 2PEF microscopy, we can study the cellular dynamics that follow these microhemorrhages with high spatial and temporal resolution as well as with the capability for long-term, chronic imaging in the same animals, up to

months if necessary. These investigations, especially those in Aim 2 that characterize changes in synaptic spine density and turnover dynamics, would not be possible with more standard, post-mortem histology based analysis methods or with non-targeted approaches to triggering microhemorrhages (such as CADASIL or hypertensive mice).

Approach: Pilot data. The experiments we propose depend on the ability to image the dynamics of inflammatory cell and synaptic spine behavior after a microhemorrhage over weeks. The femtosecond laser-induced rupture of targeted arterioles is now routine in our lab and has been used for studies of the cellular response to microhemorrhage⁷ and for pre-clinical studies of the impact of anticoagulants on hematoma size⁴¹. Long-term 2PEF imaging (up to months, when necessary) in animals with glass-covered craniotomies at the resolution necessary to resolve dendritic spines is also routine. In addition, we have performed chronic imaging experiments with both thinned-bone preparations and the newly-described PoRTS window⁴², both of which are reported to reduce the inflammatory effects of the surgery used to gain optical access to the brain. Finally, we routinely perform bone marrow transplants to introduce fluorescent protein labeled bone marrow derived cells into other animal models.

Using a mouse that is heterozygous for a replacement of the CX₃CR1 fractalkine receptor with green fluorescent protein (GFP), we recently imaged the activation and increase in microglia/macrophage density after a laser-induced microhemorrhage⁷. While we were unable to distinguish between brain-resident microglia and blood-derived macrophages in these experiments (both are labeled in this mouse), we will use bone marrow transplants between these animals and wild type mice to create chimeric animals that resolve this ambiguity in the experiments proposed in Aim 1. In a preliminary experiment, we demonstrated our ability to image the invasion of bone marrow derived cells over the days and weeks following a microhemorrhage (Fig. 2). In this experiment, we gave a wild type animal a bone marrow transplant from a mouse that expressed red fluorescent protein (RFP) under a ubiquitous promoter. Bone marrow derived cells appeared within a few days and persisted at the lesion site for over a month (Fig. 2). While we were unable to distinguish between classes of bone marrow derived cells in this mouse because RFP is ubiquitous, we will use bone marrow transplants from the CX₃CR1-GFP mice to create similar chimeric animals where only macrophages are labeled.

In a final, critical piece of preliminary data, we imaged synaptic spines in excitatory neurons near a microhemorrhage and in control regions on the contralateral side of the brain (Fig. 3; n=4 microhemorrhages and control regions across two mice). These experiments used animals that expressed GFP in a subset of the Layer V pyramidal neurons (GFP-M line)⁴³. We quantified the cumulative number of spines gained and lost, per dendritic segment, for dendrites within a few hundred micrometers of a microhemorrhage over two weeks and observed an approximately two-fold elevation in both the rate of spine gain and spine loss near the hemorrhage as compared to control regions (Fig. 4; p<0.04, analysis of covariance). There was no difference in total spine density near the hemorrhage as compared to controls. We further found that the number of spines that were stable across the full two weeks of imaging was lower near the microhemorrhage as compared to controls (~83% stable spines in the microhemorrhage region vs. ~91% stable spines in the contralateral control region; p < 0.05; student t-test). This data supports our hypothesis that microhemorrhages lead to ~2X elevated rates of spine turnover, but without changes in overall spine density.

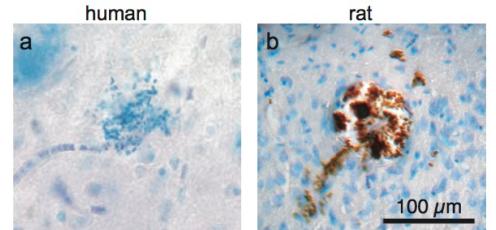


Fig. 1. Microhemorrhages in human and rat. (a) Microhemorrhage found in post-mortem human brain. Bright blue is a haem-rich deposit stained with Prussian blue. (b) Femtosecond laser-induced microhemorrhage in the cortex of a rat. Erythrocytes stained brown by diaminobenzidine while neurons are stained with Nissl. (a) from Cullen, 2005.

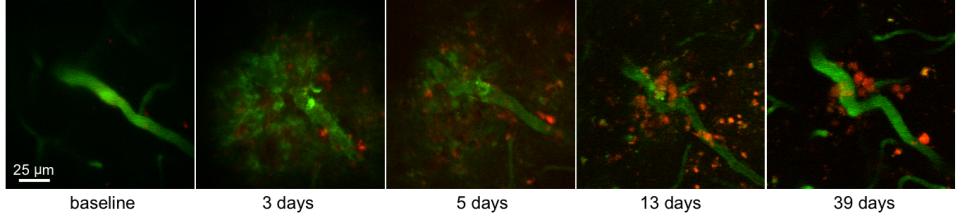


Fig. 2. Repeated 2PEF imaging of bone marrow derived cell invasion after a microvascular hemorrhage. The labeling results from a wild type mouse receiving a bone marrow transplant from a mouse ubiquitously expressing RFP (red). Vessels labeled with intravenously injected fluorescein-dextran (green).

Aim 1: Determine the relative role of microglia and macrophages in the inflammatory response to a microhemorrhage and test strategies to suppress the response of inflammatory cells to the lesion.

Mice and surgeries. These experiments will utilize heterozygous CX₃CR1-GFP mice as donors and recipients of bone marrow transplants with wild type animals. All animals receiving bone marrow transplants will recover

for at least two months before experiments begin. Mice with one functional copy of CX₃CR1 do not appear to have different microglial responses as compared to wild type animals, so one copy of the CX₃CR1-GFP knockin serves primarily as a strategy for fluorescent labeling of both microglia and macrophages. By using these animals as a bone marrow donor to a wild-type mouse, we create an animal with only labeled macrophages. We will do the bone marrow transplant both with and without lead shielding of the head. Recent work has suggested that the gamma irradiation necessary to kill the native bone marrow leads to injury to the blood brain barrier that elevates rates of invasion of bone marrow derived cells. This increased macrophage invasion is likely small compared to the invasion near a microhemorrhage, though it may still produce a background of macrophages in the brain before the lesion. On the other hand, shielding of the brain leads to only partial chimeras (about 70%), because the bone marrow in the skull does not get replaced. In the case of CX₃CR1-GFP transplants into wild type animals, this will lead only to a decrease in the fraction of macrophages that are labeled and does not result in any ambiguity in the experiment – i.e. we will still be able to cleanly distinguish the invasion of macrophages without confusion from labeled microglia. We will also transplant wild type bone marrow into the heterozygous CX₃CR1-GFP mice. Here, animals with head shielding during the transplant will retain some GFP-positive macrophages, making it harder to unambiguously resolve just activated microglia, and the unshielded animals will provide more unambiguous labeling. Reconciling experiments both with and without shielding will help rule out any impact of the gamma irradiation on the inflammatory process after a microhemorrhage. In addition, we will perform experiments with both glass-covered craniotomies (as in Figs. 2 and 3) as well as glass-reinforced thinned skull preparation (PoRTS window⁴²). The latter preparation reduces inflammation caused by the surgery itself and will enable us to rule out a baseline level of inflammation that modulates the response we see after a microhemorrhage. In all cases, animals will recover for at least four weeks after the surgery before we begin imaging experiments and only clean surgeries where baseline microglia activation is not detectable will be utilized.

Inhibition of microglia activation. Recent experiments with knockout animals and pharmacological inhibition have shown that microglia activation after focal brain injury is dependent upon extracellular ATP gradients that are sensed by the P2Y class of purigenic receptors²¹. Several topically-applied inhibitors of P2Y activity exist, including reactive blue 2 and PPADS²¹. These have been shown to inhibit the response of microglia to focal burn injuries in the brain as well as to mechanical trauma. It is likely that microhemorrhages lead to microglia response through a similar pathway. The burn injury was shown to activate astrocytes, which then release ATP, creating an extracellular gradient²¹. The subsequent

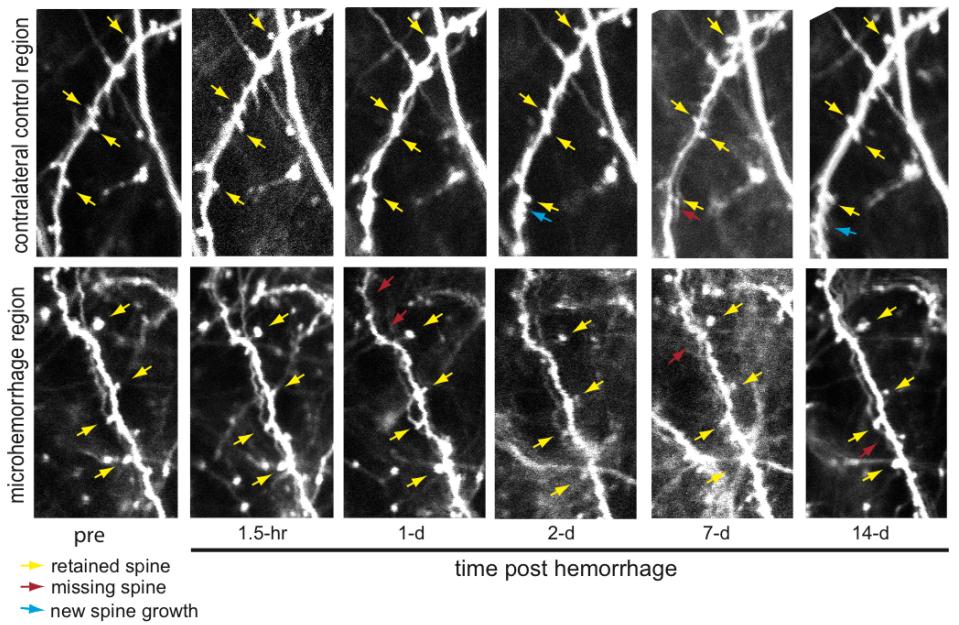


Fig 3. Time-lapse 2PEF images of dendrite spines. 2PEF images of fluorescently labeled dendrite spines, 20–100 μm beneath the cortical surface, before and up to 14 days after microhemorrhage (bottom). Control region was on the contralateral side of the brain (top). Several spines were lost near the microhemorrhage.

labeled dendrite spines, 20–100 μm beneath the cortical surface, before and up to 14 days after microhemorrhage (bottom). Control region was on the contralateral side of the brain (top). Several spines were lost near the microhemorrhage. This increased macrophage invasion is likely small compared to the invasion near a microhemorrhage, though it may still produce a background of macrophages in the brain before the lesion. On the other hand, shielding of the brain leads to only partial chimeras (about 70%), because the bone marrow in the skull does not get replaced. In the case of CX₃CR1-GFP transplants into wild type animals, this will lead only to a decrease in the fraction of macrophages that are labeled and does not result in any ambiguity in the experiment – i.e. we will still be able to cleanly distinguish the invasion of macrophages without confusion from labeled microglia. We will also transplant wild type bone marrow into the heterozygous CX₃CR1-GFP mice. Here, animals with head shielding during the transplant will retain some GFP-positive macrophages, making it harder to unambiguously resolve just activated microglia, and the unshielded animals will provide more unambiguous labeling. Reconciling experiments both with and without shielding will help rule out any impact of the gamma irradiation on the inflammatory process after a microhemorrhage. In addition, we will perform experiments with both glass-covered craniotomies (as in Figs. 2 and 3) as well as glass-reinforced thinned skull preparation (PoRTS window⁴²). The latter preparation reduces inflammation caused by the surgery itself and will enable us to rule out a baseline level of inflammation that modulates the response we see after a microhemorrhage. In all cases, animals will recover for at least four weeks after the surgery before we begin imaging experiments and only clean surgeries where baseline microglia activation is not detectable will be utilized.

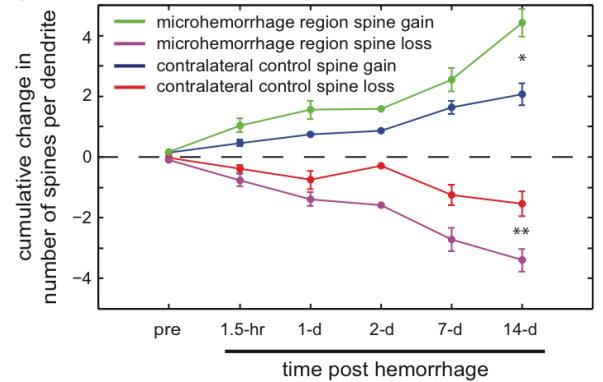


Fig. 4. Dendrite spine change is increased in microhemorrhage region. Cumulative change in the number of spines gained and lost per dendrite in the microhemorrhage and contralateral control region out to 14 days after microhemorrhage. Dendrite spine gain (loss) in the microhemorrhage region is represented by the green (purple) line. Dendrite spine gain (loss) in the contralateral control region is represented by the blue (red) line. (4 hemorrhages, 4 control regions across 2 mice).

These have been shown to inhibit the response of microglia to focal burn injuries in the brain as well as to mechanical trauma. It is likely that microhemorrhages lead to microglia response through a similar pathway. The burn injury was shown to activate astrocytes, which then release ATP, creating an extracellular gradient²¹. The subsequent

activation of microglia likely initiates additional signaling that sustains the astrocyte activation and thus maintains the inflammatory response. In the case of a microhemorrhage, the blood plasma that is pushed into the brain contains high levels of ATP and other inflammatory molecules (e.g. thrombin) and thus could initiate activation and launch this positive feedback of inflammatory signaling. Supporting this idea, we observed rapid microglia activation over the same spatial scale that blood plasma penetrated into the brain parenchyma after a microhemorrhage⁷. One intriguing possibility is that if the initial inflammatory reaction to the microhemorrhage could be blocked, this positive feedback cascade could be prevented and not just the acute phase, but also the chronic inflammation could be blocked. In support of this, we recently administered the P2Y inhibit PPADS topically to the cortex before inducing a microhemorrhage. We observed no acute microglial activation over the first few hours after the lesion, and no signs of chronic inflammation one day after the lesion (Fig. 5). In contrast, control animals that received topical infusions of artificial cerebral spinal fluid, showed the same rapid microglial activation and sustained inflammatory response we observed in previous experiments⁷. While this preliminary data supports it, if we find that this acute blockage of P2Y activity does not lead to robust long-term suppression of inflammation (we have only looked one day after the lesion so far), we will deliver the inhibitors chronically by implanting a small piece of tubing under the craniotomy that connects to a sub-cutaneously implanted osmotic pump. We will nick the dura at the edge of the craniotomy to allow the drug easy access to the brain. Control animals will have artificial cerebral spinal fluid infusions. As a final alternative strategy, we can use P2Y₁₂ knockout animals to block microglial activation¹⁷, although several generations of breeding will be required to introduce the CX₃CR1-GFP labeling into these animals. These P2Y knockout animals are commercially available from Jackson Labs.

Inhibition of macrophage recruitment. Invasion of macrophages to the brain requires circulating cells to bind to the endothelium of a blood vessel. This binding is dependent on a complex interplay of hydrodynamic forces and multiple different molecular interactions, including selectins, integrins, and adhesion molecules²². One essential mediator of macrophage endothelial adhesion is the vascular cell adhesion molecule (VCAM-1), which is strongly upregulated on the surface of inflamed endothelial cells. We expect this upregulation in the vessel that was hemorrhaged and, perhaps, in some nearby vessels. VCAM-1 binds to α_4 -integrin, a molecule expressed in macrophages that undergoes a conformational change to an active binding state when the cell is activated (such as by a cytokine signal from inflamed endothelial cells). This binding is a critical first step in the extravasation of a macrophage into the brain tissue in response to inflammation and blocking it has been shown to decrease the recruitment of macrophages into the brain after stroke⁴⁴ and severe epileptic seizures⁴⁵.

Monoclonal antibodies against the binding domain of α_4 -integrin are available and can be administered intraperitoneally on a daily basis to provide sustained blocking of macrophage recruitment.

Experiment design. First, we will repeat the experiments shown in Fig. 2 for mice with only microglia or macrophages labeled. Based on our previous data⁷, imaging the inflammatory cell behavior in two microhemorrhages and two control regions in each of five mice will provide more than enough statistical confidence in observed trends. We will image animals over several hours after the microhemorrhage, then on one, two, three, seven, 14, and 30 days after the lesion (times will be adjusted if warranted by the dynamics observed in the initial animals). We will quantify the density of microglia or macrophages as well as classify the degree of response of individual cells to the lesion using several metrics, including reduction in and morphology change in the number of processes, orientation of processes toward the lesion, and change in the extension/retraction rate and pause time of processes. These parameters will be measured as a function of time and distance from the microhemorrhage. In a smaller number of animals, we will confirm these results with the PoRTS window to rule out the effects of mild surgery-induced background inflammation. We expect to find that the rapidly responding cells we have already observed are brain-resident microglia and that blood-derived macrophages begin to enter the tissue near the

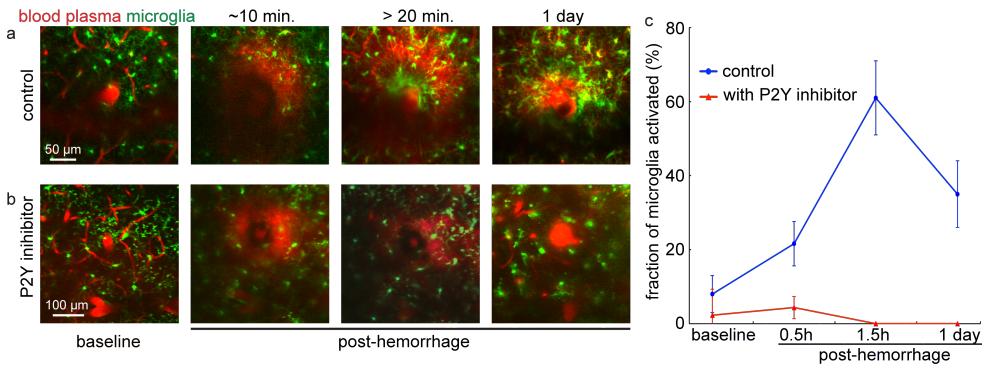


Fig. 5. Inhibition of microglial activation after a microhemorrhage with P2Y inhibitors. Imaging of microglia (green) and blood plasma (red) in animals receiving a microhemorrhage without (a) and with (b) topical application of the P2Y inhibitor PPADS. (c) Fraction of microglia within 200 μm of the microhemorrhage control regions in each of five mice that are activated as a function of time.

lesion in significant numbers after a few days. This work will clarify a critical ambiguity in our existing data and set the stage for experiments that test specific strategies for inhibiting microglia activation and macrophage recruitment. Next, we will set up animals with only microglia labeled that will be acutely administered either artificial cerebral spinal fluid or 1 mM PPADS just before the hemorrhage (or delivered chronically to the cranial window with osmotic infusion pumps, if deemed necessary from initial experiments) (5 per group). Additionally, animals with only macrophages labeled will receive daily intraperitoneal injections of 100 µg of anti- α_4 -integrin antibody or a control isotype antibody (5 per group). Finally, we will have non bone marrow transplanted heterozygous CX₃CR1-GFP that receive both treatments or both shams (5 per group). In each of these 30 mice we will induce two microhemorrhages and identify two control regions and image changes in the chronic response of microglia and/or macrophages to the lesion that result from these pharmacological manipulations. These data will serve to establish that the molecular pathways discussed above do govern the response of inflammatory cells after a microhemorrhage. Additionally, this work will set the stage to investigate how modulation of the inflammatory response affects synaptic spine turnover after a microhemorrhage.

Aim 2: Quantify the elevation of synaptic turnover after a microhemorrhage and test the hypothesis that activated inflammatory cell interactions with synapses drive this accelerated spine turnover.

Quantify spine turnover rates in excitatory and inhibitory neurons. The potential relationship between spine turnover and inflammation was first recognized it studies on neuronal structural stability that used chronic, 2PEF imaging of mice expressing fluorescent proteins in neurons^{11, 18, 46}. Different authors' results varied wildly, and one possibility that emerged was that the method of making craniotomies could influence synaptic turnover because GFAP upregulation in astrocytes and microglia morphology changes seemed to correlate with higher synaptic turnover rates. While neuroscientists seem to be generally satisfied that studies of spine dynamics require very careful window preparations regardless of window method, the systematic study of the mechanisms that underlie inflammation-mediated increases in spine turnover remains less explored. To track spine dynamics after microhemorrhage, we will use mice expressing fluorescent proteins in neurons. To compare with existing literature we will use mice expressing GFP in layer V excitatory neurons (M-line)⁴³. In addition, we will use mice with GFP labeling of somatostatin (SOM) inhibitory neurons⁴⁷. Mice will be prepared, lesioned and repeatedly imaged as in Aim 1 to generate data on spine density and turnover like that in Figs. 3 and 4 (n=5 mice per group, two hemorrhages and two control regions each). To ensure that these experiments are not confounded by effects of cranial window implantation, we will only include data from mice that show a low baseline spine turnover rate matching recent standards^{11, 18, 46}. Measurements of spine density and dynamics include identifying the same, new or disappeared dendrites and spines in each imaging session, with the researcher quantifying spine dynamics blinded as to whether the images are from hemorrhage or control sites. In addition to counts of number, we will also measure morphology such head size and lengths of spines. We expect to find elevated rates of spine turnover, with no changes in spine density, in the region where inflammatory cells are activated, consistent with our preliminary data. Because susceptibility to injury is different between inhibitory and excitatory neurons, SOM and pyramidal spines may show different dynamics.

Changes in spine turnover due to inhibiting the activation of microglia and invasion of macrophages. Using the protocols from Aim 1, we will measure spine dynamics after microhemorrhage when either microglia activation, macrophage invasion, or both are inhibited (5 mice per group). We expect that blocking the activity of inflammatory cells will reduce the elevation in spine turnover near the microhemorrhage and this result would firmly establish the overall hypothesis of this proposal. Critically, comparing these changes in spine density and turnover with microglia, macrophages, or both inhibited will reveal the cell type that is most critical for inflammation-mediated elevation of synapse turnover, suggesting possible therapeutic targets.

Interaction between microglia/macrophage processes and spines. To enable high-quality, simultaneous imaging of microglia/macrophages we will use the "Brainbow"⁴⁸ line of mice, which, if not exposed to Cre, express RFP in a subset of cortical excitatory neurons. Crossing these animals with CX₃CR1-GFP animals, then giving a bone marrow transplant from a wild-type will enable imaging of neurons and microglia. Similarly, transplants from the CX₃CR1-GFP to the Brainbow mouse will allow imaging of neurons and macrophages. The goal of these experiments is to measure the degree of interaction between spines and the inflammatory cells. We ask whether spine disappearance, maintenance, and appearance is dependent on the activation state of the microglia or macrophage after a microhemorrhage and the nature of the contact between the inflammatory cell process and the synapse. Other work has shown that prolonged contact between microglia processes and synapses after ischemia favors spine disappearance¹⁷, so it is likely that microglia/macrophage morphologies associated with activation could be associated with spines that disappear. In addition, it is possible that blood-derived and microglia-descended macrophages have different interactions with neurons.