Neurotherapeutics
https://doi.org/10.1007/s13311-021-01052-3

ORIGINAL ARTICLE

α_7-Acetylcholine Receptor Signaling Reduces Neuroinflammation After Subarachnoid Hemorrhage in Mice

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Accepted: 23 March 2021

Abstract

Aneurysmal subarachnoid hemorrhage (aSAH) causes a robust inflammatory response which leads worse brain injury and poor outcomes. We investigated if stimulation of nicotinic acetylcholine α_7 receptors (α_7-AChR) (receptors shown to have anti-inflammatory effects) would reduce inflammation and improve outcomes. To investigate the level of peripheral inflammation after aSAH, inflammatory markers were measured in plasma samples collected in a cohort of aSAH patients. To study the effect of α_7-AChR stimulation, SAH was induced in adult mice which were then treated with a α_7-AChR agonist, galantamine, or vehicle. A battery of motor and cognitive tests were performed 24 h after subarachnoid hemorrhage. Mice were euthanized and tissue collected for analysis of markers of inflammation or activation of α_7-AChR-mediated transduction cascades. A separate cohort of mice was allowed to survive for 28 days to assess long-term neurological deficits and histological outcome. Microglia cell culture subjected to hemoglobin toxicity was used to assess the effects of α_7-AChR agonism. Analysis of eighty-two patient plasma samples confirmed enhanced systemic inflammation after aSAH. α_7-AChR agonism reduced neuroinflammation at 24 h after SAH in male and female mice, which was associated with improved outcomes. This coincided with JAK2/STAT3 and IRAK-M activity modulations and a robust improvement in neurological/cognitive status that was effectively reversed by interfering with various components of these signaling pathways. Pharmacologic inhibition partially reversed the α_7-AChR agonist's benefits, supporting α_7-AChR as a target of the agonist's therapeutic effect. The cell culture experiment showed that α_7-AChR agonism is directly beneficial to microglia. Our results demonstrate that activation of α_7-AChR represents an attractive target for treatment of SAH. Our findings suggest that α_7-AChR agonists, and specifically galantamine, might provide therapeutic benefit to aSAH patients.

Key Words: Subarachnoid hemorrhage · Neuroinflammation · Galantamine · Nicotinic acetylcholine receptor

Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) affects about thirty thousand Americans every year and has a mortality rate as high as 67% [1]. As the average age of individuals affected by aSAH is 45–55 years [2], the socio-economic burden is large. Despite advances in management of aSAH, many survivors develop significant neurological and cognitive impairments [3–5].

Both experimental and clinical studies have shown that aSAH induces an intensive inflammatory reaction both peripherally and centrally [6, 7]. This excessive inflammation has been hypothesized to be a main driver of secondary brain injury after aSAH leading to worse clinical outcomes [8, 9]. Several lines of evidence suggest that acetylcholine α_7 receptors (α_7-AChR) in the brain modulate cerebral

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Published online: 10 May 2021

inflammation. For example, in animal models of aSAH, increased parasympathetic tone, either directly through stimulation of the sphenopalatine ganglion or by activation of perivascular α_7-AChR, improves outcome [10, 11]. Furthermore, acetylcholine levels in cerebrospinal fluid after aSAH are significantly lower in the acute phase of illness, suggesting that reduced Ach receptor activity may play a role in SAH injury [12].

α_7-AChR agonists have been reported to offer benefit after a number of hemorrhagic insults to the brain including intracerebral hemorrhage [13], traumatic brain injury [14], and SAH [15]. Specifically, agonism of α_7-AChR has been observed to provide anti-inflammatory and anti-apoptotic mechanisms which can improve outcome from experimental hemorrhagic stroke. Studies have indicated that α_7-AChR signaling can produce anti-inflammatory effects via JAK2 [13, 16] or IRAK-M pathways [17]. JAK2 signaling negatively regulates inflammation via STAT phosphorylation. On the other hand, IRAK-M negatively regulates TLR4-mediated inflammation via inhibition of MyD88. Since TLR4 has been implicated as a major contributor to poor outcome after SAH [18, 19], we hypothesized that α_7-AChR agonism will reduce neuroinflammation (via JAK2/STAT3 and IRAK-M/MyD88) following SAH in mice, thereby improving functional outcomes.

Materials and Methods

Human plasma samples ($n=59$ aSAH patients, $n=23$ unruptured aneurysm patients) from patients enrolled in an observational study approved by the Institutional Review Board (HSC-MS-12-0637) were used. Plasma samples were collected within 24 h after SAH and the cytokines were measured using a multiplex human immunoassay kit (Millipore) following established protocols [20].

Animal experiments were approved by the local Animal Welfare Committee, conducted in compliance with the *National Institutes of Health Guidelines for the Use of Animals in Neuroscience Research*, and are reported in compliance with the ARRIVE guidelines.

Study Design

A total of 104 male C57BL/6J mice (28–36 g), 18 female C57BL/6J mice (25–30 g), and 6 P0 C57BL/6J pups were used. Animals, housed in a temperature-controlled room with a 12-h light-dark cycle, had complete access to food and water. Mice were electronically randomized into groups before surgery. SigmaPlot 11.0 was used to estimate all sample sizes using data from previous experiments and preliminary data with $\alpha=0.05$ and $\beta=0.2$. All investigators performing functional assessment, measurement of outcomes, and/or data analysis were blinded to the experimental groups. A flowchart of each experiment is provided in the Supplemental Material.

Male and female mice were analyzed in separate groups in order to test if there were any significant differences between outcome for the SAH + Vehicle or the SAH + α_7-AChR agonist (galantamine) groups. The mice were allocated into three main groups: sham, SAH + vehicle, and SAH + intervention. The sham group (which undergoes all surgical procedures but does not experience SAH) is used to assess the injury caused by SAH alone (i.e., sham outcome is compared to SAH + vehicle outcome). To assess only the effects of the interventions (galantamine, MLA, HY-13277, and LY2784544), the solution used for dissolving the drugs (called vehicle) was given to SAH mice; comparison of the outcomes from SAH + vehicle mice and SAH + intervention mice allows for the effects of the invention alone to be determined.

Experiment 1

Male mice ($n=12$/group) were allocated into sham, SAH + vehicle, SAH + galantamine (Tocris Bioscience), or SAH + galantamine + MLA (methyllycaconitine citrate, α_7-AChR antagonist, Tocris Bioscience), to test treatment effects on functional performance and inflammation. MLA was chosen as the antagonist of α_7-AChR since it is 40-fold more selective for α_7-AChR than its other cholinergic receptors ($\alpha4\beta2$ or $\alpha6\beta2$). Sample sizes were calculated using data from previous neurobehavioral experiments [21, 22]. Female mice (assigned to sham, SAH + vehicle, or SAH + galantamine, $n=6$/group) were used to test treatment effects on behavior and inflammation. A cohort of male mice ($n=6$/group), assigned to SAH + vehicle or SAH + galantamine, were euthanized 6 h after SAH for measurement of cytokines. The sample sizes for the female and 6 h male cohorts were calculated using previous and preliminary data from ELISAs [23].

Experiment 2

To study the long-term beneficial effects of galantamine treatment, mice were allocated into sham ($n=10$), SAH + vehicle ($n=12$), or SAH + galantamine ($n=12$) and allowed to survive for 28 days post-SAH. Vehicle or galantamine was administered twice daily on days 0–2 post-SAH. Sample sizes were estimated using previous data from water maze [24].

Experiment 3

To determine the anti-inflammatory mechanism of galantamine, mice ($n=6$/group) were allocated into sham, SAH + vehicle, SAH + galantamine, SAH + galantamine + MLA,

SAH + galantamine + HY-13277 (IRAK-M inhibitor, MedChemExpress), or SAH + galantamine + LY2784544 (JAK2 inhibitor, Selleckchem). Mice were used to examine functional performance and protein expressions of the MyD88/Nf-κB-p65 and JAK2/STAT3 pathways. HY-13277 and LY2784544 were chosen as antagonists for IRAK-M and JAK2, respectively. HY-13277 has no known off-targets but there is a possibility that it interacts with other IRAKs. LY2784544 is 8-fold, 20-fold, and 14-fold more selective for JAK2 than JAK1, JAK3, and FMS-like tyrosine kinase 3 [25], respectively. Sample sizes were estimated using previous data from Western blots [16].

BV2 mouse microglial cells (ATCC) were used to assess the effect of galantamine on cytokines and protein expressions. Cells were grown in Dulbecco's modified eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum at 37 °C in 95% air and 5% CO_2 on tissue-culture plates. When the cells reached 75% confluency, MLA (10 μM) or saline (10 μL) was added to the wells. Thirty minutes later, galantamine (10 μM) [26] or saline (0.8 μL) was added, followed by hemoglobin (50 nM) [27] or saline (10 μL) 1 h later. Twenty-four hours after hemoglobin administration, the media was removed and stored at − 80 °C and cells were prepared for Western blot.

Subarachnoid Hemorrhage Model

Subarachnoid hemorrhage was induced in mice via endovascular perforation following established protocols [22]. Perforation was confirmed by respiratory distress [22, 28, 29]. Perforation was also confirmed by assessing mice during the first 6 h after recovery for functional deficits. Vehicle (normal saline for experiments 1 and 2, 50% ethanol for experiments 3 and 4), galantamine (0.5 mg/kg in normal saline (experiments 1 and 2), or 50% ethanol (experiment 3)), MLA (0.1 mg/kg in normal saline (experiments 1 and 2) or 50% ethanol (experiment 3)), HY-13277 (0.1 mg/kg in 50% ethanol), and LY2787544 (0.1 mg/kg in 50% ethanol) were intranasally administered 1 and 8 h post-SAH (BID on days 0–2 for experiment 2). Briefly, alternating each nostril, 5 μL was given for a total of 30 μL; there was 1 min between administrations.

Mice were subjected to functional testing before euthanasia. Mice allocated for histology (experiment 2) were transcardially perfused with PBS followed by 4% paraformaldehyde, and brains were carefully removed and stored in paraformaldehyde. The remaining mice, used for Western blot or ELISA, had blood collected before transcardial perfusion with PBS, followed by removal of the brains which were snap frozen. For experiments 1 and 3, immediately following removal of the brains, the brains were observed for blood clot(s) near the circle of Willis to confirm SAH.

Neurobehavioral Performance

All animals underwent functional performance evaluation using a 24-point neuroscore, beam walking, T-maze, and rotarod 1 day post-surgery. The neuroscore examines sensorimotor function using 8 sub-tests [21]. Beam walking assesses the animal's ability to traverse a round rod [30]. T-maze was used to detect working memory deficits; spontaneous alterations were recorded for 10 individual trials. Rotarod assesses the animal's motor skills. Briefly, two trials of constant 5 rpm were used to train mice. Then, mice were subjected to 2–3 trials of a 5 rpm starting speed with an 8 rpm acceleration and then 2–3 trials of a 5 rpm starting speed with a 16 rpm acceleration. Each trial was a total of 2 min and at least 2 min rest time were given between trials.

Experiment 2

All mice underwent assessment of functional performance using the neuroscore, beam walking, T-maze, and rotarod on days 1–3, 5, and 7 post-SAH. Neuroscore and rotarod were also performed on day 24. On days 25–27, water maze was performed before euthanasia on day 28. On day 25, water maze visible platform training was performed; then 3 h later, the mice began the hidden platform memory block 1. On day 26, mice were subjected to memory blocks 2 and 3 separated by 3 h. On day 27, mice performed memory block 4, and then 3 h later, the probe test was performed [31].

ELISA Plasma was obtained by spinning down mouse whole blood. Brain concentrations of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) used 50 μL of the supernatant after protein extraction (see Western blot section below). Enzyme-linked immunosorbent assay (ELISA) kits were used to assess the concentrations of IL-1β (MLB00C) and TNF-α (MTA00B) in plasma and brain samples in duplicates following the manufacturer's instructions (R&D Systems).

Histological Assessment

Brains were vibratome sectioned at −2 from bregma (30 μm thick) and stored free-floating at 4 °C. For experiment 2, 30-μm-thick brain sections, taken every mm from 1 to −3 from bregma, were used to measure white matter area and ventricle volume after H&E staining. ImageJ was used to determine the white matter area and area of the ventricles in each slice. The total white matter area is the sum of the white matter areas in each slice. The ventricle volume is determined by summing all ventricle areas and multiplying by the thickness (1 mm) of each slice. For experiment 2, sections were stained with primary antibodies against Iba1 (1:2000, ab5076, Abcam) at 4 °C overnight followed by secondary antibody (anti-rabbit Alexa Fluor 488 (1:200, 111-545-003, Jackson ImmunoResearch)) at room temperature for 1 h. Stained sections were imaged at 6 different locations and the number of

Iba1-positive cells were recorded. The cell counts are presented as the number of positive stained cells per square millimeter.

Western Blot

Frozen brains were homogenized and sonicated in Lysis buffer (Biorad) and protein concentrations were measured. Cells were sonicated in lysis buffer and protein concentrations were measured. Western blot was performed, as previously described [32], using primary antibodies for IRAK-M (ab8116, Abcam), MyD88 (4283S, Cell Signaling Technology), p-JAK2 (3776, Cell Signaling Technology), JAK2 (3230, Cell Signaling Technology), p-STAT3 (ab476315, Abcam), p-NF-κB p65 (3033T, Cell Signaling Technology), NF-κB p65 (8242T, Cell Signaling Technology), IL-10 (BioLegend), and β-actin (sc-47778, Santa Cruz Biotechnology). Signals were normalized to β-actin for each lane and then, within each gel, all lanes were normalized to the sham values [2–3 per membrane].

Statistical Analysis

Data is presented as individual data points with the mean and standard deviation shown. All tests were two sided, all data was assessed for normality and homoscedasticity, and $p < 0.05$ was considered statistically significant. Data for cell counts, ELISA, Western blot, white matter area, ventricle volume, rotarod, and water maze probe trial were analyzed using one-way ANOVAs with Tukey post hoc. Sex differences for the functional data for sham, SAH + vehicle, and SAH + galantamine groups were analyzed using unpaired *t* tests. Human cytokine data was analyzed using Mann-Whitney *U* tests. Neuroscore, beam walking, and T-maze were analyzed using Kruskal-Wallis with Dunn's post hoc. Water maze memory trials were analyzed using two-way ANOVA with Tukey post hoc. GraphPad Prism 6 (La Jolla, CA, USA) and SigmaPlot 11.0 (SysStat, Germany) were all used for analyzing and graphing data. Data is available upon reasonable request.

Results

For experiments 1 and 3, 0/18 sham male, 1/18 SAH + vehicle male, 2/18 SAH + galantamine male, 2/18 SAH + galantamine + MLA male, 1/6 SAH + galantamine + HY-13277 male, 0/6 SAH + galantamine + LY2784544 male, 0/6 sham female, 0/6 SAH + vehicle female, and 0/6 SAH + galantamine female mice died before neurobehavioral testing 1 day post-SAH. No mice (SAH + vehicle or SAH + galantamine) died for the 6 h endpoint (experiment 1). For experiment 2, 0/10 sham, 3/12 SAH + vehicle, and 5/12 SAH + galantamine mice died. One SAH + vehicle mice died within 2 days and the other 2 mice died from delayed cerebral ischemia. Three SAH + galantamine mice died within 2 days and the other 2 SAH + galantamine mice died from delayed cerebral ischemia. Details of the mortalities are reported in the Supplement Material. Samples from mice which died within 24 h were excluded from statistical analysis. Complete statistical reports are available in the Supplemental Material.

aSAH Leads to Significant Elevation of Plasma Cytokines in Patients

In humans with aSAH, a number of cytokines and chemokines, including the pro-inflammatory cytokine IL-1β, are significantly elevated within 24 h compared to unruptured aneurysm controls (Fig. 1).

Galantamine Reduces Neuroinflammation After SAH

Six hours following SAH, galantamine-treated mice had a significantly lower concentration of IL-1β in the brain than vehicle-treated mice ($p = 0.0149$). At 6 h post-SAH, there was no difference between vehicle- and galantamine-treated SAH mice for the levels of brain TNF-α, plasma IL-1β, nor plasma TNF-α (Fig. 2a). Twenty-four hours after SAH, vehicle-treated male mice had significantly elevated levels of IL-1β and TNF-α in both their plasma and brain compared to sham controls. Galantamine treatment decreased the levels of brain IL-1β ($p = 0.0070$) and brain TNF-α ($p = 0.0140$). For the plasma levels of IL-1β and TNF-α, galantamine-treated mice had levels similar to those observed in uninjured shams. When an antagonist of α_7-AChR (MLA) was coadministered with galantamine, the reduction of IL-1β in the brain by galantamine alone was reversed (Fig. 2b). In female mice, galantamine treatment significantly reduced the SAH-elevated levels of brain IL-1β, plasma IL-1β, and brain TNF-α (brain IL-1β: $p = 0.0117$; brain TNF-α: $p = 0.0009$; plasma IL-1β: $p = 0.0003$), but did not alter plasma TNF-α (Fig. 2c).

Galantamine Improves Functional Performance

One day after SAH, vehicle-treated male mice had significant functional deficits ($p < 0.05$ vs Sham). Galantamine-treated mice performed significantly better than vehicle-treated SAH mice on neuroscore and rotarod ($p < 0.05$ vs SAH + vehicle) (Fig. 3a). Notably, galantamine treatment improved the performances of SAH mice to levels comparable to those observed in sham mice in all of the behaviors tested ($p > 0.05$ vs Sham). Galantamine's improvement of behavioral deficits caused by SAH was also observed in female mice (Fig. 3b). Interestingly, there was a sex

(A)

Cytokine	Concentration (95% CI)		p-Value
	UA	aSAH	
EGF	35.75 (-4.479 to 75.97)	21.46 (12.87 to 30.05)	0.2155
Eotaxin	81.92 (61.28 to 102.6)	70.96 (52.69 to 89.23)	0.0946
FGF2	35.44 (13.19 to 57.68)	94.48 (67.01 to 121.9)	0.0021
Flt3L	1.15 (0.603 to 1.697)	33.89 (7.50 to 60.27)	**0.0006**
Fracktaline	94.49 (-12.84 to 201.8)	110.8 (73.01 to 148.6)	0.0039
GCSF	58.52 (-10.2 to 127.2)	186.3 (-33.25 to 405.8)	**0.0002**
GM-CSF	6.203 (-1.453 to 13.86)	17.82 (7.509 to 28.14)	**0.0001**
GRO	166.5 (85.21 to 247.8)	365.3 (268.2 to 462.4)	**0.0003**
IFNα2	11.78 (-1.167 to 24.72)	56.79 (5.871 to 107.7)	0.0005
IFNγ	43.26 (-0.813 to 87.33)	58.67 (29.46 to 87.87)	0.2084
IL-1α	61.23 (11.99 to 110.5)	92.08 (-11.05 to 195.2)	0.3648
IL-1β	0.64 (-0.210 to 1.49)	4.54 (1.83 to 7.251)	**<0.0001**
IL-1Ra/IL-1R1	68.27 (-16.26 to 152.8)	129.9 (-19.68 to 279.5)	**0.0006**
IL-2	1.474 (-0.014 to 2.962)	8.004 (-1.204 to 17.21)	0.0096
IL-3	0.044 (-0.026 to 0.113)	0.370 (0.078 to 0.662)	0.0059
IL-4	2.808 (-0.183 to 5.799)	4.74 (0.568 to 8.911)	0.3712
IL-5	7.853 (-3.720 to 19.43)	3.469 (0.240 to 6.698)	0.6311
IL-6	9.197 (0.051 to 18.34)	342.8 (-302.9 to 988.6)	**0.0002**
IL-7	1.877 (0.056 to 3.697)	3.607 (0.655 to 6.56)	0.0346
IL-8	21.81 (1.475 to 42.15)	30.92 (5.661 to 56.17)	0.0023
IL-9	1.912 (0.108 to 3.716)	10.25 (-5.857 to 26.35)	0.1754
IL-10	12.19 (2.009 to 22.37)	15.41 (6.543 to 24.29)	0.0501
IL-12p40	20.11 (-1.6494 to 41.91)	39.71 (3.649 to 75.77)	0.0858
IL-12p70	8.78 (-1.056 to 18.62)	22.28 (9.304 to 35.25)	0.0082
IL-13	48.16 (-12.11 to 108.4)	13.87 (-2.66 to 30.4)	0.7854
IL-15	2.205 (0.075 to 4.335)	3.99 (1.991 to 5.989)	**0.0011**
IL-17a	6.191 (1.703 to 10.68)	8.97 (5.722 to 12.22)	0.0621
IP-10	1068 (189.7 to 1947)	508.2 (325.8 to 690.6)	0.0118
MCP-1	343.3 (252 to 434.6)	428.3 (128.9 to 727.6)	0.1191
MCP-3	64.86 (-12.64 to 142.4)	32.94 (10.22 to 55.66)	0.2558
MIP-1α	11.47 (5.683 to 17.27)	8.531 (5.806 to 11.26)	0.2103
MIP-1β	35.8 (11.87 to 59.73)	35.54 (25.98 to 45.10)	0.3593
PDGF-AA	62.23 (5.402 to 119.1)	306 (151.7 to 460.2)	0.0037
PDGF-AB/BB	3.237 (2.524 to 3.95)	1355 (-262.1 to 2973)	**<0.0001**
RANTES	6.257 (3.6 to 8.913)	777.8 (524.4 to 1031)	**<0.0001**
sCD40L	45.74 (36.8 to 54.69)	84.38 (66.69 to 102.1)	0.0083
TGF-α	0.8496 (-0.085 to 1.784)	2.047 (0.941 to 3.154)	**0.0001**
TNF-α	8.937 (2.952 to 14.92)	7.941 (5.405 to 10.48)	0.4774
TNF-β	65.64 (-40.51 to 171.8)	27.22 (1.095 to 53.33)	0.5948
VEGF	238.4 (74.03 to 402.7)	243.1 (157.4 to 328.9)	0.1399

(B)

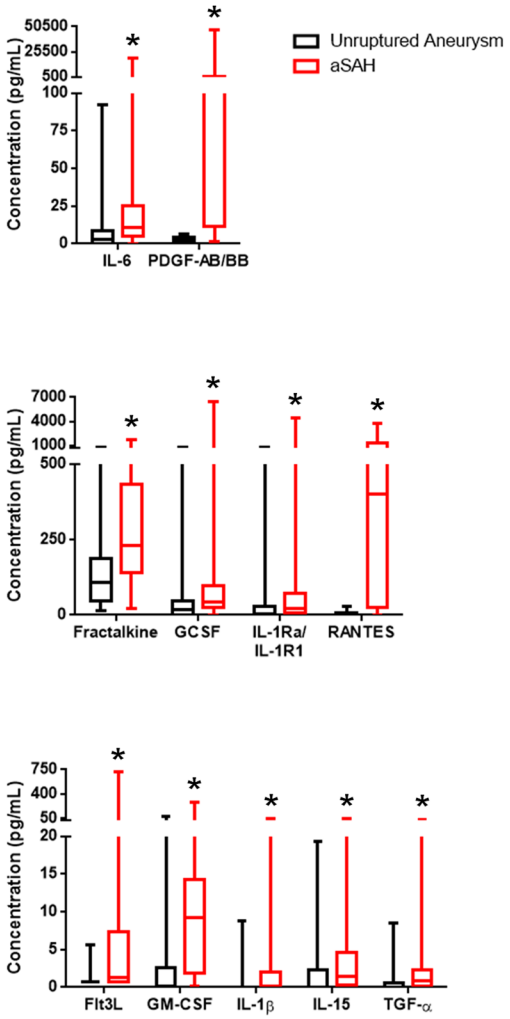


Fig. 1 Cytokines and chemokines are elevated 24 h after SAH. (**a**) In aSAH patients, 40 cytokines and chemokines were measured. (**b**) In aSAH patients, several cytokines and chemokines which were significantly different (after multiple comparison adjustment) between aSAH and unruptured aneurysm (UA) patients. $n = 23$ UA, $n = 59$ aSAH. *$p < 0.05$

difference in the protective effect of galantamine; female SAH + galantamine mice had greater behavioral improvement compared to male SAH + galantamine mice in neuroscore and T-maze (Fig. 3c).

To determine if acute treatment with galantamine offers lasting improvement in SAH outcome, long-term functional performance was assessed. Galantamine-treated mice had significantly improved memory (Fig. 4c, d) and sensorimotor function (Fig. 4e, f) 24 days post-SAH. Swimming speed did not differ across groups (Fig. 3). Histological analysis of brains from mice surviving 28 days after SAH showed that galantamine-treated mice had less white matter damage than vehicle-treated mice ($p < 0.0001$) and ventricle volumes were reduced to the sham level ($p = 0.0136$ vs SAH + vehicle) (Fig. 4g–i).

Beneficial Effects of Galantamine Occur Through α_7-AChR Signaling

When the α_7-AChR antagonist MLA is co-administered with galantamine, the beneficial effects of galantamine on brain IL-1β (Fig. 2) and functional outcome (Fig. 3) were lost.

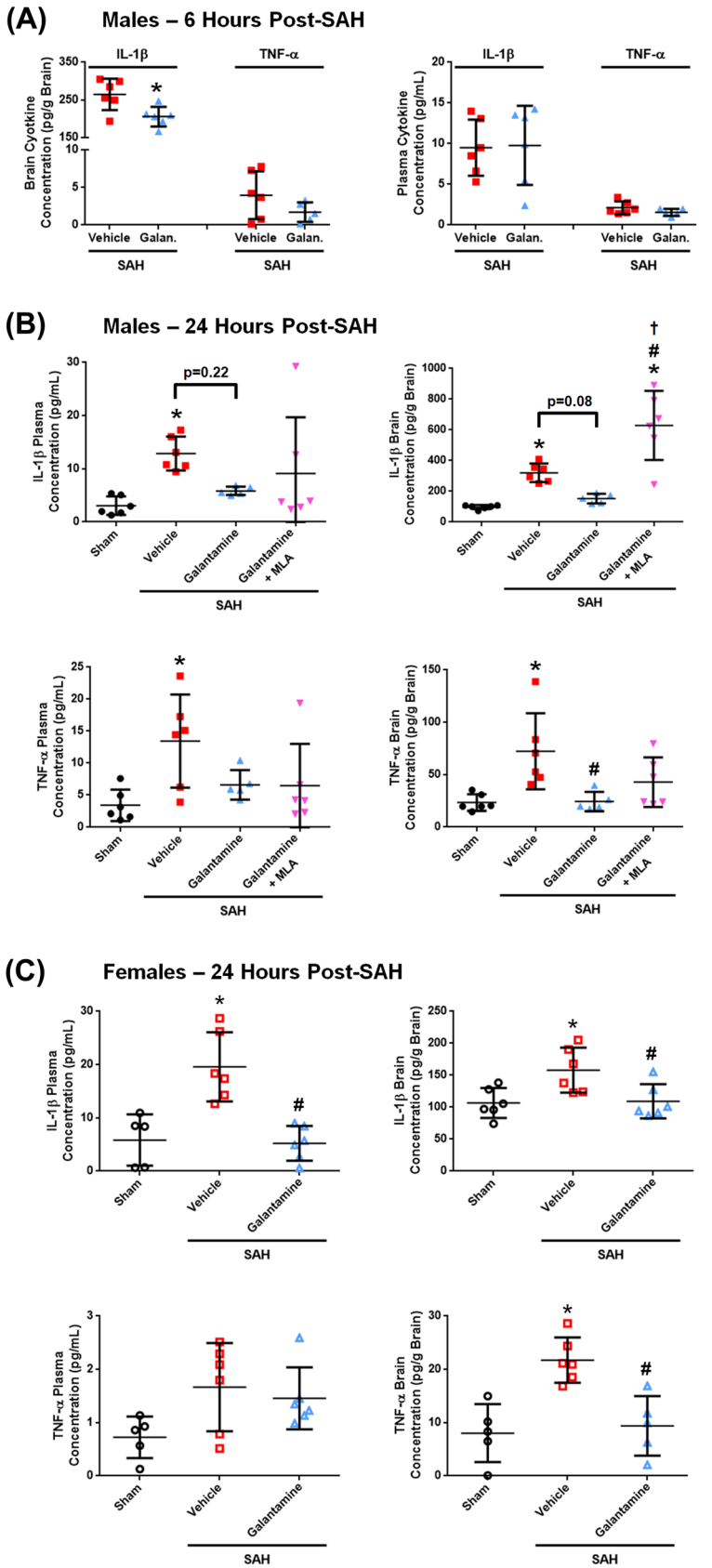


Fig. 2 IL-1β and TNF-α concentrations following SAH in mice. (**a**) Six hours post-SAH in male mice, galantamine reduces IL-1β in the brain but not brain TNF-α, plasma IL-1β, nor plasma TNF-α. One plasma sample and one brain sample from mice in the SAH + galantamine group had undetectable levels of TNF-α. For statistical purposes, these samples were assigned the minimal detectable concentration (0.36 pg/mL) as indicated by the manufacturer. (**b**) Twenty-four hours after SAH in male mice, galantamine reduces the amount of IL-1β in the plasma and brain, although neither reached statistical significance. Co-administration of MLA with galantamine increased IL-1β in the brain. Galantamine reduces brain TNF-α but not plasma TNF-α. (**c**) In female mice, galantamine reduces plasma IL-1β, brain IL-1β, and brain TNF-α, but not plasma TNF-α 24 h after SAH. One plasma sample from a sham mouse had an undetectable amount of IL-1β. One plasma sample from a sham mouse had an undetectable amount of TNF-α. For TNF-α in the brain, one sham and one SAH + galantamine mice had undetectable levels. For statistical purposes, these samples were assigned the minimal detectable concentrations (TNF-α: 0.36 pg/mL, IL-1β: 0.46 pg/mL) as indicated by the manufacturer. $n = 5–6$/group. *$p < 0.05$ vs Sham, #$p < 0.05$ vs SAH + vehicle, †$p < 0.05$ vs SAH + galantamine

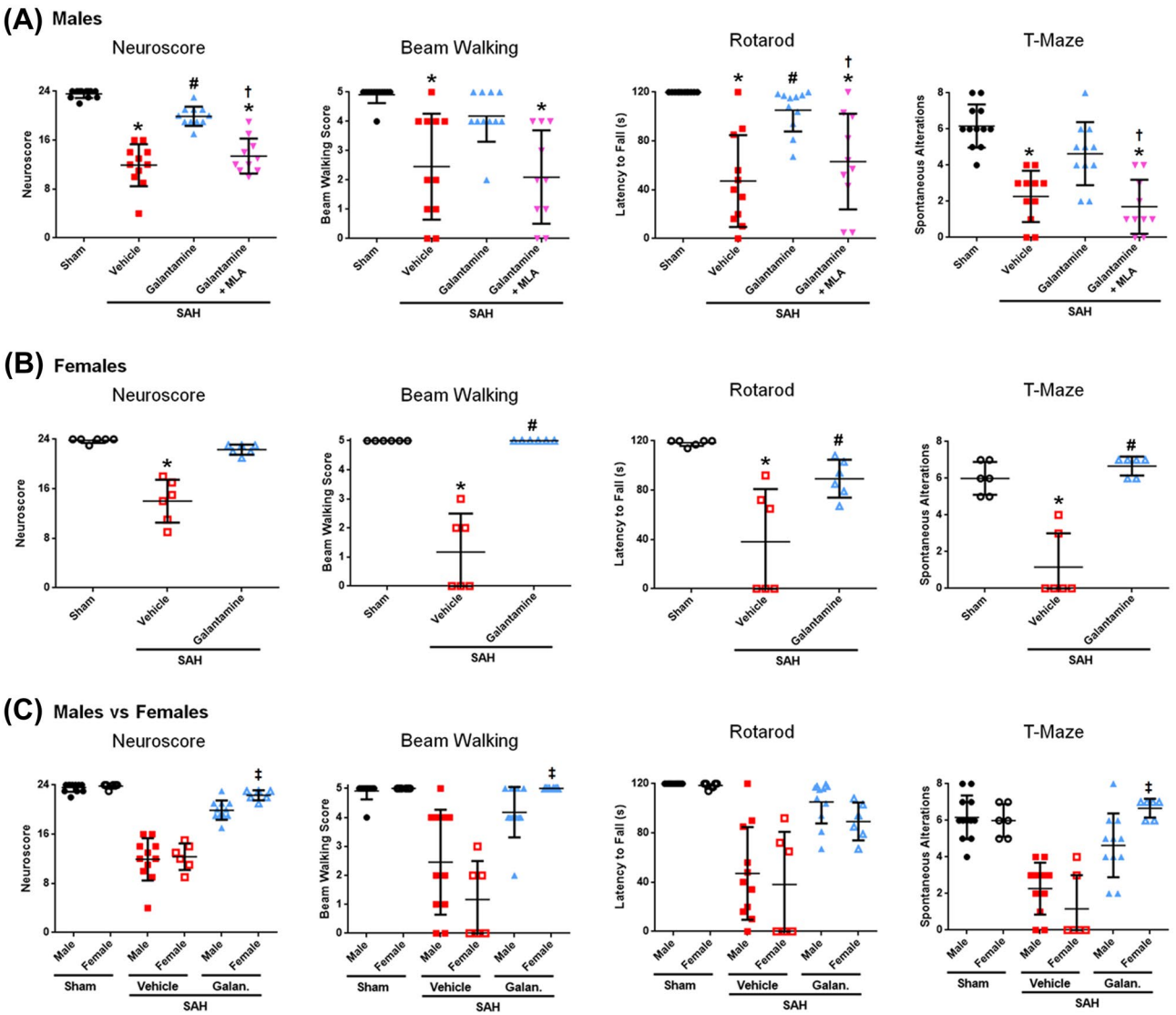


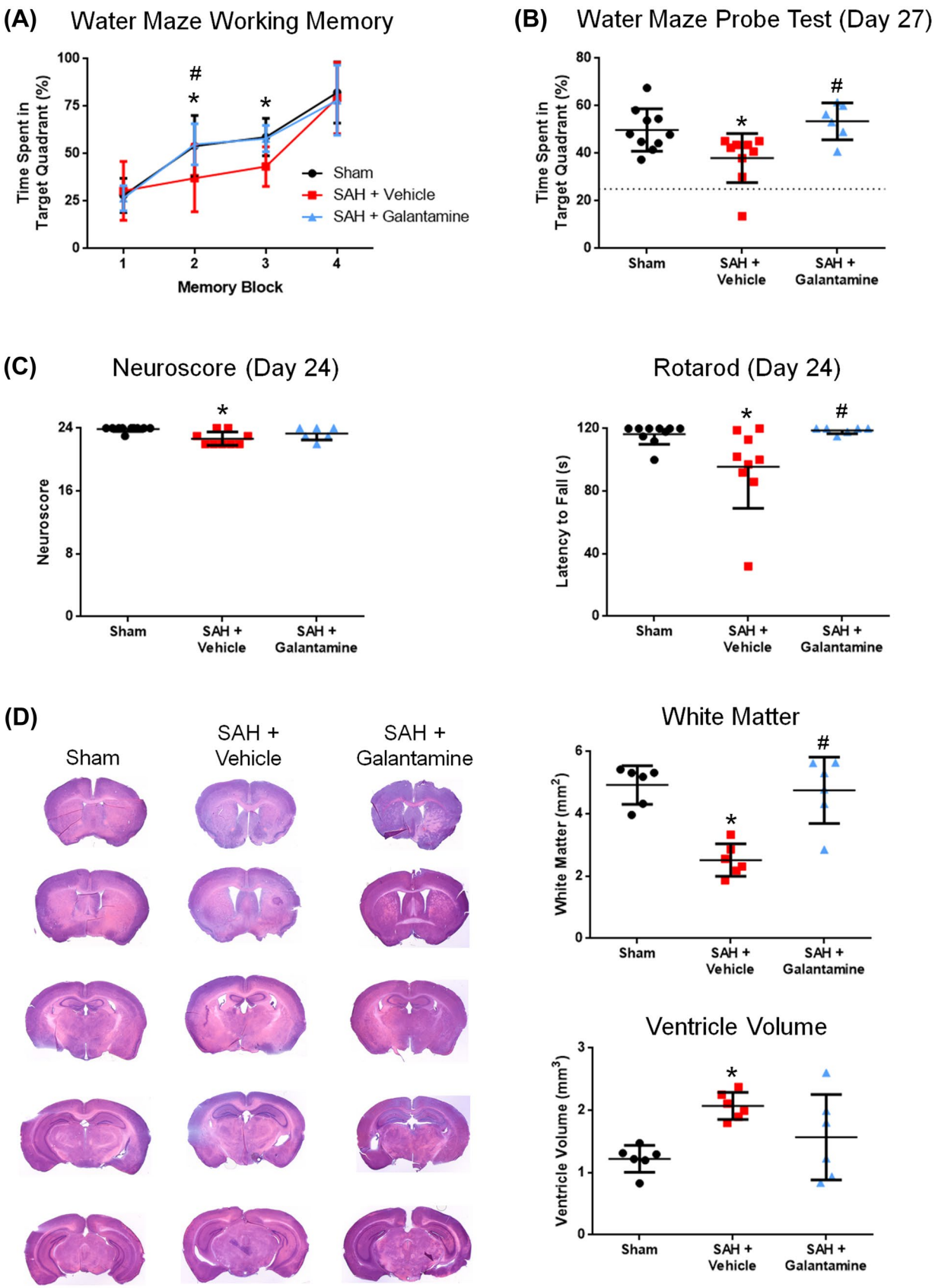
Fig. 3 Galantamine improves neurobehavioral performance on sensory, motor, and working memory tests 24 h post-SAH. (**a**) In male mice, galantamine improves performance on the neuroscore and rotarod tests. Co-administration of MLA reverses galantamine's protection. *$p < 0.05$ vs Sham, #$p < 0.05$ vs SAH + vehicle, †$p < 0.05$ vs SAH + galantamine. (**b**) In female mice, galantamine improves performance on the beam walking, rotarod, and T-maze tests. *$p < 0.05$ vs Sham, #$p < 0.05$ vs SAH + vehicle. (**c**) Females treated with galantamine after SAH have significantly better behavioral performances than their male counter-parts on the neuroscore, beam walking, and T-maze tests. Males: $n = 5$–6/group, females: $n = 5$–6/group. ‡$p < 0.05$ between the male and female mouse for the specific injury group

Anti-inflammatory Effects of α_7-AChR Stimulation

Since the α_7-AChR is expressed on immune cells, we probed the signaling pathways downstream of α_7-AChR to determine whether galantamine acts via JAK2/STAT3 or IRAK-M/MyD88 signaling.

When the antagonists of the α_7-AChR/JAK2/STAT3 and α_7-AChR/IRAK-M/MyD88 pathways are coadministered with galantamine, the functional benefits of galantamine treatment are significantly reversed (Fig. 5a). Within immune cells, α_7-AChR activation may provide anti-inflammatory effects through JAK2/STAT3 signaling leading to increased anti-inflammatory IL-10 production, or by inhibiting the pro-inflammatory TLR4/MyD88/NF-κB-p65 signaling pathway.

HY-13277 (IRAK-M antagonist) was used to attenuate the beneficial effects of galantamine on the TLR4/MyD88 signaling pathway. Treatment with galantamine did not have any effect on TLR4 expression (Fig, 6). MyD88 and phospho p-65 (NF-κB) were elevated in the brain following SAH, and galantamine reduced both MyD88 and phosphorylated p65 (NF-κB) levels to those of sham ($p < 0.05$ SAH + vehicle vs SAH + galantamine). Galantamine also increased expression of IRAK-M ($p < 0.05$ vs


(A)
Water Maze Working Memory
Time Spent in Target Quadrant (%)
Memory Block
Sham
SAH + Vehicle
SAH + Galantamine
(B)
Water Maze Probe Test (Day 27)
Time Spent in Target Quadrant (%)
Sham
SAH + Vehicle
SAH + Galantamine
(C)
Neuroscore (Day 24)
Neuroscore
Rotarod (Day 24)
Latency to Fall (s)
(D)
Sham
SAH + Vehicle
SAH + Galantamine
White Matter
White Matter (mm²)
Ventricle Volume
Ventricle Volume (mm³)

◂**Fig. 4** Galantamine prevents long-term memory, sensory, and motor deficits while preserving white matter integrity in SAH mice. (**a**) Water maze working memory test on days 24–27 post-SAH. $n=6$–10/group. *$p<0.05$ Sham vs SAH + vehicle at indicated time-point. #$p<0.05$ SAH + vehicle vs SAH + galantamine at indicated time-point. (**b**) Water maze probe test on day 27 post-SAH. Dotted line indicates random chance for being in the target quadrant (25%). $n=6$–10/group. *$p<0.05$ vs Sham, #$p<0.05$ vs SAH + vehicle. (**c**) Sensory and motor functional testing on day 24 after SAH was evaluated using the neuroscore and rotarod. $n=6$–10/group. *$p<0.05$ vs Sham, #$p<0.05$ vs SAH + vehicle. (**d**) Representative H&E stained brains from mice euthanized 28 days post-SAH. H&E staining was used to quantify the white matter area (internal capsule and corpus callosum) and ventricle volume. $n=6$/group. *$p<0.05$ vs Sham, #$p<0.05$ vs SAH + vehicle

Sham and SAH + vehicle). MLA (α7-AChR antagonist) prevented galantamine's effects on these three proteins. HY-13277 was able to block galantamine's benefit on IRAK-M and MyD88 expressions, and partially counteracted galantamine's reduction of phospho p65 (NF-κB) (Fig. 5b, c).

The JAK-2 antagonist, LY2784544, reversed galatanmine's functional benefits on neuroscore and rotarod (Fig. 5a). After SAH, p-JAK2 is elevated, but p-STAT3 and IL-10 levels were unchanged. Galantamine treatment further increases p-JAK2 levels ($p<0.05$ vs SAH + vehicle) and also elevated p-STAT3 expression, leading to more IL-10 within the brain ($p<0.05$ vs SAH + vehicle). Both MLA and LY2784544 prevented galantamine from increasing p-JAK2, p-STAT3, and IL-10 expressions. LY2784544 also prevented galantamine lowering of phosphorylated NF-κB p65 (Fig. 5b, c).

Microglia (BV2) cells were used to identify if galantamine had a direct effect on this cell type. Twenty-four hours after hemoglobin toxicity, IL-1β and TNF-α were significantly elevated in the media of BV2 cells (IL-1β: $p=0.0194$; TNF-α: $p<0.0001$). Galantamine treatment of the microglia cells partially attenuated the increase of IL-1β (Hb vs Hb + galantamine $p=0.0802$), but had no effect on TNF-α. Antagonism of α7-AChR with MLA partially prevented galantamine's protective effects (Fig. 6a, b). Protein expression from microglia shows that galantamine can provide benefit against the effects of hemoglobin toxicity in BV2 cells (Fig. 6c–i). Overall schematic of the pathway involved in galantamine's protection against neuroinflammation is presented in Fig. 6j.

Discussion

Agonism of α7-AChR has been suggested to have neuroprotective effects after brain hemorrhages via lower neuroinflammation. Herein, we investigated the therapeutic potential of galantamine, an FDA-approved cholinergic drug with α7-AChR agonist properties, for aSAH. Our findings show (1) an elevated inflammatory response following SAH in humans and mice, (2) agonism of α7-AChR can prevent neuroinflammation and improve functional outcomes in SAH mice, (3) α7-AChR is crucial for improving poor outcomes after SAH since α7-AChR null mice tend to have worse working memory after SAH, and (4) two major signaling pathways in microglia are directly affected and key in reducing neuroinflammation.

aSAH leads to a robust pro-inflammatory response as indicated by the elevation of numerous cytokines and chemokines (seen in this study and as reported by others [33–36]). Furthermore, a higher pro-inflammatory response leads to worsened delayed injury after SAH [36–38]. Previous clinical trials using drugs aimed at preventing inflammation have failed to offer significant benefit for aSAH [39]. One explanation for the failure of anti-inflammatory agents in improving SAH outcome is that the drugs only targeted inflammation. It is now accepted that injury following SAH does not just rely on a single pathological consequence, but rather multiple injury cascades, including inflammation and apoptosis [4, 5, 40]. α7-AChR has multiple downstream signaling pathways which are beneficial and can attenuate a variety of pathophysiological events [13–16, 41, 42]. In this study, we observed the potential benefit of α7-AChR agonism using galantamine, a cholinesterase inhibitor, and partial α7-AChR agonist, to improve outcome following SAH in mice through its anti-inflammatory effects. Intranasal administration of galantamine to mice after SAH was able to reduce neuroinflammation, leading to improved functional outcomes. To determine if the observed protection was via direct effects and signaling by microglia, we performed in vitro microglia cell culture experiments; we observed a direct anti-inflammatory effect by galantamine on microglia as assessed by measurement of inflammatory cytokines. Since galantamine is reported to have a number of mechanisms (i.e., agonism of α7-AChR and antagonism of acetylcholinesterase), we used α7-AChR knockout mice and observed that galantamine was unable to provide a therapeutic benefit for either neuroinflammation or functional outcome. Additionally, there is a worse trend for worse working memory (T-maze) after SAH in α7-AChR null mice compared to wild-type mice (Fig. 5). Although not powered to test for significant differences in functional outcome between α7-AChR knockout and wild-type SAH mice, a sample size estimation indicates that six to seven mice per group are needed, which suggests that there may be a significant differences in working memory (i.e., α7-AChR knockout mice have worse working memory than wild-type mice). This trend is a crucial piece of evidence that α7-AChR is a key modulator of brain injury after aSAH. Furthermore, the observed findings suggest that α7-AChR is the primary therapeutic target of galantamine for SAH injury.

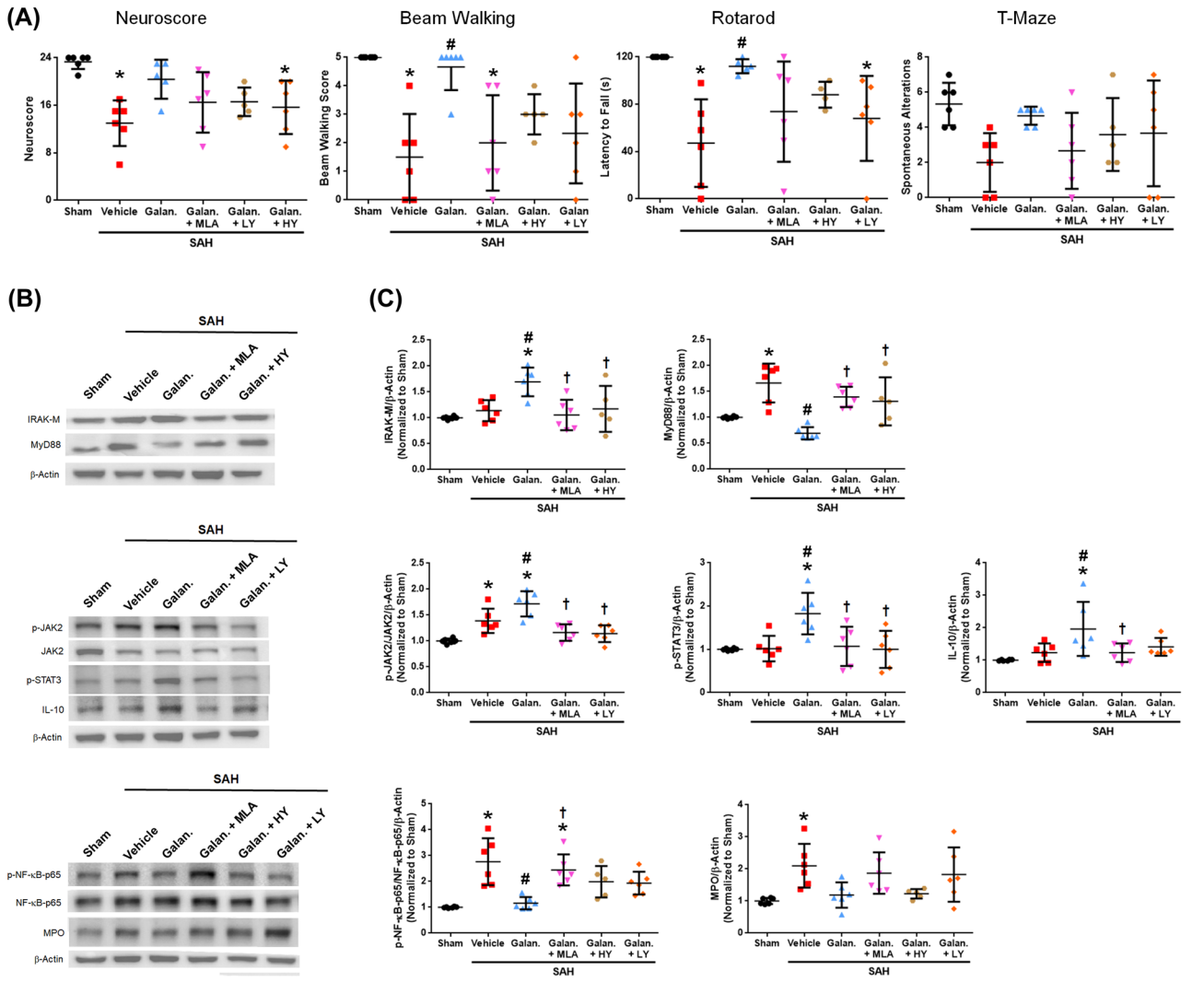


Fig. 5 Role of galantamine in the MyD88/NF-κB-p65 and JAK2/STAT3 signaling pathways to attenuate neuroinflammation 24 h after SAH in mice. (**a**) Behavioral performance of SAH mice. (**b**, **c**) Mouse protein expressions of IRAK-M, p-JAK2, MyD88, p-STAT3, p-NF-κB-p65/NF-κB-p65, and IL-10. $n = 5$–6/group. $^{*}p < 0.05$ vs Sham, $^{\#}p < 0.05$ vs SAH + vehicle, $^{\dagger}p < 0.05$ vs SAH + galantamine. Galan: galantamine. HY: HY-13277. LY: LY2784544

Mechanisms of Therapeutic Benefit by α_7-AChR Agonism

To identify the potential signaling cascades responsible for protection against secondary aSAH injury, we investigated two downstream signaling pathways. Our data indicates that neither signaling pathway was the key to the anti-inflammatory benefit offered by galantamine. Rather, each signaling pathway played a role in the overall protection provided by α_7-AChR agonism; the combination of anti-inflammatory signaling (by JAK2/STAT3) and prevention of pro-inflammation (by IRAK-M/MyD88 and JAK2/STAT3) were needed to improve functional outcome.

A major signaling pathway reported for α_7-AChR is the JAK2 pathway [16]. SAH triggers a pro-inflammatory response in part through TLR4/MyD88/p65 (NF-κB) signaling. While we found no difference in TLR4 brain expression (Fig. 6), we observed that α_7-AChR activation can inhibit MyD88 via IRAK-M and inhibit phosphorylation of p65 (NF-κB) via STAT3. Additionally, we found increased anti-inflammatory cytokine IL-10 via JAK2/STAT3 signaling. Our findings related to preventing neuroinflammation with α_7-AChR agonists are supported by others [13–16, 23, 41, 42].

Microglia have been suggested to be a target for improving outcome after SAH in mice [43]. Specifically, deactivation of microglia resulted in less inflammation and neuronal survival [44]. In order to determine if there was a direct effect of α_7-AChR agonism for neuroinflammation via microglia, we used in vitro microglia experiments and observed that

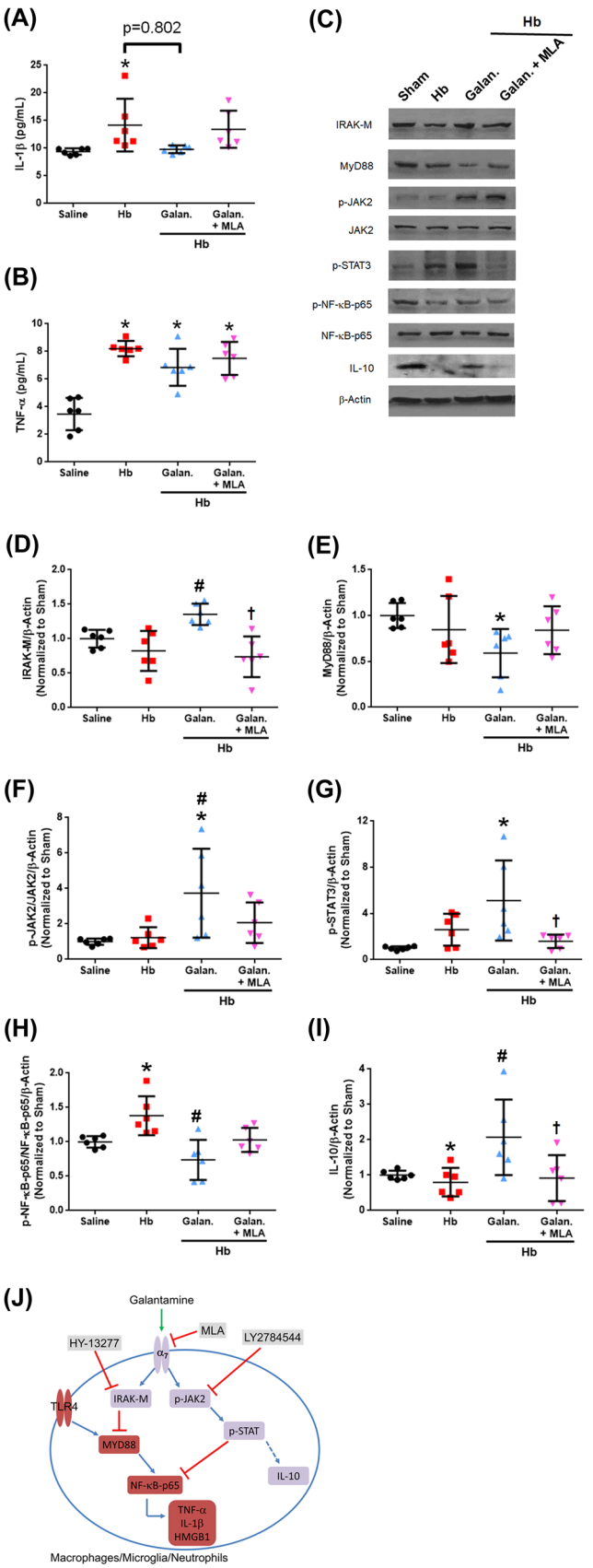


Fig. 6 Galantamine reduces inflammation via MyD88/NF-κB-p65 and JAK2/STAT3 signaling in microglia. (**a**, **b**) Levels of IL-1β and TNF-α in the media from in vitro microglia subjected to hemoglobin toxicity. $n=6$/group. (**c–i**) In vitro microglia protein expressions after hemoglobin toxicity. $n=6$/group. *$p<0.05$ vs Sham, #$p<0.05$ vs SAH + vehicle, †$p<0.05$ vs SAH + galantamine. Galan: galantamine. Hb: hemoglobin. (**j**) Schematic of galantamine's anti-neuroinflammatory mode of action

galantamine was able to reduce inflammation in culture via IRAK-M/MyD88 and JAK2/STAT3 signaling pathways. Others have also shown anti-inflammatory properties of α_7-AChR activation in immune cells [45, 46].

Implications for Human Use

Activation of α_7-AChR has been shown to be a therapeutic target capable of preventing neuroinflammation, neuronal cell loss, blood-brain barrier disruption, and brain edema in numerous rodent models of brain injuries, including SAH [15], traumatic brain injury [14, 23], and intracerebral hemorrhage [13, 16, 41, 42]. In these studies, various agonists were used, but none have been translated into clinical practice. There is also clinical evidence that acetylcholinesterase inhibitors are beneficial in traumatic brain injury [47, 48]]. Since galantamine is both a cholinesterase antagonist and an agonist for α_7-AChR, and it is FDA approved for treatment of cognitive impairments associated with Alzheimer's disease and dementia, galantamine can be quickly translated into the clinic for use to treat SAH.

Currently, a phase 2 clinical trial is underway to investigate the therapeutic benefit of galantamine for SAH (NCT02872857). This clinical trial will assess tolerability of galantamine, as well as examine if daily administration of galantamine to SAH patients can improve outcome at 90 days post-SAH.

Study Limitations

First, we did not investigate different α_7-AChR agonists. However, several α_7-AChR agonists are beneficial after brain hemorrhage. Second, we did not test different galantamine doses. Our dose was determined from the human dose [23] and was adjusted for intranasal administration. The third limitation is that α_7-AChR agonism can also promote cell survival. [42] Herein, we only investigated the effects of α_7-AChR agonism on neuroinflammation after SAH. Evidence from others [45, 46, 49–51] suggests that α_7-AChR agonism can directly affect microglia and neurons, and there may be a synergistic effect when both these cell types are receiving α_7-AChR activation [52]. Additionally, the contribution of anti-acetylcholinesterase activity was not studied here [53]. Inflammation has been suggested to lead to worse delayed injury and poor outcome [36–38, 54]. Herein, we chose to focus on the acute inflammation, but future studies will investigate the role of α_7-AChR agonism on delayed injury. Another limitation is that we used only pharmacological inhibitors which usually have off-target effects. However, the drugs used are significantly more selective for the primary target than other targets (see methods). Future studies can investigate more selective inhibitors or protein knockdown. Finally, it was assumed that all mice had similar hematomas and SAH injury severities since neuroscore values are very comparable (for SAH + vehicle) among experiments 1, 2, and 3. SAH blood was not graded since mice have an inherently rapid clearance of the extravagated blood [55, 56]. Other methods which could provide insight into injury severity (e.g., MRI hours after SAH, ICP, or CBF monitoring during surgery) were not used.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13311-021-01052-3.

Required Author Forms Disclosure Form provided by the authors are available with the online version of this article.

Disclosures H.A.C. is involved in a clinical trial (NCT02872857). All other authors declare no conflicts of interest.

Funding Funding support was provided by the Brain Aneurysm Foundation (D.W.M), a National Institute of Health K23 (S.L.B.), Center for Clinical and Translational Sciences Scholar Program (H.A.C.), and a seed grant provided by the Department of Neurosurgery at UTHealth (D.W.M).

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