

Chronic inflammation, cognitive impairment, and distal brain region alteration following intracerebral hemorrhage

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ABSTRACT: Delayed cognitive decline commonly occurs following intracerebral hemorrhage (ICH), but the mechanisms underlying this phenomenon remain obscure. We therefore investigated the potential mechanisms responsible for impaired cognitive function in a mouse collagenase model of ICH. Following recovery of motor and sensory deficits in the chronic phase of ICH, we noted significant cognitive impairment, which was assessed by the Morris water maze. This finding was accompanied by reduced dendrite spine density of ipsilateral hippocampal CA1 neurons. Reduced synaptic plasticity, manifested by impaired long-term potentiation in hippocampal neurons, was also evident in both ipsilateral and contralateral hemispheres, suggesting that ICH also induces functional alterations in distal brain regions remote from the site of injury. In addition, the accumulation of microglia, infiltration of peripheral immune cells, and generation of reactive oxygen species were observed in both contralateral and ipsilateral hemispheres up to 5 wk post-ICH. Furthermore, depletion of microglia using PLX3397, which inhibits colony stimulating factor 1 receptor, ameliorated this delayed cognitive impairment. Collectively, these results suggest that persistent and diffuse brain inflammation may contribute to cognitive impairment in the chronic stage of ICH recovery.—Shi, E., Shi, K., Qiu, S., Sheth, K. N., Lawton, M. T., Ducruet, A. F. Chronic inflammation, cognitive impairment, and distal brain region alteration following intracerebral hemorrhage. *FASEB J.* 33, 000–000 (2019). www.fasebj.org

KEY WORDS: microglia • delayed cognitive impairment • LTP

Patients suffering an intracerebral hemorrhage (ICH) are at high risk of developing cognitive dysfunction that occurs in 2 distinct phases (1). First, an early cognitive impairment emerges in 19% of patients within the first several weeks to months following a brain hemorrhage. This correlates directly with the size and location of the hematoma, suggesting that this early phase of cognitive decline relates to the primary hemorrhagic brain injury (2). A second phase of delayed cognitive decline, which is not associated with the characteristics of the acute hemorrhage, occurs as late

as 6 mo posthemorrhage (2), implying the involvement of distinct pathophysiologic mechanisms.

The hippocampus plays a central role in the formation and maintenance of learning and memory. Evidence suggests that physical injury and inflammation alters hippocampal neurotransmission and synaptic plasticity, engendering cognitive dysfunction (3, 4). Studies of neurodegenerative disorders such as Alzheimer and Parkinson diseases demonstrate that persistent brain inflammation may promote neurodegeneration (5), suggesting that chronic neuroinflammation following acute brain injuries may shape long-term neurofunctional outcomes. Acute brain insults including ischemia and traumatic brain injury elicit an inflammatory response that persists through the late phase of these diseases (6–8). In the setting of ICH, the initial hematoma causes a primary mechanical injury as well as secondary injury mechanisms, which promote neuroglial activation and the infiltration of immune cells into the brain. However, it remains unclear whether neuroinflammation persists during the late stage of ICH, and a definitive link between neuroinflammation and delayed cognitive impairment has not been established.

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CSF1R, colony stimulating factor 1 receptor; fEPSP, field excitatory postsynaptic potential; Iba1, ionized calcium-binding adapter molecule 1; ICH, intracerebral hemorrhage; IVIS, *In Vivo* Imaging System; LTP, long-term potentiation; mNSS, modified neurologic severity score; MWM, Morris water maze; ROS, reactive oxygen species; TBS, theta-burst tetanic stimulation

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To address these questions, we evaluated neural plasticity and cognitive function in mice during the late stage of ICH recovery. In addition, we assessed delayed immune responses in the brain as well as their potential contributions to delayed cognitive impairment following ICH.

MATERIALS AND METHODS

Animals and ICH model

Male C57Bl/6J mice (3–4 mo old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Female mice were not used in order to minimize the influence of sex steroids on recovery. Experiments were conducted according to the *Guide for the Care and use of Laboratory Animals* [National Institutes of Health (NIH), Bethesda, MD, USA]. All protocols were approved by Barrow Neurologic Institute Animal Care and Use Committee. All animal experiments were designed, performed, and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. ICH was induced by intracerebral injection of collagenase as previously described by Zhang *et al.* (9). In brief, the mouse's skull was fixed in a stereotactic frame after anesthesia, and 0.03 U collagenase (type IV-S; MilliporeSigma, Burlington, MA, USA) was injected to the right basal ganglia *via* microinfusion pump. The injection coordinates were 2.3 mm lateral to midline and 0.5 mm anterior to bregma, and the needle was inserted to a depth of 3.7 mm beneath the skull. Collagenase was dissolved in 0.5 μ L saline and infused at a rate of 0.1 μ L/min. Sham surgery involved the injection of saline only. Following infusion, the needle was removed after a 5 min pause to minimize overflow. During the surgery, mouse body temperature was maintained by a heating pad to $37 \pm 0.5^\circ\text{C}$. The burr hole was closed with bone wax, and the incision was sutured after surgery. In total, 500 μ L of saline was injected subcutaneously to each mouse to avoid dehydration.

Neurologic function assessment

The modified neurologic severity score (mNSS) of mice was continuously evaluated from d 0–35 post-ICH as previously reported by Sun *et al.* (10). Mice underwent a battery of sensorimotor tests including rotarod, cylinder, corner, and beam tests (11) at 35 d following ICH to evaluate residual motor dysfunction.

Cognitive function was evaluated using the Morris water maze (MWM). This test was conducted in 2 periods. During the training period, mice introduced into a circular pool could escape by finding a submerged hidden platform in the northeast quadrant. In each trial, mice were introduced to the pool through south or west sides and allowed to swim a maximum of 120 s to find the platform. Mice that successfully found the platform within 120 s were allowed to stay on the platform for 10 s, whereas mice that failed to locate the platform were placed on the platform and were also allowed to stay for 10 s. The training was conducted in 2 trials per session, with 2 sessions/d for 4 d. The swimming velocity, escape latency, and total distance swum during a trial were recorded. The water temperature in the pool was maintained at 24°C during the test. On the fifth day, the platform was removed, and a single trial was conducted by introducing the mice to the pool through the southwest quadrant. Each mouse was allowed to swim for 120 s, during which the time spent in the target quadrant and crossing times of the target platform were recorded. An EthoVision 3.1 tracking system (Noldus Information Technology, Wageningen, The Netherlands) was used for recording and analyzing data.

Neuroimaging

MRI images were obtained 3 d after surgery using a 7 Tesla small-animal MRI (Bruker Daltonics, Billerica, MA, USA), with scan parameters as previously described (12). Hematoma volume was measured using T2 images. In total, 2 mice that showed extension of the hemorrhagic injury into the hippocampus were excluded from cognitive evaluation *a priori*.

The generation of reactive oxygen species (ROS) in the brain was detected by Xenogen *In Vivo* Imaging System (IVIS) 200 imager (PerkinElmer, Waltham, MA, USA) after intraperitoneal injection of 200 mg/kg sodium luminol (Thermo Fisher Scientific, Waltham, MA, USA) (13) at 1 d, 2 wk, and 5 wk after surgery. Two regions of interest from both hemispheres were used to measure chemiluminescent intensity. Data were collected as photons per second per square centimeter using Living Image software (PerkinElmer).

Morphologic evaluation of hippocampal neurons

Five weeks after model induction, mice were euthanized, and fresh brain slices cut at the level of hippocampus were incubated in 0.25% biocytin in a K^+ -based internal electrode solution and dialyzed at 500-pA current for 10 min. The slices were then fixed overnight in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100, followed by incubation with avidin–Alexa Fluor 488 (Thermo Fisher Scientific) for 48 h. CA1 neuron dendritic arborization was traced using a Zeiss (Oberkochen, Germany) Axiophot microscope equipped with fluorescent light source, filters, and a monochrome digital camera (2000R; Teledyne Qimaging, Surrey, BC, Canada). The X, Y, and Z dimensions were registered using Neurolucida software (MBF Bioscience, Williston, VT, USA). Morphometric parameters, including Sholl analysis of dendritic length and intersection numbers, were extracted offline using Neurolucida Explorer. Both neurons from contralateral and ipsilateral hemispheres were evaluated.

Electrophysiological recordings

Electrophysiological recordings were performed at 5 wk after ICH. Following anesthesia, mice were euthanized, and brain tissue was quickly removed and bathed in artificial cerebrospinal fluid (ACSF; containing NaCl, 119 mM; KCl, 2.5 mM; NaHCO_3 , 26 mM; MgSO_4 , 1.3 mM; NaH_2PO_4 , 1.0 mM; CaCl_2 , 2.5 mM; and glucose, 11 mM). The ACSF was continuously bubbled with 95% O_2 –5% CO_2 (carbogen). The brain slices containing dorsal hippocampus were cut by vibratome (The Vibratome Company, St. Louis, MO, USA) at a thickness of 400 μm . The brain slices were then incubated in ACSF at room temperature for at least 1 h prior to recording. For recording, 1 brain slice was transferred to a liquid-air interface chamber (Harvard Bioscience, Holliston, MA, USA) and suspended on nylon net in a bath of continuously dripping oxygenated ACSF (2.0–2.5 ml/min). Humidified carbogen was passed along the upper surface of the slice, and the temperature was maintained by a feedback circuit to $31 \pm 0.5^\circ\text{C}$.

Standard field potential was recorded in the hippocampal CA1 region. A bipolar platinum wire electrode (0.08 mm diameter) was placed at the Schaffer collateral pathway, and stimulation was delivered using a Model 2100 A-M Systems Isolated Pulse Stimulator (Sequim, WA, USA). A recording glass electrode filled with 2 M NaCl was placed at the CA1 apical dendrite region. The stimulus intensity that can induce half maximal intensity in input-output relationship was used. The baseline synaptic potentials were first recorded for 20 min; then, a theta-burst tetanic stimulation (TBS, 15 burst trains at 5 Hz, each train contained 5

pulses at 100 Hz) was delivered to induce long-term potentiation (LTP), and the baseline intensity-evoked field excitatory post-synaptic potentials (fEPSPs) were recorded for 60 min with 0.33 Hz. All evoked responses were recorded by an Axoclamp-2B amplifier (Axon Instruments, Union City, CA, USA), and data were acquired by pClamp 10.2 software (Molecular Devices, Sunnyvale, CA, USA) (14). LTP was recorded in both contralateral and ipsilateral hippocampus of sham-treated or ICH mice.

Histology

Histology was assessed at 5 wk after ICH. After euthanizing the mice, brain tissue was harvested and fixed in 4% paraformaldehyde and embedded in paraffin with 5- μ m slices cut for staining. Hematoxylin and eosin stains were performed as previously described (12). For specific staining, goat anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:500; Abcam, Cambridge, MA, USA) primary antibody was incubated at 4°C overnight followed by incubation with Alexa Fluor 594-conjugated donkey anti-goat secondary antibody (1:1000; Thermo Fisher Scientific) for 1 h at room temperature. Pictures were captured by a confocal microscope and analyzed by ImageJ (NIH).

Flow cytometry

Single-cell suspensions were prepared from brains as previously described by Liu *et al.* (15) and stained using fluorochrome-conjugated antibodies. The following antibodies were used: phycoerythrin anti-CD45, phycoerythrin-Cyanine7 anti-CD11b, allophycocyanin-Cyanine7 anti-F4/80, allophycocyanin

anti-Ly6G, FITC anti-CD3, and peridinin chlorophyll protein complex anti-B220. The cells were detected by BD Fortessa flow cytometry analyzer (BD Biosciences, San Jose, CA, USA), and data were analyzed using Flowjo X (BD Biosciences).

Administration of PLX3397 and FTY720

The survival of microglia is dependent on colony stimulating factor 1 receptor (CSF1R) signaling, and inhibition of CSF1R for 3 wk eliminates nearly all microglia without impacts on cognition and other behaviors in healthy mice (16). In the present study, a CSF1R inhibitor (PLX3397) was used to deplete microglia during the late stage of ICH. PLX3397 (Selleckchem, Houston, TX, USA) was dissolved in DMSO and then diluted with PBS. PLX3397 was administered *via* oral gavage at a dosage of 40 mg/kg daily. Fingolimod (FTY720), a sphingosine-1-phosphate receptor modulator, inhibits lymphocytes' egress from secondary lymphoid organs (17). Here, we used FTY720 to block the migration of lymphocytes as an additional approach to reduce chronic brain inflammation. FTY720 (MilliporeSigma) was dissolved in DMSO and then diluted in PBS. FTY720 was given at a dosage of 1 mg/kg *via* oral gavage daily. Mice were randomly divided into 3 groups according to random numbers generated in Excel (Microsoft, Redmond, WA, USA). Mice of each group received treatment with PBS, PLX3397, or FTY720 daily starting 7 d post-ICH induction until the end of the experiments.

Statistical analysis

Data were analyzed by investigators blinded to experimental groups. All results were expressed as means $\pm$ SEM. Statistical

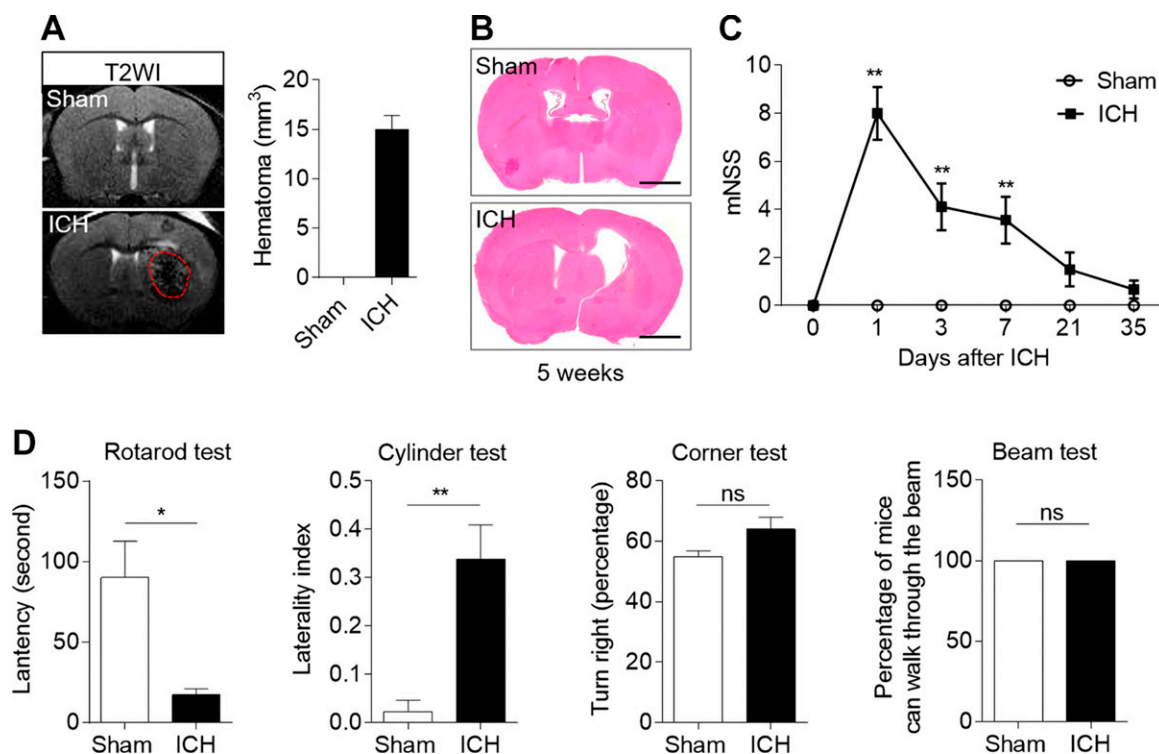


Figure 1. Residual sensorimotor dysfunction of mice at 5 wk post-ICH. **A**) Images from a 7 Tesla rodent MRI T2WI (T2 weighed imaging) scanning 3 d after 0.03 U collagenase injection showing the hemorrhagic lesion of ICH mice and calculated hematoma volume. **B**) Hematoxylin and eosin staining of coronal brain slices at the bregma level 5 wk post-ICH depicting complete hemorrhage resorption. Scale bars, 1 mm. **C**) mNSS scores from baseline to 5 wk after ICH. **D**) At 5 wk after ICH, a battery of tests were used to evaluate the sensorimotor function of ICH mice. Ns, no significance. Data are shown as means $\pm$ SEM; $n = 15$ in each group. * $P < 0.05$, ** $P < 0.01$ by 2-way ANOVA (**C**) and Student's *t* test (**D**).

analysis was performed using Prism 7 (GraphPad Software, La Jolla, CA, USA). D'Agostino and Pearson omnibus normality tests were performed to determine normal distribution. Two-way ANOVA was used for multiple comparisons. Two-tailed unpaired Student's *t* test was used for comparison of 2 groups.

RESULTS

Cognitive impairment in mice during chronic phase of ICH recovery

We first sought to determine whether the phenomenon of delayed cognitive impairment observed in patients with ICH can be observed in our ICH model. A murine model of collagenase-induced ICH was used, and the mean volume of hemorrhage at 3 d as measured by MRI T2 images was $\sim 15 \mu\text{l}$ (Fig. 1A, B). In this model, the acute neurologic deficit assessed *via* the mNSS gradually recovered, returning to sham level by 3–5 wk post-ICH (Fig. 1C). At this delayed time point, no differences in sensorimotor function between sham-treated and ICH mice was observed using the corner and beam tests; however, the rotarod and cylinder tests still showed a low level of residual motor dysfunction in ICH mice (Fig. 1D).

We next evaluated spatial learning and memory ability in these mice at 5 wk post-ICH using the MWM. Because

subtle residual motor dysfunction might influence the behavior of these mice in the water maze, we first evaluated whether swimming velocity was impacted in mice following ICH. We noted no apparent difference of swim velocity between ICH and sham-treated mice (Fig. 2A). Using the water maze, we showed that mice subjected to ICH exhibited impaired learning (Fig. 2B) and memory relative to sham-treated animals (Fig. 2C, D) at 5 wk. This suggests that cognitive impairment can be detected at a late stage of recovery following ICH and that this cognitive dysfunction occurs independent of motor deficits.

Dendritic morphology is altered in hippocampal CA1 neurons during the late stage of ICH

As hippocampal structure and electrophysiologic properties are closely related to cognitive function (18), we next evaluated whether the hippocampal structure and electrophysiologic activity are affected in the late stage of ICH. We utilized MRI imaging at 3 d post-ICH to confirm that the hemorrhage did not extend into hippocampus directly (Fig. 3A). CA1 hippocampal neurons were filled with an internal electrode solution containing biocytin using patch-clamp electrode in mouse brain slices at 5 wk post-ICH. CA1 neuron morphology was revealed by *post*

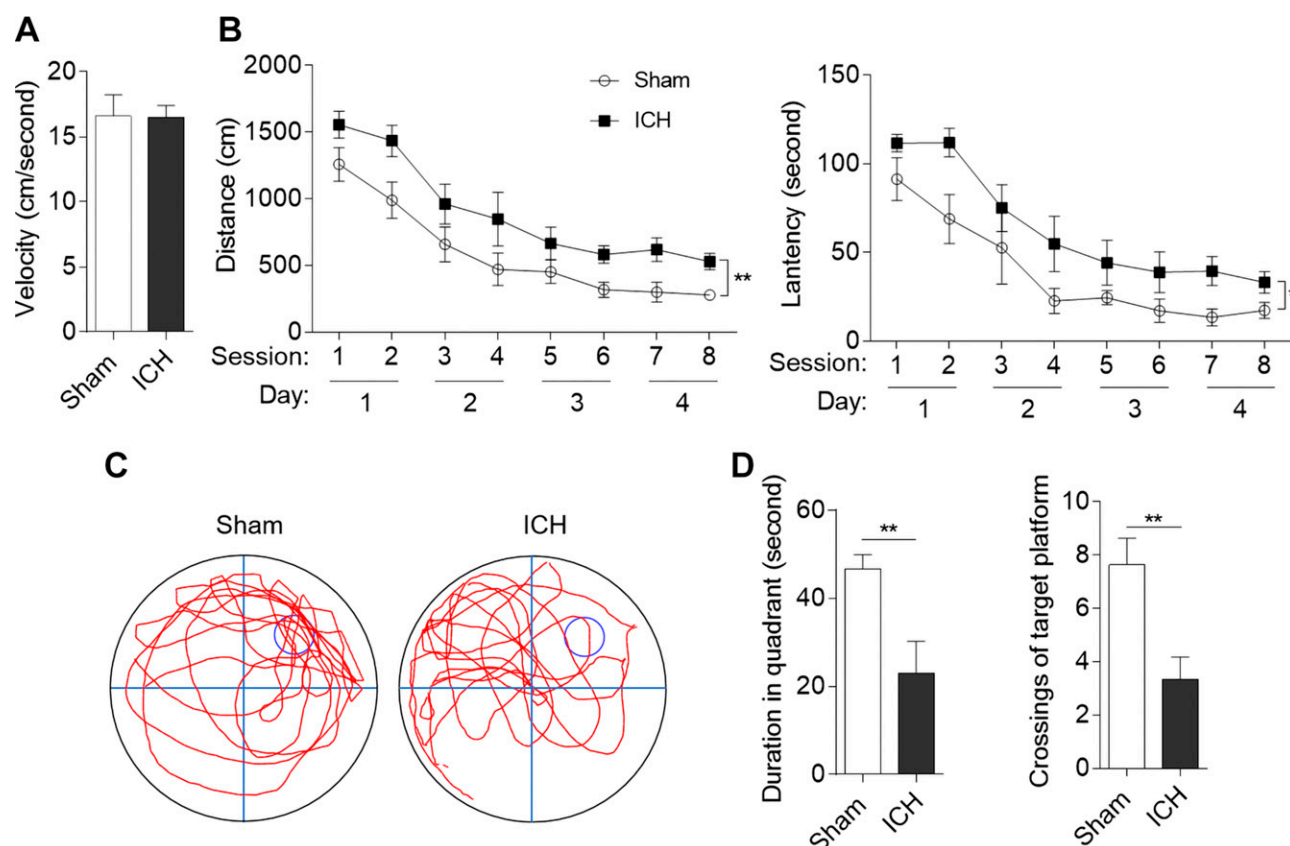


Figure 2. Cognitive decline of mice at 5 wk after ICH. MWM was used to evaluate cognitive function of mice 5 wk after ICH. A) Comparison of the velocity of mice swimming in the pool. B) The distance and latency from trial beginning to end at each training session from d 1–4. C) Pictures show the tracing of sham-treated and ICH mice during the probe test at d 5 of test. D) Left: duration in the target quadrant in the probe test; right: times crossing the target platform during probe test; $n = 15$ in each group. Mean $\pm$ SEM, 2-way ANOVA test was used for comparison in B, and Student's *t* test was used in D. * $P < 0.05$, ** $P < 0.01$.

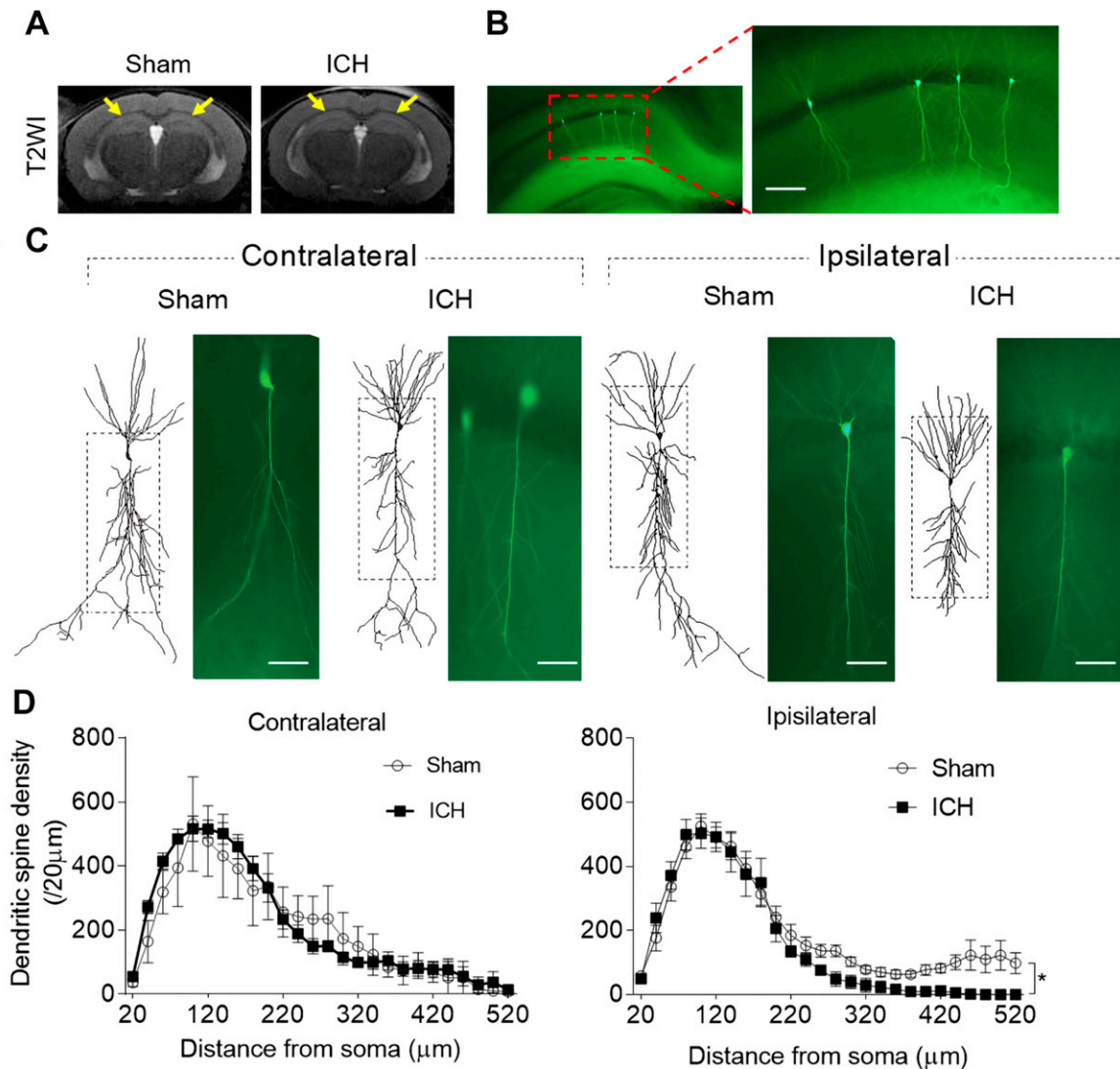


Figure 3. Altered dendritic morphology in hippocampal CA1 neurons at 5 wk after ICH. **A)** MRI T2 images depicting the hippocampi (yellow arrow) of sham-treated and ICH mice at d 3 after onset. No discernable hippocampal alteration was seen after ICH. T2WI, T2 weighted imaging. **B)** CA1-region neuronal structures after filling neurons with biocytin and processing *post hoc* with avidin–Alexa Fluor 488. Scale bar, 200 μm. **C)** Schematic images showing the structures of both contralateral and ipsilateral CA1 neurons from sham-treated and ICH mice. Scale bars, 100 μm. **D)** Left: comparison of dendritic length as a function of distance from soma in contralateral CA1 neurons of sham-treated and ICH mice; $n = 5$ in sham, $n = 7$ in ICH; right: comparison of dendritic length of ipsilateral CA1 neurons of sham and ICH mice; $n = 6$ in sham, $n = 5$ in ICH. Two-way ANOVA test was used for comparison; mean $\pm$ SEM. * $P < 0.05$.

hoc processing with avidin–Alexa Fluor 488, and their dendritic arborization was traced in 3 dimensions using Neurolucida. As shown in Fig. 3, CA1 neuron distal dendritic length was significantly reduced in the ipsilateral hippocampus of ICH mice relative to sham-treated animals, whereas no significant morphologic changes were noted in the contralateral hippocampus.

Impaired LTP in bilateral hippocampi during the late stage of ICH

LTP reflects synaptic plasticity and has long been considered one of the major mechanisms responsible for learning and memory (19). We next evaluated whether hippocampal LTP was impacted by ICH (Fig. 4). Consistent with

the observed morphologic change, the induction and maintenance of LTP of the ipsilateral hippocampus was significantly impaired at 5 wk post-ICH. Of interest, although the introduction of LTP of the contralateral hippocampus was not impacted in these mice, the maintenance was significantly inhibited. This suggests that hippocampal electrophysiological injury in the late stage of ICH was not limited to the ipsilateral hemisphere, as it was also detected in the contralateral hemisphere in a brain region remote from the hemorrhage.

ROS generation in the late stage of ICH

Following ICH, intraparenchymal blood elicits a host of biologic responses, including the generation of cytotoxic

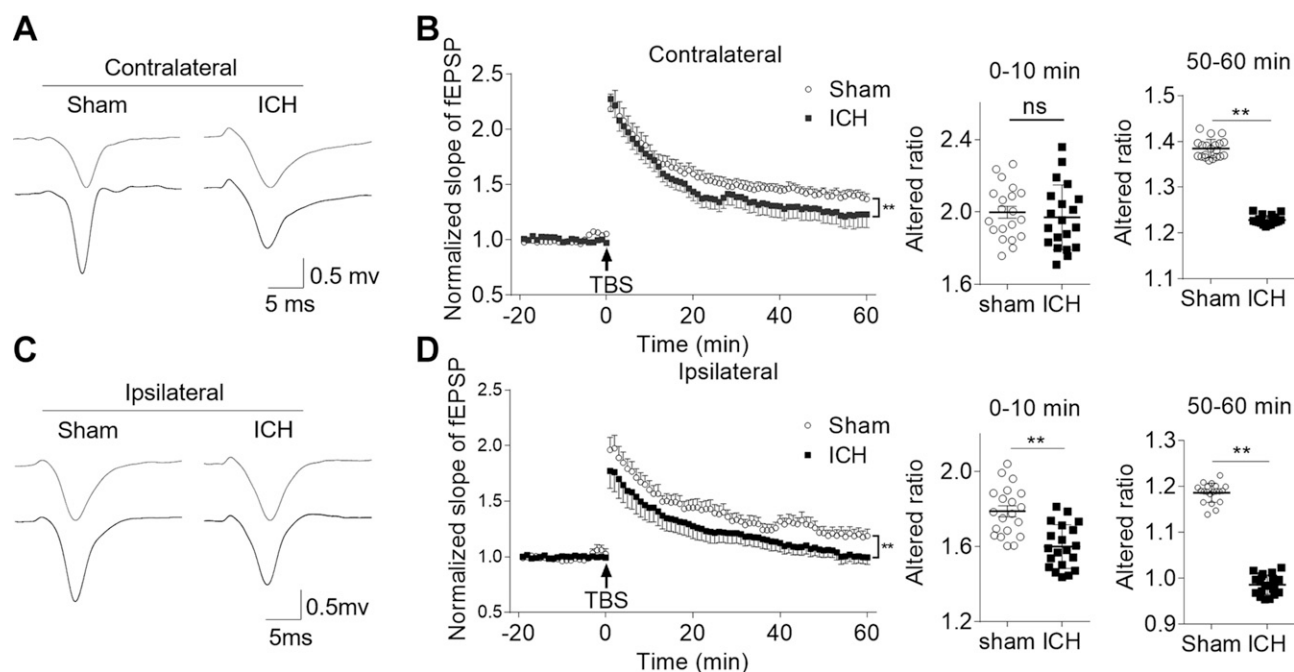


Figure 4. Hippocampal LTP was reduced 5 wk after ICH. *A*) fEPSP curves recorded before (top) and after (bottom) TBS from contralateral hippocampus of sham-treated and ICH mice at 5 wk post-ICH onset. *B*) Left: summary of normalized slope of fEPSP of contralateral hippocampus from baseline to 60 min after TBS. Right: comparing the quantification of first and last 10 min after TBS, which indicate the induction and maintenance of LTP. $n = 16$ in sham, $n = 15$ in ICH. ns, no significance. *C*) fEPSP curves recorded before (top) and after (bottom) TBS from ipsilateral hippocampus. *D*) Left: summary of normalized slope of fEPSP of ipsilateral hippocampus from baseline to 60 min after TBS. Right: comparison of first and last 10 min after TBS. Mean $\pm$ SEM, $n = 13$ in sham, $n = 15$ in ICH. Comparisons were performed by 2-way ANOVA or Student's *t* test. $**P < 0.01$.

blood breakdown products as well as the overproduction of free radicals and proinflammatory molecules (20). ROS can exacerbate brain injury *via* several pathways, including the induction of DNA damage, which leads to cell apoptosis and inflammation (21). Oxidative brain injury has been reported in animal models of ICH, and antioxidant treatment ameliorates neurologic deficits in an ICH model (20). Whether oxidative stress persists in later stages postinjury is unknown. Here, we demonstrate continuous ROS production in the brain of mice following ICH in both hemispheres beginning early and persisting to the late phases posthemorrhage (Fig. 5). This suggests a global and continuous overproduction of ROS in the brain following ICH.

Accumulation of microglia and infiltrating peripheral immune cells in bilateral brain hemispheres in the late stage of ICH

Acute brain hemorrhage incites brain inflammation, characterized by glial activation, infiltration of peripheral immune cells including monocytes and lymphocytes, and the production of several inflammatory mediators (9, 10). Brain inflammation also exacerbates the secondary brain injury following ICH, but whether brain inflammation persists to the chronic stage of ICH remains unclear. We therefore evaluated the immune cell profile during the late stage of ICH. Significant accumulation of both myeloid cells and lymphocytes were seen in the brain at 5 wk after

ICH. This occurred most prominently in the ipsilateral hemisphere but also, to a lesser extent, contralaterally (Fig. 6*A, B*). Next, the distribution of microglia was evaluated using immunohistochemistry at 5 wk post-hemorrhage. As compared with sham-treated controls, the number of Iba1⁺ microglia became elevated, with morphologically larger cell bodies observed in hippocampi at both hemispheres at 5 wk after ICH (Fig. 6*C, D*). In addition, the number of microglial processes was reduced after ICH (contralateral, sham *vs.* ICH: 5.5 ± 0.3 *vs.* 4.3 ± 0.3 , $P < 0.01$; ipsilateral, sham *vs.* ICH: 5.1 ± 0.4 *vs.* 3.8 ± 0.4 , $P < 0.01$). These findings suggest that chronic brain inflammation occurs following ICH and that inflammatory cells distribute globally across both hemispheres.

Depletion of microglia preserves cognitive function in the late stage of ICH

To examine the influence of persistent brain inflammation on delayed cognitive impairment following ICH, we evaluated whether inhibiting brain inflammation improves cognitive function in the late stage of ICH. We employed 2 approaches, including the depletion of microglia using a CSF1R inhibitor, PLX3397 (16, 22), and the blockade of lymphocyte egress using a sphingosine-1-phosphate receptor modulator, FTY720 (23). In the present study, PLX3397 or FTY720 was administered orally to mice starting at 7 d post-ICH (Fig. 7*A*), at which point both acute brain

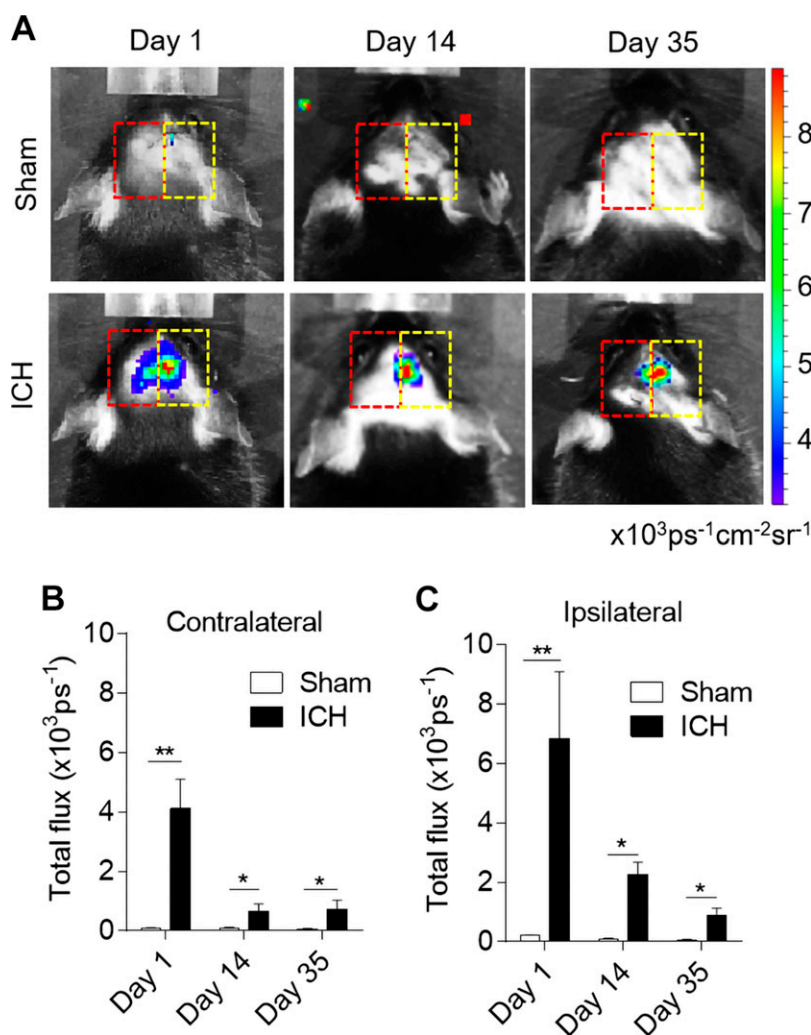


Figure 5. Increased ROS production after ICH. ROS were detected by bioluminescent imaging *in vivo*. Images were captured for 1 min by IVIS (Xenogen IVIS-200) after luminol intraperitoneal injection. **A**) Representative bioluminescent images show intrabrain ROS production of sham-treated and ICH mice; red rectangle shows the region of interest of contralateral hemisphere; yellow indicates ipsilateral hemisphere to measure the intensity. **B**, **C**) quantification and statistical analysis; $n = 10$ in sham-treated and 8 in ICH group at each time point; 2-way ANOVA test; mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

inflammation and brain edema have waned (24, 25). PLX3397 treatment reduced brain microglia cells by $\sim 80\%$ compared with PBS-treated mice. In addition, myeloid cells were also reduced in PLX3397-treated mice compared with PBS, whereas the brain-infiltrated lymphocytes are not apparently impacted. In addition, ICH mice treated with FTY720 showed reduced lymphocyte infiltration in the brain without a significant impact on myeloid cells and microglia (Fig. 7B, C). These findings are in line with prior reports (10, 17, 26). Relative to ICH mice receiving PBS, mice receiving PLX3397 exhibited improved spatial learning and memory performance on the water maze test (Fig. 7D–G). However, no significant improvement for learning and memory performance was seen in mice receiving FTY720 (Fig. 7D–G). These data suggest that microglia depletion but not blockade of lymphocyte egress ameliorates cognitive decline during the late stage of ICH in mice.

DISCUSSION

In this study, we report delayed cognitive impairment accompanied by morphologic and functional changes of hippocampal neurons in a murine collagenase model of

ICH. Additionally, we provide novel evidence supporting the concept that chronic brain inflammation in brain regions remote from the hemorrhage focus may contribute to this delayed cognitive impairment.

Although delayed cognitive decline in patients with ICH has long been recognized in the clinical setting, evidence for post-ICH cognitive impairment in animal models of ICH has not been well established. This may in part be due to deficiencies in animal models of ICH. For instance, in a mouse model utilizing intrastriatal injection of thrombin, cognitive decline assessed by the water maze test accompanied by impaired dentate gyrus neurogenesis was detected at 5 d (27). This suggests that the activation of thrombin by the breakdown of blood products following ICH may contribute to cognitive impairment in the acute phase. However, this study did not evaluate long-term functional and cognitive outcome, and direct injection of thrombin does not accurately model the complex pathophysiology of ICH. Furthermore, autologous blood and collagenase ICH models differ in many facets of initial and delayed pathophysiology. For example, autologous blood causes greater mass effect and early mortality, whereas collagenase-induced ICH produces more brain edema, inflammation, and cell death (28). Because the collagenase model exhibits more severe secondary injury and

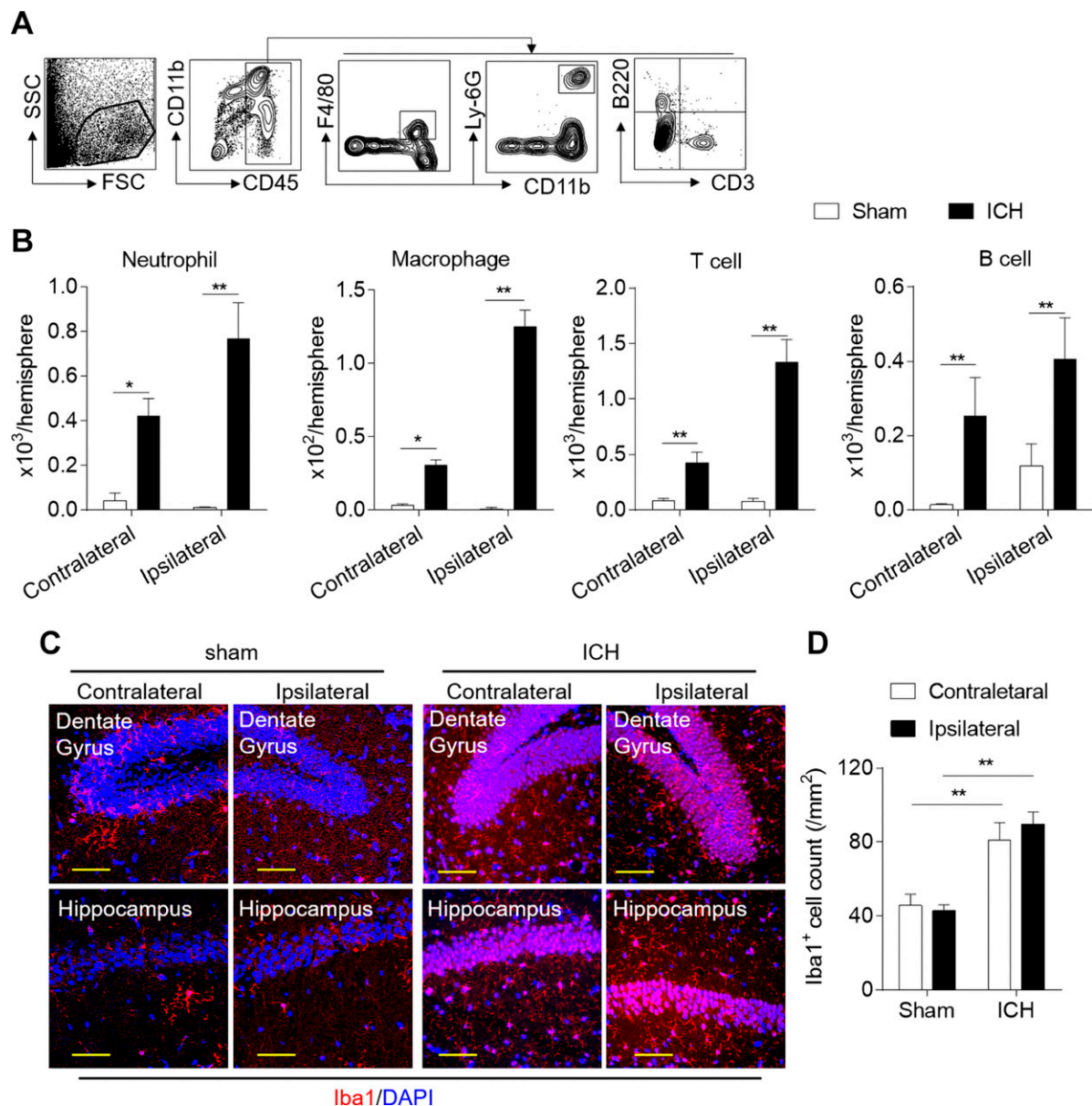


Figure 6. Accumulation of microglia and infiltrating immune cells in the late stage of ICH. **A**) Flow cytometry of brain tissue for infiltrating peripheral immune cells (CD45⁺) at 5 wk after ICH. Representative gating of macrophage (CD11b⁺F4/80⁺), neutrophil (CD11b⁺Ly6G⁺), B cell (B220⁺), and T cell (CD3⁺). FSC, forward scatter; SSC, side scatter. **B**) Quantification of cell counts of each cell subset. $n = 10$ in sham-treated and 8 in ICH group; mean $\pm$ SEM; 2-way ANOVA test; * $P < 0.05$, ** $P < 0.01$. **C**) Immunohistochemistry images show the distribution of microglia (Iba1⁺, red) in bilateral hippocampal area of mice at 5 wk after ICH. Scale bars, 100 μ m. **D**) Counts of Iba1⁺ cells in indicated groups of mice; $n = 16$ in sham, $n = 20$ in ICH; mean $\pm$ SEM. ** $P < 0.01$ by 2-way ANOVA.

inflammation, as well as persistent functional deficits, in the present study we utilized the mouse collagenase model to evaluate cognitive dysfunction. In another recent study utilizing an autologous blood injection model by Xiong *et al.* (29), cognitive impairment was detected 28 d posthemorrhage using the radial arm water maze. However, in 2 studies using a rat collagenase model of ICH, delayed cognitive deficit was not identified by MWM test (30, 31), although one of them did show learning and memory dysfunction on the water maze test at 2 wk posthemorrhage (31). Although rat ICH models parallel mouse ICH models in many ways, including a similar time course of edema, neuronal loss, and inflammation (32), these models may differ in the observed

behavioral outcomes. For example, motor deficit persists for a longer period in rat models than in mouse models (28). Rat models of ICH may therefore not be ideal for testing of long-term cognitive outcome.

The presence of delayed cognitive decline following ICH (2) suggests the existence of distinct pathophysiologic mechanisms underlying this phenomenon. In the present study, we show that cognitive decline occurs in mice after their recovery from ICH-induced acute neurologic deficits, mirroring findings observed in human patients. Additionally, we show that even after mice recover from the acute brain hemorrhage, remote brain structures such as the hippocampus can be injured *via* secondary mechanisms during the late stage.

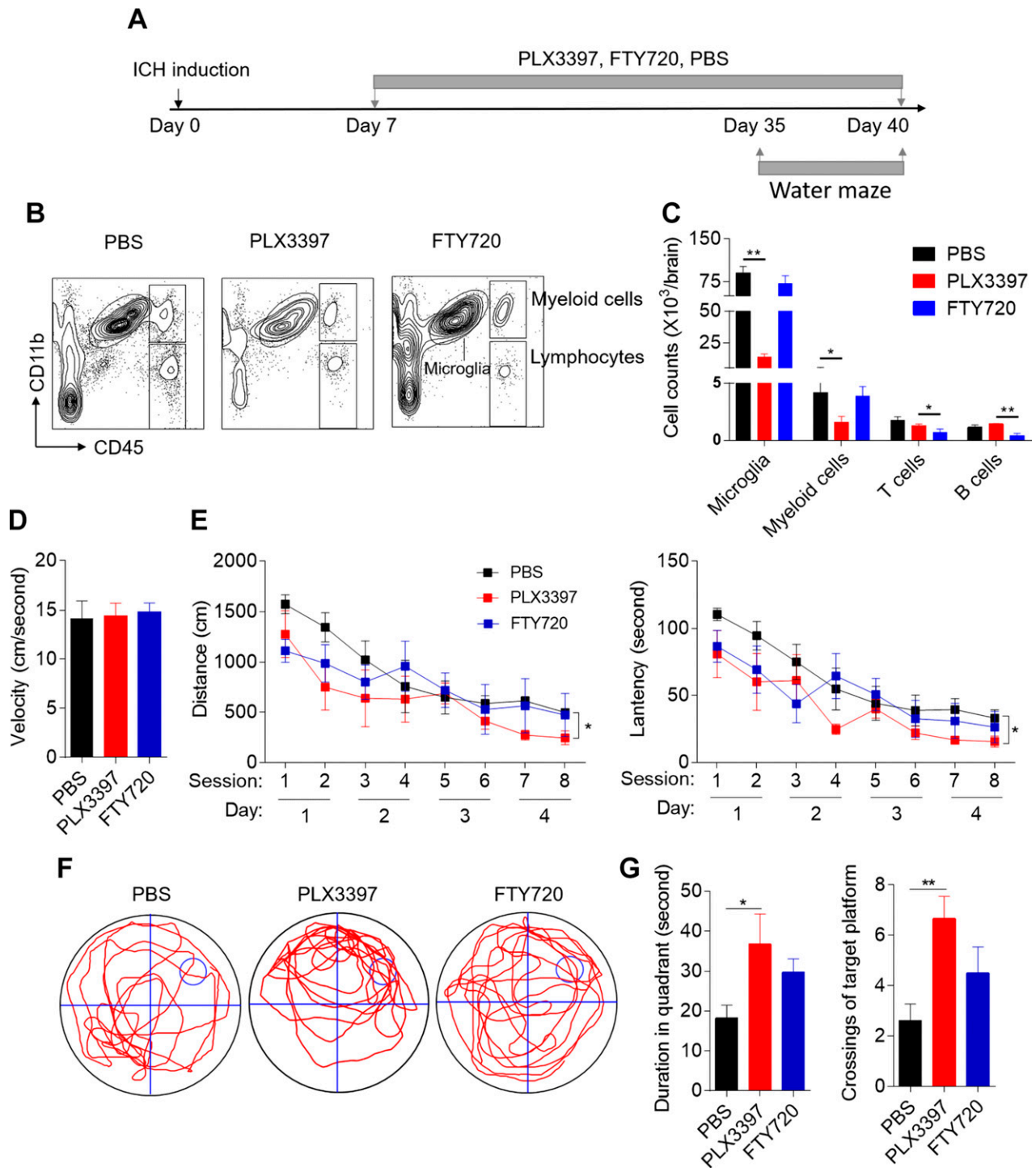


Figure 7. Inhibition of brain inflammation improved cognitive function in the late stage of ICH. **A)** Flow chart shows the experiment design. ICH mice randomly received daily treatment of PLX3397, FTY720, or PBS from d 7 after ICH until the end of experiment. MWM was used to evaluate spatial learning and memory performance at 5 wk after ICH. **B)** Representative flow cytometry gating images show microglia ($\text{CD11b}^+\text{CD45}^{\text{inter}}$), myeloid cells ($\text{CD11b}^+\text{CD45}^{\text{high}}$), and lymphocytes ($\text{CD11b}^-\text{CD45}^{\text{high}}$) from mouse brain at 5 wk after ICH in indicated groups. **C)** Summarized data of immune cell counts in the brain; $n = 6$ in each group. **D)** Swimming velocity of mice in a water-filled pool. **E)** Graphs show the total distance (left) and duration (right) from beginning to end of each trial in the training period. **F)** Images show the swim path of mice during the probe test. **G)** Statistical results of time spent in northeast quadrant (left) and the times of crossing the target platform area (right) during the probe test. $n = 10$ in PBS group, 9 in PLX3397 group, and 10 in FTY720 group. Data are presented as means $\pm$ SEM; 2-way ANOVA was used for comparison in **C** and **E**, and 1-way ANOVA was used in **G**. $*P < 0.05$, $**P < 0.01$.

This injury develops in or spreads to the contralateral hemisphere, suggesting that morphologic and functional changes in the hippocampus during the late

phase of ICH may underlie the observed delayed cognitive decline. Furthermore, we show bilateral accumulation of peripheral immune cells in the brain as well

as activation of brain intrinsic microglia during the late phase after ICH, suggesting the existence of chronic, diffuse neuroinflammation after ICH. Inhibition of this brain inflammation *via* depletion of microglia preserved the cognitive function of mice during the late stage of ICH. These findings suggest that brain inflammation may be one of the underlying causes of delayed cognitive impairment following ICH. In support of this notion, a growing body of evidence has suggested a link between neuroinflammation and cognitive impairment. For example, an experimental study by Garber *et al.* (33) shows that astrocyte-derived IL-1 impairs memory function by inhibiting neurogenesis during viral infection. Several reports have also suggested that brain inflammation induces cognitive dysfunction independent of structural brain damage (34). In addition, proinflammatory cytokines such as TNF- α can impair synaptic structure and function (35).

Microglia have multiple roles in the brain, including phagocytosis, production of proinflammatory or anti-inflammatory factors, and orchestration of the activity of infiltrating immune cells (36). Previous studies using ICH animal models suggest a beneficial role for microglia through phagocytosis of debris and erythrocyte residue in the brain after hemorrhage as well as through the generation of anti-inflammatory factors to tune down brain inflammation (11, 37, 38). Therefore, depletion of microglia using PLX3397 at a given stage of ICH may not provide a comprehensive view of their function, particularly during the recovery phase of ICH.

Although acute hemorrhage was not noted in the hippocampus on MRI images at 3 d, we cannot exclude the possibility of secondary hippocampal injury occurring during the early phase of ICH. We did not assess intracranial pressure in our ICH mouse model, and a previous report by Chen *et al.* (39) indicates that increased intracranial pressure was associated with hippocampus tissue loss. This suggests the possibility that the propagation of elevated intracranial pressure from the hematoma within the brain may potentially cause damage to the contralateral hemisphere. In addition, the precise inflammatory mechanisms underlying distal brain structure change and cognitive decline have not been determined. Nevertheless, we have demonstrated a delayed cognitive impairment accompanied by morphologic and functional changes of hippocampus in the late stage of ICH in mice. We have also shown brain inflammation as well as generation of ROS in brain regions distant from original injury site during the chronic stage of ICH, and that targeting this chronic brain inflammation preserves cognitive function of mice. These findings suggest that persistent brain inflammation may contribute to impaired brain recovery after ICH, providing a basis for future pursuing of the mechanisms underlying delayed cognitive impairment after ICH. FJ

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AUTHOR CONTRIBUTIONS

K. N. Sheth, M. T. Lawton, and A. F. Ducruet formulated the concept and study design; E. Shi, K. Shi, and S. Qiu conducted the experiments; and E. Shi, K. Shi, and A. F. Ducruet analyzed data and drafted the manuscript.

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