

# Class IIa HDAC Downregulation Contributes to Surgery-Induced Cognitive Impairment Through HMGB1-Mediated Inflammatory Response in the Hippocampi of Aged Mice

Chen-miao Huang<sup>1,2</sup>

Jia-jing Cai<sup>1,2</sup>

Shao-wu Jin<sup>1,2</sup>

Qi-cheng Lin<sup>1,2</sup>

Qian-juan Fang<sup>1,2</sup>

Ke Nan<sup>1,2</sup>

Yuan Han<sup>1,2</sup>

Wen-wei Ge<sup>1,2</sup>

Yu Liu<sup>1,2</sup>

Yuan-xiang Tao<sup>1,3</sup>

Hong Cao<sup>1,2</sup>

Jun Li<sup>1,2</sup>

<sup>1</sup>Department of Anesthesiology and Perioperative Medicine, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325027, People's Republic of China; <sup>2</sup>Zhejiang Province Key Laboratory of Anesthesiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325027, People's Republic of China; <sup>3</sup>Department of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ, 07103, USA

**Objective:** Perioperative neurocognitive disorders (PND) are a common complication in the elderly. Histone deacetylases (HDACs) are a class of enzymes that control the acetylation status of intracellular proteins. Thus, we explored whether HDACs trigger the release of high mobility group box 1 (HMGB1) through altering the acetylation status in the hippocampi of aged mice.

**Materials and Methods:** The effect of the Class IIa HDAC in PND was explored using an in vivo form of splenectomy. Sixteen-month-old healthy male C57BL/6J mice were randomly divided into five groups: control, anesthesia plus sham surgery, anesthesia plus splenectomy, LMK235 treatment, and PBS treatment. The hippocampi were harvested on either first, third, or seventh postoperative day. Cognitive function was assessed via a Morris water maze (MWM) test. Quantitative RT-PCR, Western blots and ELISAs were carried out to assess the targeted gene expression at transcriptional and translational levels.

**Results:** Splenectomy led to a significant deficiency in spatial memory acquisition, marked decreases in mRNA and protein levels of HDAC4 and HDAC5 in the hippocampus, and increases in the levels of total HMGB1 and acetylated HMGB1. In a similar fashion to splenectomy, treatment with the HDAC4/5 inhibitor LMK235 produced impaired spatial memory and an increase in the expression of HMGB1 and its acetylated counterpart in the hippocampus.

**Conclusion:** These results suggest that surgery leads to PND through class IIa HDAC downregulation-triggered HMGB1 release in hippocampus of aged mice. HDACs may be a potential therapeutic target for postoperative cognitive dysfunction.

**Keywords:** postoperative cognitive dysfunction, hippocampus, HDAC4, HDAC5, HMGB1, neuroinflammation

## Introduction

Postoperative neurocognitive dysfunction (PND) is characterized by a deficit in memory and cognitive function following surgery or anesthesia.<sup>1</sup> PND can manifest through the impairment of memory, attention, and information processing and can last for several days to several years.<sup>41</sup> Risk factors for PND include advancing age, low level of education, duration of anesthesia, multiple surgeries, and postoperative infections. Additionally, age is also a vital risk factor for PND.<sup>42</sup> The incidence of PND varies widely in previous reports, possibly due to differences in methodology.

Correspondence: Jun Li; Hong Cao  
Department of Anesthesiology and Perioperative Medicine, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325027, People's Republic of China  
Tel/Fax +86 577-88002925  
Email lijun0068@163.com;  
caohong1955@21cn.com

The International Study of PND (ISPND) reported that 25.8% of the patients older than 60 years suffered PND at 7 days after surgery and 9.9% still had PND at 3 months.<sup>2</sup>

Until recently, researchers have attempted to investigate the pathophysiology of PND, but the mechanisms underlying pathogenesis remain unclear. Neuroinflammation triggered by surgical trauma may be a key component in the pathogenesis of PND. Reports have demonstrated that peripheral surgery could impact processes in the central nervous system.<sup>3,4</sup> An aseptic injury (such as sterile surgery, where the damage of tissue was caused by mechanical injury) or a non-aseptic injury (such as bacterial or viral invasion) can cause peripheral inflammation.<sup>5</sup> Tumor necrosis factor alpha (TNF- $\alpha$ ), Interleukin-1 $\beta$  (IL-1 $\beta$ ) and other cytokines derived from peripheral inflammation have adverse effects on the integrity of the blood-brain barrier, allowing it to become permeable and permitting the entry of proinflammatory factors into the brain and cause neuroinflammation.<sup>1</sup>

High mobility group box 1 (HMGB1), a highly conserved protein found in eukaryotic nuclei, is described as a nuclear DNA-binding protein and a pathogenic inflammatory mediator associated with sepsis, stroke, and other disease syndromes.<sup>6</sup> A previous study revealed that the level of HMGB1 was increased significantly after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (CRS-HIPEC) in adults, accompanied by the development of perioperative short-term cognitive dysfunction.<sup>7</sup> Administration of neutralizing anti-HMGB1 monoclonal antibodies or glycyrrhizin significantly reduced the severity of neuroinflammation and improved postoperative cognitive decline.<sup>8,9</sup> HMGB1 can shuttle between the nucleus and cytoplasm and be released into the extracellular space through a passive process (where HMGB1 is released from damaged cells following sterile insult) or an active process (after acetylation of its lysine residues).<sup>10,11</sup> These mechanisms can cause the release of a large amount of extracellular HMGB1, with the active process contributing to the pathogenesis of both infection and injury, and can activate the pattern-recognition receptors for Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE).<sup>12,13</sup>

Histone deacetylases (HDACs) are a family of enzymes that act synergistically with histone acetyltransferases to remove acetyl groups from lysine residues. Human HDAC enzymes are divided into four major classes: Class I HDAC (including HDAC1, 2, 3 and 8);

Class II HDAC (including HDAC4, 5, 6, 7, 9 and 10); Class III HDAC (alternatively named Sirtuins; including SIRT1 to SIRT7); and Class IV HDAC (the only member is HDAC11).<sup>14</sup> Class III HDAC also have a nicotinamide adenine dinucleotide (NAD<sup>+</sup>) dependence. Accumulating evidence shows that pan-HDAC inhibitors exhibit complicated properties in animal models of various neurological diseases.<sup>15</sup> Recent results demonstrated that HDAC inhibition promoted HMGB1 hyperacetylation and subsequently its extracellular release in liver ischemia/reperfusion (I/R) injury.<sup>16,17</sup> However, the role of individual Class IIa HDACs (HDAC4 and HDAC5) in PND remains unclear. The aim of the present study was to examine whether surgery contributed to neuroinflammation-induced PND through Class IIa HDAC downregulation-triggered HMGB1 in the hippocampi of aged mice.

## Materials and Methods

### Animals

In view of the neuroprotective effect of estrogen, we chose male mice as the experimental subjects to avoid the interference of estrogen to the experiment.<sup>43</sup> Sixteen-month C57-BL6 male mice (body weight 20–30 g, the total sample size is about 120) were purchased from the Animal Use Committee of Wenzhou Medical University and were grouped housed in a standard environment (temperature 24  $\pm$  1 °C, a 12 h light-dark cycle, and 50%  $\pm$  10% humidity) with access to food and water ad libitum. Animals were acclimated to the environment for 7 days prior to the experiment. All experimental operations were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (protocol number: SYXK2015-0009), which operates according to the recommendations for the care and use of experimental mice. The mice were randomly divided into five major groups (n = 24/group): 1) control, in which the mice do not undergo anesthesia and surgery; 2) anesthesia, in which mice were only anesthetized with 1% pentobarbital solution (7 mL/kg, i.p.); 3) anesthesia plus splenectomy; 4) treatment with LMK235 only and (5) treatment with phosphate-buffered saline (PBS, a vehicle solution). Mice were sacrificed on days 1, 3, and 7 after intervention. Each major group contains three subgroups (n=8/subgroup) according to the intervention time (1 day after intervention; 3 days after intervention; 7 days after intervention).

## Surgical Procedures

For splenectomy, a midline incision was performed, and the two primary sources of blood flow (splenic artery and gastric artery) were tied off with sterile suture proximal to the spleen. The spleen was then isolated and removed. Lincomycin lidocaine gel was applied to the wound every 8 hours within 24 hours after surgery.

## Drugs Application

Ten milligrams/kilogram of LMK235 (an HDAC4/HDAC5 inhibitor, dissolved in PBS) was administered intraperitoneally. PBS served as a control. The dosage and route of LMK235 administration were based on previous studies and slightly adjusted according the experimental condition, this method could increase histone acetylation in hippocampal neurons.<sup>44</sup>

## Morris Water Maze (MWM) Test

The MWM test was used to evaluate spatial learning and memory through a computerized video track system (Xinruan Information Technology Co., Ltd., shanghai). Briefly, a nonvisible round platform (10-cm diameter) was placed 1 cm below the water surface located in the center of the target quadrant (SW quadrant) of a circular pool. The mice were placed on the platform for 30 seconds preceding the start of each trial and was then released into the water facing the tank wall in one of the four present stating locations. Mice were trained for five consecutive days with four trials per day (60 seconds maximum for a trial). The mice were given 60 seconds to locate the platform, otherwise they were gently guided to the platform and remained for 15 seconds. On day 6, animals underwent surgery, general anesthesia, or LMK235 treatment. On post-treatment days 1, 3, and 7, mice were subjected to a probe test where they were released into the water in the quadrant opposite to the target quadrant. The time to locate the platform (latency), the percentage of time spent in the target quadrant and the numbers of platform crossings were recorded.

## Open Field Trial

On days 1, 3, and 7 following treatment, the locomotor activity was tested with the open field, prior to this, all mice had been subjected to a probe test in MWM test. Briefly, all mice were adapted to the environment for 1.5 hours prior to the trial. The mouse was placed directly into the center of the open field (dimensions of 50 × 50 cm)

and a computerized video track system (Logitech Suzhou, China) recorded total movement distance of the mice in the box, the mice were monitored for 10 minutes. The total traveled distance and mean speed were measured as the parameter of locomotor activity.

## Cytokine Analysis

On days 1, 3, and 7 following treatment, the blood of four mice in each subgroup was collected transcardially, and then the hippocampus of the four mice were isolated. We divided the hippocampus of a mice into two parts, which are used to extract protein and RNA, respectively. Other four mice in each subgroup were used for immunofluorescence. Blood samples were centrifuged at 2500 g for 25 minutes and the plasma was collected. After the plasma was stand for 2 hours, the serum is extracted to measure the content of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 with the use of ELISA kits (Haixi Tang Biotechnology Co., Ltd., Shanghai, China). Blood samples from mice without intervention served as controls, and samples were processed as described above.

## Protein Extraction and Western Blot Analysis

Mice hippocampi were homogenized in cold RIPA buffer and centrifuged at 4 °C at 12,000 g for 30 minutes. A BCA protein assay kit (Thermo Fisher, USA) determined the concentration of protein in the supernatants. Protein samples of 35  $\mu$ g were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. A TBS buffer of 10% non-fat dried milk blocked membranes for 2 hours and incubated with primary antibodies: rabbit anti-HMGB1 monoclonal (1:3000; ab-18256; Abcam, Inc., MA, USA), rabbit anti-RAGE polyclonal (1:1000; DF6618; Affinity, UK), rabbit anti-TNF- $\alpha$  polyclonal (1:1000; sc-12744; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-IL-1 $\beta$  polyclonal (1:1000; sc-7884; Santa Cruz Biotechnology Inc.), rabbit anti-HDAC4 (1:2000; ab-79521; Abcam, Cambridge, UK), rabbit anti-HDAC5 polyclonal (1:2000; sc-133225; Santa Cruz Biotechnology Inc.), rabbit anti-TLR4 polyclonal (1:1000; sc-293072; Santa Cruz Biotechnology Inc.), and mouse anti-MyD88 monoclonal antibody (1:1000; sc-74532; Santa Cruz Biotechnology Inc.) overnight at 4 °C. The anti- $\beta$  actin (1:3000; TA-09; Beijing Zhongshan Jinqiao Biotechnology) was used as a loading control.

Membranes were incubated with goat anti-mouse (1:3000; Biosharp) or goat anti-rabbit (1:3000; Biosharp) secondary antibodies at room temperature for 2 hours. Immune complexes were assessed with peroxidase and an enhanced chemiluminescence system (ECL; Thermo Fisher Scientific); band intensity was quantified with densitometry.

## Co-Immunoprecipitation

Co-immunoprecipitation was performed as previously described.<sup>45</sup> Immunoprecipitation was performed to investigate the status of acetylated HMGB1 in hippocampus tissue. A total of 2.5 mg rabbit monoclonal anti-HMGB1 (ab18256; Abcam, Cambridge, UK) or rabbit IgG (Beyotime, China) was added to 500 mg of hippocampal lysates with gentle rotation for 1.5h at 4 °C. After the addition of 20 µL of Protein A/G Plus beads (Santa Cruz Biotechnology, SC-2003), the samples were incubated at 4 °C overnight with gentle rotation. Pellet beads were centrifuged at 2500 rpm for 5 minutes and the pellets were washed four times with PBS. Finally, the samples were resolved on SDS-PAGE and analyzed by Western Blot with anti-acetyl-lysine antibody (1:500; sc-32268; Santa Cruz Biotechnology Inc.).

## Quantitative PCR (RT-qPCR)

The hippocampus tissue samples were homogenized with TRIzol reagent, the total RNA was extracted, and cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). HDAC1, 2, 4, 5, 11 were amplified with qPCR using SYBR-Green qPCR Master mix (TOYOBO, Japan). The PCR amplification consisted of a 1 min denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C and 20 s at 60 °C on an iCycler real-time PCR detection system (Bio-Rad Laboratories, CA, USA). Primer sequences and amplification sizes are presented in Table 1.  $\beta$ -Actin was used as an internal reference to determine the relative expression levels of mRNA using the  $2^{-\Delta\Delta Ct}$  method. All samples were analyzed in triplicate.

## Brain Tissue Preparation and Double Immunofluorescent Staining

Mice were perfused with normal saline followed by a fixative reagent containing 4% paraformaldehyde in 0.1 M PBS; brain samples were harvested and protected in sucrose solution (30%) overnight and embedded in

**Table 1** The Primers Used for qPCR Analysis

Name		Sequences (5'→3')
HDAC1	Forward primer	TTCTATACTACAGACCGGGTCA
	Reverse primer	TGGACATGACTGGCTTAAAGAT
HDAC2	Forward primer	TGATGGAGATGTACCAGCCTAGCG
	Reverse primer	ACCGAGCATCAGCAATGGCAAG
HDAC4	Forward primer	GACACACTGATGTTGAAGCATC
	Reverse primer	CAGTTTCTGTCTGTTGAGAGGA
HDAC5	Forward primer	CAAAAAGTTGTTCCGAGATGC
	Reverse primer	ACTTGAGGAGTGCATCTCATT
HDAC11	Forward primer	GGCGGAAGGTGGAATTGGAGTG
	Reverse primer	CGTCCGTGCCAGCGTTGTAC

optimal cutting temperature compound (Solarbio, China) at  $-80^{\circ}\text{C}$ . Then, 4- $\mu\text{m}$ -thick coronal sections from of the brains were made in a cryostat, we sliced the entire brain but just focused on the hippocampus in this experiment. Sections were blocked in 5% BSA for 1.5 hours at room temperature and incubated in primary antibodies at 4 °C overnight. For double immunofluorescent staining, rabbit anti-NeuN (1:200; ab177487; Abcam) and mouse anti-NeuN (1:200; Millipore; MAB377) for neurons, rabbit anti-IBA1 (1:50; 10904-1-AP; Proteintech) and mouse anti-IBA1 (1:200; GT10312; GeneTex) for microglia, mouse anti-GFAP (1:200; SC-33637; Santa Cruz Biotechnology) and rabbit anti-GFAP (1:200; ab33922; Abcam) for astrocytes were used as specific cellular markers. The sections were then incubated with Alexa Fluor 488 (1:200) and Alexa Fluor 594 (1:200; Invitrogen) for 1.5 hours at 37 °C. The staining was examined with a fluorescence microscope (OLYMPUS, BX53 MicroPublisherTM 5.0 RTV, Japan).

## Statistical Analysis

All data were presented as the means  $\pm$  SEM. The Western blot band densities were quantified by Quantity one software (Bio-Rad, CA, USA). The results were analyzed by one-way ANOVA, after ANOVA showed a significant difference, pairwise comparisons between mean were tested by Bonferroni multiple comparison. A value of  $p < 0.05$  was considered to manifest a statistically significant difference. All statistical tests were performed using SPSS software 19.0 (IBM, Armonk, NJ, USA). The results were presented using GraphPad Prism software 7.0 (GraphPad, San Diego, CA, USA).

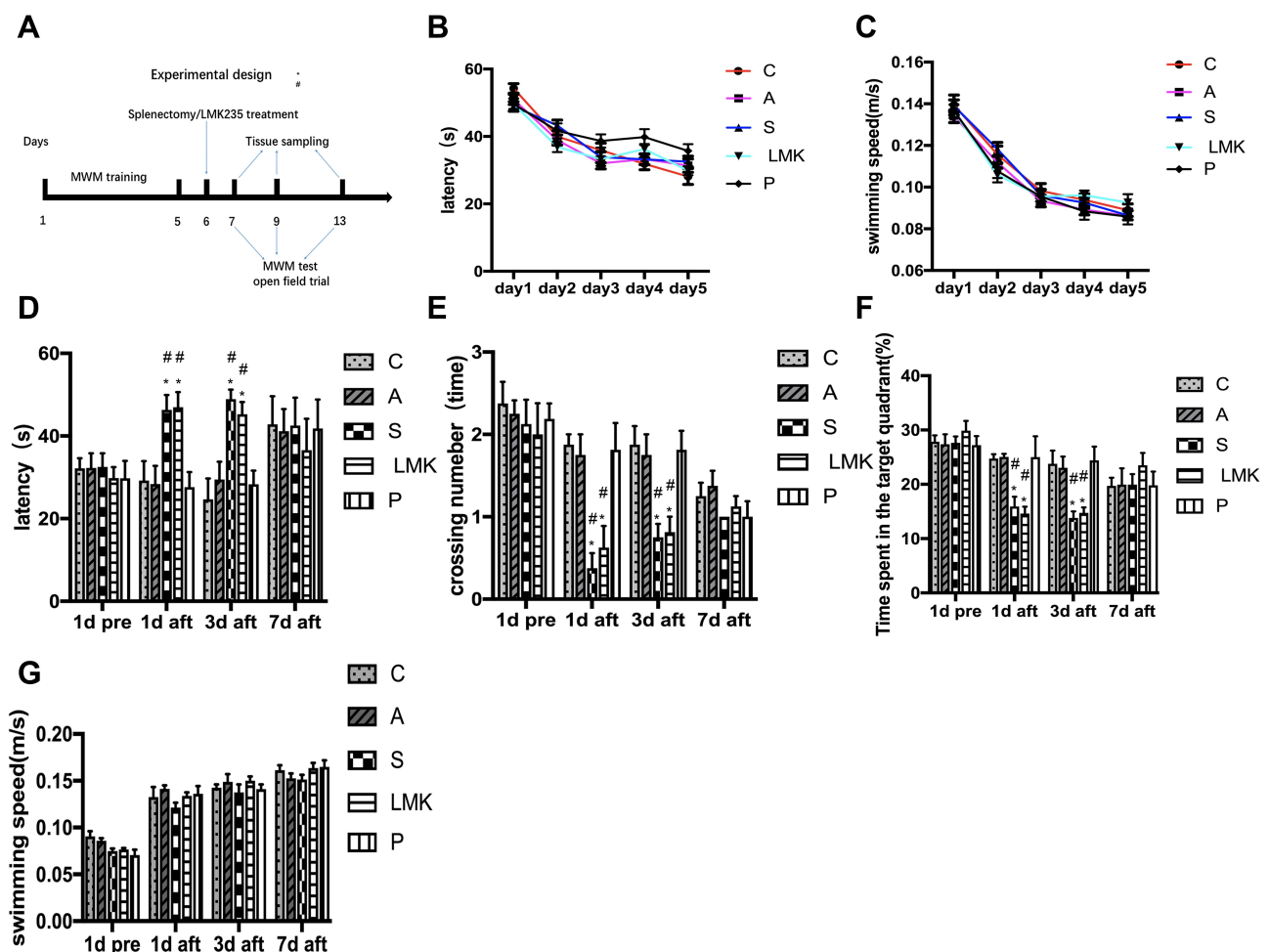
## Results

### Splenectomy Induced Spatial Memory Impairment without Alteration of Locomotor Activity

To investigate the effects of splenectomy on cognitive function, we tested mice in the Morris Water Maze, which assesses spatial working memory. Comparisons by one-way ANOVA showed no significant difference either in latency or in swimming speeds among the groups on every training day before the surgery (Figure 1B and C,  $p > 0.05$ ). The result indicated that all mice showed the same improvement in spatial learning and memory over time. The spatial probe test was conducted postoperatively. Compared with the control group, the latency for the first time to the

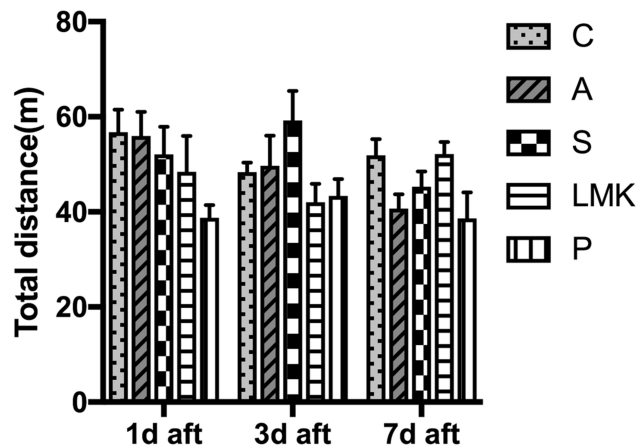
platform increased in the splenectomy group (Figure 1D, 1d aft:  $F_{(4,35)}=6.191$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,35)}=8.731$ ,  $P < 0.05$ ). The time spent in the target quadrant (the third quadrant) (Figure 1F, 1d aft:  $F_{(4,35)}=7.154$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,35)}=6.927$ ,  $P < 0.05$ ) and the number of platform crossings (Figure 1E, 1d aft:  $F_{(4,35)}=9.161$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,35)}=7.026$ ,  $P < 0.05$ ) decreased in the splenectomy group at days 1 and 3 after surgery. The results of the MWM revealed no differences between the control group and the anesthesia group in the aforementioned indices (Figure 1B–F,  $p > 0.05$ ).

In the open field trial, there was no difference in the total distance traveled by the groups in the arena (Figure 2,  $p > 0.05$ ), indicating that the observed cognitive deficits in the MWM were not caused by reduced spontaneous locomotor activity. Comparisons by one-way ANOVA showed no



**Figure 1** Effect of surgery and LMK235 treatment on spatial learning in the Morris Water Maze. **(A)** Experimental design. **(B)** Mean escape latency of the five major groups in the acquisition phase,  $n = 24$  per group. **(C)** Average swimming speed of the major groups in the acquisition phase,  $n = 24$  per group. **(D)** Latency for the first time get to the platform of each group at 1 d before surgery and 1, 3, and 7 d after surgery in the probe trials,  $n=8$  per group. **(E)** The number of platform crossings with a 60 s limit of each group at 1 d before surgery and 1, 3, and 7 d after surgery in the probe trials,  $n = 8$  per group. **(F)** Time spent in the target quadrant of each group at 1 d before surgery and 1, 3, and 7 d after surgery in the probe trials,  $n = 8$  per group. **(G)** Average swimming speed of each group at 1 d before surgery and 1, 3, and 7 d after intervention in the probe trials,  $n=8$  per group. All data are expressed as the means  $\pm$  SEM. # $p < 0.05$  versus the day-matched control group. \* $p < 0.05$  versus the day-matched anesthesia group.

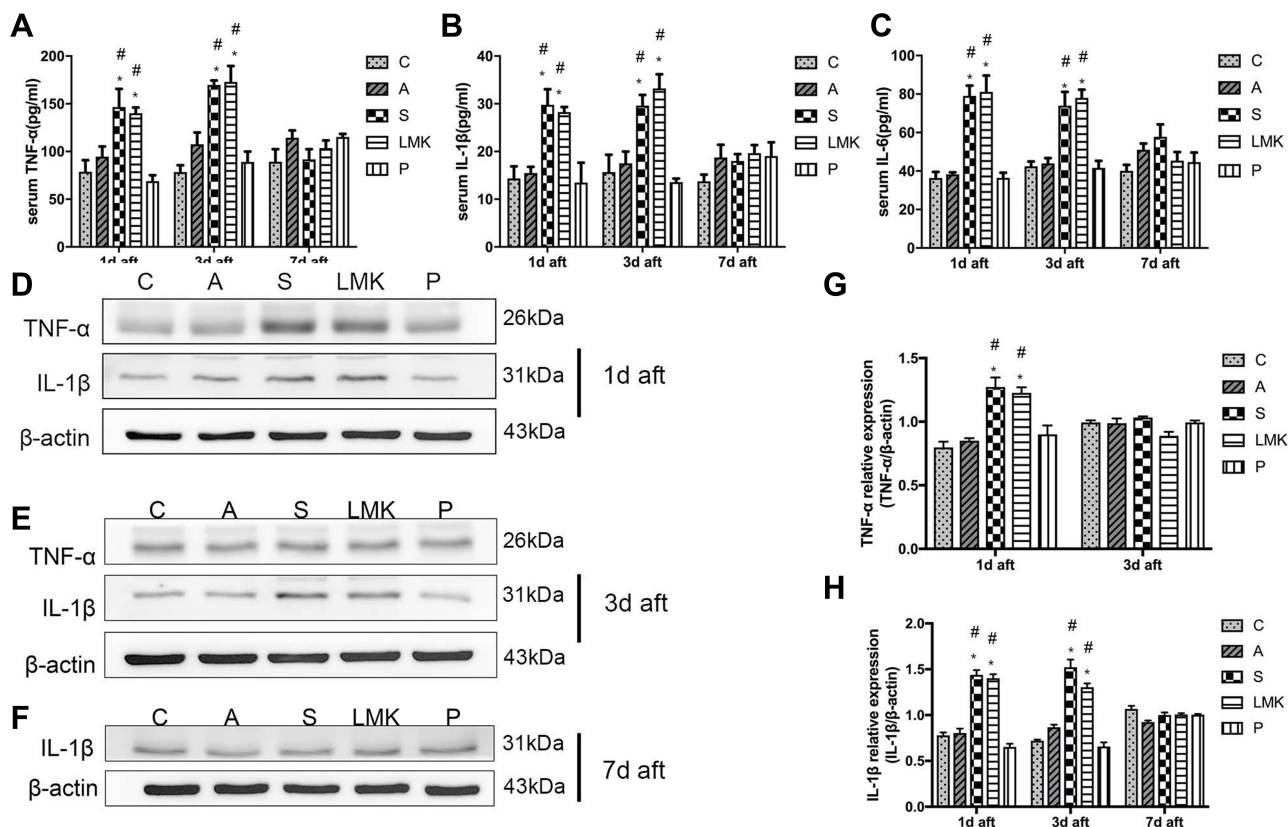
significant difference in swimming speeds among the groups after the surgery on the corresponding date (Figure 1G,  $p > 0.05$ ).



**Figure 2** Motor dysfunctions was not evident in each group in open field. Total distance travelled of each group during monitoring in an open field box,  $n = 8$  per group.

## Early Inflammatory Response Following Surgery

Evidence for peripheral inflammation and neuroinflammation was seen at postoperative day 1 and day 3, as demonstrated by the increase in the levels of proinflammatory cytokines in the plasma and the hippocampal tissues of the splenectomy groups compared with the control and anesthesia groups (Figure 3A: 1d aft:  $F_{(4,15)}=10.552$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=16.900$ ,  $P < 0.05$ ; Figure 3B 1d aft:  $F_{(4,15)}=8.679$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=11.695$ ,  $P < 0.05$  Figure 3C, 1d aft:  $F_{(4,15)}=23.226$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=17.382$ ,  $P < 0.05$ ). Representative Western blots of TNF- $\alpha$  and IL-1 $\beta$  on postoperative days 1, 3, and 7,  $n = 4$  per group. Each sample repeated the experiment 3 times. (Figure 3D–F) Analysis of Western blots showed that the expression levels of IL-1 $\beta$  and TNF- $\alpha$  were increased in the hippocampi of the splenectomy groups. Expression of TNF- $\alpha$  only increased significantly on postoperative day 1, while expression of IL-1 $\beta$  decreased on day 7 (Figure 3G 1d aft:  $F_{(4,15)}=16.549$ ,  $P < 0.05$ , Figure 3H 1d aft:  $F_{(4,15)}=75.519$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=61.744$ ,  $P < 0.05$ ).

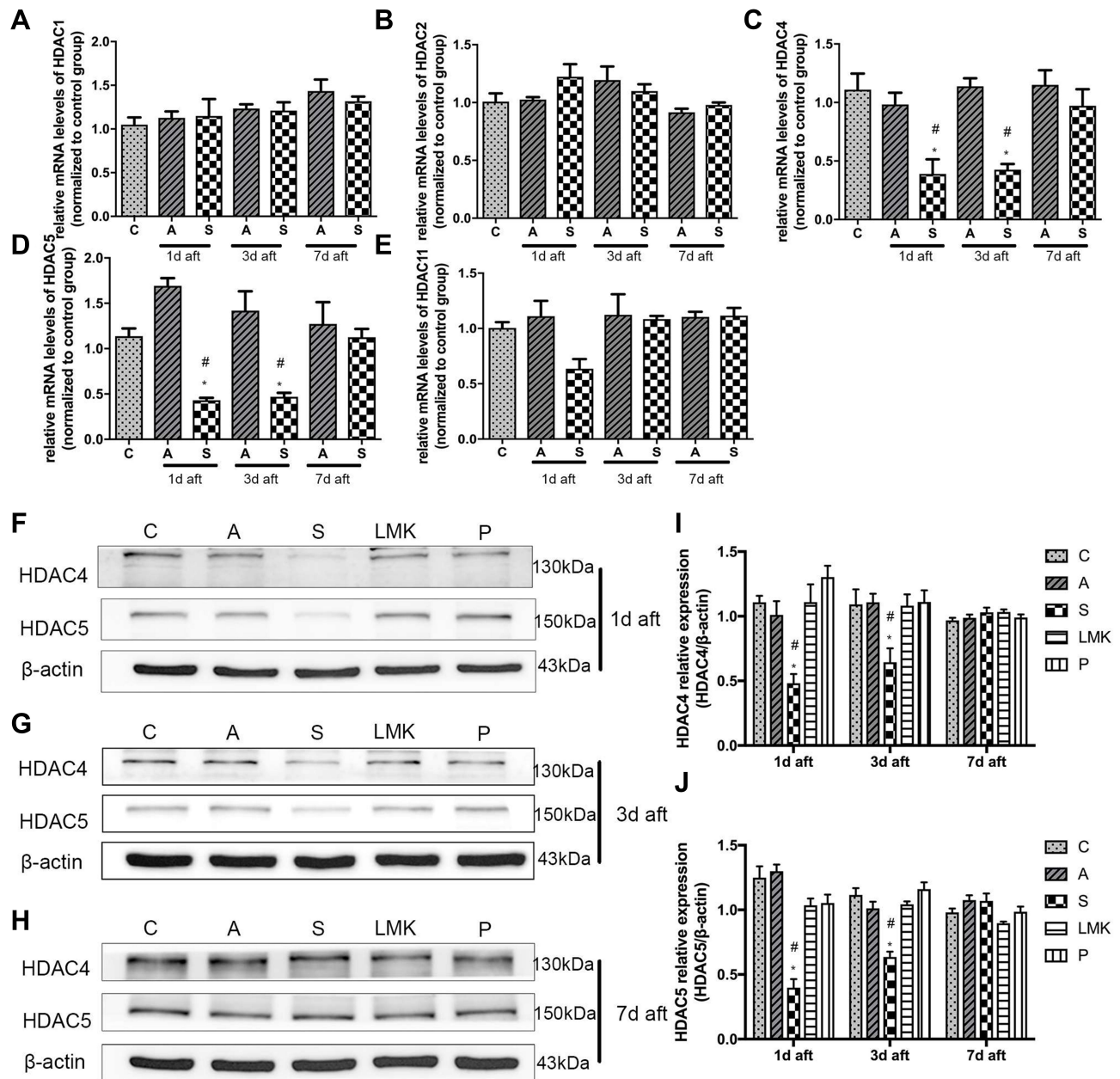


**Figure 3** Early peripheral-inflammatory response and neuroinflammation following surgery or LMK235 treatment. (A–C) The serum level of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  on postoperative day 1, 3, and 7 were measured by ELISA,  $n = 4$  per group. Each sample repeated the experiment 3 times. (D–F) Representative Western blots of TNF- $\alpha$  and IL-1 $\beta$  on postoperative days 1, 3, and 7,  $n = 4$  per group. Each sample repeated the experiment 3 times. (G, H) Densitometric analysis of Western blot data from D–F,  $n = 4$  per group. # $p < 0.05$  versus the day-matched control group. \* $p < 0.05$  versus the day-matched anesthesia group.

## Expression Patterns of HDACs in the Hippocampi of Mice That Developed Postoperative Cognitive Disorders

To determine the expression patterns of the HDAC family in brains with postoperative cognitive dysfunction, RT-qPCR and Western blot analyses were performed. Among  $Zn^{2+}$  dependent HDACs, the expression of Class IIa HDACs were significantly decreased at postoperative day 1 and day 3 in surgery group compared with the anesthesia

group and naive control (Figure 4A–E, C:  $F_{(6,21)}=8.885$ ,  $P < 0.05$  Figure 4D:  $F_{(6,21)}=12.109$ ,  $P < 0.05$ ). The levels of HDAC1, 2, and 11 had no significant difference at this experimental condition. The protein expression level of HDAC4 and HDAC5 were also significantly decreased at postoperative day 1 and day 3 in the hippocampus (Figure 4F–J, I: 1d aft:  $F_{(4,15)}=10.582$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=4.556$ ,  $P < 0.05$  Figure 4J: 1d aft:  $F_{(4,15)}=30.298$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}=20.684$ ,  $P < 0.05$ ).



**Figure 4** Expression patterns of HDACs in hippocampi from mice that developed postoperative cognitive disorders. (A–E) RT-qPCR analysis of mRNA levels of HDAC1, 2, 4, 5, and 11 from total RNA extracted from the hippocampi of each group,  $n = 4$  per group. Each sample repeated the experiment 3 times. (F–H) Representative Western blots of HDAC4 and HDAC5 on postoperative days 1, 3, and 7,  $n = 4$  per group. Each sample repeated the experiment 3 times. (I, J) Densitometric analysis of Western blot data from (F–H),  $n=4$  per group. # $p < 0.05$  versus the day-matched control group. \* $p < 0.05$  versus the day-matched anesthesia group.

We performed dual immunofluorescent staining in the hippocampus from group C to identify the cell types in which HDAC4 and HDAC5 were expressed. Surprisingly, HDAC4 and HDAC5 were predominantly expressed in neurons and there was no colocalization of HDAC4 (Figure 5) or HDAC5 (Figure 5) in astrocytes or microglia. In conclusion, these results suggest that the neuron is a major source of HDAC4 and HDAC5 expression in the hippocampus.

## Splenectomy Induced Acetylation of HMGB1 in the Hippocampus

Anesthesia did not lead to the upregulation of HMGB1 expression at any time point compared with the control group (Figure 6A–C). However, compared with the control group, the expression of HMGB1 increased significantly in the surgery group at postoperative days 1 and 3, reached a peak after day 1, and largely decreased by 7 days after surgery (Figure 6D, 1d aft:  $F_{(4,15)}=12.789$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}= 22.248$ ,  $P < 0.05$ ). We performed fluorescent immunostaining on the hippocampus of group S on the first day after surgery to assess the cellular location of HMGB1. As expected, the surgery induced robust release of HMGB1 (Figure 6F). Recently, research revealed that active secretion of HMGB1 from the cytoplasm into the extracellular space requires post-translational modification such as acetylation.<sup>18</sup> In order to assess the acetylation status of released HMGB1, the immunoprecipitation was performed in group S on the first day after surgery. We found that the majority of released HMGB1 was acetylated (Figure 6E), which indicates that the HMGB1 liberation may be HDAC related.

## Predominant Neuronal Expression and Release of HMGB1 in the Hippocampus

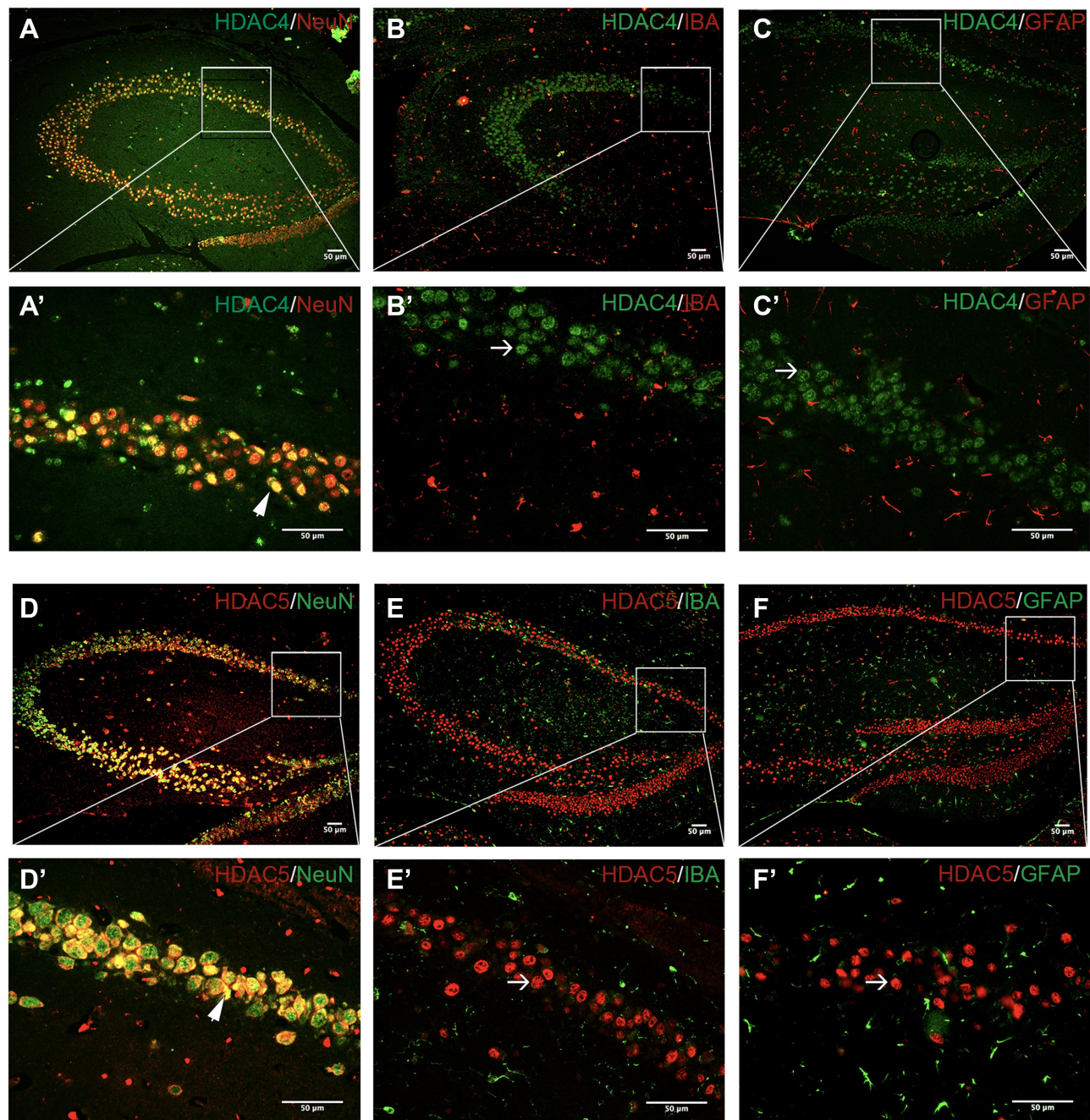
To investigate the cell-type expression of HMGB1 in the hippocampus, we performed double immunofluorescent staining in group C. Interestingly, we found that HMGB1 colocalized mainly with neurons and rarely with Iba-1 +microglia or GFAP (Figure 7). These results suggest that HMGB1 is highly expressed in hippocampal neurons. This data suggests that the predominant neuronal source of HMGB1 in this model and HMGB1 is the active performers of neuroinflammation in neurons.

## The HMGB1 Induces Proinflammatory Cytokine Expression Through TLR4/RAGE Activation

Previous research has found that the oral pretreatment of glycyrrhizin (a natural inhibitor of HMGB1) reduced post-operative neuroinflammation<sup>9</sup> and suggested that HMGB1 may function to prime the neuroinflammatory response. However, we do not know if the HMGB1-mediated inflammatory effect in vivo is partly achieved by modulating TLR expression and activation. Therefore, we analyzed the expression of TLR4 in vivo following splenectomy. Our data showed that the expression levels of TLR4 in the hippocampi of the splenectomy groups were increased on postoperative day 1 (Figure 8A) and day 3 (Figure 8B) compared with the control and anesthesia groups (Figure 8D 1d aft:  $F_{(4,15)}=61.986$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}= 32.588$ ,  $P < 0.05$ ). We next analyzed the expression level of the TLR4 adaptor and signaling protein, MyD88, and found the same degree of increase (Figure 8A–C, E 1d aft:  $F_{(4,15)}=20.455$ ,  $P < 0.05$ ; 3d aft:  $F_{(4,15)}= 7.665$ ,  $P < 0.05$ ). Splenectomy also resulted in increased RAGE levels in hippocampus postoperative day 1 (Figure 8A, B, F 1d aft:  $F_{(4,15)}=10.157$ ,  $P < 0.05$ ). Our findings show that HMGB1 causes neuroinflammation in a manner that depends on TLR4 and RAGE.

## Exogenous Administration of LMK235 Triggers PND-Like Features

We evaluated the role of HDAC-HMGB1 signaling in PND by administering LMK235, which is a specific Class II HDAC inhibitor. We investigated whether LMK235 treatment caused spatial memory impairment. First, compared with the naive control, the latency for the first time to the platform increased in LMK235 treatment group (Figure 1D,  $P < 0.05$ ). The time spent in the target quadrant (Figure 1F,  $P < 0.05$ ) and the number of platform crossings (Figure 1E,  $P < 0.05$ ) also decreased on the first and third days after administration. On the seventh day after administration, these parameters returned to normal (Figure 1D–F, compared with group C,  $P < 0.05$ ). Furthermore, LMK235 treatment caused both peripheral inflammation and neuroinflammation. The levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 were increased in both the serum and hippocampus, and largely decreased at 7 days after administration (Figure 3A–H). The administration also led to the upregulation of acetylated HMGB1 expression in the hippocampus (Figure 6E), which resulted in increased expression of TLR4, MyD88, and RAGE (Figure 8). Overall, the results



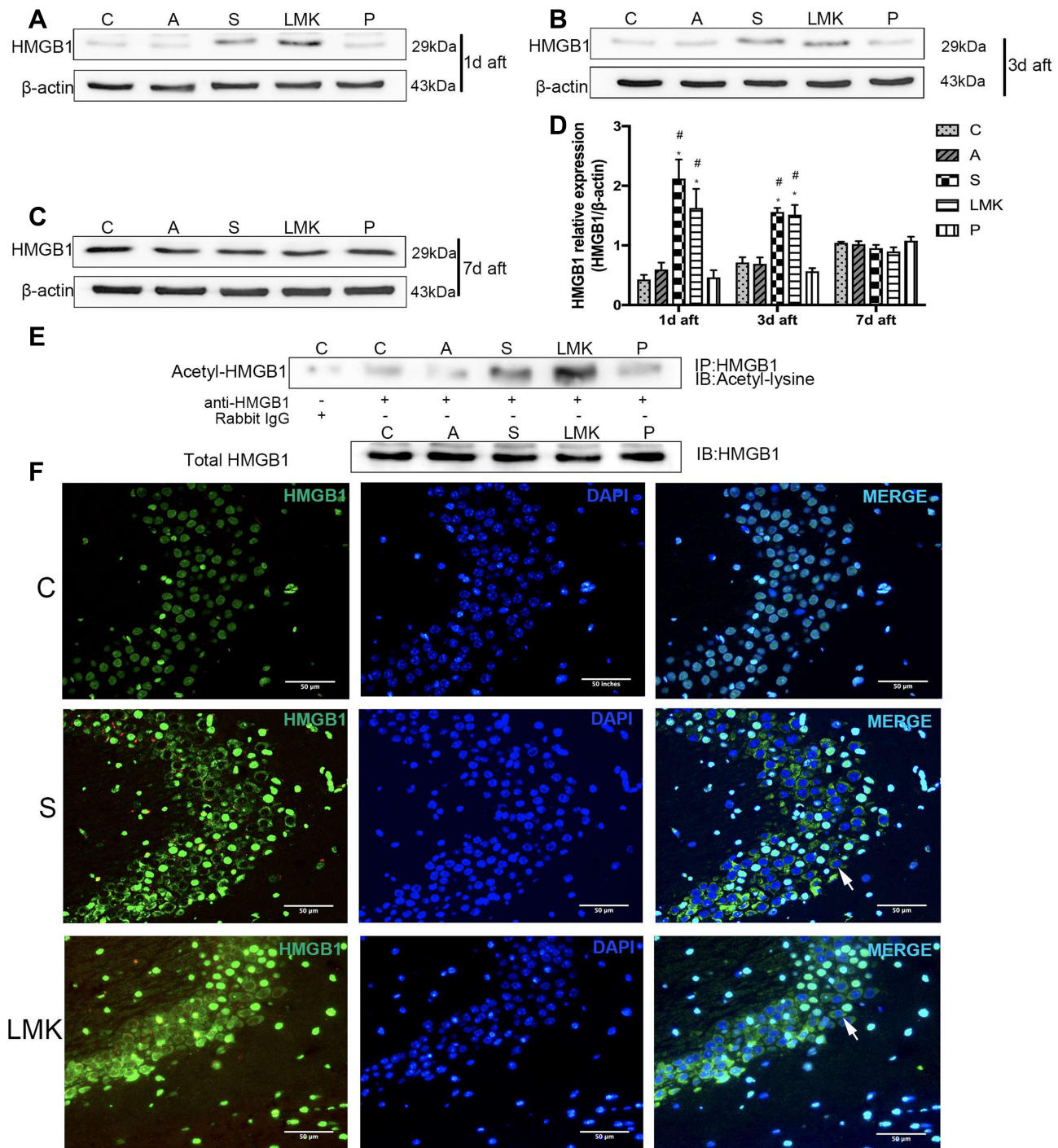
**Figure 5** Neurons are a major source of HDAC4 and HDAC5 expression in the hippocampus. Colocalization of HDAC4 with NeuN (part A), IBA (part B), and GFAP (part C). Colocalization of HDAC5 with NeuN (part D), IBA (part E), and GFAP (part F). The colocalization is in yellow in the image. The arrowhead indicates double-labelled cells (part A'; part D'), the arrow shows the protein of interest that is not colocalized with the cell (part B'; part C'; part E'; part F'). The magnified image of the regions is marked with boxes. Scale bar = 100 μm.

suggest that LMK235 treatment mimics the surgery-induced prolonged inflammation response and cognitive impairment.

## Discussion

HDACs are a family of enzymes involved in determining the post-translational acetylation status of non-histone proteins. The Class IIa HDACs (HDAC4, HDAC5) have been

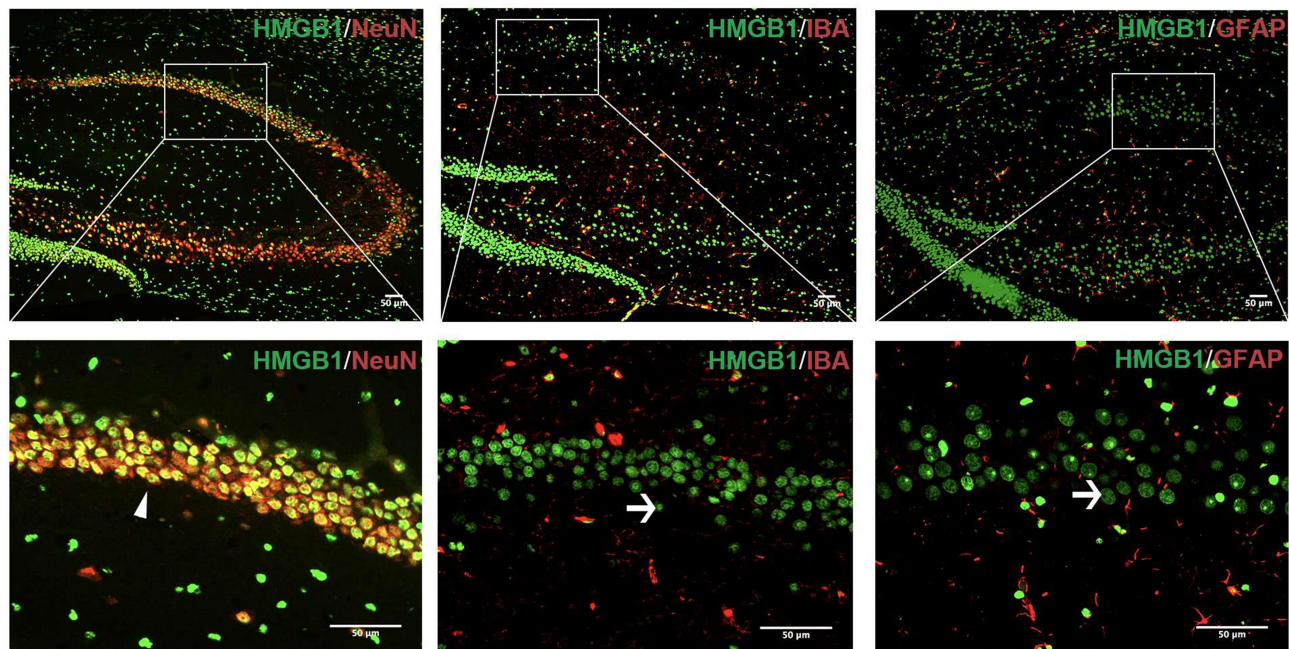
found to have a positive effect on the nervous system. Recent data suggest that mice generated with a conditional brain-specific HDAC4 deletion show alterations in motor coordination, accompanied by significant deficits in learning tasks.<sup>19</sup> HDAC5 is equally necessary for memory consolidation in mice, and attenuation of HDAC5 does not improve cognition in a mouse model



**Figure 6** Splenectomy-induced acetylation of HMGB1 expression in the hippocampus. (A–C) Representative Western blots of HMGB1 postoperative day 1, 3, and 7, n = 4 per group. (D) Densitometric analysis of Western blot data from (A–C), n = 4 per group. Each sample repeated the experiment 3 times. (E) Co-immunoprecipitation analysis of hippocampus tissue for acetylated HMGB1 level. Anti-rabbit IgG was used as a negative control. (F) Representative images of double immunofluorescent staining with HMGB1 (green) and DAPI (blue); the arrow indicates the nuclear translocation and release of HMGB1. Scale bar=100 mm. #p < 0.05 versus the day-matched control group. \*p < 0.05 versus the day-matched anesthesia group.

of AD.<sup>20</sup> In our study, both the RNA and protein levels of HDAC4 and HDAC5 were significantly reduced in the hippocampi of mice suffering from postoperative cognitive disorders, while the mRNA levels of HDAC1, 2, and 11

had no significant difference at this experimental condition. This suggests that both HDAC4 and HDAC5 play a key role in the development of PND. A recent study found that anesthesia plus surgery increased HDAC2



**Figure 7** Predominant neuronal expression and release of HMGB1 in the hippocampus. Colocalization of HMGB1 with NeuN, IBA, and GFAP. The co-localization is in yellow in the image. The arrowhead indicates double-labelled cells, the arrow shows the HMGB1, which is not colocalized with the cell. The magnified images of the regions are marked with boxes. Scale bar = 100 μm.

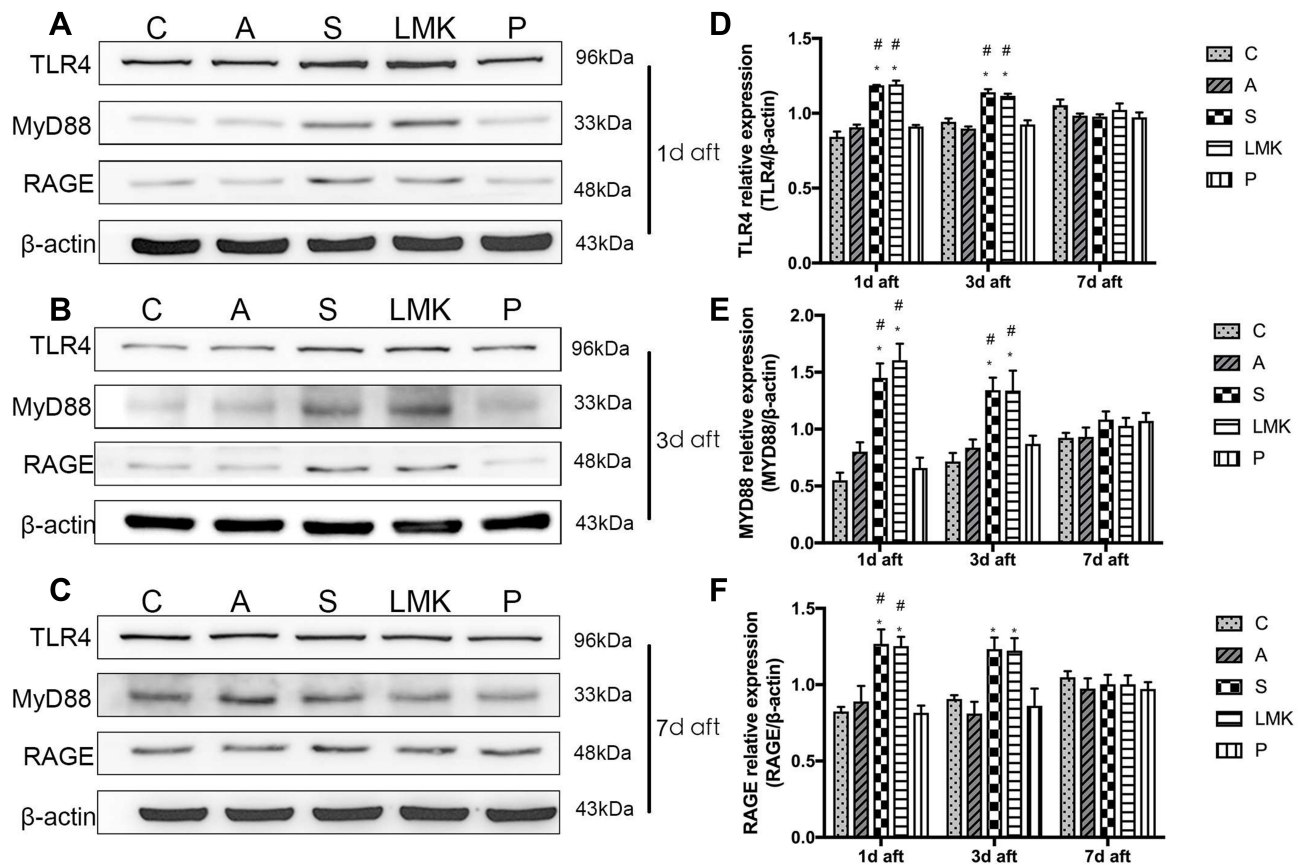
expression in hippocampus,<sup>46</sup> the discrepancy may be due to the different species of mice, and on the other hand, the surgical procedures performed are also different. The splenectomy we performed is more traumatic for exploratory laparotomy, which may cause differences in experimental results. Previous studies have shown that the HDAC4/5 can be localized in nucleus and cytoplasm, the cellular localization of HDAC4/5 is mediated by 14-3-3 protein. Similar to HMGB1, HDAC4/5 contains NES to ensure accumulation in the cytoplasm, and contain NLS to direct the nuclear localization, phosphorylation of key residues on HDAC will cause it to bind to 14-3-3, and the combination of the two will mask the NLS site on HDAC4/5, leading to nuclear export. The separation from 14-3-3 will allow HDAC to re-enter the nucleus and perform deacetylation.<sup>31</sup> In our study, the reduction in the total amount of HDAC also weakens the activity of the enzyme.

HMGB1 is now generally described as a damage-associated molecular pattern involved in a sterile inflammatory response. Recent studies affirmed the hypothesis that HMGB1 is linked to surgery-induced neuroinflammation.<sup>21</sup> Protein shuttling between the nucleus and cytoplasm depends on nuclear localization sequences (NLS) and nuclear export sequences (NES). HMGB1 contains two NLS sites that direct the nuclear localization of the vast majority of HMGB1 in normal

conditions.<sup>22</sup> Post-translational modification such as lysine residue acetylation within NLS promotes HMGB1 release towards cytoplasm. Although previous studies demonstrated that abundant HMGB1 was discharged after surgery, our data further indicate that surgery-induced expression of HMGB1 was acetylated, and ultimately, demonstrates a HDAC-related mechanism.<sup>23</sup>

Previous studies demonstrated that the induction of HDAC activation resulted in suppression of acetylation of HMGB1 in human intestinal epithelial HT-29 cells.<sup>24</sup> Trichostatin A (TSA, a pan-HDAC inhibitor) treatment promoted nucleocytoplasmic mobilization of HDAC1/4, leading to increased total HMGB1 and acetylated HMGB1 release in a model of rat hippocampal-entorhinal cortex brain slices.<sup>25</sup> Consistent with these findings, pharmacological inhibition of HDAC4 and HDAC5 regulates HMGB1 release from hippocampal neurons in our *in vivo* model. Renet al showed that post-insult treatment with the pan-HDAC inhibitor valproic acid improved behaviors in a middle cerebral artery occlusion stroke rat model.<sup>26</sup> These mixed results indicate the possible impact of distinct histone modification in different diseased states and that HDAC subtypes may have specific roles.

Together with recent findings that surgery triggers neuroinflammation via HMGB1 release, we propose three critical steps here for the formation of prolonged



**Figure 8** TLR4 and RAGE play a critical role in HMGB1-mediated neuroimmune activation. (A–C) Representative Western blots of TLR4, MyD88, and RAGE on postoperative day 1, 3, and 7,  $n = 4$  per group. Each sample repeated the experiment 3 times. (D–F) Densitometric analysis of Western blot data from (A–C),  $n = 4$  per group. # $p < 0.05$  versus the day-matched control group. \* $p < 0.05$  versus the day-matched anesthesia group.

neuroinflammation. In the first step, abdominal surgery triggers peripheral inflammation. We observed an increase in TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the serum on postoperative day 1 and day 3. The content of these proinflammatory factors were similar to those of mice suffering from sepsis.<sup>27</sup> The blood–brain barrier (BBB) is known as a highly specialized endothelial layer and was believed to separate the peripheral immune system from the CNS in normal conditions; however, inflammatory insult allows the BBB to become more permeable.<sup>1</sup> Recent studies also demonstrated that the peripheral surgery interferes with the integrity of the BBB.<sup>28,29</sup> Our data shows that proinflammatory factors in the serum were increased after surgery. It was previously shown that TNF- $\alpha$  and IL-1 $\beta$  are capable of crossing the BBB, resulting in neuroinflammation.<sup>30</sup> In the second step, expression levels of HDAC4 and HDAC5 in neurons are remarkably decreased in the inflammatory environment. The total amount of Class IIa HDACs varies in several pathogenesises such as in ischemia/reperfusion injury.<sup>17</sup> We found that both

HDAC4 and HDAC5 expression levels were decreased in postoperative hippocampal neurons. These effects led to a highly acetylated state of the nuclei and increase the acetylation of HMGB1. In the final step, hyperacetylation of HMGB1 shifted its equilibrium from a predominantly nuclear location towards extracellular release. We provide evidence that the inhibition of HDAC4 and HDAC5 resulted in increased acetylated HMGB1, accompanied by the rise of TLR4 and RAGE levels. A prior study claims that TLR4 and RAGE are highly probable HMGB1 receptors.<sup>32</sup> HMGB1 has three cysteines residues at positions 23, 45, and 106. Three different redox isoforms of HMGB1 have been detected in vivo: 1) fully reduced HMGB1 (fr-HMGB1), where thiol groups are present on each cysteine residue; 2) disulfide HMGB1 (ds-HMGB1), where the disulfide bridge is present between C23 and C45, while C106 remains reduced; and 3) sulphonyl HMGB1 (ox-HMGB1), where all cysteine residues are eventually oxidized.<sup>34</sup> When a tissue becomes damaged, the fr-HMGB1 is released from the cell. The extracellular environment is more oxidizing

than the cytosol and nucleus, which facilitates the formation of the C23-C45 disulfide bridge and conversion of fr-HMGB1 to ds-HMGB1.<sup>33</sup> The ds-HMGB1 could bind the MD2-TLR4 complex and facilitates the activation of NF- $\kappa$ B as well as secretions of various inflammatory mediators.<sup>35</sup> Additionally, damage-associated factors can fundamentally increase the expression of TLRs.<sup>36</sup> A recent study found that the oral pretreatment of glycyrrhizin inhibits postoperative HMGB1 extracellular release and decreases TLR4 and noted that the treatment reduced postoperative neuroinflammation.<sup>9</sup> Treatment using a TLR4 antagonist as well as TLR4 siRNA knockdown remarkably reduced the exogenous HMGB1-induced proinflammatory cytokine release in hippocampal slices.<sup>25</sup> We further demonstrated that surgery induced the expression of RAGE. HMGB1 could be rapidly endocytosed by RAGE and transferred to lysosomes. HMGB1 damages the lysosomal membrane within the lysosomes and allows HMGB1 and its chaperones to enter the cytoplasm. The end result is inflammatory body activation, pyrophosphorylation, and subsequent release of proinflammatory mediators.<sup>37</sup>

Many studies have demonstrated that the hippocampal memory function can be damaged by surgery in aged animals compared with their counterpart controls.<sup>38</sup> Surgery induces different degrees of neuroinflammation in adult and aged animals and the age is considered basis for PND. A chronic inflammatory status is the typical characteristic of the aging process and emerged as a crucial onset of several age-related diseases, such as Alzheimer's disease.<sup>39</sup> In the aging hippocampus, microglia become sensitized and primed. During peripheral insult, the inflammatory response in the hippocampi of aged mice is exaggerated and prolonged compared with the young mice.<sup>40</sup> Our data suggest that the aged hippocampus is susceptible to a change in acetylation status, which leads to neuroinflammation.

Our study does have limitations. We did not perform a treatment group for HDAC agonists because the current technology fails to synthesize specific Class IIa HDAC agonists. Although the cognitive function was impaired by the LMK235 treatment, it remains unclear whether the HDAC agonists attenuate PND.

Taken together, decrease in Class IIa HDAC after splenectomy contributes to postoperative neuroinflammation and cognitive disorders, enhanced expression and activation of Class IIa HDAC may represent a promising target for PND. Our findings could guide the development of better treatment

options for PND patients, eventually leading to the development of prevention and treatment strategies for PND.

## Ethical Approval

All experimental operations were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (protocol number: SYXK2015-0009) and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## Consent for Publication

Not applicable.

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## Disclosure

The authors declare that they have no competing interests.

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