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Neutrophil depletion after subarachnoid hemorrhage improves memory via NMDA receptors

Jose Javier Provencio^{1,2,3}, Valerie Swank¹, Haiyan Lu¹, Sylvain Brunet², Selva Baltan², Rohini V. Khapre¹, Himabindu Seerapu¹, Olga N Kokiko-Cochran², Bruce T Lamb², and Richard M Ransohoff¹

¹Neuroinflammation Research Center, Neuroscience, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave. Cleveland, OH, USA 44195

²Neuroscience, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave. Cleveland, OH, USA 44195

³Department of Neurology and Neuroscience, Brain Immunology and Glia Center, University of Virginia, PO Box 800394, Charlottesville, VA USA 22908

Abstract

Cognitive deficits after aneurysmal subarachnoid hemorrhage (SAH) are common and disabling. Patients who experience delayed deterioration associated with vasospasm are likely to have cognitive deficits, particularly problems with executive function, verbal and spatial memory. Here, we report neurophysiological and pathological mechanisms underlying behavioral deficits in a murine model of SAH. On tests of spatial memory, animals with SAH performed worse than sham animals in the first week and one month after SAH suggesting a prolonged injury. Between three and six days after experimental hemorrhage, mice demonstrated loss of late long-term potentiation (L-LTP) due to dysfunction of the NMDA receptor. Suppression of innate immune cell activation prevents delayed vasospasm after murine SAH. We therefore explored the role of neutrophil-mediated innate inflammation on memory deficits after SAH. Depletion of neutrophils three days after SAH mitigates tissue inflammation, reverses cerebral vasoconstriction in the middle cerebral artery, and rescues L-LTP dysfunction at day 6. Spatial memory deficits in both the short and long-term are improved and associated with a shift of NMDA receptor subunit composition toward a memory sparing phenotype. This work supports further investigating suppression of innate immunity after SAH as a target for preventative therapies in SAH.

Corresponding Author: Jose Javier Provencio, MD, Neurology, PO Box 800394, Charlottesville, VA USA 22903, 1-434-924-8371, jp3b@virginia.edu.

Change of address: Ransohoff, RM, Neuroimmunology, Biogen Idec, 250 Binney St. Cambridge, MA, USA 02142

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AUTHOR CONTRIBUTIONS

JJP contributed to study design, conduct of experiments, acquiring and analyzing data, and manuscript preparation. VS, HL, SBr, RVK, ONK-C and HS contributed to acquiring and analyzing data and review of the manuscript. SBa contributed to study design, acquiring and analyzing data and review of the manuscript. BTL contributed to study design, analyzing data and review of the manuscript. RMR contributed to study design, analyzing data, and manuscript review and revision.

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INTRODUCTION

Subarachnoid hemorrhage (SAH) due to the rupture of an intracranial aneurysm accounts for 3% of all strokes but makes up a disproportionate amount of the morbidity (25%)¹, and affects a population that is younger and healthier than those who suffer ischemic stroke. Delayed deterioration associated with vasospasm (also called delayed cerebral vasospasm or delayed cerebral ischemia) is a curious secondary complication of SAH with the hallmark of neurological dysfunction that begins 3–13 days after the hemorrhage and is associated with confusion, decreased level of consciousness, and stroke. Treatment of delayed deterioration has focused on the treatment of the vasculopathy and prevention of stroke with little success.^{2,3,4}

A number of important issues remain to be resolved. Although ischemic stroke leads to the greatest measured disability in patients with delayed deterioration, permanent cognitive deficits account for an important, but less-well recognized disability⁵. Cognitive deficits occur in 73% of patients with SAH 3 months after the hemorrhage and remain double the incidence of the general population 18 months after SAH^{6,7,8}. In mice, memory deficits are localized to spatial memory tasks, but not activity levels, suggesting that hippocampal memory function is involved⁹. Similar behavioral deficits are implicated in other memory disorders such as Alzheimer's-type dementia^{10, 11–13}.

Entrainment of spatial memory is dependent on late long-term potentiation (L-LTP) mediated by the N-methyl-D-aspartate receptor (NMDAR) and α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA). In SAH, pH-dependent chemical blockade of one subunit of the NMDAR (NR2B) improves neurobehavioral scores in mice but the mechanism for these memory deficits has not been explored¹⁴.

The question of the time of onset of delayed deterioration is particularly vexing. In patients with SAH, delayed deterioration occurs between the 3rd and 14th day after the hemorrhage¹⁵. It is not uncommon for a patient to recover full consciousness in the first days after the initial hemorrhage only to slip away into a coma again after a week. A number of unsatisfying hypotheses have been investigated. Red cell break down has been hypothesized as a delayed instigator of damage although extracts of lysed cells do not recapitulate the syndrome¹⁶. Likewise, the development of reactive oxidant species (ROS) from bilirubin degradation has been investigated (Bilirubin oxidation products or BOX's) but, in samples collected from patients, bilirubin and ROS production develop early^{17,18}. Alterations in nitric oxide (NO) and endothelin-1 (ET-1) have also been studied in the development of vascular spasm, but not in the context of cognitive or behavioral dysfunction.

Early inflammation has been shown to be a marker for delayed deterioration, but the mechanisms underlying the delay in manifestations are not yet understood^{19, 20}. Mice depleted of myeloid cells using an antibody against Ly6G/C (anti-Gr1, which depletes neutrophils, activated monocytes, and some populations of macrophages) *prior* to SAH, do not develop vascular spasm or short-term spatial memory deficits suggesting that early inflammation plays a role²¹⁹.

In this set of studies, we investigate the mechanism of spatial memory deficits in relationship to the timing of inflammation in delayed deterioration. We find that dysfunction of the N-methyl-D-aspartate receptor (NMDAR) is associated with impaired performance on spatial memory tasks. We also show that depletion of neutrophils at a specific time after SAH recovers both NMDAR function and the behavioral deficits associated with SAH.

MATERIALS AND METHODS

All studies were done under the supervision of and with the approval of the Lerner Research Institute Institutional Animal Care and Use Committee (IACUC).

SAH model

C57BL/6 male mice 8–12 weeks of age were exposed to either experimental SAH (SAH) using a modification of a protocol previously described or sham surgery²². In addition, 8–12 week old male C57BL/6 EGFP-LysM reporter mice (Lyz2^{tm1.1Graf}) that exhibit green fluorescence on the cell surface of bone marrow derived myeloid cells were exposed to SAH to evaluate myeloid cell infiltration into the brain. Briefly, mice were anesthetized using 2% isoflurane and positioned under the surgical microscope on a heated pad to maintain body temperature. Bupivacaine was injected at the site of surgery and a small incision was made in the midline between the strap muscles. All the muscles in the neck were separated to reveal the dura mater overlying the cerebellum. A 30G needle was inserted through the dura mater and a conserved subarachnoid vein located in the subarachnoid space (just below the dura mater) was located and carefully transected with the beveled edge of the needle tip. The mice were then positioned with the head down at a 30° angle for 5 minutes to allow the blood to migrate to the subarachnoid spaces in the basilar cisterns and base of the brain. Sham operated mice had a similar incision and were injected with 10–20 microliters of sterile phosphate buffered saline into the subarachnoid space also with a 30G needle. In both treatments, the site of incision was sutured and the mice were transferred to a recovery chamber prior to returning to their respective cages with appropriate analgesia.

The model has been previously described in detail²². Mice recover quickly from surgery with few deficits²². Although the mice exhibit impaired spatial memory function on initial testing, they recover by day 5 post hemorrhage. The animals have no motor deficits by rotorod testing and survive long-term. Gross evaluation of the brain after surgery shows blood in the subarachnoid space predominantly at the base of the brain but there is no evidence of brain discoloration or bleeding²².

Hippocampal Slice Electrophysiology

Experiments were performed in the CA1 region of the hippocampus with 450 μ M thick transverse slices from mice after SAH or sham three or six days after surgical intervention. Mice were decapitated after anesthesia, the brains were immediately removed and placed in iced saline. The hippocampi were quickly dissected and sliced. The slices were stabilized in carbogenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF, in Mm: 126 NaCl, 3.5 KCl, 1.3 MgCl₂, 2 CaCl₂, 1.3 NaH₂PO₄, 25 NaHCO₃ and 10 glucose at pH 7.4) for 1–2 hours at room temperature.

Experiments were done in tissue fully submerged in warm ACSF (at 33–34°C) continually flowing at a rate of 3–3.5 ml/min. For simultaneous extracellular recordings of excitatory post-synaptic potentials (EPSPs) and population spikes (PSs), glass microelectrodes filled with 2 M NaCl (resistance 1–2 MΩ) were placed in the CA1 stratum radiatum and stratum pyramidale, respectively. The responses were evoked by stimulation of Schaffer collateral fibers by a bipolar tungsten wire electrode, with 100μs pulses, at intervals of 30 seconds. Tetanic LTP was elicited by two 1-second bursts of high frequency stimulation (HFS) at 100-Hz delivered 20 seconds apart, repeated after another 20–25 min to obtain maximal LTP. Depotentiation²³ was induced by stimulating at 1 Hz for 15 min with low frequency stimulation (LFS). To obtain more comprehensive data, responses were recorded at stimulus intensities covering a wide range before and after applications of HFS and LFS. Results from several slices were pooled and normalized to the maximal values of stimulus intensity and the corresponding amplitude of the afferent volley, EPSP slope, or population spike.

To test whether the NMDA receptor is necessary and sufficient to produce the observed LTP, a modified ACSF (containing 30 μM CNQX, 10μM Picrotoxin, 10 μM Glycine, 4 mM CaCl₂ and 0 mM Mg²⁺) was applied 30 min before the onset of HFS and was continued 10 min after the end of the second HFS.

Assessment of vasospasm: animals were anesthetized with pentobarbital (6 mg/kg i.p.) and underwent transcardiac perfusion with 20 ml saline, followed by 20 ml of 4% paraformaldehyde, and subsequently, 10 ml of warmed 5% India ink in gelatin. Animals were then decapitated and the brains were carefully removed preserving the vasculature. The Circle of Willis and the brain stem vasculature were examined under a surgical microscope and relevant pictures were captured using a microscope-mounted camera (Leica, DM4000M, Wetzlar, Germany). Image analysis was conducted with Adobe Photoshop CS5 version 12 (San Jose CA). The diameter of the MCA segment was measured at the point 1 mm from the posterior wall of the carotid artery into the MCA, and the values from SAH and sham animals are compared. We compared the incidence of vasospasm between control and SAH.

Cell depletions

Animals were treated with the neutrophil-depleting anti-ly6G antibody (1A8) at a dose of 4mg/ kg i.p., or 50 μl of saline (saline control) at the time points described^{24,25}. The time course of neutrophil depletion with antibody 1A8 has been described by other groups^{26,21}. The depletion of approximately 90% of neutrophils is achieved within 6 hours and remains suppressed for more than 72 hours. By one week, neutrophils have repopulated the blood stream.

The mechanism by which neutrophils are depleted is not completely understood but likely involves activation of the reticuloendothelial system to scavenge opsonized neutrophils which, in turn, activates complement. As a control to evaluate whether this non-specific effect contributes to the findings, we exposed a subset of mice to an antibody against Glycerol Kinase (GK 1.5) at 3 mg/kg i.p. to deplete a non-neutrophil leukocyte subset (CD4⁺ Lymphocytes) that are not suspected to be involved in this type of inflammation.

Immunoblotting

Brains were extracted from SAH and Sham treated mice and hippocampi were carefully isolated for electrophoresis and western blot analysis. Isolated hippocampi were lysed in ice-cold modified RIPA buffer (1M Tris pH 7.5, NP40 10%, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1mM, PMSF 1mM, Na₃VO₄ 1mM, protease inhibitor and phosphatase inhibitor). To isolate cell membranes, lysates were centrifuged for 5 minutes at 1000 rpm and supernatant was collected. The supernatant was centrifuged at 12000 rpm for 30 minutes and the separated pellet was homogenized for 2 minutes in ice-cold modified RIPA buffer. Protein concentration was determined by protein BCA assay. For each lysate, 37µl of brain tissue (containing 7 µg protein) was loaded to a 4–20% Tris-Glycine gels (SDS-PAGE) and transferred on Invitrolon PVDF membranes using a wet transfer method (Invitrogen, Carlsbad, CA). Proteins were detected using specific primary antibodies as follows: Anti-NR1, NR2A, and NR2B (Cell Signaling, Danvers, MA), anti-PKC, anti-Phospho-PKC, anti-CaMKII and β-actin (ThermoFisher Scientific, Waltham, MA), and anti-CaMKII (Phospho-T286)(AbCam, Cambridge, UK). Band detection and quantification was done using Li-COR Odyssey CLx imaging system with IRDye Secondary antibodies (IRDye 680 RD anti-rabbit (Li-COR), IRDye 800 CW anti-mouse). Results were normalized against β-actin. Intensity values were converted to relative units using the mean intensity of the sham cohort to represent 1.

Immunohistochemistry

Wild type C57BL/6 mice were subjected to SAH and sham surgery with and without neutrophil depletion and sacrificed at specific time points after surgery. Brains were fixed in 4% paraformaldehyde for 24 h at 4°C after perfusion with PBS. They were cryoprotected in 30% sucrose at 4 °C overnight and embedded in OCT solution and sectioned into 40-µm cryosections. Free floating sections were incubated for 45 min in TBS containing 0.1% Triton X-100 and 5% normal goat serum (NGS) and incubated with monoclonal antibody Ly6G (for neutrophils) or Iba-1 (Wako labs, 1:1,000 dilution)] overnight at 4 °C. Sections were incubated in Alexa Fluor 488–goat anti-rabbit IgG (Invitrogen; 1:500 dilution) and Alexa Fluor 594–goat anti-mouse IgG (Invitrogen; 1:500 dilution) for 1 h at room temperature, rinsed, and air dried before mounting. Confocal imaging was done using Leica DM 6000 CSF confocal microscope with Leica Microsystems LAS AF-TCS SP5.

C57BL/6 EGFP-LysM (*Lyzz^{tm1.1Graf}*) mice were subjected to SAH and slices were evaluated 3 days after SAH using the same preservation and staining technique with the exception that the staining was performed in the dark to prevent loss of fluorescent signal. A secondary anti-GFP antibody (ThermoFisher Scientific, Waltham, MA) was used to enhance the signal. Confocal imaging was done using Leica DM 6000 CSF confocal microscope with Leica Microsystems LAS AF-TCS SP5.

Behavior tests

Barnes maze test—SAH and Sham-treated mice were tested using the Barnes maze test to evaluate spatial memory and cognitive performance in the immediate period after the intervention. After surgery, mice were rested for two days after which they underwent two days of twice-a-day training. Visual cues were placed around the maze that remained

constant throughout the experiment. Training was aimed at teaching mice to reach a hidden goal box by guiding them towards the box. During testing trials, mice were placed at the center of the table and allowed to locate the escape box. Performance was evaluated once a day for 7 days. The test was recorded using a video tracking system (Columbus Instruments, Columbus, OH) and latency was defined as the time taken for each mouse to find the goal box. The test was terminated when the mouse found the goal box or if five minutes expired without the mouse finding the box.

Water maze—Morris water maze (MWM) was performed for the same experimental conditions as the Barnes maze test to evaluate spatial memory and cognitive performance one month after the intervention. Because the Barnes maze and MWM test similar cognitive domains but are unique experiences, we chose MWM testing at the later time point to limit confounding learning effects.

The MWM test was conducted in a circular water-filled tank with a goal platform in a room with visual spatial cues, which remained constant throughout the experiment. Mice were recovered after the initial surgical intervention, underwent BMT then were allowed to survive under normal conditions to the one-month time point. At that time, training using a visible platform was undertaken for 3 days with 4 trials per day. After training, the mice were evaluated in opaque milky water for five days to test visual memory. Mice were placed in the pool from one of four start locations (i.e., north, south, east, west) and given one minute to locate a goal platform. Mice were subjected to four trials a day and their movement was recorded with a video tracking system (Noldus Information Technology, Leesburg, VA).

Novel Object Recognition—Animals were placed in an arena where they are habituated to several objects for 10 minutes and removed from the arena. Recognition memory was tested by replacing one of the known objects with a novel object and recording the amount of time spent exploring the novel object compared to the previously explored object.

Statistics—Statistical analysis was performed using GraphPad PRISM software (Carlsbad, CA). For experiments with values measured over time (Electrophysiology, Barnes maze and MWM), 2-way ANOVA analysis was used. We used Student's T-tests for normally distributed data and Welch corrections if the data was not normally distributed. P values <0.05 were considered significant.

Multiple slices of brain from each animal were used for electrophysiology experiments. The mean value from each animal was compared to other animals. Standard deviations were calculated for all the slices in each experiment.

RESULTS

After SAH, mice have long-term spatial memory impairment due to impairment of L-LTP. Six days after SAH in mice, there is a change in the ability of mice to perform spatial memory tasks (the Barnes Maze Test or BMT)⁹. After SAH and delayed deterioration, patients report similar long-term cognitive deficits⁷. To investigate whether changes seen

early in SAH are persistent, mice were tested with BMT over the first 9 days of injury, and with the Morris Water Maze test (MWM) and novel object recognition (NOR) at 1 month after the SAH or sham. Mice initially showed longer times to find the goal box on the BMT after SAH compared to sham (Figure 1A). The increased time was attributable to increased wandering around the open field as evidenced by longer path lengths in SAH than sham (Supplemental Figure 1A) suggesting that memory, not physical impairment, leads to the poor performance.

To test whether these memory deficits are long lasting, the same mice were tested one month after SAH or sham in a MWM. MWM was chosen to examine performance after the BMT because both mazes test similar spatial memory performance but are different enough environments to limit task specific memory. On MWM, mice with SAH performed worse than sham animals in the time required to find the platform (Figure 1B). Again, the delay in finding the platform was associated with longer path lengths suggesting that spatial memory was impaired (Supplemental Figure 1B). A test of novel object recognition one month after SAH did not reveal differences between groups suggesting that the loss of spatial memory is due to a specific domain loss and not part of global poor mental function (Supplemental Figure 1C).

To investigate the neurophysiological underpinnings of the memory deficits, specifically whether the NMDA/AMPA receptor system is altered during the development of delayed deterioration, mice were sacrificed after experimental SAH or sham surgery at day 3 and day 6 for electrophysiological evaluation. The six-day time-point was chosen as it corresponds with the initiation of vasospasm and impairment of spatial memory on BMT⁹. The day 3-time point was chosen to be before memory impairment.

The Shaffer collateral fibers were stimulated and transynaptic potentials in the CA1 pyramidal cells were sensed (Figure 1C). Stimulus amplitude plots in sham and SAH show normal transduction patterns suggesting that stimulus generation from CA3 is not impaired. Excitatory post-synaptic potentials (EPSP) slope vs. amplitude plots show normal potentiation across successive tetanic volleys in sham but loss of LTP in SAH (**Data not shown**).

In animals evaluated three days after sham or SAH, EPSP slope remains consistently positive over time after the potentiating stimulus (Figure 1D). In contrast, animals tested six days after sham or SAH, only the sham maintained consistent EPSP slope through the time of the experiment; the SAH animals failed to develop L-LTP (Figure 1E). These data suggest that impairment of L-LTP develops between 3 and 6 days after SAH and is consistent with the development of memory impairment.

To better define the receptor responsible for this effect, slices from mice 6 days after sham or SAH were exposed to 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a competitive antagonist of the AMPA receptor), picrotoxin (a blocker of inhibitory Cl⁻ channels), and excess Ca²⁺ in a Mg²⁺-free bath. This excludes any facilitating effects from the AMPAR and the effects of inhibitory receptors, allowing investigation of the NMDAR. Isolation of the NMDAR failed to protect the SAH mice from impairment of L-LTP suggesting that the

NMDAR is responsible for changes in L-LTP after SAH (Figure 1F). These experiments show that L-LTP is impaired between 3 and 6 days after SAH through the impairment of the NMDAR (**Shown graphically in Figure 1G**).

Neutrophil depletion after SAH improves delayed deterioration

In previous work, we showed that selective depletion of myeloid cells (neutrophils, monocytes, and activated macrophages) using an antibody against the Ly6G/C antigen *prior* to the hemorrhage improves vasospasm and behavioral deficits associated with SAH ⁹. To investigate whether specific inhibition of neutrophils improves vasospasm and behavioral function, neutrophils were selectively depleted using an antibody against Ly6G.

Although depletion of inflammatory cells prior to SAH reveals important information about the physiology involved, it is not a reasonable treatment option as SAH is an unpredictable occurrence. To investigate if there is a role for neutrophil depletion after SAH, anti-Ly6G antibody was administered at four time points: 1 day before SAH, 12 hours, 1 day and 3 days after SAH or sham. SAH animals not treated with anti-Ly6G showed significantly smaller diameter of the MCA implying vasospasm. Interestingly, depletion of neutrophils *prior* to SAH did not prevent spasm of the middle cerebral artery (MCA) 6 days after SAH unlike depletion of a more general population of myeloid cells with Ly6G/C ⁹. Administration of anti-Ly6G 12 hours (0.5 days) and 1 day after SAH failed to prevent vasospasm. In contrast, administration 3 days after SAH prevented vasospasm (MCA diameter not significantly different than sham) (Figure 2A). Subsequent experiments focused on this 3-day neutrophil depletion time point.

To evaluate whether nonspecific activation of the reticuloendothelial system during scavenging of opsonized neutrophils and complement activation is responsible for the effect on vasospasm, mice were administered an antibody against Glycerol Kinase (GK 1.5) to deplete CD4⁺ lymphocytes. This antibody did not prevent vasospasm in SAH mice. Since CD4⁺ lymphocytes are not understood to be involved in this inflammation, this finding suggests that the effect is neutrophil specific (Supplemental Figure 2A).

To test spatial memory, mice with neutrophil depletion at 3 days were evaluated with BMT in the first 9 days after SAH and MWM one month after SAH (the day 3 administration of anti-Ly6G antibody was completed 6 hours before behavioral training was initiated). In both tests, depletion of neutrophils returned the animals to performance significantly improved from SAH alone and indistinguishable from sham (Figure 2B).

To investigate the mechanism of improvement in behavioral testing, mice treated with anti-Ly6G antibody three days after SAH or sham were sacrificed three days later (at day 6) and brain slices were tested for L-LTP. Evaluation of EPSP slope over the time of the experiment shows that L-LTP is not impaired in neutrophil depleted animals. Although the response to stimulation in the SAH animals after neutrophil depletion was improved from the 15th to the 60th minutes compared to sham animals, the final slope at 180 minutes was not different (Figure 2C). A comparison of final EPSP slopes at 3 days after SAH, 6 days after SAH, 6 days after SAH with NMDAR isolation, and 6 days after SAH with neutrophil depletion at day 3 highlights the improvement in L-LTP with neutrophil depletion.

A number of mechanisms may account for this impairment of L-LTP after SAH and improvement with neutrophil depletion. Unchanged action potential signal intensity of EPSP by electrophysiology makes significant hippocampal neuronal loss unlikely, but changes in the two downstream second messenger systems, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) signaling, and/or changes in NMDAR subunit composition are possible etiologies. Levels of CaMKII, PKC and phosphorylated CaMKII by electrophoresis and Western blotting of hippocampus showed no differences between sham and SAH (Supplemental Figure 2B).

Changes in subunit composition of the NMDAR have been implicated in a number of disorders of memory and are altered in the setting of ischemia reperfusion induced inflammation²⁷²⁸. NR1 is constitutively expressed in NMDAR but the relative levels of NR2A and NR2B are variable. The expression of NR1, NR2A and NR2B is not significantly different between SAH and sham.

The level of NR2A is significantly decreased in neutrophil depleted mice and subsequently, the ratio of NR2A/2B is significantly decreased after depletion of neutrophils at day 3 (Figure 2D). Although the subunit change is not the associated with deterioration, it is a likely candidate for the improvement in behavioral testing in mice undergoing neutrophil depletion. A decreased ratio of NR2A/NR2B has been associated with improved memory function in models of brain trauma and chronic stress²⁹²⁷.

Microglial morphology change is associated with improved memory function after neutrophil depletion

We hypothesized that neutrophils increase brain inflammation, which then leads to vasospasm and poor performance on spatial memory tasks. Interestingly, neutrophils do not enter the hippocampus in significant quantity at either day 1, day 3 or day 6 after SAH suggesting that the action of neutrophils is not from direct invasion as is seen in other inflammatory disorders of the central nervous system such as experimental autoimmune encephalitis (EAE) (Figure 3A). To better visualize myeloid cells, we tested C57Bl/6-EGFP LysM mice (Lyz2^{tm1.1Graf}). These mice express GFP on the surface of monocytes, neutrophils and macrophages. We found occasional perivascular myeloid cells in the hippocampus and few intraparenchymal myeloid cells (Figure 3B). In wild type mice, microglial staining intensity was significantly increased after SAH in the hippocampus and significantly improved after neutrophil depletion (Figure 3C). There were more inflammatory cells in SAH hippocampi than sham or SAH after neutrophil depletion. The association of increased microglia/monocyte activation and memory dysfunction suggests that neutrophils act through increasing microglial brain inflammation.

DISCUSSION

Patients with SAH who experience delayed deterioration associated with vasospasm after SAH show impairment of executive function tasks such as prose recall as long as 18 months after the injury^{7,8}. Current therapeutic strategies to ameliorate the vasculopathy associated with delayed deterioration have not made measurable impact on patient cognitive outcomes suggesting that non-stroke injury mechanisms may play a role^{2,30}. The findings presented

here implicate delayed alterations in the NMDAR-generated L-LTP as the cause of long-term memory deficits seen in experimental SAH. Mice in our model had changes in L-LTP six days after the injury that were not apparent at day 3.

In addition, behavioral abnormalities in spatial memory tasks coincided with this change. Importantly, novel object interest did not change significantly suggesting that the cognitive dysfunction is domain specific and selective, a finding also seen in patients with SAH⁸.

There are other reports that implicate the NMDAR in behavioral task dysfunction in SAH, but our study expands on these previous studies in important ways^{14,31, 32, 33}. Huang and colleagues administered memantine, a low-affinity NMDA antagonist immediately after SAH. In a more severe model of SAH that shows significant motor dysfunction, animals given memantine had improved motor function three days after hemorrhage³¹. More specifically, Chen and colleagues administered a specific NR2B inhibitor, Ro 25-6981, which improved vasospasm but showed no improvement in MWM performance at day 3 after SAH. Wang and colleagues showed that selectively blocking the NR2B subunit of the NMDA receptor improved global neurological scores up to 28 days but the improvements were in a composite motor/sensory score and were apparent 3 days after SAH making them unlikely to be due to delayed deterioration¹⁴. These studies used a more severe form of experimental SAH and found early results that were possibly attributed to early injury after SAH. By contrast, our data suggests that important brain damage after mild SAH occurs between 3 and 6 days similar to delayed deterioration seen in humans and inhibits specific spatial memory tasks.

There has been debate over the relative contributions of early brain injury after SAH caused by acute intracranial hypertension and cerebral perfusion impairment and effects of delayed deterioration^{34,35,36}. This study calls into question the findings of groups who have suggested that early brain injury is responsible for the deficits seen after SAH^{34,19,37}. Early brain injury, although likely an important factor in the outcome of patients (and in more severe models of SAH in rodents), is difficult to prevent because the events typically occur outside the hospital. It is clear that patients with SAH experience delayed worsening associated with angiographic vasospasm that may not be well modeled in more severe models of SAH. Our model allows the evaluation of delayed deficits due to the absence of significant early injury found in other models.

An important finding in this work is the durability of the behavioral deficits. In patients with SAH, cognitive impairment lasts far after physical rehabilitation^{7,38}. Findings from this study show that NMDA mediated delayed injury chronically affects memory function. The time delay in expression of the L-LTP abnormality and the delay of worsened performance suggest that there may be a treatment window to prevent memory impairment. Additionally, this time window may be days, not minutes or hours^{9,32}.

The mechanism of delayed deficits is poorly understood. Focus over the last three decades has been on blood degradation in the subarachnoid space leading to ischemia from the cerebral vasculopathy, vasospasm (Reviewed by Tani)³⁹. A number of studies suggest that the vasculopathy may not drive all of the pathology in humans calling into question whether

ischemia is the predominant driver for injury^{40,41}. Inflammation has been postulated as a mechanism of delayed injury but the type of inflammation is still not defined⁴². Elevation of CSF neutrophils in patients with SAH on the third day after the hemorrhage are associated with delayed deterioration⁴³. Inhibition of myeloid cells in a murine model through the CD11b receptor has been shown to improve vessel spasm^{44,45}. In addition, depletion of myeloid cells using anti-Ly6G/C antibodies mitigates spatial memory dysfunction seen and delayed spasm⁹. We were therefore interested in whether modulating innate immunity after SAH could be a therapy for the prevention of delayed deterioration.

The second part of this study was aimed at exploring the time window for possible intervention against this secondary injury. In these experiments, we tested a specific neutrophil depleting antibody both before and after SAH. Interestingly, depletion of neutrophils before SAH does not appear to have the same effect on vasospasm as depletion of myeloid cells more broadly. This implicates other myeloid cells such as monocytes and activated macrophages as important mediators of the early inflammation, which occurs directly after the hemorrhage. Nam and colleagues have found that early after SAH in patients, the expression of IL-1 β by *ex vivo* stimulation of monocytes with lipopolysaccharide predicted patients who would later go on to develop vasospasm⁴⁶. Additionally, Schneider and colleagues found that CSF from patients with SAH increased monocyte migration across an experimental blood brain barrier early after SAH⁴⁷.

The important finding from a therapeutic standpoint is the resolution of both vasospasm and spatial memory deficits after depletion of neutrophils 3 days after SAH. This suggests strongly that inflammation after SAH continues to have deleterious effects long after the hemorrhage and defines the innate immune response as critical in that ongoing damage. Neutrophils (and their associated products), as late actors of damage in acute illness, have become better recognized recently, particularly in non-infectious pathologies^{48,49}. Their role here is curious because, although they are clearly important in activating microglia in the area of the hippocampus, they are not present in that part of the brain. Previous studies from our laboratory have shown neutrophil accumulation in the CSF and scattered sparsely throughout the parenchyma of the brain; particularly adjacent to the CSF space containing blood clots but it is unclear how these neutrophils interact with distant intraparenchymal cells⁹.

A number of possible mechanisms exist for neutrophils to exert distant effects on brain tissue in a delayed manner. Systemic depletion of neutrophils may affect signaling to other infiltrating immune cells which may act as effectors of inflammation. Conversely, neutrophils might be acting through secretory signaling mechanisms that enter the brain either through the CSF brain barrier or blood brain barrier. The cytokines IL-1 β and IL-6, but not TNF α , have been shown to be important in L-LTP production and maintenance⁵⁰. IL-6 reduces calcium entry through NMDA receptors and inhibits L-LTP production^{51,52}. Neutrophil initiated release of brain-derived cytokines is a possible mechanism to tie inflammation to impairment in L-LTP.

Understanding the mechanism by which cognitive deficits improve is valuable. The NMDAR is a tetrameric heterodimer of the subunits NR1, NR2A and NR2B that forms

NR1/NR2A, NR1/NR2B, or NR1/NR2A/NR2B calcium channels stimulated by glutamate. The receptor signals downstream CAMKII and PKC, which in turn, leads to the development of L-LTP which is critical to the development and maintenance of memories⁵³. The subunit composition of the NMDAR plays an important role in the function of the receptor and has been implicated in a number of memory disorders^{13,54}.

We explored the NMDAR subunit composition over the first 3 days after SAH and found no change in subunit concentration. Surprisingly, we found a significant decrease in the NR2A/NR2B ratio with neutrophil depletion where we find improved spatial memory function. In our study, we found that NR2A levels and the NR2A/NR2B ratio are significantly decreased at day 6 with neutrophil depletion after day 3 from SAH. A shift to decreased NR2A/NR2B ratio should result in improved L-LTP and is potentially the mechanism for improvement in the animals treated with neutrophil depletion¹⁴. Although this finding does not lead us closer to understanding the cause of L-LTP impairment, it does open a potential new avenue of research for a treatment to the delayed deficits seen after SAH.

This study begins to scratch the surface of cognitive deficits after SAH and their interaction with inflammatory processes in the brain. We focused our attention on spatial memory tasks because there is evidence in patients that deficits occur in this domain⁷. This work suggests that NMDA receptors may be a target for future intervention to mitigate the cognitive deficits associated with SAH and delayed deterioration. The incongruous findings in the NMDA subunit composition between the disease state and the treatment state are intriguing. Further work to better understand the stimuli that lead to loss of LTP is necessary to develop rational treatment strategies. In addition, interrupting late innate immune responses appears to be a potent treatment strategy. Future treatments could exploit this relationship. Finally, the time window for intervention engenders hope that patients with SAH who survive the initial injury may benefit from later therapies particularly as it concerns cognitive recovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Delayed deterioration after SAH is associated with poor cognitive outcomes.
- In a murine model, SAH leads to delayed loss of NMDA mediated L-LTP.
- Changes in L-LTP are associated with changes in NMDA NR2 subunit proportions.
- Selective neutrophil depletion 3 days after SAH recovers NMDA function.
- Behavioral outcomes are improved by late neutrophil depletion.

Figure 1. Subarachnoid hemorrhage leads to long term spatial memory loss due to NMDA receptor mediated abnormalities in late long-term potentiation (L-LTP) that occur between the third and sixth day

Barnes maze (BMT) and Morris water maze (MWM) analysis completed on the same cohort of animals over the first 9 days (BMT) and one month after (MWM) SAH or Sham. (A) There was significantly worse performance on BMT in animals in the SAH group. (B) This was consistent in the MWM performed at one month after SAH/Sham suggesting that the spatial memory deficits after SAH are present early and sustained as late as a month. (C) The diagram shows the experimental design of the electrophysiology experiments performed with stimulation of Schaffer collateral fibers between the CA3 region of the hippocampus

and the CA1 region with acquisition of data from synapses between Shaffer collateral axons and pyramidal cell bodies. (D) The EPSP slope vs. time graph shows significantly preserved L-LTP on day 3 in SAH animals compared to Sham (n=3 animals/group with 7 slices). (E) At day 6, SAH animals lose L-LTP while Sham do not (n=3 animals/group with 9 slices). (F) Isolation of the NMDA receptor using bath conditions shown in the figure reveal continued loss of L-LTP suggesting the NMDAR (and not the AMPAR) is responsible for the effect (n=3 animals/group with 6 total slices). (G) shows a graphical display of the difference in L-LTP between SAH and sham at day 3, 6 and at day 6 with NMDA isolation.

Figure 2. Neutrophil depletion three days after SAH Improves vasospasm and spatial memory task performance through changes in the proportion of NMDA subunit composition
 (A) Neutrophil depletion using the 1A8 antibody prior to SAH does not significantly improve vasospasm measured 1mm from the carotid terminus in the middle cerebral artery 6 days after SAH (unpaired Student's t-test). The bar on the left shows the experimental design and a picture of the measurement point on the middle cerebral artery. Depleting neutrophils 12 hours (0.5 days) and 1 day after SAH also does not significantly improve vasospasm (retained significant difference between SAH and Sham) (unpaired Student's t-test). Conversely, depletion of neutrophils 3 days after SAH does improve vasospasm. (B) Mice depleted of neutrophils 3 days after SAH (red line) show significant improvement in Barnes Maze test (BMT) and Morris Water maze (MWM) performance compared to SAH. (C) The EPSP slope vs. time graph shows preserved L-LTP on day 6 in SAH animals compared to Sham ($n=3$ animals/group). The bar graph adds the neutrophil depletion to the Sham/SAH data presented in Figure 1 and shows no significant difference in L-LTP between SAH and Sham animals. (D) Western blot analysis of NR1, NR2A and NR2B NMDAR subunits in hippocampal cell membrane fractions show that NR1, NR2A and NR2B are not significantly altered after SAH but NR2A is significantly decreased after depletion of

neutrophils 3 days after SAH. Likewise, the NR2A/NR2B ratio is also significantly decreased after neutrophil depletion.

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Figure 3. Microglial activation is increased after SAH but few neutrophils enter the brain
 (A) Neutrophil staining with the Ly6G antibody 1A8 (TO-Pro-3 stains nuclei) shows no neutrophils in the area of the hippocampus on day 1, 3 or 6 or SAH. For comparison, naïve spinal cord (SC), SC at onset after EAE (experimental autoimmune encephalitis, an experimental model of spinal cord inflammation and demyelination) or at peak EAE: the later two showing neutrophils in the parenchyma (arrows). White bar represents 200 μ m and yellow bar represents 25 μ m. (B) In a LysM-EGFP mouse (Lyz2^{tm1.1Graf}), we compared SAH and sham mice. In the SAH mice, we found few Anti-GFP⁺, Iba-1⁻, cells that represent non-macrophage perivascular infiltrating myeloid cells in the hippocampus. (C) Microglial staining using Iba-1 (TO-Pro-3 nuclei) shows increased size and decreased ramifications six days after SAH (at the same time as changes in behavior). Depletion of neutrophils increases

microglial ramification and decreases the number of Iba-1 positive cells. Solid bars represent 250 μm and dotted lines represent 50 μm . The graph shows total fluorescence intensity of Iba-1 positive cells in the hippocampus 6 days after SAH.