Activation of the High-Mobility Group Box 1 Protein-Receptor for Advanced Glycation End-Products Signaling Pathway in Rats During Neurogenesis After Intracerebral Hemorrhage

Chunyan Lei, MD; Bo Wu, MD, PhD; Tian Cao, MD; Shuting Zhang, MD, PhD; Ming Liu, MD, PhD

Background and Purpose—Following intracerebral hemorrhage (ICH), high-mobility group box 1 protein (HMGB1) may promote neurogenesis that supports functional recovery. How HMGB1 regulates or participates in this process is unclear, as are the pattern recognition receptors and signaling pathways involved.

Methods—ICH was induced by injection of collagenase in Sprague–Dawley rats, which were treated 3 days later with saline, with the HMGB1 inhibitor ethyl pyruvate or with FPS-ZM1, an antagonist of the receptor for advanced glycation end-products. A Sham group was treated with saline solution instead of collagenase and then treated 3 days later with saline again or with ethyl pyruvate or N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1). Expression of the following proteins was measured by Western blot, immunohistochemistry, or immunofluorescence: HMGB1, receptor for advanced glycation end-products, toll-like receptor (TLR)-2, TLR4, brain-derived neurotrophic factor, and matrix metalloproteinase-9. The number of cells positive for 5-bromo-2-deoxyuridine or doublecortin was determined by immunohistochemistry and immunofluorescence.

Results—Levels of HMGB1, receptor for advanced glycation end-products, TLR4, TLR2, brain-derived neurotrophic factor, and matrix metalloproteinase-9 were significantly higher 14 days after ICH than at baseline, as were the numbers of 5-bromo-2-deoxyuridine- or doublecortin-positive cells. At the same time, HMGB1 moved from the nucleus into the cytoplasm. Administering ethyl pyruvate significantly reduced all these ICH-induced increases, except the increase in TLR4 and TLR2. Administering FPS-ZM1 reduced the ICH-induced increases in the expression of brain-derived neurotrophic factor and matrix metalloproteinase-9 and in the numbers of 5-bromo-2-deoxyuridine- or doublecortin-positive cells.

Conclusions—These findings suggest that HMGB1 acts via the receptor for advanced glycation end-products signaling pathway to promote neurogenesis in later phases of ICH. (Stroke. 2015;46:500-506. DOI: 10.1161/STROKEAHA.114.006825.)

Key Words: advanced glycosylation end-product receptor | box protein 1, high mobility group | intracerebral hemorrhage | neurogenesis
of HMGB1 in post-ICH neuronal remodeling has never been demonstrated directly.

HMGB1 is known to recruit stem cells and to trigger tissue remodeling after various types of brain injury, such as cerebral ischemia and damaged white matter.11,12 It does so by activating pattern recognition receptors, such as the receptor for advanced glycation end-products (RAGE), toll-like receptor (TLR)-2, and TLR4. TLR2 and TLR4 are well known for mediating innate immune responses to pathogens,13,14 whereas RAGE is a multiligand receptor of the immunoglobulin superfamily.15 It is possible that HMGB1 also triggers these pattern recognition receptors to promote neurogenesis after ICH, but previous studies have not examined this question.

Here we examined whether HMGB1 may be involved in restoring neurological function in a rat model of collagen-induced ICH and whether the protein works via the same RAGE, TLR2, and TLR4 receptors involved in recovery after other types of brain injury.

Materials and Methods

Animals and Treatment Groups

All experiments were performed in accordance with the guidelines for animal research at Sichuan University. Adult male Sprague–Dawley rats were randomly divided into the following 6 groups: ICH, ethyl pyruvate (EP), N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1), Sham, Sham+EP, and Sham+FPS-ZM1. How each group was treated is described in detail in Methods I in the online-only Data Supplement. ICH was induced by administering collagenase VII (Sigma C0773, 5.0 ul), as previously described.16 Further details of ICH induction are given in Methods II in the online-only Data Supplement.

Administration of Ethyl Pyruvate and FPS-ZM1

The HGMB1 inhibitor ethyl pyruvate (EP; Sigma) and the RAGE antagonist FPS-ZM1 (Millipore, USA) were dissolved in 0.9% saline solution and administered at respective doses of 60 and 1.5 mg/kg via intraperitoneal injection (IP) once daily starting 3 days after ICH induction.

5-Bromo-2-Deoxyuridine Labeling

To label S-phase cells after ICH induction, 5-bromo-2-deoxyuridine (BrdU; Sigma) in 0.9% saline was administered intraperitoneally at a dose of 100 mg/kg once daily for 14 days after ICH induction.

Western Blot Analysis

Western blotting was performed as previously described16 to measure levels of HMGB1, RAGE, TLR-2, TLR4, brain-derived neurotrophic factor (BDNF), matrix metalloproteinase-9 (MMP-9) in the animal groups (n=5 for each group). Complete details are provided in Methods III in the online-only Data Supplement.

Immunohistochemistry

Immunohistochemistry of ipsilateral striatum around the hematoma was performed as previously described16 to assess abundance of HMGB1 and RAGE, as well as determine numbers of cells positive for BrdU (1:100; Abcam) or doublecortin (DCX, 1:50; Abcam). Multiple sections were analyzed from 3 animals randomly selected from each group. Complete details are provided in Methods IV in the online-only Data Supplement.

Immunofluorescence

After deparaffinization and rehydration, sections were incubated overnight at 4°C with antibody against BrdU, DCX, or RAGE, followed by secondary anti-rabbit antibody for 1 hour at room temperature. The striatum was observed by light microscopy and analyzed using Image-Pro Plus 6.0.

Behavioral Tests

At 14 days after ICH or Sham induction, all animals were subjected to a battery of behavioral tests that allowed calculation of the modified neurological severity score.17,18 Eight animals were randomly selected from each group for analysis. Details of the behavioral tests are provided in Methods V in the online-only Data Supplement.

Statistical Analysis

After confirming that data were normally distributed, intergroup differences were assessed for statistical significance using analysis of variance, followed by post hoc testing based on least squares differences. All data were presented as mean±SD, and differences were considered significant at the 5% level.

Results

Expression of HMGB1 in Rat ICH

To examine whether HMGB1 may be involved in rat ICH, we used immunohistochemistry and Western blotting to probe levels of the protein in the ipsilateral striatum in the presence and absence of ICH. Brain sections were stained with hematoxylin-eosin to help ensure that we analyzed ipsilateral striatum around the site of tissue damage (Figure 1A). In the Sham group, HMGB1 was present in nuclei throughout the striatum. After ICH induction, HMGB1 moved from the nucleus into the cytoplasm (Figure 1B). Immunohistochemistry showed a significantly higher number of HMGB1-positive cells in the ipsilateral striatum of ICH rats than in the Sham group (P<0.001; Figure 1B). These results were confirmed by Western blotting (Figure 2; Table I in the online-only Data Supplement).

Expression of HMGB1 Receptors in Rat ICH

Next we examined whether HMGB1 receptors may be involved in how the brain responds to ICH in later stages. Using Western blotting, we measured expression levels of RAGE, TLR2, and TLR4 in ipsilateral striatum on day 14 after ICH induction. Compared with Sham animals, ICH animals showed significant increases in levels of RAGE, TLR4, and TLR2 (Figure 3; Table I in the online-only Data Supplement).

Neurogenic Effects of HMGB1 After ICH

Compared with Sham animals, ICH animals showed higher levels of HMGB1, BDNF, and MMP-9 in the ipsilateral striatum on day 14 after ICH induction, as well as higher numbers of BrdU-positive cells and DCX-positive cells. EP administration led to levels of BDNF in the ipsilateral striatum that were significantly lower than those in the ICH group, but still significantly higher than those in the Sham group on day 14 (Figure 4; Table I in the online-only Data Supplement). EP also led to levels of MMP-9 that were significantly lower than those in the ICH group on day 14, but still significantly higher than those in the Sham group (Figure 4; Table I in the online-only Data Supplement). Similarly, EP administration led to significantly lower numbers of BrdU-positive cells and DCX-positive cells in ipsilateral striatum than in ICH animals on day 14 (both P<0.001; Figure 5).
Both immunohistochemistry and immunofluorescence showed much higher numbers of BrdU-positive cells and DCX-positive cells in the ipsilateral striatum and subventricular zone in ICH animals than in Sham animals on day 14 (Figure 5). To confirm whether neuroblasts were being newly generated in our rat model of collagen-induced ICH, we performed immunofluorescence double staining to determine the overlap in BrdU-positive and DCX-positive cell populations. Our results indicated that BrdU-positive cells were also positive for DCX.

**HMGB1-RAGE Axis and Neurogenesis After ICH**

Our analysis of HMGB1 expression in ICH and Sham animals suggested that HMGB1 promotes neurogenesis after ICH. This raised the question of which receptor(s) may mediate this HMGB1-induced neurogenesis. To test this hypothesis, we compared post-ICH expression of RAGE and TLR4 in the presence and absence of EP. Administration of this HMGB1 inhibitor led to RAGE levels on Western blots that were much lower than those in the ICH group on day 14, but still significantly higher than those in the Sham group (Figure 3; Table I in the online-only Data Supplement). Similar results were observed by immunohistochemistry (Figure 1C, P<0.001). However, N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) administration did not markedly affect HMGB1 expression. Cells showing cytoplasmic HMGB1 were counted as HMGB1-positive.

**Figure 1.** Immunohistochemistry of high-mobility group box 1 protein (HMGB1) in sections taken from around the hematoma in the ipsilateral striatum at 14 days after intracerebral hemorrhage (ICH) induction or Sham treatment. **A**, Complete section from the brain of a Sham rat stained with hematoxylin-eosin and shown at different magnifications to indicate how ipsilateral striatum was identified and analyzed. **B**, In Sham animals, the protein showed a primarily nuclear localization. After ICH induction, HMGB1 moved from the nucleus into the cytoplasm, and the levels were significantly higher than in Sham animals on day 14 (P<0.001). Ethyl pyruvate (EP) administration at 3 days after ICH significantly reduced this ICH-induced increase (P<0.001). However, N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) administration did not markedly affect HMGB1 expression. Cells showing cytoplasmic HMGB1 were counted as HMGB1-positive. **C**, Immunohistochemistry of receptor for advanced glycation end-products (RAGE) in ipsilateral striatum at 14 days after ICH induction or Sham treatment. Receptor levels were significantly higher in the ICH group than in the Sham group (P<0.001). EP administered 3 days after ICH markedly reduced these ICH-induced increases. **D**, Immunofluorescence double staining was used to identify the localization of RAGE (green) relative to 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue). Representative results are shown.
Figure 2. Western blotting of high-mobility group box 1 protein (HMGB1) levels in the ipsilateral striatum at 14 days after intracerebral hemorrhage (ICH) induction or Sham treatment. Levels of the protein were significantly higher in the ICH group than in the Sham group (P<0.001). Ethyl pyruvate (EP) administered 3 days after ICH led to significantly lower levels than in ICH animals (P<0.001), whereas N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) did not affect HMGB1 expression. Representative results are shown in the left panel and quantified in the right panel, which shows means±SD values for band intensity normalized to the intensity for β-actin. *P<0.001, ICH group vs EP group; †P<0.001, Sham, Sham+EP, and Sham+FPS-ZM1 groups vs Sham, Sham+EP, and Sham+FPS-ZM1 groups.

the ICH group, but still significantly higher than those in the Sham group (Figure 5). These data suggest that HMGB1 can act through RAGE to promote neurogenesis after ICH.

Controlling for Effects of EP and FPS-ZM1 not Mediated by HMGB1 or RAGE
To control for the possibility that some or all of the observed effects of EP or FPS-ZM1 on our animals reflected processes not specifically mediated by HMGB1 or RAGE, including general toxic effects, we performed all experiments with Sham animals that we treated with EP or FPS-ZM1 at 3 days after Sham induction of ICH. Protein levels in these 2 groups were similar to those in the Sham group that was given saline 3 days after Sham induction (Table I in the online-only Data Supplement). These results suggest that the observed effects of EP or FPS-ZM1 on protein levels were mostly or entirely mediated by HMGB1 or RAGE.

Behavioral Tests
Neurological function was lower in ICH animals than in Sham animals based on behavioral tests. This function gradually recovered in ICH animals that did not receive any additional treatments; in contrast, the recovery was significantly inhibited by EP and FPS-ZM1 (Figure IA in the online-only Data Supplement). Animals treated with either substance showed significantly lower recovery on day 14 than ICH animals or Sham animals (both P<0.001; Figure IB in the online-only Data Supplement). All 3 Sham groups showed similar results on behavioral tests, suggesting that the observed effects of EP and FPS-ZM1 on functional recovery were mostly or entirely mediated by HMGB1 or RAGE.

Discussion
In this study, we observed a stroke-induced increase in HMGB1 levels that was associated with increases in BDNF and MMP-9 levels, numbers of BrdU-positive cells and DCX-positive cells, and recovery of neurological function. All these effects were suppressed by the HMGB1 inhibitor EP. EP also inhibited the expression of RAGE, but not of TLR4 or TLR2, all 3 of which can be activated by HGMB1 to trigger downstream signaling pathways. Similar to EP, the RAGE antagonist FPS-ZM1 suppressed ICH-induced increases in BDNF and MMP-9 levels, numbers of BrdU- and DCX-positive cells, and recovery of neurological function. Together, these findings suggest that HMGB1 acts via RAGE-dependent pathways to trigger neurogenesis after ICH.

The nuclear transcription factor HMGB1 is a crucial regulatory molecule that stimulates inflammation after traumatic brain injury and acute stroke.1,7,8,19 In previous work, we showed that HMGB1 is released into the cytoplasm during the acute phase after ICH, which resulted in neuronal apoptosis, cerebral edema, and neurological impairment.7 Taken together, these results suggest that HMGB1 acts as an early proinflammatory cytokine within the neurovascular unit to mediate inflammation during the acute phase of ICH.

Subsequent to this detrimental proinflammatory activity, the same HMGB1 has been proposed to promote restorative tissue remodeling in later stages of ICH. A study in mice showed that HMGB1 helps drive neurovascular remodeling and recovery of neurological function after cerebral ischemia.7 Another study found that HMGB1 from reactive astrocytes attracts endothelial progenitor cells to sites of white matter injury in order to promote recovery.12 The present study extends these findings by showing that the HMGB1-RAGE axis plays a crucial role in neurogenesis in later stages after ICH.

The present work also extends previous findings that RAGE plays a critical role in determining pathological outcomes in trauma. RAGE is also expressed in neurons and glial cells of the brain, and its expression can be upregulated by activated astrocytes and microglia cells.8 In a mouse model of ischemia/reperfusion injury in heart, HMGB1 binds to RAGE soon after injury and thereby activates proinflammatory pathways and exacerbates myocardial injury.11,21 In a human pancreatic tumor cell culture model, RAGE and HMGB1 coordinately enhance mitochondrial complex I activity, ATP production, tumor cell proliferation, and migration.2,21 In fact, extracellular HMGB1, which acts as a strong macrophage-activating factor, binds to RAGE, activates endothelial cell proliferation, and induces endothelial cell migration and sprouting, thereby promoting tumor
progression and propagation. The present study adds to the number of pathways, both physiological and pathophysiological, that the HMGB1-RAGE axis helps to regulate.

Our finding that HMGB1 may promote the expression of MMP-9 is consistent with previous studies implicating this enzyme in tissue recovery after injury. In a mouse model of cerebral ischemia, HMGB1 upregulated MMP-9 expression in neurons and astrocytes. Upregulation of MMP-9 is believed to promote tissue recovery because the high levels of enzyme degrade extracellular matrix and allow new neuronal cells to migrate toward damaged tissue, where they differentiate into mature neurons that compensate for the loss of neuronal function. This implies that upregulation of MMP-9 should be associated with neuronal proliferation, which can be measured using the S-phase marker BrdU, and with the appearance of new neurons, which can be measured using DCX. We previously reported that ICH induction in the same rat model used here led to significant increases in the levels of MMP-9 and in the numbers of BrdU- and DCX-positive cells in the ipsilateral brain during the later phase after ICH. Intracerebroventricular injection of MMP-9 siRNA reduced these ICH-induced increases. In the present study, we found that ICH induced increases in MMP-9 levels and in the numbers of BrdU- and DCX-positive cells in the subventricular zone and ipsilateral striatum on day 14. Furthermore, we
found essentially complete overlap between BrdU- and DCX-positive cells, suggesting that they are the same cells. These results lead us to propose that after ICH, MMP-9 promotes the migration of BrdU- and DCX-positive cells from the subventricular zone toward the ipsilateral striatum.

An advantage of our study is that we correlate changes in protein levels in the ipsilateral striatum with functional recovery using the modified neurological severity score. Although this scoring system has proven effective for assessing functional recovery, it does not capture all aspects of behavioral

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**Figure 4.** Western blotting of brain-derived neurotrophic factor (BDNF) and matrix metalloproteinase (MMP)-9 levels in the ipsilateral striatum at 14 days after intracerebral hemorrhage (ICH) induction or Sham treatment. The levels of both proteins were significantly higher in the ICH group than in the Sham group (both P<0.001). Administering either ethyl pyruvate (EP) or N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) at 3 days after ICH significantly reduced these ICH-induced increases (all P<0.001). Representative results are shown in the upper panel and quantified in the lower panels, which show mean±SD values for band intensity normalized to the intensity for β-actin. *P<0.001, ICH group vs EP and FPS-ZM1 groups; #P<0.001, ICH, EP, and FPS-ZM1 groups vs Sham group.

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**Figure 5.** Immunohistochemistry to track number of 5-bromo-2-deoxyuridine (BrdU)-positive cells and doublecortin (DCX)-positive cells at 14 days after intracerebral hemorrhage (ICH) induction or Sham treatment. Upper panels show that both populations of cells in the ipsilateral striatum were significantly larger in the ICH groups than in the Sham group (both P<0.001). Ethyl pyruvate (EP) or N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) treatment at 3 days after ICH significantly reduced these ICH-induced increases. The bottom panel shows immunofluorescence double labeling of sections from the ipsilateral subventricular zone. The images show colocalization of BrdU- and DCX-positive cells. Representative results are shown.
function. Another limitation of our study is that we measured neurogenesis using only DCX staining, which is an indirect method because DCX-positive cells may not be viable. Nevertheless, we did find that rat groups with greater numbers of DCX-positive cells also showed greater functional recovery. This suggests that the DCX-positive cells in our study were indeed functional neurons. Future work should examine neurogenesis using more direct methods.

Neurogenesis after brain injury is highly complex and presumably plays a key role in ameliorating the damage and providing at least partial recovery of neurological function. Understanding the pathways responsible for this postinjury recovery will help us understand basic brain biology, and they may also provide clues to molecules and processes that we can manipulate in the clinic to improve and accelerate recovery. The current study provides strong evidence that HMGB1, RAGE, and RAGE-dependent pathways deserve further investigation for their role in promoting neurogenesis after ICH.

Sources of Funding
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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL
Activation of the HMGB1-RAGE signaling pathway in rats during neurogenesis after intracerebral hemorrhage

Supplemental Methods I
Adult male Sprague-Dawley rats were randomly divided into (1) an ICH group, in which ICH was induced by injection of collagenase VII; (2) an ethyl pyruvate (EP) group, in which ICH was induced and then the specific HMGB1 inhibitor EP was administered 3 days later; (3) an FPS-ZM1 group, in which ICH was induced and then the RAGE antagonist FPS-ZM1 was administered 3 days later; (4) a Sham group, which underwent the same procedures as the other groups, except that 0.9% saline solution was administered instead of collagenase VII and again 3 days later instead of EP or FPS-ZM1; and (5) Sham+EP and (6) Sham+FPS-ZM1 groups, which underwent the same procedures as the Sham group, except that EP or FPS-ZM1 was administered 3 days after mock induction of ICH. The rats that died of anesthesia were excluded (n=6). Moreover, the rats that had not any neurological deficits when waked up were excluded (n=7).

Supplemental Methods II
Induction of ICH
Rats in the ICH group were anesthetized with 3.6% chloral hydrate (1 ml/100 g; ZSGB, China) and placed in a stereotaxic frame. After disinfection and incision, a hole was drilled in the skull, and collagenase VII (5.0ul, Sigma C0773) was injected via trace syringe into the striatum at 0.2 mm anterior, 3.0 mm lateral, and 5.5 mm ventral from bregma. The syringe was held in position for 2 min to prevent backflow of collagenase, and slowly removed. A thermo-regulated heating pad was used to maintain epidermal temperature at 37 ºC.

Supplemental Methods III
Western blot analysis
Briefly, brain samples (n= 5 for each group) were homogenized at 4 ºC for 30 min in lysis buffer containing protease inhibitor cocktail (Roche, USA), ultrasonicated, and centrifuged at 13300 rpm at 4 ºC for 40 min. The supernatant was boiled for 10 min. Proteins (40ug) were separated by 10-12% SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were incubated with antibodies against the following proteins: HMGB1(1:1000 dilution; Abcam, USA), RAGE (1:1000; Abcam), TLR4 (1:500; Abcam), TLR2 (1:500; Abcam), brain-derived neurotrophic factor (BDNF) (1:500; Santa Cruz Biotechnology, USA), and matrix metalloproteinase (MMP)-9 (1:500; Santa Cruz). All membranes were simultaneously incubated with antibody against β-actin (1:1000; ZSGB, China) to provide an internal control. Membranes were washed 3 times for 10 min in TBST, then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37 ºC. Bands were visualized by enhanced chemiluminescence (Millipore), and signal intensity was
Supplemental Methods IV

Immunohistochemistry
Three rats from each group (n= 3 for each group) were anesthetized and subjected to intracardiac perfusion with 400 ml of 0.9% normal saline, followed by 300 ml of 4% paraformaldehyde. Brains were isolated and fixed overnight at 4 °C in 4% paraformaldehyde. Brain tissue was prepared, embedded in paraffin and cut into sections 16µm. Sections from around the hematoma in the ipsilateral striatum were incubated overnight at 4 °C with primary antibody against HMGB1 (1:200, Abcam; USA), doublecortin (DCX; 1:100; Abcam), or BrdU (1:200; Abcam). Then sections were incubated for 60 min at room temperature with anti-rabbit secondary antibody. Three rats were randomly selected from each group, their brains were removed and sectioned, and three sections containing ipsilateral striatum were randomly selected from each brain. Neurogenesis in the ICH border region was evaluated by counting the number of BrdU and DCX-positive cells. For multi-stage random sampling, four fields per brain section were randomly chosen in ICH border region under light microscope. These sections were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Supplemental Methods V

Behavioral Tests
A battery of behavioral tests (n= 11 for each group) was performed in order to calculate the modified neurological severity score (mNSS) \(^2,3\). Motor ability was tested by raising the animal by the tail and scoring his response on a 3-point scale: 1 point if he flexed his forelimb, 1 point if he flexed his hind limb, and 1 point if he moved his head >10° from the vertical axis within 30 sec (maximum score = 3 points). Walking ability was assessed by placing the animal on the floor and scoring his walk on a 3-point scale: 0, normal walk; 1, inability to walk straight; 2, circling toward the paretic side; 3, falling down on the paretic side.

Sensory ability was assessed using a placing test, which examined both visual and tactile responses, as well as a proprioceptive test to induce deep sensation, in which the paw was pushed against the table edge to stimulate limb muscles. One point was given for the inability to perform each test, for a total possible score of 2 points.

Balance was assessed using a beam balance test, which was graded on a 6-point scale: 0, balances with steady posture; 1, grasps side of beam; 2, hugs the beam and one limb falls down from the beam; 3, hugs the beam and two limbs fall down from the beam, or spins on the beam for >60 sec; 4, attempts to balance on the beam but falls off after 40 sec; 5, attempts to balance on the beam but falls off after 20 sec; 6, falls off after 20 sec without making any attempt to balance or hang on to the beam.

Animal reflexes and the presence of abnormal movements were assessed by testing
the pinna reflex, which examines whether the head shakes when the auditory meatus is touched; the corneal reflex, which tests whether the eye blinks when the cornea is lightly touched with cotton; and the startle reflex, which assesses motor response to a brief noise generated by snapping a clipboard. One point was given for the absence of each tested reflex. An additional point was given for the presence of seizures, myoclonus, or myodystony.

The total points from all these behavioral tests were summed for each animal (highest possible overall score = 18) and used to calculate mean ± SD scores for each group.

Supplemental Table I
Relative expression of selected proteins in brains of rats subjected to intracerebral hemorrhage (ICH) or mock ICH (Sham), followed by administration of HMGB1 inhibitor EP or RAGE antagonist FPS-ZM1.

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<thead>
<tr>
<th>Protein</th>
<th>Expression relative to β-actin (mean ± SD) in each experimental group</th>
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<tr>
<td>HMGB1</td>
<td>ICH 1.260±0.034&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; EP 0.842±0.037&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 1.464±0.035&lt;sup&gt;b&lt;/sup&gt; Sham 0.122±0.019 Sham+EP 0.116±0.011 Sham+FPS-ZM1 0.0117±0.009</td>
</tr>
<tr>
<td>RAGE</td>
<td>ICH 0.846±0.038&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; EP 0.532±0.032&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 ND Sham 0.128±0.019 Sham+EP 0.124±0.023 Sham+FPS-ZM1 0.127±0.018</td>
</tr>
<tr>
<td>TLR4</td>
<td>ICH 0.668±0.032&lt;sup&gt;b&lt;/sup&gt; EP 0.652±0.038&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 ND Sham 0.134±0.017 Sham+EP 0.126±0.018 Sham+FPS-ZM1 0.128±0.018</td>
</tr>
<tr>
<td>TLR2</td>
<td>ICH 0.238±0.026&lt;sup&gt;b&lt;/sup&gt; EP 0.218±0.013&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 ND Sham 0.062±0.013 Sham+EP 0.070±0.016 Sham+FPS-ZM1 0.064±0.011</td>
</tr>
<tr>
<td>BDNF</td>
<td>ICH 1.422±0.093&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; EP 0.878±0.056&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 0.904±0.037 Sham 0.196±0.030 Sham+EP 0.194±0.035 Sham+FPS-ZM1 0.200±0.016</td>
</tr>
<tr>
<td>MMP-9</td>
<td>ICH 0.840±0.042&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; EP 0.340±0.041&lt;sup&gt;b&lt;/sup&gt; FPS-ZM1 0.049±0.039 Sham 0.120±0.027 Sham+EP 0.124±0.027 Sham+FPS-ZM1 0.129±0.028</td>
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ND, not done

<sup>a</sup>p<0.001, ICH group vs EP and FPS-ZM1 groups
<sup>b</sup>p<0.001, ICH, EP, and FPS-ZM1 groups vs Sham, Sham+EP, and Sham+FPS-ZM1 groups
<sup>c</sup>p<0.001, ICH group vs FPS-ZM1 group
<sup>d</sup>p<0.05 Sham group vs Sham+EP and Sham+FPS-ZM1 groups.
**Supplemental Figures I:** (A) ICH induction caused neurological deficits from which the animals gradually recovered, based on a battery of behavioral tests used to determine a modified neurological severity score (mNSS). (B) Treatment with either EP or FPS-ZM1 at 3 days after ICH inhibited this recovery. Overall scores across all tasks are shown as mean ± SD (n=11 animals from each group). ※ p<0.001, ICH group vs EP and FPS-ZM1 groups; # p<0.001, ICH, EP, and FPS-ZM1 groups vs Sham, Sham+EP, and Sham+FPS-ZM1 groups.

**Supplemental References**