Understanding postoperative cognitive dysfunction: *Novel insights*

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List of abbreviations

AD Alzheimer’s disease
BBB blood brain barrier
BZD benzodiazepines
BM-DM bone marrow-derived macrophage
CCR2 C-C chemokine receptor type 2
CSF cerebral spinal fluid
DAMP damage-associated molecular pattern
ELISA enzyme-linked immunosorbent assay
HMGB1 high-molecular group box 1 protein
ICU Intensive Care Unit
IKKβ Ikappa B kinase
IL interleukin
LPS lipopolysaccharide
MCP-1 monocyte chemotactic protein-1
MetaS metabolic syndrome
nAchR nicotinic acetylcholine receptor
qPCR quantitative polymerase chain reaction
NF-κB nuclear factor kappa B
NREM non-rapid eye movement
PAMP pathogen-associated molecular pattern
POCD postoperative cognitive dysfunction
POD postoperative delirium
PRR pattern recognition receptors
REM rapid eye movement
SF sleep fragmentation
TLR toll-like receptor
TNF tumor necrosis factor
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BACKGROUND

Impairment of cognition after surgery is a disturbing reality. Postoperative delirium (POD), listed in the *Diagnostic and Statistical Manual of Mental Disorders*, DSM-5, is characterized by inattention, disorganized thinking and altered level of consciousness with acute onset and fluctuating course. While some patients develop POD, others develop a later onset form of postoperative cognitive decline known as postoperative cognitive dysfunction (POCD). POCD is diagnosed by the International Society of Postoperative Cognitive Dysfunction as subtle deficits in one or more discrete domains of cognition, which include attention, concentration, executive function, verbal memory, visuospatial abstraction and psychomotor speed. The diagnosis requires sensitive presurgical and postsurgical neurocognitive testing. This condition typically develops over weeks to months and is long-lasting. As a consequence, patients can lose their livelihood or independence, which can seriously reduce their quality of life.

It is estimated that POCD occurs in more than 10% of non-cardiac surgical patients over 60 years old and is independently associated with poor short-term and long-term outcomes including an increased risk of mortality. Some reports describe cognitive decline persisting for up to one year after surgery. Both because the number of major surgical interventions (requiring anaesthesia) exceeds 230 million world-wide and because of the increasing prevalence of surgical interventions in patients with comorbidities, if current rates hold steady, we can expect that many millions of patients will run the risk of developing POCD. This possibility raises the stakes considerably: not only on an individual level, but also on a societal scale.

Although several risk factors have been identified, the exact pathophysiology that underlies POCD remains undefined. Data from preclinical studies support the concept that inflammation is a possible pathogenic mechanism for post-operative cognitive dysfunction. Increased expression of interleukins in mice hippocampus following surgery was associated with cognitive decline, corroborating the view that surgery-
induced neuroinflammation can result in cognitive impairment. Surgical patients exhibit elevations of proinflammatory cytokines in both the central nervous system and the systemic circulation, the extent of which may relate to the degree of cognitive decline\textsuperscript{18-20}. Assuming that neuroinflammatory changes noted postoperatively in rodents also occur in humans, reasons must be sought why POCD is a relatively infrequent clinical event (± 10\%)\textsuperscript{5} whereas neuroinflammation always occurs\textsuperscript{12,19}. Among the possibilities include the fact that the neuroinflammatory changes are usually evanescent and do not normally cause a long-lasting consequence in the animal models\textsuperscript{21}. Several clinical conditions can transform the self-limiting post-surgical neuroinflammatory response into one that is persistent\textsuperscript{22-24}. Causes for the persistence in neuroinflammation may be due to dysfunction in the inflammation \textit{initiation} or \textit{resolving} mechanisms.
Surgery

Tissue trauma, such as in surgical insult, releases damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PRR), which then trigger an immune response in a manner remarkably similar to that of microbial-derived pathogen-associated molecular patterns (PAMPs)\textsuperscript{25-27}. Among PRRs, Toll-like receptors (TLRs) are of critical importance, recognizing various ligands (including PAMPs and DAMPs) and activating TLR signals along different pathways, thereby increasing the synthesis and release of pro-inflammatory mediators. Although the function of TLR4 during lipopolysaccharide (LPS) endotoxemia\textsuperscript{28} has been deeply explored, the pathways of infection-mediated neuroinflammation and cognitive decline seem to be distinct from that of aseptic surgical trauma\textsuperscript{14}. One of the most important DAMPs (released from dead or dying cells through non-apoptotic processes\textsuperscript{29}) is high-mobility group box 1 protein (HMGB1). HMGB1 can bind and signal through a family of PRRs that are evolutionarily conserved\textsuperscript{30}. Clinical conditions such as sepsis, arthritis, and stroke, all release massive amounts of HMGB1\textsuperscript{31}. Both DAMPs and PAMPs converge on NF-κB to increase synthesis and release of pro-inflammatory cytokines\textsuperscript{32}, including TNF-α, which disrupt blood brain barrier (BBB) integrity\textsuperscript{12,14,15,33}. Early activation of the innate immunity through DAMPs (HMGB1 and cytokines) will introduce the initial response to surgery resulting in neuroinflammation and concomitant cognitive decline (figure 1)\textsuperscript{14}.

\textbf{Figure 1.} Neuroinflammatory response to surgery\textsuperscript{1}
Following injury, a transient inflammation is necessary for tissue repair processes that promote healing. Neuroinflammation after surgery is likely to include a pro-inflammatory phase and an anti-inflammatory phase (neural and humoral pathways mediate the switch between these two phases)\textsuperscript{34,35}. Regarding the resolution phase of the inflammatory state, this is mediated by neural factors, termed the cholinergic anti-inflammatory pathway. Release of acetylcholine mediates inhibition of macrophage NF-κB activity by signalling through the α7 subtype of nicotinic acetylcholine receptors (α7 nAChR). Ultimately it inhibits synthesis and release of pro-inflammatory cytokines from circulating immunocompetent cells\textsuperscript{32,36,37}. The neural cholinergic reflex is very important in resolving the inflammatory pathogenesis of several diseases including sepsis\textsuperscript{38}, rheumatoid arthritis\textsuperscript{39}, and colitis\textsuperscript{40}. Furthermore, the cholinergic anti-inflammatory pathway also modulates the function of T regulatory cells\textsuperscript{41}, which influences the production of anti-inflammatory cytokines (IL-10 and IL-4)\textsuperscript{42} and alternative macrophage activation that promotes the resolution of inflammation\textsuperscript{43}. Abnormalities of the switching mechanism may cause a non-resolving chronic inflammatory state that could create the circumstances for persistent cognitive decline.

Studies have shown the importance of this reflex for resolving DAMP-induced neuroinflammation, pro-inflammatory cytokine release, neuroinflammation, and cognitive decline; stimulating the α7 nAChR in macrophages, inhibited NF-κB activity while in vivo, α7 nAChR agonists prevented postoperative monocyte migration into the hippocampus as well as memory impairment\textsuperscript{44}. Advanced age is associated with decline in cholinergic function\textsuperscript{45}, which may be relevant in explaining the high prevalence of postoperative cognitive dysfunction in elderly patients\textsuperscript{44}.

When inflammation does not subside, it can contribute to the pathogenesis of diseases\textsuperscript{46}. Through a permeable BBB, CCR2-expressing bone marrow-derived macrophages (BM-DM) are attracted, by the newly-expressed chemokine, MCP-1, into the brain parenchyma. The macrophages synthesize and release a variety of pro-inflammatory cytokines that interfere with processes required for memory. Macrophage-specific Ikappa B kinase (IKK)β coordinates activation of NF-κB; when it is deleted it prevents BBB disruption
and BM-DM infiltration into the hippocampus following surgery. Transgenic mice that overexpress Hsp72 and inhibit NF-κB activity have attenuation of postoperative neuroinflammation and cognitive decline.

Learning and memory processes rely on the hippocampus, a region of the brain that contains a large number of proinflammatory cytokine receptors. The hippocampus has the highest density of IL-1 receptors, and although IL-1β is required for normal learning and memory processes, higher levels can also produce diminished cognitive function. Recent studies have suggested a role for cytokines such as IL-1 as well as IL-6 in the genesis of POCD. The relative prevalence of the TNF-α receptor, and other PRR, on the endothelium of this brain region may account for its vulnerability to systemic proinflammatory cytokines. Surgical trauma in animal models is associated with the persistent activation of macrophages in the CNS, as well as, high hippocampal levels of IL-1β, TNF-α and IL-6. These changes are correlated with cognitive dysfunction seen in animal models (contextual fear memory, spatial learning or reversal learning). Sub-clinical inflammation following administration of LPS substantially increases IL1-β levels and cognitive deterioration after surgery. In addition, several studies suggest that the marked and sustained expression of inflammation-related enzymes, such as cyclooxygenase-2, plays an important role in secondary events that amplify cerebral injury after ischemia. Patients also exhibit a robust neuroinflammatory response to peripheral surgery with an initial rise in pro-inflammatory cytokines in the CSF.
**Sleep**

Sleep is crucial for the repair of many types of injury and disease, especially with regard to the central nervous and immune systems; it also has anabolic, restorative properties that improve both neurocognitive and immune function. During non rapid eye movement (NREM) sleep slow wave activity performs a homeostatic function to reduce the strength of synapses that has been acquired during wakeful activity\(^5\). This synaptic homeostasis improves subsequent cognitive function by allowing new changes in synaptic strength. For example, both NREM and REM sleep are necessary for the consolidation of learning and memory while sleep deprivation results in cognitive dysfunction\(^5\).

Sleep disturbance is commonly observed in the hospital setting and include changes to sleep patterns and quality (especially sleep fragmentation), as well as sleep architecture. Polysomnographic studies revealed extreme sleep disruption in Intensive Care Unite (ICU) patients with decreases in total sleep-time, altered sleep architecture (predominance of stage 1 and 2 sleep, decreased or absent stage 3 NREM and REM sleep), and sleep fragmentation\(^5\)\(^,\)\(^5\); also, up to 50% of the total sleep-time occurred during daytime. Studies have shown that fragmented sleep is prevalent due to frequent arousals and awakenings, and that sleep architecture is altered with an increase in light sleep, and a decrease in restorative slow wave sleep\(^6\).

Environmental factors and health care practices further contribute to sleep disruption in critically ill patients; these include disturbances like inappropriately high noise levels, continuous ambient light, and the near constant performance of medical tests procedure and procedures. Lack of sleep hygiene results in cognitive dysfunction\(^6\)\(^,\)\(^6\), contributes to delirium\(^6\), adversely affects immunity\(^6\)\(^,\)\(^6\), and independently increases both morbidity and mortality\(^6\). Sleep disruption during hospital care has the potential to adversely impact patients’ outcome and also provides a direct financial cost with respect to the length of hospital stay and depletion of healthcare resources.
Additionally, many sedative and analgesic agents potently suppress slow wave sleep. Sedative practices have also shown to be a main causative factor for this disruption. Anaesthetics have different action targets and ultimately different consequences. The pivotal work of the MENDs trial indicated the benefits of a specific sedative agent, dexmedetomidine, in the outcome of ICU population. α2 adrenergic agonists converge on sleep pathways within the brainstem while those that act by modulating the GABA<sub>A</sub> receptor converge at the level of the hypothalamus. Several studies have now demonstrated the association between the use of benzodiazepine (BZD) and increased incidence and duration of delirium in ICU patients. A recent prospective study has also shown that patients with sleep disorders have an increased likelihood of exhibiting postoperative delirium.

Despite the common occurrence of both ICU delirium and sleep disruption in critically ill patients, a causal relationship has, as of yet, not been well described. Still, the question remains if we are doing all in our power to avoid the development of POCD.
Cerebral Ischemia

Stroke is the leading cause of disability in adults and an important risk factor for bone fracture. In the United States, ≈ 70,000 stroke victims suffer from bone fracture within the first year after their stroke. A small proportion of these stroke patients experience bone fracture within the first 24h after the ischemic stroke, but the impact of bone fracture on acute stroke lesion is unknown. Symptomatic pre-operative neurologic diseases, including dementia and any disease of the central nervous system, are often considered as exclusion criteria for POCD studies. Interestingly, cerebral vascular accidents (without residual deficit) were associated with risk factors for POCD, suggesting a pre-operative ischemic brain insult could influence the possibility of POCD.
**Metabolic Syndrome**

Roughly 25% of the 45 million surgical patients in the US have MetaS\textsuperscript{76}, a constellation of conditions whose precise definition and diagnostic criteria continue to evolve\textsuperscript{77}. MetaS, comprising of insulin resistance, visceral obesity, hypertension, and dyslipidemia increases the risk of postoperative complications contributing to a significant higher mortality rate\textsuperscript{78-81}. While each of the subphenotypes that define the MetaS have a strong genetic component, lifestyle factors that contribute to this cluster of conditions include sedentary behaviour and a diet with a high caloric content from saturated fats and/or simple carbohydrates\textsuperscript{82}. Many complications of MetaS (including atherosclerosis) are inflammatory in nature and the pathologic metabolism in adipose stores may be the source of pro-inflammatory adipokines\textsuperscript{83}. Conversely, with little adiponectin to attenuate activation of the transcription factor NF-κB in macrophages, expression of genes for pro-inflammatory cytokines are increased\textsuperscript{84}; up-regulated NF-κB activity in morbid obesity can be rectified with adiponectin\textsuperscript{85}. Recent evidence indicates patients suffering from MetaS may be particularly susceptible to POCD\textsuperscript{23,80}.
AIMS OF THE THESIS

Studies have sought to identify factors that may contribute to POCD, which include surgery, in-patient care factors, and patient-related factors. This work considers the possible role of inflammation in the development of postoperative cognitive dysfunction in the setting of underlying systemic diseases/conditions.

The specific aims are:

1) To identify the role of hippocampal recruitment of BM-DM in the pathogenesis of POCD.

2) To determine the mechanism by which HMGB1 regulates the activation and trafficking of circulating BM-DM to the brain.

3) To investigate the contribution of perioperative SF to the neuroinflammatory and cognitive responses of surgery.

4) To determine whether bone fracture, shortly after ischemic stroke, enhances stroke-related injuries by augmenting the neuroinflammatory response.

5) To investigate whether surgery induces a more severe and persistent form of cognitive decline in a rat model of MetaS.
METHODS AND RESULTS
Surgery
Depletion of Bone Marrow-derived Macrophages Perturbs the Innate Immune Response to Surgery and Reduces Postoperative Memory Dysfunction

Participated in study design, conducted qPCR and ELISA experiments and participated in data analysis and interpretation and final preparation of the manuscript.
Depletion of Bone Marrow-derived Macrophages Perturbs the Innate Immune Response to Surgery and Reduces Postoperative Memory Dysfunction

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ABSTRACT

Background: According to rodent models of postoperative cognitive decline, activation of the innate immune response following aseptic surgical trauma results in the elaboration of hippocampal proinflammatory cytokines, which are capable of disrupting long-term potentiation, the neurobiologic correlate of memory. The authors hypothesize that hippocampal recruitment of bone marrow–derived macrophages plays a causal role in these processes, resulting in memory dysfunction.

Methods: Clodrolip injection (liposomal formulation of clodronate) before stabilized tibial fracture under general anesthesia was used to deplete bone marrow–derived macrophages. Systemic inflammation and neuroinflammation were studied on postoperative day 1, and memory in a fear trace conditioning paradigm was assessed on postoperative day 3. CX3CR1GFP/+ CCR2RFP/+ mice were used to identify bone marrow–derived macrophages.

Results: Clodrolip effectively depleted splenic CCR2+ bone marrow–derived macrophages. It also attenuated the surgery-induced increase of interleukin-6 in the serum and the hippocampus, and prevented hippocampal infiltration of CCR2+ cells without affecting the number of CX3CR1+ microglia. It did not alter the surgery-induced increase in hippocampal monocyte chemoattractant protein-1, the recruitment signal for CCR2+ cells. Clodrolip prevented surgery-induced memory dysfunction, as evidenced by a significant increase in freezing time (29% [95% CI, 21–38%] vs. 48% [95% CI, 38–58%]), n = 20, P = 0.004), but did not affect memory in nonsurgical mice.

Conclusion: Depletion of bone marrow–derived macrophages prevents hippocampal neuroinflammation and reduces postoperative memory deficits.

What We Already Know about This Topic

- Animal models of postoperative cognitive dysfunction implicate an innate immune response, with increased circulating inflammatory mediators and migration of bone marrow–derived cells into the brain.
- Inhibitors of inflammatory mediators reduce postoperative memory deficits in mice but also reduce wound healing.

What This Article Tells Us That Is New

- In mice, treatment with a drug that depletes bone marrow–derived macrophages reduced circulating inflammatory mediators after surgical orthopedic injury, reduced migration of immune cells into the brain, and reduced postoperative memory deficits.

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memory dysfunction after experimental tibial fracture. These data suggest that the hippocampal recruitment of bone marrow–derived macrophages is a necessary mechanism in murine postoperative cognitive dysfunction. Interventions designed to prevent its activation and/or migration into the brain may represent a feasible preemptive strategy.

Acute postsurgical memory deterioration leads to persistent cognitive decline that can result in considerable morbidity and increased mortality. The specter of memory dysfunction, including acute delirium, postoperative decline, and dementia, is a source of anxiety for patients and their families. Knowledge about the molecular and cellular pathways involved in postoperative memory dysfunction may provide a launching pad for the development of biomarkers to identify the most vulnerable patients as well as preventive strategies.

Using a murine model of aseptic surgical trauma with a long-bone fracture, we previously demonstrated that postoperative cognitive decline requires the engagement of the innate immune response. This engagement includes increased systemic expression of alarmins and proinflammatory cytokines such as interleukin (IL)-6 in the blood; increased ratio of CD11b+ cells corresponding to macrophages/microglia cells, and specifically the ratio of CCR2+ bone marrow-derived macrophages; and elaboration of proinflammatory cytokines that are capable of disrupting hippocampal long-term potentiation, a neurobiologic correlate of learning and memory.

Strategies designed to block the effect of proinflammatory cytokines with IL-1 receptor antagonist (anakinra) or tumor necrosis factor (TNF)-α antibody (etanercept) prevented murine postoperative memory dysfunction. These interventions also prevented inflammation-dependent wound healing.

In this study, we tested the hypothesis that mediation of postoperative memory decline requires recruitment of systemic bone marrow–derived macrophages into the brain, using a specific pharmacologic strategy to acutely deplete systemic phagocytes before an aseptic surgical trauma with an experimental tibial fracture.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the University of California, San Francisco Institutional Animal Care and Use Committee, and conformed to National Institutes of Health guidelines. Twelve 8- to 12-week-old CCR2RFP+/CX3CR1GFP/+ male mice (fig. 1A) were used to identify bone marrow–derived macrophages. CCR2 and CX3CR1 are acronyms for chemokine (C-C motif) receptor 2 (whose cognate ligand is monocyte chemoattractant protein [MCP]-1) that is highly expressed in bone marrow–derived macrophages; and CX3C chemokine receptor 1 (CX3CR1, fractalkine receptor) that is highly expressed in resident microglia. Eighty-nine

Fig. 1. Study design and splenic macrophage depletion with clodrolip. (A) First experiment with 12 CCR2RFP+/CX3CR1GFP/+ mice divided into two groups of six mice each treated with intraperitoneal (IP) injection of clodrolip versus CT-lip 1 h before the tibia fracture model and 25 h before tissue collection. (B) Second experiment with 24 C57BL/6J mice divided into four groups of six mice treated with IP injection of clodrolip versus CT-lip 1 h before the tibia fracture and sacrificed 12 or 24 h after the tibia fracture. (C) Third experiment with 70 C57BL/6J mice divided into four groups treated with IP injection of CT-lip versus clodrolip 1 h before the tibia fracture (20 mice per group) versus sham procedure (15 mice per group). The training session of the memory test was performed 30 min after the IP injection and 30 min before surgery, and the context session was performed 72 h after surgery. CT-lip = control liposome.
wild-type male mice (C57BL/6J, 10–12 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME): 29 for the cytokine expression (fig. 1B) and 70 for the behavior tests (fig. 1C). Mice did not experience unexpected lethality in the study and were euthanized according to our institutional animal care and use committee guidelines.

**In Vivo Systemic Phagocyte Depletion with Clodrolip**

Clodrolip is a liposomal formulation of clodronate (dichloromethylene bisphosphonic acid), a nontoxic bisphosphonate. Liposomes are lipid vesicles consisting of concentric phospholipid bilayers surrounding aqueous compartments. In this case, liposomes are used as “Trojan horses” encapsulating clodronate, which are then ingested and digested by phagocytes, followed by an intracellular release and accumulation of clodronate. At a certain intracellular concentration, clodronate induces apoptosis of the phagocytes. Clodronate liposomes were obtained from clodronateliposomes.org* *(Vrije Universiteit, Amsterdam, The Netherlands) at a concentration of 7 mg/ml and prepared as described previously.14 Clodrolip (200 μl, approximately 100 mg/kg) was injected intraperitoneally 60 min before the bone fracture. Control animals received 200 μl of control liposomal solution (CT-lip). No intraperitoneal or extraperitoneal damage was observed after clodrolip intraperitoneal administration.

**Long-bone Fracture with Tibia Fracture Surgery**

Anesthesia was induced and maintained with isoflurane by inhalation. We used a dedicated chamber for induction with 5% isoflurane for 3 min, and the operation was performed under 2% isoflurane for 10–12 min. Under aseptic surgical conditions, an open tibial fracture of the right hind limb with intramedullary fixation was performed as described previously.4 Body temperature was maintained at 37° ± 0.5°C using a thermal blanket throughout the surgical procedure, and analgesia was provided by injection of buprenorphine (0.3 mg in 100 μl of saline). Sham mice for bone fracture (sham group) received the same anesthesia and analgesia as the bone fracture mice.

**Measurement of IL-6 in Serum**

Mouse blood was collected using cardiac puncture under general anesthesia (isoflurane, 3%) in separate cohorts 12 and 24 h after the bone fracture procedure (fig. 1B). Blood samples were centrifuged at 1300 rpm for 10 min at room temperature, and the serum was collected and frozen at −80°C. IL-6 is secreted by bone marrow–derived macrophages in response to alarmins,16 and the IL-6 level in the serum is increased within the first 24 h after the tibia fracture.4,5 The IL-6 level in the serum is also associated with the postoperative memory dysfunction phenotype1 and is affected by clodrolip in response to lipopolysaccharide infusion.17 For these reasons, we decided to quantify IL-6 levels in the serum of mice exposed to clodrolip or CT-lip using the IL-6 enzyme-linked immunosorbent assay kit (KMC0062; Invitrogen, Grand Island, NY). Results are expressed as fold increase compared with that measured in five control mice that did not receive any treatment or surgery.

**Measurement of Cytokines in the Hippocampus**

The hippocampi of the mice were collected rapidly under a dissecting microscope, 12 and 24 h after the tibia fracture (fig. 1B), and placed in RNAlater solution (Qiagen, Valencia, CA). To avoid blood contamination, mice were perfused with saline for 5 minutes before sample collection. Total RNA was extracted using the RNasy Lipid tissue Kit (Qiagen) and reverse-transcribed to complementary DNA with a High Capacity RNA to Complementary DNA Kit (Applied Biosystems, Bedford, MA). TaqMan Fast Advanced Master Mix (Applied Biosystems) and gene-specific primers and probes used for quantitative polymerase chain reaction are as follows: β-actin (NM_007393.1), IL-6 (Mm00446190_m1), TNF-α (Mm00443258_m1), IL-1β (Mm01336189_m1), and MCP-1 (Mm00441242_m1). Quantitative polymerase chain reaction was performed using StepOnePlus (Applied Biosystems). Each RNA sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle (ACT) method and normalized to β-actin. Results are expressed as fold increase compared with that observed in five control mice that did not receive any treatment or surgery.

**Quantification of Bone Marrow–derived Macrophages and Microglia**

Twenty-four hours after the tibia fracture surgery, the brain and spleen of the CCR2200GFP/CX3CR1200GFP mice were collected after intracardiac perfusion with paraformaldehyde 4% (fig. 1A). Spleen and brain (bregma, −1.0 to −1.4 mm, corresponding to interaural 2.7 to 2.3 mm in coronal orientation) were sectioned into 20-μm-thick slices and mounted with Vectashield DAPI (Vector Laboratories, Burlingame, CA). The expression of CCR2-RFP and CX3CR1-GFP cells was assessed using confocal images, performed with a Spectral Confocal microscope (Nikon Instruments, Melville, NY) using three laser lines (405, 488, and 561 nm). Z-stacks were rendered into a three-dimensional image using the NIS-Elements AR 3.0 software (Nikon), and the expression of CCR2-RFP and CX3CR1-GFP cells was quantified using ImageJ (National Institutes of Health, Bethesda, MD), with three different photographs per mouse taken with a 20× objective. Data are expressed as relative cell percentages normalized to the average value of the CT-lip group.

**Behavioral Test for Hippocampus-dependent Memory with Trace Fear Conditioning**

Fear conditioning is used to assess memory in rodents, which are trained to associate a conditional stimulus, such as a conditioning chamber, with an aversive, unconditional stimulus,
such as a foot shock. Freezing behavior is an indicator of aversive memory that is measured when subjects are reexposed to the conditional stimulus. With this model, lesions of the hippocampus disrupt recall of fear responses to the presentation of the context, resulting in a diminution in freezing.18,19

For this study, we used a previously published paradigm.4–6 Briefly, the behavioral study was conducted using a conditioning chamber (Med Associates, Inc., St. Albans, VT) and an unconditional stimulus (two periods of foot shock of 0.75 mA during 2 s). An infrared video camera, mounted in front of the chamber, captured motion speed (Video Freeze; Med Associates).

All of the animals underwent the same training session, regardless of the specific intervention, and received their training 30–40 min after the liposomal intraperitoneal injections (whether clodrolipid or CT-lip) that occurred 30 min before surgery (fig. 1C). Three days after conditioning, mice were returned to the same chamber where training had occurred for a context test. During the context test, mice were exposed just to the context and no tones or foot shocks were delivered. Freezing was recognized by the software as a total lack of movement, excluding breathing and movement of vibrissae (linear detection with a minimal freeze duration of 20 frames corresponding to 0.7 s and a motion threshold of 20 arbitrary units).5–6 Decrease in the percentage of time spent freezing indicated impairment of memory.

Body Weight and Maximal Motion Speed

The body weight of the animals was measured 3 days after surgery, following assessment of freezing behavior. An infrared video camera (Video Freeze) captured and quantified motion speed during the context test, and the maximal motion speed was recorded for each mouse.

Statistical Analysis

Data are presented as mean ± 95% CI. Normality was tested with the d’Agostino–Pearson omnibus normality test. Equality of variances was tested with the F test. For two-sample comparisons, Student t tests were used (using the Welch correction if necessary); Mann–Whitney U tests were used if data were not normally distributed. For comparisons of more than two groups, means were compared using one-way ANOVA followed by Student t tests with a Bonferroni-corrected alpha level.

We used the two-way ANOVA procedure to determine whether or not time and treatment were significant factors in predicting IL-6 concentration in the serum, and IL-6, IL-1β, TNF-α, and MCP-1 messenger ribonucleic acid (mRNA) expression in the hippocampi. Given the highly skewed nature of the mRNA expression, we checked the distribution of the residuals. We applied a log transformation (ln[X]) to the response of the mRNA expression before performing analysis to better adhere to the ANOVA model’s assumptions of normally distributed residuals and homoscedasticity of residuals.

For the behavior tests, animals were tagged and allocated randomly to each group before any treatment, and researchers were blinded to the group assignment that was revealed only after the analysis phase. A repeated measures ANOVA was performed to determine whether treatment (CT-lip and clodrolip) and the three time periods (baseline, first shock, and second shock) were significant predictors of percentage freezing time during the training session.

For this study, our primary outcome was percentage of freezing time during the context session. Based on previous freezing time data,4 we estimated that a sample of 18 C57BL/6J surgical mice per group was necessary to demonstrate a 20% increase in percentage freezing time, with 80% power at the 0.017 alpha level (after adjusting for three comparisons) to find a significant difference between clodrolip and CT-lip.

A two-tailed value of $P < 0.05$ was considered statistically significant for two-group comparisons, and the significance threshold was adjusted for multiple comparisons with a Bonferroni correction. Prism 5 (GraphPad Software, Inc., La Jolla, CA) was used to conduct the statistical analyses.

Results

Clodrolip Depletes Splenic Bone Marrow–derived Macrophages and Prevents Hippocampal Bone Marrow–derived Macrophage Infiltration

Using CCR2<sup>−/−</sup> X3CR1<sup>+/−</sup> mice (fig. 1A), in which RFP<sup>+</sup> bone marrow–derived macrophages and GFP<sup>+</sup> resident microglia can be tracked,6,5 we found that clodrolip depleted splenic macrophages and surgery-induced bone marrow–derived macrophage infiltration into the hippocampus. The CCR2<sup>+</sup> cells, which are mainly present in the splenic red pulp (fig. 2A), decreased by 96% in the clodrolip-exposed mice (fig. 2B) (95% CI, 95–97%; $P < 0.001$). As shown in figure 3, the number of CCR2<sup>+</sup> cells was also significantly reduced in the hippocampi of clodrolip-treated mice compared with CT-lip–treated mice 24 h after surgery (decrease of 76% for the dentate gyrus and 87% in the cornu ammonis 3). However, clodrolip treatment did not change the number of CX3CR1<sup>+</sup> cells in the dentate gyrus and cornu ammonis 3 hippocampal regions (fig. 3).

Clodrolip Reduces Systemic and Hippocampal Proinflammatory Cytokines

We previously showed that proinflammatory cytokines in the blood and hippocampus increased within the first day after surgery.2 To test whether clodrolip treatment would reduce the proinflammatory cytokines, we studied serum and hippocampal expression 12 and 24 h after surgery (fig. 1B). Twelve hours after surgery, the rise in IL-6 in the serum was significantly attenuated in mice exposed to clodrolip (two-way ANOVA, $P = 0.004$ for the treatment, $P = 0.003$ for the time effect, and $P = 0.19$ for interaction) (fig. 4).

Between 12 and 24 h after surgery, the increase in mRNA hippocampal expression of IL-6, TNF-α, and IL-1 induced by
Fig. 2. Effects of systemic macrophage depletion with clodrolip on the CCR2⁺ splenic cells. (A) Representative photographs of spleen section showing CCR2⁺ cell repartition mainly in the red pulp (RP) and less in the white pulp (WP), 24h after tibia fracture. Top photographs are of low magnification (scale bar = 100 μm) and bottom photographs are highly magnified images (scale bar = 50 μm) in the CT-lip and the clodrolip mice. (B) Quantification of the relative percentage of CCR2⁺ cells in the spleen after clodrolip (n = 6, **P < 0.001 with unpaired Student t test). CCR2 = chemokine (C-C motif) receptor 2; CT-lip = control liposome; DAPI = 4′,6′-diamidino-2-phenylindole; IP = intraperitoneal (bars = mean ± 95% CI).

Fig. 3. Effects of systemic macrophage depletion with clodrolip on the hippocampal CCR2⁺ and CX3CR1⁺ cells. (A) Representative photographs of the section of interest corresponding to bregma, −1.2 mm (scale bar = 500 μm), showing the dentate gyrus (DGyrus) and the cornu ammonis subdivision 3 (CA.3). (B) Representative highly magnified photograph (scale bar = 20 μm) of a ramified CX3CR1⁺ green cell and an amoeboid CCR2⁺ red cell in the hippocampus. (C) Bar graph shows quantification of the relative percentage of CCR2⁺ cells in the dentate gyrus and the CA.3 regions after clodrolip treatment (n = 6, significant F test for both comparisons, *P = 0.02; **P = 0.002 with unpaired Student t tests with Welch’s correction). (D) Representative photographs of the dentate gyrus hippocampal sections in the CT-lip (D1) and the clodrolip (D2) mice (scale bar = 100 μm) showing the decrease of CCR2⁺ cells after clodrolip treatment. (E) Representative photographs of the dentate gyrus hippocampal section in the CT-lip (E1) and the clodrolip (E2) mice (scale bar = 100 μm) showing the absence of CX3CR1⁺ cell depletion after clodrolip treatment. (F) Bar graph shows quantification of the relative percentage of CCR2⁺ cells in the dentate gyrus and the CA.3 regions after clodrolip (n = 6, P = 0.51 for dentate gyrus and P = 0.51 for CA.3). CT-lip = control-liposome; CCR2 = chemokine (C-C motif) receptor 2; DAPI = 4′,6′-diamidino-2-phenylindole (bars = mean ± 95% CI).
surgery returned to almost baseline values at 24 h (fig. 5). Clodrolip exposure significantly inhibited the surgery-induced increased expression of mRNA IL-6 (two-way ANOVA, \( P < 0.001 \) for the treatment, \( P = 0.002 \) for the time effect, and \( P = 0.51 \) for interaction), and interacted with the time-dependent decrease for TNF-\( \alpha \) (two-way ANOVA, \( P = 0.03 \) for interaction). Clodrolip treatment did not change IL-1\( \beta \) mRNA expression (two-way ANOVA, \( P = 0.42 \) for the treatment, \( P < 0.001 \) for the time effect, and \( P = 0.66 \) for interaction) (fig. 5).

Systemic macrophages are recruited into tissues by the chemoattractant MCP-1 that binds to CCR2, which is expressed on the surface of bone marrow–derived macrophages.21,22 Following surgery, MCP-1 mRNA expression increases and is unaffected by prior exposure to clodrolip (two-way ANOVA, \( P = 0.64 \) for the treatment, \( P < 0.001 \) for the time effect, and \( P = 0.67 \) for interaction) (fig. 5D).

**Clodrolip Prevents Surgery-induced Memory Impairment**

During the preoperative training period, learning was similar in the clodrolip-exposed and the control (nonexposed) groups, with the percentage of freezing being highly associated with time (fig. 6). During the context session, surgery significantly decreased percentage of freezing time in comparison with the sham group (52% [95% CI, 41–63%] vs. 29% [95% CI, 21 to 37%], \( P = 0.0012 \)); preoperative exposure to clodrolip resulted in significantly greater freezing time than in the nonexposed surgical cohort (29% [95% CI, 21–38%] vs. 48% [95% CI: 38–58%], \( P = 0.004 \)), reaching a level similar to that observed in the sham-operated clodrolip-exposed mice (49% [95% CI, 36–63%], \( P = 0.86 \)) (fig. 7, A and B).

Clodrolip did not affect the body weight of the mice 3 days after the injection (fig. 7C). As for maximal motion speed, the clodrolip-treated groups were no different from the CT-lip groups, even though the maximal motion speed of the surgical groups was significantly slower than the sham groups (fig. 7D).

**Discussion**

In this study, we report for the first time that bone marrow–derived macrophages are required in the pathogenesis of the neuroinflammatory and memory dysfunction induced by surgery. Also, we report that a possible hippocampal signal through MCP-1 is involved in the recruitment of bone marrow–derived macrophages to this brain region. Data from rodent surgical models have provided insight into the neuroinflammatory basis for postoperative cognitive decline. This usually transient process appears to be part of a motivational system that reorganizes the organism’s priorities to facilitate recovery. To date, we have established a pivotal early role for the proinflammatory cytokine TNF-\( \alpha \), and our study demonstrated that hippocampal infiltration of bone marrow–derived macrophages also plays a role in the initiation of neuroinflammation.

**Hippocampal Infiltration of Bone Marrow–derived Macrophages after Surgery**

Monocyte infiltration into the brain is mainly described in acute brain injuries such as stroke23 and traumatic brain injuries, as well as chronic inflammatory brain injuries such as multiple sclerosis.15 Using long-bone fracture as a surrogate for a peripheral orthopedic surgical insult, we previously reported that CCR2+ cells were present in the hippocampus.6 Because microglia can also express CCR2 under certain conditions,25,26 we could not ascertain whether these CCR2-expressing cells arose from the resident macrophage population (microglia) or through an infiltration from outside of the central nervous system. Using clodrolip to specifically deplete the systemic pool of phagocytes, including bone marrow–derived macrophages, we were able to demonstrate that the CCR2+ cells in the hippocampus are a result of the recruitment of bone marrow–derived macrophages into the brain.

For passage into the brain, monocytes are required to overcome the blood–brain and/or blood–cerebral spinal fluid barrier27,28; these barriers can be disrupted by direct acute brain injury.29,30 Interestingly, after peripheral surgery, the blood–brain barrier is disrupted, although there is no discernible brain lesion.6 Now we show that after surgery, the hippocampus expresses MCP-1, which is capable of attracting CCR2+ expressing cells migrating through the disrupted blood–brain barrier. This increased expression of MCP-1 is unaffected by clodrolip treatment, indicating that bone marrow–derived macrophages are not a self-perpetuating source of this chemoattractant for its own recruitment. Future understanding of the source and the triggers for hippocampal MCP-1 following peripheral surgery may result...
in interventional strategies designed to prevent recruitment of bone marrow–derived macrophages into the brain.

**Hipocampal Bone Marrow–derived Macrophage Infiltration and Memory Dysfunction**

Our recent data suggest that transient hippocampal inflammation is the key element in postoperative memory dysfunction because (1) hippocampal areas are known to be involved in memory tasks; (2) hippocampal neuroinflammation profile correlates with the level of memory dysfunction; and (3) hippocampal neuroinflammation leads to long-term potentiation disruption.\(^{2,3}\) Now we report here that the absence of bone marrow–derived macrophage infiltration, produced by systemic depletion by clodrolip, decreases surgery-induced hippocampal inflammation and memory dysfunction. Therefore, postoperative bone marrow–derived macrophage recruitment into the hippocampus plays a key role in the initiation of postoperative memory dysfunction.

In the context of postoperative cognitive decline, determining which cells are involved in the initiation of the inflammation response is important because, when exaggerated, this could overwhelm resolving responses and produce persistent postoperative cognitive decline. Earlier, we described that surgical trauma induces systemic release of alarmins (i.e., high-mobility group protein 1) and proinflammatory cytokines (i.e., TNF-α and IL-6).\(^{4,5}\) Improving our knowledge of cellular and molecular initiation mechanisms will allow insight into an _ex vivo_ bioassay to prospectively determine whether patients are at risk.

**Limitations of the Study**

We used experimental tibial fracture to generate animal postoperative memory acute dysfunction. With this model, we traumatized the bone marrow directly, which could play
a key role. However, other models that did not damage bone marrow with a splenectomy also showed that surgery generated postoperative cognitive dysfunction.

Fibrin is deposited in the hippocampus after tibia fracture, suggesting that the blood–brain barrier becomes disrupted and may allow the passage of clodrolip to act directly on the microglia population. However, we found that the systemic administration of clodrolip acts only on the number of CCR2+ cells without significantly affecting the number of CX3CR1+ cells (fig. 3); if clodrolip has an effect on microglia, it may be to functionally modify them. For this reason, we cannot exclude the possibility that clodrolip does not affect the function of microglia, and that microglia do not play a key role in postoperative cognitive dysfunction.

For this study, we used a pharmacologic strategy to quickly deplete the pool of systemic macrophages. However, because clodrolip is highly toxic for monocytes and macrophages, it can increase the risk of postsurgical infections, generating a phenotype of its own. With a single dose, we did not observe loss of weight or other signs of sickness within the

Fig. 6. Clodrolip effect on the training session. (A) Representative record of a training session showing the motion (motion index expressed in arbitrary units) of the mouse according to the time. The two green bars represent the two shocks and the three red rectangles represent the 40-s periods used to quantify the baseline, first, and second shock freezing responses. Bar graph (B) quantifies the percentage of freezing time (n = 35), and two-way ANOVA shows a significant effect of time (P < 0.0001), no significant effect of treatment (P = 0.68), and no interaction (P = 0.61).

Fig. 7. Depletion of systemic macrophages reduces surgery-induced memory dysfunction. (A) Representative records of context sessions of tibia fracture mice treated with CT-lip (A1) and with clodrolip (A2) showing the motion (motion index expressed in arbitrary units) of the mice according to the time. (B) Quantification of the freezing time percentage according to the four groups (n = 15–20, *P = 0.0012 and $P = 0.004$, respectively, with one-way ANOVA and Bonferroni post hoc analysis). (C) Quantification of the body weight in the four groups 3 days after surgery (n = 15–20, $P = 0.02$ and $P = 0.03$, respectively, not significant after adjustment for multiple comparisons with one-way ANOVA and Bonferroni post hoc analysis and no significant effect of the clodrolip treatment). (D) Quantification of the maximal motion speed in the four groups 3 days after surgery (n = 15–20, **P = 0.012 and ###P = 0.0003, respectively, with one-way ANOVA and Bonferroni post hoc analysis and no significant effect of the clodrolip treatment; bars = mean ± 95% CI). AU = arbitrary units; CT-lip = control-liposome.
3 days. We performed a very short-term study, focusing on the acute exaggeration phase of neuroinflammation, and did not perform any long-term study with clodrolip. Clodrolip should be considered as a tool for mechanistic studies but cannot be proposed for clinical therapy.

To distinguish whether these recruited cells were resident or recruited systemic macrophages, we used CCR2<sup>Cre<sup>ERT2</sup></sup>-CX3CR1<sup>GFP<sup>+</sup></sup> mice. CCR2 is receptor for MCP-1 and is mainly expressed in bone marrow–derived monocytes–macrophages. We previously showed that CD11b<sup>+</sup> macrophages–microglia cells were recruited in the hippocampus after tibial fracture.4 In this study, however, we did not determine that CCR2<sup>+</sup> cells were only bone marrow–derived macrophages. Further study on the role of other systemic phagocytes, including neutrophils, in our phenotype will be of most interest.

Hippocampal cytokine expression was performed with mRNA and not with protein. This is a limitation, but we considered that the potential extravasation from blood may affect protein levels. Indeed, in this model we found blood–brain barrier leakage after the tibia fracture,5 and the increase of circulating IL-6 protein in the serum could contaminate the hippocampal samples with passive movement in the parenchyma (this phenomenon could be amplified by the perfusion itself). By analyzing mRNA expression in the brain collected after purging blood from the vessels, we ensured that hippocampal cells were the source of proinflammatory cytokines.

In conclusion, we showed in this study that bone marrow–derived macrophage activation after experimental tibial fracture is directly involved in the tibia fracture–induced hippocampal bone marrow–derived macrophage infiltration and animal memory dysfunction. Understanding the cellular and biologic pathways involved in postoperative cognitive decline is a key element in designing interventions to prevent this disease. Reducing activation and/or migration of innate immune cells, such as systemic macrophages, into the brain represents a viable preemptive strategy.

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HMGB1 Initiates Postoperative Cognitive Decline by Engaging Bone Marrow-derived Macrophages
ABSTRACT

Background: Aseptic trauma engages the innate immune response to trigger a neuroinflammatory reaction that results in postoperative cognitive decline. The authors sought to determine whether high-mobility group box 1 protein (HMGB1), an ubiquitous nucleosomal protein, initiates this process through activation and trafficking of circulating bone marrow–derived macrophages to the brain.

Methods: The effects of HMGB1 on memory (using trace fear conditioning) were tested in adult C57BL/6J male mice; separate cohorts were tested after bone marrow–derived macrophages were depleted by clodrolip. The effect of anti-HMGB1 neutralizing antibody on the inflammatory and behavioral responses to tibial surgery were investigated.

Results: A single injection of HMGB1 caused memory decline, as evidenced by a decrease in freezing time (52 ± 11% vs. 39 ± 5%; n = 16–17); memory decline was prevented when bone marrow–derived macrophages were depleted (39 ± 5% vs. 50 ± 9%; n = 17). Disabling HMGB1 with a blocking monoclonal antibody, before surgery, reduced postoperative memory decline (52 ± 11% vs. 29 ± 5%; n = 15–16); also, hippocampal expression of monocyte chemotactic protein-1 was prevented by the neutralizing antibody (n = 6). Neither the systemic nor the hippocampal inflammatory responses to surgery occurred in mice pretreated with anti-HMGB1 neutralizing antibody (n = 6).

Conclusion: Postoperative neuroinflammation and cognitive decline can be prevented by abrogating the effects of HMGB1. Following the earlier characterization of the resolution of surgery-induced memory decline, the mechanisms of its initiation are now described. Together, these data may be used to preoperatively test the risk to surgical patients for the development of exaggerated and prolonged postoperative memory decline that is reflected in delirium and postoperative cognitive dysfunction, respectively.

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interferes with cognition) or no change (short-lived initia-
tion and resolution of aseptic trauma-induced inflamma-
tion), we have explored, in rodent models, the process that
mediates persistent postoperative cognitive decline.1,2,5 After
tissue injury the innate immune response is engaged, result-
ing in penetration of bone marrow–derived macrophages
(BM-DM) into the brain through a disrupted blood–brain
barrier.2 Within the hippocampus these activated macro-
phages release proinflammatory cytokines that are capa-
bile of attenuating long-term potentiation that is the neurobi-
ologic correlate of learning and memory.6,7 These processes
are reversed within days through inflammation-resolving
mechanisms involving both neural and humoral path-
ways.2 Failure to resolve the neuroinflammatory response
results in exaggerated and persistent postoperative cognitive
decline.1,8,9 In an attempt to devise strategies that can detect
and mitigate this risk, the most vulnerable patients need to
be identified; in pursuit of this goal we sought to precisely
define the initiating processes in order to devise a preopera-
tive functional assay that is predictive of the patient’s likely
immune response to aseptic trauma.

Alarmins, a family of damage-associated molecular pat-
terns, are capable of activating the innate immune response
through their interaction with pattern recognition receptors
on circulating monocytes.10 In particular, high-mobility
group box 1 protein (HMGB1) is an alarmin that is pas-
sively released into the circulation from traumatized necrotic
cells; also, HMGB1 can be rapidly secreted by stimulated
leukocytes and epithelial cells.10,11 We previously demo-
strated that circulating HMGB1 increases after surgery in
humans and also in a murine aseptic trauma model12,13; fur-
thermore, we reported that this species of alarmin is required
for trauma-induced exacerbation of the morphological and
functional consequences of stroke.12

Now we describe data from experiments designed to test
the hypothesis that the early release of HMGB1 triggers the
neuroinflammatory and behavioral responses to trauma.
These data set the stage for the development of a functional
assay that assesses the initiation and resolution of inflam-
matory processes that are pivotal in postoperative cognitive
decline.

Materials and Methods

Animals
All experimental procedures involving animals were approved
by the Institutional Animal Care and Use Committee of the
University of California, San Francisco, San Francisco, Cali-
ifornia, and conformed to the National Institutes of Health
Guidelines. All animals were fed standard rodent food and
water ad libitum, and were housed (five mice per cage) in
sawdust-lined cages in an air-conditioned environment with
12-h light/dark cycles. Wild-type male mice (C57BL/6J,
12–14 weeks old) were purchased from Jackson Laboratory
(Bar Harbor, ME) for the behavior tests (fig. 1, A and B) and
for the cytokine expression (fig. 1C).

![Fig. 1. Study design. (A) First experiment: mice were divided in five groups treated with intraperitoneal injection of control lipo-
some versus clodrolip 1 h before high-mobility group box 1 protein (HMGB1) Ag versus saline injection. Control animals received
saline injections. The training session of the memory test was performed 30 min after the clodrolip/control liposome injection and
30 min before HMGB1 Ag/saline injection; and the context session was performed 72 h later. (B) Second experiment: mice were
divided in four groups treated with anti-HMGB1 versus saline 1 h before tibia fracture. The training session of the memory test
was performed 30 min after the intraperitoneal injection and 30 min before tibia fracture. (C) Third experiment: mice were divided
in four groups treated with anti-HMGB1 versus saline 1 h before tibia fracture and sacrificed 1 and 24 h after the tibia fracture.
Anti-HMGB1 = neutralizing HMGB1 monoclonal antibody; CT-lip = control liposome; HMGB1 Ag = HMGB1 antigen.](image-url)
Animals were tagged and randomly allocated to each group before any treatment or procedure. Researchers were blinded to the group assignment, which was revealed only after the analysis phase.

Body weight was measured before any procedure or treatment and 3 days later, after assessment of freezing behavior.

**Surgical Trauma**

Under aseptic conditions, groups of mice were subjected to an open tibia fracture of the left hind paw with an intramedullary fixation as previously described. Briefly, mice received general anesthesia with 2% isoflurane and analgesia was achieved with buprenorphine 0.1 mg/kg administered subcutaneously, immediately after anesthetic induction and before surgical insult. Warming pads and temperature-controlled lights were used to maintain body temperature at 37°C ± 0.5°C. The entire procedure from induction of anesthesia to end of surgery lasted 12 ± 5 min.

**Trace-fear Conditioning**

Fear conditioning is used to assess learning and memory in rodents, which are trained to associate a conditional stimulus, such as a tone, with an aversive, unconditional stimulus, such as a foot-shock. Freezing behavior is an indicator of aversive memory that is measured when subjects are reexposed to the conditional stimulus.

For this study we used a previously published paradigm. In brief, the behavioral study was conducted by using a conditioning chamber (Med. Associates Inc., St. Albans, VT) and an unconditional stimulus (two periods of 2-s foot-shock of 0.75 mAmp). Behavior was captured with an infrared video camera (Video Freeze; Med. Associates Inc.). Thirty minutes after a particular intervention, animals underwent the training session after which they were returned to their housing cage. Three days after training, mice underwent a context test, during which no tones or foot-shocks were delivered. Freezing behavior, recognized as lack of movement, was recorded by video and analyzed by software. A decrease in the percentage of time spent freezing indicated impairment of memory.

**Systemic Inflammatory Response to Surgery**

One hour and then 24 h after aseptic surgical trauma, blood was collected transcardially after thoracotomy under terminal isoflurane anesthesia and placed into heparin-coated syringes. Samples were centrifuged at 3,400 rotations per minute for 10 min and plasma was collected and stored at −80°C until these were assayed. Blood samples taken from animals without intervention served as controls. Plasma interleukin (IL)-6 and HMGB1 were measured using commercially available enzyme-linked immunosorbent kits, according to the manufacturer’s instructions (Invitrogen, Grand Island, NY, and IBL International, Toronto, Ontario, Canada, respectively).

**Neuroinflammatory Response to Surgery**

Twenty-four hours after surgery, mice were perfused with saline and the hippocampus was then rapidly extracted, placed in RNAlater™ solution (Qiagen, Valencia, CA) and stored at 4°C overnight. Total RNA was extracted using RNeasy Lipid tissue Kit (Qiagen). Extracted RNA was treated with recombinant DNase I by using a RNase-Free DNase set™ (Qiagen). Messenger RNA (mRNA) concentrations were determined with a ND-1000 Spectrophotometer (NanoDrop®; Thermo Fisher Scientific, Wilmington, DE) and mRNA was reverse transcribed to complementary DNA with a High Capacity RNA to-cDNA Kit (Applied Biosystems, Carlsbad, CA).

TaqMan Fast Advanced Master Mix (Applied Biosystems) and specific gene-expression assays were used for quantitative polymerase chain reaction as follows: actin beta (NM_007393.1), IL-6 (Mm00446190_m1), tumor necrosis factor-α (Mm00443258_m1), IL-1β (Mm01336189_m1), and monocyte chemotactic protein-1 (MCP-1) (Mm00441242_m1). Quantitative polymerase chain reaction was performed using StepOnePlus™ (Applied Biosystems).

Each sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle \( \Delta \Delta C_t \) and normalized to β-actin. Results are expressed as fold increases relative to controls.

**Interventions**

**Depletion of BM-DM.** Clodrolip is a liposomal formulation of clodronate (dichloromethylen bisphosphonic acid), a nontoxic bisphosphonate. Liposomes (lipid vesicles consisting of concentric phospholipid bilayers surrounding aqueous compartments) encapsulate clodronate, which are then ingested and digested by phagocytes, followed by an intracellular release and accumulation of clodronate. At a certain intracellular concentration, clodronate induces apoptosis of the phagocytes. Clodrolip was obtained from clodronateliposomes.org (Vrije Universiteit, Amsterdam, The Netherlands) at 7 mg/ml concentration and prepared as previously described. Clodrolip (200 μl, about 100 mg/kg) was injected intraperitoneally 60 min before aseptic surgical trauma. Control animals received 200 μl of control liposomal solution.

**Administration of Reagents to Simulate or Block the HMGB1 Response to Trauma.** Fifty microgram per kilogram (100 μl) of recombinant HMGB1 (R&D System, Minneapolis, MN) was administered intraperitoneally. To neutralize trauma-released HMGB1, 50 μg of anti-HMGB1 neutralizing monoclonal antibody (2G7, mouse IgG2b supplied by Dr. Tracey’s Laboratory, Manhasset, NY) in 100 μl of saline was administered intraperitoneally, 60 min before bone fracture. Control animals received the same volume (100 μl) of the vehicle (saline).

**Statistical Analysis**

Data are presented as mean ± SD. Normality was tested with the Kolmogorov–Smirnov normality test. Equality of variances was tested with the F test. We applied a log transformation (ln(X)) to the response of HMGB1 and IL-6 blood
concentrations and mRNA expression before performing analyses to better adhere to ANOVA model’s assumptions of normally distributed residuals and homogeneity of variance.

For comparisons of more than two groups, means were compared using one-way ANOVA followed by t tests with a Bonferroni-corrected α level. We used the two-way ANOVA procedure to determine whether or not time and antibody treatment were significant factors in predicting HMGB1 and IL-6 concentrations in the serum; this was followed by Bonferroni post hoc analyses.

For our study, the primary outcome was the percentage of freezing time during the context session observed in anti-HMGB1 neutralizing monoclonal antibody and control groups. On the basis of previous freezing time data, we estimated that a sample of 13 C57BL/6J surgical mice per group was necessary to demonstrate a 20% increase in percentage freezing time, with 80% power at the 0.0125 α level (after adjusting for four comparisons) to reach a significant difference.

A two-tailed P value less than 0.05 was considered statistically significant for two-group comparisons and the significance threshold was adjusted for multiple comparisons with a Bonferroni correction. Prism 6 (GraphPad Software Inc, La Jolla, CA) was used to conduct the statistical analyses.

Results

**HMGB1 Antigen Is Sufficient to Cause Cognitive Decline through the Participation of BM-DM**

A single administration of HMGB1 produced cognitive decline as evidenced by a significant reduction in freezing time (52 ± 11% vs. 39 ± 5%, n = 16 in control group, n = 17 in HMGB1 antigen group; P = 0.012; fig. 2).

Recently, we showed that depletion of BM-DM by clodrolip exposure blocks surgery-induced neuroinflammation and cognitive decline. To determine whether HMGB1-induced cognitive decline requires the participation of BM-DM, mice were exposed to clodrolip or its vehicle before administration of HMGB1. Training was unaffected by the clodrolip exposure (data not shown). We lost one animal in the clodrolip group and another in the control liposome group on day 2. According to the manufacturer this may happen if there is transference of microorganisms from the skin or by injection of a nonhomogeneous suspension of liposome. We did not experience any death in the surgical groups. The HMGB1-induced decline in contextual freezing time was prevented by previous clodrolip exposure (39 ± 5% vs. 50 ± 9%, n = 17; P = 0.039; fig. 2).

**HMGB1 Antibody Prevented Postsurgical Cognitive Decline**

During the preoperative training period, learning was similar in the anti-HMGB1–exposed group and the control (nonexposed) groups (data not shown). Surgery significantly decreased the percentage of freezing time when compared with the control group (52 ± 11% vs. 29 ± 5%, n = 16 in control group, n = 15 in surgical group; P < 0.001); preoperative exposure to anti-HMGB1 attenuated the surgery-induced freezing behavior, rendering the response to be no different from that of the nonsurgical control group (52 ± 11% vs. 47 ± 11%, n = 16 in control group, n = 15 in anti-HMGB1 + surgery group; ns; fig. 3).

**HMGB1 Antibody Reduces Systemic and Neuroinflammatory Response to Surgery**

As expected, we observed a significant decrease of the early increase of HMGB1 blood level between the first hour and 24 h after surgery (two-way ANOVA; P = 0.002 for the time effect; fig. 4). However, the neutralizing anti-HMGB1 reduced the systemic levels of HMGB1 (two-way ANOVA; P = 0.005 for the treatment effect and P = 0.010 for interaction between time and treatment), and this reduction was significant 1 h after the trauma (P = 0.04 with Bonferroni’s post hoc analysis; fig. 4A). With regard to IL-6 increase after surgery, we observed a significant decreased within the first day (two-way ANOVA; P = 0.015 for the time effect) HMGB1 neutralizing antibody was also a significant predicting factor for IL-6 concentration (two-way ANOVA; P < 0.001 for treatment effect). Using HMGB1 neutralizing antibody, we observed a reduction in IL-6 concentration 1 h after trauma (P = 0.006 with Bonferroni’s post hoc analysis; fig. 4B).

Twenty-four hours after surgery, the increase in hippocampal mRNA expression of IL-6 (n = 6; P = 0.009) and
tumor necrosis factor-α (n = 6; P < 0.001) was blocked by exposure to the neutralizing anti-HMGB1 antibody. Treatment did not significantly change mRNA transcription of IL-1β (n = 6; P = 0.085; fig. 5, A–C).

Circulating chemokine C-C motif receptor 2-expressing BM-DM are recruited into the brain by the chemoattractant, MCP-1.12 After surgery there was an increase of hippocampal mRNA transcription of MCP-1 (n = 6; P = 0.003); treatment with the anti-HMGB1 neutralizing antibody prevented surgery-induced expression of MCP-1 (n = 6; P = 0.005; fig. 5D).

**Conclusion**

This study posits that HMGB1, when released from a sterile traumatic injury, plays a pivotal role in postoperative memory dysfunction. Together with the detection of the cell type involved in the initiation of the surgery-induced inflammatory cascade these findings establish both the precise elements of the immune response that need to be interrogated for establishing risk of dysregulated trauma-induced inflammation as well as putative targets for interventions designed to limit or reverse persistent postoperative cognitive decline.

The independent role of the alarmin HMGB1 in disrupting cognitive processing was established by the fact that a single injection of HMGB1 was capable of reproducing a deficit in an hippocampal memory test similar to the surgical phenotype that we previously described.1 Furthermore, its dependence on bone marrow–derived monocytes was evidenced by the attenuation of HMGB1-induced cognitive decline if clodrolip was first administered to induce apoptosis of these circulating phagocytes (fig. 2); this is similar to findings of a previous report in the setting of surgery.1,5

After the aseptic trauma of elective surgery, either experimentally (fig. 4A) or clinically, HMGB1 is released into the circulation where it can interact with pattern recognition...
HMGB1 Role on Postoperative Cognitive Dysfunction

receptors (toll-like receptors 2 and 4 as well as receptor for advanced glycation end products) on immunocytes. By neutralizing the early release of alarmins, HMGB1 antibody both decreased surgery-induced inflammation (figs. 4 and 5) as well as cognitive decline (fig. 3), further establishing the importance of hippocampal inflammation for the development of postoperative memory dysfunction. After peripheral surgery, chemokine C-C motif receptor 2-expressing cells migrate to the brain, attracted by signaling from hippocampal MCP-1, a chemokine that regulates migration and infiltration of monocytes/macrophages. Interestingly, by depleting BM-DM, the expression of MCP-1 in the surgical model remained unaffected, indicating that BM-DM are not the self-perpetuating source of this chemoattractant for its own recruitment. We now show that treatment with HMGB1 antibody was able to prevent the synthesis of MCP-1 in the hippocampus, establishing the dependence of its increased expression on the release of HMGB1. Taking our previous report and our current findings together, it follows that HMGB1 is signaling to the hippocampus to produce the chemoattractant, MCP-1, through a mechanism that is independent of BM-DM; this HMGB1-dependent hippocampal expression of MCP-1 could involve either a neural or humoral pathway.

Accumulating evidence indicates that HMGB1 can stimulate migration of not only monocytes, but also various types of cells including neurite, smooth muscle cells, tumor cells, mesoangioblast stem cells, dendritic cells, and neutrophils. This could explain how blocking the effect of the release of HMGB1 may have blocked signaling of other HMGB1-derived factors. Although BM-DM play a necessary role, they may not be sufficient to completely explain the cognitive decline seen after surgery; there may be other cells and factors involved in the genesis of postoperative cognitive dysfunction.

The following caveats apply when interpreting our findings. Our surgical model involves disruption of the bone marrow with an intramedullary pin for internal fixation of the broken bone. The bone marrow itself produces soluble factors that affect immune cells, such as a proliferation-inducing ligand A (APRIL), B-cell activating factor (BAFF), both belonging to the tumor necrosis factor family, CXCL12, IL-6, IL-7, and macrophage inhibitory factor. The possibility exists that surgical trauma that does not involve damage to the bone marrow may not use the same panoply of alarmins. In addition to its role as a primary lymphoid organ, the bone marrow can act as a host for various mature lymphoid cell types. Several subsets of

![Fig. 5. Effects of high-mobility group box 1 protein (HMGB1) neutralizing monoclonal antibody on hippocampal transcription of interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, and monocyte chemotactic protein (MCP)-1 24 h after tibia surgery (Arm C).](image-url)
bone marrow cells have been shown to support immune cell function. 29

Given that HMGB1 resides in the nucleus and functions as an essential nonhistone chromatin-binding protein, there are no HMGB1 knockout animals. For this study we then decided to use a pharmacologic strategy to quickly deplete the pool of systemic macrophages. However, because clodrolip is highly toxic to all phagocytes, it can increase the risk of post-surgical infections, generating a phenotype of its own. 30 With a single dose, we did not observe loss of weight or other signs of sickness within 3 days. As this was a short-term study, focusing on the acute exaggeration phase of neuroinflammation, we are unable to extrapolate from these data the long-term effects and did not perform any long-term study with clodrolip. Clodrolip should be considered a tool for mechanistic studies.

In previous reports using this model of surgical trauma, we documented the effectiveness of preoperative administration of interventions such as IL-1 receptor antagonist, anti-tumor necrosis factor monoclonal antibody and activation of the α7 subtype of nicotinic acetylcholine receptor in preventing postoperative memory dysfunction. 1,2,5 Each of these affect important host defense mediators, and risk of infection needs to be evaluated before considering these agents.

These studies on the initiation of trauma-induced cognitive decline, coupled with our previous reports on the resolution of postoperative cognitive decline, 1,2,5 sets the stage for the development of an ex vivo bioassay that can test the function of the innate immune response to trauma. Such an assay may be capable of prospectively identifying surgical patients at increased risk for the development of exaggerated and persistent cognitive decline; stratification of a surgical cohort, enriched for development of cognitive decline, can result in a randomized trial to test efficacy of interventions using fewer surgical patients.

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References

The Neuroinflammatory Response of Postoperative Cognitive Decline
The neuroinflammatory response of postoperative cognitive decline

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Background: Aseptic surgical trauma provokes a homeostatic neuroinflammatory response to promote healing and protect the organism from further injury. When this response is dysregulated, harmful consequences can follow, including postoperative cognitive decline.

Sources of data: We performed a comprehensive search on PubMed related to postoperative cognitive dysfunction (POCD).

Areas of agreement: Although the precise pathogenic mechanisms for POCD remain unclear, certain risk factors are known.

Areas of controversy: The mechanisms that lead to exaggerated and persistent neuroinflammation and the best way to counteract it are still unknown.

Areas for developing research: It is imperative that we identify the underlying processes that increase the risk of cognitive decline in elderly surgical patients. In this review we explore non-resolution of inflammation as an underlying cause of developing exaggerated and persistent POCD. If interventions can be developed to promote resolution of neuroinflammation, the patient’s postoperative recovery will be enhanced and long-term consequences can be prevented.

Keywords: neuroinflammation/cognitive decline/neurodegeneration/surgery, sleep

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Introduction

Impairment of cognition after surgery is a disturbing reality. Postoperative delirium (POD) is characterized by inattention, disorganized thinking and altered level of consciousness with acute onset and fluctuating course. While some patients develop POD, others develop a later onset form of postoperative cognitive decline known as postoperative cognitive dysfunction (POCD).

It is estimated that POCD occurs in >10% of non-cardiac surgical patients over 60 years old, and is independently associated with poor short-term and long-term outcomes, including an increased risk of mortality. Although several risk factors have been identified, the exact pathophysiology that underlies POCD remains undefined.

The thesis that neuroinflammation is a possible cause of POCD has recently been tested. Data from preclinical studies support the concept that inflammation is a possible pathogenic mechanism for postoperative cognitive dysfunction. Increased expression of interleukins in mouse hippocampus following minor surgery was associated with cognitive decline, corroborating the view that surgery-induced neuroinflammation can result in cognitive impairment. Surgical patients exhibit elevations of pro-inflammatory cytokines in both the central nervous system and the systemic circulation, the extent of which may relate to the degree of cognitive decline. Assuming that neuroinflammatory changes noted postoperatively in rodents also occur in humans, reasons must be sought why POCD is a relatively infrequent clinical event whereas neuroinflammation always occurs. Among the possibilities include the fact that the neuroinflammatory changes are usually evanescent and do not normally cause a long-lasting consequence in animal models. Several clinical conditions can transform the self-limiting postsurgical neuroinflammatory response into one that is persistent. The persistence in neuroinflammation may be due to dysfunction in the inflammation-resolving mechanism. Alternatively, a normal neuroinflammatory response to surgery may have long-lasting detrimental effects in settings of neurological pathology, whether clinically evident or not. In a recently completed prospective study, patients who had previously suffered a stroke were more at risk of POCD even though they had no neurological sequelae from the remote stroke event. Epidemiologic studies have suggested that neurodegenerative disorders, such as Alzheimer’s disease (AD), may be accelerated by surgery and that exacerbates dementia in AD patients while increasing the occurrence of dementia. However, this relationship has recently been challenged.
This review considers the possible role of inflammation in the development of POCD in the setting of underlying systemic and neurologic diseases; it will also discuss future research possibilities that might help identify vulnerable patients with whom interventional strategies could be invoked.

**Clinical condition**

Clinical studies distinguish POD from POCD. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV-TR)\(^2\) defines the standards necessary for a diagnosis of delirium. These include disturbance of consciousness, change in cognition, inattentiveness and a fluctuating time course. Clinically, this diagnosis is often made using the confusion assessment method (CAM), a simple four-question screening tool that has a sensitivity of 94% and a specificity of 89%. A variation known as the CAM-ICU is often used in the intensive care unit (ICU) setting and is useful in sedated or intubated patients. Diagnosis of delirium in the cases of dementia can also be accomplished with the appropriate tools.\(^2\)

There are three subtypes of delirium: hyperactive (25%), hypoactive (50%) and mixed (25%). The hypoactive subtype is the most frequently missed and may actually be associated with greater mortality than the hyperactive subtype.\(^2\)\(^4\) The incidence of POD is between 10 and 55% in postoperative patients, depending on the type of procedure the patient underwent, with a higher percentage in orthopedics compared with general surgery patients.\(^2\)\(^5\) Additionally, the incidence of POD is significantly higher in elderly patients. It is estimated that up to 50% of elderly patients suffer from delirium after surgery.\(^2\)\(^6\) Furthermore, over 40% of hospitalized patients with delirium suffer from psychotic features, including visual hallucinations. It typically manifests itself within 24–48 h postoperatively, with exacerbation of symptoms at night, perhaps due to circadian disturbances. The implications of POD are significant. It is associated with increased morbidity and a 1-year mortality that approaches 40%.\(^2\)\(^7\) The estimated healthcare-associated costs related to delirium are astronomic. They made up nearly $7 billion of Medicare expenditures in 2004.\(^2\)\(^8\)

Persistent cognitive decline is predominantly seen in the elderly\(^2\)\(^9\) and is termed POCD. POCD is diagnosed by the International Society of Postoperative Cognitive Dysfunction as subtle deficits in one or more discrete domains of cognition, e.g. attention, concentration, executive function, verbal memory, visuospatial abstraction and psychomotor speed.\(^3\)\(^0\) This condition typically develops over weeks to months, and is long-lasting. The diagnosis requires sensitive presurgical and
postsurgical neuropsychiatric testing. As a consequence of this complication, patients can lose their employment or independence, which can seriously reduce their quality of life.

An international multicenter study of POCD (ISPOCD) reported memory impairments in more than a quarter of the patients 1 week after non-cardiac surgery and in 10% after 3 months in patients older than 60 years. Follow-up studies have shown similar incidences with some reports describing cognitive decline persisting for up to 1 year after surgery. Both because the number of major surgical interventions (requiring anesthesia) exceeds 230 million worldwide and because of the increasing prevalence of surgical interventions in patients >65 years old, this age group will become the largest segment of surgical patients by 2020. If current rates hold steady, we can expect that millions of elderly patients will run the risk of developing POCD. This possibility raises the stakes considerably: not only on an individual level, but also on a societal scale.

Risk factors

Studies have sought to identify factors that may contribute to POCD, some of which include surgery, anesthesia and patient-related factors.

Non-modifiable patient factors

Patient-related risk factors include: advanced age, education, genetic polymorphism (apolipoprotein E4) and several other comorbidities.

Advanced age is associated with infirmities, many of which can be successfully treated with surgery. Unfortunately, persistent cognitive impairments can develop as a side effect of these surgical procedures. An increase in the aging population and improvements in anesthesia and surgery have led to increases in the number of elderly patients undergoing surgery. Therefore, it is likely that postoperative central nervous system dysfunction will become increasingly common.

Systemic disease: metabolic syndrome

Roughly 25% of the 45 million surgical patients in the US have metabolic syndrome (MetaS), though its precise definition and diagnostic criteria continue to evolve. MetaS, comprising insulin resistance, visceral obesity, hypertension and dyslipidemia, increases the risk of
postoperative complications contributing to a significant higher mortality rate.\textsuperscript{48–51} While each of the subphenotypes that define MetaS has a strong genetic component, lifestyle factors that contribute to this cluster of conditions include sedentary behavior and a diet with a high caloric content from saturated fats and/or simple carbohydrates.\textsuperscript{52} Many complications of MetaS (including atherosclerosis) are inflammatory in nature and the pathologic metabolism in adipose stores may be the source of pro-inflammatory adipokines.\textsuperscript{53} Conversely, with little adiponectin to attenuate activation of the transcription factor NF-$\kappa$B in macrophages, expression of genes for pro-inflammatory cytokines is increased;\textsuperscript{54} up-regulated NF-$\kappa$B activity in morbid obesity can be rectified with adiponectin.\textsuperscript{55} Roughly a quarter of the American adult population have MetaS; 50\% of cardiac surgery patients are affected with MetaS.\textsuperscript{56} Recent evidence indicates that patients suffering from MetaS may be particularly susceptible to POCD.\textsuperscript{50,57}

**Neurologic disease**

The two most common causes of dementia are vascular dementia and AD, although most cases of dementia have both types of pathology. Pre-operative cognitive impairment, such as mild cognitive impairment (possible prodrome for AD), may already exist in many elderly patients who incidentally present at surgery. Although perioperative cognitive decline and AD may share certain neuropathologic and biochemical mechanisms, there is no direct evidence linking the involvement of AD-type pathogenic mechanisms and POCD in humans and only weak epidemiological evidence associating surgery with onset of AD.\textsuperscript{58} Epidemiological studies have suggested that neurodegenerative disorders, including AD, may be accelerated by surgery.\textsuperscript{15} However, large retrospective studies have thus far not associated surgery or anesthesia with further dementia and AD.\textsuperscript{21} Evidence from animal models suggests that inhaled anesthetic exposure increases pathology normally associated with AD, including increase in $\beta$-amyloid peptide and $\beta$-acting cleavage enzyme;\textsuperscript{59} anesthesia-induced hypothermia increased tau hyperphosphorylation by decreasing phosphatase 2A activity.\textsuperscript{60}

Symptomatic pre-operative neurologic diseases, including dementia and any disease of the central nervous system, are often considered exclusion criteria for POCD studies.\textsuperscript{14} Interestingly, cerebral vascular accidents (without residual deficit) were associated with risk factors for POCD, suggesting for the first time that a pre-operative ischemic brain insult could influence the possibility of POCD. However, no causal link between pre-operative cerebral vascular accident and POCD has been established yet in experimental or human investigations.
Postoperative neurodegeneration, akin to AD, is observed in aged rats; while inflammation-resolution mechanisms have not been investigated, it is known that these mechanisms decline with advancing age. AD-type neurodegeneration is accelerated by neuroinflammation, raising the possibility that failure to resolve neuroinflammation may provoke neurodegenerative changes that cause persistent cognitive decline. It is noteworthy that anesthesia alone, at higher concentrations and for more prolonged periods, has been reported to produce AD-like neurodegeneration although this has been challenged.

The anesthetized state

There are several risk factors directly related to anesthesia that may be involved in the pathogenesis of POCD. Intra-operative hypotension, hypoxia, embolism, medications and postoperative infections have all been described as risk factors for POCD. Although general anesthetics are capable of producing long-lasting cognitive dysfunction under certain circumstances, the incidence of POCD is similar after regional and general anesthesia, the reason why attention has been focused on the role of the surgical intervention itself in the genesis of this condition. Postoperative pain is a possible etiologic factor in POCD. Epidural analgesia with local anesthetics and/or opioids may be better than parenteral analgesics for the control of postoperative pain and the prevention of early POCD. Furthermore, patients who are prescribed postoperative oral analgesics experience less POCD compared with those receiving parenteral medication. Even though studies have shown the potential benefit of intra-operative monitoring of anesthetic depth and cerebral oxygenation as a pragmatic intervention to reduce postoperative cognitive impairment, this factor still remains a controversial issue as some studies have shown no relation between deeper states of anesthesia and the emergence of POCD. In support of the latter position, a number of recent studies show that animals exposed to short-duration isoflurane do not develop memory impairment.

Sleep

Sleep is crucial for the repair of many types of injury and disease, especially with regard to the central nervous and immune systems; it also has anabolic, restorative properties that improve both neurocognitive and immune function. During non-rapid eye movement (NREM) sleep, slow-wave activity performs a homeostatic function
to reduce the strength of synapses that has been acquired during wakeful activity. This synaptic homeostasis improves subsequent cognitive function by allowing new changes in synaptic strength. For example, both NREM and REM sleep are necessary for the consolidation of learning and memory while sleep deprivation results in cognitive dysfunction.

Polysomnographic studies revealed extreme sleep disruption in ICU patients with decreases in total sleep-time, altered sleep architecture (predominance of stage 1 and 2 sleep, decreased or absent stage 3 NREM and REM sleep) and sleep fragmentation; also, up to 50% of the total sleep-time occurred during daytime. Lack of sleep hygiene results in cognitive dysfunction, contributes to delirium, adversely affects immunity and independently increases both morbidity and mortality. Sleep disruption during hospital care has the potential to adversely impact on patients’ outcome and also provides a direct financial cost with respect to the length of hospital stay and depletion of healthcare resources.

Preclinical studies have shown the detrimental effect of lack of sleep on cognition. In addition, perioperative sleep deprivation induced significant neuroinflammatory changes. The exact mechanism for the deleterious consequences of a ‘double hit’ (aseptic surgical trauma and sleep deprivation) is still poorly understood, though it has been shown to increase the expression of inflammatory cytokines in the brain. Sedative practices have also shown to be a main causative factor for this disruption.

Anesthetics have different action targets and ultimately different consequences. The pivotal work of the MENDs trial indicated the benefits of a specific anesthetic agent, dexmedetomidine, in the outcome of ICU populations. α2 adrenergic agonists converge on sleep pathways within the brainstem, while those that act by modulating the GABA receptor converge at the level of the hypothalamus. Several studies have now demonstrated the association between the use of benzodiazepine (BZD) and increased incidence and duration of delirium in ICU patients, although the relationship of the development and duration of delirium to sleep disruption has not yet been thoroughly ascertained. Acute withdrawal from long-term sedation with BZDs and opiate narcotics results in profound sleep disruption. Thus, thoughtful attention must be paid in selecting an anesthetic agent that best mimics natural sleep in order to decrease the decline of cognitive function. These efforts have to be maintained for the entire perioperative period.
Neuroinflammatory response to surgery

Activation of the immune system after surgery is associated with cognitive decline.5,7 Tissue trauma releases damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PRRs), which then trigger an immune response in a manner remarkably similar to that of microbial-derived pathogen-associated molecular patterns (PAMPs).97–99 Among PRRs, Toll-like receptors (TLRs) are of critical importance, recognizing various ligands (including PAMPs and DAMPs) and activating TLR signals along different pathways, thereby increasing the synthesis and release of pro-inflammatory mediators. Although the function of TLR4 during lipopolysaccharide (LPS) endotoxemia100 has been deeply explored, the pathways of infection-mediated neuroinflammation and cognitive decline seem to be distinct from that of aseptic surgical trauma.7 One of the most important DAMPs (released from dead or dying cells through non-apoptotic processes101) is high-mobility group box 1 (HMGB1). HMGB1 can bind and signal through a family of PRRs that are evolutionarily conserved.102 Clinical conditions such as sepsis, arthritis and stroke all release massive amounts of HMGB1.103 Both DAMPs and PAMPs converge on NF-κB to increase synthesis and release of pro-inflammatory cytokines104, including TNF-α, which disrupt blood brain barrier (BBB) integrity.5,7,8,105 Early activation of the innate immunity through DAMPs (HMGB1 and cytokines) will introduce the initial response to surgery resulting in neuroinflammation and concomitant cognitive decline.7

Following injury, this ‘transient’ inflammation is a necessary tissue repair process that promotes healing. Macrophages are highly heterogeneous hematopoietic cells found in nearly every tissue in the body and have been defined as the sentinels of the innate immune system. They are also key players in the resolution of inflammation and are critical to tissue repair, wound healing and restoration of homeostasis.106 In addition, macrophages are responsible for sensing, integrating and appropriately responding to a bewildering array of stimuli from its local microenvironment. Macrophage responses are mediated through two distinct and mutually exclusive activation programs, termed classical (or M1) and alternative (or M2).107 These activation programs were initially defined by their antimicrobial activities; classical activation occurs in response to bacterial infections and results in highly inflammatory macrophages with high phagocytic and bactericidal potential.107 In contrast, alternative activation occurs in response to parasitic infections and promotes antiparasitic functionalities as well as those involved in tissue repair and remodeling.108 Both programs promote differentiation of neighboring macrophages to their same activation state and potently inhibit maturation of the other.
Neuroinflammation after surgery is likely to include a pro-inflammatory phase and an anti-inflammatory phase (neural and humoral pathways mediate the switch between these two phases). With respect to the humoral factors, resolvins, lipoxins and maresins (macrophage mediators in resolving inflammation), derived from polyunsaturated fatty acids (PUFAs), are novel lipid mediators that promote the resolution of inflammation. Protective actions of D-series resolvins have been observed in both acute and chronic inflammatory diseases, such as peritonitis, ischemia/reperfusion injury and sepsis. Resolvins both limit PMN infiltration and enhance macrophage phagocytosis by transducing signaling mechanisms that originate at specific receptors on human PMN, monocytes and macrophages. Lipoxins were the first mediators recognized to have both anti-inflammatory and pro-resolving actions. Maresins are newly identified macrophage mediators with the same properties. They are capable of dampening the pro-inflammatory response by inhibiting macrophage NF-κB activity and polarizing macrophages into an M2 phenotype. Dietary supplementation with PUFAs in patients with MetaS corrects many of the metabolic derangements as well as the pro-inflammatory markers. Regarding the resolving inflammatory state mediated by neural factors, DAMPs activate the efferent arc of the inflammatory reflex via NF-κB, termed the cholinergic anti-inflammatory pathway. At its splenic nerve terminus, vagal outflow releases adrenergic agonists (rather than the usual cholinergic neurotransmitter); these catecholamines activate β2 adrenergic receptors on CD3 T lymphocytes that are capable of synthesizing and releasing acetylcholine needed to mediate inhibition of macrophage NF-κB activity by signaling through the α7 subtype of nicotinic acetylcholine receptors (α7 nAChR). Ultimately, it inhibits synthesis and release of pro-inflammatory cytokines from circulating immunocompetent cells.

The neural cholinergic reflex is very important in resolving the inflammatory pathogenesis of several diseases including sepsis, rheumatoid arthritis and colitis. Furthermore, the cholinergic anti-inflammatory pathway also modulates the function of T regulatory cells, which influences the production of anti-inflammatory cytokines (IL-10 and IL-4) and alternative macrophage activation that promotes the resolution of inflammation. IL-4 is the cytokine responsible for polarizing macrophage from the pro-inflammatory classically activating (M1) to the reparative alternatively activating (M2) phenotype. In the mouse models of type II diabetes, there is a relative lack of T regulatory cells and an imbalance of M1/M2 macrophages, which might contribute to persistent low-grade inflammation. Abnormalities of the switching mechanism may cause a non-resolving chronic inflammatory state that could create the
circumstances for persistent cognitive decline. Recently, it has been shown that MetaS will contribute to exaggerated and persistent POD in a rat model of tibial fracture.\textsuperscript{127} In addition, dysfunctional resolution of inflammation was found to be associated with behavioral deficits after surgery in metabolic syndrome rats.\textsuperscript{128} However, the detailed mechanisms are still unclear and more research is warranted.

Studies have shown the importance of this reflex for resolving DAMP-induced neuroinflammation, pro-inflammatory cytokine release, neuroinflammation and cognitive decline; stimulating the \( \alpha_7 \) nAChR in macrophages, inhibited NF-\( \kappa \)B activity which in the quiescent state precludes postoperative memory impairment by preventing monocyte migration into the hippocampus.\textsuperscript{129} Drugs used clinically in the perioperative period, including anesthetics, exert anti-cholinergic activity that may translate into non-resolution of inflammation and PCD in the form of delirium.\textsuperscript{130} Advanced age is associated with decline in cholinergic function, which may be relevant in explaining the high prevalence of POCD in elderly patients.

When inflammation does not subside, it can contribute to the pathogenesis of diseases.\textsuperscript{106} Through a permeable BBB, CCR2-expressing bone marrow-derived macrophages (BM-DM) are attracted, by the newly expressed chemokine, MCP-1, into the brain parenchyma. The macrophages synthesize and release a variety of pro-inflammatory cytokines that interfere with processes required for memory. Macrophage-specific Ikappa B kinase (IKK)_\( \beta \) coordinates activation of NF-\( \kappa \)B; when it is deleted, it prevents BBB disruption and BM-DM infiltration into the hippocampus following surgery.\textsuperscript{129} Transgenic mice that overexpress Hsp72 and inhibit NF-\( \kappa \)B activity have attenuation of postoperative neuroinflammation and cognitive decline.\textsuperscript{131,132}

Learning and memory processes rely on the hippocampus, a region of the brain that contains a large number of pro-inflammatory cytokine receptors.\textsuperscript{133,134} The hippocampus has the highest density of IL-1 receptors, and although IL-1\( \beta \) is required for normal learning and memory processes, higher levels can also produce diminished cognitive function.\textsuperscript{135,136} Recent studies have suggested a role for cytokines such as interleukins-1 and -6 in the genesis of POCD. The relative prevalence of the TNF-\( \alpha \) receptor, as well as other PRR, on the endothelium of this brain region may account for its vulnerability to systemic pro-inflammatory cytokines.\textsuperscript{137}

Surgical trauma in animal models is associated with the persistent activation of macrophages in the CNS that are capable of maintaining elevated levels of IL-1\( \beta \), and other pro-inflammatory cytokines, such as TNF-\( \alpha \) and IL-6. These changes are correlated with cognitive dysfunction seen in animal models (contextual fear memory,\textsuperscript{5,7,9} spatial learning\textsuperscript{99,138} or reversal learning\textsuperscript{98}). IL-1 is released in response to a wide
range of infectious, inflammatory or toxic insults, IL-1.\textsuperscript{99,139} Sub-clinical inflammation following administration of LPS substantially increases IL1-\(\beta\) levels and cognitive deterioration after surgery.\textsuperscript{8} In addition, several studies suggest that the marked and sustained expression of inflammation-related enzymes, such as cyclooxygenase-2, plays an important role in secondary events that amplify cerebral injury after ischemia.\textsuperscript{140} Patients also exhibit a robust neuroinflammatory response to peripheral surgery with an initial rise in pro-inflammatory cytokines in the CSF\textsuperscript{12,141} as well as release of reactive oxygen species and endothelins.\textsuperscript{102,142,143}

Can we identify vulnerable patients pre-operatively?

We believe that non-resolution of inflammation is a factor that contributes to the pathogenesis of POCD, which in turn significantly increases morbidity and mortality in surgical patients. We might be witnessing a perfect and unfortunate storm of factors with regard to POCD: to put it another way, given the rise in surgeries and increasing number of elderly patients worldwide, the stakes could not be higher.

Vulnerable patients need to be identified and risk/benefit should be considered before contemplating the efficacy of surgical intervention. Advanced age, MetaS, patients prone to AD and poor selection of sedative agents may each result in an exaggerated and persistent neuroinflammatory response to surgery. Further studies are needed to understand which patients will suffer from exacerbated inflammation with the aim of developing a biomarker that is quick to assay for clinicians and easy to comprehend for patients and their families. Concurrently, clinical interventions need to be further developed to promote the resolution of neuroinflammation in postoperative patient populations. Following both tracks, we anticipate that postoperative recovery for vulnerable patients will be greatly enhanced and possible long-term consequences, such as postoperative neurodegeneration, can be significantly reduced.

Conclusions

In the majority of patients, postoperative neuroinflammation is part of the normal protective mechanism to peripheral trauma and resolves properly with no residual cognitive consequences. Indeed, it is also possible that surgery for a chronic inflammatory disease may result in cognitive improvement by eliminating disease-inducing cognitive impairment that may be associated with chronic inflammatory disease. That
said, some risk factors, such as MetaS, patients prone to AD and poor selection of sedative agents, may each promote the intractable persistence of neuroinflammatory response to surgery. For an increasing number of patients with advanced age, POCD is alarmingly common, making postoperative central nervous system dysfunction a looming public health crisis, given the world’s rising elderly population.

Additional study is essential to elucidate the risk factors, preventative strategies and the underlying pathophysiology of this disorder. If these studies can succeed in identifying patients prospectively, or early enough in the advent of persistent inflammation, interventions can be judiciously and appropriately launched.

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Sleep
Consequences of Sleep Fragmentation in the Perioperative Setting
Consequences of Sleep Fragmentation in the Perioperative Setting
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Short Title: Sleep fragmentation and surgery
ABSTRACT (400 words)

Background: Sleep is integral to biological function and sleep disruption can result in both physiological and psychological dysfunction. The acute cognitive consequences of sleep loss have been an active field of recent investigation, evidence suggests that sleep disruption in critically ill older adults can result in acute decrements in cognitive functioning. Surgery activates the innate immune system, inducing neuroinflammatory changes that interfere with cognition. The fact that patients with sleep disorders have an increased likelihood of exhibiting postoperative delirium encourages us to investigate the contribution of perioperative SF to the neuroinflammatory and cognitive responses of surgery.

Methods: The effects of 24h sleep fragmentation (SF) and surgery were explored on adult C57BL/6J male mice. SF procedure started at 7 am with the home-cages being placed on a large platform orbital shaker cycled every 120 seconds (30 sec on/90 sec off). This procedure lasted for 24h. Stabilized tibia fracture was performed either before or after the 24h SF procedure. Separate cohorts of mice were tested for systemic and hippocampal inflammation and cognition.

Results: Twenty-four hours of SF induced non-hippocampal memory dysfunction and increase in systemic IL-6. SF and surgery caused hippocampal-dependent memory impairment, although memory impairment was not exacerbated by combining SF with surgery. One day after either SF or surgery there was a significant increase in IL6 mRNA and TNF-alpha mRNA. These increments were more pronounced when either pre or post operative SF was combined with surgery.

Conclusions: We show that while SF and surgery can independently produce significant memory impairment, perioperative SF significantly increased hippocampal inflammation without further cognitive impairment. The dissociation between neuroinflammation and cognitive decline may relate to our use of a sole memory paradigm that does not capture other aspects of cognition, especially learning.
INTRODUCTION

Importance of sleep for memory
Sleep restores and repairs several mechanisms related to learning and memory. Slow wave activity weakens the synaptic strengthening that occurs during wakefulness and restores the brain to a state that is capable of appropriately processing sensory input in succeeding periods of wakefulness.

Importance of continuous sleep
Unlike sleep deprivation, sleep fragmentation (SF) does not necessarily affect total sleep time, but reduces the total amount of time spent in the deeper levels of sleep. Further, the brain’s capacity to successfully respond to cognitive challenges through compensatory recruitment becomes overwhelmed if the patient is not presented with appropriate and continual sleep. Optogenetic activation of orexinergic neurons, which play a key role in arousal processes, demonstrate that a minimal unit of uninterrupted sleep is crucial for memory consolidation. Interrupted, fragmented or restricted sleep is a consequence of many diseases, including the respiratory disorder Obstructive Sleep Apnea (OSA).

OSA consequences
OSA is not just highly disruptive to steady breathing but also to constant and continuous sleep. It is also the most pervasive sleep disorder, increasing the risk of heart failure, stroke, coronary heart disease, and cognitive impairment. Studies also suggest that the cognitive and structural deficits in OSA may be secondary to SF and repetitive nocturnal intermittent hypoxemia. Further, it is an independent risk factor for the development of postoperative delirium.

Surgery and sleep
Cognitive dysfunction, either alone or as part of delirium, is an adverse outcome for surgical/ICU patients that result in serious consequences, including higher mortality rates. Surgery activates the innate immune system, inducing neuroinflammatory changes that cause subsequent decline in cognitive function. These changes are necessary in order to defend the organism from further injury, but when this response is dysregulated, they can lead to cognitive decline. SF by itself can also induce activation of inflammatory mechanisms, more specifically TNF-α-dependent pathways, which can itself impair the immune system. Polysomnographic studies have revealed profound sleep disruption in intensive care unit patients, with decreases in total sleep-time, altered sleep architecture, and SF. The fact that patients with sleep disorders have an increased likelihood of exhibiting postoperative delirium encourages us to investigate the contribution of perioperative SF to the neuroinflammatory and cognitive responses of surgery.
METHODS

Animals

All the experiments were conducted under approval of the Institutional Animal Care and Use Committee of the University of California, San Francisco, and conformed to the National Institutes of Health Guidelines. Experiments were performed using 12-14 weeks old male C57BL/6J mice (Jackson Laboratories, Bay Harbor, ME), under a 12:12 hours light-dark cycle, in a constant temperature and humidity controlled environment, with free access to food and water. Animals were tagged and randomly allocated to each group before any treatment or procedure (fig. 1 Study design). Researchers blinded to the group assignment performed all neurobehavioral tests. For those animals in which behavior was assessed, no blood harvesting or tissue sampling was performed, in order to obviate possible confounding effects of fear conditioning testing on inflammatory markers.16

Body weight was measured before any procedure or treatment and daily after that.

Sleep fragmentation

Sleep was interrupted for 30 sec every 2 min as previously described.17 Briefly, animals were kept in their home cage and placed on an analog orbital shaker (OS-500, VWR, Champaign, IL). Repetitive on/off cycling of the shaker (30 sec on/90 sec off), set at 100 rpm, was controlled by a timer (traceable controller, Fisher Scientific, Pittsburg, PA). A metal cage cardholder was suspended from the top of the cage, creating an additional audible stimulus when the holder knocked against the side of the cage. Standard laboratory chow was supplemented with aqueous gel.

Surgical trauma

Under aseptic conditions, groups of mice were subjected to a tibial fracture as previously described.18 Mice were anesthetized with 2% isoflurane, and analgesia was achieved with buprenorphine 0.1 mg/kg subcutaneously immediately after anesthetic induction and before skin incision. Warming pads and temperature-controlled lights were used to maintain body temperature at 37 ±0.5ºC. Aseptic conditions were maintained throughout the procedure. The entire procedure from induction of anesthesia to end of surgery lasted 10 ± 3 min.

Novel object recognition (NOR)

NOR is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of non-hippocampal learning and recognition memory. For our study we used a previously described protocol.19 Briefly, animals were familiarized for two days to the testing environment that differed from the home cage. In a first phase, animals were presented with the to-be familiarized object in the testing environment (10 min). Following the sample-object exposure, animals were returned to the home-cage for a retention period (1 hour). In the second phase, animals were returned to the environment and presented with two-objects: the previously experienced sample object and a novel object (3-5 min). Object recognition was distinguished by the animal spending more time exploring the novel object. To score time of object interaction from video, XNote Stopwatch® was used.

Trace-fear Conditioning (TFC)

Fear conditioning is used to assess learning and memory in rodents, which are trained to associate a conditional stimulus, such as a tone, with an aversive, unconditional stimulus, such as a foot-shock.20 Freezing behavior is an indicator of aversive memory that is measured when subjects are re-exposed to the conditional stimulus. For this study we used a previously published paradigm.11,21,22 Briefly, the behavioral study was conducted using a conditioning chamber (Med. Associates Inc., St. Albans, VT) and an unconditional stimulus (two periods of 2-second foot-shock of 0.75 mAmp). Three days after the training session, mice were returned into the same chamber where training had occurred for a context test, during which no tones or foot-shocks were delivered. Freezing behavior in response to the context was
recognized by the software as a total lack of movement excluding breathing but including movement of fur, vibrissae and skeleton. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities.

**Systemic Inflammatory response**

After termination of both SF procedure and surgery (7 am on day one), blood was collected transcardially under terminal isoflurane anesthesia into heparin-coated syringes, Samples were centrifuged at 3400 rpm for 10 minutes and plasma was collected and stored at -80°C until assaying. Blood samples taken from animals without intervention served as controls. Plasma IL-6 was measured using a commercially available ELISA kit (Invitrogen, Grand Island, NY).

**Neuroinflammatory response to surgery**

After termination of both SF procedure and surgery (7 am on day one), the hippocampus was rapidly extracted under a dissecting microscope and placed in RNAlater™ solution (Qiagen, Valencia, CA, USA). Total RNA was extracted using RNeasy Lipid tissue Kit (Qiagen). Extracted RNA was treated with recombinant DNase I using a RNase-Free Dnase set™ (Qiagen). mRNA concentrations were determined with a ND-1000 spectrophotometer (NanoDrop® Thermo Fisher Scientific, Wilmington, DE) and mRNA was reverse transcribed to cDNA with a High Capacity RNA to-cDNA Kit (Applied Biosystems, Carlsbad, CA).

TaqMan Fast Advanced Master Mix (Applied Biosystems) and specific gene expression assays were used for qPCR as follows: beta-actin (ACTB) (NM_007393.1), IL-6 (Mm00446190_m1), TNF-a (Mm00443258_m1) and IL-1b (Mm01336189_m1). qPCR was performed using StepOnePlus™ (Applied Biosystems). Each sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle ΔΔCt and normalized to ACTB. Results are expressed as fold-increases relative to controls.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD).

For comparisons of more than two groups, means were compared using one-way analysis of variance (ANOVA) followed by t-tests with a Bonferroni-corrected alpha level. Based on previous freezing time data, we estimated that a sample of 10 C57BL/6J surgical mice per group was necessary to demonstrate a 20% increase in percentage freezing time, with 80% power at the 0.0125 alpha level (after adjusting for 4 comparisons) to reach a significant difference. A two-tailed p value < 0.05 was considered statistically significant for 2-group comparisons and the significance threshold was adjusted for multiple comparisons with a Bonferroni correction. Prism 6 (GraphPad Software Inc, La Jolla, CA) was used to conduct the statistical analyses.
RESULTS

Twenty-four hours of sleep fragmentation induces non-hippocampal memory dysfunction and increase in systemic IL-6

NOR test was assessed after 24h of SF. Sleep fragmented animals were unable to differentiate between the familiar and novel object (time of object interaction $6.02\pm3.43$ sec versus $5.56\pm3.08$ sec, ns) as opposed to the control group spending significantly more time at the novel object relative to the familiar ($7.23\pm2.61$ sec versus $11.93\pm5.88$ sec, $p<0.01$) (fig.2 NOR control vs SF). This dysfunction was associated with an increase in systemic IL-6 ($p<0.01$). (Fig.3 ELISA IL6 control vs SF).

Pre and postoperative SF enhanced neuroinflammatory response to surgery

Twenty-four hours after surgery or initiation of SF we observed a significant increase in hippocampal mRNA expression of IL-6 ($n=5$, $p<0.05$ and $p<0.01$) and TNF-α ($n=5$; $p<0.01$ and $p<0.001$, respectively). These changes were even more pronounced when pre or post operative SF was combined with surgery (fig. 4).

Perioperative SF did not affect surgery-induced memory impairment

Surgery significantly decreased the percentage of freezing time when compared to the control group ($49.30\pm5.77\%$ versus $30.26\pm3.27\%$ n=10, $p<0.001$). SF insult also induced cognitive decline ($49.30\pm5.77\%$ versus $32.90\pm5.80$, n=10, $p<0.01$). Memory impairment was not further exacerbated when SF is applied either pre ($43.91\pm13.12\%$) or postoperatively ($38.47\pm12.32\%$) (fig. 5).
CONCLUSIONS

Summary of results: dissociation between neuroinflammation and cognitive test

This study posits that SF induces significant changes that underlie memory, indicating a loss of cognitive function in non-hippocampal and hippocampal dependent domains. We show that while SF and surgery can independently produce significant memory impairment, perioperative SF significantly increased hippocampal inflammation (fig. 4) without further cognitive impairment (fig. 5). This peculiar dissociation between neuroinflammation and cognitive decline may relate to our use of memory paradigms that do not capture all aspects of cognition.

Sleep rebound and preconditioning effect

We chose a SF model that is characterized by a decrease in REM and NREM sleep. This type of sleep disturbance closely resembles both patients with OSA and also the type of sleep disorder reported in acute care facilities. During recovery, mice exhibit a rebound in REM sleep time and an increase in the depth of NREM sleep as measured by delta (1-4Hz) power in the EEG. Although SF and surgery separately produced memory dysfunction, when combined, cognitive function reverted back to the control state. It may be possible that by day three the rebound REM and NREM sleep is enough to restore the brain to a state that is capable of appropriately processing sensory input. It has been shown that rebound sleep shortly after stroke onset may be causally associated with neuroprotection through an ischemic preconditioning mechanism. The neuroprotective effects of acute sleep deprivation are in agreement with previous reports of neuroprotection and attenuation of microglial responses in rats subjected to global ischemia.

Caveats: SF method may induce stress

One of the caveats of our study is that sleep deprivation/SF methods can induce stress responses, which may affect memory, while some studies have shown that SF methods only modestly elevate plasma corticosterone. It could be argued that stress is a confounding factor, producing immobility and thus influencing the amount and extent of freezing in fear conditioning. Our study’s design attempts to address this concern by delaying the context session in the trace fear paradigm test by three days after surgery and/or SF; we also chose a SF method where animals are kept in their original environmentally enriched home cages, thus mitigating the amount of stress. From our previous work we have demonstrated that the surgical memory dysfunction phenotype is still present at day three while inflammatory markers are back to normal levels.

Stress corticosteroids may underlie some of the deficits identified in both sleep-fragmented humans as well as the animals in our model. Even if our model does have a stress response associated, it has been shown that this is also a normal response to sleep deprivation in humans. Cortisol levels are high early in the morning and low at the time of sleep onset and partial acute sleep loss, which delays the recovery of the hypothalamus-pituitary-adrenal from early morning circadian stimulation, is thus likely to involve an alteration in negative glucocorticoid feedback regulation. These data support the need to adopt interventions that can provide patients with appropriate rest in order to mitigate neuroinflammation. While we realize that the etiology of cognitive dysfunction in surgical/ICU patients is multi-factorial, if the restorative and reparative benefits of sleep mitigate the development of inflammation, this may result in shorter ICU or postoperative lengths of stay. Subsequent studies are warranted in order to both understand the mechanisms involved in the reversion of memory function after SF but also to improve our knowledge of POCD and ameliorate its adverse effects.
Study design. (A) First experiment; mice were divided in 2 groups: control versus 24h SF. The training session of the NOR memory test was performed immediately after cessation of SF procedure. (B) Second experiment; mice were divided in 5 groups and tested according to trace fear conditioning paradigm: control; 24h SF; surgery; preoperative SF; postoperative SF. The training session of the memory test was performed right before any intervention and the context session was performed 3 days later. (C) Third experiment; Mice were divided in 5 groups and sacrificed 24h after the start of the intervention: control; 24h SF; surgery; preoperative SF; postoperative SF. Downwards blue arrow represents tibia surgery. SF = sleep fragmentation, NOR = Novel Object Recognition.

Figure 2

Effect of 24h SF on non-hippocampal memory function assessed by using novel object recognition test.

Figure 3
Effect of 24h SF on systemic IL-6 serum concentration (n=5; ** p<0.01, with two tailed t-test). SF = sleep fragmentation.

**Figure 4**

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Effects of perioperative SF on hippocampal transcription of IL-6, TNF-α, IL-1β and MCP-1 24h after tibia surgery. (n=5; * p<0.05; ** p<0.01; *** p<0.001 with one-way ANOVA and Bonferroni post hoc analysis). SF = sleep fragmentation.

**Figure 5**

Effects of Perioperative SF on Cognitive Decline. Contextual fear response reveals hippocampal-dependent memory impairment at day 3. Quantification of the freezing time percentage according to the five groups (n=10; ** p<0.01, *** p<0.001, with one-way ANOVA and Bonferroni post hoc analysis).
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Sleep and Anesthesia: Common Mechanisms of Action
INTRODUCTION

“You are going to go to sleep now” is an oft-repeated colloquialism in every anesthesiologist’s daily practice. The phrase might be useful in allaying the fears of nervous patients, but does general anesthesia actually mimic sleep? Do they travel on the same neural pathways? To what degree does the comparison accurately reflect the underlying mechanisms involved?

Sleep, especially non–rapid eye movement (NREM) sleep, and anesthesia may use common neuronal and genetic substrates. Anesthetics act through sleep neural circuits but not necessarily in the same way.

AROUSAL PATHWAYS

To promote and sustain cortical arousal, neuronal pathways have developed two parallel ascending neuronal pathways. The first branch activates the thalamic relay neurons that are crucial for transmission of information to the cortex. Cholinergic signaling originating from the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei and the basal forebrain promote the cortically activated states of wakefulness and rapid eye movement (REM) sleep. The second branch bypasses the thalamus, activating neurons in the lateral hypothalamic area and basal forebrain (BF), and throughout the cortex. This pathway originates from monoaminergic neurons in the upper brainstem and caudal hypothalamus. The locus coeruleus (LC) provides norepinephrine-mediated inhibition of the ventrolateral preoptic (VLPO) nucleus in the hypothalamus. Therefore, γ-aminobutyric acid (GABA₉)-mediated and galanin-mediated inhibition of the ascending arousal circuits by the VLPO nucleus is inhibited and the awake state is promoted.

SLEEP PATHWAYS

Sleep is under the control of two processes, a circadian clock that regulates the appropriate timing of sleep and wakefulness across the 24-hour day and a homeostatic process (sleep homeostasis) that regulates sleep need and intensity according to the time spent awake or asleep. Sleep is a nonhomogeneous state that can be divided into NREM sleep and REM (paradoxical) sleep. The brain areas identified as important in sleep fall into 2
general groups: those with an arousing influence and active during wakefulness, namely the LC, dorsal raphe (DR), tuberomammillary (TMN), and BF; and those active primarily during sleep, namely the VLPO. The median preoptic area contains both wake-active and sleep-active neurons.

Discrete neurochemical changes accompany the different types of sleep with cholinergic (in brainstem and forebrain), noradrenergic (LC), and serotonergic (DR) all becoming less active in NREM sleep, whereas cholinergic activity increases in REM sleep. Activity in the VLPO is increased in NREM sleep and the GABAergic/galanin input from VLPO inhibits the histaminergic TMN nucleus. Orexinergic pathways from the perifornical nucleus are inactive during NREM sleep (Fig. 1).

The relatively quiescent LC facilitates a series of changes that includes activation of the galanin/GABA-containing neurons of the VLPO nucleus that terminate on and inhibit aminergic neurons within the tuberomammillary nucleus.

Anesthetic-induced unconsciousness results from specific interactions of anesthetics, with the neural circuits regulating sleep and wakefulness.

WHERE DO ANESTHETICS COLLIDE IN SLEEP PATHWAYS?

The currently available imaging techniques can only indirectly measure neuronal activity, for example through changes in blood flow, glucose metabolism, or oxygen concentration. One can then understand the difficulty in fully comprehending the mechanisms by which anesthesia induces sleep/unconsciousness. A common finding between NREM sleep and anesthesia in imaging studies is the deactivation of the thalamus leading to cortical inhibition. Anesthetic drugs induce unconsciousness by altering neurotransmission at multiple sites in the cerebral cortex, brainstem, and thalamus. The different actions of the different available anesthetics have made the understanding of the exact mechanism even more difficult. Effects of modern anesthetics on subsequent sleep behavior are known for some, but not all, anesthetics. No one electroencephalogram (EEG) pattern characterizes the anesthetized state. Different anesthetics and doses have distinct profiles with respect to the EEG activity.

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**Fig. 1.** Simplified NREM sleep-promoting pathway. An inhibition of noradrenergic neurons in the LC, which accompanies endogenous NREM sleep, releases a tonic noradrenergic inhibition of the VLPO. The activated VLPO is believed to release GABA into the TMN which inhibits its release of arousal-promoting histamine into the cortex, and thus induces loss of consciousness. A number of pathways are involved in NREM sleep; the sleep-active VLPO projects to all the ascending monoaminergic, cholinergic and orexinergic arousal nuclei (TMN, LC, DR, PPTg, LDTg, PeF), which project to the cortex where they release arousal-promoting neurotransmitters to promote wakefulness. (From Nelson LE, Guo TZ, Lu J, et al. The sedative component of anesthesia is mediated by GABA(A) receptors in an endogenous sleep pathway. Nat Neurosci 2002;5(10):979–84; with permission.)
Although some agents act on excitatory synapses, others act through potentiation of inhibitory synaptic receptors. The GABA\textsubscript{A} receptors are neurotransmitter-gated chloride channels that exist on cells that may also contain nicotinic acetylcholine receptors, glycine receptors, and serotonin type 3 receptors. Anesthetics such as propofol, etomidate, and barbiturates exert their effect through enhancement of GABA-mediated channel activation and prolong postsynaptic inhibitory currents, suppressing neuronal excitability. In brain regions containing neurons that promote wakefulness, GABAergic inhibition has been shown to cause an increase in sleep. These brain regions include the DR nucleus, TMN, medial preoptic area, and ventrolateral periaqueductal gray.\textsuperscript{6,7} In a series of studies involving GABAergic agents, it was reported that unlike in NREM sleep, these hypnotic agents did not alter noradrenergic activity in the LC (see Fig. 1).\textsuperscript{1} Instead these agents converged on the NREM sleep pathway at the level of the hypothalamus.\textsuperscript{8} However, short-term administration of the GABAergic agent propofol permitted normal recovery after a period of sleep deprivation.\textsuperscript{9,10}

At clinical concentrations, drugs such as N\textsubscript{2}O, xenon, and ketamine have little or no effect on GABA\textsubscript{A} receptors. Instead, these anesthetics potently inhibit N-methyl-D-aspartate (NMDA) receptors, which are excitatory cation channels activated by glutamate. These agents reduce excitatory signals in critical neuronal circuits, causing unconsciousness. Glutamate levels in the PPT are greater during wakefulness as opposed to NREM and REM sleep.\textsuperscript{11,12} The dissociative state that is produced by ketamine anesthesia can be in part attributed to the different regions to which ketamine promotes glutamate release (nucleus accumbens,\textsuperscript{13} prefrontal cortex,\textsuperscript{14} and anterior cingulate\textsuperscript{15}). It has been shown that isoflurane and sevoflurane reduce glutamate release\textsuperscript{16–18} and inhibit its uptake,\textsuperscript{19} but few in vivo studies elaborated on understanding the exact mechanism by which isoflurane modulates glutamatergic transmission.

REM sleep rebound after exposure to volatile anesthetics suggests that these volatile anesthetics do not fully substitute for natural sleep.\textsuperscript{20} In humans, isoflurane anesthesia alone (without surgery) results in no change in subsequent REM or NREM sleep, but a shift in NREM sleep from slow-wave sleep to lighter (I and II) stages.\textsuperscript{21} On the other hand, it has been shown that wake-active orexinergic neurons are inhibited by isoflurane and sevoflurane, and that waking up from anesthesia uses neural circuits distinct from those necessary to become anesthetized but similar to the neural circuitry that promotes arousal.\textsuperscript{22} Furthermore, isoflurane depresses serotonin levels on hypoglossal motoneurons in dogs\textsuperscript{23} and on mice hippocampus.\textsuperscript{24}

The molecular targets for dexmedetomidine are central \(\alpha\textsubscript{2}\)-adrenergic receptors. It has been shown that \(\alpha\textsubscript{2}\)-agonists transduce its hypnotic response after binding to the \(\alpha\textsubscript{2A}\)-receptor subtype\textsuperscript{25} in the LC.\textsuperscript{26} Through signaling processes that involve both pertussis toxin–sensitive G proteins\textsuperscript{27} and effector mechanisms that include inhibition of adenylyl cyclase\textsuperscript{27} and ligand-gated calcium channels as well as activation of inwardly rectifying potassium channels,\textsuperscript{28} the noradrenergic neurons become hyperpolarized and are less likely to achieve an action potential. \(\alpha\textsubscript{2}\)-Agonists such as dexmedetomidine are associated with similar changes in neuronal activity, as is seen in deeper stages of NREM sleep\textsuperscript{2,29} apart from the absence of inhibitory effect on the orexinergic neurons in the perifornical nucleus.\textsuperscript{8} A functional magnetic resonance study showed that a thalamic nucleus, which receives afferent input from orexinergic neurons, is activated during an arousal stimulus in \(\alpha\textsubscript{2}\)-agonist–sedated subjects.\textsuperscript{30} Children sedated with dexmedetomidine showed an EEG pattern that was identical to that seen in stage 2 NREM sleep.\textsuperscript{31}

Although acetylcholine (ACh) plays a primary role in generating the brain-activated states of wakefulness and REM sleep, cholinergic receptors are not a main target of common anesthetics. Nonetheless, ACh interacts with other transmitter systems that are targets of sleep pharmacology, for example the GABAergic agents. The clinical finding that physostigmine (acetylcholinesterase inhibitor) reverses propofol sedation, causing arousal, suggests that propofol produces unconsciousness, in part, by disrupting cholinergic neurotransmission.\textsuperscript{32} In vitro studies showed that propofol, isoflurane, sevoflurane, and ketamine inhibit muscarinic and nicotinic ACh receptors,\textsuperscript{33–37} providing support that these agents cause sedation, in part, by inhibiting cholinergic neurotransmission in brain regions that regulate arousal.

**CIRCADIAN RHYTHM**

The 2-process model of sleep homeostasis as described by Borbely and Wirz-Justice\textsuperscript{38} integrates sleep debt (“process-s”) and circadian rhythm (“process-c”). This model implicates circadian rhythm and sleep as intertwined, codependent processes. Experimentally, distinguishing process-c from process-s presents a challenge in deconstructing causative factors in sleep disorders. Nevertheless, there is a growing body of evidence that suggests circadian rhythm can be altered independent of sleep deprivation and that anesthetics can specifically change circadian rhythm.
Vacas et al

Circadian rhythmicity is thought to be controlled by the suprachiasmatic nucleus and is established by processing external cues (zeitgebers), such as light, into systemic mediators, such as temperature, adrenergic signaling, and circulating hormones (eg, cortisol and melatonin). This process serves to maintain a diurnal pattern, presumably to coordinate intracellular or intersystem function by resynchronizing intrinsic cellular molecular clocks. In brief, the molecular core of the circadian clock involves the heterodimerization of CLOCK and BMAL1, which act as the canonical arm of the clock, and the heterodimerization of PER1/2 and CRY1/2, which are critical components of the negative feedback arm; stabilizing proteins RORα, REV-ERB, DEC, DBP, and E4BP4 act as additional repressors or activators of the canonical arm, and these proteins oscillate through the day and translocate from the cytoplasm to the nucleus in a highly coordinated fashion to provide a reliable rhythm of approximately 24 hours. Disruption of circadian processes is being studied as a relevant contributing factor to multiple human conditions altering, among others, immunity.

CIRCADIAN RHYTHM AND ANESTHESIA

An initial indication that circadian rhythm is important in anesthetic delivery is the time-of-day variance in susceptibility to general anesthetics. Indeed, the greatest therapeutic effect of general anesthetics in animal models occurs during the animals’ rest phase for propofol and ketamine. A volatile anesthetic effect may also vary according to a diurnal pattern; halothane administration in rats varied with a lower minimum alveolar concentration requirement during the rest phase in comparison with the active phase. Recently, using bees as a model system, 6-hour isoflurane administration during the rest phase failed to alter the circadian activity patterns of the hive, whereas isoflurane administered in the active phase significantly altered the circadian activity of the hive. Taken together, the time-of-day administration of anesthesia is likely important in both the dose-dependent effect and the maintenance of circadian rhythm.

As parameters for outlining circadian rhythm, cortisol and melatonin levels can be used to make assumptions about the effect of general anesthesia on daily cycling in human subjects. Most human studies following these variables involve general anesthesia with the confounding aspect of surgery, and do not incorporate the natural underlying cycling of these hormones adequately. Given these and other significant caveats, a propofol-based anesthetic appears to decrease the amount of plasma cortisol during surgery in comparison with sevoflurane. Postoperatively, following a thio- pental/propofol-based or thiopental/isoflurane-based anesthetic, cortisol levels are elevated in men who underwent long-duration surgery for larynx or pharynx cancer. Conversely, in women undergoing laparoscopic procedures for pelvis surgery, anesthesia with thiopental/propofol reduced amounts of cortisol 2 to 4 hours after surgery compared with thiopental/isoflurane anesthesia. Given that these studies were designed to investigate the anesthetic effects on stress responses and not on circadian rhythm directly, there remains only a suggestion that altered cortisol levels may interfere with circadian rhythm after anesthesia and surgery.

Investigation into the effect of anesthesia on the cycling of melatonin points more directly to a circadian rhythm effect. A comparison of general anesthesia (thiopental/isoflurane) with spinal anesthesia for orthopedic procedures showed a significant reduction in melatonin levels in the first postsurgical night compared with baseline levels; interestingly, there was no significant difference in the reduction of melatonin between the experimental groups, indicating that the effect was independent of the anesthesia, and pointing to the possibility of a significant surgical influence of postoperative opiate use on melatonin secretion. In patients undergoing general anesthesia (thiopental/isoflurane/fentanyl) for laparoscopy, a modest reduction in 13-hour average melatonin secretion was noted in the evening after surgery compared with the presurgical night, with a large increase in melatonin secretion the second night after surgery. Corroborating these data, in patients undergoing major abdominal surgery with general anesthesia and concomitant use of a thoracic epidural, there was a similar modest reduction in basal melatonin secretion on the first postoperative day, followed by a significant increase on the second postoperative day. Following urine metabolites of melatonin (aMT6s), general anesthesia (thiopental/propofol) decreased the maximal concentration and delayed the peak of aMT6s. Short mask-inhalation anesthetics (21 minutes average duration) for dilation and curettage showed no difference in melatonin secretion compared with nonsurgical controls. Experimental models allow for more precise examination of melatonin secretion and general anesthesia apart from surgical effect. Rats anesthe- tized with propofol for 25 to 30 minutes around the peak of serum melatonin secretion showed a significant reduction in melatonin secretion for the 3 hours following anesthesia, a subsequent increase 20 hours after the anesthetic, and a phase advance of cyclical melatonin secretion of 40 minutes.
consistent with the approximate duration of anesthesia. Whether in humans or rats, it seems consistent that melatonin levels are reduced in the immediate postoperative/anesthetic period with a rebound phenomenon observed thereafter, although it remains unclear in human subjects which component of the observed effect can be attributable to either surgery or anesthesia.

The effect of anesthetics on intrinsic molecular circadian clocks is beginning to be explored in experimental models. In rats, 6 hours of sevoflurane anesthesia changed the expression pattern of approximately 1.5% of 10,000 genes surveyed. Of interest, the expression of Per2 was the only circadian protein in the brain that was significantly reduced after the anesthetic. The effect of reduced expression of Per2 and an auxiliary clock geneDbp persists for 24 hours. Infusions of both propofol and dexmedetomidine likewise reduced the expression of Per2 in rat brain 6 hours after anesthetic delivery, but the effect persisted for only 24 hours in the case of dexmedetomidine. Further investigation demonstrated that a 4-hour sevoflurane anesthetic blunted Per2 mRNA production in the suprachiasmatic nucleus in response to a light stimulus, and created a delayed activity rhythm in anesthetized mice. Repression of Per2 expression by sevoflurane anesthesia was most significant when administered between the hours of 8 AM and 12 PM in comparison with other time points, but activity patterns of anesthetized animals were delayed for all time points of anesthetic administration. Bees anesthetized with a 6-hour course of isoflurane during the day had a reduction in the amplitude of Cry expression and phase delay of both Cry and Per2 expression in whole bee brains in comparison with bees anesthetized during the evening, leading to alterations in circadian governed homing patterns and foraging times. With respect to the molecular clock, general anesthesia appears to significantly affect critical clock proteins in a time-of-day–dependent fashion, and corresponds to changes in activity patterns consistent with circadian disruption.

CIRCADIAN CONTROL OF IMMUNE FUNCTION

A separate line of investigation has focused on the influence of circadian rhythm on immune function. Clinically, timing is relevant in terms of susceptibility to infection, asthma attacks, or flares of rheumatic arthritis, all of which suggest circadian principles underlying these immune-mediated processes.

Circulating pools of many immune cells’ cycle throughout the day indicate the influence of circadian timing. Natural killer (NK) cells peak in both activity and numbers in the human circulation in the early morning, and likely marginate away from circulation during other times of the day. In mouse models, after lipopolysaccharide (LPS) administration, macrophages circulate cyclically to the spleen. Of importance, the circadian molecular clock exists and functions in NK cells, macrophages, dendritic cells, and B cells. Indeed, approximately 8% of the macrophage genome is classified as falling under the control of circadian transcription factors and critical immune transcription factors such as signal transducer and activator of transcription family (STATs) and nuclear factor κB (NF-κB) are regulated by clock proteins. Study of the functional consequence of disturbing circadian molecular clock proteins in immune subsets is generating interesting data. Injection of LPS at specific time points caused significantly elevated cytokine production in both macrophage-restricted Bmal1 knockout (KO), and systemic Rev-Erbα KO mice, compared with the chronometric 12-hour opposite time point (antiphasic control). Cry1/2 double KO mice have elevated NF-κB activity causing increased baseline inflammatory cytokine expression in vitro, and generated greater inflammation when challenged with LPS in vitro and in vivo. Examining lymphocyte function has similarly elucidated at least some aspect of circadian gating. Per2 KO mouse lymphocytes had a robust increase in proliferative capacity after being immunized in vivo when compared with their antiphasic control. Isolated T cells cultured from mouse lymph nodes proliferated after stimulation in a circadian manner, an effect that was abolished in Clock mutant mice. Whether by observation or direct experimentation these data showed that certain immune cells traffic according to apparent circadian parameters, possess oscillating intrinsic molecular clocks, have critical transcription factors controlled by clock proteins, and have altered function when clock proteins are perturbed.

SLEEP DISRUPTION AND COGNITIVE DYSFUNCTION IN SEDATED PATIENTS IN THE INTENSIVE CARE UNIT

Sleep disruption in critically ill patients is a common occurrence in the intensive care unit (ICU), with the potential to adversely affect patients’ outcome and also to provide a direct financial detriment with respect to the length of hospital stay and depletion of health care resources. Early polysomnographic studies had revealed extreme sleep disruption in ICU patients with decreases in total sleep time, altered sleep
architecture (predominance of stage 1 and 2 sleep, decreased or absent stages 3 and 4 NREM and REM sleep), and sleep fragmentation\textsuperscript{72,73}; also, up to 50\% of the total sleep time occurred during daytime. Among the possible causes that contribute to sleep disruption in the ICU are those related to the patient’s acute illness and comorbidities, environmental factors (including noise and inappropriate light), and iatrogenic factors including frequent care-related interruptions and medications prescribed for analgesia and sedation.\textsuperscript{74,75} Among those that are potentially amenable to modification, excessive noise does not contribute as much as was anticipated,\textsuperscript{76} and attention has focused on sedative practices.\textsuperscript{77} Sedative-hypnotic agents are widely used to facilitate sleep in the ICU; however, depending on the sedative agent, it may not produce appropriate sleep hygiene and instead will aggravate the problem by producing fewer of the restorative properties of natural sleep.

Several studies have now demonstrated the association between the use of benzodiazepines (BZDs) and increased incidence\textsuperscript{78} and duration\textsuperscript{79} of delirium in medical ICU patients, although the relationship of the development and duration of delirium with sleep disruption was not ascertained. Acute withdrawal from long-term sedation with BZDs and opiates results in profound sleep disruption.\textsuperscript{80} The pivotal work of the MENDs trial\textsuperscript{78,81,82} indicated the benefits of a specific anesthetic agent, dexmedetomidine, in the outcome of the ICU population.

**SUMMARY**

Appropriate sleep hygiene is crucial for repair in states of disease and injury, and in restoring function especially in the central nervous and immune systems. Lack of sleep hygiene results in cognitive dysfunction, contributes to the delirium that is prevalent in patients within the ICU, adversely affects immunity, and independently increases both morbidity and mortality. Anesthetics used in hospital care have different action targets and, ultimately, different consequences. Those which act by modulating the GABA\(_A\) receptor converge at the level of the hypothalamus, whereas \(\alpha_2\)-adrenergic agonists converge on sleep pathways within the brainstem. Thus, thoughtful attention must be paid to selecting an anesthetic agent that best mimics natural sleep. Future studies will further elucidate the benefits of dexmedetomidine as a good anesthetic/sedative candidate to mimic natural sleep.

While the fields of investigation into the anesthetic effect on circadian rhythm and the circadian influence on immune function remain disparate, there exists the plausible concatenation that anesthetics, by altering circadian rhythm (possibly independent of sleep deprivation), affect immune function. Given that anesthetics are often used adjuvatively to facilitate therapeutic interventions, it would be worthwhile to elucidate whether more precise applications of anesthesia could improve immunologic outcomes for patients who require them.

Anesthesia is not the same as sleep. The actions of anesthetics on sleep pathways and the restorative properties of natural sleep for the central nervous system are undeniable and essential, yet they also advance a concomitant advantage to the immune system, with fewer infections and a greater likelihood of survival from sepsis.

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Cerebral Ischemia
Bone Fracture Exacerbates Murine Ischemic Cerebral Injury

Participated in study design, conducted qPCR experiments and participated in data analysis and interpretation and final preparation of the manuscript.
Bone Fracture Exacerbates Murine Ischemic Cerebral Injury


ABSTRACT

Background: Bone fracture increases alarmins and proinflammatory cytokines in the blood, and provokes macrophage infiltration and proinflammatory cytokine expression in the hippocampus. We recently reported that stroke is an independent risk factor after bone surgery for adverse outcome; however, the impact of bone fracture on stroke outcome remains unknown. We tested the hypothesis that bone fracture, shortly after ischemic stroke, enhances stroke-related injuries by augmenting the neuroinflammatory response.

Methods: Tibia fracture (bone fracture) was induced in mice one day after permanent occlusion of the distal middle cerebral artery (stroke). High-mobility-group box chromosomal protein-1 (HMGB1) was tested to mimic the bone fracture effects. HMGB1 neutralizing antibody and clodrolip (macrophage depletion) were tested to attenuate the bone fracture effects. Neurobehavioral function (n = 10), infarct volume, neuronal death, and macrophages/microglia infiltration (n = 6–7) were analyzed after 3 days.

What This Article Tells Us That Is New

- In mice, infarct volume from experimental stroke was 2.5-fold larger if a bone fracture was performed 1 day after stroke
- Depleting macrophages and neutralizing high-mobility-group box chromosomal protein-1 attenuated the aggravating effects of bone fracture on stroke

What We Already Know about This Topic

- As many as 250,000 patients annually throughout the world will suffer a bone fracture within a day after stroke, but the impact of bone fracture on recovery from stroke has not been examined

Results: We found that mice with both stroke and bone fracture had larger infarct volumes (mean percentage of ipsilateral hemisphere ± SD: 30 ± 7% vs 12 ± 3%, n = 6, P < 0.001), more severe neurobehavioral dysfunction, and more macrophages/microglia in the periflaxt chart region than mice with stroke only. Intraperitoneal injection of HMGB1 mimicked, whereas neutralizing HMGB1 attenuated, the bone fracture effects and the macrophage/microglia infiltration. Depleting macrophages with clodrolip also attenuated the aggravating effects of bone fracture on stroke lesion and behavioral dysfunction.

Conclusions: These novel findings suggest that bone fracture shortly after stroke enhances stroke injury via augmented inflammation through HMGB1 and macrophage/microglia infiltration. Interventions to modulate early macrophage/microglia activation could be therapeutic goals to limit the adverse consequences of bone fracture after stroke.

S TROKE is the leading cause of disability in adults¹ and an important risk factor for bone fracture.² In the United States, ~70,000 stroke victims suffer from bone fracture within the first year after their stroke.³ A small proportion of these stroke patients experience bone fracture within the first 24 h after the ischemic stroke, but the impact of bone fracture on acute stroke lesion is unknown. In a database cohort study including more than 270,000 European patients hospitalized for stroke from 1987 to 1996, the authors found that overall, 9% of the stroke patients had a subsequent fracture.³ Interestingly, the increase in risk was most evident immediately
after the stroke. Based on the data provided by Kanis et al., the estimated incidence of having a bone fracture within 24 h of a stroke is 2.4–3.6/100,000, corresponding to 1–1.5% of stroke patients. Thus, about 7,000–11,000 patients in the United States and 167,000–250,000 worldwide will experience a fall-fracture within the first day of the stroke. More recently, an American study confirmed that the hazard ratio of suffering from a hip fracture during the first 24 h after the stroke diagnosis was significantly increased by 3.9 (95% CI of 2.1–7.3), when compared to that of a nonstroke population.3

Our retrospective review of more than 400,000 surgical patients revealed that stroke is an independent risk factor for poor outcome after orthopedic bone surgery but not abdominal aortic surgery,5 suggesting a specific interaction between stroke and bone surgery. Understanding the impact and underlying mechanism of this interaction will enable clinicians to intervene appropriately and to design target-selective neuroprotective strategies perioperatively.

In a model of aseptic bone fracture, circulating alarmins, including high-mobility-group box chromosomal protein-1 (HMGB1) and proinflammatory cytokines, increase; in addition, aseptic bone fracture provokes macrophage infiltration and proinflammatory cytokine expression in the hippocampus. HMGB1, a nonhistone DNA-binding protein, stabilizes nucleosome formation and DNA repair; additionally, it activates pattern recognition receptors (e.g., toll-like receptors 2 and 4, and the receptor for advanced glycation end-products) on bone marrow-derived monocytes and macrophages to initiate an innate immune response. Because systemic and local inflammation in the acute phase of ischemic stroke may have deleterious effects on stroke outcome, and because the risk of bone fracture in the stroke population is mainly just after the brain insult, we tested the hypothesis that bone fracture, one day after ischemic stroke, aggravates brain damage and functional consequences of stroke.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, and conformed to the National Institutes of Health Guidelines. All animals were fed standard rodent food and water ad libitum, and were housed (5 mice per cage) in sawdust-lined cages in an air-conditioned environment with 12-h light/dark cycles.

Wild-type male mice (C57BL/6J, 10–12 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). C-C motif Chemokine receptor-2 red fluorescent protein (CCR2RFP/+) CX3C chemokine receptor 1 (fractalkine receptor) fluorescein (CX3CR1GFP/+), mice (10–12 weeks old) were provided by Katerina Akasoglou, Ph.D.; Israel F. Chao, M.D., Ph.D.; and Kim Baeten, Ph.D. (Associate Investigator, Associate Director, and Postdoctoral Fellow, respectively, University of California, San Francisco Gladstone Institute, San Francisco, CA). CCR2 and CXCR1 are acronyms for chemokine (C-C motif) receptor 2 (monocyte chemotactic protein-1), highly expressed in bone marrow-derived macrophages, and CX3C chemokine receptor 1 (fractalkine receptor), highly expressed in resident microglia, respectively.

Animals were tagged and randomly allocated to each group before any treatment. Researchers blinded to the group assignment performed all neurobehavioral tests, infarct volume, and cell counting. Based on preliminary data, in corner tests, there was a standard deviation of 15% in the percentage of left turns 3 days after permanent middle cerebral artery occlusion (pMCAO). We estimated that a sample of nine mice per group was necessary to find a significant difference between the pMCAO mice and the pMCAO+bone fracture mice with 80% power if the difference was 20%. For this reason, we included n = 10 mice per group for each behavior test’s comparison.

Human Blood Samples

Under an approved protocol by the University of California, San Francisco Committee on Human Research (Study number: H5636-20263-09), four individuals presenting with osteoarthritis elective for total knee replacement under spinal anesthesia were enrolled. Blood was drawn immediately before and after the tourniquet was released using an uncoated tube. Blood samples were centrifuged at 1300 rpm for 10 min at room temperature and the serum samples were immediately frozen at −80°C.

pMCAO for Stroke Model

Following anesthesia (isoflurane, 2%), under aseptic surgical condition, animals received a left craniotomy and a dissection of the dura. The left middle cerebral artery was permanently occluded (pMCAO) using electrical coagulation just proximal to the pyriform branch. Rectal temperature was maintained at 37 ± 0.5°C using a thermal blanket throughout the surgical procedure. Surface cerebral blood flow was monitored during the procedure using a laser Doppler flowmeter (Vasamedics Inc., Little Canada, MN). Mice were excluded from further analysis when the surface cerebral blood flow in the ischemic core was more than 15% of the baseline after pMCAO, or if the artery injuries with the coagulator generated a massive bleeding. Animals were allowed to recover spontaneously from the anesthetic under warm conditions and received one intraperitoneal injection of buprenorphine (0.3 mg in 100 μl saline). Control mice were subjected to craniotomy without arterial occlusion but with the same amount and duration of anesthesia and the same amount of buprenorphine (0.3 mg in 100 μl saline) used for stroke mice. In this study, a total of 6 C57BL/6J mice were euthanized during the pMCAO procedures due to massive bleeding induced by vascular surgical injury, and were replaced by other mice from the same cage. No mouse was lost during the experiment’s 3-day duration.

Tibia Fracture Surgery for Bone Fracture Model

Twenty-four hours after the pMCAO procedure, animals were given general anesthesia with 2% isoflurane inhalation. Under aseptic surgical conditions, animals received an open tibia
fracture of the right hind limb with an intramedullary fixation, as previously described.6 Animals were allowed to recover spontaneously from the anesthetic under warm conditions and received one intraperitoneal injection of buprenorphine (0.3 mg in 100 μl saline). Rectal temperature was maintained at 37 ± 0.5°C using a thermal blanket throughout the surgical procedure. Repeated measurements of arterial systolic blood pressure were performed using the tail-cuff method (ML125M, AD Instruments, Colorado Springs, CO) as previously described.15

The mice subjected to craniotomy and pMCAO had similar tail arterial systolic blood pressure before the bone fracture procedure (data not shown). Control mice for bone fracture received hind limb hair shaving with the same amount and duration of anesthesia and analgesia (buprenorphine, 0.3 mg in 100 μl saline) as for the bone fracture mice. Body weight of the animals was measured before pMCAO and immediately after the neurobehavioral tests. Mice with pMCAO alone presented a significant loss of weight when compared to control mice, but the groups of mice with pMCAO with and without bone fracture were not different. HMGB1 neutralized antibody and clodrolip did not influence the loss of body weight (data not shown). The tibia fracture surgery did not present any lethality.

Chemical Reagents
Based on serum levels of HMGB1 after mice tibia fracture,7 we injected 50 μg/kg (100 μl) of recombinant HMGB1 (R&D System, Minneapolis, MN) intraperitoneally 24 h after the stroke. Control animals received the same volume (100 μl) of the vehicle (saline). To neutralize HMGB1 in the blood, we injected anti-HMGB1 antibodies (chicken IgG, IBL International, Toronto, Canada), 200 μg in 100 μl (corresponding to 10 mg/kg) intraperitoneally, 60 min before the bone fracture. Control animals received the same volume (100 μl) of the control chicken IgG antibodies (IBL International, 10 mg/kg). Clodronate liposomes (clodrolip) were obtained from clodronate liposomes.org (Vrije Universiteit, Amsterdam, Netherlands) at 7 mg/ml concentration and prepared as previously described elsewhere.16,17 Clodrolip (200 μl, about 100 mg/kg) was injected intraperitoneally 60 min before the bone fracture. Control animals received 200 μl of control liposomal solution.

Behavioral Tests
All tests were conducted 3 days after the pMCAO. Corner Test. As previously described,18 the corner test was used to detect sensorimotor and postural asymmetries. Mice were placed between two boards with identical dimensions (30 × 20 cm). When mice near the corner, both sides of their vibrissae were stimulated. The mice would rear forward and upward, and then turn back to face the open end. Normal mice would turn to the left or right side with equal frequency, whereas the stroke mice would turn more often to the ipsilateral side of the lesion (left). The percentage of left turns was recorded in three different sets of 10 trials. Turning movements not incorporated in a rearing movement were not recorded.

Adhesive Removal Test. To assess the forepaw lateral sensitivity and point out a possible somatosensory neglect, we performed the adhesive removal test.19 Briefly, adhesive tape (0.3 × 0.3 cm) was applied on each paw. The time that it took for the mice to remove the tape from each paw was recorded with a maximum testing time of 120 s. Mice were trained three times daily for 4 days before the surgery to obtain an optimal level of performance.

Evaluation of Infarct Volume
Three days after the pMCAO, brain samples were collected after paraformaldehyde 4% perfusion. A series of 20-μm-thick coronal sections was obtained. One in every 10 sections was stained with cresyl violet (one section per 200-μm-thick tissue) and sections were digitized. After binary imaging, the infarct and the ipsilateral hemisphere areas were outlined using Image J software (National Institutes of Health, Bethesda, MD) and then measured.20 The infarct and ipsilateral hemisphere volumes were estimated as the sum of each area multiplied by 200 μm. The ratio of infarct volume versus ipsilateral hemisphere volume was calculated.

Measurement of Mice HMGB1 in Serum
Six hours after the bone fracture procedure, blood was collected by cardiac puncture under general anesthesia (isoflurane, 3%). Blood samples were centrifuged at 1300 rpm for 10 min at room temperature and the serum was collected and frozen at –80°C. HMGB1 levels in the serum of the mice and the human samples were quantified using the HMGB1 ELISA kit (IBL International).

Measurement of Cytokines in the Brain Lesion
The left frontal-parietal cortical region of the mice was rapidly collected under a dissecting microscope 6 h after the bone fracture, and placed in RNAlater solution (Qiagen, Valencia, CA). To avoid blood contamination, mice were perfused with saline for 5 minutes before sample collection. Total RNA was extracted using RNeasy lipid tissue kit (Qiagen) treated with recombinant DNase I using a RNase-Free DNase Set (Qiagen), and reverse-transcribed to complementary deoxyribonucleic acid with a High Capacity RNA to cDNA Kit (Applied Biosystems, Carlsbad, CA). TaqMan Fast Advanced Master Mix (Applied Biosystems, CA) and gene specific primers and probes used for real-time polymerase chain reaction are beta-actin (NM_007393.1), interleukin-6 (IL-6, Mm00443258_m1), and tumor necrosis factor-α (TNF-α, Mm00443258_m1). Real-time polymerase chain reaction was performed using StepOnePlus (Applied Biosystems). Each RNA sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle ΔCT and normalized to beta-actin. Results are expressed as fold increases relative to controls.

Histological Analysis 3 Days after Ischemia Onset
Immunohistochemical staining was performed using a series of 20-μm-thick coronal sections. All the quantifications were

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performed using the sections in the same anatomical region (bregma 1.2–1.4 mm). For immunostaining, sections were incubated with the following primary antibodies: CD68 (1:50, AbD Serotec, MCA1957, Raleigh, NC) and neuronal nuclei (1:500, MAB377, Millipore, Bedford, MA). Sections were then incubated with Alexa Fluor 647-conjugated, Alexa Fluor 594-conjugated, and Alexa Fluor 488-conjugated IgG (1:500, Invitrogen, Carlsbad, CA). Negative controls were performed by omitting the primary or the secondary antibodies in the staining procedures. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay was performed using the dedicated kit (ApopTag, Millipore) per instructions in the manual. The neuronal nuclei-TUNEL double staining was verified with confocal imaging and quantified using image J at the periflament region inside the cortical infarct border, using three different pictures per mice under 40× objective (fig. 1). CD68+ cells, CCR2+ cells, CX3CR1+ cells, and CCR2+ with CX3CR1+ double-positive cells were counted separately, using three different pictures per mice taken under 40× objective (fig. 1) at the periflament region outside the infarct border. CD68 staining and expression of CCR2-RFP and CX3CR1-GFP were verified using confocal images (data not shown) and quantified using Image J with three different pictures/mice taken under 40× objective. The effectiveness of clodrolip in the depletion of macrophages was verified by CD68 staining of 20-μm-thick spleen sections.

Statistical Analyses
Data are presented as mean ± SD. Gaussian distribution was tested with d’Agostino and Pearson omnibus normality test. Equalities of variances were tested with the F test. For dual comparisons, t tests (Student and Mann–Whitney for non-Gaussian distribution, and Welch correction of unequal variances) were used when appropriate. For multiple comparisons, means were compared using one-way ANOVA followed by Bonferroni post hoc correction.

Comparison of the human and mice HMGB1 expressions before and after bone fracture was performed with two-way ANOVA. Correlations were analyzed using the Pearson r coefficient. A two-tailed P value < 0.05 was considered statistically significant. Prism 5 (GraphPad Software Inc., La Jolla, CA) was used to conduct the statistical analyses.

Results
Bone Fracture Aggravates Functional and Morphological Consequences of Stroke
Mice with stroke alone exhibited neurobehavioral deficits, e.g., increased time to remove adhesive on the contralateral right paw and more turns to the lesion side (left turn) in the corner test, than sham-operated mice subjected to craniotomy only (fig. 1, A and B). These neurobehavioral abnormalities of stroke were significantly worsened by bone fracture, with longer latency times to remove the adhesive on the contralateral (P < 0.001, fig. 1A) paw and higher percentage of left turns in the corner test (P < 0.001, fig. 1B). Bone fracture alone did not affect neurobehavioral function, and the time to remove adhesive on the ipsilateral paw was not increased in the groups of mice with stroke alone and bone fracture alone (data not shown).

Compared to mice with stroke alone, mice with both stroke and bone fracture had infarct volumes almost three times larger (P < 0.001, fig. 1, C and D) and more TUNEL positive neurons in the periflament region right inside the border of the cortical infarct area (fig. 1, E and F).

Bone Fracture Shortly after Stroke Exacerbates Brain Inflammation
Thirty hours after occlusion, mice with stroke alone exhibited higher transcript expression than controls in the left hemisphere (lesion side) for IL-1β and tumor necrosis factor-α, but not for IL-6 (fig. 2, A–C). Six hours after the bone fracture (corresponding to 30 h after the stroke), mice with both injuries had higher transcript levels of IL-6 in the left hemisphere than mice with stroke (P = 0.01, fig. 2A). The IL-1β level also trended higher in mice with both injuries than mice with stroke alone (fig. 2B, P = 0.09). Furthermore, mice with both injuries presented higher transcript expression for IL-6, IL-1β, and tumor necrosis factor-α than mice with bone fracture only (fig. 2, A–C).

Using CCR2<sub>GFP</sub>+/CX3CR1<sub>GFP</sub> mice, we demonstrated that bone fracture increased both bone marrow-derived CCR2<sup>+</sup> macrophages (P = 0.01) and resident CX3CR1<sup>+</sup> microglia in the periflament region (P < 0.001), compared to mice with stroke only (fig. 2, D and E). About 20% of the CCR2<sup>+</sup> cells and ≈10% of the CX3CR1<sup>+</sup> cells expressed both CX3CR1 and CCR2 (data not shown). Mice with both stroke and bone fracture showed a trend toward more double-positive cells than mice with stroke only. The number of CCR2<sup>+</sup> cells positively correlated with CX3CR1<sup>+</sup> cell number (r = 0.40, P = 0.03). CCR2<sup>+</sup> macrophages and active CX3CR1<sup>+</sup> microglia in the periflament region expressed CD68, whereas CX3CR1<sup>+</sup> cells in the contralateral hemisphere (inactive microglia) did not (data not shown)<sup>12</sup>; mice with both stroke and bone fracture had more CD68<sup>+</sup> cells in the periflament region than mice with stroke alone (P < 0.001, fig. 3, A and B). The ratio of CD68-positive cells correlated with the ratio of neuronal nuclei TUNEL-positive cells (fig. 3C; r<sup>2</sup> = 0.70, P < 0.001).

HMGB1 Plays a Causative Role in the Exacerbating Effects of Bone Fracture on Stroke Injury
HMGB1 increased in the serum within 6 h of bone fracture in both patients and mice (fig. 4, A and B). HMGB1 (50 μg/
Fig. 1. Bone fracture enhances neuronal injury. A, Quantification of the time used to remove the tape from the right paw (contralateral) in adhesive removal test. (n = 10, # P < 0.001 vs. mice subjected to sham procedures for stroke and bone fracture, § P < 0.001 vs. bone fracture group + P < 0.001 vs. stroke group). B, Quantification of corner test. (n = 10, # P < 0.001 vs. mice subjected to sham procedures for stroke and bone fracture, § P < 0.001 vs. bone fracture group, * P < 0.001 vs. stroke group). C, Representative images of cresyl violet-stained brain sections (bregma 1.3 mm, scale bar: 1 mm) of stroke (C1) or stroke and bone fracture mice (C2). The red squares correspond to the three regions used to quantify neuronal nuclei (NeuN)-terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end-labeling (TUNEL)–positive cells. The green squares correspond to the three regions used to quantify the CX3CR1, CCR2, and CD68 cells. D, The bar graph shows the quantification of infarct volumes (n = 7, * P < 0.001). E, Representative NeuN (red) and TUNEL (green) costained pictures of stroke (E1) or stroke and bone fracture mice (E2). Insert in E2 is a 3 dimensional reconstructed confocal image showing a nucleus stained positively for both NeuN and TUNEL (yellow). F, Bar graph shows the quantification of TUNEL+ neurons (n = 7, * P < 0.001).

kg), injected intraperitoneally 1 day after stroke, produced significant larger infarct volumes (fig. 4C) and more TUNEL-positive neurons in the infarct area than the saline-treated stroke mice (13 ± 2% vs. 43 ± 10%, P < 0.001, n = 6). HMGB1 treatment increased CD68+ cells in the perifract region (fig. 4D). HMGB1-treated stroke mice demonstrated more severe neurobehavioral dysfunction with increased adhesive removal time (fig. 4E) and percentage of left turns in the corner test (fig. 4F).

Intraperitoneal administration of HMGB1 neutralizing antibodies (10 mg/kg) immediately before bone fracture attenuated fracture-enhanced infarct volume (fig. 5A). HMGB1 neutralizing antibodies also attenuated TUNEL-positive neurons in the infarct area (47 ± 9% vs. 17 ± 4%, P < 0.001, n = 6), the number of CD68+ cells in the perifract region (fig. 5B), and the behavioral dysfunction (fig. 5, C and D).

Systemic Macrophages Play a Causative Role in the Exacerbating Effects of Bone Fracture on Stroke Injury

We selectively depleted macrophages through intraperitoneal injection of liposomal formulation of clodronate (100 mg/kg) (clodrolip) immediately before bone fracture. This intervention drastically reduced the number of CD68+ cells in the spleen (data not shown) and decreased by three times the number of CD68+ cells in the perifract region (P < 0.001, fig. 6A). Clodrolip treatment prior to the bone
Fig. 2. Bone fracture exacerbates brain inflammation. Relative mRNA expression of interleukin (IL)-6 (A), IL-1β (B), and tumor necrosis factor (TNF)-α (C) 6h after bone fracture and 30h after the stroke in the stroke lesion (n = 5; [A] * P = 0.01 vs. stroke group without fracture, § P = 0.01 vs. bone fracture group; [B] # P < 0.001 vs. sham procedures for stroke § P = 0.002 vs. bone fracture group). The mice with stroke and bone fracture showed a trend toward higher IL-1β in the brain tissue than the mice with stroke only (P = 0.09).

(D) Representative picture taken in the periinfarct region of CCR2^{RFP+}/CX3CR1^{GFP+} mice with stroke and bone fracture. D1. Low-magnified (scale bar: 100 μm) and D2, high-magnified (scale bar: 50 μm) images show that there are many CCR2+ (red) and CX3CR1+ (green) cells. Some cells are CCR2–CX3CR1 double positive (yellow).

(E) The bar graph shows quantification of the percentage of CCR2+, CX3CR1+, or CCR2&CX3CR1+ cells amount total (4',6-diamidino-2-phenylindole [DAPI]-positive nuclei) cells in the periinfarct region (n = 5, * P = 0.01 and ** P < 0.001).

Fig. 3. Bone fracture exacerbates the recruitment of activated macrophages. A, Representative images of CD68 antibody stained section. A1, A low magnified picture showing the border zone (B.Z.) and the periinfarct region outside (P.I.) and inside the border (core). CD68+ cells formed a band just outside the infarct border. scale bar: 100 μm A2 and A3 are representative pictures taken in the periinfarct region outside the infarct border of mice with stroke (A2) or stroke plus bone fracture mice (A3). Scale bar: 50 μm.

B, Bar graph shows the quantification of CD68+ cells in the periinfarct region (n = 7, * P < 0.001). C, Correlation between the numbers of CD68+ cells and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end-labeling (TUNEL)+ neurons (r² = 0.70, P < 0.001). DAPI = 4',6-diamidino-2-phenylindole.
Fig. 4. High-mobility-group box chromosomal protein-1 (HMGB1) injection mimics the negative impact of bone fracture on stroke injury. A, HMGB1 levels in the human serum before, and 3, 6, 9, 12, and 24 h after total knee replacement surgery. Red dots represent the levels in individual patients. The green curve shows the global shape of the time course (n = 4). B, Comparison of HMGB1 in human and mice serum before and after bone fracture (n = 4 for human and n = 5 for mice *P < 0.001 vs. before bone fracture). C, Intraperitoneal injection of HMGB1 increased the infarct volume of stroke mice (n = 6, *P = 0.003). D, Intraperitoneal injection of HMGB1 increased CD68+ cells in the perinfarct region (n = 6, *P < 0.001). E, Intraperitoneal injection of HMGB1 increased the time for stroke mice to remove the tape from the right paws (n = 10, *P < 0.001). F, Intraperitoneal injection of HMGB1 increased the percentage of left turn in the corner test (n = 10, *P < 0.001). DAPI = 4',6-diamidino-2-phenylindole; ip = intraperitoneal.

Fig. 5. Neutralized high-mobility-group box chromosomal protein-1 (HMGB1) antibodies attenuate the negative impact of bone fracture on stroke injury. A, Representative images of cresyl violet-stained brain sections (bregma 1.3 mm, scale bar: 1 mm) of stroke mice with bone fracture procedure receiving control antibodies (A1, control antibody [CTab]) or anti-HMGB1 antibodies (A2, HMGB1 neutralized antibody [HMGB1ab]). B, The bar graph shows quantification of the infarct volumes (n = 7, *P < 0.001). C, Quantification of CD68+ cells in the perinfarct region (n = 6, *P < 0.001). D, Adhesive removal time of the right paw of mice treated with CTab or HMGB1ab (n = 10, *P < 0.001). E, Percentage of left turn in the corner test of mice treated with CTab or HMGB1ab (n = 10, *P < 0.001). DAPI = 4',6-diamidino-2-phenylindole.
Fig. 6. Depletion of macrophage reduces the negative impact of bone fracture on stroke injury. A, Representative picture taken in the perinfarct region (bregma 1.3 mm) of mice treated with control liposome (A1, control liposome [CT-lip]) and clodrolip (A2). Scale bar: 100 μm. B, A bar graph shows the quantification of CD68+ cells in the perinfarct region (n = 7, * P < 0.001). C, Quantification of infarct volume (n = 7, * P < 0.001). D, Quantification of adhesive removal time of the right paw (n = 10, * P < 0.001). E, Quantification of the percentage of left turn (n = 10, * P < 0.001). F, Synthesis of possible mechanisms underlying the negative impact of bone fracture on stroke injury: 1, Alarmins including high-mobility-group box chromosomal protein-1 (HMGB1) is released into blood after bone fracture. 2, HMGB1 interacts with its receptors on innate immune cells including macrophages. 3, Systemic macrophages are recruited to the stroke lesion site in the brain; together with activated microglia, they release neurotoxic molecules. 4, Exacerbation of neuroinflammation, neuronal cell death, and behavioral dysfunction. 5, Increased neuronal death further increases the release of alarmins and chemokines, triggering a vicious cycle through HMGB1-macrophage activation. DAPI = 4',6-diamidino-2-phenylindole.
fracture significantly reduced infarct volume (fig. 6B), neu- ronal injury (neuronal nuclei-TUNEL-positive cells of 37 ± 6% vs. 17 ± 4%, \( P = 0.001, n = 6 \)), and neurobehavioral dysfunction (fig. 6, C and D) in mice subjected to stroke and bone fracture.

Discussion

Sterile tissue injury, caused by surgery (fig. 4, A and B), ischemia–reperfusion, \(^{23}\) or hemorrhagic shock, \(^{24}\) provokes release of the alarmin HMGB1 that can initiate systemic inflammation and cause remote organ injury. \(^{25}\) Previously, we reported that circulating levels of HMGB1 in the serum of stroke mice with tibia fracture; additionally, some of the monocytes were positive for both CCR2 and CX3CR1. We postulate that after bone fracture, HMGB1 is released into the blood and interacts with pattern recognition receptors (toll-like receptors 2 and 4 as well as the receptor for advanced glycation end-products) on immunocytes, including macrophages. Together with activated microglia, systemic macrophages are recruited to the site of the stroke lesion where they are capable of releasing proinflammatory cytokines, which then exacerbate neuroinflammation that causes neuronal cell death and worsening behavior dysfunction in a feed-forward manner. Supporting these interpretations are data from the “sufficiency/necessity” experiments involving HMGB1 (figs. 4 and 5) and the necessity experiments involving systemic macrophages (fig. 6). Thus, our data demonstrate that bone fracture aggravates the functional and morphological consequences of stroke, and further suggest that this occurs through engagement of the innate immune response by the alarmin HMGB1 that likely enhances neuroinflammation in the peri-infarct region (fig. 4D).

Stroke is associated with increased risk of severe fall-related bone fracture, \(^{26,27}\) with 4–7% of patients suffering from bone fracture within the first year of their stroke. \(^{4}\) Previously, we reported that circulating levels of HMGB1 increase after aseptic tibia fracture in mice, and that this is accompanied by an influx of macrophages and expression of proinflammatory cytokines in the hippocampus \(^{6,7}\); others have shown that inflammation is an important modulatory factor in stroke-related injuries and poststroke recovery. \(^{11-13,28,29}\) Circulating levels of IL-6 in humans correlated positively with imaged brain infarct volume assessed by imaging analysis, and negatively with 1-yr survival. \(^{30}\) In our study, IL-6 transcript did not significantly increase in the ipsilateral hemisphere of stroke mice. However, subsequent bone fracture significantly increased messenger RNA expression of IL-6 in the ipsilateral hemisphere of stroke mice, which was accompanied by increased infarct volume and more severe neurobehavioral dysfunction. Our data support enhanced inflammation for mediating the negative impact of bone fracture on the functional and morphological consequences of stroke.

We acknowledge that the direct link between the number of CD68\(^*\) cells and the number of TUNEL\(^*\) neurons could not be determined by this study. However, we found that the depletion of the CD68\(^*\) cells with clodrolip reduced the ratio of TUNEL\(^*\) neurons to the total number of neurons. We have also found a significant correlation between the number of CD68\(^*\) cells and the TUNEL\(^*\) neurons (fig. 3C). Moreover, in vitro studies about macrophage/microglia’s activation showed that excitotoxic and/or inflammation stressors can induce neuronal toxicity. \(^{31,32}\) All together, the evidence suggests that the activation of macrophages/microglia can be involved in early neuronal cell death and thus, exacerbate lesions and behavioral dysfunction.

We found that both CCR2-RFP\(^*\) bone marrow-derived macrophages and CX3CR1-GFP\(^*\) microglia increased in the peri-infarct region of stroke mice with tibia fracture; additionally, some of the monocytes were positive for both CCR2 and CX3CR1. We showed that bone fracture increased CD11b-positive cells in the hippocampus. \(^{6,8}\) Using CCR2\(^{RFP^+}\)CX3CR1\(^{GFP^+}\) mice, we found that a majority of the infiltrated CD11b-positive cells are bone marrow-derived macrophages. \(^{8}\) In our study, both species of monocytes increased in the peri-infarct lesions and behavioral dysfunction. We do not know if intraperitoneal injection of clodrolip affects resident microglia in the brain. However, our data show that both macrophages and activated microglia are capable of playing important roles in bone fracture-induced exacerbation of stroke injury.

Limitations of the Study

We showed that bone fracture shortly after stroke enhances stroke injury via augmented inflammation through HMGB1 and macrophage/microglia infiltration. This experimental study mimics a relevant rare scenario, in which ischemic stroke patients suffer bone fracture within the first 24 h of the ischemic insult. \(^{3,4}\) Kanis et al. \(^{4}\) showed that ≈9% of stroke patients experienced a bone fracture after stroke and that 10–15% of the fracture occurs on the first day of stroke, which means that 1–1.5% of stroke patients will have a fracture on the first day of stroke. Interestingly, more than 80% of them would be older than 60 yrs. Because the elderly patient subgroup is on the rise in Western countries, this incidence should increase in the next decades.

These results give rise to questions and issues that need to be addressed in future studies. Because we report on only one time point, is it possible that bone fracture occurring at different time points, both before or after stroke, would have a different impact on stroke outcome? Could our neutralizing strategies regarding HMGB1 and systemic macrophage have blocked the alarmin and inflammatory response of the stroke itself? Given that HMGB1 has not only a neurotoxic effect in the acute stage of ischemic stroke, \(^{33}\) but also a protective effect in the latter stages of stroke, \(^{11,12}\) could an alternative timing of the...
administration of the neutralizing antibody have produced a different response? Shichita et al. recently showed that blocking the release of HMGB1 with neutralized antibody was only protective when it was given during the MCAO procedure but not 6 h after the ischemic injury. Even if these data argue against the use of neutralizing HMGB1 as a neuroprotective strategy for delayed stroke lesion, our data show that inhibiting HMGB1 appears to be effective in preventing a second increase of HMGB1 induced by a bone fracture surgery 24 h after the stroke lesion. Further studies are needed to evaluate the effect of bone fracture occurring at different time points (before the stroke or a longer time period after).

In this study, we found that two treatments (neutralization of HMGB1 and systemic macrophage depletion) were neuroprotective in our model of pMCAO+bone fracture, and our data suggest that this neuroprotection was induced by the reduction of the macrophage recruitment. However, these two strategies can also indirectly affect other proinflammatory pathways, such as trauma-induced hyperthermia, that could provide neuroprotection as well.

We measured the ratio of the immune cells (CD68, CX3CR1, and CCR2) in the perinfarct region just outside the infarct border, as shown in figures 1C and 3A. These measures were performed at the same coordinates for all the mice (bregma +1.3 mm) for consistency. It is important to note that the variation in the infarct size can affect the location of the infarct border. We cannot exclude the possibility that the quantification of the macrophages was influenced by the location of the infarct border.

Using CX3CR1-GFP; CCR2-RFP mice, we found that bone fracture increased the numbers of both CCR2+ and CX3CR1+ cells in the brain. For this reason, we considered that the increase in the two cell populations was associated with the phenotype, and thus decided to focus on the CD68+ cells. CD68 antibody stains for a lysosomal protein that is mainly expressed in the activated phagocytosis cells. In the normal brain, there are few CD68+ cells, whereas a significant number of CD68+ cells is present in the perinfarct side of the border zone of pMCAO. Although we cannot distinguish CCR2+ and CX3CR1+ cells by CD68 antibody staining, we were able to show that the neutralized antibody and the clodrolip treatments reduced CD68+ cells in the perinfarct side of the border zone.

We selected two well-established behavior tests that exhibit reproducibility in a pMCAO model. As shown in figure 1, tibia fracture alone did not significantly influence mouse performance in these tests. However, we could not completely rule out the effect of bone fracture on this function in mice with pMCAO and bone fracture, as these mice may have more severe hind-limb dysfunction due to increased inflammation in the fracture. Furthermore, even if we show that the frequency of neuronal cell death increases in mice with pMCAO and bone fracture, we cannot rule out that cortical edema induced by the activation of the neuroinflammation does not play a role in increased behavioral dysfunction.

In summary, we demonstrated that bone fracture shortly after ischemic stroke increases stroke-related neuronal injury and neurobehavioral dysfunction in mice. HMGB1 and macrophage/microglia play a causal role in the negative impact of bone fracture on stroke outcomes. Our findings regarding modulation of HMGB1 level and macrophage/microglia activities pose possible intervention opportunities for patients with both stroke and bone fracture.

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References


Metabolic Syndrome
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Surgery Results in Exaggerated and Persistent Cognitive Decline in a Rat Model of the Metabolic Syndrome

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ABSTRACT

Background: Postoperative cognitive decline can be reproduced in animal models. In a well-validated rat model of the Metabolic Syndrome, we sought to investigate whether surgery induced a more severe and persistent form of cognitive decline similar to that noted in preliminary clinical studies.

Methods: In rats that had been selectively bred for low and high exercise endurance, the low capacity runners (LCR) exhibited features of Metabolic Syndrome (obesity, dyslipidemia, insulin resistance, and hypertension). Tibial fracture surgery was performed under isoflurane anesthesia in LCR and high capacity runner (HCR) rats and cognitive function was assessed postoperatively in a trace-fear conditioning paradigm and Morris Water Maze; non-operated rats were exposed to anesthesia and analgesia (sham). Group sizes were n = 6.

Results: On postoperative D7, LCR rats had shorter freezing times than postoperative HCR rats. Five months postoperatively, LCR rats had a flatter learning trajectory and took longer to locate the submerged platform than postoperative HCR rats; dwell-time in the target quadrant in a probe trial was shorter in the postoperative LCR compared to HCR rats. LCR and HCR sham rats did not differ in any test.

Conclusion: Postoperatively, LCR rats diverged from HCR rats exhibiting a greater decline in memory, acutely, with persistent learning and memory decline, remotely; this could not be attributed to changes in locomotor or swimming performance. This Metabolic Syndrome animal model of surgery-induced cognitive decline corroborates, with high fidelity, preliminary findings of postoperative cognitive dysfunction in Metabolic Syndrome patients.

What We Already Know about This Topic
- The contributions of surgery, anesthesia and pathophysiological factors in postoperative cognitive decline are difficult to resolve in clinical studies
- Metabolic syndrome might enhance inflammation-mediated postoperative complications, including cognitive dysfunction

What This Article Tells Us That Is New
- Using a rat model, metabolic syndrome produced greater memory impairment and persistent learning and memory decline following tibial fracture surgery under isoflurane anesthesia
- Further studies are necessary to determine the mechanisms of these effects, and how metabolic syndrome exacerbates postoperative cognitive dysfunction
operative cognitive decline. We, and others, have shown that more severe and persistent cognitive decline occurs in >10% of non-cardiac surgical patients over the age of 65 years. With neither standardized cognitive domain testing nor appropriate controls, some have challenged whether the frequency of persistent postoperative cognitive decline is beyond that expected from “natural,” age-dependent deterioration. In order to overcome the lack of appropriate controls in clinical studies (i.e., surgical patients who are randomized to a cohort that does not receive surgery), we have set up models of rodents in which to explore the independent effects of surgery and anesthesia, and to address the molecular and cellular mechanisms that contribute to this condition. For animal models to be valid, these models need to reproduce salient mechanisms that can contribute to these and other inflammation-mediated postoperative complications.

The induction of short-lived neuroinflammation, following the release of damage-associated molecular patterns from traumatized tissue, appears to be necessary for the organism’s central nervous system-mediated “sickness behavior,” this is comprised of a fever, anorexia, somnolence, and cognitive impairment, and injured animals in this state remain sedentary promoting healing rather than risking further injury. Our recent preclinical studies have revealed that the transient cognitive decline that follows aspetic surgical trauma occurs by engaging the innate immune system through nuclear factor-κB-dependent signaling to release cytokines that disrupt blood brain barrier integrity. Through a permeable blood brain barrier, bone marrow-derived macrophages migrate into the brain parenchyma promoting neuroinflammation that is capable of interfering with processes required for learning and memory.

Two recent preliminary reports from Hudetz et al. have drawn attention to an exacerbation of early postoperative cognitive decline, as well as a greater likelihood of the persistence of postoperative cognitive decline in patients with Metabolic Syndrome (MetaS) who undergo either cardiac or non-cardiac surgery, respectively. In order to investigate the possible mechanisms, we reverted to a validated animal model of MetaS.

Most of the mouse models of MetaS are created by the extinction or modification of a single gene ± a dietary perturbation. These gene knock-out/knock-in approaches reveal the dependence on that gene, together with the subsequent adaptations, for the resulting loss or gain of function. These approaches may fail to model the human phenotype because they do not reconstitute complex gene-gene interaction. Amongst the several rat models of MetaS, we have chosen one that not only has the appropriate phenotype but also has been generated in a manner that is “lifestyle-related.” Starting in 1995, Koch and Britton applied divergent artificial selection for intrinsic low and high endurance running capacity starting with a founder population of genetically heterogeneous rats. Thirty generations of selection have produced lines of low capacity runners (LCRs) and high capacity runners (HCRs) that differ by 7-fold in treadmill running capacity. The LCR rats contain features of the MetaS, including elevated low density lipoproteins, cholesterol, blood pressure, triglycerides, fasting glucose, insulin, C-reactive protein, and visceral adiposity being 100 g heavier than the HCR rats at 12 weeks. The LCR rats have a low intrinsic aerobic capacity that results in easy fatigability; this may contribute to their sedentary behavior which is thought to be causally related to the development of the MetaS.

Using this rat model of MetaS, we have explored whether cognitive decline is more severe in the early postoperative period and whether it is likely to be more persistent in the LCR rats that have the features of MetaS; establishment of this an abnormal immune response to elective surgery can set the stage for a thorough exploration of the mechanisms that can contribute to these and other inflammation-mediated postoperative complications.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the University of California, San Francisco Institutional Animal Care and Use Committee, and conformed to National Institute of Health guidelines. Animals were handled in strict accordance with good animal practice. LCRs and HCRs were developed by Koch and Britton. The LCR rats are maintained as genetically heterogeneous rats. Both lines by using a rotational mating paradigm that minimizes inbreeding, thereby maintaining genetic complexity and allowing combinations of allelic variants at multiple interacting loci to be enriched by selection pressure. Only male rats aged between 4 and 5 months were used. All animals were fed standard rodent chow and water ad libitum, and were housed (2 rats per cage) in sawdust-lined cages in an air-conditioned environment with 12-h light/dark cycles. Animals were tagged and randomly allocated to the surgery or sham group before any procedure was undertaken; researchers were blinded to the group assignment during assessments, prior to the analysis phase. Because of the difference in color and weight between the LCR (brown, approximately 400 g) and HCR (white approximately 300 g) rats, it is not possible for the observer to be blinded for the phenotype, but they were blinded for the treatment group. Forty-eight rats were included with no lethality or exclusion.

Surgery

Under general anesthesia with 2.1% isoflurane in 0.30 FiO₂, rats underwent an open tibial fracture of the left hind paw...
with an intramedullary fixation under aseptic surgical conditions as previously described.7–9 The surgical field was maintained as sterile throughout the procedure. Briefly, the left hind paw of surgical animals was meticulously shaved and disinfected with povidone iodine. Following a median incision, a 20 G pin was inserted into the intramedullary canal, the periosteum was stripped, and osteotomy was performed. The wound was irrigated, the skin was sutured, and the animals were allowed to recover spontaneously from the anesthetic. During the procedure, temperature was monitored and maintained at 36.5–37.5°C with the aid of warming pads (Harvard Apparatus, Holliston, MA) and a temperature-controlled light. Buprenorphine (0.1 mg/kg) was given subcutaneously to provide analgesia after the induction of anesthesia and before skin incision. The sham rats were exposed to anesthesia and analgesia as above and had the paw shaved.

Twenty-four rats were used for trace-fear conditioning (TFC) assessed 7 days after surgery and an additional 24 rats were used for Morris Water Maze (MWM) assessed 5 months after surgery.

**Behavioral Studies**

**Trace Fear Conditioning.** TFC was used to assess hippocampal-dependent memory in rodents as previously described.7–9 The clear acrylic TFC chamber (Med Associates Inc., St Albans, VT), with dimensions of 32 cm long, 25 cm wide, and 25 cm high, included a floor constructed of stainless steel bars that was connected to a shock delivery system (Med Associates). The chamber was wiped with a pine-scented cleaner (5% Pine Scented Disinfectant; Midland, Inc., Sweetwater, TN) before and after each session, and training and assessment was performed in a room illuminated with overhead fluorescent bulbs with a ventilation fan providing background noise (65 db). During the training, rats were allowed to explore the context for 3 min, after which they were presented with an auditory cue (75–80 dB, 5 kHz, conditional stimulus for 20 s). The unconditional stimulus, a 2-s foot shock (0.8 mAmp), was administered 20 s after termination of the auditory tone. Rats were removed from the chamber after an additional 30 s. Rats anticipate the shock by “freezing,” which is defined as the absence of all movement except for respirations; this defensive posture reflects learned fear. When placed in the same context on a subsequent occasion, the learned fear is recalled, and the amount of learning and recall is measured by the amount of freezing. Surgery was performed within 30 min after training. Memory of the learned fear was assessed 7 days later by returning the rat into the same chamber in which it was trained, in the absence of tone and shock. Behavior was recorded by a Polaris digital video recorder (Cohu Electronics Division, Poway, CA). Each animal’s behavior was scored every 5 s during the 5 min observation period and a percentage was calculated using the formula 100 × f/n, where f is the number of freezing events per rat and n is the total number of observations per rat.

**Open Field.** A standardized measure of general motor function is spontaneous activity. At the conclusion of the contextual fear response, rats were placed in a wooden open field apparatus (45° square; 18” high) in which the floor was subdivided into 25 blocks (9” square) with thin white stripes, and activity was recorded by the Polaris digital video recorder (Cohu Electronics Division, Poway, CA). The number of line crossings and rearings performed in a 5 min epoch was scored by an observer that was unaware of group assignment.22

**Morris Water Maze**

Five months after surgery, separate cohorts of rats were investigated in the MWM in the following manner.23

**Cuing Procedure.** A platform (diameter, 10.3 cm) was placed one inch above the level of warm (24°C), opaque water in a circular pool (diameter, 180 cm; depth, 50 cm). The platform was rendered more visible from the surface by marking its edge with bright, yellow tape. For each of the three sessions, the rat was placed in the water and emerged from the water onto the platform within 60 s; if not, the rat was guided towards the raised platform. A different platform site was used for each session. This cuing procedure enables the rat to realize that they can escape the water by locating a platform.

**Spatial Reference Memory.** The pool is surrounded by visual cues and the platform is submerged below the surface. Daily, two training sessions spaced 7 h apart, were performed; for each session, the rat was released from one of three assigned locations facing the wall of the tank, resulting in one short, one medium, and one long swim per session, in random order. Rats were given 90 s to locate the hidden platform; if the rat failed to locate the hidden platform within the allotted time, the rat was guided to the platform. In either case, the rat was removed from the platform after 15 s. To minimize any bias associated with platform location, equal numbers of rats in each group were assigned one of the four quadrant locations of the platform for the duration of training. The time to reach the platform (latency), path length, swimming speed, and time-integrated distance to the platform were analyzed using an EthoVision video tracking system (Noldus Instruments, Wageningen, Holland) that was set to analyze 10 samples per second. The mean difference of decrease in the escape latency to the submerged platform per session, as well as the escape latency for the final session (session number 10), were analyzed.

**Probe Trials.** To assess memory retention for the hidden platform location, a probe trial, with the platform removed from the tank, was performed immediately after the last training session. During the 60-s probe trial, the proportion of time spent in the quadrant in which the platform previously resided (“target quadrant”), as well as each of the other quadrants, was determined for a 60-s interval.

**Statistical Analysis**

Data are presented as a mean ± SD. We tested for normal distribution of the data with the d’Agostino and Pearson
omnibus test and the equality of variances with the F-test. For comparisons of the two independent variables (strain [HCR/LCR] and surgery [fracture/sham]), we performed two-way ANOVA; this was followed by four pairwise student t tests with a Bonferroni correction (Bonferroni corrected alpha = 0.05/4 = 0.0125).

For TFC, we performed two-way ANOVA, testing the percentage of freezing time followed by four pairwise t tests (Bonferroni corrected alpha = 0.05/4 = 0.0125).

Regarding the MWM test, based on previous data (SD of the percentage of dwell time in target quadrant = 15), we estimated that a sample of 6 rats per group was necessary to find a significant difference between the LCR and the HCR surgery groups, with 80% of power if the difference was 25%. For the spatial reference memory experiments, the escape latency to the submerged platform of the 4 groups in the last session was analyzed with two-way ANOVA followed by four pairwise t tests (Bonferroni corrected alpha = 0.05/4 = 0.0125). To analyze the mean difference of decrease per session within the 10 sessions, we used a mixed-effects linear regression model with a three-way interaction involving session number, intervention (surgery or sham), and strain (LCR/HCR) with all lower order terms as predictors. We performed four additional post hoc models for pairwise comparisons of groups to determine whether there was a faster improvement in the latency time to platform amongst pairings across sessions; a pairing was deemed significantly different if the interaction term for the session and group was significant in the model (Bonferroni corrected alpha = 0.05/4 or 0.0125). For the probe trial, we compared the proportions of dwell time spent in each of the quadrants for the groups with a two-way ANOVA followed by four pairwise t tests (Bonferroni corrected alpha = 0.05/4 or 0.0125).

A two-tailed P value < 0.05 was considered statistically significant and data were analyzed using Stata 11.2 software (StataCorp, College Station, TX).

Results
Animals were assessed by open field testing after the contextual fear response on day 7, in order to exclude possible locomotor impairments that could confound the TFC assessment. Spontaneous movement was not different in the LCR versus HCR rats with or without surgery (fig. 1). Acute postoperative memory, assessed by the percentage of time spent freezing when the rat was placed in the same context as the pre-operative TFC training, was impaired in both cohorts of postoperative rats on postoperative day 7. Two-way ANOVA revealed a significant interaction between the strain and intervention (P = 0.02); the degree of memory impairment, as reflected by the percentage freezing, was greater in the postoperative LCR than the postoperative HCR rats (20 ± 4, vs. 33 ± 6, P = 0.006, fig. 2).

In the MWM test, the swimming speed was similar in all groups tested at 5 months (fig. 3A). Although the strain of the rats presented a significant effect on the weight recorded one week prior to the MWM (P < 0.001), surgery had no significant effect, suggesting that all groups continued to thrive throughout the course of the study (fig. 3B).

LCR surgery rats had significantly less overall improvement with successive tests compared to HCR surgery, as evidenced by a significantly longer time required to locate the submerged platform after the final, 10th session (P = 0.007 for two-way ANOVA strain effect). Pairwise post hoc t tests revealed that this strain effect was significant in the surgery rats (LCR surgery vs. HCR surgery, 34 ± 21 vs. 15 ± 6, P = 0.001, fig. 4A). A three-way interaction in the mixed-effects model was significant (P = 0.010), indicating that the effects of surgery and strain could not be considered in isolation when explaining variation in the rate of change in time to the submerged platform over successive trials. The mean rate of improvement (seconds/per session) was significantly less (P < 0.001) for the postoperative LCR rats (2 s/session) than for the sham-LCR rats (6 s/session) in a pairwise comparison of groups (fig. 4B). The difference in improvement per session between postoperative LCR rats and postoperative HCR rats (3 s/session, P = 0.010) was also significant after applying a Bonferroni correction.
The modeled curves reveal that the improvement in the escape latency per session to locate the hidden platform in the LCR surgery group was smaller than the LCR sham group (fig. 4B), whereas the improvements of HCR surgery and HCR sham rats were similar (fig. 4C).

In the probe trial, two-way ANOVA revealed a significant interaction between strain and intervention \( (P = 0.02) \) for the target quadrant (in which the platform formerly resided); post hoc analyses revealed that the percentage of time that the postoperative LCR rats spent in the target quadrant was significantly shorter than for each of the other groups (for example—LCR surgery vs. HCR surgery 24 ± 7 vs. 40 ± 6, \( P = 0.007 \), fig. 5). Apart from the postoperative LCR group, groups spent significantly more time in the target quadrant than in each of the others, while the postoperative LCR rats spent equivalent time in each of the four quadrants.

**Discussion**

Cognitive decline after surgery, whether temporary in the form of Postoperative Delirium or persistent, possibly in the form of Postoperative Cognitive Dysfunction, appears to be associated with long-term adverse outcomes including withdrawal from the workplace, loss of independent living, and an increase in mortality rate.\(^2,26\) Therefore, it is vital to understand the risk factors and pathogenesis that may contribute to these postoperative complications.

In a series of preclinical studies, we had noted that postoperative cognitive decline is due to the engagement of the innate immune system in response to aseptic trauma.\(^5,9,27,28\) The expression of cognitive decline is due to neuroinflammation that produces a constellation of symptoms, referred to as “sickness behavior,” which is thought to protect the traumatized organism from further injury and to facilitate the healing process.\(^29\) This is a short-lived process in which neuroinflammation is curtailed by feedback mechanisms that involve both neural (especially the parasympathetic nervous system), as well as humoral (including oxygenated and nitrogenated lipid) factors.\(^30,31\) Disruption of these precisely regulated processes may contribute to abnormal quantitative and qualitative responses to aseptic trauma.\(^3\) The existence of a disease that causes either exacerbation of the acute postoperative decline and/or the persistence of cognitive impairment, validates the animal model as one in which to explore pathogenic mechanisms and, thereby, possible therapeutic interventions.\(^14,15\)

MetaS, comprised of insulin resistance (hyperglycemia that can progress to Type 2 diabetes mellitus), visceral obesity, hypertension, and dyslipidemia increases the risk of postoperative complications contributing to a significantly higher mortality rate.\(^14,15,32\) In a recent review of a case series of coronary artery bypass surgical patients, those suffering
with MetaS had a longer postoperative stay although overall outcome was not affected. Many of the complications of MetaS (including atherosclerosis) are inflammatory in nature with the pathological adipose stores being the source of pro-inflammatory adipokines. It is estimated that more than a quarter of the American adult population has MetaS; its prevalence is probably over-represented in the surgical population, with half of all patients undergoing cardiac surgery being afflicted by this syndrome. Recent evidence indicates patients suffering from MetaS may be particularly susceptible to postoperative cognitive decline.

Starting in 1995, Koch and Britton applied divergent artificial selection for intrinsic low and high endurance running capacity starting with a founder population of genetically heterogeneous rats. Thirty generations of selection have produced lines of LCRs and HCRs that differ 7-fold in treadmill running capacity; the LCR rats contain features of the MetaS, including elevated low density lipoproteins, cholesterol, blood pressure, triglycerides, fasting glucose, insulin, C-reactive protein, and visceral adiposity being approximately 100 g heavier than the HCR rats at 12 weeks. The LCR-HCR rats represent polygenic substrate that can be used for exploring medically-relevant features, such as postoperative cognitive decline, that may be associated with the MetaS. The major genetic hypothesis is that contrasting alleles, causative of the trait differences, have been enriched or fixed differentially between the lines.

In the behavioral paradigms that we have used to interrogate cognitive domains of learning and memory, non-surgical LCR and HCR rats did not differ in either TFC or in the MWM. For the MWM experiments, we applied a mixed-effects model that took into account the strain, the type of surgery and the repeated measures. Other tests, which are perhaps more “subtle” than the ones that we employed, do show a behavioral phenotype in non-surgical rats. Postoperatively, the LCR rats exhibit both an early exacerbation of memory decline as well as a persistent abnormality in both learning and memory. Non-cognitive factors that could have contributed to the behavioral

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**Fig. 4.** Effect of remote surgery and phenotype on learning. (A) Five months after surgery, rats were tested twice daily for 5 consecutive days, for their ability to locate a submerged platform in a Morris Water Maze. For each of the ten sessions, the latency (s) to escape the water and find the platform was the average of three scored trials. Data were analyzed at session 10 with two-way ANOVA showing a significant effect of the strain ($P = 0.007$ for strain), but not for surgery, or for the interaction by strain and surgery. Post hoc pairwise t test comparisons with Bonferroni correction revealed that escape latency to the submerged platform in LCR surgery rats was significantly longer than for the HCR surgery rats ($P = 0.01$). Mixed-effects linear regression model of the effect of surgery on learning in LCR (B) and HCR (C) rats. The average raw values for each session from A were analyzed, taking into account the three-way interaction involving session number, surgery/sham and a session-squared term. Pair-wise comparisons with Bonferroni correction revealed that the slope was significantly less in LCR surgery rats compared to the LCR sham rats ($P < 0.001$); additionally, there was a significant difference in slope between LCR surgery rats and HCR surgery rats ($P = 0.010$). The curves for sham and surgery HCR rats were similar (C). HCR = high capacity runner phenotype; LCR = low capacity runner phenotype; surg = surgery.
assessments, such as altered spontaneous movement in TFC or the swim speed in MWM, are not different between the LCR and HCR rats.

It is noteworthy that the HCR rats may not represent “normal” rats and are more akin to organisms that are performing at a higher level of efficiency for energy expenditure compared to “normal” rats. As eight strains of rats formed the original cross-breeding reagents, it is not possible to ascertain which strain of rat is the normal one to be used as a control. Therefore, we can only comment that postoperatively, the LCR rats diverge from the HCR rats and are not able to opine whether this difference relates to deterioration in function from normal for the LCR rats and/or an improvement from normal for the HCR rats. However, it is noteworthy that the HCR rats do not appear to recover from acute postoperative memory decline faster than that noted in our earlier study involving male Sprague Dawley rats in a Y-maze test.6

Our findings set the stage for a thorough exploration of the reasons why MetaS induces differences in postoperative cognitive function. Recently, we reported that when the cholinergic neural feedback mechanism for surgery-induced neuroinflammation is disrupted in wild-type mice, an exacerbation of postoperative cognitive decline follows.8 It is noteworthy that patients with MetaS have a disorder in cholinergic function.6,37 Further exploration in these rat reagents may reveal the mechanism for this abnormality in cholinergic function and, thereby, reveal targets for interventions.

While we have concentrated on the reasons why the brain is a target for postoperative inflammatory complications, it will be important to consider whether other postoperative complications with a putative inflammatory basis, including conversion from acute to chronic postoperative pain and thrombo-embolic complications, may have a similar propensity to occur in MetaS possibly on the basis of dysregulation of mechanisms involved in the initiation and/or resolution of inflammation.

The authors acknowledge the insights on linear mixed effects modeling provided by Pedro Gambus, M.D., Visiting Professor, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California.

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3. Avidan MS, Evers AS: Review of clinical evidence for persistent cognitive decline or incident dementia attributable to surgery or general anesthesia. J Alzheimers Dis 2011; 24:201–16
POCD has long-term consequences, including higher mortality rate and costs associated with the care of the cognitively impaired. According to rodent models of postoperative cognitive decline, activation of the innate immune response following aseptic surgical trauma results in the elaboration of hippocampal proinflammatory cytokines, which are capable of disrupting long-term potentiation, the neurobiologic correlate of memory. We used a specific pharmacologic strategy to acutely deplete systemic phagocytes before an aseptic surgical trauma with an experimental tibia fracture. Depletion of BM-DM reduced the circulating inflammatory mediators, reducing migration of immune cells into the brain, and postoperative memory deficits. We also showed that the recruitment of BM-DM is mediated by hippocampal signaling through MCP-1. Following the characterization of the resolution of surgery-induced memory decline, we sought to describe the mechanisms involved in its initiation. We found that a single injection of HMGB1 antigen is sufficient to cause cognitive decline through the activation and trafficking of circulating BM-DM to the brain. HMGB1 antibody reduced the systemic and neuroinflammatory response to surgery, preventing post-surgical cognitive decline.

Studies have sought to identify factors that may contribute to POCD, which include surgery, as well as in-patient care factors, and patient-related factors. We tried to understand the causal relationship between a common occurrence during in-hospital care, sleep disruption, and cognitive impairment. We found that SF independently produced significant memory impairment, although perioperative SF significantly increased hippocampal inflammation without further cognitive impairment.

If we divide the possible risk factors into categories of modifiable/non-modifiable and patient related/environmental, we can both disentangle the causes of the neuroinflammatory cascade and also to focus on possible clinical adjustments and applications to stave it off. Using a rodent model, we demonstrated that surgery aggravates functional and morphological
consequences of stroke by depleting macrophages. The effects of bone fracture on stroke were attenuated by neutralizing HMGB1. The fact that patients with MetaS have a significantly greater likelihood of exhibiting cognitive decline than a cohort of patients without MetaS, encouraged us to investigate the extent and severity cognitive decline in MetaS patients. Using a genetically heterogenous lineage of MetaS rat model, we showed that MetaS produced greater memory impairment and persistent learning and memory decline following tibia fracture surgery.

In the majority of patients, postoperative neuroinflammation is part of the normal protective mechanism to peripheral trauma and resolves properly with no residual cognitive consequences. Indeed, it is also possible that surgery for a chronic inflammatory disease may result in cognitive improvement by eliminating disease-inducing cognitive impairment that may be associated with chronic inflammatory disease. That said, some risk factors, such as MetaS, patients prone to neurological disease, and poor selection of sedative agents, may each promote the intractable persistence of neuroinflammatory response to surgery. For an increasing number of patients with advanced age, POCD is alarmingly common, making postoperative central nervous system dysfunction a looming public health crisis given world's rising elderly population.

Understanding the cellular and biologic pathways involved in postoperative cognitive decline is a key element in designing interventions to prevent this disease. Reducing activation and/or migration of innate immune cells, such as systemic macrophages into the brain, represents a viable preemptive strategy. We demonstrated that BM-DM activation after experimental tibia fracture is directly involved in the tibia fracture–induced hippocampal BM-DM infiltration and animal memory dysfunction. HMGB1, when released from a sterile traumatic injury, plays a pivotal role in postoperative memory dysfunction. Together with the detection of the cell type involved in the initiation of the surgery-induced inflammatory cascade, these findings establish both the precise elements of the immune response that need to be interrogated for establishing the risk of dysregulated trauma-induced inflammation as well as
putative targets for interventions designed to limit or reverse persistent postoperative cognitive decline.

These studies on the initiation of trauma-induced cognitive decline, coupled with previous reports on the resolution of postoperative cognitive decline\textsuperscript{12,14,44} set the stage for the development of an \textit{ex-vivo} bio-assay that can test the function of the innate immune response to trauma. Such an assay may be capable of prospectively identifying surgical patients at increased risk for the development of exaggerated and persistent cognitive decline; stratification of a surgical cohort, enriched for the development of cognitive decline, can result in a randomized trial to test with efficacy of interventions using fewer surgical patients.

Clearly, the surgical effect of this neuroinflammatory trigger is just one possible mechanism. Indeed, environmental culprits can also offer a window into the postoperative cognitive decline conundrum. One great suspect in this complex puzzle is that of sleep fragmentation: SF induces significant changes that underlie memory, indicating a loss of cognitive function in non-hippocampal and hippocampal dependent domains. We show that while SF and surgery can independently produce significant memory impairment, perioperative SF significantly increased hippocampal inflammation without further cognitive impairment. This peculiar dissociation between neuroinflammation and cognitive decline may relate to our use of memory paradigms that do not capture all aspects of cognition. These data support the need to adopt interventions that can provide patients with appropriate rest in order to mitigate neuroinflammation. While we realize that the etiology of cognitive dysfunction in surgical/ICU patients is multi-factorial, if the restorative and reparative benefits of sleep mitigate the development of inflammation, this may result in shorter ICU or postoperative lengths of stay. If the restorative and reparative benefits of sleep mitigate the development of cognitive dysfunction, this will result in shorter ICU and postoperative lengths of stay for critically ill patients with a concomitant reduction in healthcare costs. Furthermore, it is possible that the restorative properties of sleep for
cognition in the central nervous system can extend to the immune system with less infection and/or greater likelihood of survival from sepsis \(^{68,86}\).

In a recently completed prospective study, patients who had previously suffered a stroke were more at risk for POCD even though they had no neurological sequelae from the remote stroke event \(^{5,87}\). We have demonstrated that bone fracture shortly after ischemic stroke increases stroke-related neuronal injury and neurobehavioral dysfunction in mice. HMGB1 and macrophage/microglia play a causal role in the negative impact of bone fracture on stroke outcomes. By modulating the level of HMGB1 level and macrophage/microglia activities we could be opening for possible intervention opportunities for patients with both stroke and bone fracture.

We have also tried to explore that modifiable patient related factors also bear a great influence on POCD development. In a rat model of MetaS we report that these animals have greater memory impairment and persistent learning and memory decline following tibia fracture surgery. These findings set the stage for a thorough exploration of the reasons why MetaS induces differences in postoperative cognitive function and if appropriate patient optimization before surgery could mitigate the development of POCD.

We believe that non-resolution of inflammation is a factor that contributes to the pathogenesis of POCD, which in turn significantly increases morbidity and mortality in surgical patients. We might be witnessing a perfect and unfortunate storm of factors with regard to POCD: to put it another way, given the rise in surgeries and increasing number of patients with chronic conditions worldwide, the stakes could not be higher.

Vulnerable patients need to be identified and risk/benefit should be considered before contemplating the efficacy of surgical intervention. Advanced age, MetaS, patients prone to neurological disease, and poor selection of sedative agents may each result in exaggerated and persistent neuroinflammatory response to surgery. Further studies are needed to understand which patients will suffer from exacerbated inflammation with an aim toward developing a biomarker that is quick to assay for clinicians and
easy to comprehend for patients and their families. Concurrently, clinical interventions need to be further developed to promote the resolution of neuroinflammation in the postoperative patient population. Following both tracks, we anticipate that postoperative recovery for vulnerable patients will be greatly enhanced and possible long-term consequences, such as postoperative neurodegeneration, can be significantly reduced.

Additional study is essential to elucidate the risk factors, preventative strategies, and underlying pathophysiology of this disorder. If these studies can succeed in identifying patients prospectively, or early enough in the advent of persistent inflammation, interventions can be judiciously and appropriately launched.
**Table 1 – Risk Factors for Postoperative Delirium**

<table>
<thead>
<tr>
<th>Risk factors for POD</th>
<th>Incidence of delirium (%)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient Related factors</strong></td>
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<td></td>
</tr>
<tr>
<td>Advanced age</td>
<td>42-92</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>42.3</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>?</td>
<td>63,92,104</td>
</tr>
<tr>
<td>Education</td>
<td>?</td>
<td>94,102,103</td>
</tr>
<tr>
<td>Male sex</td>
<td>?</td>
<td>96,106</td>
</tr>
<tr>
<td>Apolipoprotein E4</td>
<td>?</td>
<td>104</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative depression</td>
<td>?</td>
<td>105,107,108</td>
</tr>
<tr>
<td>Metabolic Syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obstructive Sleep Apnea</td>
<td>24</td>
<td>89</td>
</tr>
<tr>
<td>Preoperative cognitive impairment</td>
<td>24</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>55.6</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>?</td>
<td>86,92,93,100,104,106,108,112</td>
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<tr>
<td>Other comorbidities</td>
<td>18.3</td>
<td>89</td>
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<tr>
<td></td>
<td>?</td>
<td>63,98,96,95,94,97,95,106,109,113</td>
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<tr>
<td><strong>Surgery and anesthesia</strong></td>
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<td></td>
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<tr>
<td>Duration of surgical procedure</td>
<td>40</td>
<td>90</td>
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<tr>
<td></td>
<td>?</td>
<td>63,97</td>
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<tr>
<td>Pain management</td>
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<td>98,114</td>
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<td>Metabolic derangement</td>
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<td>89</td>
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<tr>
<td></td>
<td>13-41</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>?</td>
<td>63,98,102,110</td>
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<tr>
<td><strong>Other</strong></td>
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<tr>
<td>Tobacco use</td>
<td>53.3</td>
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</tr>
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<td></td>
<td>?</td>
<td>97</td>
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<td>History of alcohol or drug abuse</td>
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<td></td>
<td>?</td>
<td>92,96,100,102,103,105,107,110</td>
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<tr>
<td>Preoperative anxiety</td>
<td>?</td>
<td>115,116</td>
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<td>Sleep deprivation</td>
<td>?</td>
<td>69</td>
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### Table 2 – Risk Factors for Postoperative Cognitive Dysfunction

<table>
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<tr>
<th>Risk factors for POCD</th>
<th>Assessment in days</th>
<th>Incidence of POCD (%)</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Patient Related factors</strong></td>
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<td></td>
</tr>
<tr>
<td>Advanced age</td>
<td>≤ 1wk</td>
<td>41.4</td>
<td>5, 29, 11.4, 5.8, ?</td>
</tr>
<tr>
<td></td>
<td>1wk – 3mo</td>
<td>12.7-14</td>
<td>5, 8, 117, 11.4, 118, 119-124</td>
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<tr>
<td></td>
<td>3mo – 6mo</td>
<td>?</td>
<td>119, 122, 11.4, 117, 119, 120, 122, 124, 126</td>
</tr>
<tr>
<td></td>
<td>&gt;1yr</td>
<td>16</td>
<td>8, 118, 119, 122, 126</td>
</tr>
<tr>
<td>Education (&lt; high school)</td>
<td>≤ 1wk</td>
<td>27</td>
<td>5, 124, 13.2, 5, 120, 122</td>
</tr>
<tr>
<td></td>
<td>1wk – 3mo</td>
<td>9</td>
<td>9, 9, 9</td>
</tr>
<tr>
<td></td>
<td>&gt;1yr</td>
<td>10</td>
<td>8, 122, 124, 126</td>
</tr>
<tr>
<td>Male sex</td>
<td>≤ 1wk</td>
<td>?</td>
<td>123, 127</td>
</tr>
<tr>
<td>Menopause</td>
<td>1wk – 3mo</td>
<td>?</td>
<td>127, 127</td>
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<tr>
<td>Apolipoprotein E4</td>
<td>≤ 1wk</td>
<td>11.7</td>
<td>128, 21.8, 128</td>
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<td></td>
<td>1wk – 3mo</td>
<td>10.3</td>
<td>128, 41.7, 130</td>
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<tr>
<td><strong>Comorbidities</strong></td>
<td>≤1wk</td>
<td>?</td>
<td>120, 121, 131, 120, 121, 131</td>
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<tr>
<td>Preoperative depression</td>
<td>1wk – 3mo</td>
<td>?</td>
<td>120, 121, 131</td>
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<tr>
<td></td>
<td>3mo – 6mo</td>
<td>?</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>6mo -1yr</td>
<td>?</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>&gt;1yr</td>
<td>?</td>
<td>126, 129, 122, 129, 126</td>
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<tr>
<td>Metabolic Syndrome</td>
<td>≤1wk</td>
<td>?</td>
<td>22, 22, 22, 22</td>
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<td></td>
<td>1wk – 3mo</td>
<td>?</td>
<td>22, 22, 22, 22</td>
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<tr>
<td>Preoperative cognitive impairment</td>
<td>≤ 1wk</td>
<td>?</td>
<td>120</td>
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<td></td>
<td>1wk – 3mo</td>
<td>?</td>
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<td>Other Comorbidities</td>
<td>≤1wk</td>
<td>3.2-6.3</td>
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<td>122, 123, 122, 123, 122, 123</td>
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<td></td>
<td>1wk – 3mo</td>
<td>11</td>
<td>122, 122, 120, 122</td>
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<tr>
<td></td>
<td>&gt;1yr</td>
<td>?</td>
<td>122, 122, 120</td>
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<tr>
<td><strong>Surgery and Anesthesia</strong></td>
<td>≤ 1wk</td>
<td>43</td>
<td>8, 54</td>
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<td>Second operation</td>
<td>1wk – 3mo</td>
<td>20</td>
<td>8, 14</td>
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<tr>
<td>Long duration of surgical procedure/anesthesia</td>
<td>≤ 1wk</td>
<td>33</td>
<td>5, 9, 124</td>
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<tr>
<td></td>
<td>1wk – 3mo</td>
<td>29</td>
<td>9, 123, 122</td>
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<tr>
<td></td>
<td>6mo -1yr</td>
<td>?</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>&gt;1yr</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>≤ 1wk</td>
<td>1wk – 3mo</td>
<td>&gt;1yr</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------</td>
<td>-----------</td>
<td>------</td>
</tr>
<tr>
<td>Postoperative infection</td>
<td>39</td>
<td>14-19</td>
<td>19</td>
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<tr>
<td>Respiratory complication</td>
<td>59</td>
<td>14-15</td>
<td>14</td>
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<tr>
<td>Anesthetic type</td>
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<td>?</td>
</tr>
<tr>
<td>Pain management</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Tobacco use</td>
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<td>?</td>
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<td>History of alcohol or drug abuse</td>
<td>26.8</td>
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Other

References: 6, 9, 124, 134

History of alcohol or drug abuse

References: 6, 124, 134
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