



Article

Extracellular Histones Associate with Blood–Brain Barrier Disruption and Astrocyte-Mediated Neuroinflammation During Polymicrobial Sepsis

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Abstract

Histones, normally confined to nucleosomes, are released into the bloodstream during sepsis due to cell damage and NETosis, contributing to organ dysfunction. In sepsis-associated encephalopathy (SAE), histones may worsen neurological outcomes. Using a cecal ligation and puncture (CLP)-induced polymicrobial sepsis model, we evaluated histone release, blood–brain barrier (BBB) disruption, complement activation, and glial responses in the brain. Immunofluorescence revealed histone accumulation and increased soluble histone levels in the brain 8–24 h post-CLP. BBB permeability increased, confirmed by FITC-inulin and Texas Red-dextran clearance assays. Complement activation, along with increased GFAP-positive astrocytes and Iba1-positive microglia, occurred post-CLP. Histones were detected in astrocytes and microglia. In vitro, stimulated astrocytes released histones upon activation and also demonstrated the ability to uptake extracellular FITC-labeled histones. Histone exposure elevated intracellular calcium levels and triggered cytokine secretion in astrocytes. Notably, histone stimulation activated the NLRP3 inflammasome, amplifying inflammation. These findings suggest that histone release during sepsis drives neuroinflammation, BBB disruption, and glial activation, positioning extracellular histones as potential therapeutic targets for sepsis-related brain manifestations like SAE.

Keywords: sepsis-associated encephalopathy (SAE); blood–brain barrier (BBB) permeability; glial fibrillary acidic protein (GFAP); neutrophil extracellular traps (NETs)



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1. Introduction

Sepsis is a critical, life-threatening condition caused by an overwhelming and dysregulated immune response to infection, which can lead to dysfunction in multiple organs, including the brain. The brain involvement may present as sepsis-associated encephalopathy (SAE), a major neurological complication of sepsis that affects up to 70% of patients and is associated with increased mortality [1–3]. In the U.S., both sepsis and septic shock remain leading causes of death in intensive care units (ICUs), with an estimated annual economic burden of approximately \$16.7 billion [4]. As sepsis progresses to septic shock and multi-organ failure, the likelihood of fatal outcomes increases significantly [5,6].

Neurological symptoms such as delirium, confusion, and cognitive dysfunction are well-documented consequences of sepsis, contributing to elevated mortality, prolonged ICU stays, and greater healthcare resource use [7–10]. SAE manifests across a broad clinical spectrum, from hallucinations and confusion to cognitive impairment and coma, all of which can severely impact quality of life [7–13]. Reported prevalence of SAE varies widely depending on the diagnostic criteria applied [10,11,14,15]. Many survivors experience ongoing cognitive and behavioral difficulties that affect long-term functioning [16]. Despite its high prevalence, SAE continues to be diagnosed mainly on clinical presentation and EEG patterns, as validated biological markers for sepsis-related brain injury are lacking [17,18].

The pathophysiology of SAE remains poorly understood but is thought to involve neuroinflammation, immune cell infiltration, and dysfunction of the blood–brain barrier (BBB), which is formed by specialized brain endothelial cells supported by pericytes, astrocyte endfeet, and perivascular macrophages, together constituting the neurovascular unit [19]. Research by our group [20] and others [21–24] has used the cecal ligation and puncture (CLP) model of polymicrobial sepsis, which replicates many hallmarks of human sepsis, including cognitive and neuroinflammatory changes. In this model, septic mice display delayed somatosensory responses [25], memory deficits, and anxiety-like behavior, along with elevated brain cytokine levels such as TNF, IFN- γ , IL-1 β , and IL-6 [26–28].

Importantly, these cytokines, along with reactive oxygen species, can disrupt BBB endothelial tight junctions and increase permeability, allowing infiltration of harmful blood-derived factors and further amplifying neuroinflammation [3,19,29]. A hallmark of sepsis is neutrophil activation, which drives the formation of neutrophil extracellular traps (NETs) and release of extracellular histones, highly proinflammatory molecules capable of causing tissue damage. Normally confined to the nucleus, histones are released into circulation during severe infection or trauma, contributing to endothelial injury, coagulopathy, and multi-organ failure [2,30]. NETs, composed of DNA, histones, and proteolytic enzymes, amplify this process by increasing oxidative stress and triggering inflammatory mediator release [2].

Recent studies have shown that NET products and extracellular histones can directly disrupt the BBB, causing site-specific barrier opening and endothelial injury [30,31]. Together with complement activation (e.g., C5a) [32–34], these factors may synergistically worsen BBB dysfunction and neuroinflammation during sepsis.

While extracellular histones are well documented to damage peripheral vascular permeability, their specific effects on the brain microvasculature and neurovascular unit are less clearly defined. The BBB, which protects the brain from systemic insults through highly specialized endothelial tight junctions and supportive cells, becomes particularly vulnerable during sepsis. Histones can weaken its structure and disrupt its integrity and also interact with pattern recognition receptors such as Toll-like receptors (TLRs), triggering downstream inflammatory cascades that may further impair BBB function [2,30,31,35].

In this study, we explored how extracellular histones contribute to brain injury during sepsis, focusing on their effects on BBB integrity and glial cells activation. Although histone-mediated tissue damage is well characterized in peripheral organs, their role in the central nervous system is not well understood. Given that BBB breakdown is pivotal in SAE pathogenesis [36], we investigated both its onset and underlying mechanisms. Our findings reveal that BBB disruption, along with the appearance of extracellular histones in the brain, occurs as early as 8 h post-CLP in mice and appears complement dependent. We also identified significant astrocyte activation, characterized by elevated glial fibrillary acidic protein (GFAP) expression and the release of proinflammatory mediators [37], suggesting an active neuroinflammatory response during sepsis [38].

Taken together, these findings provide new insight into the role of extracellular histones in SAE, highlighting their contribution to BBB breakdown and astrocyte activation, and underscoring the urgent need to define the mechanisms of histone-induced brain injury to guide development of therapeutic targets in sepsis [32].

2. Results

2.1. Appearance of Histones in Mouse Brain at Different Time Points After CLP

Frozen brain sections from the frontal cortex were stained for histones at baseline (sham control, further referred to as Ctrl) and at various time points after CLP (8–24 h). No histone signal was observed in control brains, indicating that histones were not detectable in the brain under normal conditions. In contrast, diffuse histone staining appeared in the frontal cortex beginning at 8 h after CLP and was similarly observed at 18 and 24 h (Figure 1A). There were no marked differences in staining intensity or distribution across these time points after CLP.

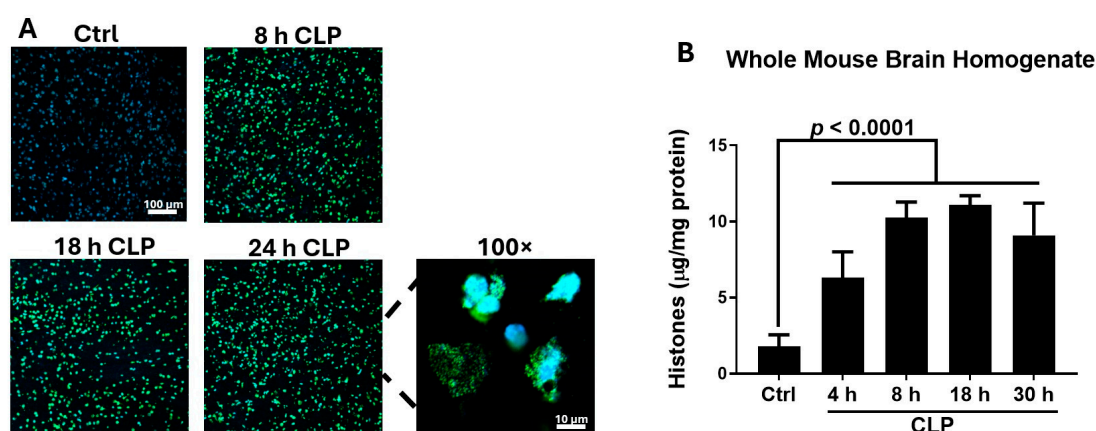


Figure 1. Appearance of Histones in Mouse Brain at Different Time Points After CLP. (A) Immunofluorescence staining of frozen frontal cortex sections from Ctrl mice, and 8, 18, and 24 h post-CLP using BWA3 monoclonal antibody detecting histones H2A and H4. Ctrl shows no histone staining, while diffuse histone staining appears after CLP. Scale bar = 100 μm. Higher-magnification images of histone-stained sections. Scale bar = 10 μm. Data representative of tissue from ≥ 4 mouse brains. (B) ELISA quantification of soluble histone levels in whole-brain homogenates from Ctrl and CLP-treated mice, showing peak histone concentrations at 8–30 h post-CLP with a gradual decrease at 30 h. Values are averages $\pm$ SD of $n \geq 4$ mouse brains. $p < 0.0001$ compared to Ctrl.

To quantify histone accumulation, an enzyme-linked immunosorbent assay (ELISA) was performed on brain extracts from Ctrl and CLP-treated mice at 4–30 h post-CLP (Figure 1B). Histone levels began to rise at 4 h, peaked at approximately 12 μg/mg protein by 8 h, and remained elevated by 18 h before slightly decreasing at 30 h ($p < 0.0001$ vs. Ctrl). This increase may reflect uptake of circulating extracellular histones due to a compromised BBB, or release by brain-resident cells such as glial cells. While our current data cannot distinguish between these two sources, both possibilities are explored in the following sections.

2.2. BBB Disruption Following Polymicrobial Sepsis Induced by CLP

We assessed BBB permeability over time in wild-type C57BL/6 mice following CLP-induced polymicrobial sepsis. Figure 2A presents the quantitative analysis of BBB permeability using influx rate constants (K_i), reflecting tracer passage from blood into brain tissue. Increased BBB permeability to the small molecular weight tracer Fluorescein Isothiocyanate (FITC)-inulin (5 kDa) was evident as early as 8 h post-CLP ($p < 0.0001$). In contrast, signifi-

cant permeability to the larger tracer, Texas Red (TR)-dextran (20 kDa), was only observed at 24 h post-CLP ($p < 0.0001$), indicating a time-dependent increase in barrier permeability, which suggests a progressive BBB injury corresponding to the severity of CLP.

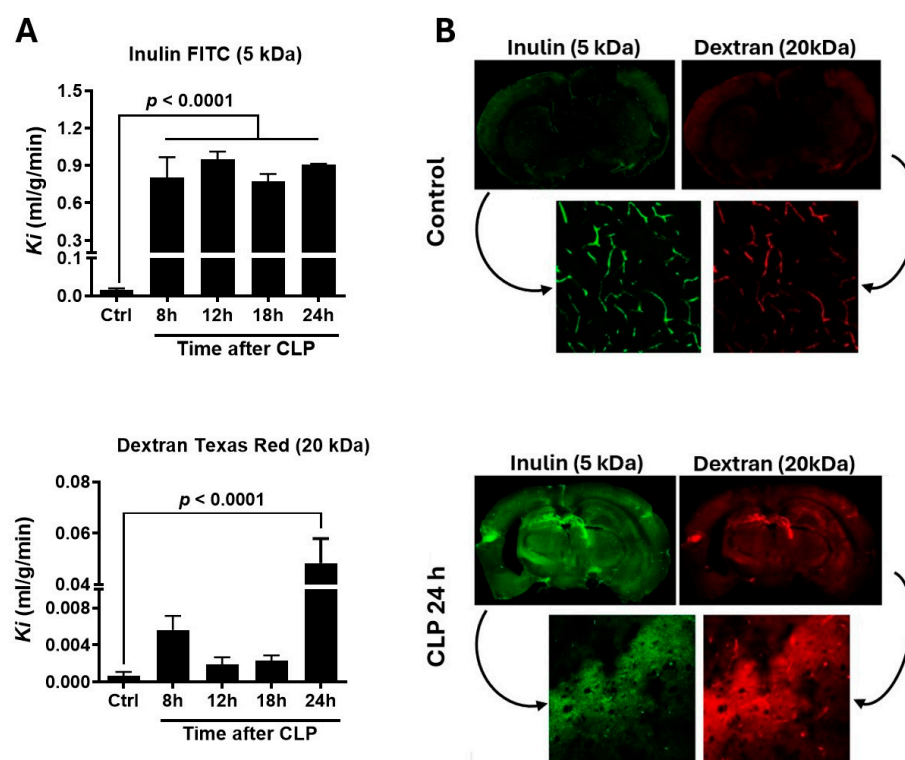


Figure 2. Blood–brain barrier Disruption Following Polymicrobial Sepsis Induced by CLP. (A) BBB permeability assessed by clearance rate (K_i) of FITC-inulin (5 kDa, top graph) and TR-dextran (20 kDa, bottom graph) shows increased tracer passage following CLP. Inulin permeability increased as early as 8 h post-CLP and remained elevated, while dextran permeability significantly increased at 24 h, indicating size-dependent and time-dependent BBB disruption. Values are averages $\pm$ SD of $n = 5$ mouse brains per group. $p < 0.0001$ compared to Ctrl. (B) Representative coronal brain sections (montage images) from Ctrl and CLP-treated mice 24 h post-CLP demonstrate minimal tracer leakage in Ctrl and prominent extravascular fluorescence of FITC-inulin (green) and TR-dextran (red) in septic mice, confirming BBB breakdown. Upper panels ($2.5\times$) and lower panels ($20\times$); curved arrows indicate the higher-magnification views.

Figure 2B illustrates tracers, inulin (5 kDa) and dextran (20 kDa) accumulation. In Ctrl brains (top panels), a minimal tracer signal was observed in the brain parenchyma, consistent with an intact BBB. In contrast, septic brains at 24 h post-CLP (bottom panels) show diffuse leakage of both inulin and dextran throughout the brain parenchyma without regional restriction, indicating widespread BBB hyperpermeability. The late increase in permeability for 20 kDa dextran reinforces the idea that BBB permeability evolves over time after septic insult.

Together, these findings demonstrate that BBB integrity is compromised early following CLP-induced sepsis, with increased permeability detectable by 8 h and sustained through at least 24 h. The differential tracer leakage underscores both the timing and extent of BBB disruption in sepsis.

2.3. Complement Activation Responses in Mouse Brain at Different Time Points After CLP

We further investigated complement activation by examining complement components and their receptor expression levels in the brain over time following CLP (Figure 3A–D). Using whole-brain extracts from Ctrl and septic mice at 4–18 h post-CLP, we assessed mRNA

levels for complement components C3, C5, and their receptors (C5aR, C3aR) by RT-qPCR. Importantly, to ensure that the brain tissue was properly analyzed, we performed brain perfusion prior to harvesting, as this is essential for distinguishing brain-derived expression. Among the complement components, C5aR1 mRNA exhibited the most substantial increase, showing an approximately 8-fold elevation at 8 and 12 h and to a lesser extent at 18 h post-CLP (Figure 3A), which was statistically significant compared to Ctrl ($p < 0.01$). C5 mRNA levels also increased, with a 2.2-fold elevation at 12 h and a less pronounced increase at 18 h post-CLP (Figure 3B, both $p < 0.01$). C3aR mRNA showed a moderate but statistically significant increase (1.7-fold) at 8 and 12 h post-CLP (Figure 3C, $p < 0.01$). C3 mRNA expression was significantly upregulated at 18 h post-CLP, reaching a 3.5-fold increase (Figure 3D, $p < 0.01$). These findings indicate that CLP robustly upregulates C5aR1 expression in the brain, while other complement-related components show more modest increases.

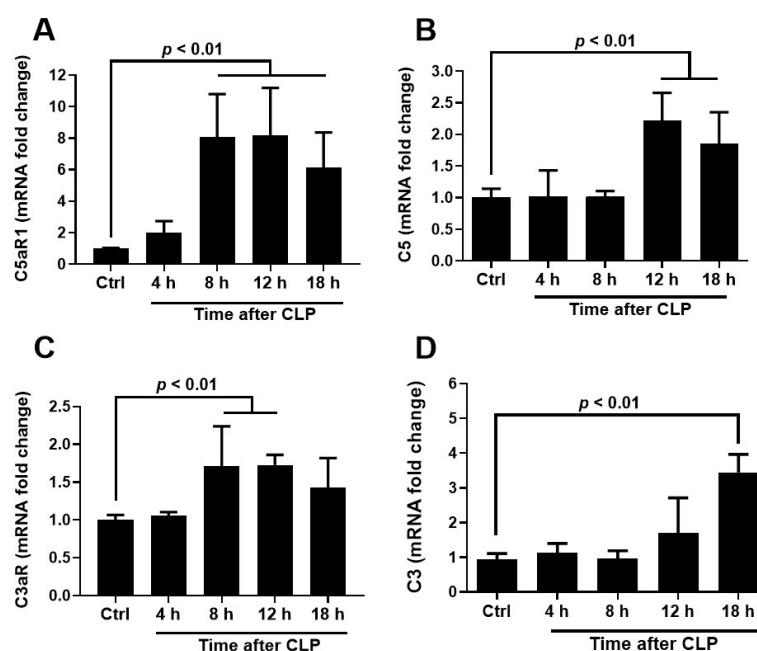


Figure 3. Complement Activation in Mouse Brain at Different Time Points After CLP. RT-qPCR analysis of complement-related gene expression in brain extracts post-CLP. (A) C5aR1 mRNA shows an 8-fold increase at 8–12 h post-CLP. (B) C5 mRNA peaks at 12 h. (C) C3aR shows a modest increase at 8 h, and (D) C3 expression rises at 18 h post-CLP. $n \geq 4$ mouse brains. All graphs represent the values $\pm$ SD from 3 independent experiments.

2.4. Increased Activation of Astrocytes and Glial Cells Markers Following CLP and/or Histone Injection in Mouse Brain

We examined the frontal cortex of mice in Ctrl, post-CLP, and after intraperitoneal infusion of histone. H&E staining (Figure 4A) and GFAP staining (Figure 4B) were performed. In contrast to the Ctrl brain, there was a marked increase in GFAP-positive astrocytes, indicative of astrocyte activation, in the brains of mice after CLP or histone mix infusion (Figure 4B).

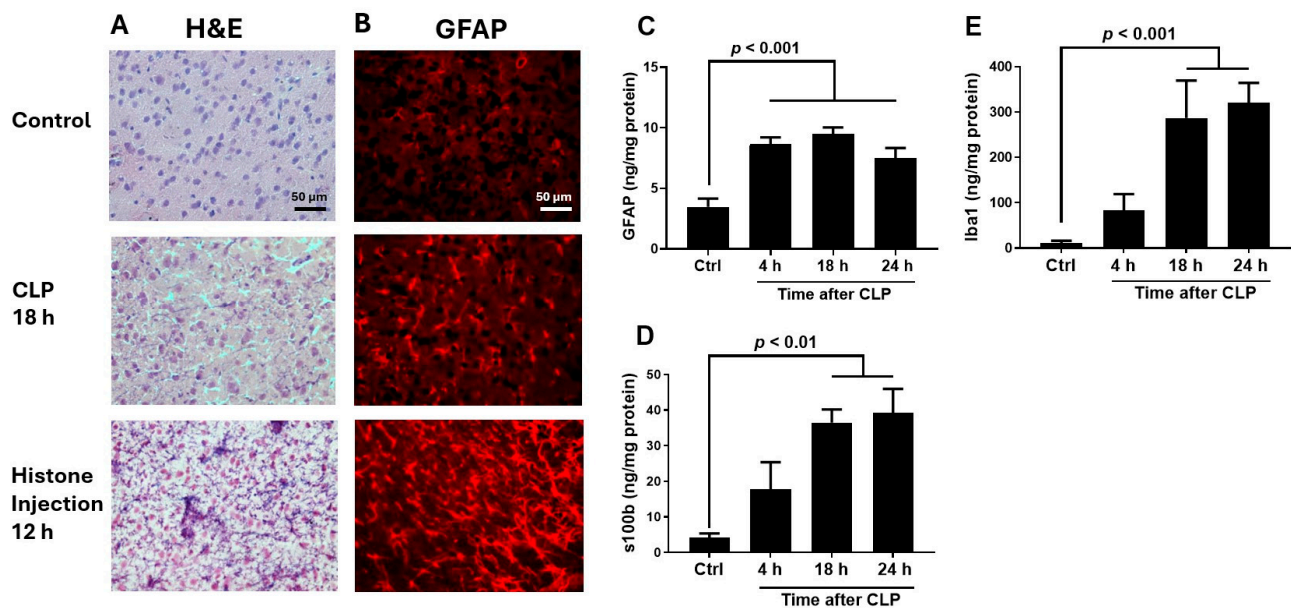


Figure 4. Increased Activation of Astrocytes and Glial Cells Markers Following CLP and/or Histone Injection in Mouse Brain. (A) H&E-stained frontal cortex sections from Ctrl, CLP-treated, and histone-infused mice. Scale bar = 50 μ m. (B) GFAP immunostaining shows astrocyte activation in CLP-treated and histone-infused mice compared to Ctrl. (C–E) ELISA quantification of GFAP, Iba1, and S100B protein levels in brain homogenates confirms astrocyte and microglial activation post-CLP. Values are averages $\pm$ SD of $n = 4$ mouse brains. $p < 0.01$ compared to Ctrl.

GFAP levels were quantified by ELISA in whole brain homogenates collected from Ctrl mice and from mice at 4–24 h following CLP. As shown in Figure 4C, GFAP protein levels significantly increased as early as 4 h after CLP and remained elevated through 24 h ($p < 0.001$). This increase is consistent with our immunofluorescence findings, which show enhanced GFAP expression in astrocytes, suggesting astrocyte activation following CLP. We also measured other glial activation markers, including S100B (a marker of activated astrocytes) and Iba1 (a marker of microglial activation). At 18 h post-CLP, we observed significant increases in both S100B (Figure 4D) and Iba1 (Figure 4E), ($p < 0.01$ and $p < 0.001$, respectively), indicating that both astrocytes and microglia are activated in response to polymicrobial sepsis-induced by CLP. These findings, taken together, suggest that astrocyte activation and microglial activation are integral to the neuroinflammatory response following CLP in the mouse brain.

2.5. Appearance of Histones in Astrocytes and Microglial Cells of Mouse Brain After CLP

At 18 h after CLP, a strong intracellular accumulation of histones was observed in the frontal cortex. Astrocytes, visualized by GFAP staining, showed an activated morphology. Nuclear labeling with DAPI defined cell nuclei in the same region. The merged image confirmed histone presence within the cytoplasm of activated astrocytes, including cells located around blood vessels (Figure 5A). Similarly, histone accumulation was detected in microglial cells, which were identified by Iba1 staining, with corresponding DAPI-stained nuclei. The merged image showed histones colocalizing in the cytoplasm of these activated microglia. These results suggest that 18 h after CLP, both astrocytes and microglia incorporate extracellular histones, potentially contributing to their activation (Figure 5B).

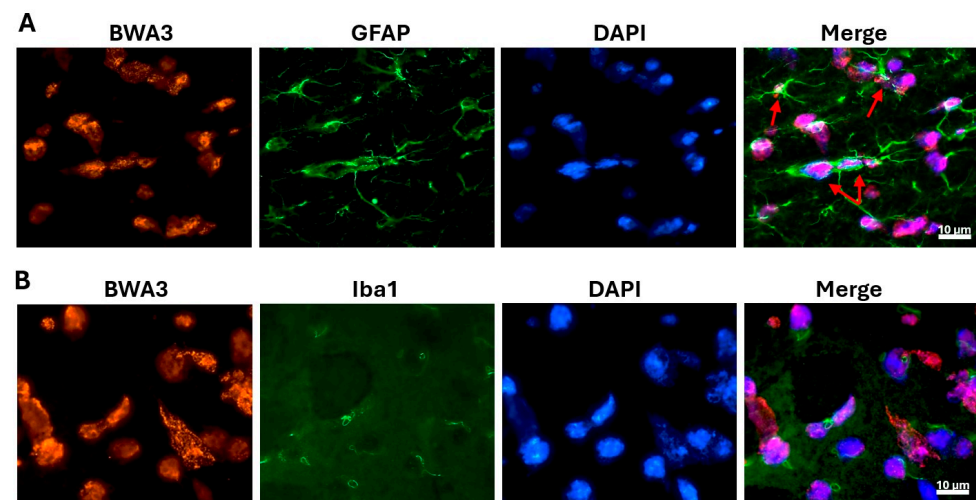


Figure 5. Appearance of Histones in Astrocytes and Microglial Cells of Mouse Brain After CLP. (A) Immunofluorescence staining of frozen frontal cortex sections from CLP-treated mice at 18 h post-CLP. Histones (BWA3, red) colocalize with astrocytes stained for GFAP (green) and DAPI-labeled nuclei (blue), as shown in the merged image (red arrow). Scale bar = 10 μ m. (B) Similarly, histones (BWA3, red) colocalize with the microglial marker Iba1 (green) and DAPI-labeled nuclei (blue), as confirmed by the merged image, indicating the presence of histones in both astrocytes and microglia. Scale bar = 10 μ m.

2.6. Ability of Astrocytes to Release Histones After Activation and to Uptake FITC-Labeled Histones

We investigated the ability of mouse astrocytes to release extracellular histones after stimulation with different agonists. Based on our previous findings regarding the role of complement C5a in brain dysfunction during sepsis [32], we first examined its effect on histone release by astrocytes. For this, astrocytes were stimulated with recombinant mouse C5a (1 μ g/mL) and showed histone accumulation within the cytoplasm and cellular projections (Figure 6A). Quantification of histone release (Figure 6B) showed increased levels after stimulation with C5a, LPS, or PMA compared to Ctrl (non-treated cells) ($p < 0.01$). These data suggest that, similar to neutrophils and macrophages [39], astrocytes, once activated, can release histones in response to inflammatory stimuli.

Because astrocytes might alternatively acquire histones from the circulation (potentially derived from NETs released during CLP and able to cross a disrupted BBB), we considered this possibility. To test it, we examined whether primary mouse and human astrocytes could internalize FITC-labeled histone H1 (50 μ g/mL) over short incubation periods using fluorescence microscopy. In mouse astrocytes, histone uptake was rapid and detectable as early as 1 min, showing a diffuse cytoplasmic distribution of green fluorescence extending into cytoplasmic projections. In human astrocytes histone labeling initially appeared as spotty fluorescence on the cell surface at 1 min, though this was less prominent than in mouse astrocytes. After extending the incubation to 15 min, human astrocytes displayed more extensive fluorescence on the cell surface, in cytoplasmic projections, and within nuclei, as confirmed by DAPI staining for nuclear colocalization (Figure 6C). These findings suggest that astrocytes internalize extracellular histones through active cellular uptake and trafficking, with eventual accumulation in both cytoplasm and nucleus, demonstrating a similar ability for phagocytes to take up extracellular histones [40].

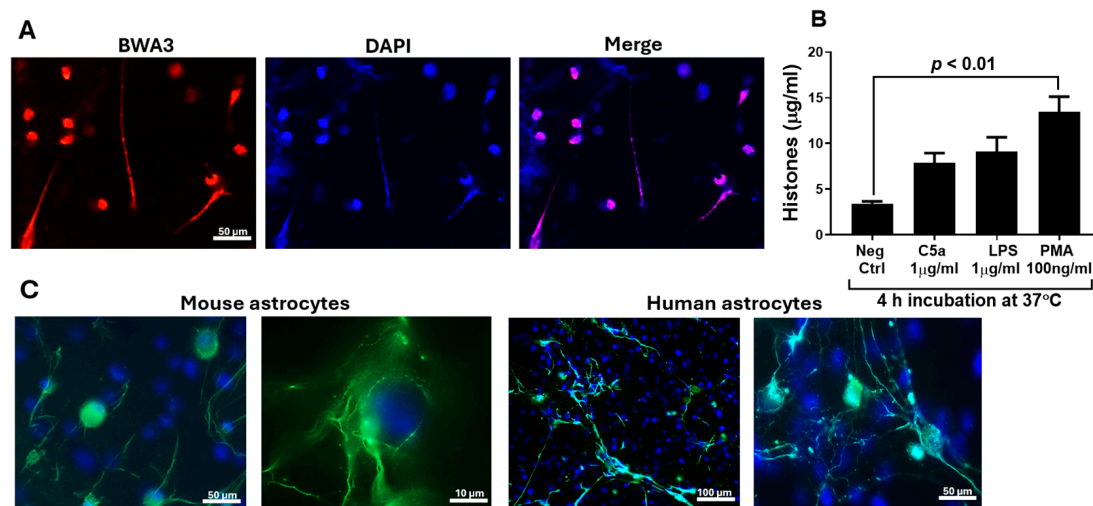


Figure 6. Ability of Astrocytes to Release Histones After Activation and to Uptake FITC-Labeled Histones. (A) Representative image of the mouse astrocyte cell culture stimulated with rmC5a (1 µg/mL) for 90 min shows cytoplasmic histone (BWA3) accumulation in the cell body and processes. Scale bar = 50 µm (B) ELISA-based quantification of histone release after 4 h of stimulation with rmC5a, LPS, or PMA indicates dose-dependent histone release. Values are averages ± SD of $n = 3$ independent experiments. $p < 0.01$ compared to Ctrl (non-stimulated cells). (C) Representative images of primary mouse astrocytes and human astrocytes incubated with FITC-labeled histone H1 (50 µg/mL) at 37 °C. In mouse astrocytes, histone uptake was observed as early as 1 min, with diffuse cytoplasmic distribution and labeling of cell processes. Scale bar = 50 µm and zoom image 10 µm. In human astrocytes, prominent fluorescence was evident after 15 min, including on the cell surface, in cytoplasmic projections, and within nuclei. Scale bar = 100 µm and zoom image scale bar = 50 µm.

2.7. Intracellular Calcium Release, Inflammatory Cytokine Secretion, and NLRP3 Inflammasome Activation in Mouse Astrocytes

To assess the impact of histones on intracellular calcium ($[Ca^{2+}]_i$) release, a key indicator of astrocyte activation, we examined this response in primary mouse astrocytes. Isolated astrocytes from neonatal mice [41] were preloaded with Fluo-3 AM and then exposed to purified histones (50 µg/mL). Intracellular $[Ca^{2+}]_i$ changes were measured by flow cytometry, acquiring a minimum of 10,000 events per sample. A rapid increase in $[Ca^{2+}]_i$ was observed within seconds of histone exposure (Figure 7A), indicating a robust calcium influx upon histone treatment, consistent with our previous observations [42,43].

Astrocytes exposed to histones showed a significant increase in IL-6 and TNF-α secretion (Figure 7B,C). The release of inflammatory cytokines from mouse astrocytes was further investigated following incubation with individual histones (Supplemental Figure S1A,B). Histones H2A, H2B, and H1 were the most potent inducers, triggering cytokine release from mouse astrocytes after 4 h of incubation, consistent with our previous findings in both mouse and human macrophages [42].

We next examined the release of IL-1β from mouse astrocytes using the NLRP3 inflammasome activation with the traditional inflammasome agonists LPS and ATP according to our previous studies on phagocytes [39,42]. Treatment with LPS followed by ATP induced a marked increase in IL-1β release, providing strong evidence for NLRP3 inflammasome activation in mouse astrocytes. Although NLRP3 is expressed in multiple immune and selected non-immune cell types, standardized LPS + ATP-based inflammasome activation protocols are best established in macrophages and monocytes. Nevertheless, this paradigm has been applied in prior studies to other immune cells and certain non-immune cells [44]. Consistent with our prior work in phagocytes [39] and cardiomyocytes [45], we employed this approach here to assess the capacity of astrocytes to activate the NLRP3 inflamma-

some under inflammatory conditions, rather than to model a specific physiological trigger *in vivo*.

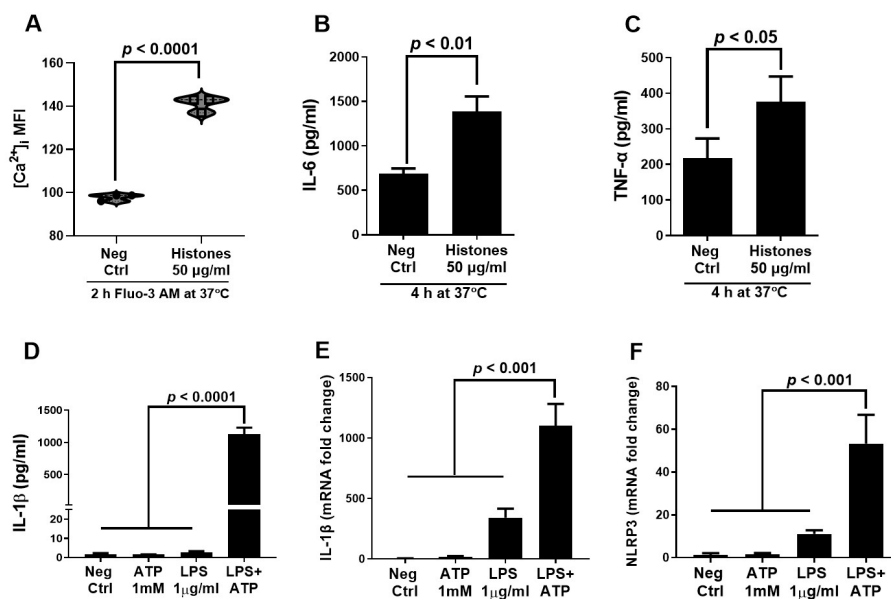


Figure 7. Histone-Induced Calcium Flux, Cytokine Release, and NLRP3 Inflammasome Activation in Mouse Astrocytes. (A) Intracellular calcium ([Ca²⁺]_i) release was evaluated in Fluo-3-loaded primary mouse astrocytes immediately (within seconds) after exposure to purified histones (50 µg/mL), and changes were measured by flow cytometry. A minimum of 10,000 events per sample were analyzed. (B,C) IL-6 and TNF-α secretion was quantified in mouse astrocytes by ELISA after 4 h stimulation with histones (50 µg/mL) at 37°C. (D) IL-1β release was assessed after LPS priming (1 µg/mL, 4 h) followed by ATP stimulation (1 mM, 45 min). (E,F) IL-1β and NLRP3 mRNA expression was quantified by RT-qPCR using parallel astrocyte cultures treated under the same conditions. Data represent mean ± SD from ≥2 independent experiments using astrocytes isolated from different litters.

To further confirm NLRP3 inflammasome involvement, we evaluated mRNA expression changes for NLRP3 besides the IL-1β using qPCR. In Figure 7E, IL-1β mRNA expression increased 340-fold with LPS alone and approximately 900-fold with the LPS + ATP combination. Similarly, NLRP3 mRNA (Figure 7F) exhibited a significant increase (10-fold) with LPS alone, while LPS + ATP induced a 50-fold rise. These results align with expected NLRP3 inflammasome activation patterns, as evidenced by IL-1β release in astrocytes. These results collectively demonstrate that mouse astrocytes respond to inflammatory stimuli such as histones by releasing intracellular calcium, secreting proinflammatory cytokines, and activating the NLRP3 inflammasome, supporting their potential role in neuroinflammatory processes.

3. Discussion

In this study, we demonstrated elevated levels and accumulation of extracellular histones in the brain following CLP-induced polymicrobial sepsis. We showed that sepsis disrupts BBB integrity, with increased permeability detectable as early as 8 h post-CLP and progressing through 24 h, accompanied by complement activation, particularly involving C5a and its receptor. These interacting events are summarized in Figure 8.

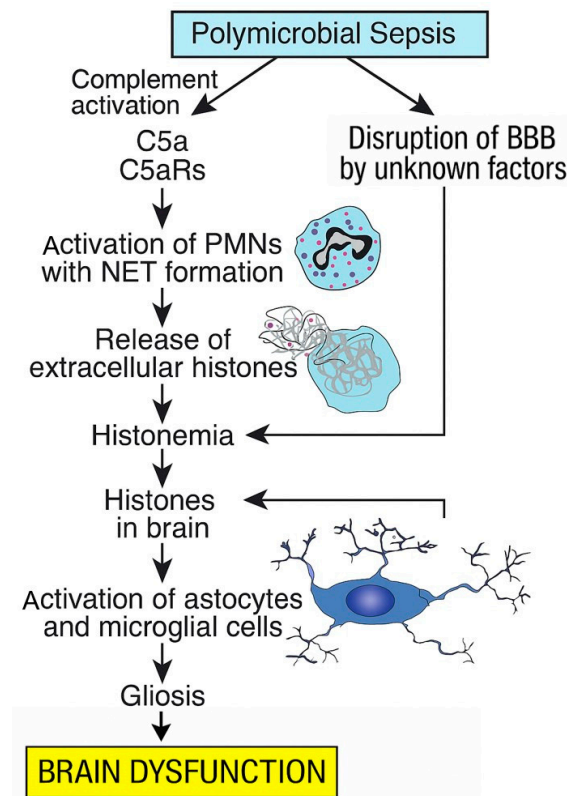


Figure 8. Schematic Summary of Brain Dysfunction During Polymicrobial Sepsis and the Interacting Roles of Complement Activation and Extracellular Histones, and BBB disruption. Polymicrobial sepsis leads to complement activation, generating C5a–C5aR signaling that triggers polymorphonuclear neutrophil (PMN) activation and NET formation, resulting in the release of extracellular histones into the circulation (histonemia). In parallel, BBB disruption facilitates histone entry into the brain. Circulating histones accumulate in the brain and activate astrocytes and microglial cells, leading to gliosis and ultimately brain dysfunction.

Although our *in vivo* data cannot definitively distinguish whether the brain-resident cells such as astrocytes released histones locally or took them up from the circulation, where histones are mainly sourced from phagocytes, especially neutrophils undergoing NETosis during sepsis [43,46–50], it is evident they contain histones after sepsis. Our *in vitro* experiments further showed that astrocytes can actively release histones upon inflammatory stimulation, as well as internalize extracellular histones. Given the positive charge of histones and their approximate size of 11–15 kDa, their passive diffusion across the BBB is unlikely, even with permeability increased to ~20 kDa during sepsis. Therefore, their entry may instead involve receptor-mediated transcytosis or severe paracellular leakage, process described previously [19,51].

We also found increased expression of glial cell markers including GFAP, S100B and Iba1 following sepsis. These findings together support a feed-forward amplification of neuroinflammation in the septic brain, where reactive glial responses, consistent with early stages of gliosis including astrocyte reactivity, microglial activation, and GFAP upregulation, contribute to ongoing BBB dysfunction and neuroinflammatory injury [52–56].

Previous studies by our group and others have established that extracellular histones play a significant role in the pathophysiology of sepsis [43,50], with elevated plasma levels persisting for 24 h, followed by reduction [43]. Histones released into circulation during sepsis originate primarily from dying or activated neutrophils via NETosis [57,58], which may contribute to BBB dysfunction (leakage).

Proinflammatory cytokines and reactive oxygen species (ROS) are also critical mediators in BBB disruption during sepsis. Elevated levels of TNF- α , IL-1 β , IL-6, and ROS contribute to endothelial cell damage and increased BBB permeability [26–29]. These inflammatory mediators trigger the expression of adhesion molecules and matrix metalloproteinases (MMPs), which degrade the extracellular matrix and tight junction proteins, leading to BBB breakdown [59–61]. The compromised BBB then allows harmful circulating substances, including histones, to enter the brain parenchyma, exacerbating neuroinflammation and tissue injury. Furthermore, this increased permeability is not only a cause of brain injury but also a consequence of ongoing neuroinflammation, as BBB breakdown activates glial cells, which in turn produce cytotoxic mediators that reinforce barrier dysfunction [60]. These events should be interpreted as overlapping and mutually amplifying rather than strictly linear. Histone accumulation in the brain was detectable as early as 4 h post-CLP, whereas measurable BBB permeability changes and complement activation became evident around 8 h. We propose that early histone exposure and complement activation occur in parallel and synergistically promote BBB dysfunction and glial activation. Our findings demonstrate a temporal pattern of histone accumulation in the brain, peaking by 8–12 h and declining by 30 h, which parallels the upregulation of C5a receptor (C5aR1) expression observed in brain homogenates. Given that C5aR is involved in complement-driven inflammatory pathways, this upregulation supports a mechanistic link between complement activation and BBB disruption in sepsis. Our prior studies showed an increase in Evans Blue accumulation in brain after CLP, which was reversed with anti-C5a antibody treatment, suggesting that BBB injury is closely tied to C5a bioavailability [32]. The compromised BBB could facilitate passage of complement factors like C5a into the brain, further exacerbating neuroinflammation. These results together suggest a coordinated response between histone release and complement activation during sepsis, consistent with our findings in another organ (i.e., heart) [43,62]. Another aspect not addressed in the current study is the contribution of endothelial tight junction proteins to BBB disruption. Future studies are warranted to determine whether histone- and complement-mediated BBB dysfunction is associated with structural alterations in tight junction components such as claudin-5, occludin, and ZO-1.

Our data also show that histones may not only enter the brain through a damaged BBB but also localize within astrocytes and microglia. Their intracellular presence likely results from receptor-mediated endocytosis or macropinocytosis, processes that warrant further investigation. Although this study primarily focused on astrocytes, our immunofluorescence analyses demonstrate histone accumulation in both astrocytes and microglia *in vivo*, indicating that microglia may also participate in histone uptake and downstream inflammatory responses. While histone localization within astrocytes and microglia was demonstrated qualitatively by immunofluorescence, future studies incorporating quantitative colocalization analyses (e.g., Pearson's correlation coefficients) will enable more rigorous comparison of histone association between glial subtypes. Astrocytes were prioritized for mechanistic studies because of their abundance, strategic localization at the neurovascular unit, and well-established role in regulating BBB integrity and complement-mediated signaling. The functional capacity of microglia to internalize extracellular histones and initiate downstream signaling cascades represents an important area for future investigation. To our knowledge, the detection of extracellular histones within brain-resident glial cells has not been previously reported, making this a novel observation. This concept complements findings by Warford et al., who reported increased astrocyte activation, reflected by elevated GFAP expression, in frontal brain biopsies from three individuals who died of sepsis compared with brain tissue from five non-septic controls [63], supporting the clinical relevance of astrocyte activation in sepsis-associated encephalopathy. Additionally, we

observed upregulation of GFAP and increased secretion of proinflammatory cytokines (IL-6, TNF- α) by astrocytes in response to histone exposure, responses similar to those observed in activated phagocytes [42]. Together, these findings underscore a potentially expanded role for astrocytes in SAE pathogenesis, functioning as both targets and sources of histone-mediated neuroinflammatory signals.

These findings are highly relevant to SAE, which remains a leading cause of delirium and cognitive impairment among ICU survivors [13,15,64]. Given the lack of standardized diagnostic criteria and the current reliance on symptomatic and supportive care [65], our study offers valuable insights into the mechanisms of BBB leakage and neuroinflammation that could guide the development of new therapeutic targets. Since histones are known to induce significant tissue damage and inflammation [37,40,43,50], reducing extracellular histones during sepsis could potentially decrease neuroinflammation and cognitive decline observed in septic survivors.

Understanding the pathways driving BBB disruption and neuroinflammation in sepsis is crucial for developing targeted therapies. Our findings suggest that extracellular histones and complement components (e.g., C5a/C5aR1) could represent promising targets to protect the CNS in sepsis. Given the strong association of sepsis with ICU delirium and long-term cognitive deficits [13,15,64], targeting extracellular histones and complement components may offer therapeutic potential. We have shown in our earlier studies that targeting C5a signaling decreases BBB disruption. Furthermore, while our data did not directly test whether histone neutralization preserves BBB integrity, previous studies from our group showed that systemic neutralization of histones with monoclonal antibodies can mitigate sepsis-induced organ dysfunction and improve survival [43]. Future studies are warranted to determine if similar strategies might protect BBB function and reduce neurocognitive injury following sepsis. Neutralizing antibodies against histones or complement inhibitors may help preserve BBB integrity, which is particularly important given the burden of SAE on ICU patients and its long-term cognitive consequences [13,15,64]. Future studies are also suggested to explore the contributions of individual histone subtypes (H1, H2A, H2B, H3, H4) to BBB dysfunction and assess whether blocking histone released from astrocytes or targeting complement pathways offers therapeutic benefit. In addition, understanding the crosstalk among histones, complement factors (C3a, C5a, C5b-9), and molecular mechanisms such as the NLRP3 inflammasome [66,67] will be essential for developing therapies that address SAE.

4. Materials and Methods

4.1. Animals and Anesthesia

All procedures were performed in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University Committee on Use and Care of Animals. Mice were housed under standard laboratory conditions (12 h light/dark cycle, controlled temperature and humidity) with free access to food and water. Male C57BL/6 mice (8–10 weeks old, 20–25 g; The Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized via intraperitoneal injection of a ketamine-xylazine mixture (ketamine from Hospira, Lake Forest, IL, USA; xylazine from Lloyd Laboratories, Shenandoah, IA, USA).

4.2. Polymicrobial Sepsis Model

Polymicrobial sepsis was induced using the CLP technique, as we previously described [20]. Sham-operated animals underwent identical handling and bowel manipulation but without the CLP step. Both Ctrl and CLP groups received fluid resuscitation with 1 ml of lactated Ringer's solution (Fisher Scientific, Waltham, MA, USA), administered

subcutaneously in the nuchal area. Animals were euthanized at the designated time points after the CLP procedure. Animals were monitored at least twice daily for clinical signs of distress, including reduced mobility, ruffled fur, hypothermia, and weight loss. Humane endpoints were defined according to institutional guidelines, and animals exhibiting severe distress were humanely euthanized. No unexpected adverse events occurred during the study.

4.3. Quantification of Histone Levels in Brain Tissue

To measure histone content, brain tissues from Ctrl and septic mice were first perfused with cold PBS to remove residual intravascular blood, and then mechanically homogenized in PBS containing protease inhibitors (Roche, Indianapolis, IN, USA). Total protein concentration was determined using the BCA assay (Sigma-Aldrich, St. Louis, MO, USA). Histone levels were then quantified by ELISA (Roche), following methods detailed in our prior studies [40]. Standard curves were generated using purified mixed calf thymus histones, as described previously [39,40,43].

4.4. BBB Integrity and Permeability Assessment

BBB permeability was determined *in vivo* using molecular tracers of different sizes, FITC-inulin (5 kDa) and TR-dextran (20 kDa), in the brains of Ctrl mice and mice with polymicrobial sepsis at various time points following CLP. Permeability was assessed by calculating the influx rate constant (K_i ; units: $\mu\text{L/g/min}$) for each tracer using the Patlak equation [68], which reflects the clearance of the tracer from blood into brain tissue and estimates how much passes through the BBB and becomes trapped in the brain. We conducted the *in vivo* BBB permeability assessment as we previously described [69,70]. Briefly, TR-dextran and FITC-inulin (both at a concentration of 1 mg/mL from Sigma-Aldrich) were injected as a bolus into a femoral vein 20 min prior to the end of the experiment. At the end of the experiment, arterial blood samples were collected from a femoral artery to determine the plasma tracer profile. Brain tissues were also collected and homogenized in 50 mM Tris buffer solution (pH 7.4) [70,71]. The fluorescence intensity of brain tissue was measured using a fluorescent plate reader (Tecan, Morrisville, NC, USA; excitation: 485 nm, emission: 540 nm), and BBB permeability index (K_i) was quantified using the Patlak equation [68].

BBB leakage was also visualized by fluorescent angiography [72,73]. At 24 h after CLP or sham induction, FITC-inulin or TR-dextran was intravenously injected as a single bolus and allowed to circulate for 60 min before mouse sacrifice. Brains were then harvested, fixed in 4% paraformaldehyde for 18 h, cryoprotected in 20% sucrose solution, and sectioned into 50- μm -thick coronal slices. The sections were imaged using a Zeiss confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) to assess tracer extravasation.

4.5. Astrocyte Isolation from Mouse Brain

Primary astrocytes were isolated from the brains of postnatal day 1–2 (P1–P2) C57BL/6 mouse pups using a protocol previously described by our group [73]. Briefly, cerebral cortices were dissected and enzymatically digested in HBSS with $1 \times$ trypsin (Invitrogen, Carlsbad, CA, USA) and 10 U/ μL DNase I (Sigma-Aldrich) for 45 min at 37 °C. The dissociated cells were then washed in HBSS and resuspended in astrocyte culture medium containing DMEM supplemented with 10% heat-inactivated FBS, $1 \times$ glutamine, and $1 \times$ antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA). Cells were plated at a density of 2.5×10^7 per 75 cm^2 flask and incubated at 37 °C in a humidified atmosphere of 5% CO_2 .

After two weeks, cultures were shaken gently at 0.2 g for 2 h at 4 °C to remove microglia, followed by an 18 h shaking at 37 °C to eliminate neurons. Immunocytochemical analysis confirmed that the cultures were approximately 99% astrocytes, as indicated by

GFAP staining. Cells from the first or second passage were used for subsequent experiments. For cytokine secretion, calcium flux, and qPCR assays, astrocytes were replated in poly-D-lysine-coated 6-well plates at approximately 3×10^5 cells per well and cultured overnight to reach 70–80% confluence before subsequent stimulation with agonists (histones, LPS, ATP) according to established protocols [74,75].

4.6. Histone Uptake and Release in Astrocytes

Mouse astrocytes were plated on sterile 22 mm poly-D-lysine-coated coverslips in 6-well plates and incubated overnight to allow optimal cell adherence. To assess histone uptake, FITC (Sigma-Aldrich) was conjugated to 1 mg of purified histone, as previously described by our group and others for histone labeling [40,76]. Mouse or human astrocytes were incubated with the FITC-conjugated histones for up to 30 min at 37 °C. The labeled histones were then used to assess uptake by astrocytes in vitro, with uptake visualized through fluorescence imaging.

To evaluate histone release, astrocytes were treated with either sham buffer or activators, including recombinant mouse (rm) C5a (1 µg/mL), LPS (1 µg/mL), or PMA (100 ng/mL), and incubated for 90 minutes at 37 °C. Cells were then fixed in 4% paraformaldehyde (PFA) and processed for immunofluorescence (IF) staining using an anti-histone H2A/H4 antibody (clone BWA3) [77]. BWA3 is a monoclonal anti-histone antibody that recognizes a shared epitope on histones H2A and H4, as previously described for anti-histone antibodies generated from autoimmune mice [77]. The antibody was generated in-house and purified from ascites fluid by protein A/G chromatography as described previously [39,40,43]. The antibody binding was visualized with a TRITC-conjugated anti-mouse IgG secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA).

4.7. Immunofluorescence of Brain Tissue

Fresh-frozen brain tissues were fixed in 4% paraformaldehyde, cryoprotected, and sectioned at 15 µm. Paraffin-embedded brain tissues were sectioned at 4 µm. The brain samples from septic and Ctrl mice were incubated with primary antibodies; anti-GFAP antibody (1:500; Sigma-Aldrich), anti-Iba1 antibody (1:100; Abcam) [73]. The reaction was visualized with the following secondary antibodies: anti-mouse IgG-TRITC (1:200; Jackson ImmunoResearch) and anti-mouse IgG-Alexa Fluor 488 (1:200; Jackson ImmunoResearch). Slides were mounted with ProLong Gold antifade reagent containing DAPI (Life Technologies, Grand Island, NY, USA). Images were captured using a Nikon A-1 confocal microscope and processed with Nikon Elements software (version 3.2, Nikon Instruments Inc., Tokyo, Japan).

4.8. Enzyme-Linked Immunosorbent Assay

Levels of the cytokines TNF, IL-6, and IL-1β were measured in treated mouse astrocytes in vitro using DuoSet sandwich ELISA kits from R&D Systems (Minneapolis, MN, USA), following the manufacturer's protocol.

The levels of extracellular histone levels in mice were quantified using a cell death detection ELISA kit, which detects all histone types (Roche). A standard curve was generated with a histone mixture containing H2, H2A, H2B, H3, and H4, following our previously established methods [39,43].

The levels of the soluble glial-specific marker S100B and the central nervous system marker glial fibrillary acidic protein (GFAP) were measured in brain homogenates using ELISA kits from Aviva Systems Biology Corporation (San Diego, CA, USA), following the manufacturer's instructions. Similarly, the soluble microglial marker ionized calcium-binding adapter molecule 1 (Iba1) was quantified in brain homogenates

using an ELISA kit from LifeSpan BioSciences, Inc. (Newark, CA, USA), according to the manufacturer's guidelines.

4.9. Reagents

The reagents used in our studies included a mixed histone (type II-A), as well as purified individual histones H1 and H3, all derived from calf thymus and obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse C5a was sourced from R&D Systems (Minneapolis, MN, USA), while recombinant histones H2A, H2B, and H4 were acquired from Cayman Chemical (Ann Arbor, MI, USA). Anti-histone antibody (clone BWA3) [77] was purified from ascites fluid by protein A/G chromatography, as in our earlier studies [39,40,43]. Additional reagents, including PMA, LPS, ATP, Poly-L-lysine solution, FITC-inulin, and TR-dextran, were also purchased from Sigma-Aldrich. Calcium indicator Fluo-3 AM, used to evaluate intracellular calcium levels, was purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA).

4.10. RT-qPCR

Total RNA was isolated from snap-frozen mouse brain tissue and cultured astrocytes using TRIzol reagent (Sigma-Aldrich) and treated with DNase I to remove residual genomic DNA. cDNA was synthesized from total RNA using oligo(dT) primers and reverse transcriptase according to the manufacturer's instructions (Life Technologies). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystems). Reactions were run in triplicate and melt-curve analysis was used to confirm amplification of a single product. Relative mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method with GAPDH as the internal reference gene. Primers (Integrated DNA Technologies, Ann Arbor, MI) used for amplification were as follows: C3, 5'-CGCAACGAACAGGTGGAGATCA-3' (forward) and 5'-CTGGAAGTAGCGATTCTTGGCG-3' (reverse); C5, 5'-CCTGTTACCAGTGATGAAGGCAG-3' (forward) and 5'-TCGTTAGTGAGTCAGGCAGCGT-3' (reverse); C3aR, 5'-TGTTGGTGGCTCGCAGAT-3' (forward) and 5'-GCAATGTCTTGGGGGTTGAAA-3' (reverse); C5aR1, 5'-TATAGTCCTGCCCTCGCTCAT-3' (forward) and 5'-TCACCACTTTGAGCGTCTTGG-3' (reverse); IL-1B, 5'-CCTGCTGGTGTGTGACGTTTC-3' (forward) and 5'-CAGCACGAGGCTTTTTTGTGT-3' (reverse); NLRP3, 5'-GAGTTCTTCGCTGCTATGT-3' (forward) and 5'-ACCTTCACGTCTCGGTTTC-3' (reverse); and GAPDH, 5'-CTTCAACAGCAACTCCCACTCTTCC-3' (forward) and 5'-GGTGGTCCAGGGTTTCTTACTCC-3' (reverse).

4.11. Statistical Analysis

Data are presented as means $\pm$ SD. Analyses were performed using one-way ANOVA followed by Tukey's post hoc tests, or by Student's *t*-test as appropriate. A *p*-value of less than 0.05 was considered indicative of statistical significance. Statistical evaluations were conducted using GraphPad Prism version 10.0 (GraphPad Software, Inc., San Diego, CA, USA).

5. Conclusions

Our study highlights the critical role of extracellular histones in disrupting BBB integrity and driving neuroinflammation during sepsis. These findings offer valuable insight into potential therapeutic strategies to protect the CNS from sepsis-induced damage, with future research needed to define precise molecular mechanisms and therapeutic targets.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052126/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

ATP	Adenosine triphosphate
BBB	Blood–brain barrier
BCA	Bicinchoninic acid
C3	Complement component C3
C3a	Complement component C3a
C3aR	Complement component C3a receptor
C5	Complement component C5
C5a	Complement component C5a
C5aR	Complement component C5a receptor
C5aR1	Complement component C5a receptor 1
C5aR2	Complement component C5a receptor 2
CLP	Cecal ligation and puncture
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNase	Deoxyribonuclease
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein

H&E	Hematoxylin and eosin
HBSS	Hanks' balanced salt solution
IF	Immunofluorescence
IFN- γ	Interferon gamma
Iba1	Ionized calcium-binding adapter molecule 1
IgG	Immunoglobulin G
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
Ki	Influx rate constant
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MMPs	Matrix metalloproteinases
NETs	Neutrophil extracellular traps
NLRP3	NOD-, LRR-, and pyrin domain-containing protein 3
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
rm	Recombinant mouse
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SAE	Sepsis-associated encephalopathy
S100B	S100 calcium-binding protein B
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor alpha
TR-dextran	Texas Red dextran
TRITC	Tetramethylrhodamine isothiocyanate

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