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Authors

Feng, Xiaomei
Valdearcos, Martin
Uchida, Yosuke
[et al.](#)

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Microglia Mediate Postoperative Hippocampal Inflammation and Cognitive Decline in Mice

Xiaomei Feng,^{1*} Martin Valdearcos,^{2*} Yosuke Uchida,¹ David Lutrin,¹ Mervyn Maze,¹⁺ and Suneil K. Koliwad^{2,3+}

¹Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California, USA

²The Diabetes Center, University of California, San Francisco, San Francisco, California, USA

³Department of Medicine, University of California, San Francisco, San Francisco, California, USA

*Denotes equal contribution; +Denotes equal contribution

Correspondence:

Suneil K. Koliwad, MD, PhD

Diabetes Center

University of California, San Francisco

S1230 Medical Sciences; 513 Parnassus Avenue

San Francisco, CA 94143, USA

Phone: 415-479-9623

Email: skoliwad@diabetes.ucsf.edu

Mervyn Maze, MB, ChB

Department of Anesthesia and Perioperative Care

University of California, San Francisco

1001 Potrero Avenue, Box 1363

San Francisco, CA 94143, USA

Phone: 415-206-8906

Email: mervyn.maze@ucsf.edu

Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

Surgery can induce cognitive decline, a risk that increases with advancing age. In rodents, postoperative cognitive decline (POCD) is associated with the inflammatory activation of hippocampal microglia. To examine the role of microglia in POCD, we inhibited the colony-stimulating factor 1 receptor (CSF1R) in adult mice, effectively depleting CNS microglia. Surgical trauma (tibial fracture) reduced the ability of mice to remember a conditioned response learned preoperatively, a deficit more pronounced and persistent in mice with diet-induced obesity (DIO). Whereas microglial depletion by itself did not affect learning or memory, perioperative microglial depletion remarkably protected mice, including those with DIO, from POCD. This protection was associated with reduced hippocampal levels of inflammatory mediators, abrogation of hippocampal recruitment of CCR2⁺ leukocytes, and higher levels of circulating inflammation-resolving factors. Targeting microglia may thus be a viable strategy to mitigate the development of POCD, particularly in those with increased vulnerability.

Introduction

Postoperative cognitive decline (POCD) can complicate surgery and increase morbidity and mortality, especially in the elderly (1, 2). Efforts to identify causes of, and treatments for, POCD have focused on the innate immune response to the aseptic trauma of surgery, and consequent neuroinflammation (3, 4). Neuroinflammation may be a common precursor of cognitive decline, as it is also implicated in more chronic forms of neurodegeneration (5). However, because the stimulus for POCD is discrete, limiting the acute neuroinflammatory impact of surgery may afford an opportunity to preserve cognitive function, thus greatly improving surgical risk-benefit.

Though rodent models suggest that hippocampal neuroinflammation contributes to POCD (6-9), how this process is triggered and orchestrated remains unclear. Initial work focused on the idea that macrophages derived from circulating monocytes can drive postoperative neuroinflammation. Evidence for this concept comes from studying monocyte chemoattractant protein 1 (MCP-1/CCL2) and its cognate receptor (CCR2), which facilitate monocyte recruitment into tissues, including the CNS, under infectious and sterile inflammatory conditions (10). For example, CCR2-expressing macrophages accumulate in the hippocampi of mice experiencing experimental surgical trauma (11). Moreover, manifestations of POCD are reduced in mice with attenuated MCP-1 expression (12). Furthermore, depleting macrophages with clodronate reduced POCD in mice (13).

There is also evidence that postoperative hippocampal neuroinflammation involves more than just blood-borne macrophages. In particular, aseptic surgical trauma in preclinical models is associated with the accumulation and potential pro-inflammatory

activation of hippocampal cells expressing anti-ionized calcium binding adaptor molecule 1 (Iba1), which include macrophages and microglia, the CNS analogs of macrophages (7, 8, 11, 13-15). However, the importance of microglia in POCD is unclear, as is the nature of any crosstalk between microglia and CCR2⁺ immune cells that infiltrate the hippocampus through a transiently permeable blood brain barrier (BBB) (11).

Most peripheral myeloid cell types proliferate in response to multiple inputs, one of which is colony-stimulating factor 1 (CSF1) (16, 17). CNS microglia, on the other hand, depend on CSF1 receptor (CSF1R) stimulation for their survival (18, 19). Indeed, germline deletion of CSF1 or the CSF1 receptor (CSF1R), which responds to both CSF1 and IL-34, prevents microglia from seeding the developing brain and leads to early death in mice (19, 20). By contrast, inhibiting the CSF1R in adult mice depletes microglia without impacting mortality (18, 21, 22). Here we take advantage of this property, inhibiting the CSF1R in order to deplete ~98% of microglia from the brains of adult mice (18, 22), and demonstrate the impact of doing so on neuroinflammation and POCD in both healthy and vulnerable mice. Our studies reveal the critical role of microglia in orchestrating postoperative neuroinflammation and memory loss, and a heretofore-unrecognized interplay between microglia and circulating monocytes. We thus posit that microglia could be targeted to lessen POCD, as they have been to limit neurodegenerative forms of cognitive decline (23).

Results

Perioperative depletion of microglia attenuates surgery-induced cognitive decline in mice. We depleted microglia from adult mice by feeding them a standard chow diet containing PLX5622, which suppresses the tyrosine kinase activity of CSF1R (18, 22). Consuming a diet incorporating PLX5622 for 7 days produced a profound depletion of microglia vs. control, including in the hippocampus (Figures 1A & B), consistent with prior findings (22). By contrast, PLX5622 treatment did not affect the numbers of hippocampal astrocytes (GFAP+ cells), indicating specificity for microglia (Supplemental Figure 1). Control and PLX5622-treated mice were then subjected to either an aseptic tibial fracture with internal fixation or an anesthesia-matched sham procedure, after which they were assessed for markers of hippocampal and systemic inflammation, and for memory of an association learned preoperatively in a trace-fear conditioning (TFC) paradigm (Figure 1C). TFC memory assessed in rodents within days following surgery correlates well with performance in a Morris Water Maze, a well-validated assay of learning and memory often used in clinical models of cognitive dysfunction (24, 25).

Mice display a highly variable freezing response to contextual cues designed to evoke memories of TFC when tested immediately after initial associative learning (Figure 1D). Microglial depletion on its own impacted neither this learning, nor the ability of mice to freeze in response to contextual cues, an indication of their TFC memory (Figure 1B). Mice undergoing surgery, on the other hand, were markedly less responsive than sham-treated controls to cues evoking memories of preoperative TFC when tested on postoperative day 3 (Figure 1E). By comparison, mice treated perioperatively with PLX5622 retained a memory of preoperative TFC equal to that of

sham-treated controls (Figure 1E). These data indicate that perioperative microglial depletion can completely prevent the development of this experimental indicator of POCD.

Microglial depletion specifically reduces surgically-induced hippocampal neuroinflammation. Surgical trauma induces inflammation in brain regions involved in memory formation, such as the hippocampus (3). In control mice, surgical trauma produced an accumulation of hippocampal microglia that had transitioned from having a lacy, highly ramified morphology indicative of a quiescent state, to cells with shortened processes and rounded cell bodies, indicative of inflammatory activation (Supplemental Figure 2A). These microglia had increased expression of Iba1 (Supplemental Figure 2A), and reduced expression of the purinergic receptor P2Y₁₂ (Supplemental Figure 2B), characteristic of a pro-inflammatory polarization state.

Surgical trauma also significantly increased hippocampal levels of the pro-inflammatory cytokines IL-6 and MCP-1 at 6 and 24 hours following surgery (Figure 2A). Of these, IL-6 levels reached a plateau at 6 hours and fell thereafter, whereas MCP-1 levels rose progressively during the 24-hour period. PLX5622 treatment for 7 days and then perioperatively, however, dramatically lowered the postoperative hippocampal levels of both of these inflammatory mediators (Figure 2A), indicating that microglial depletion lessens postoperative hippocampal neuroinflammation.

Surgery also induced a rapid postoperative rise in hippocampal levels of the inflammatory cytokines TNF α and IL-1 β . However, this rise was more transient than that seen for IL-6 and MCP-1, measurable at 6 hours but no longer at 24 hours after surgery

(Supplemental Figure 2C). Regardless, perioperative PLX5622 treatment reduced the postoperative hippocampal induction of both of these mediators as well.

By contrast, surgery increased circulating cytokine levels in both untreated and PLX5622-treated mice; any impact of PLX5622 treatment on postoperative systemic inflammation was mild as compared to the hippocampus, and was totally absent for MCP-1 at 24 hours post-surgery (Figures 2B and Supplemental Figure 2D). Thus, the anti-inflammatory impact of perioperative microglial depletion is relatively specific to the brain.

Microglia are required for postoperative hippocampal infiltration by bone marrow-derived CCR2-expressing mononuclear cells. Previously, we showed that surgical trauma induces hippocampal MCP-1, the ligand for CCR2, and promotes hippocampal infiltration by CCR2-expressing monocytes (12). Moreover, treating mice with a neutralizing monoclonal antibody against HMGB1, a member of the “alarmin” family of damage-associated molecular patterns (DAMPs) that is released following trauma, is sufficient to abrogate this surgically-induced increase in MCP-1, thus mitigating the neuroinflammatory impact of surgery (12). Since PLX5622 treatment protected mice from surgically-induced increases in hippocampal MCP-1 levels, we assessed its effect on the recruitment of CCR2-expressing cells into the brain using mice expressing fluorescent fusion proteins of both CCR2 and fractalkine (CCR2^{RFP/+}: CX3CR1^{GFP/+}) to distinguish monocyte-derived macrophages (RFP⁺) from resident microglia (GFP⁺).

Whereas we did not see RFP⁺ cells in the hippocampi of any control (sham-treated) mice (Figure 3A), RFP⁺ cells consistently appeared in the choroid plexus,

around the 3rd ventricle, and began to enter the hippocampus within 24 hours of surgery (Figure 3B). To examine these cells further, we co-stained postoperative hippocampal sections for Iba1, which marks resident microglia, macrophage populations, and monocytes (Supplemental Figure 3). Focusing on the choroid plexus, we saw two populations of Iba1⁺ cells in this context. Some Iba1⁺ cells had a stellate morphology and did not co-express CCR2 (RFP), as would be expected for resident microglia and other long-lived resident myeloid cells populations, such as perivascular and subdural macrophages. However, another population of cells co-expressed both Iba1 and RFP (Supplemental Figure 3). Probing this population revealed that indeed all of the CCR2-expressing (RFP⁺) cells seen postoperatively in the hippocampus and associated choroid plexus also express Iba1. Moreover, these CCR2-expressing cells had either a rounded morphology or an elongated one, typical of cells in the process of vascular extravasation. Together these findings support the concept that the CCR2-expressing cells infiltrating the hippocampus in response to surgery are monocyte-derived.

Remarkably, microglial depletion abolished this surgically-induced recruitment of RFP⁺ cells (Figures 3B and C), indicating that microglia are responsible for the influx of circulating CCR2⁺ monocytes into the hippocampus following surgical trauma, and suggesting that they are therefore the likely source of the chemoattractant MCP-1. By contrast, perioperative PLX5622 treatment did not deplete circulating CCR2-expressing cells (Figure 3D), suggesting that PLX5622-dependent prevention of infiltration in this setting occurs through an on-target effect on microglia.

We used two other approaches to more definitively demonstrate that PLX5622 treatment abolishes postoperative infiltration of CCR2⁺ cells into the hippocampus

through its effect on microglia, rather than through a direct effect on monocytes. We first tested the possibility that PLX5622 treatment reduces the number of infiltrative, pro-inflammatory monocytes. To do so, we performed multi-parameter FACS analysis on circulating leukocytes from postoperative mice. Circulating CCR2⁺ cells were gated using markers for myeloid cells (CD11b⁺/CD45⁺), and the resulting pool of cells were then gated to identify individual monocyte subsets and neutrophils, respectively (Supplemental Figure 3A).

Doing so (Supplemental Figure 3B) clearly showed that PLX5622 does not deplete infiltrative pro-inflammatory (Ly6C^{Hi}) monocytes, Ly6C-intermediate (Ly6C^{Int}) monocytes, or neutrophils (Ly6C^{Int}/Ly6G⁺). On the other hand, monocytes expressing little detectable Ly6C (Ly6C^{Lo}), which have been identified as endothelium-adherent “patrolling” monocytes (26), were depleted by ~30% (Supplemental Figure 3B). However, patrolling monocytes are not implicated in tissue infiltration, suggesting strongly that PLX5622-treatment does not abolish hippocampal immune cell infiltration by depleting relevant monocyte subsets.

Second, we tested whether PLX5622 treatment impairs the ability of pro-inflammatory monocytes to infiltrate the brain. To do so, we injected CCR2^{RFP/+}: CX3CR1^{GFP/+} mice with TNF α by tail vein, a treatment that disrupts BBB integrity and increases CNS permeability to circulating monocytes (27, 11). We found that despite depleting microglia, PLX5622 treatment did not reduce monocyte infiltration into the hippocampi of TNF α -treated mice (Supplemental Figure 3C). This finding indicates that PLX5622 treatment does not fundamentally impair the capacity of monocytes to infiltrate

the brain and supports the concept that our findings in postoperative mice result from microglial depletion, rather than a direct effect on monocytes.

Microglial depletion remains highly protective in mice with heightened vulnerability to POCD. Metabolic frailty and advanced age enhance the risk for postoperative cognitive decline (28, 29), and we previously modeled this heightened vulnerability in LCR rats that spontaneously develop obesity, dyslipidemia, insulin resistance, and hypertension (30). Here we found that mice with diet-induced obesity (DIO) have normal TFC memory, with the expected degree of individual variability, when tested immediately after initial learning (Figure 4A). However like LCR rats, mice with DIO have a more pronounced and persistent loss of the freezing response to contextual testing than do strain-matched healthy lean controls when tested postoperatively (Figure 4B). Remarkably, perioperative PLX5622 treatment normalized postoperative memory in mice with DIO (Figure 4B), indicating that perioperative microglial depletion prevents postoperative memory loss even in mice with a heightened vulnerability to POCD.

In exploring the mechanism for this vulnerability, we followed levels of eicosanoid-derived specialized pro-resolving mediators (SPMs) of inflammation (31); whereas surgery increased plasma levels of the SPM Lipoxin A₄ (LXA₄) in healthy mice (vs. sham controls), mice with DIO had paradoxically lower postoperative LXA₄ levels than did corresponding controls (Figure 4C). These findings indicate that DIO fundamentally impairs the pro-resolving response to aseptic surgical trauma in mice. Consistent with this, mice with DIO had markedly higher postoperative hippocampal IL-6 levels than control mice (Figure 4D).

Remarkably, however, PLX5622 treatment prevented the postoperative abrogation in circulating LXA₄ levels otherwise seen in the context of DIO, restoring these levels to those more on par with healthy control mice. It also prevented the increase in postoperative hippocampal IL-6 levels induced by DIO (Figures 4E & F). Together, these data indicate that depleting microglia abolishes the ability of DIO to either attenuate the pro-resolving response to surgical trauma, or potentiate postoperative hippocampal inflammation. Of note, PLX5622 treatment led some mice to have relatively low circulating LXA₄ levels both under control (sham-treated) conditions and following surgery, and increased the inter-individual variability in these levels (Figure 4F). However, the qualitative capacity of perioperative PLX5622 treatment to restore normal LXA₄ responsiveness in mice with DIO was present despite this variability.

Discussion

We demonstrate that microglia are required for the development of hippocampal neuroinflammation in response to aseptic peripheral trauma, and that preemptively depleting mice of these cells by CSF1R inhibition prior to an experimental tibial fracture, and maintaining this depletion postoperatively, prevents not only neuroinflammation, but also the ability of surgery to impair memory.

Specifically, surgery increases hippocampal levels of pro-inflammatory cytokines in association with an accumulation of microglia displaying prominent features of inflammatory activation, and induces circulating CCR2⁺ cells to enter the hippocampus. Here we show that resident microglia are responsible for both of these processes. Indeed, perioperative microglial depletion not only abrogated the surgical induction of hippocampal cytokine levels, it also abolished CCR2⁺ cell influx into the hippocampus.

With this in mind, we were encouraged to see that whereas PLX5622 treatment effectively depletes CNS microglia, it does not reduce the number of circulating CCR2⁺ monocytes capable of tissue infiltration (Ly6C^{Hi}), consistent with prior work (22). Moreover, PLX5622 treatment did not alter the number of either circulating intermediate monocytes (Ly6C^{Int}/Ly6G^{Lo}) or neutrophils (Ly6C^{Int}/Ly6G⁺), or impair the ability of monocytes to infiltrate the hippocampus in response to a non-surgical stimulus (systemic TNF α treatment). On the other hand, CSF1R inhibition reduced the number of (Ly6C^{Lo}) “patrolling” monocytes by ~30%, although these monocytes are not implicated in tissue infiltration (26). It is also worth noting that 2-15% of T cells may express the CSF1R, and we did not monitor the impact of CSF1R inhibition on T cell numbers.

What stimulates microglial activation following surgical trauma? The alarmin HMGB1 engages circulating mononuclear cells and increases mRNA levels of the gene encoding MCP-1 (*Cc12*) in the hippocampus (4, 12). HMGB1-activated monocytes signal through NF- κ B and secrete TNF α , which is implicated in disrupting BBB integrity, allowing these cells to infiltrate the brain, including the hippocampus (11). Considering this work, the data from our study supports the possibility that HMGB1 may stimulate hippocampal microglia to secrete MCP-1, enabling monocyte recruitment. These data, however, do not preclude the possibility that other mechanisms may also contribute to activating hippocampal microglia during peripheral inflammation. These include vagal afferents that can regulate microglia (32), cytokines crossing the BBB by diffusion or active transport and regulating microglia under feedback control (33), and receptors present on endothelial cells lining the BBB or CNS-penetrating blood vessels that may control microglial polarization (34). Future work should attempt to dissect the relative contribution of these putative mechanisms in the context of surgically-induced trauma.

Examining surgically-induced hippocampal inflammation provided insight into the kinetics with which key cytokines rise and fall. For example, IL-6 levels rose over 6 hours following surgery, and then drifted slightly towards baseline. IL-1 β and TNF α levels were induced even more transiently (8), and were indistinguishable from those in sham-treated controls within 24 hours of surgery. By contrast, hippocampal levels of MCP-1 rose sharply and progressively throughout the entire 24-hour period following surgery. Despite these kinetic differences, microglial depletion reduced postoperative levels of each of these factors. Thus, resident microglia orchestrate a temporally complex hippocampal cytokine response to surgical trauma.

Beyond cognitive dysfunction, trauma also triggers fever, anorexia, fatigue, and a diminution in procreative instincts, together referred to as “sickness behavior” (35). Sickness behavior may protect an organism against further tissue damage by limiting excessive movement and risky behaviors in favor of rest, and by activating innate immunity. Our findings suggest that microglia may help mediate the cognitive manifestations of sickness behavior.

When tissue healing occurs, SPMs (36) such as LXA₄ help end the initial inflammatory response at the site of insult, systemically, and potentially within the brain. LCR rats do not adequately resolve postoperative inflammation and develop cognitive decline that persists for months (37). We now show that LXA₄ levels do not rise appropriately following aseptic trauma in C57BL/6 mice with DIO, which also have reduced glycemic control and other features of metabolic syndrome (38). This finding indicates that like LCR rats, mice with DIO fail to properly resolve postoperative inflammation. Moreover, mice with DIO also develop exaggerated and persistent postoperative neuroinflammation and memory loss. Our development of a mouse model of high-risk POCD will enable the use of mouse genetics to dissect how microglia, infiltrating monocytes and macrophages, and SPMs contribute to POCD.

Remarkably, PLX5622-treatment of mice with DIO not only prevented both surgically-induced neuroinflammation and cognitive dysfunction, it also prevented the ability of DIO to impair the postoperative rise in circulating LXA₄ levels seen in healthy lean mice without affecting inflammatory cytokine levels in the circulation. Together, these findings suggest that microglia may, at least in part, mediate the mechanism by which DIO impacts the resolution of acute inflammation. It is worth noting, though, that

PLX5622 treatment itself increased the variability and tended to lower the levels of circulating LXA₄, even in sham-treated controls. One possibility for this observation is that CSF1R inhibition could independently affect the capacity of leukocytes to synthesize and/or secrete LXA₄ or other eicosanoid-derived SPMs, however this has not been directly tested.

How might microglia regulate systemic LXA₄ levels? One possibility is that acutely activated microglia could down-regulate their capacity to produce LXA₄. If so, the exaggerated post-surgical microglial activation seen in mice with DIO could limit the contribution of CNS-derived LXA₄ to rising plasma LXA₄ levels after surgery. Indeed, human microglia express key LXA₄ enzymes, including 5-lipoxygenase (5-LOX) and 5-LOX activating protein (FLAP), involved in LXA₄ synthesis (39).

Another possibility is that activated microglia could, through secretion of another factor that is able to enter the circulation, indirectly reduce LXA₄ production or secretion by peripheral immune cells. This possibility remains to be tested. On the other hand, preclinical models of neuroinflammation suggest that circulating LXA₄ can feedback to inhibit microglial activation (40) or induce an alternatively activated (“M2”) state (41), suggesting the possibility of a regulatory feedback loop between LXA₄ and microglia.

Importantly, our approach avoids key off-target effects. For example, PLX5622 treatment in absence of surgery did not impact associative learning or memory. This point is important, as any acute application of CSF1R inhibition to limit POCD should not affect cognitive function on its own. The modest impact of PLX5622 treatment on circulating inflammatory markers is also potentially beneficial, as any effort to target microglia in the context of surgery or injury should preserve one’s capacity to mount a

systemic inflammatory response (42). Our observation that PLX5622 targets microglia while sparing pro-inflammatory monocytes underscores the concept that the host response to peripheral infections might remain robust in the context of perioperative CSF1R inhibition. Despite these reassuring aspects of the currently study, future studies should functionally modulate microglia without depleting them in order to confirm the mechanistic interpretation of our study.

There are other aspects of our approach to microglial depletion that have translational relevance. For example, microglial numbers return to normal in less than 36 hours after stoppage of PLX5622 treatment (18), a reversibility which bodes well under clinical circumstances. Moreover, others have shown that CSF1R inhibition with PLX3397, an agent closely related to PLX5622, did not impact key elements of tibial fracture repair in relatively young rodents, and actually improved aspects of fracture repair in older animals (40). This suggests that perioperative CSF1R inhibition may not fundamentally impair bone healing in the context of orthopedic surgeries. Finally, CSF1R inhibitors are already in clinical trials for diseases ranging from cancer to inflammatory forms of arthritis (43-45). Perioperative CSF1R inhibition would be focused and short-lived by comparison, thus minimizing patient risk.

Our study highlights the concept that targeting microglia may effectively limit the negative impact of surgery on cognition in especially vulnerable individuals. Prior work indicated that rats with metabolic syndrome have accentuated manifestations of POCD, akin to humans with advanced age (24). Here we show that mice with DIO, an established model of metabolic syndrome, are also prone to more pronounced POCD. We thus used DIO as a surrogate for advanced aging in mice, as it would have been

difficult to know what chronological age is best to study the impact of aging on POCD in mice without studying multiple cohorts of mice across the aging spectrum.

With this in mind, our findings may be extrapolated to surgical patients with advanced age or associated metabolic diseases that may fail to readily resolve trauma-induced inflammation, placing them at high risk for persistent postoperative cognitive impairments (29, 30). The health significance of this is noteworthy, given the increasing size of these high-risk patient populations.

By identifying microglia as critical components of the process by which surgical trauma induces neuroinflammation and consequent cognitive dysfunction, we may also inform strategies to lessen cognitive decline in chronic neurodegenerative conditions (23), or prevent further cognitive decline when patients with preexisting neurodegenerative conditions undergo surgery.

Methods

Study approval

All experiments were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco (AN111420-02 and AN097767-03) and adhered to the highest standards of animal care.

Animals.

Male C57BL/6J mice were used in all studies. Five-week-old mice were group-housed with 12-hour light/dark cycles and fed standard rodent chow (control) or a HFD with 42% of calories from fat (TD.09682, Envigo-Teklad) for 8 weeks. Dr. Israel Charo (J. David Gladstone Institutes, San Francisco, CA) provided CCR2-RFP and CX3CR₁-GFP mice. Microglia were depleted in all cases by feeding mice a diet containing the CSF1R inhibitor PLX5622 (Plexxikon, Emeryville, CA, USA) formulated in either a standard low-fat AIN-76A chow diet (RD) or a high-fat Western diet (D12079B, RD) at a dose of 1.2 g/kg for 7 days. In order to disrupt the BBB independently of surgery, mice were given recombinant murine TNF α (0.15 mg/kg; Peprotech; Cat. No. 315-01A) by i.v. Injection, and then studied 16 hours later. Anesthesia was with 2.1% isoflurane in 0.30 FiO₂.

Fear conditioning tests

Mice adopt a characteristic “freezing” posture when anticipating an aversive stimulus, the memory of which is evoked by contextual cues related to a fear-inducing stimulus-response pairing learned previously. The percentage of time that mice spend freezing when in the presence of standardized contextual cues is thus a reliable index of

their memory of an experimental bout of prior associative learning. The extent of initial learning (training) and memory of this learning (contextual testing) was recorded in a trace fear condition (TFC) chamber (Med Associates, Inc.).

Training: Training consisted of 2 trials of tone and foot-shock pairings. Mice were placed in the TFC chamber and allowed to explore it for 100 seconds. Mice were then exposed to an auditory cue (75-80 Db, 5 kHz, conditional stimulus) for 20 seconds followed 20 seconds later by a 2-second foot-shock (0.8 mAmp; unconditional stimulus). The tone and foot-shock pairing was repeated with an inter-trial interval of 100 seconds, and the mice were removed from the chamber for 30 seconds in between trials.

Contextual Testing: Contextual test was first performed 3 days after the training protocol. At this time, the mice were placed back into the same TFC chamber in which training had occurred previously, but without any tone or shock for 5 minutes. Movement of the mice was monitored by an attached tracking system, and Video Freeze software recorded the time spent freezing as a fraction of total time in the chamber. Contextual testing was later repeated postoperatively as a means to quantify residual memory at time points following surgical interventions.

Surgery

Surgery was performed on mice within 30 minutes after the TFC training, and consisted of an aseptic open tibial fracture with intramedullary fixation performed under general anesthesia, including 2.1% isoflurane in 0.30 FiO₂ and buprenorphine as previously described (1). Briefly, the left hind paw was shaved and disinfected. A median paw incision was then performed, followed by the insertion of a 0.38-mm pin in

the tibial intramedullary canal. The periosteum was then stripped and osteotomy performed. After producing the fracture, the wound was irrigated and the skin sutured with 5-0 Vicryl sutures; thereafter, animals were allowed to recover spontaneously from the anesthetic. During the procedure, temperature was monitored and maintained between 36 and 37°C with the aid of warming pads (Harvard Apparatus). Subcutaneous analgesia (buprenorphine, 0.1 mg/kg) was administered after anesthetic induction and before skin incision. Control (sham-treated) mice received anesthesia and analgesia.

Cytokine measurement

ELISA assays (R&D Systems) were used to measure TNF α , IL-1 β , IL-6, and MCP-1 levels in the plasma and hippocampus.

Plasma lipoxin A4 (LXA₄) measurement

An ELISA assay (Oxford Biochemical Research, Michigan) was used to measure plasma levels of LXA₄, following manufacturer's instructions.

Immunofluorescence histology

WT mice and CCR2-RFP : CX3CR1-GFP mice were sacrificed and perfused with saline followed by 4% paraformaldehyde in 100 mM phosphate buffer. Their brains were removed, post-fixed in the same fixative overnight (4°C), and then immersed in 30% sucrose for 48 hours. Brains were then embedded in optimal cutting temperature compound, immediately frozen on dry ice, and stored (-80°C). 35 μ M-thick coronal sections were cut on a cryostat, mounted on glass slides using Vectashield antifade

mounting media (Vector Labs; Cat. No. H-1200), blocked for 1 hr with 5% BSA in PBS containing 0.1% Triton X-100, and incubated with an anti-Iba1 antibody (1:500 rabbit polyclonal; Wako). Immunofluorescence was performed with Alexa Fluor 488-labeled anti-rabbit DAPI Vectashield solution (Vector Labs) to identify cell nuclei. Sections were. Images were acquired by confocal laser-scanning microscopy (Leica TCS SP5).

Flow cytometry

To analyze whether, in addition to depleting microglia, PLX5622 treatment reduces the total number of circulating CCR2-expressing cells, blood samples were taken from drug-treated and control CCR2-RFP : CX3CR1-GFP mice. After red blood cell lysis (RBC lysis buffer, eBioscience), plasma leukocyte single cell suspensions were acquired on a BD LSR II flow cytometer to measure RFP+ cells, and analyzed with BD FACSDiva v6 software.

Statistics

Data are expressed as means \pm standard error. Statistical analyses were performed using the Prism 5.01 (GraphPad Software, Inc, Lo Jolla, CA) statistical package and Stata 11.2 software (StataCorp, College Station, TX). We performed 2-tailed Student's t-tests for single comparisons and ANOVA (1-way and 2-way) in the context of multiple comparisons, as appropriate (see figure legends), with *post hoc* Newman Keuls testing following ANOVA analysis of nonparametric samples. We tested for normal distribution of the data with the d'Agostino and Pearson omnibus test, and for equality of variances with the F-test. 2-way ANOVA was specifically used whenever

there was a possibility of interaction between independent treatment variables, and prior to performing individual pairwise comparisons. In each case, p values < 0.05 were considered significant.

Author contributions

XF and MV contributed equally to designing and conducting relevant experiments, analyzing data, preparing figures, and drafting the manuscript. YU and DL assisted in conducting relevant experiments. MM and SKK contributed equally to conceiving of the studies, supervising their design and completion, and writing the final manuscript. SKK prepared the final figures. MM's laboratory conducted the neurocognitive testing in mice, and SKK's laboratory performed all fluorescence microscopy.

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Figure Legends

Figure 1. Perioperative microglial depletion prevents surgically induced memory loss in mice. **A.** Representative hippocampal immunofluorescence images including Iba1⁺ cells (green) and nuclei (DAPI; blue) from mice following tibial fracture, showing marked depletion of hippocampal microglia in response to PLX5622 treatment for 7 days before surgery and 3 days following surgery. Scale bars=50 μ m. **B.** Quantification of the extent of microglial depletion in A (n = 6/group; *p<0.01 vs. control by two-tailed Student's t-test). **C.** Schematic, depicting the protocol for preoperative trace-fear conditioning (TFC) training, TFC testing, experimental surgery, and postoperative analyses of both hippocampal and systemic inflammation, and the memory of preoperative associative learning. **D and E.** Freezing in response to contextual cues, expressed as a percentage of total time in the testing environment prior to surgery (D), and relative to that of appropriate sham-treated controls 3 days following surgery (E). Data in D were analyzed by 2-way ANOVA. Data in E were analyzed by 2-tailed Student's t-test (**p=0.0002 for surgery vs. sham-treated control). For both D and E, n = 10/group.

Figure 2. Perioperative microglial depletion abrogates surgically induced hippocampal inflammation. **A.** Tissue ELISA, showing the rise in hippocampal levels of both IL-6 and MCP-1 in response to surgery, and the prevention of this postoperative rise by perioperative PLX5622 treatment as analyzed by 2-way ANOVA (p=0.006 at 24 hours for IL-6, and p<0.01 at both 6 and 24 hours for MCP- 1). **B.** Plasma ELISA, showing the comparative lack of effect of PLX5622 treatment on postoperative plasma

IL-6 and MCP-1 levels. Data were analyzed by 2-way ANOVA. In all cases, n=7-8/group, and ***p<0001; **p<0.001; and *p<0.02 for surgery vs. corresponding sham-treated control.

Figure 3. Perioperative PLX5622 treatment prevents surgery induced entry of CCR1-expressing cells into the hippocampal region of the brain in mice. A.

Representative immunofluorescence images of hippocampal sections from CCR2^{RFP/+} : Cx3CR1^{GFP/+} mice (n=4, 3 sections per sample), showing that sham-treated mice do not have CCR2-expressing (RFP⁺) cells in the hippocampus or associated choroid plexus.

B. Similar images in mice 24 hours after surgery, showing that depletion of microglia (green) by perioperative PLX5622 treatment is associated with the complete disappearance of CCR2-expressing cells otherwise present in the choroid plexus and entering the hippocampus at this time point. Scale bars=50mm. **C.** Quantification of the prevention of surgically induced CCR2^{RFP/+} cell infiltration by PLX5622 treatment in mice

from B (n = 8 mice/group; *p<0.05 vs. control by 2-tailed Student's t-test). **D.** FACS data from blood samples taken from control and PLX5622-treated mice 3 days following surgery, showing no effect of perioperative PLX5622 treatment on the number of circulating CCR2^{RFP/+} cells (n=8 mice/group; analyzed by 2-tailed Student's t-test).

Figure 4. Perioperative microglial depletion prevents the inflammatory and cognitive consequences of surgery in mice with DIO. A.

The freezing response to preoperative TFC testing (% of total time) of healthy (chow-fed) controls, mice with DIO, and mice with DIO treated with PLX5622 (PLX), showing no difference between groups.

B. Freezing times measured from the mice in A (% vs. corresponding sham-treated mice) during TFC testing 3 and 7 days after surgery. Whereas the impact of surgery on TFC memory is transient and returns to sham levels by day 7 in control mice (* $p < 0.001$ vs. control at day 3), mice with DIO have a more pronounced (* $p < 0.001$ vs. control at day 3) and persistent ($^{\$}$ $p < 0.001$ vs. control mice at day 7) memory loss. Perioperative PLX5622 treatment protects mice with DIO from surgery induced memory loss ($^{\#\#}$ $p < 0.005$ vs. DIO alone at day 7). **C and D.** Analysis of LXA₄ and IL-6 levels, showing that DIO paradoxically lowers plasma LXA₄ levels that otherwise rise by 3 days after surgery in healthy control mice (C), and potentiates the postoperative (day 3) rise in hippocampal IL-6 levels seen in control mice (D). **E and F.** Corresponding measurements from plasma (LXA₄) and hippocampus (IL-6), showing that perioperative PLX5622 treatment abolishes the impact of surgery on circulating LXA₄ levels in mice with DIO (E), and prevents hippocampal IL-6 levels from rising in mice with DIO (F). All data are from $n = 12-19$ /group, and were analyzed by 2-way ANOVA (A, C, and D), 1-way ANOVA (B), and Student's t-test (E-F); ** $p < 0.001$ for comparisons shown.

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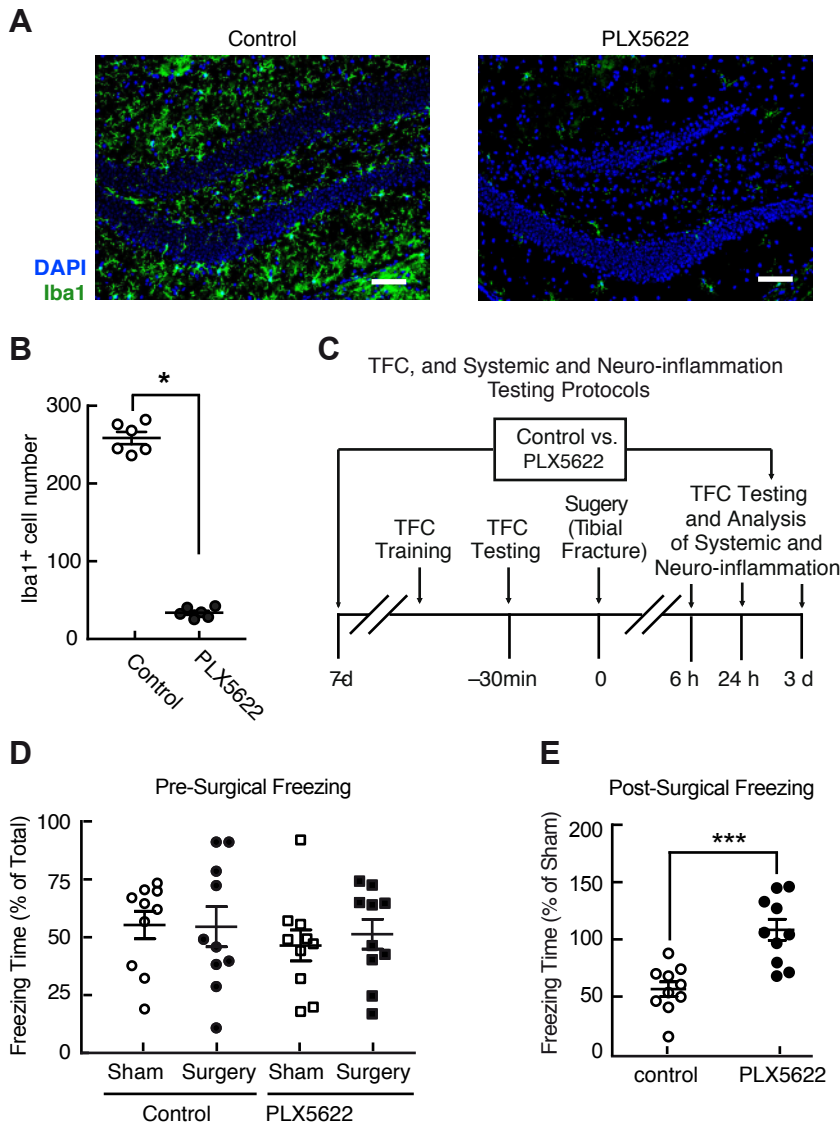


Figure 1. Perioperative microglial depletion prevents surgically induced memory loss in mice.

A. Representative hippocampal immunofluorescence images including Iba1⁺ cells (green) and nuclei (DAPI; blue) from mice following tibial fracture, showing marked depletion of hippocampal microglia in response to PLX5622 treatment for 7 days before surgery and 3 days following surgery. Scale bars=50 μ m. **B.** Quantification of the extent of microglial depletion in A (n = 6/group; *p<0.01 vs. control by two-tailed Student's t-test). **C.** Schematic, depicting the protocol for preoperative trace-fear conditioning (TFC) training, TFC testing, experimental surgery, and postoperative analyses of both hippocampal and systemic inflammation, and the memory of preoperative associative learning. **D and E.** Freezing in response to contextual cues, expressed as a percentage of total time in the testing environment prior to surgery (D), and relative to that of appropriate sham-treated controls 3 days following surgery (E). Data in D were analyzed by 2-way ANOVA. Data in E were analyzed by 2-tailed Student's t-test (***p=0.0002 for surgery vs. sham-treated control). For both D and E, n = 10/group.

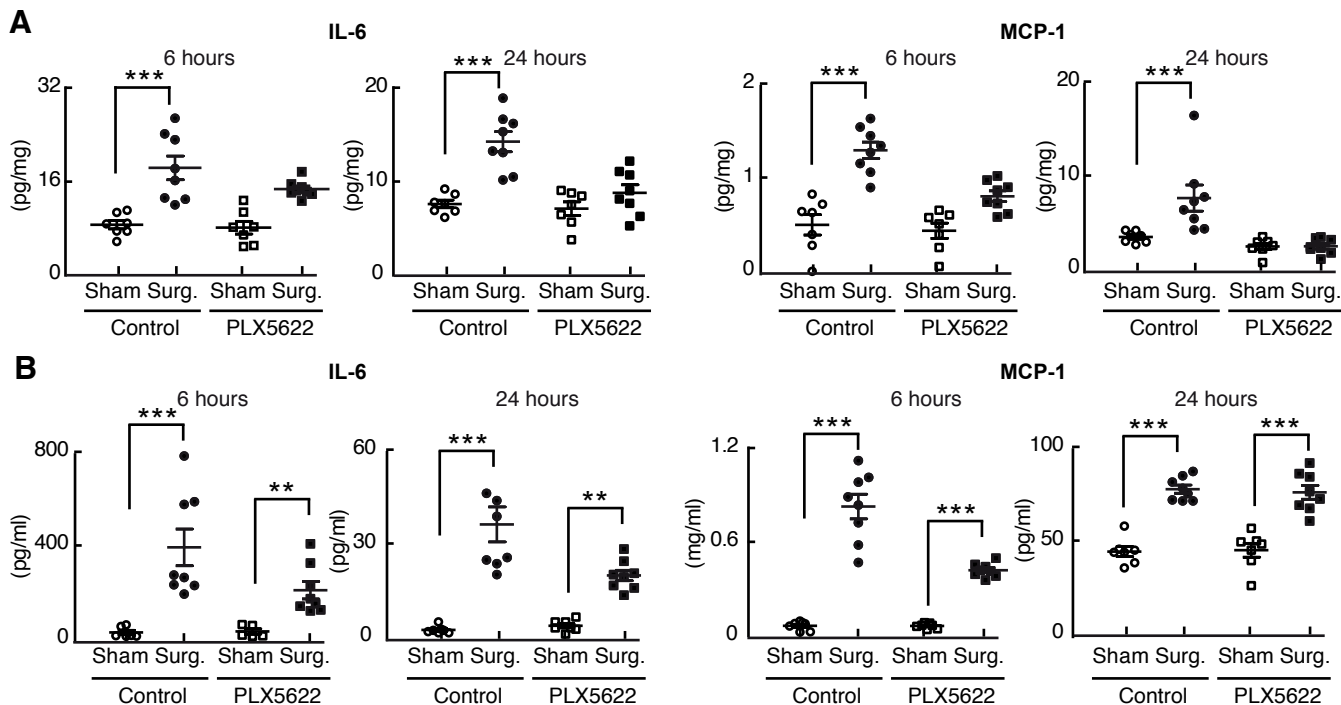


Figure 2. Perioperative microglial depletion abrogates surgically induced hippocampal inflammation.

A. Tissue ELISA, showing the rise in hippocampal levels of both IL-6 and MCP-1 in response to surgery, and the prevention of this postoperative rise by perioperative PLX5622 treatment as analyzed by 2-way ANOVA ($p=0.006$ at 24 hours for IL-6, and $p<0.01$ at both 6 and 24 hours for MCP-1). **B.** Plasma ELISA, showing the comparative lack of effect of PLX5622 treatment on postoperative plasma IL-6 and MCP-1 levels. Data were analyzed by 2-way ANOVA. In all cases, $n=7-8/\text{group}$, and $***p<0.001$; $**p<0.001$; and $*p<0.02$ for surgery vs. corresponding sham-treated control.

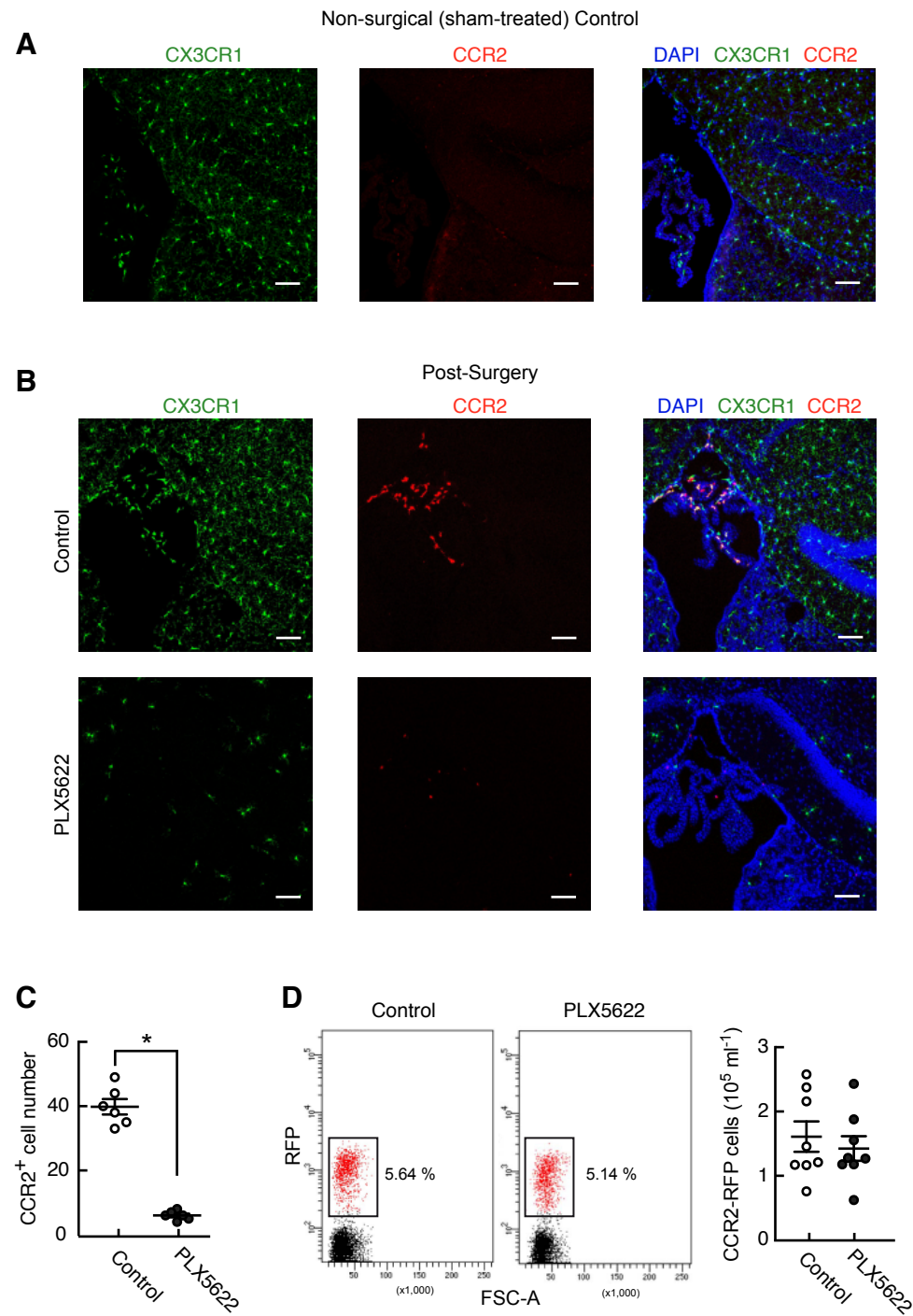


Figure 3. Perioperative PLX5622 treatment prevents surgery induced entry of CCR1-expressing cells into the hippocampal region of the brain in mice. **A.** Representative immunofluorescence images of hippocampal sections from $CCR2^{RFP/+} : Cx3CR1^{GFP/+}$ mice ($n=4$, 3 sections per sample), showing that sham-treated mice do not have CCR2-expressing (RFP^+) cells in the hippocampus or associated choroid plexus. **B.** Similar images in mice 24 hours after surgery, showing that depletion of microglia (green) by perioperative PLX5622 treatment is associated with the complete disappearance of CCR2-expressing cells otherwise present in the choroid plexus and entering the hippocampus at this time point. Scale bars= $50\mu m$. **C.** Quantification of the prevention of surgically induced $CCR2^{RFP/+}$ cell infiltration by PLX5622 treatment in mice from B ($n = 8$ mice/group; $*p<0.05$ vs. control by 2-tailed Student's t-test). **D.** FACS data from blood samples taken from control and PLX5622-treated mice 3 days following surgery, showing no effect of perioperative PLX5622 treatment on the number of circulating $CCR2^{RFP/+}$ cells ($n=8$ mice/group; analyzed by 2-tailed Student's t-test).

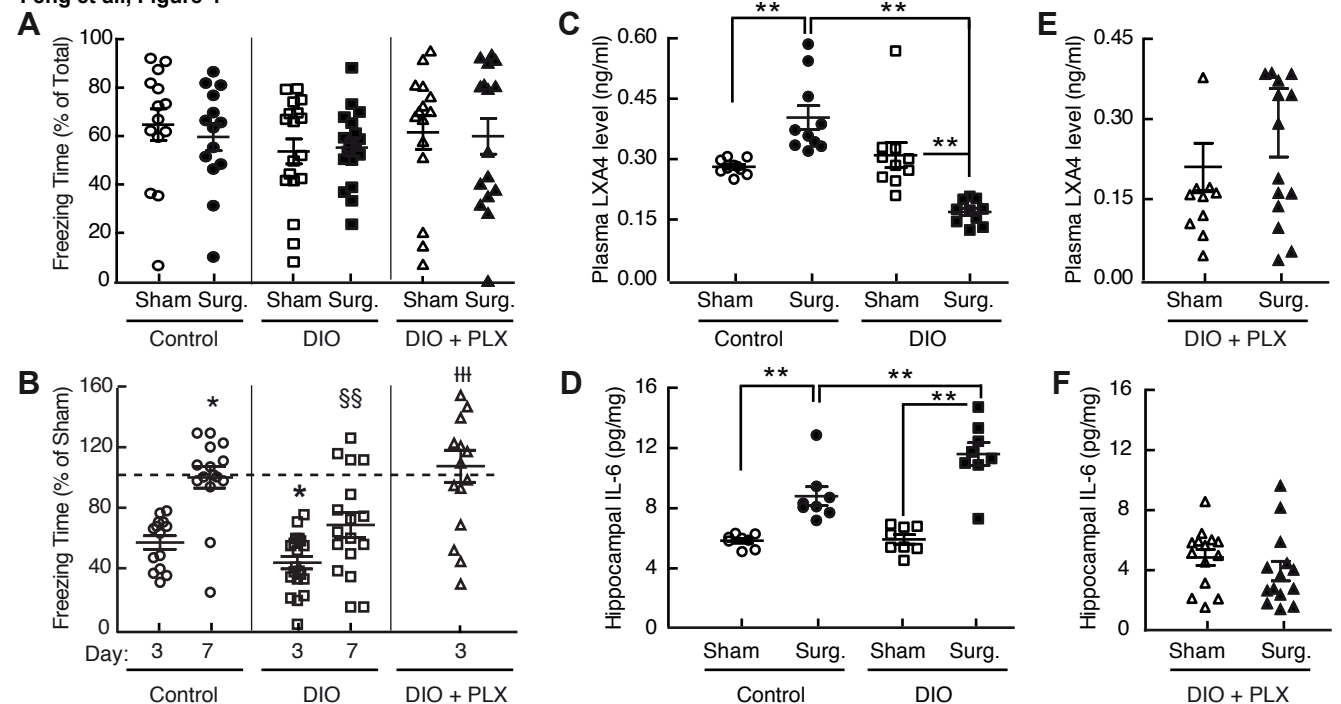


Figure 4. Perioperative microglial depletion prevents the inflammatory and cognitive consequences of surgery in mice with DIO.

A. The freezing response to preoperative TFC testing (% of total time) of healthy (chow-fed) controls, mice with DIO, and mice with DIO treated with PLX5622 (PLX), showing no difference between groups. **B.** Freezing times measured from the mice in A (% vs. corresponding sham-treated mice) during TFC testing 3 and 7 days after surgery. Whereas the impact of surgery on TFC memory is transient and returns to sham levels by day 7 in control mice (*p<0.001 vs. control at day 3), mice with DIO have a more pronounced (*p<0.001 vs. control at day 3) and persistent (\$\$p<0.001 vs. control mice at day 7) memory loss. Perioperative PLX5622 treatment protects mice with DIO from surgery induced memory loss (##p<0.005 vs. DIO alone at day 7). **C and D.** Analysis of LXA4 and IL-6 levels, showing that DIO paradoxically lowers plasma LXA4 levels that otherwise rise by 3 days after surgery in healthy control mice (C), and potentiates the postoperative (day 3) rise in hippocampal IL-6 levels seen in control mice (D). **E and F.** Corresponding measurements from plasma (LXA4) and hippocampus (IL-6), showing that perioperative PLX5622 treatment abolishes the impact of surgery on circulating LXA4 levels in mice with DIO (E), and prevents hippocampal IL-6 levels from rising in mice with DIO (F). All data are from n=12-19/group, and were analyzed by 2-way ANOVA (A, C, and D), 1-way ANOVA (B), and Student's t-test (E-F); **p<0.001 for comparisons shown.