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# Resolving postoperative neuroinflammation and cognitive decline

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## Abstract

**Objective—**Cognitive decline accompanies acute illness and surgery, especially in the elderly. Surgery engages the innate immune system that launches a systemic inflammatory response which, if unchecked, can cause multiple organ dysfunction. We sought to understand the mechanisms whereby the brain is targeted by the inflammatory response and how this can be resolved.

**Methods—**C57BL/6J, *Ccr2*$^{RFP/+}$ *Cx3cr1*$^{GFP/+}$, *Ikk*$^{F/F}$ mice and *LysM-Cre/Ikk*$^{F/F}$ underwent stabilized tibial fracture operation under analgesia and general anesthesia. Separate cohorts of mice were tested for systemic and hippocampal inflammation, integrity of the blood brain barrier (BBB), and cognition. The putative resolving effects of the cholinergic pathway on these postoperative responses were also studied.

**Results—**Peripheral surgery disrupts the BBB via release of tumor necrosis factor-alpha (TNFα), which facilitates the migration of macrophages into the hippocampus. Macrophage-specific deletion of IKKβ, a central coordinator of TNFα signaling through activation of NF-κB, prevents BBB disruption and macrophage infiltration in the hippocampus following surgery. Activation of the α7 subtype of nicotinic acetylcholine receptors, an endogenous inflammation-resolving

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pathway, prevents TNFα-induced NF-κB activation, macrophage migration into the hippocampus and cognitive decline following surgery.

**Interpretation—**These data reveal the mechanisms for bidirectional communication between the brain and immune system following aseptic trauma. Pivotal molecular mechanisms can be targeted to prevent and/or resolve postoperative neuroinflammation and cognitive decline.

## Introduction

Cognitive decline accompanies acute illness and surgery, especially in the elderly, a growing segment of patients worldwide.[1] Postoperative cognitive decline appreciably increases mortality and results in morbidity requiring considerable healthcare resources.[2, 3] Surgery and other forms of aseptic trauma initiate a systemic inflammatory response that, if unchecked, can disrupt the function of multiple organs.[4] We recently reported a key role for pro-inflammatory cytokines, including interleukin (IL)-1 and tumor necrosis factor-alpha (TNFα), in mediating surgery induced neuroinflammation and subsequent cognitive decline following orthopedic surgery.[5, 6] Tracey *et al* have highlighted the importance of a cholinergic reflex in resolving the inflammatory pathogenesis of several diseases including sepsis,[7] rheumatoid arthritis,[8] and colitis.[9] Therefore, we wondered how peripheral surgery produces neuroinflammation and how endogenous inflammatory mechanisms are capable of dampening this response. Using a variety of mouse reagents, which provide insights into the causal roles of discrete molecular species, we demonstrated that peripheral surgery disrupts the blood brain barrier (BBB) facilitating the migration of macrophages into the brain parenchyma through activation of TNFα/nuclear factor (NF)-κB signaling pathway. Macrophage-specific deletion of IKKβ, a central coordinator of TNFα activation of NF-κB, prevents BBB disruption and macrophage infiltration in the hippocampus following surgery. Engaging the cholinergic reflex by stimulating the α7 subtype of nicotinic acetylcholine receptors (α7 nAChR) in macrophages, inhibited NF-κB activity; *in vivo,* α7 nAChR agonists prevented macrophage migration into the hippocampus and cognitive decline following surgery. Now that we have elucidated the mechanisms for both the activation and resolution of the neuroinflammatory response to peripheral surgery, we can devise strategies to either disable activation, and/or enhance resolution, of neuroinflammation in patients at risk for developing disabling postoperative cognitive decline.

## Material and Methods

### Chemicals

Nicotine, choline, methyllycaconitine (α7 nAChR antagonist; MLA), and TNFα-neutralizing antibody (clone TN3) were purchased from Sigma (St. Louis, MO). PHA 568487 (PHA), a selective agonist of α7 nAChR, was purchased from Tocris Bioscience (Ellisville, MO). Each was dissolved in 0.9% saline before the appropriate experiment. Control animals were injected with the same volume of saline.

### Animals

Animals were handled in strict accordance with good animal practice and all animal work was approved by the appropriate committee, IACUC University of California, San

Francisco. Wild-type mice (C57BL/6J, 12-14 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Ccr2*$^{RFP/+}$ *Cx3cr1*$^{GFP/+}$, *Ikk*$^{F/F}$ mice (- CRE) and *LysM-Cre/Ikk*$^{F/F}$ mice were used as previously described.[10, 11] All animals were fed standard rodent chow 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.

## Surgery

Animals were given a general anesthetic at 2.1% isoflurane in 0.30 $FiO_2$. Under aseptic surgical conditions, animals received an open tibial fracture of the left hind paw with an intramedullary fixation as previously described.[5] The surgical field was maintained sterile throughout the procedure and autoclaved instruments were used throughout. Briefly, the left hind paw of surgical animals was meticulously shaved and disinfected with povidone iodine. A median incision was performed on the left hind paw followed by the insertion of a 0.38-mm pin in the intramedullary canal, the periosteum was then stripped and osteotomy performed. After producing the fracture the wound was irrigated and the skin sutured with 8/0 Prolene sutures; thereafter, animals were allowed to recover spontaneously from the anesthetic. During the procedure, temperature was monitored and maintained between 36 and 37°C with the aid of warming pads (Harvard Apparatus) and a temperature-controlled light. Analgesia (Buprenex, 0.1 mg/kg) was given s.c. after anesthetic induction and before skin incision. In some experiments either MLA (4 mg/kg) or PHA 568487 (0.4 mg/kg) was administered i.p. 30 minutes prior to surgery or anesthesia. In other experiments nicotine (0.25 mg/kg) and choline (50 mg/kg) were given i.p. immediately after anesthesia induction. TNFα-neutralizing antibody (100 μg per mouse) was administered i.p. 18 h preoperatively.

## Cytokine measurements

Blood was collected by cardiac puncture under deep isoflurane anesthesia and then centrifuged at 2,000g for 7 min at 4 °C. Plasma cytokines were stored at −80 °C for further analysis. Levels of TNFα, IL-1β, IL-10, and HMGB1 were measured using commercially available ELISA kits from Biosource, R&D (IL-10) and Shino-test Corporation, respectively. The sensitivities of the assays were <3 pg/mL for TNF-α, <7 pg/mL for IL-1β, <4pg/mL for IL-10 and 1 ng/mL for HMGB-1.

## Immunohistochemistry

Under terminal anesthesia the thoracic cavity was opened to reveal the heart, and the right atrium was severed. A solution of ice-cold 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS at pH 7.4 (VWR International) was perfused into the left ventricle. The brains were harvested and postfixed in 4% paraformaldehyde in 0.1 M PBS at 4 °C and cryoprotected in 0.1 M PBS solutions containing 15% sucrose (Sigma, MO) for 24 h and then 30% sucrose for a further 48 h. Brain tissue was freeze-mounted in OCT embedding medium (VWR International) and 25-μm thick coronal sections of the hippocampus were cut sequentially and mounted on Superfrost plus slides (Menzel-Glaser). After primary antibody incubation with anti-CD11b (clone M1/70.15, 1:200, Serotec) sections were incubated with biotinylated secondary antibody (goat anti-rat, 1:200, Chemicon International), followed by the ABC Elite system and lastly developed in 3,3-

diaminobenzidine (Vector Laboratories). The sections were dehydrated and coverslipped with Permount (Fisher). A negative control omitting the primary antibody was performed in all experiments. Quantitative image analysis was performed in three non-overlapping fields randomly acquired in hippocampal subregions using a counting frame size of 0.4mm$^2$. Three slides per mouse were imaged and the number of pixels per image with intensity above a predetermined threshold level was quantified using ImageJ software (NIH). Total immunoreactivity was calculated as percentage area density defined as the number of pixels (positively stained areas) divided by the total number of pixels (sum of positively and negatively stained area) in the imaged field.[12] All quantitative analyses were performed in a blinded manner. For the fibrinogen staining, animals were killed and perfused transcardially as previously described. Immunofluorescence for fibrinogen was performed as previously described.[13] Sections were permeabilized in 0.1% Triton X-100, blocked with 5% BSA, and incubated for 24 h at 4°C with the rabbit anti-fibrinogen (1:1000; a gift from Jay Degen, University of Cincinnati College of Medicine, Cincinnati, Ohio). Sections were then washed in PBS with 0.1% Triton X-100 and incubated with secondary antibodies conjugated with Alexa Fluor 594 (1:200; Invitrogen) for 1 h in the dark. After washing in PBS, sections were then mounted on glass slides and coverslipped with Prolong Gold antifading agent (Invitrogen). Images were acquired with an Axioplan II epifluorescence microscope with an Axiocam HRc CCD camera (Carl Zeiss) and fluorescence in tissue sections was compared by ImageJ software.

### Bone marrow-derived macrophage (BMDM) culture

Bone marrow derived macrophages (BMDMs) were prepared as described previously.[14] In brief, bone marrow (BM) was isolated from tibia and femur of 10-week-old mice. BM cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 10 ng/ml murine M-CSF (eBioscience). On day 6, adherent BMDMs were harvested from plates by the addition of PBS containing 5 mM EDTA for experiments. BMDMs were plated and cultured with 1% FBS in RPMI 1640 overnight in Nunc plates. Cells were was pre-incubated with PHA 568487 (10 mg/ml) for 30 min followed by mouse TNFα (40 ng/ml) stimulation for 2 hr. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature, rinsed in PBS, and incubated with blocking buffer (PBS containing 5% normal goat serum and 0.3% Triton X-100) for 60 min. Cells were stained with anti-phospho-NF-κB p65 (1:200, Cell Signaling) for 24 hr at 4 °C. Cells were washed in PBS and incubated with anti-rabbit Cy3-conjugated secondary antibody (1:200; Jackson ImmunoReseach) for 1.5 hr at room temperature in dark. After washing with PBS, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen). Five representative images per well were taken using an Axioplan II epifluorescence microscope (Carl Zeiss) with a dry Plan-Neofluar 40 × 0.75 NA objective and an Axiocam HRc CCD camera. Images were acquired and analyzed with Axiovision image analysis software. Quantification was determined as the proportion of total cells exhibiting nuclear phospho-NF-κB p65. All experiments were repeated three times.

### Behavior

**Trace fear conditioning—**The behavioral study was conducted using a dedicated trace fear conditioning chamber (Med Associates Inc.). The fear conditioning paradigm was used as previously described.[6] Fear conditioning is used to assess learning and memory in rodents.[15] Training of trace fear conditioning consisted of placing the mouse in the conditioning chamber and allowing exploration of the context for 100 s. The mice were then presented with an auditory cue (75–80 dB, 5 kHz, conditional stimulus (CS) for 20 s. The unconditional stimulus (US), a 2-s foot-shock (0.75 mAmp) was administered 20 s after termination of the tone. This procedure was repeated with an inter-trial interval of 100 s, and mice were then removed from the chamber 30 s later, for a total trial time of 270 s. Both the low-affinity nAChR antagonist (MLA 4 mg/kg) and the selective agonist (PHA 568487 0.4 mg/kg) were administered ip 30 min prior to training of trace fear conditioning; because of their short half-lives nicotine (0.25 mg/kg) and choline (50 mg/kg) were given immediately after fear conditioning training but prior to surgery. Surgery was performed within 30 min after the fear conditioning paradigm. Contextual test was performed 3 days after training and mice were returned to the same chamber for 5 min without an electric shock. Freezing behavior in response to context was automatically recorded by the software.

**Rotarod test—**The rotarod apparatus (Ugo Basile, Biological Research Apparatus, Varese, Italy) was used to measure motor coordination and balance, with minor modifications.[16, 17] In the accelerating mode, speed ranged from 3 to 35 rpm over 5 min. Fixed speed rotarod was performed at a constant speed of 20 rpm with a 300-s maximum time limit and means were collected for at least three trials. The mean latency to fall off the rotarod was automatically recorded and used in subsequent analysis.

### Data Analysis

GraphPad v3.0 (GraphPad Software) was used to calculate the mean and SEM, and perform statistical analysis. We analyzed multiple group means by one-way or two-way analysis of variance, followed by Newman-Keuls *post hoc* test wherever appropriate. The nonparametric test of Kruskal-Wallis followed by the Dunn's multiple comparison test was used for categorical data. P values less than 0.05 were considered significant.

## Results

### NF-κB activation in myeloid cells mediates BBB disruption and neuroinflammation following peripheral surgery

Neuroinflammation, microglia activation and release of pro-inflammatory cytokines, including TNFα and IL-1, are critically regulated by NF-κB activity.[18] We selectively assessed the role of NF-κB in myeloid cells in the development of inflammation following peripheral surgery using *LysM-Cre/Ikkβ*$^{F/F}$ mice. In these mice *LysM-Cre* deletes the *IKKβ*$^{F}$ allele in bone-marrow-derived macrophages (BMDMs) and microglia.[19] Neither systemic inflammation nor CD11b immunoreactivity in the hippocampus occur postoperatively in *LysM-Cre/Ikkβ*$^{F/F}$ mice as compared to *Ikk*$^{F/F}$ mice (- CRE) (*Fig 1A,B*). We assessed the integrity of the BBB by measuring fibrinogen deposition;[20] 24 h after orthopedic surgery and there was significant perivascular deposition confined to the hippocampus ($P < 0.001$).

Notably, BBB disruption did not occur in *LysM-Cre/Ikkβ*$^{F/F}$ mice (*Fig 1C*). Stimulation of the "cholinergic anti-inflammatory pathway" *via* activation of the α7 nAChR subtype is a pivotal regulator of NF-κB activity.[21, 22] We tested whether pharmacologic activation of this receptor could prevent surgery-induced neuroinflammation. Animals treated with a selective α7 agonist (PHA 568487) had no signs of perivascular fibrinogen deposition in the hippocampus similarly to *LysM-Cre/Ikkβ*$^{F/F}$ mice. Conversely, disabling the cholinergic reflex with a selective α7 nAChR antagonist (MLA) exacerbated BBB disruption suggesting a key role for cholinergic signaling in the resolution of postoperative neuroinflammation (*Fig 1C, P* < 0.05).

## Surgery-induced neuroinflammation is associated with infiltration of peripheral macrophages

To understand the cellular origin of the enhanced CD11b immunoreactivity in operated mice we used Ccr2$^{RFP/+}$ Cx3cr1$^{GFP/+}$ mice, which distinguish monocyte-derived macrophages from resident microglia.[10] Following surgery, an influx of CCR2 (red fluorescent protein; RFP) positive monocytes was observed within the parenchyma of the hippocampus as well as in periventricular areas but not in other brain regions (*Fig 2A*). In contrast, resident microglia labeled by CX3CR1 (green fluorescent protein; GFP) were unchanged at 24 h following surgery (*Fig 2B*). Because TNFα disrupts BBB function and plays a key role in triggering surgery-induced cognitive decline,[6, 23] we explored the effect of pre-operative administration of anti-TNF antibody on the influx of CCR2 positive monocytes. Disabling TNFα prevented postoperative disruption of BBB and migration of CCR2 positive cells into the CNS, suggesting that this mechanism is TNFα-dependent (*Fig 2A*). Engaging the cholinergic reflex with preoperatively administered selective α7 nAChR agonist (PHA 568487) prevented the migration of monocyte-derived macrophages into the CNS (*P* < 0.01); also, non-selective nAChR agonists that are either BBB-permeable (nicotine) or not (choline) prevented surgery-induced neuroinflammation (*Fig 3A, P* < 0.001). Conversely, RFP$^{+}$ monocyte-derived macrophages were significantly higher in the hippocampus of surgical animals in which the cholinergic reflex was disabled with the nAChR antagonist, MLA (*P* < 0.05); consistent with this finding CD11b immunoreactivity was also enhanced in the hippocampus of operated animals pretreated with MLA (*P* < 0.01).

## α7 nAChR stimulation prevents NF-κB activation in BMDMs and prevents postoperative cognitive decline

TNFα activated NF-κB signaling in BMDMs was assessed by nuclear translocation of the phosphorylated p65 subunit. Pretreatment with the selective nAChR agonist prevented these changes (*Fig 4A*). Preoperative administration of a single bolus dose of the selective α7 nAChR agonist, PHA 568487, blocked the pro-inflammatory response to surgery (*P* < 0.01, *P* < 0.001). Similarly, the non-selective cholinergic agonists nicotine and choline markedly reduced the pro-inflammatory cytokine response to surgery at each time point (*Fig 4B-D*). In addition to the marked reduction of pro-inflammatory cytokines, all three nAChR agonists increased levels of the anti-inflammatory cytokine, IL-10 at 24 h (*Fig 4E*, *P* < 0.001).

Next, we explored the effects of cholinergic modulation on postoperative cognitive decline assessing hippocampal-dependent memory function using trace fear conditioning (TFC).[6]

Surgery decreased freezing behavior, the index for memory retention in rodents; the memory impairment was worsened when the cholinergic reflex was disabled with the nAChR antagonist, MLA. Conversely, nicotine, choline or PHA 568487 ameliorated memory impairment (*Fig 5A, B*). Because cholinergic compounds including PHA 568487 have been designed to directly enhance memory function,[24] we explored whether this obtained in non-surgical animals; single dose administration of the nAChR agonists, alone or in combination with anesthesia and analgesia did not affect memory (*Fig 5C*).[25] To further exclude possible locomotor impairments that could have confounded the TFC assessment, animals were tested on a rotarod after contextual fear response at day 3; no significant differences were observed amongst groups (*Fig 5D, E*).

## Discussion

Surgical trauma engages the innate immune system through NF-κB dependent signaling to release cytokines that disrupt BBB integrity. Through a permeable BBB, peripheral macrophages migrate into the hippocampus promoting neuroinflammation that impairs memory. Stimulation of the anti-inflammatory cholinergic pathway prevents surgery-induced pro-inflammatory cytokine release, neuroinflammation, and cognitive decline after surgical injury (*Fig 5F*).

Pro-inflammatory cytokines including TNFα can readily compromise endothelial function and permeabilize the BBB.[23] The modest increase in systemic TNFα following surgery appears sufficient to disrupt the BBB, allowing deposition of fibrinogen, a glycoprotein of 340 KDa. The disruption of the barrier function also contributes to the monocyte-derived macrophage migration into the parenchyma, and these inflammatory cells, rather than microglia, comprise the up-regulation of CD11b immunoreactivity detected in the surgical phenotype. While macrophages had previously been observed to leave the circulation at regions in the nervous system that had been traumatized,[26] we are unaware of previous studies showing macrophage migration into the brain when the injury had not directly involved the nervous system. The hippocampus is known to highly express pro-inflammatory cytokine receptors; the relative prevalence of the TNFα receptor, and other pattern recognition receptors, on the endothelium of this brain region may account for its vulnerability to systemic pro-inflammatory cytokines.[27] Soluble cytokines can also stimulate afferent C-fibers to open the blood-spinal cord barrier.[28] This may represent an alternative pathway for peripheral macrophages and pro-inflammatory cytokines to access the CNS. Interestingly patients undergoing total hip arthroplasty display elevated pro-inflammatory cytokines in the cerebrospinal fluid after surgery and this could contribute to the neuroinflammatory phenotype and postoperative cognitive decline.[29] While the mechanism by which the influx of macrophages putatively disrupts cognitive processing is beyond the scope of this report, it is notable that in several CNS diseases, migration of macrophages is pivotal to both the morphological and functional consequences of disease.[30, 31]

Activation of the nAChR by PHA 568487, nicotine or choline resolved the neuroinflammatory and behavioral responses to surgery while antagonism by MLA worsened this surgical phenotype. Because exposure to the non-selective choline, a charged precursor of acetylcholine that does not easily penetrate the BBB, attenuated the response to

surgical trauma, it is possible that this effect is exerted at extra-CNS sites. Thus cholinergic stimulation may be targeting α7 nAChR signaling in the peripheral macrophage.[21] We recapitulated this aspect of the surgical phenotype by observing the effect of a selective α7 nAChR agonist on activation of the NF-κB pathway in cultured BMDMs. The TNFα-enhanced nuclear translocation of the phosphorylated form of the NF-κB p65 subunit was blocked by the α7 nAChR selective agonist suggesting a mechanism whereby the surgical phenotype can be negated. Notably, cholinergic agonists induced systemic IL-10, a potent cytokine inhibitor of macrophages and NF-κB inactivation may represent an important inflammation-resolving "*switch*".[32, 33]

The neuroinflammatory response to aseptic trauma may have developed as a survival mechanism in which an injured animal experiences CNS-derived anorexia, fatigue, and impaired cognition;[34] these features of "*sickness behavior*" could promote healing *in lieu* of hunting in an injured state. The neuroinflammatory response to injury appears to be self-limiting through the cholinergic reflex because interruption of the reflex with the nAChR antagonist worsened outcome (*Fig 2A, 3A*).

In previous reports using this model we documented the effectiveness of preoperative administration of existing approved therapeutics such as IL-1 receptor antagonist (IL-1Ra) or anti-TNF monoclonal antibody;[5, 6] further documentation is provided for the efficacy of TNFα blockade in this report. There are potential hazards of using IL-1Ra or anti-TNF antibody that need to be considered before these agents can be used to prevent postoperative cognitive decline in patients. As TNFα and IL-1 are important host defense mediators against microbiologic agents, risk of infection will need to be evaluated; furthermore, low levels of TNFα are needed for fracture repair.[35, 36] Alternatively, mechanisms that can enhance resolution of the inflammatory response may also be beneficial in mitigating postoperative cognitive decline. Given the importance of NF-κB signaling in mediating postoperative inflammation, it will also be important to address the role of *IKKβ* regulation on memory processes and cognitive decline.

Cholinergic signaling, in particular α7 nAChR dependent pathways, are critically involved in several CNS disorders, including schizophrenia, depression, anxiety, Alzheimer's disease (AD) and Parkinson's disease (PD).[24] The data demonstrating worsening of cognitive decline when the cholinergic reflex is disabled (*Fig 5A*) raise the possibility that cholinergic dysfunction is a risk factor for the development of postoperative cognitive decline by interfering with resolution of the neuroinflammatory response. This could be particularly relevant in explaining the high prevalence of postoperative cognitive dysfunction in elderly patients;[37] as it is notable that advancing age is associated with declining cholinergic function;[38] this association should now be sought in the clinical setting. The contribution of cholinergic dysfunction, especially by advanced age, diabetes and obesity (also key determinants of cognitive decline)[39], needs to be further explored and could become an important biomarker in identifying high-risk patients. If cholinergic dysfunction is established as a risk factor, its presence can be used to select a cohort for a clinical trial investigating prophylactic and therapeutic strategies to prevent and/or abort postoperative cognitive dysfunction.

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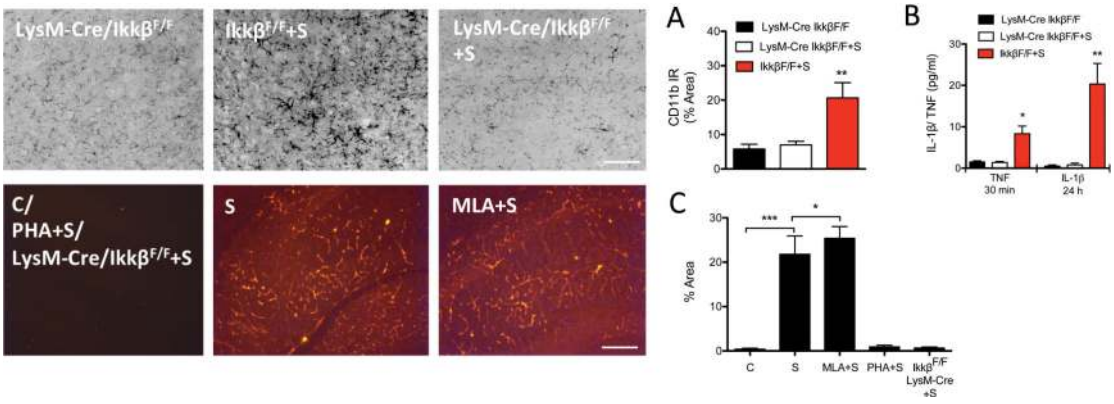


**Figure 1. NF-κB activation in myeloid cells mediates BBB disruption and macrophage migration into the hippocampus following surgery**

In *LysM-Cre/Ikk*β$^{F/F}$ mice surgery produced neither CD11b immunoreactivity (A) as measured with the avidin-biotin and 3,3'-diaminobenzidene technique, nor signs of peripheral inflammation (B) as reflected by normal levels of TNFα and IL-1β (* $p<0.05$, ** $p<0.01$ vs *LysM-Cre/Ikkβ*$^{F/F}$+S). Compared to naïve animals (c) surgery increased expression of immunolabeled fibrinogen in the hippocampus at 24 h postoperatively, *** $p<0.001$ (C). *LysM-Cre/Ikkβ*$^{F/F}$ had no fibrinogen deposition in the brain compared to operated wild-type mice. Immunolabeling for fibrinogen shows significant deposition in the hippocampus 1 day following surgery. Perivascular fibrinogen was also significantly elevated following MLA administration and surgery (MLA+S). Notably, quantification revealed that control mice, surgical mice treated with PHA 568487 and *LysM-Cre/Ikk*$^{F/F}$ had no fibrinogen deposition in the brain (C). Data are expressed as mean ± SEM (n = 4) and compared by one-way analysis of variance and Student-Newman-Keuls method. Scale bars: 30μm. Abbreviations: C=control, MLA=methyllycaconitine, PHA=PHA 568487, S=surgery.

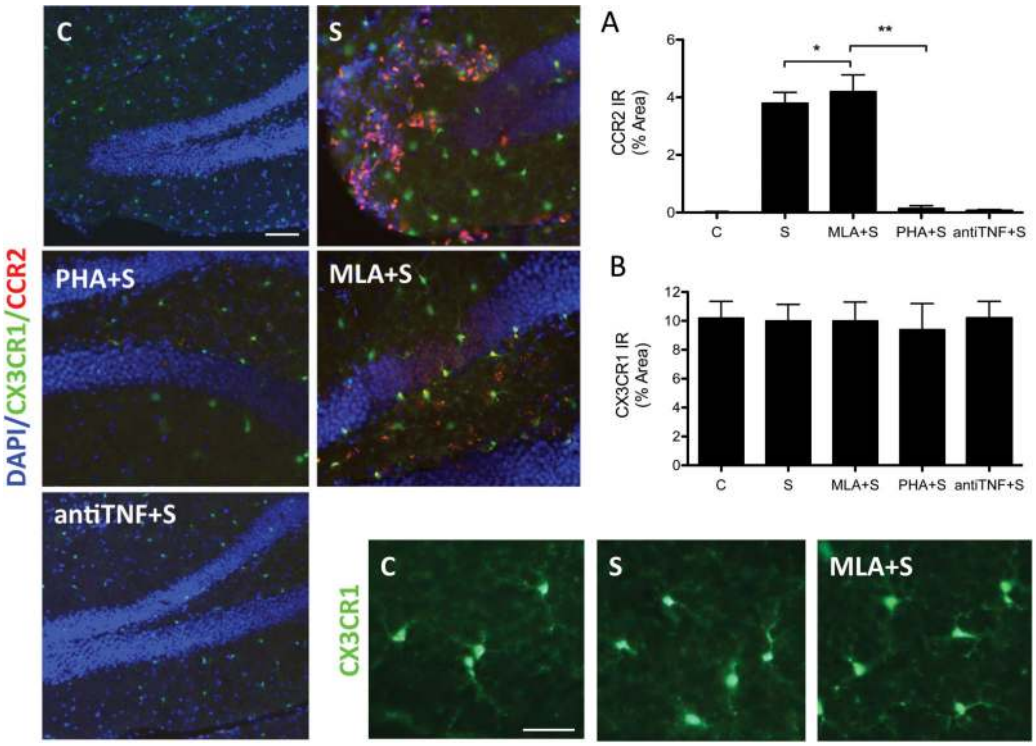


**Figure 2. Surgery-induced neuroinflammation associates with migration of peripheral macrophages**

Epifluorescence of $Ccr2^{RFP/+}Cx3cr1^{GFP/+}$ shows no $RFP^+$ cells in controls (c) or surgical animals treated with the selective α7 agonist (PHA+S) or anti-TNF antibody (antiTNF+S). One day after surgery a significant increase in $RFP^+$ cells was observed around the periventricular lining into and into the parenchyma of the hippocampus (S), but not in other brain regions. Blockade of the cholinergic pathway exacerbated neuroinflammation and infiltration of macrophages in the hippocampus, * $p<0.05$ (MLA+S). Resident microglia (CX3CR1) maintained long and ramified pseudopodia in all groups, suggesting that this subset of resident cells was not involved in surgery induced neuroinflammation or its modulation by nAChRs. Data are expressed as mean ± SEM (n = 4) and compared by one-way analysis of variance and Student-Newman-Keuls method. Abbreviations: C=controls, S=surgery, MLA=methyllycaconitine, PHA=PHA 568487

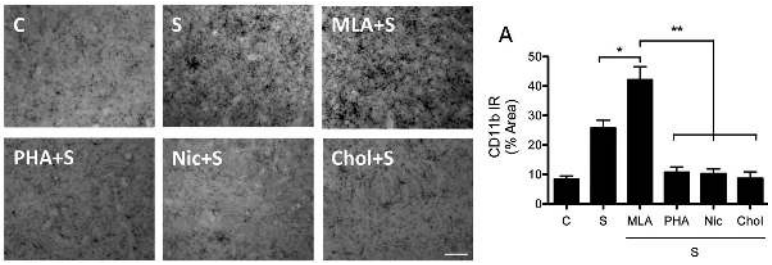


**Figure 3. Effects of cholinergic modulation on neuroinflammation**

Hippocampi were extracted 1 day after surgery and CD11b immunoreactivity was measured with the avidin-biotin and 3,3'-diaminobenzidene technique. Representative photomicrographs show control animals, tibial surgery under general anesthesia and analgesia (S), surgery with preoperative administration of either a bolus dose of a nAChR antagonist (MLA+S), a selective α7 agonist (PHA+S), nicotine (Nic+S) or choline (Chol +S). Densitometry of CD11b immunostaining revealed significant higher immunoreactivity as characterized by hypertrophy of cell bodies and clumping of the ramifications following surgery and surgery in the presence of the cholinergic antagonist, * $p<0.01$ vs S. On the other hand, administration of PHA 568487 and the other cholinergic agonists were able to rescue the normal phenotype, ** $p<0.001$ vs S. Data are expressed as mean ± SEM (n = 4) and compared by one-way analysis of variance and Student-Newman-Keuls method. Scale bars: 30μm. Abbreviations: C=controls, MLA=methyllycaconitine, PHA=PHA 568487, S=surgery, Nic=nicotine, Chol=choline

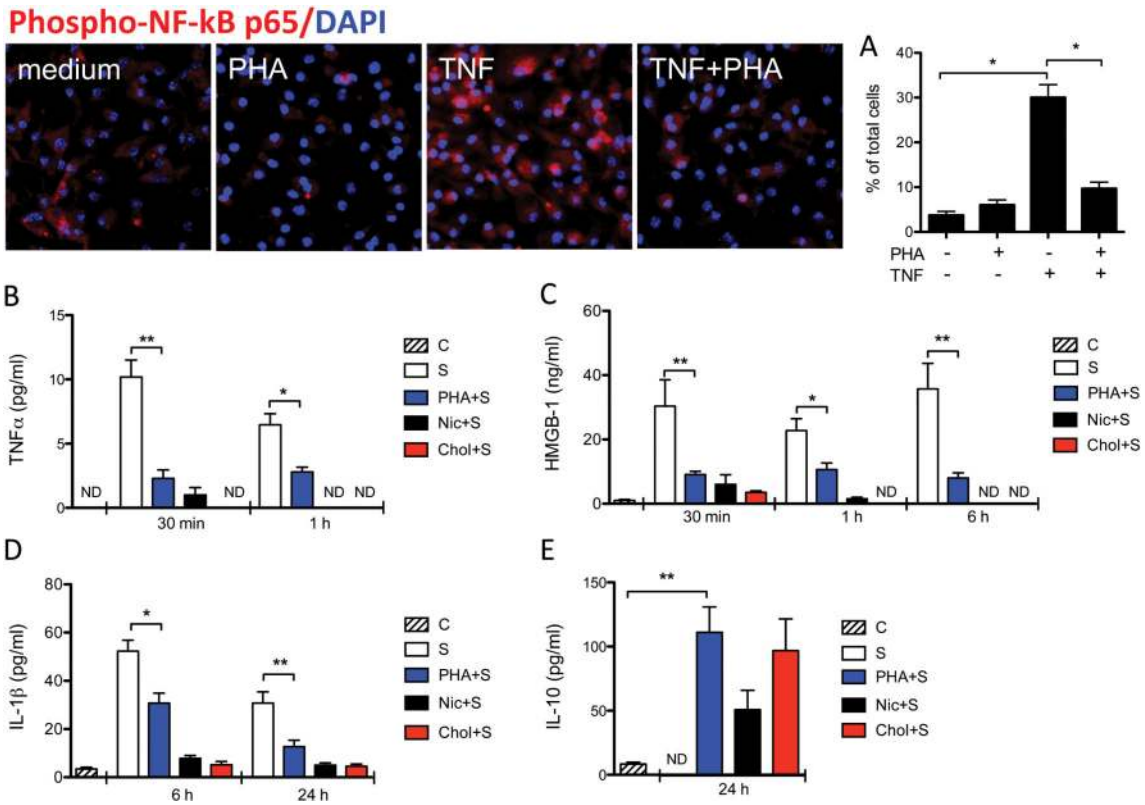


**Figure 4. Stimulation of α7 nAChR blocks TNF-induced NF-κB activation in BMDMs in vitro and postoperative systemic inflammation**

Bone marrow was extracted from long bones of the hind limbs of C57BL6/J mice and BMDMs isolated and differentiated for 7 days (A). Cells were stimulated with TNFα (40 ng/ml) for 2 h and immunostained for nuclear phosphorylated NF-κB subunit p65, this was significantly attenuated when pre-incubated with PHA 568487 (10 μg/ml) for 30 min (TNF +PHA) (* $p<0.01$ vs PHA and control). NF-κB activation was undetectable in unexposed cells (medium) or in cells exposed only to PHA 568487 (PHA). Systemic levels of cytokines were measured by ELISA at different time points; each cohort underwent surgery (tibial fracture under general anesthesia and analgesia) with the exception of naïve controls. Stimulation of the cholinergic signaling pathway with the non-selective BBB permeant (nicotine), impermeant (choline) nAChR agonist, and the selective α7 agonist (PHA) all significantly reduced levels of TNFα (B) and HMGB1 (C) and at all time points (* $p<0.01$, ** $p<0.001$ vs S). Delayed production of IL-1β starting from 6 hours following surgery was also reduced by each of the nAChR agonists (D). 24 hours after surgery the nAChR agonists increased levels of anti-inflammatory IL-10, ** $p<0.001$ vs control (E). Results are expressed as mean ± SEM (n = 5) and compared by one-way analysis of variance followed by Bonferroni's multiple comparison test and Student-Newman-Keuls test. Abbreviations: C=controls, S=surgery, PHA=PHA 568487, Nic=nicotine, Chol=choline, ND=not detected

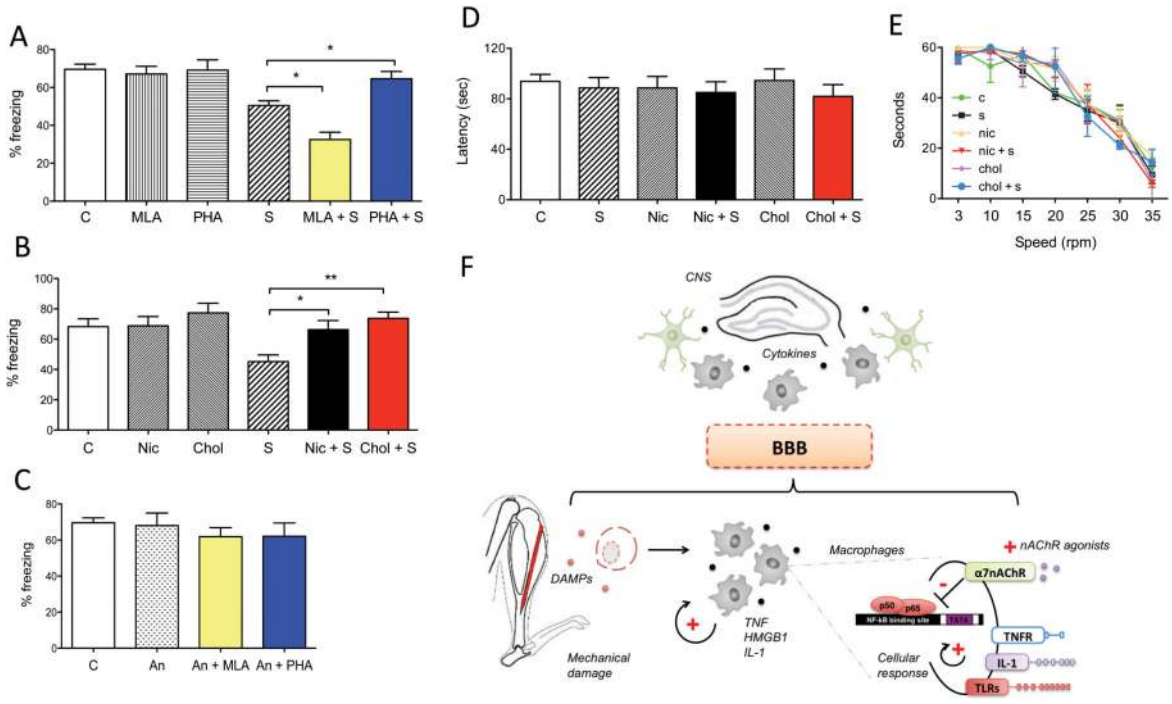


**Figure 5. Effects of cholinergic modulation on memory function following surgery**

Contextual fear response reveals hippocampal-dependent memory impairment at postoperative day 3. Preoperative treatment with a selective α7nAChR agonist (PHA+S) prevented postoperative memory impairment, * $p<0.05$ vs S (A). Conversely, preoperative blockade of cholinergic signaling (MLA+S) exacerbated the deficiency in freezing behavior following surgery. Preoperative administration of nicotine and choline also prevented surgery-induced cognitive decline (* $p<0.05$, ** $p<0.01$ vs S, respectively) (B). Mice were treated with MLA or PHA 568487 before undergoing general anesthesia with 2.1% isoflurane for 15 minutes (C). There were no additive effects by cholinergic treatment following exposure to isoflurane anesthesia on memory function 3 days after exposure. Following contextual fear assessment 3 days after surgery, mice were tested on a rotarod to address possible motor impairments and general activity. Fixed speed rotarod: latencies (average of three trials at 15 rpm) were not significantly different between groups, whether exposed to surgical trauma or cholinergic stimulation only (D). Accelerating rotarod: no changes between groups were observed during the accelerating phase (E). Working model (F): damage to the cells following orthopedic surgery promotes rapid release of damage-associated molecular pattern (DAMP) mediators. Within the peripheral macrophages, NF-κB is activated to enhance transcription and subsequent synthesis and release of pro-inflammatory cytokines including TNFα, HMGB1, IL-1 *etc*, further augmenting NF-κB transcription *via* activation of different receptors (PRRs). Systemic cytokines, in particular TNFα, augment BBB permeability, ultimately allowing peripheral immunocompetent cells to invade the CNS. Macrophages once in the brain parenchyma account for the neuroinflammatory changes, affecting hippocampal networks, synapses and neurons and ultimately affecting memory function. Cholinergic agonists activate the nAChR on peripheral macrophages to inhibit NF-κB-induced inflammation, BBB disruption and postoperative cognitive decline. Data are expressed as mean ± SEM (n = 10) and compared

by one-way or two-way (rotarod) analysis of variance and Student-Newman-Keuls method. Abbreviations: N=naïve, MLA=methyllycaconitine, PHA=PHA 568487, S=surgery, Nic=nicotine, Chol=choline