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# Hexokinase 2 Inhibition Attenuates Microglial Activation and Neuronal Degeneration Following Traumatic Brain Injury

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**Research Article**

**Keywords:** traumatic brain injury, neuroinflammation, microglia, hexokinase 2, 3-bromopyruvic acid, immunometabolism, neuronal degeneration

**Posted Date:** September 17th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-10824954/v1

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**Additional Declarations:** No competing interests reported.

# Abstract

**Background:** Traumatic brain injury (TBI) initiates a secondary injury cascade characterized by persistent neuroinflammation, metabolic dysfunction, and progressive neuronal degeneration. Activated microglia undergo immunometabolic reprogramming toward aerobic glycolysis, accompanied by increased expression of hexokinase 2 (HK2), a major regulator of glycolytic metabolism. However, whether pharmacological targeting of HK2-associated metabolism can attenuatemicroglial activation and secondary brain injury after TBI remains unclear.

**Methods:** Adult rats underwentcontrolled cortical impact and received the glycolytic inhibitor 3-bromopyruvic acid (3-BP) immediately after injury. Histological, immunohistochemical, biochemical, and behavioral analyses were conducted to evaluate HK2 expression, microglial activation, neuroinflammation, hippocampal neuronal injury, and neurological outcomes.

**Results:** TBI markedly increasedhippocampal HK2 expression, whereas 3-BP treatment attenuated this increase. Treatment also reduced neuronal degeneration in the hippocampal CA1 region, preserved NeuN-positive cell counts and MAP2 immunoreactivity, and suppressed microglial activation, as indicated by reduced Iba-1 immunoreactivity and lower expression of CD68,CD86, and interleukin-1β. These histological and molecular effects were accompanied by improved neurological function, spatial-learning performance, and selected measures of spatial-memory retention after TBI.

**Conclusions:** Post-injury administration of 3-BP attenuated microglial activation and neuroinflammatory-marker expression, preservedhippocampal neuronal integrity, and improved selected neurological and cognitive outcomes after experimental TBI. These findings suggest that targeting HK2-related metabolic pathways may offer a potential therapeutic approach for limiting secondary neuroinflammation and neuronal injury after TBI.


# 1. Background

Traumatic brain injury (TBI) is a leading cause of death and long-term neurological disability worldwide, affecting more than 69 million individuals annually (Maas et al., 2017). Although advances in emergency and neurosurgical care have improved survival, no effective pharmacological therapy is currently available to limit secondary brain injury (Beauchamp, Mutlak, Smith, Shohami, & Stahel, 2008; Lerouet, Marchand-Leroux, & Besson, 2021). The primary mechanical insult occurs at the time of trauma and is largely irreversible; however, a secondary injury cascade develops over the ensuing hours to weeks and contributes substantially to neurological deterioration (Loane & Kumar, 2016; Xiong, Mahmood, & Chopp, 2013). This delayed cascade involves persistent neuroinflammation, oxidative stress, metabolic dysfunction, blood–brain barrier disruption, and progressive neuronal degeneration, which collectively contribute to cognitive impairment and poor functional recovery (Jarrahi et al., 2020; Loane & Kumar, 2016; Xiong et al., 2013). Elucidating the molecular mechanisms that drive secondary injury is therefore essential for developing disease-modifying therapies for TBI.

Among the processes that contribute to secondary injury, neuroinflammation is a major determinant of disease progression (van Erp et al., 2023). Microglia, the resident innate immune cells of the central nervous system, rapidly respond to traumatic injury by adopting activation states that coordinate inflammatory and reparative responses (Kwon & Koh, 2020; Shields, Haque, & Banik, 2020). Under physiological conditions, microglia survey the neural microenvironment, clear cellular debris, remodel synaptic networks, and maintain tissue homeostasis (Kwon & Koh, 2020). After TBI, however, excessive or prolonged microglial activation promotes sustained production of proinflammatory cytokines, reactive oxygen species, nitric oxide, and other cytotoxic mediators that aggravate neuronal injury and impede functional recovery (Cherry, Olschowka, & O'Banion, 2014; Loane, Kumar, Stoica, Cabatbat, & Faden, 2014; L. Wang et al., 2021). Although transient microglial activation supports debris clearance and tissue repair, persistent activation contributes to chronic neurodegeneration and neurological dysfunction (Cherry et al., 2014; L. Wang et al., 2021). Therapeutic strategies that limit pathological microglial activation while preserving essential homeostatic and reparative functions have therefore attracted considerable interest (Scott, Bedi, Olson, Sears, & Cox, 2021; Xu et al., 2017).

Advances in neuroimmunology have shown that microglial inflammatory responses are closely coupled to cellular metabolism. Metabolic pathways not only provide energy but also regulate immune-cell function through immunometabolic reprogramming (M. Fang et al., 2025; Yu et al., 2023). Homeostatic microglia primarily rely on oxidative phosphorylation to meet their energy requirements, whereas inflammatory activation induces a rapid shift toward aerobic glycolysis, resembling the Warburg effect described in cancer cells (Bernier, York, & MacVicar, 2020; Huang, Wang, Chen, & Liang, 2024; Orihuela, McPherson, & Harry, 2016). This glycolytic state generates ATP and biosynthetic intermediates that support cytokine production, phagocytosis, proliferation, and sustained inflammatory signaling (Lauro & Limatola, 2020). Glycolytic reprogramming is therefore increasingly recognized as an important regulator of microglial inflammatory responses in neurological disease.

Increasing evidence indicates that dysregulated glycolytic metabolism contributes to neuroinflammation across a broad range of central nervous system disorders, including ischemic stroke, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and TBI (Huang et al., 2024; Yu et al., 2023). Beyond meeting cellular energy demands, enhanced glycolysis can regulate inflammatory gene expression, inflammasome activation, and cytokine production through interconnected immunometabolic pathways (Lonkar, Latz, & McManus, 2025; Yu et al., 2023). Targeting metabolic pathways may therefore modulate several inflammatory processes upstream of cytokine production and could offer broader effects than strategies directed at individual inflammatory mediators (Palsson-McDermott & O'Neill, 2020; Vizuete et al., 2022).

Hexokinase 2 (HK2), which catalyzes the first committed step of glycolysis, is an important regulator of immunometabolic reprogramming. In addition to controlling glycolytic flux, HK2 participates in inflammatory signaling through mechanisms that extend beyond glucose metabolism (Chen, Li, Sun, Li, & Chen, 2024; Wolf et al., 2016). HK2 expression increases in activated microglia and other innate immune cells, where it has been associated with glycolytic reprogramming, inflammasome activation,

cytokine production, and sustained inflammatory responses (Chen et al., 2024; J. Fang, Luo, & Lu, 2023; S. Wang et al., 2024; Wolf et al., 2016). These findings position HK2 as a potential link between cellular metabolism and innate immune activation. However, its contribution to post-traumatic neuroinflammation and secondary brain injury remains incompletely defined (Strogulski, Portela, Polster, & Loane, 2023).

Pharmacological targeting of HK2-associated metabolism has therefore emerged as a potential strategy for modulating neuroinflammation. Among available glycolytic inhibitors, 3-bromopyruvic acid (3-BP) inhibits HK2 and other metabolism-related targets and was initially investigated in highly glycolytic cancer cells (J. Li, Pan, et al., 2022; Shoshan, 2012). More recent studies suggest that 3-BP can suppress inflammatory activation in immune cells by limiting glycolytic reprogramming and reducing inflammatory-mediator production (H. Chen et al., 2025; Chen et al., 2024). These observations raise the possibility that 3-BP may attenuate pathological microglial activation after TBI. However, whether post-injury 3-BP treatment reduces secondary neuroinflammation and improves neurological recovery after TBI remains unclear.

Secondary injury after TBI involves interactions among neurons, glial cells, infiltrating immune cells, and components of the neurovascular unit (O'Neill, Kishton, & Rathmell, 2016; Simon et al., 2017). Within this network, activated microglia integrate injury-associated signals and coordinate inflammatory responses that influence neuronal survival, synaptic remodeling, and tissue repair (Strogulski et al., 2023). Because metabolic remodeling supports sustained microglial activation, targeting HK2-associated immunometabolic pathways may help limit pathological inflammation. Unlike approaches directed at individual cytokines or downstream signaling molecules, metabolic modulation may affect several interconnected inflammatory pathways (Palsson-McDermott & O'Neill, 2020; Vizuete et al., 2022).

In this study, we investigated whether pharmacological targeting of HK2-associated metabolism with 3-BP attenuates microglial activation and secondary brain injury after experimental TBI in rats. Using a controlled cortical impact model, we assessed HK2 expression, microglial activation, neuroinflammatory responses, hippocampal neuronal degeneration and integrity, and neurological recovery. We hypothesized that TBI-associated increases in HK2 expression contribute to pathological microglial activation and secondary neuroinflammation and that post-injury 3-BP treatment would attenuate these responses, preserve hippocampal neuronal integrity, and improve neurological outcomes.

# 2. Materials and Methods

### 2.1. Ethics statement and animal care

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Hallym University (approval no. Hallym 2023-70; February 22, 2024). The study was designed, conducted, and reported in accordance with the ARRIVE 2.0 guidelines.

Eight-week-old male Sprague–Dawley rats weighing 300–350 g were obtained from DBL Co. (Eumseong, Republic of Korea). The animals were housed in standard polypropylene cages under controlled environmental conditions of 20 ± 2°C, 55 ± 5% relative humidity, and a 12-h light/12-h dark cycle. Standard laboratory chow and water were available ad libitum. The rats were acclimatized for at least 1 week before experimentation.

An investigator who was not involved in outcome assessment generated the allocation sequence and assigned the animals to the experimental groups. Investigators responsible for treatment administration and outcome assessment were blinded to group allocation. The animals were monitored daily to minimize pain and distress, and humane endpoints were applied in accordance with institutional guidelines.

The sample size was set at a minimum of five animals per group, based on our previous experimental experience and the need to minimize the number of animals used while maintaining the ability to detect treatment-related effects. When additional animals were required, they were added only to the minimum extent necessary. The number of biological replicates was n = 5 or 8, with 3-11 technical replicates per biological replicate, as applicable. No animals were excluded following group allocation. The final sample size for each analysis is reported in the corresponding figure legend.

### 2.2. Drug preparation and administration

3-Bromopyruvic acid (3-BP; MedChemExpress, Monmouth Junction, NJ, USA), a glycolytic inhibitor with activity against hexokinase 2 (HK2), was freshly dissolved in sterile phosphate-buffered saline (PBS) immediately before administration. Immediately after traumatic brain injury (TBI), rats received a single intraperitoneal injection of 3-BP at 10 mg/kg in a total volume of 3 mL. Control animals received an equivalent volume of sterile PBS intraperitoneally.

To select an appropriate dose, a dose-ranging experiment was performed using 3-BP at 5, 10, 20, 30, 40, or 50 mg/kg, with five animals assigned to each dose group. All animals treated with 30–50 mg/kg died within 24 h, whereas all animals receiving 5, 10, or 20 mg/kg survived beyond 24 h. Among the doses that did not cause treatment-related mortality, both 10 and 20 mg/kg significantly reduced neuronal degeneration in the CA1 region. Moreover, 10 mg/kg produced a greater reduction in neuronal degeneration in the CA3 and dentate gyrus (DG) regions than 20 mg/kg. Based on its efficacy and tolerability, 10 mg/kg was selected as the final dose for all subsequent experiments (Figure 1F).

### 2.3. Induction of traumatic brain injury

TBI was induced using a controlled cortical impact (CCI) model in 8-week-old male Sprague–Dawley rats. Animals were anesthetized with 1.0%–1.5% isoflurane delivered in a gas mixture of 25% oxygen and 75% nitrous oxide and secured in a stereotaxic frame. After a midline scalp incision, a 3-mm-diameter craniotomy was created over the right parietal cortex, 2.8 mm lateral and 3.0 mm posterior to bregma, using a high-speed micromotor drill. The dura mater was preserved.

A controlled cortical impact was delivered using an Impact One™ stereotaxic impactor equipped with a 3-mm-diameter tip (Leica Biosystems, Buffalo Grove, IL, USA). The impact velocity was 5.0 m/s, the deformation depth was 3.0 mm, and the dwell time was 100 ms. After injury induction, the scalp incision was sutured, and the animals recovered in a temperature-controlled environment. Body temperature was maintained at 36.0°C–37.5°C throughout surgery using a homeothermic monitoring system (Harvard Apparatus, Holliston, MA, USA).

Immediately after CCI, rats received a single intraperitoneal injection of 3-BP at 10 mg/kg or an equivalent volume of PBS vehicle. Animals were euthanized 24 h or 7 days after injury, according to the experimental protocol, for histological, biochemical, or behavioral analyses.

### 2.4. Brain tissue collection

Brain tissues were collected at 24 h or 7 days after TBI, according to the experimental protocol. Tissues collected at 24 h were used for Fluoro-Jade B (FJB) staining, western blotting, and analyses of microglial markers. Tissues collected at 7 days, following completion of the behavioral assessments, were used for NeuN and MAP2 analyses.

#### 2.4.1. Tissue preparation for histological and immunofluorescence analyses

For histological and immunofluorescence analyses, animals were deeply anesthetized with urethane (1.5 g/kg, intraperitoneally) and transcardially perfused with normal saline followed by 4% paraformaldehyde (PFA). The brains were removed and post-fixed in 4% PFA for 1 h at room temperature. The tissues were then cryoprotected in 30% sucrose until they sank completely, which required approximately 48 h. Coronal sections of 30-μm thickness were prepared using a cryostat and stored as free-floating sections in cryoprotectant solution at −80°C until analysis.

#### 2.4.2. Tissue preparation for western blot analysis

For western blot analysis, animals were deeply anesthetized with urethane (1.5 g/kg, intraperitoneally) and transcardially perfused with ice-cold normal saline. The brains were rapidly removed, and the ipsilateral hippocampi were dissected on ice in ice-cold normal saline. The tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C until protein extraction.

### 2.5. Immunofluorescence

Free-floating coronal brain sections (30 μm thick) were washed in phosphate-buffered saline (PBS) and incubated overnight at 4°C with the following primary antibodies: rabbit anti-Iba-1 (1:500; Biocare Medical; Cat. No. ACR290B; RRID: AB_10578940), goat anti-Iba-1 (1:200; Abcam; Cat. No. ab5076; RRID: AB_2224402), mouse anti-CD68 (1:100; Bio-Rad; Cat. No. MCA341R; RRID: AB_2291300), rabbit anti-CD86 (1:200; Proteintech; Cat. No. 13395-1-AP; RRID: AB_2074882), goat anti-CD206 (1:500; R&D Systems, Cat. No. Af2535; RRID: AB_2063012), mouse anti-NeuN (1:500; Millipore; Cat. No. MAB377; RRID: AB_2298772), and rabbit anti-MAP2 (1:200; Abcam; Cat. No. ab32454; RRID: AB_776174).

For immunocytochemistry, cells were incubated overnight at 4°C with primary antibodies diluted in a bovine serum albumin (BSA)-based blocking buffer. The primary antibodies used were rabbit anti-Iba-1 (1:500; Biocare Medical; Cat. No. ACR290B; RRID: AB_10578940) and mouse anti-CD68 (1:500; Bio-Rad; Cat. No. MCA341R; RRID: AB_2291300). Subsequent procedures, including secondary antibody incubation and washing, were performed as described for tissue immunofluorescence. The immunocytochemical specimens were coverslipped using Dako Faramount Aqueous Mounting Medium, Ready-to-Use (Agilent Technologies).

After primary antibody incubation, the brain sections were washed with PBS and incubated with the appropriate fluorophore-conjugated secondary antibodies (1:250; Invitrogen) for 2 h at room temperature in the dark: donkey-anti-mouse Alexa Fluor 488 (Cat. No. A-21202; RRID: AB_141607), donkey-anti-rabbit Alexa Fluor 488 (Cat. No. A-21206; RRID: AB_2535792), donkey-anti-goat Alexa Fluor 488 (Cat. No. A-11055; RRID: AB_2534102), donkey-anti-mouse Alexa Fluor 594 (Cat. No. A-21203; RRID: AB_141633), donkey-anti-rabbit Alexa Fluor 594 (Cat. No. A-21207; RRID: AB_141637) and donkey-anti-goat Alexa Fluor 594 (Cat. No. A-11058; RRID: AB_2534105). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:1,000; Invitrogen). The sections were then mounted on gelatin-coated slides and coverslipped using DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

Fluorescence images were acquired using an LSM 900 confocal microscope equipped with Airyscan 2 (Carl Zeiss, Oberkochen, Germany; RRID: SCR_022263). Images from all experimental groups were acquired using identical laser power, detector gain, pinhole size, and image-acquisition settings. The immunoreactive area and mean fluorescence intensity were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA; RRID: SCR_003070) within standardized regions of interest in the hippocampal CA1 region. Background fluorescence was subtracted before quantification. Investigators responsible for image acquisition and analysis were blinded to group allocation.

### 2.6. Assessment of microglial activation

The composite microglial activation criteria were adapted from previously published methods and modified for the experimental conditions used in this study (Kauppinen et al., 2008; Kauppinen & Swanson, 2005; Park et al., 2025). Analyses were conducted within standardized regions of interest (ROIs) measuring 1,600 × 1,200 μm in the hippocampal CA1 region using a 10× objective. All image analyses were performed using ImageJ by investigators blinded to group allocation.

A composite activation score ranging from 0 to 9 was calculated as the sum of three components, each scored from 0 to 3:

1) Number of immunoreactive microglial cells: 0 cells, 0 points; 1–5 cells, 1 point; 6–10 cells, 2 points; and ≥11 cells, 3 points.

2) Predominant microglial morphology within each ROI: ramified, 0 points; hyper-ramified, 1 point; bushy, 2 points; and amoeboid, 3 points.

3) Fluorescence intensity: Mean fluorescence intensity was normalized to the mean value in the Sham + Vehicle group and scored as follows: ≤1.0, 0 points; 1.1–1.5, 1 point; 1.6–2.0, 2 points; and ≥2.1, 3 points.

The three component scores were summed to produce the composite microglial activation score, with higher values indicating greater activation. Immunoreactive area and mean fluorescence intensity were also quantified and analyzed as separate continuous outcomes.

### 2.7. SIM-A9 microglial cell culture

The murine microglial cell line SIM-A9 (ATCC, Manassas, VA, USA) was used for the in vitro experiments. Cell line authentication by short tandem repeat (STR) profiling or other independent authentication methods was not performed in our laboratory. The cells were maintained and handled according to the supplier's recommended culture conditions. Glass coverslips were placed in 24-well culture plates and coated overnight at 4°C with poly-L-lysine (R&D Systems, Minneapolis, MN, USA). Before cell seeding, the coating solution was removed, and the coverslips were rinsed thoroughly with sterile distilled water and allowed to air-dry.

SIM-A9 cells were seeded onto the coated coverslips at a density of $1 \times 10^4$ cells per well and maintained in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12; ATCC) supplemented with 5% horse serum (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin–glutamine (Gibco). The cells were cultured for 24 h at 37°C in a humidified atmosphere containing 5% $CO_2$ before the scratch assay. Following scratch induction, the cells were treated with 3-BP and incubated for an additional 24 h before fixation.

For immunocytochemical analysis, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature.

### 2.8. In vitro injury model using a scratch assay

A mechanical scratch assay was used as an in vitro injury model. After 24 h of culture, mechanical injury was induced using a sterile 100-μL pipette tip. Scratches were generated in a 5 × 5 grid pattern, with approximately 3-mm spacing between adjacent lines, to standardize the injured area.

Immediately after scratching, the culture medium containing detached cells and cellular debris was aspirated carefully and replaced with fresh medium. Cells in the treatment group received 3-BP at a final concentration of 2.5 μM immediately after injury, whereas control cells received an equivalent volume of vehicle.

At 24 h after injury, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and processed for immunocytochemical assessment of microglial activation.

### 2.9. Assessment of neuronal degeneration

Neuronal degeneration was assessed 24 h after TBI using Fluoro-Jade B (FJB) staining (Histo-Chem Inc., Jefferson, AR, USA). Coronal brain sections of 30-μm thickness were mounted on gelatin-coated slides, dehydrated in 100% ethanol for 3 min and 70% ethanol for 1 min, and rinsed in distilled water. The sections were then incubated in 0.06% potassium permanganate for 15 min to reduce background fluorescence, followed by staining with 0.001% FJB for 30 min in the dark.

After staining, the sections were rinsed three times with distilled water, cleared in xylene for 2 min, and coverslipped using DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence images were acquired using an Olympus BX53 fluorescence microscope equipped with a DP74 digital camera (Olympus, Tokyo, Japan) and a 450–490-nm excitation filter.

FJB-positive neurons were quantified within standardized ROIs in the hippocampal CA1, CA3, and dentate gyrus using ImageJ by an investigator blinded to group allocation. The number of FJB-positive neurons was used as an index of neuronal degeneration.

### 2.10. Western blot analysis

Hippocampal tissue was homogenized in radioimmunoprecipitation assay buffer (iNtRON Biotechnology, Seongnam, Republic of Korea) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). The lysates were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were collected for protein analysis.

Protein concentrations were determined using the Bradford assay. Equal amounts of protein (25 μg) were mixed with sodium dodecyl sulfate sample buffer, denatured at 100°C for 10 min, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes.

The membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. They were then incubated overnight at 4°C with the appropriate primary antibodies. After washing, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (anti-rabbit IgG for IL-1β and HK2; anti-mouse IgG for β-actin) for 1 h at room temperature. The following secondary antibodies were used: anti-rabbit IgG (1:5,000; Cell Signaling Technology; Cat. No. 7074S; RRID: AB_2099233) and anti-mouse IgG (1:5,000; Cell Signaling Technology; Cat. No. 7076S; RRID: AB_330924).

The following primary antibodies were used: anti-HK2 (1:1,000; Proteintech; Cat. No. 22029-1-AP; RRID: AB_11182717), anti-interleukin-1β (IL-1β; 1:1,000; GeneTex; Cat. No. GTX74034; RRID: AB_378141), and anti-β-actin (1:10,000; Cell Signaling Technology; Cat. No. 8H10D10; RRID: AB_2242334). β-Actin was used as the loading control.

Immunoreactive bands were detected using EZ-Western Lumi Pico Alpha chemiluminescent substrate (DoGenBio, Seoul, Republic of Korea) and imaged with an Amersham Imager 680 (GE Healthcare, Chicago, IL, USA). Band intensities were quantified using ImageJ. Images were converted to 8-bit

grayscale, and the integrated optical density of each band was measured after subtraction of the adjacent local background. Only bands at the expected molecular weight were included in the densitometric analysis. Protein expression was normalized to the corresponding β-actin signal from the same membrane and expressed relative to the mean value in the Sham + Vehicle group. Investigators performing densitometry were blinded to group allocation. Uncropped western blot images, including molecular-weight markers, are provided in the supplementary material, and the quantified bands correspond to those shown in the original images.

### 2.11. Behavioral assessments

#### 2.11.1. Modified neurological severity score

Neurological function was evaluated using the modified neurological severity score (mNSS), a composite assessment of motor, sensory, balance, and reflex function. Scores range from 0 to 18, with higher scores indicating greater neurological impairment.

Assessments were performed once daily for 7 consecutive days after TBI by an investigator blinded to group allocation. The absolute mNSS was recorded at each time point. Functional improvement was additionally expressed as the change in mNSS (ΔmNSS), calculated by subtracting the score at each subsequent time point from the score obtained 24 h after TBI. Thus, a larger ΔmNSS value indicates greater neurological improvement (Y. Li et al., 2002). Videos of the beam-balance assessments for each experimental group are provided in Additional Files 1–4.

Eight animals were included in each experimental group.

#### 2.11.2. Morris water maze

Spatial learning and memory were assessed using the Morris water maze. Testing was performed once daily for 6 consecutive days after TBI. The apparatus consisted of a circular pool divided conceptually into four quadrants and filled with water. A circular escape platform was submerged approximately 1 cm below the water surface in the target quadrant and remained in the same position throughout acquisition training. The platform was positioned in quadrant 1, and the opposite, nonadjacent quadrant was designated as quadrant 3.

During the 5-day acquisition phase, rats were released facing the pool wall from predetermined starting positions. Each trial lasted for a maximum of 120 s. Rats that did not locate the platform within this period were guided to it and allowed to remain there for 20 s before being returned to their home cage. Escape latency and path length to the hidden platform were recorded.

A probe trial was conducted on day 6 to assess spatial-memory retention. After the platform was removed, the rats were allowed to swim freely for 120 s. The recorded outcomes included the number of crossings over the former platform location, time spent in the target quadrant, latency and distance

traveled to the former platform location, entries into the target zone, and time spent in the opposite quadrant (zone 3).

Behavior was recorded and analyzed using SMART video-tracking software (Panlab, Barcelona, Spain). Investigators conducting the assessments and analyses were blinded to group allocation (Barnhart, Yang, & Lein, 2015).

Five animals were included in each sham group, whereas eight animals were included in each TBI group.

### 2.12. Statistical analysis

Data are presented as the mean ± standard deviation (SD). For all in vivo experiments, the individual animal was considered the experimental unit.

Parametric analyses were performed when both normality and homogeneity of variance were satisfied ($P > 0.05$ for the Shapiro-Wilk and Brown-Forsythe tests). Differences among multiple groups were analyzed using one-way or two-way analysis of variance, as appropriate, followed by Tukey's multiple-comparison test. Longitudinal behavioral outcomes, including Morris water maze acquisition measures and mNSS, were analyzed using repeated-measures two-way analysis of variance followed by Tukey's or Bonferroni's multiple-comparison test, as appropriate.

Exact sample sizes, statistical tests, and P values are reported in the corresponding figure legends. Statistical significance was defined as $P < 0.05$. Analyses and graph generation were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA; RRID: SCR_002798).

## 3. Results

### 3.1. 3-Bromopyruvic acid reduces neuronal degeneration and HK2 expression after traumatic brain injury

To evaluate the effects of 3-bromopyruvic acid (3-BP) after traumatic brain injury (TBI), rats received an intraperitoneal injection of 3-BP immediately after injury induction. Behavioral assessments were conducted for 7 consecutive days, and brain tissue was collected at 24 h or 7 days post-injury for histological and molecular analyses (Figure 1A).

Neuronal degeneration was assessed 24 h after TBI using Fluoro-Jade B (FJB) staining. No FJB-positive neurons were detected in the Sham + Vehicle or Sham + 3-BP groups. In contrast, the TBI + Vehicle group showed a marked increase in FJB-positive neurons in the hippocampal CA1, CA3, and dentate gyrus regions. Compared with vehicle treatment, administration of 3-BP at 10 or 20 mg/kg significantly reduced the number of FJB-positive neurons in the CA1 region ($P < 0.05$). Although fewer FJB-positive neurons were also observed in the CA3 and dentate gyrus after 3-BP treatment, these differences were not statistically significant (Figures 1B–E). Because the clearest treatment-associated difference was observed in CA1, representative high-magnification images from this region are presented.

To select a dose for subsequent experiments, survival was assessed following administration of increasing doses of 3-BP after TBI. No deaths occurred at doses of 5–20 mg/kg, whereas doses of 30–50 mg/kg resulted in 100% mortality within 24 h (Figure 1F). Based on the observed effect in the CA1 region and the absence of treatment-related mortality, 10 mg/kg was selected for the remaining experiments. The 10 mg/kg dose was selected instead of 20 mg/kg because it produced a comparable effect in the CA1 region while yielding numerically fewer FJB-positive neurons in the CA3 and dentate gyrus regions.

Hippocampal HK2 protein expression was assessed by western blotting 24 h after injury. HK2 expression was significantly higher in the TBI + Vehicle group than in the Sham + Vehicle group. Treatment with 3-BP significantly reduced HK2 expression compared with that in the TBI + Vehicle group (Figures 1G and 1H).

Together, these findings demonstrate that post-injury treatment with 3-BP reduced neuronal degeneration in the hippocampal CA1 region and attenuated the TBI-associated increase in hippocampal HK2 protein expression.

### 3.2. 3-Bromopyruvic acid attenuates microglial activation and neuroinflammatory-marker expression after traumatic brain injury

We next examined microglial activation in the hippocampal CA1 region following TBI and evaluated whether this response was altered by 3-BP treatment. After TBI, microglia exhibited morphological features associated with activation, including hypertrophic, bushy, and amoeboid forms (Figure 2A). Iba-1 immunostaining revealed significantly greater fluorescence intensity and immunoreactive area in the TBI + Vehicle group than in the sham groups, accompanied by prominent morphological activation. Both Iba-1 fluorescence intensity and immunoreactive area were significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group (Figures 2B and 2C).

A composite microglial activation score incorporating immunoreactive cell number, morphology, and fluorescence intensity was also determined. Each component was scored from 0 to 3, yielding a total score ranging from 0 to 9. The composite score was significantly higher in the TBI + Vehicle group than in the sham groups and significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group (Figure 2D).

We next examined CD68, a marker associated with phagocytic microglial activation. Both the CD68-positive area and fluorescence intensity were significantly increased after TBI and were significantly lower following 3-BP treatment (Figures 2E and 2F). Similarly, the CD86-positive area and fluorescence intensity were significantly greater in the TBI + Vehicle group and significantly lower in the TBI + 3-BP group (Figures 2G–I).

CD206 immunoreactivity was assessed as an additional marker associated with microglial activation states (Figure 2J). The CD206-positive area and fluorescence intensity were significantly increased after

TBI and were significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group (Figures 2K and 2L). Thus, 3-BP treatment was associated with reduced expression of multiple microglial activation-associated markers rather than a selective change in a single marker profile.

To determine whether a similar pattern occurred *in vitro*, SIM-A9 microglial cells were subjected to scratch injury and treated with 3-BP (Figure 2M). Representative images showed lower Iba-1 and CD68 immunoreactivity in 3-BP-treated cells than in vehicle-treated cells.

Hippocampal interleukin-1β (IL-1β) protein expression was subsequently assessed by western blotting (Figure 2N). IL-1β expression was significantly higher in the TBI + Vehicle group than in the Sham + Vehicle group and significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group (Figure 2O).

Collectively, these findings indicate that post-injury 3-BP treatment attenuated microglial activation-associated changes and reduced hippocampal IL-1β expression following TBI.

### 3.3. 3-Bromopyruvic acid improves neurological function and selected measures of spatial learning and memory after traumatic brain injury

To determine whether 3-BP treatment was associated with functional recovery after TBI, neurological and cognitive performance was assessed using the modified neurological severity score (mNSS) and Morris water maze.

During the acquisition phase of the Morris water maze, sham-operated rats progressively learned the location of the hidden platform, as shown by decreasing escape latency across training days. TBI impaired acquisition performance, whereas 3-BP treatment significantly shortened escape latency relative to vehicle treatment on training days 2–5 (Figures 3A and 3B). Similarly, the distance traveled to reach the hidden platform was significantly shorter in the TBI + 3-BP group than in the TBI + Vehicle group on training days 3–5 (Figure 3C).

Spatial-memory retention was evaluated in the probe trial. Representative swim trajectories showed preferential searching near the former platform location in sham-operated animals, whereas vehicle-treated TBI animals displayed a less spatially focused search pattern. Rats treated with 3-BP showed greater searching near the former platform location than vehicle-treated TBI rats (Figure 3D). The latency and distance traveled to the former platform location were significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group. The number of entries into zone 1 was also significantly higher after 3-BP treatment (Figures 3E–G). By contrast, the number of target crossings, time spent in the target quadrant, and time spent in zone 3 did not differ significantly between the TBI + 3-BP and TBI + Vehicle groups, although the numerical values favored 3-BP treatment (Figure S1).

Neurological impairment was assessed using the mNSS. Scores improved over time in vehicle-treated TBI animals, indicating spontaneous neurological recovery. However, mNSS values were significantly lower in the TBI + 3-BP group than in the TBI + Vehicle group from day 2 after injury onward (Figure 3H).

Consistently, ΔmNSS values indicated significantly greater improvement in the TBI + 3-BP group during the recovery period (Figure 3I).

Together, these findings indicate that post-injury 3-BP treatment improved neurological function, spatial-learning performance during Morris water maze acquisition, and selected measures of spatial-memory retention following TBI.

### 3.4. 3-Bromopyruvic acid preserves hippocampal neuronal markers after traumatic brain injury

To determine whether the behavioral improvements associated with 3-BP treatment coincided with preservation of hippocampal neuronal markers, brain sections collected after completion of behavioral testing were immunostained for NeuN and MAP2 (Figure 4A).

TBI significantly reduced the MAP2-positive area and fluorescence intensity in the hippocampal CA1 region, indicating impaired dendritic and neuronal structural integrity (Figures 4B and 4C). Both measures were significantly higher in the TBI + 3-BP group than in the TBI + Vehicle group, consistent with attenuation of TBI-associated structural injury.

The number of NeuN-positive cells in the hippocampal CA1 region was also significantly lower in the TBI + Vehicle group than in the sham groups. Compared with vehicle treatment, 3-BP treatment significantly preserved the number of NeuN-positive cells in this region (Figure 4D).

These findings indicate that the functional improvements associated with 3-BP treatment coincided with preservation of neuronal and dendritic markers in the hippocampal CA1 region following TBI.

## 4. Discussion

This study demonstrated that post-injury administration of 3-bromopyruvic acid (3-BP) attenuated several indices of secondary brain injury following experimental traumatic brain injury (TBI). Treatment with 3-BP attenuated the TBI-associated increase in hippocampal hexokinase 2 (HK2) expression, reduced the expression of microglial activation markers and interleukin-1β (IL-1β), preserved hippocampal neuronal and dendritic markers, and improved neurological function and selected cognitive outcomes. Collectively, these findings support a potential relationship among HK2-associated metabolic signaling, post-traumatic neuroinflammation, and neuronal injury (Hu et al., 2022; Wood, 2017).

Secondary injury after TBI involves persistent neuroinflammation, oxidative stress, mitochondrial dysfunction, blood–brain barrier disruption, and progressive neuronal degeneration (Kumar & Loane, 2012; Simon et al., 2017). Sustained microglial activation can contribute to tissue injury through the release of cytokines, reactive oxygen species, nitric oxide, and other potentially neurotoxic mediators (Hu et al., 2022; Mander & Brown, 2005; Tsankova, Renthal, Kumar, & Nestler, 2007). These inflammatory responses are closely linked to cellular metabolism (O'Neill et al., 2016). Glycolysis not only generates energy but also supports immune-cell activation through metabolic reprogramming (M. Fang et al., 2025; Yu et al., 2023). In the present study, lower microglial activation-marker expression after 3-BP treatment

coincided with lower hippocampal HK2 expression. This pattern is consistent with the proposed involvement of HK2-associated metabolism in post-traumatic microglial activation, although metabolic flux was not measured directly.

HK2 catalyzes the first committed step of glucose metabolism and can also influence inflammatory signaling through its relationships with mitochondrial function and inflammasome activation (Chen et al., 2024; Hu et al., 2022; Y. Li et al., 2018). Increased HK2 expression has been reported in activated microglia in several neurological conditions, including cerebral ischemia, Alzheimer's disease, Parkinson's disease, and autoimmune neuroinflammation (Leng et al., 2022; Y. Li et al., 2018). Consistent with these reports, hippocampal HK2 protein expression increased following controlled cortical impact in the present study, and this increase was attenuated by 3-BP treatment (Zhou et al., 2017).

A principal finding of this study was that 3-BP treatment reduced several indices of microglial activation. Treatment decreased Iba-1 immunoreactivity, the composite microglial activation score, and the expression of CD68 and CD86 (Leng et al., 2022; Y. Li et al., 2018). CD206 immunoreactivity was also reduced following 3-BP treatment. Although CD206 has traditionally been associated with reparative or M2-like microglial states, transcriptomic and single-cell studies indicate that the binary M1/M2 framework does not adequately represent the diversity of microglial activation states (Kumar, Alvarez-Croda, Stoica, Faden, & Loane, 2016; Morganti, Riparip, & Rosi, 2016; Ransohoff, 2016). Microglia may simultaneously express markers associated with inflammatory, phagocytic, and reparative programs. The concurrent reductions in CD68, CD86, and CD206 are therefore more consistent with a generalized attenuation of injury-associated microglial activation than with selective modulation of a single polarization state.

The reduction in microglial activation-marker expression was accompanied by lower hippocampal IL-1β expression, further supporting attenuation of inflammatory signaling following 3-BP treatment. IL-1β is an important mediator of post-traumatic neuroinflammation and can contribute to neuronal dysfunction, blood–brain barrier disruption, and amplification of innate immune responses (Mander & Brown, 2005; Tsankova et al., 2007). Glycolytic metabolism can promote inflammasome activation and cytokine production through several interconnected signaling pathways (Codocedo et al., 2024; Hu et al., 2022; Y. Li et al., 2018).

The attenuation of neuroinflammatory markers coincided with preservation of hippocampal neuronal and dendritic markers. The hippocampus, particularly the CA1 region, is vulnerable to secondary injury because of its high metabolic demand and susceptibility to excitotoxic and oxidative stress (Royo et al., 2006; Simon et al., 2017; Witgen et al., 2005). Consistent with previous reports, TBI increased the number of Fluoro-Jade B-positive cells in the CA1 region. Treatment with 3-BP reduced acute neuronal degeneration in CA1 at 24 h and preserved NeuN-positive cell counts and MAP2 immunoreactivity at 7 days after TBI. These findings indicate that reduced expression of microglial and inflammatory markers following treatment was accompanied by less acute neuronal degeneration and greater preservation of neuronal and dendritic markers during the subacute period (Y. Li et al., 2018; Simon et al., 2017).

Several processes may account for the association between attenuated microglial activation and neuronal preservation. Activated microglia can release cytokines, reactive oxygen species, nitric oxide, and excitotoxic mediators that disrupt neuronal homeostasis and promote secondary injury (Hu et al., 2022; Mander & Brown, 2005; Tsankova et al., 2007). Persistent inflammatory signaling may also impair synaptic integrity and neuronal-network function. By interfering with metabolic pathways that support sustained inflammatory activation, 3-BP may attenuate this self-amplifying response and thereby limit secondary neuronal injury (Codocedo et al., 2024; Hu et al., 2022; Simon et al., 2017).

The behavioral findings generally paralleled the histological observations. Rats treated with 3-BP exhibited lower neurological-deficit scores and improved Morris water maze acquisition compared with vehicle-treated TBI rats. Differences in the modified neurological severity score emerged from day 2 onward, indicating that treatment-associated improvements became evident during the post-injury recovery period (Chen et al., 2001; Werner & Engelhard, 2007).

During the Morris water maze probe trial, 3-BP treatment reduced the latency and distance traveled to the former platform location and increased the number of entries into the target zone. In contrast, the number of target crossings, time spent in the target quadrant, and time spent in the opposite zone did not differ significantly between the TBI + 3-BP and TBI + Vehicle groups (Hamm et al., 1992; Morris, Garrud, Rawlins, & O'Keefe, 1982; Park et al., 2026). Preservation of NeuN-positive cells and MAP2 immunoreactivity provides a structural correlate for these behavioral effects, although synaptic plasticity and electrophysiological function were not assessed (Xiong et al., 2013).

These findings have broader implications for immunometabolic regulation in neurological disease. Enhanced glycolysis and increased HK2 activity have been associated with inflammatory activation across diverse neurological disorders, including ischemic stroke, neurodegenerative diseases, epilepsy, and multiple sclerosis (Baik et al., 2019; Hu et al., 2022; Y. Li et al., 2018). Unlike strategies targeting individual cytokines or downstream signaling molecules, modulation of immunometabolism may influence several interconnected inflammatory pathways by acting on upstream metabolic processes (Palsson-McDermott & O'Neill, 2020; Vizuete et al., 2022). This approach may be relevant to TBI, in which inflammatory responses evolve over time and involve interactions among resident glia, infiltrating immune cells, neurons, and vascular components.

Nevertheless, complete suppression of microglial activation is unlikely to be beneficial because microglia also contribute to debris clearance, synaptic remodeling, angiogenesis, and tissue repair (Kwon & Koh, 2020; Mander & Brown, 2005; Tsankova et al., 2007). Therapeutic strategies should therefore aim to attenuate sustained pathological activation while preserving essential homeostatic and reparative functions (Codocedo et al., 2024; Hu et al., 2022).

Several limitations should be considered. First, this study did not directly examine the signaling pathways linking HK2-associated metabolism to inflammatory responses. Second, 3-BP is not cell type-specific and may affect metabolic targets other than HK2. The relative contributions of microglia, neurons, astrocytes, endothelial cells, and infiltrating immune cells to the observed effects therefore

remain unclear (C. Chen et al., 2025; Kalyanaraman, 2017; J. Li, Chen, et al., 2022). Third, neither glycolytic flux nor mitochondrial metabolism was directly measured. Fourth, only male rats were studied; therefore, the extent to which these findings apply across sexes remains unknown. Finally, treatment was administered immediately after injury, outcomes were assessed over a relatively short period, and higher doses of 3-BP were associated with mortality. These limitations should be addressed before the translational potential of targeting HK2-associated metabolism in TBI can be established.

# 5. Conclusion

Post-injury administration of 3-BP attenuated the TBI-associated increase in hippocampal HK2 expression, reduced the expression of microglial activation markers and IL-1β, decreased neuronal degeneration in the CA1 region, preserved NeuN-positive cell counts and MAP2 immunoreactivity, and improved neurological function and selected cognitive outcomes. These findings support a potential relationship among HK2-associated metabolic signaling, post-traumatic neuroinflammation, and hippocampal injury. the results provide a rationale for further investigation of HK2-associated metabolic pathways as potential therapeutic targets for limiting secondary injury following TBI.

# List of abbreviations

TBI: traumatic brain injury

ROS: reactive oxygen species

ARRIVE: Animal Research: Reporting of In Vivo Experiments

mNSS: modified neurological severity score

MWM: Morris water maze

PFA: paraformaldehyde

FJB: Fluoro-Jade B

CA1: cornu ammonis 1

CA3: cornu ammonis 3

GCL: granule cell layer

Iba-1: ionized calcium-binding adaptor molecule 1

NeuN: neuronal nuclear protein

DG: dentate gyrus

CD68: cluster of differentiation 68

CD86: cluster of differentiation 86

CD206: cluster of differentiation 206

HK2: hexokinase 2

3-BP: 3-bromopyruvic acid

BBB: blood–brain barrier

CNS: central nervous system

TNF-α: tumor necrosis factor alpha

IL-1β: interleukin-1 beta

OXPHOS: oxidative phosphorylation

ATP: adenosine triphosphate

CCI: controlled cortical impact

PBS: phosphate-buffered saline

DAPI: 4',6-diamidino-2-phenylindole

SEM: standard error of the mean

ANOVA: analysis of variance

RIPA: radioimmunoprecipitation assay

PVDF: polyvinylidene difluoride

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

TBS-T: Tris-buffered saline containing Tween 20

APC: antigen-presenting cell

MAP2: microtubule-associated protein 2

NIH: National Institutes of Health

IACUC: Institutional Animal Care and Use Committee

DMEM/F-12: Dulbecco’s modified Eagle medium/nutrient mixture F-12

BSA: bovine serum albumin

ROI: region of interest

HRP: horseradish peroxidase

IOD: integrated optical density

STR: short tandem repeat

# Declarations

### Ethics approval and consent to participate

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Hallym University (approval no. Hallym 2023-70; February 22, 2024).

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This work was supported by a National Research Foundation of Korea grant funded by the Korean government through the Ministry of Science and ICT (RS-2025-00520396 to S.W.S.); the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Education (RS-2025-25399649 to H.H.J.); and the Hallym University Research Fund (HRF-202504-002 to S.W.S.).

### Authors’ contributions

S.W.S. and H.H.J. conceptualized the study. H.H.J. developed the methodology, performed validation and formal analysis, curated the data, prepared the visualizations, and drafted the manuscript. H.H.J., M.K.P.,

S.Y.W., H.W.Y., and S.J.S. conducted the experiments. M.K.P., D.S.S. and S.W.S. reviewed and edited the manuscript. S.W.S. supervised the study. All authors read and approved the final manuscript.

**Acknowledgements**

Not applicable.

**Use of large language models**

No large language models were used in the preparation of this manuscript.

**Authors' information**

Not applicable.

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