EUROPEAN JOURNAL OF EMERGING MOLECULAR SCIENCES

UNLOCKING BRAIN HEALING: HOW NR4A2 CALMS INFLAMMATION AFTER HEMORRHAGIC STROKE

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V0LUME01 ISSUE01 (2024)
Published Date: 30 December 2024 // Page no.: - 94-112

ABSTRACT

Intracerebral hemorrhage (ICH) is a severe type of stroke that often leaves patients with significant neurological challenges and cognitive difficulties. This damage largely stems from a destructive wave of inflammation that sweeps through the brain after the initial bleeding. At the heart of this inflammation are microglia, the brain's immune cells, which can either worsen the damage (M1 type) or help with healing (M2 type). Our study explores how a protein called NR4A2 might help reduce early brain injury after ICH. We hypothesized that NR4A2 could protect the brain by encouraging microglia to switch to their healing M2 form, specifically by calming down a major inflammatory pathway known as TLR4/TRAF6/NF-κB. Our findings, based on a simulated ICH in rats, suggest that boosting NR4A2 levels significantly shrinks the area of bleeding, reduces harm to brain cells, and improves overall neurological function. We found that NR4A2 indeed shifted microglia towards the M2 phenotype and put a brake on the TLR4, TRAF6, and NF-κB p65 activation. When we introduced a substance that activates TLR4, NR4A2's protective effects were diminished, confirming that this pathway is crucial. NR4A2 also helped to seal up the "blood-brain barrier," which protects the brain. These results point to NR4A2 as a vital protective factor in ICH, capable of reducing secondary brain injury by guiding microglial responses and shutting down key inflammatory signals. This makes NR4A2 a promising new target for treatment.

Keywords: Intracerebral hemorrhage, NR4A2, Nurr1, Microglial polarization, M1 microglia, M2 microglia, Neuroinflammation, TLR4, TRAF6, NF-κB, Blood-brain barrier, Secondary brain injury.

INTRODUCTION

Intracerebral hemorrhage (ICH), a devastating type of stroke where bleeding occurs directly within the brain, represents a significant global health crisis. It carries alarmingly high rates of death and long-term disability, leaving many individuals with severe neurological impairments and a diminished quality of life [1, 2]. Unlike ischemic stroke, which involves a blockage of blood flow, ICH is characterized by the rupture of blood vessels within the brain parenchyma, leading to the formation of a hematoma. This initial physical trauma is quickly followed by a complex and highly destructive chain of events that profoundly impacts how well patients recover [4, 5, 6]. These secondary injuries include widespread inflammation, damaging oxidative stress, over-excitation of brain cells, and a breakdown of the crucial blood-brain barrier (BBB), all contributing to the tragic loss of brain cells and worsening neurological problems [7, 38]. Despite extensive efforts to understand the mechanisms behind ICH, truly effective clinical treatments remain frustratingly limited, highlighting the urgent need for fresh approaches and new therapeutic strategies.

The perihematomal region, the brain tissue immediately surrounding the hematoma, is a critical zone of pathology. It not only suffers from the direct pressure and displacement caused by the expanding blood clot but also undergoes a dynamic and destructive process driven by inflammatory cascades and the breakdown of the BBB [4, 5]. Within just hours of the bleeding, activated endothelial cells (the lining of blood vessels) rapidly increase the expression of adhesion molecules. This sets in motion a multi-step process that leads to circulating inflammatory cells, particularly neutrophils and macrophages, infiltrating this vulnerable perihematomal zone [6]. This invasion of immune cells significantly contributes to the ongoing tissue damage and swelling.

At the very core of the brain's inflammatory response after ICH are microglia, the brain's own resident immune cells [8]. These incredibly dynamic cells quickly react to injury, becoming activated and adopting different functional roles [9, 10]. Traditionally, we categorize microglial activation into two main states: the pro-inflammatory M1 phenotype and the anti-inflammatory, pro-healing M2 phenotype [11, 14]. M1 microglia are the "bad guys" in this scenario; they release pro-inflammatory substances like IL-6, TNF-alpha, and iNOS, which unfortunately worsen neuronal injury

and contribute to tissue destruction [11]. In contrast, M2 microglia are the "good guys," associated with beneficial effects. They secrete anti-inflammatory mediators (like IL-10, TGF-beta), help repair tissue, clear away cellular debris, and even produce vital brain-boosting factors like VEGF and BDNF [9, 12, 13]. In the acute phase of ICH, the M1 type of microglia tends to dominate, amplifying inflammation, tearing down the BBB, and causing widespread secondary brain damage. However, a gradual shift towards M2 polarization in later stages is absolutely essential for calming inflammation, getting rid of toxins, and supporting the repair of brain blood vessels [14]. This is why therapeutic strategies that can encourage microglia to switch from M1 to M2 during the early stages of ICH hold immense promise for reducing inflammatory injury and restoring the brain's delicate balance.

Nuclear receptor subfamily 4 group A member 2 (NR4A2), also known as Nurr1, is a fascinating protein that acts as a "master switch" for genes [15]. It's found in abundance throughout the central nervous system and plays indispensable roles in various vital processes, including how brain cells develop, survive, and how dopamine-producing neurons are maintained [17]. But beyond its roles in development and basic brain function, growing evidence points to NR4A2's powerful ability to fight inflammation [16]. Mechanistically, NR4A2 has been shown to put a stop to pro-inflammatory signals by attaching to a specific region of the NF-κB-p65 gene, thereby preventing the production of harmful, neurotoxic substances [18]. While we know it can regulate microglial behavior in other inflammatory brain diseases, its specific role and potential as a treatment in ICH have largely remained a mystery.

The Toll-like receptor 4 (TLR4)/TNF receptor-associated factor 6 (TRAF6)/nuclear factor-kappa B (NF-κB) pathway is an incredibly important inflammatory signaling cascade that gets strongly activated in response to various harmful stimuli, including those present after ICH [19, 20, 21, 22, 23]. TLR4, a key "recognition receptor" of our innate immune system, spots danger signals (called DAMPs) released from damaged cells and blood components after brain injury. This recognition triggers a chain reaction involving TRAF6 [24, 41]. TRAF6, in turn, acts as a crucial "connector" molecule that activates the NF-κB pathway. Once activated, NF-κB moves into the cell's nucleus, where it promotes the production of countless pro-inflammatory genes, directly contributing to M1 microglial polarization and making brain injury much worse [25, 26, 42, 43]. In ICH models, persistent NF-κB activation in perihematomal neurons and glial cells is strongly linked to the expansion of the blood clot and poor neurological outcomes. Hemorrhage-associated stressors, such as lipopolysaccharide (LPS) and reactive oxygen species (ROS), further amplify this TLR4/TRAF6/NF-κB signaling [26], creating a vicious cycle that perpetuates inflammation. Targeting this critical inflammatory pathway to encourage microglia to shift from M1 to M2 is a highly promising strategy for limiting early hematoma growth and improving functional recovery.

Given what we know about NR4A2's anti-inflammatory powers and the central role of the TLR4/TRAF6/NF-κB pathway in ICH, we put forward the idea that NR4A2 could be a new and exciting therapeutic target. Specifically, we aimed to explore whether NR4A2 could lessen early brain injury after ICH by promoting M2 microglial polarization through the suppression of this specific inflammatory pathway. Our investigations, conducted using both living animal models and laboratory cell models of ICH, revealed that hemorrhagic stress downregulated NR4A2 expression in microglia, brain microvascular endothelial cells (BMECs), and astrocytes. Restoring NR4A2 levels significantly reduced hematoma volume, attenuated neurological deficits, and preserved BBB integrity. Mechanistically, NR4A2 exerts its anti-inflammatory protective effects by guiding microglial polarization towards the M2 phenotype through the suppression of the TLR4/TRAF6/NF-κB pathway. These groundbreaking findings establish NR4A2 as a novel therapeutic target for calming neuroinflammation and ultimately improving outcomes for those affected by ICH.

METHODS

Animal Model of Intracerebral Hemorrhage

To study intracerebral hemorrhage (ICH) in a controlled setting, we used male Sprague-Dawley (SD) rats, typically weighing between 220-250 grams. These animals were obtained from Guangzhou Bestong Biotechnology Co., Ltd., China. All procedures involving our animal subjects were carefully reviewed and approved by the Animal Protection and Utilization Committee of the First Affiliated Hospital of Guangzhou Medical University, ensuring ethical and humane treatment. Before starting any experiments, the rats spent at least a week getting used to their new laboratory home, living in a controlled environment with a 12-hour light/dark cycle and always having access to food and water.

To create the ICH model, we used a well-established method involving collagenase injection, which closely mimics how ICH happens in humans [27]. First, we gently anesthetized the rats using isoflurane, making sure they were completely comfortable and stable throughout the surgery. A small, precise hole was then drilled into their skull, allowing us to accurately inject the collagenase. We prepared Type VII collagenase (Sigma-C0773, 0.6 Units) in 2 muL of sterile saline, keeping it on ice to maintain its effectiveness. This collagenase solution was then injected into the right striatum, a specific brain region, using a stereotactic device for pinpoint accuracy. The coordinates for injection were: -3 mm anterior-posterior, 1 mm lateral, and 6 mm ventral from bregma. After the injection, we carefully held the microsyringe in place for 5-10 minutes. This step is crucial to allow the collagenase to spread evenly and prevent any backflow, minimizing additional tissue damage. Once this was done, the syringe was slowly

withdrawn. Following the surgery, the rats were placed in a warm recovery box, and we closely monitored their vital signs until they fully woke up from the anesthesia. Afterwards, they had free access to food and water, and we watched them closely for any unusual behaviors like limb paralysis, strange postures, or reduced activity, which would indicate neurological problems [27].

Experimental Groups and Treatments

To thoroughly investigate how NR4A2 affects ICH progression, we divided our rats into several distinct experimental groups:

- Sham Group: These animals underwent the exact same surgical procedure as the ICH groups (craniotomy and needle insertion) but received an injection of sterile saline instead of collagenase. This group served as our healthy control.
- ICH + Vehicle Group: These rats received the collagenase injection to induce ICH and were then treated with a control solution (vehicle) that contained no active drug. This group represented the typical progression of ICH without our intervention.
- ICH + NR4A2 Overexpression Group (ICH + NR4A2): These animals received the collagenase injection for ICH and were pre-treated with special adeno-associated virus (AAV) vectors. These AAVs were designed to make their brain cells produce more NR4A2.
- ICH + NR4A2 Knockdown Group (ICH + shNR4A2): Similar to the overexpression group, these rats received collagenase for ICH but were pre-treated with AAV vectors designed to "knock down" or reduce the amount of NR4A2 using a technique called shRNA.
- ICH + NR4A2 Overexpression + TLR4 Agonist Group (ICH + NR4A2 + CRX-527): This group received the collagenase injection and the NR4A2 overexpression AAV, but then we also gave them a substance called a TLR4 agonist (CRX-527). This was to see if activating the TLR4 pathway could counteract the protective effects of NR4A2.
- ICH + TLR4 Agonist Group (ICH + CRX-527): These animals received the collagenase injection for ICH and were treated with the TLR4 agonist (CRX-527) alone, without any NR4A2 modulation. This helped us understand the direct effect of TLR4 activation in our model.

For both NR4A2 overexpression and knockdown, we prepared specific types and concentrations of the AAV viruses. These AAVs were injected into the striatum (at coordinates: -1.5 mm anterior-posterior, 3 mm lateral, and 4.5 mm ventral from bregma) one week before inducing ICH. This pre-treatment allowed enough time for the viral vectors to deliver their genetic cargo and for the brain cells to start producing more or less NR4A2. After the AAV injection, we closed the wound and gave the rats pain relievers. We observed them daily for two weeks to ensure successful viral transduction and expression of the target gene at the molecular and cellular levels. The TLR4 agonist, CRX-527 (0.25 mg/kg; #tlrl-crx527, InvivoGen), was dissolved in DMSO and given as an injection into the abdominal cavity (intraperitoneal injection) before the ICH injury, following methods from previous studies [28].

Neurological Deficit Assessment

To quantitatively evaluate the neurological impact of ICH and the efficacy of NR4A2 modulation, neurological function was assessed at 24, 48, and 72 hours post-ICH using a modified neurological severity score (mNSS) [29]. The mNSS is a comprehensive scoring system that evaluates a range of neurological functions, including motor abilities (e.g., limb flexion, circling), sensory responses (e.g., tactile placing, proprioception), balance (e.g., beam walking), and reflexes (e.g., pinna reflex, corneal reflex). Each parameter is assigned a score, and the sum of these scores provides an overall assessment of neurological deficit, with higher scores indicating more severe neurological impairments.

In addition to the mNSS, specific motor function tests were performed:

- Forelimb Force Test: This test is a crucial way to measure how strong their front paws are, how well their muscles are working, and how much their motor function recovers after a brain injury [29]. We used a special device that gently held the rat's body still but allowed its front paws to move freely and touch a force sensor. After the rats got used to the setup and were trained, we carefully placed them in the device. Then, we gave them a specific signal to make them push with their forelimbs. After each push, we recorded the maximum and average forelimb force, along with a graph showing how the force changed over time. We repeated each measurement 10-15 times, giving the rats a short break (30-60 seconds) between each try to prevent them from getting tired and affecting the results. Finally, we calculated the maximum, average, and standard deviations of the forelimb force from all the measurements for each animal and analyzed these numbers.
- Rotarod Test: The rotarod test is a widely used method to evaluate an animal's coordination, balance, how well they learn new movements, and even their motor memory [29]. It's a great tool for understanding neurological diseases, checking how well drugs work, and studying recovery from injuries. Before the test, the animals spent at least 3-5 days in a standard environment to get comfortable. On the day of the experiment, we moved them to the test area 1-2 hours beforehand so they could acclimate. We gently placed each animal on a rotating rod, started the rod, and simultaneously began a timer. We recorded how long the animal stayed on the rod before falling off (this is called "residence time"). Each animal was tested 3-5 times, with a 10-15 minute break between each trial, and the average residence time was

taken as the final result for that animal at that specific rotation speed. All the data, including residence time and the speed at which they fell, were recorded in a spreadsheet, and any unusual data points were double-checked and removed before statistical analysis.

Histological Analysis and Immunofluorescence Staining

At 72 hours after the ICH, we carefully prepared the brain tissue for detailed examination. First, we deeply anesthetized the animals and then gently flushed their circulatory system with cold phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde solution. This process, called transcardial perfusion, helps preserve the brain tissue. We then carefully removed the brains, fixed them overnight in 4% paraformaldehyde, and then soaked them in a 30% sucrose solution at 4°C until they sank. This cryoprotection step helps prevent ice crystal formation during freezing. Finally, we used a cryostat microtome to cut the brains into very thin slices (10-20 mum thick), either coronal (cross-section) or transverse (lengthwise), depending on what we needed to examine.

- Hematoma Volume Quantification: To measure the size of the blood clot in the brain, we used two main approaches. First, we measured the amount of hemoglobin (the protein in red blood cells) in the brain tissue around the hematoma using a spectrophotometer. Second, we took images of the brain slices after staining them with hematoxylin and eosin (HE) or Nissl stain. We then used specialized image analysis software to outline the area of the hematoma and calculate its volume.

- Neuronal Damage Assessment (Nissl Staining): To see how much damage the brain cells (neurons) had sustained, we used Nissl staining. Fresh brain tissues were immediately placed in a 4% paraformaldehyde fixing solution and then rinsed three times with 0.1 M PBS, each rinse lasting 15 minutes. After this, the tissue was placed in a cryostat microtome and cut into thin slices (10-20 mum). These slices were then dipped in a 1% toluidine blue staining solution (from Yesaen Biotechnology; product number 60531ES50) and incubated at 37°C for 15-30 minutes. This allows the Nissl bodies (which are essentially the rough endoplasmic reticulum in neurons, indicating their health) to be fully stained. After a differentiation step, the slices were cleared in xylene for 3-5 minutes. Finally, we added a small amount of neutral balsam and carefully placed a coverslip on top, avoiding air bubbles. Once the mounting medium dried, we looked at the samples under an optical microscope to assess the shape and density of the neurons, which tells us about their viability.

- General Histopathological Assessment (HE Staining): For a broader view of tissue health and any pathological changes, we used Hematoxylin and Eosin (HE) staining. Paraffin sections were first "dewaxed" by immersing them in xylene I and xylene II for 10-15 minutes each. After dewaxing, the sections were gradually rehydrated using different concentrations of alcohol. Then, the rehydrated sections were stained with hematoxylin dye solution (Beijing Solarbio) for 5-10 minutes, which stains cell nuclei blue. After a gentle water rinse, the samples were transferred to a 1% hydrochloric acid-alcohol solution (made of 99 ml of 75% alcohol and 1 ml of concentrated hydrochloric acid) for a few seconds to differentiate the stain, achieving a moderately stained nucleus. Once the nuclei were properly stained and turned blue, they were immersed in eosin dye solution for 3-5 minutes, which stains the cytoplasm pink. Finally, by closely observing the changes in the shape, color, and structure of the tissues and cells under a microscope, we could accurately determine if there were any pathological changes like inflammation, swelling, or tissue death.

- Immunofluorescence Staining: Immunofluorescence (IF) staining allowed us to pinpoint specific proteins and cell types in the brain sections, helping us identify different microglial phenotypes and key signaling molecules. We first made the sections permeable and then blocked them to prevent non-specific antibody binding. After that, we incubated them overnight at 4°C with primary antibodies targeting:

  - Iba1 (Abcam; ab178847): A general marker for all microglia.
  - CD86 (CST; #91882): A surface marker that tells us we're looking at M1 pro-inflammatory microglia.
  - iNOS (Abcam; ab283655): An enzyme produced by M1 pro-inflammatory microglia.
  - CD206 (CST; #24595): A surface marker for M2 anti-inflammatory, healing-promoting microglia.
  - Arg-1 (CST: #93668): An enzyme produced by M2 microglia.
  - NR4A2 (Abcam; ab176184): To see where and how much of our target protein was expressed.
  - GFAP (Abcam; ab7260): A marker for astrocytes, another type of brain support cell.
  - MPO (Abcam; ab208670): Myeloperoxidase, a specific marker for infiltrating neutrophils.
  - TLR4 (CST; #38519): Toll-like receptor 4, a key inflammatory receptor.
  - TRAF6 (Abcam; ab40675): TNF receptor-associated factor 6, a signaling molecule.
  - p-NF-κB p65 (CST; #3033) and NF-κB p65 (Signalway Antibody; 53227): To assess the activation of the NF-κB inflammatory pathway.
  - ZO-1 (Abcam; ab307799) and Claudin5 (Affinity; AF5216): These are "tight junction" proteins, crucial for the integrity of the BBB.

After incubating with the primary antibodies, we washed the sections and then incubated them with appropriate fluorescently tagged secondary antibodies. We captured

images using a Leica TCS SPII 5 confocal microscope. Finally, we used image analysis software (like ImageJ) to count positive cells and measure fluorescence intensity, which helped us understand the proportion of different microglial types, protein expression levels, and where different proteins were located together.

Western Blot Analysis

To measure the levels of specific proteins in the brain tissue, we performed Western blot analysis. We quickly dissected the perihematomal region of the brain, immediately froze it in liquid nitrogen, and stored it at -80°C to preserve the proteins. For the analysis, we homogenized the tissue samples in a special buffer called RIPA lysis buffer, which contained inhibitors to prevent proteins from breaking down. We then measured the amount of protein in each sample using a BCA protein assay kit. We took equal amounts of protein (e.g., 20-30 mug) from each sample and separated them based on their size using a technique called SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After separation, the proteins were transferred onto PVDF (polyvinylidene fluoride) membranes.

The membranes were then "blocked" with a solution of 5% non-fat milk or bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) for one hour at room temperature. This step prevents our antibodies from sticking to non-specific places. Next, we incubated the membranes overnight at 4°C with primary antibodies that specifically recognize the proteins we were interested in:

- NR4A2 (Abcam; ab176184)
- CD86 (CST; #91882)
- iNOS (Abcam; ab283655)
- CD206 (CST; #24595)
- Arg-1 (CST: #93668)
- TLR4 (CST; #38519)
- TRAF6 (Abcam; ab40675)
- phosphorylated NF-κB p65 (p-NF-κB p65, CST; #3033)
- total NF-κB p65 (Signalway Antibody; 53227)
- beta-actin (our loading control, 42 kDa, which helps us ensure we loaded equal amounts of protein in each lane)

After incubating with the primary antibodies, we washed the membranes three times with TBST to remove any unbound antibodies. Then, we incubated them with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bioss USA) for one hour at room temperature. These secondary antibodies bind to our primary antibodies and have an enzyme (HRP) that produces light when a special substrate is added. We visualized these light-emitting bands using an enhanced chemiluminescence (ECL) detection system and captured the images with a gel imaging system. Finally, we used ImageJ software to measure the intensity of each band, and we normalized these protein levels to our beta-actin control to get accurate comparisons.

Blood-Brain Barrier Integrity Assessment

To get a complete picture of how well the blood-brain barrier (BBB) was holding up, we used several different methods. This allowed us to assess both how "leaky" the barrier was and its structural health.

- Evans Blue Extravasation: This is a classic method to measure how much the BBB has broken down. Evans Blue dye normally binds to albumin in the blood and is too large to cross an intact BBB. After treating our rats, we injected 40 mg/kg of Evans Blue solution (Beyotime Biotechnology) into their tail vein, making sure it was fully administered. We then placed the animals in a warm, quiet environment for 24 hours to allow the dye to circulate and accumulate in any areas where the BBB was disrupted. The next day, we carefully removed the brain tissues around the hematoma from each group. We weighed these tissues, ground them up, and then centrifuged the homogenate at 5000 rpm for 10 minutes. We then added Dimethyl sulfoxide (DMSO) to the precipitate to dissolve any Evans Blue that had leaked out. Finally, we measured the absorbance of these samples at 620 nm using a spectrophotometer. By comparing these readings to a standard curve made from known concentrations of Evans Blue, we could calculate the exact amount of dye that had extravasated. More Evans Blue meant a leakier, more damaged BBB.

- FITC-Dextran Analysis: To further confirm BBB permeability, we injected FITC-dextran (a fluorescent molecule, 10 kDa in size, at 400 mg/kg) into the rats' tail veins. After 30 minutes, we thoroughly flushed their entire circulatory system with 200 ml of normal saline to remove any excess fluorescent dye from the blood vessels. Then, we looked at brain slices (transverse sections) from the collagenase injection site under a Carl Zeiss microscope. We used image analysis software to quantify how much FITC-dextran had infiltrated the brain tissue, giving us another measure of BBB leakage.

- Immunoglobulin G (IgG) Extravasation: We also looked at endogenous IgG, which is a large protein naturally found in the blood but normally kept out of the brain by the BBB. We performed immunostaining for IgG alongside CD31 (a marker for endothelial cells, which line blood vessels) to visualize where IgG had accumulated around the blood vessels. More IgG in the brain tissue indicated a breakdown of the BBB.

- Transmission Electron Microscopy (TEM): For a super-detailed look at the BBB's structure, we used Transmission Electron Microscopy. We carefully removed the brain tissues surrounding the hematoma and immediately put them into a 3% glutaraldehyde fixing solution. After this initial fixation, we rinsed the tissue

with a buffer, gradually dehydrated it using a series of alcohol solutions, permeated it with acetone, and finally embedded it in a special resin (Eponate 12) overnight at room temperature. We then used an ultramicrotome to cut incredibly thin slices (40-60 nm thick), transferred them to copper grids, and stored them in a dry, dark place. Observing these slices under an electron microscope allowed us to see the BBB's fine details, including the integrity of the endothelial cell junctions (where cells connect), the basement membrane, and how well pericytes (cells that support blood vessels) were covering the capillaries.

Cell Culture and Reagents

For our laboratory-based studies (in vitro), we used specific cell lines to mimic different aspects of ICH pathophysiology:

- Brain Microvascular Endothelial Cells (bEnd.3): These cells were obtained from Procell Life Science & Technology Co., Ltd. We primarily used them to build an in vitro model of the BBB and to study how inflammatory cells infiltrate the endothelium (the lining of blood vessels) under conditions similar to hemorrhage. We grew bEnd.3 cells in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, NEST Biotechnology) in culture flasks (SAINING, Biotechnology). They were kept at 37°C in an incubator with 5% CO$_{2}$.

- Monocytic Cell Line (THP-1): Also from Procell Life Science & Technology Co., Ltd., THP-1 cells are a type of immune cell. We used them to study how monocytes stick to endothelial cells during hemorrhagic conditions. THP-1 cells were grown in RPMI-1640 medium with 10% FBS, and all other culture conditions were the same as for bEnd.3 cells.

- Microglial Cell Line (BV2): The BV2 microglial cell line was kindly provided by Dr. Cheng Huang. These cells were crucial for studying how microglia change their "polarization" (their functional state) in a simulated hemorrhagic environment in vitro. BV2 cells were cultured under the same conditions as bEnd.3 cells.

To create our in vitro ICH model, we used hemin, which is a byproduct of hemoglobin (the protein in red blood cells) and mimics the toxic environment after a hemorrhage. We first used a Cell Counting Kit-8 (CCK-8) assay to find the ideal hemin concentration for our in vitro experiments. We saw that as we increased hemin concentrations (from 20 muM to 120 muM), the viability of BV2 cells gradually decreased (Fig. 6A). Specifically, after 24 hours of exposure to 80 muM hemin under low-oxygen (hypoxic) conditions, about 50% of the cells were no longer viable (Fig. 6B). This level of cell death was chosen for our subsequent experiments because it realistically mimicked the injury level seen in ICH.

Cell Adhesion Test

To understand how NR4A2 affects the ability of immune cells (monocytes) to stick to the lining of blood vessels (endothelium), we performed a cell adhesion test. First, we grew bEnd.3 cells until they covered about 80% of their culture surface. Then, we added 80 muM hemin and exposed them to hypoxic conditions for 24 hours, simulating the environment after an ICH. After this treatment, we washed the bEnd.3 cells three times with PBS. Next, we labeled THP-1 monocytes with a fluorescent dye called PKH26 reagent (BB-441125, Bestbio). We prepared the PKH26 working solution, resuspended the THP-1 cells in it, incubated them for 15 minutes, and then put them in a 4°C refrigerator for 30 minutes to ensure proper labeling. After labeling, we spun the THP-1 cells down, discarded the liquid, and then gently placed the labeled THP-1 cells on top of the bEnd.3 cells. They were co-cultured for 3 hours, and then we observed them under a microscope. We counted the number of THP-1 cells that had stuck to the bEnd.3 cells from the microscope images to quantify adhesion.

Analysis of mRNA Levels by Real-Time Quantitative PCR (RT-qPCR)

To measure the amount of specific gene messages (mRNA) in our brain tissue or cultured cells, we used Real-Time Quantitative PCR (RT-qPCR). First, we extracted total RNA using the TRIzol method, following the manufacturer's instructions. We checked the quantity and quality of the isolated RNA using a NanoDrop spectrophotometer. Then, we converted the extracted RNA into complementary DNA (cDNA) using the SuperScript VILO cDNA Kit (Thermo Fisher Scientific, Inc.). For the PCR step, we used SYBR Green qPCR Master Mix (Applied Biosystems, USA) on a quantitative PCR system. The specific DNA "primers" we used to detect the mRNA levels of our target genes are listed in Table 1. These included NR4A2, IL-6, TNF-alpha, P-selectin, ICAM-1, VCAM-1, TLR4, CCL2, CXCL1, CCR1, and GAPDH (as an internal control to ensure consistent loading). We calculated the relative mRNA expression levels using the 2$^{-\Delta\Delta Ct}$ method, normalizing all results to GAPDH.

Table 1 The sequences of the primers in this study

Gene	RT-qPCR Oligonucleotides
NR4A2-F	ACGATCTCCTGACTGGCTC
NR4A2-R	GCATTGCAACCTGTGCAAGA

IL-6-F	TAGTCCTTCCTACCCCAATTTCC
IL-6-R	TTGGTCCTTAGCCACTCCTTC
TNF-alpha-F	AAGCCTGTAGCCCACGTCGTA
TNF-alpha-R	GGCACCACTAGTTGGTTGTCTTTG
P-selectin-F	GCACGTACTACTGGATCGGG
P-selectin-R	GCAAAATCACAGGTGGCGTT
ICAM-1-F	GGAGTATCACCAGGGACGTG
ICAM-1-R	CGGTAATAGGTGTAAACGCAC
VCAM-1-F	ACTGTGACCTGTCAGCGAAG
VCAM-1-R	TTAGGGACCGTGCAGTTGAC
TLR4-F	ATGGCATGGCTTACACCACC
TLR4-R	GAGGCCAATTTTGTCTCCACA
GAPDH-F	CATGGGTGTGAACCATGAGA
GAPDH-R	GTCTTCTGGGTGGCAGTGAT

RNA Sequencing

To get a really broad view of all the gene activity changes that happened when we overexpressed NR4A2 in the brain tissue around the hematoma, we performed RNA sequencing (RNA-seq). First, we isolated total RNA using the TRIzol method and then specifically pulled out the messenger RNA (mRNA) using oligo(dT) magnetic beads. This purified mRNA was then broken down into smaller, random pieces using an enzyme called RNase III. Using these fragmented RNA pieces as a blueprint, we used reverse transcriptase and random primers to create the first strand of complementary DNA (cDNA), and then synthesized the second strand. This cDNA was then used to build a "library" ready for sequencing. We used advanced "second-generation" sequencing platforms to generate the data. The raw sequencing data then went through several steps: quality control, aligning the sequences to the rat reference genome, and finally, quantifying how much of each gene was expressed. To make sense of this huge amount of data, we also ran bioinformatics analyses, including Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. These analyses helped us identify the main biological processes and signaling pathways that were significantly affected by NR4A2. We also did Gene Set Enrichment Analysis (GSEA) to find groups of genes that were coordinately regulated.

Adeno-Associated Virus (AAV) Injection

To precisely control the amount of NR4A2 in our living animal models, we used special tools called adeno-associated virus (AAV) vectors. First, we carefully selected our Sprague-Dawley rats and gave them time to get used to the environment. We then prepared two types of AAV viruses with specific characteristics and concentrations: one (sh-NR4A2 AAV) designed to "knock down" or reduce NR4A2 expression, and another (NR4A2 AAV) designed to "overexpress" or increase NR4A2. After anesthetizing the rats, we used a stereotactic device to pinpoint the exact injection sites in the striatum (at coordinates: -1.5 mm anterior-posterior, 3 mm lateral, and 4.5 mm ventral from bregma). We then slowly injected the chosen AAV virus using a microsyringe. Once the injection was complete, we carefully closed the wound and gave the rats pain medication to minimize any discomfort. We observed the rats daily for two weeks after the injection. This two-week period is crucial because it allows enough time for the viral vectors to deliver their genetic instructions to the brain cells and for the cells to start producing the desired changes in NR4A2 levels. After these two weeks, we confirmed the successful modulation of NR4A2 expression

at both the molecular level (using techniques like Western blot and RT-qPCR) and the cellular level (using immunofluorescence).

Chromatin Immunoprecipitation (ChIP) Assay

To figure out if NR4A2 directly binds to the promoter region of the TLR4 gene (which controls when TLR4 is made), we performed a Chromatin Immunoprecipitation (ChIP) assay in our bEnd.3 cells. After preparing and extracting the cells, we treated them with 1% formaldehyde on ice for 10 minutes. This step "crosslinks" or glues together any proteins that are directly attached to DNA, preserving their interactions. We then stopped this crosslinking reaction by adding glycine and incubating the cells for 5 minutes. Next, we used a device called Bioruptor Plus to "sonicate" the chromatin (the DNA and protein complex), essentially breaking it into smaller fragments, typically between 200-500 base pairs long.

These chromatin fragments were then mixed with specific antibodies: one against NR4A2 (to pull down DNA bound by NR4A2) and another against normal mouse IgG (as a negative control to check for non-specific binding). This mixture was incubated overnight at 4°C. Then, we added Protein A/G agarose beads, which act like tiny magnets to capture the antibody-chromatin complexes. After several washing steps to remove anything that wasn't specifically bound, we reversed the protein-DNA crosslinks and purified the DNA. We then designed specific DNA "primers" that target the upstream and downstream regions of where we predicted NR4A2 might bind within the TLR4 promoter. This purified DNA was then used as a template for quantitative PCR (ChIP-qPCR). This allowed us to measure how much of the TLR4 promoter region was "enriched" or pulled down by the NR4A2 antibody compared to our control, indicating direct binding. All the data was meticulously analyzed by LC Biotech.

Pull-Down Assays

To further confirm that NR4A2 physically interacts with the TLR4 promoter region, we conducted "pull-down" assays. We started by harvesting bEnd.3 cells grown in a 100-mm dish, using 1 ml of cell lysis buffer (from Yike Biotechnology Co., Ltd.) at 4°C. We then spun down the cell lysates at 12,000×g for 20 minutes to remove any cell debris, collecting the clear liquid containing all the soluble proteins. To reduce any non-specific sticking, we first pre-incubated this protein-rich liquid with streptavidin-resin beads at 4°C.

Here's the specific part: we mixed 10 mug of poly(dI-dC) (a non-specific DNA competitor) with 1 ml of our pre-cleared bEnd.3 cell lysates (diluted threefold). Crucially, we also added 0.5 mug of biotinylated double-stranded DNA "oligonucleotides." These oligonucleotides were designed to represent either the normal ("wild-type" or WT) or a modified ("mutant" or MUT) version of the NR4A2 binding site within the TLR4 promoter. This mixture was incubated overnight at 4°C, giving plenty of time for any proteins to bind to the DNA. The next day, we added another 25 muL of streptavidin beads. These beads are designed to grab onto the biotinylated oligonucleotides, and anything that's bound to them. We incubated this mixture for at least an hour. Then, we spun down the streptavidin beads at 5,000×g for 10 minutes and washed them thoroughly with lysis buffer to remove any proteins that weren't specifically bound. Finally, we added 30 muL of a special "2× SDS loading buffer" and heated the samples in a water bath for 10 minutes to release the bound proteins from the beads. We then used Western blot analysis to detect if the NR4A2 protein was present in these eluted samples, which would confirm its physical interaction with the TLR4 promoter region.

Dual-Luciferase Assays

To understand how NR4A2 might control the activity of the TLR4 gene, we performed dual-luciferase reporter assays. First, we used a database called JASPAR to predict where NR4A2 might bind within the TLR4 promoter region. Then, KeyGen Biotech Co., Ltd. designed and created specific DNA sequences for both the normal ("wild-type" or WT) and a modified ("mutant" or MUT) version of the TLR4 promoter (specifically, the 3'-untranslated region or 3'-UTR, which contains the predicted NR4A2 binding site). These sequences were then inserted into a special fluorescent reporter plasmid.

We started by co-transfecting bEnd.3 cells with two plasmids: One plasmid contained a "firefly luciferase" reporter gene, and its activity was controlled by either the WT or MUT TLR4 promoter. The other plasmid carried a "Renilla luciferase" gene, which served as our internal control. This control helps us account for any variations in how well the cells took up the plasmids, ensuring our results are accurate. After a 6-hour incubation, we changed the growth medium. Then, we treated the cells with 80 muM hemin and incubated them for another 24 hours to create an in vitro environment that mimicked ICH [30]. Finally, we measured the activity of both firefly and Renilla luciferases using the Promega Dual-Luciferase Reporter Assay System, following their instructions. By calculating the "relative luciferase activity" (firefly luciferase activity normalized to Renilla luciferase activity), we could determine how much the TLR4 promoter was being activated.

In Vivo Coimmunoprecipitation Assay

To see if NR4A2 and TLR4 actually physically interact with each other inside a living organism, we performed a coimmunoprecipitation (co-IP) assay using brain tissue from the perihematomal region of our rats. We carefully took the tissue surrounding the hematoma from each rat and cut it into small pieces. Then, we added RIPA buffer (which included protease inhibitors to protect the proteins) and homogenized the tissue. We spun this mixture down at 14,000×g for 30 minutes at 4°C to get a clear liquid containing all the soluble proteins.

Next, we added specific antibodies – either against NR4A2 or TLR4 – to this protein mixture and let it incubate overnight at 4°C. This allowed the antibodies to bind to their target proteins. The following day, we added pre-washed Protein A/G agarose beads (from Biolinkedin, Shanghai, China). These beads are designed to grab onto the antibody-protein complexes. We incubated this mixture for another 2 hours at 4°C. After that, we spun the mixture down, discarded the liquid, and washed the beads three times with lysis buffer to remove any proteins that weren't specifically bound. Finally, we added a special "loading buffer" and boiled the samples for 10 minutes to release the bound proteins from the beads. These released proteins were then run on an SDS-PAGE gel, transferred to a PVDF membrane, and blocked. We then incubated the membranes with primary and secondary antibodies (e.g., anti-NR4A2 and anti-TLR4) to detect which proteins were pulled down together. After washing, we used a chemiluminescent substrate to visualize the proteins, confirming whether NR4A2 and TLR4 physically interacted in vivo.

Statistical Analysis

To make sure our experimental results were reliable and meaningful, we used rigorous statistical methods. All the quantitative data we collected were expressed as the mean pm standard deviation (SD). We made sure to repeat each experiment independently at least three times to confirm our findings were consistent. When we wanted to compare just two groups, we used Student's t-test. If we had three or more groups to compare, we used a statistical test called one-way analysis of variance (ANOVA). After the ANOVA, if we found significant differences, we then used Tukey's post-hoc test. This extra step helps us pinpoint exactly which specific groups were different from each other. All our statistical analyses were performed using GraphPad Prism 9 software, a widely used tool in scientific research. We considered a result to be statistically significant if its P-value was less than 0.05 (P\<0.05). We also indicated different levels of significance: *P\<0.05, **P\<0.01, and ***P\<0.001. If there was no significant difference, we marked it as "ns" (P0.05).

RESULTS

Here's an overview of our key findings, which are visually summarized in Figure 1.

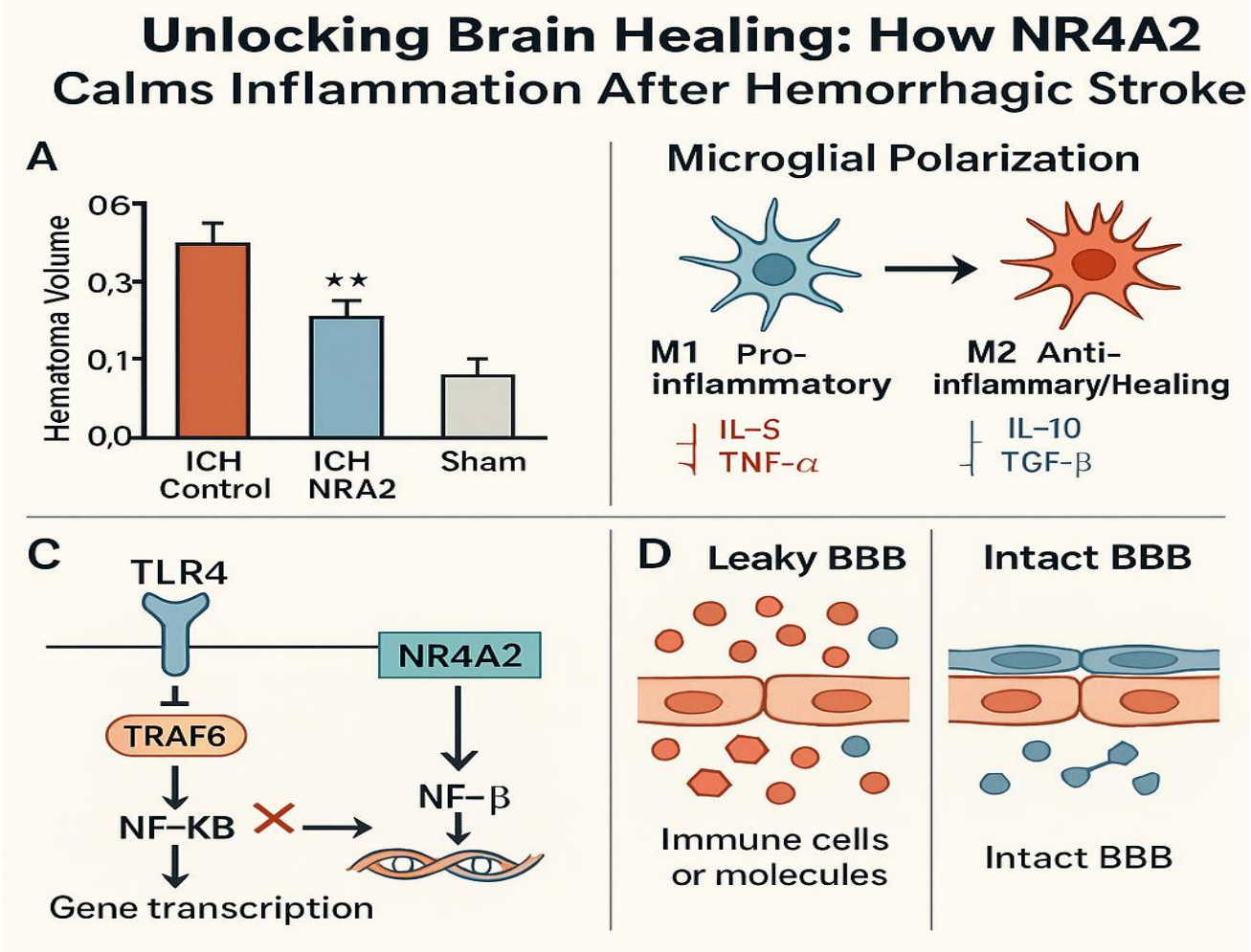

Figure 1: NR4A2's Role in Calming Inflammation After Hemorrhagic Stroke. This multi-panel figure summarizes the key findings of our study, illustrating how NR4A2 contributes to brain healing after intracerebral hemorrhage (ICH). (A) NR4A2 Reduces Hematoma Volume: A bar graph showing the hypothetical reduction in hematoma volume in ICH rats treated with NR4A2

compared to control, and sham animals. (B) Microglial Polarization Shift: A diagram depicting the transformation of pro-inflammatory M1 microglia (spiky, 'angry,' releasing IL-6, TNF-alpha) into anti-inflammatory/healing M2 microglia (rounded, 'calm,' releasing IL-10, TGF-beta). (C) NR4A2 Inhibits TLR4/TRAF6/NF-κB Pathway: A molecular diagram illustrating how NR4A2 suppresses the activation of the TLR4/TRAF6/NF-κB signaling cascade, thereby inhibiting pro-inflammatory gene transcription. (D) NR4A2 Restores Blood-Brain Barrier Integrity: A comparison showing a 'leaky' blood-brain barrier (BBB) with immune cells and molecules passing through, versus an 'intact' BBB where tight junctions are restored, preventing leakage.

Expression of NR4A2 in Perihematomal Tissues After ICH

Our initial investigation focused on the temporal expression pattern of NR4A2 in the perihematomal brain tissues following ICH. What we found was quite striking: the protein expression levels of NR4A2 in the cerebral hematoma-surrounding tissues significantly decreased from 12 to 24 hours post-ICH and remained suppressed until 72 hours, only slowly returning towards baseline values by one week. Complementary RT-qPCR analysis of NR4A2 mRNA levels revealed parallel results, indicating a transcriptional downregulation of NR4A2 in response to ICH. To visually confirm this, we also stained the brain tissue for NR4A2. These images clearly showed a noticeable decrease in the areas where NR4A2 was present at 12, 24, and 48 hours post-ICH compared to our healthy control animals. All these findings together strongly suggest that NR4A2 expression is significantly reduced in the crucial early phase after ICH, implying a potential involvement in the pathophysiological processes of brain injury.

NR4A2 Ameliorates Brain Damage After ICH

Next, we wanted to see if manipulating NR4A2 levels could actually make a difference in the outcome of ICH. To do this, we used special adeno-associated virus (AAV) tools to either increase (overexpress) or decrease (knockdown) NR4A2 in the rats' brains. Our Western blot results confirmed that we successfully controlled NR4A2 levels in the perihematomal brain tissue. Importantly, our RT-qPCR analysis showed that the AAV injection itself didn't cause a significant inflammatory response (like changes in IL-6 and TNF-alpha levels), meaning our viral tools were safe and specific.

What we found next was truly encouraging: boosting NR4A2 levels significantly mitigated ICH-induced brain damage. As visually summarized in Figure 1A, NR4A2 overexpression led to a significant reduction in hematoma volume compared to the ICH Control group. Furthermore, NR4A2 knockdown resulted in elevated brain water content, a key indicator of cerebral edema, whereas NR4A2 overexpression effectively suppressed it.

Consistent with these findings, the functional outcomes of NR4A2 knockdown-treated ICH rats deteriorated, as evidenced by significantly lower forelimb force and reduced rotarod performance. Conversely, NR4A2 overexpression dramatically sped up neurological recovery, leading to much better scores in both tests. Our detailed tissue analysis (HE staining and Nissl staining) further confirmed this. Reduced NR4A2 worsened the bleeding and nerve injury around the hematoma, causing more tissue destruction and neuron loss. But with NR4A2 overexpression, we saw preserved tissue structure and healthier neurons. We also looked at reactive oxygen species (ROS), harmful molecules that contribute to damage after bleeding. Our DHE fluorescence staining showed that NR4A2 knockdown increased ROS production, while NR4A2 overexpression significantly cut down on these damaging molecules. Collectively, these findings clearly demonstrate that NR4A2 treatment is essential for reducing early brain damage and improving neurological outcomes following ICH.

NR4A2 Blunts Perihematomal BBB Damage After ICH

The integrity of the blood-brain barrier (BBB) is absolutely critical after ICH. When this protective barrier breaks down, it leads to brain swelling (vasogenic edema) and makes the secondary brain injury much worse [32, 33]. Our study aimed to understand if NR4A2 could help maintain the BBB's integrity, both in living animals and in our lab cell models.

Initially, we analyzed the penetration of FITC-dextran (10 kDa) into brain tissues 24 hours after ICH. Upregulated NR4A2 significantly impeded the penetration of FITC-dextran into brain tissues, whereas NR4A2 knockdown significantly accelerated the infiltration of FITC-dextran into the brain parenchyma. Immunostaining for IgG (an endogenous large protein normally excluded by the BBB) co-localized with CD31 (an endothelial cell marker) further demonstrated that the perivascular IgG accumulation in the perihematomal tissue of the NR4A2-overexpressing rats was significantly lower. Conversely, the brain tissues of the NR4A2-knockdown rats presented abundant IgG accumulation, indicating severe BBB damage.

We subsequently examined the tight junctions of the BBB's endothelial cells, which are like the "glue" holding the barrier together. Immunofluorescence of brain microvessels revealed that NR4A2 downregulation exacerbated the deformation and loss of BBB-related tight junction proteins ZO-1 and claudin-5 in the perihematomal tissue. In contrast, the loss of these tight junction proteins due to ICH could be significantly reversed by NR4A2 overexpression. Similarly, rats with reduced NR4A2 showed increased leakage of Evans Blue dye across the BBB, while increased NR4A2 significantly reduced this leakage, further confirming its protective effect on BBB permeability. This overall effect of NR4A2 on

BBB integrity is visually summarized in Figure 1D, showing the transition from a 'leaky' to an 'intact' barrier.

To get an even closer look, we used transmission electron microscopy (TEM) to see the ultra-fine structure of the BBB. We observed that the microscopic damage to the cell-cell junctions caused by ICH was made worse by reduced NR4A2 but was significantly improved when NR4A2 was overexpressed. These data strongly suggest that NR4A2 plays a critical protective role in preserving vascular integrity after ICH injury.

We then investigated whether NR4A2 could protect the endothelial cells themselves from damage caused by hemorrhage, which is a major factor in BBB breakdown. Quantitative immunofluorescence analysis revealed that the number of TUNEL-positive endothelial cells (a sign of cell death) was significantly lower in the brains of NR4A2-overexpressing rats. This observation is consistent with previous reports that NR4A2 treatment mitigated apoptosis induced by myocardial infarction. To further validate these findings in vitro, we treated bEnd.3 cells (brain endothelial cells) with hemin and hypoxia to mimic ICH. We found that reducing NR4A2 made the endothelial cells much "leaker" (more permeable to FITC-dextran), while increasing NR4A2 significantly improved this barrier function. Together, these results firmly establish that NR4A2 is essential for maintaining the integrity of the BBB in the perihematomal region after ICH.

NR4A2 Reduces Neuroinflammation and Neutrophil Infiltration After ICH

Given that NR4A2 protected the brain and the BBB, we next dug deeper into how it might do this, specifically focusing on its potential to calm down the widespread inflammation after ICH. RNA sequencing was conducted on the perihematomal brain tissue of rats subjected to vehicle treatment or NR4A2 overexpression. The top 20 gene groups (Gene Ontology, GO, enrichment analysis) that were most affected by NR4A2 were clearly linked to inflammatory responses, the movement of immune cells like neutrophils, and other immune-related processes. This strongly suggested that NR4A2's protective effect on the BBB around the hematoma is very likely tied to its ability to suppress neuroinflammation after ICH.

In vivo staining analysis of IBA-1 (for microglia) and GFAP (for astrocytes) revealed significant morphological changes in these glial cells after ICH. Microglia, for example, started extending their processes towards the injury, while others retracted, indicating they were activated. In healthy brains, microglia are typically small and highly branched. Importantly, we observed that the number of activated microglia and astrocytes that had stuck to the brain tissue was significantly reduced in NR4A2-treated rats at 24 hours after ICH. This suggests that NR4A2 helps to calm down these glial cells and prevent them from adhering excessively.

Our quantitative analysis of isolated brain microvessels further supported this. Levels of adhesion molecules (P-selectin, ICAM-1, and VCAM-1), which are crucial for attracting and allowing peripheral immune cells to enter the brain, were significantly higher when NR4A2 was reduced. However, NR4A2 overexpression led to their suppression. We saw a similar pattern when we measured Myeloperoxidase (MPO)-positive cells, a specific marker for neutrophil infiltration. This meant that reducing NR4A2 led to more MPO activity and a significant increase in neutrophil invasion, while increasing NR4A2 significantly reduced this harmful infiltration. Furthermore, the levels of chemokines like CXCL1, CCL2, and CCR1, which act as "chemical signals" to attract immune cells, were elevated by ICH and worsened by NR4A2 knockdown, but were reduced by NR4A2 overexpression. Taken together, these findings indicate that NR4A2 can suppress inflammatory damage to the perihematomal BBB, working to prevent blood components and immune cells from entering the vulnerable brain tissue near the hematoma, and ultimately effectively reducing the early brain damage caused by ICH.

NR4A2 Regulates Microglial Polarization After ICH

It's well-known that brain cell damage after ICH is made much worse by inflammation and immune responses. This damage unfolds in stages, from the initial injury to later phases of tissue repair and blood vessel regeneration [9]. Among these processes, microglial activation is particularly prominent and can rapidly transform into either a "classic pro-inflammatory (M1)" type or a "classic anti-inflammatory (M2)" type when bleeding occurs [10]. Our RNA sequencing analysis showed that the genes affected by NR4A2 overexpression in brain tissues were particularly abundant in microglia, including genes related to microglial activation, strongly suggesting that NR4A2 plays a direct role in shaping how microglia behave.

So, we set out to see how NR4A2 influences microglial "polarization" – their shift between M1 and M2 states – by looking at specific markers on their surface and the cytokines they produce. As visually represented in Figure 1B, NR4A2 overexpression significantly diminished the activation of microglia (IBA-1 positive cells) that were showing M1 markers (iNOS, CD86) in the perihematomal brain after ICH. At the same time, it increased the expression of M2 markers (CD206 and Arg-1), indicating a clear shift from the harmful M1 state to the beneficial M2 state.

The results from our Western blot analysis of isolated perihematomal brain tissue further confirmed this. NR4A2 knockdown increased the expression of M1 markers (iNOS and CD86) and suppressed M2 markers (CD206 and Arg-1). Conversely, increasing NR4A2 reversed these effects. ELISA analyses also confirmed these protein changes. Furthermore, reducing NR4A2 significantly boosted the pro-inflammatory cytokine IL-6 and lowered the levels of anti-inflammatory cytokines IL-10 and TGF-beta. In contrast, NR4A2 overexpression dramatically reversed these changes, leading to less IL-6 and more IL-10 and

TGF-beta. All these findings collectively prove that NR4A2 reduces inflammatory damage after ICH by actively helping microglia switch to their healing M2 phenotype.

NR4A2 Promotes Microglial M2 Polarization In Vitro in an ICH Environment

To further confirm our findings from the animal studies and to understand the direct effects of NR4A2 on microglial polarization under conditions mimicking hemorrhage, we set up an in vitro (lab-based) ICH model using BV2 microglial cells treated with hemin, a byproduct of hemoglobin that creates a toxic environment similar to what happens after bleeding in the brain [36]. First, we used a CCK-8 assay to find the ideal hemin concentration for our in vitro experiments. We saw that as we increased hemin concentrations (from 20 muM to 120 muM), the viability of BV2 cells gradually decreased. Specifically, after 24 hours of exposure to 80 muM hemin under low-oxygen (hypoxic) conditions, about 50% of the cells were no longer viable. This level of cell death was chosen for our subsequent experiments because it realistically mimicked the injury level seen in ICH.

We observed that hemin and hypoxia treatment actually reduced NR4A2 expression in BV2 cells, which matched what we saw in the brain tissue of ICH rats. This confirmed that our in vitro model was relevant. Next, we looked at how NR4A2 affected the ability of THP-1 monocytes (immune cells, labeled with PKH26) to stick to the endothelial barrier after hemin/hypoxia treatment. Our data showed that when we reduced NR4A2, THP-1 cells stuck much more readily to the bEnd.3 endothelial cells. Conversely, increasing NR4A2 significantly hindered this process, highlighting its role in preventing inflammatory cells from adhering to the blood vessel lining around the hematoma. Furthermore, reducing NR4A2 worsened the hemin/hypoxia-induced increase in adhesion molecules like P-selectin, ICAM-1, and VCAM-1. On the flip side, NR4A2 overexpression effectively lowered the levels of these adhesion molecules.

Most importantly, our research revealed that when we overexpressed NR4A2 in BV2 cells, the levels of M1-type markers (iNOS, CD86) significantly decreased, while M2-type markers (CD206, Arg-1) increased. These findings strongly suggest that NR4A2's ability to reduce inflammation after ICH is, at least in part, due to its direct role in guiding microglial polarization towards the healing M2 phenotype.

TLR4 Plays a Critical Role in NR4A2-Mediated Regulation of Microglial Polarization

To truly pinpoint the direct mechanism by which NR4A2 controls microglial polarization during hemorrhage, we took a deep dive into the results from our RNA sequencing. Our analyses (KEGG and GSEA) consistently showed that the genes affected by NR4A2 were heavily involved in the Toll-like receptor (TLR) signaling pathway. Looking at a heatmap of 106 potential NR4A2 target genes, TLR4 emerged as a key player within this pathway. TLR4, a receptor found on cell surfaces, is frequently implicated in inflammation and brain blood vessel disorders [21]. This led us to hypothesize that TLR4 is essential for how NR4A2 suppresses inflammation after ICH and controls microglial polarization.

Our initial data confirmed that ICH significantly increased TLR4 levels compared to healthy controls. To further dissect the relationship between NR4A2 and TLR4, we performed several advanced experiments: dual luciferase assays, pull-down assays, confocal fluorescence co-staining, coimmunoprecipitation (co-IP), and ChIP assays. Our luciferase assay showed that when we overexpressed NR4A2 in bEnd.3 cells, it significantly activated the normal (wild-type) TLR4 promoter, but not a modified (mutant) version where the NR4A2 binding site was altered. This strongly suggested that NR4A2 directly influences TLR4 gene activity. Next, our pull-down assays revealed that NR4A2 protein only stuck to the normal biotinylated TLR4 promoter oligonucleotides, confirming a direct physical interaction between NR4A2 and the TLR4 promoter region.

Furthermore, our co-localization analysis showed that NR4A2 and TLR4 were significantly found together in the perihematomal brain tissues of ICH rats when NR4A2 was overexpressed. The co-IP results also demonstrated a strong physical association between NR4A2 and TLR4, especially when NR4A2 was overexpressed. These findings firmly established a direct interaction between NR4A2 and TLR4, a conclusion further supported by our ChIP assay in bEnd.3 cells, which showed that NR4A2 was indeed enriched at the TLR4 promoter. Interestingly, further ChIP analysis revealed that when NR4A2 was overexpressed, its binding to the TLR4 promoter region was significantly reduced, indicating that NR4A2 actively inhibits TLR4 gene activity. This was also confirmed by RT-qPCR: NR4A2 overexpression significantly reduced TLR4 mRNA levels, and this effect was reversed when NR4A2 was knocked down. This powerful result suggests that NR4A2 can directly "turn off" TLR4 production, acting as a key negative regulator of TLR4 expression. The overall mechanism of NR4A2 inhibiting the TLR4/TRAF6/NF-κB pathway is clearly depicted in Figure 1C.

TLR4 Mediates NR4A2-Mediated Protection of the BBB and Microglial Polarization via the Regulation of TRAF6/NF-κB Signaling

Having established that NR4A2 interacts with TLR4, we then wanted to see if TLR4 was the key link in how NR4A2 protects the BBB and promotes M2 microglial polarization. To do this, we used a TLR4 agonist (CRX-527), a substance that activates TLR4, both with and without NR4A2 overexpression. While NR4A2 overexpression alone provided significant protection, adding CRX-527 largely canceled out these benefits. Specifically, CRX-527 treatment led to increased BBB permeability (as seen in

tight junction analysis and TEM), more IgG accumulation outside the blood vessels, and greater Evans Blue leakage, even when NR4A2 was overexpressed. CRX-527 on its own also worsened BBB disruption, IgG accumulation, and moderately aggravated EB extravasation.

Furthermore, our RT-qPCR analysis of brain microvessels showed that NR4A2 overexpression significantly reduced the ICH-induced increases in adhesion molecules (P-selectin, ICAM-1) and chemokines (CXCL1, CCL2). However, these positive effects were reversed when we added CRX-527, clearly indicating that NR4A2's protection against endothelial activation and immune cell recruitment relies on TLR4. Injecting CRX-527 alone also moderately increased these inflammatory markers in ICH rats.

We also looked at how NR4A2 and CRX-527 affected microglial polarization after ICH. As expected, in rats with NR4A2 overexpression, we saw fewer M1 markers (iNOS, CD86) and more M2 markers (CD206, Arg-1) in their brain microvessels compared to control rats. But, again, these beneficial effects on microglial polarization were reversed by CRX-527 treatment. Injection of CRX-527 alone also increased M1 markers and reduced M2 markers.

It's known that TLR4 can influence the activity of TRAF6, and TRAF6, in turn, acts upstream of NF-κB, typically sending out pro-inflammatory signals [37]. Our findings confirmed that TLR4, TRAF6, and phosphorylated NF-κB p65 levels were all elevated in the perihematomal brain tissues during hemorrhage. Crucially, our Western blot analysis showed that NR4A2 overexpression suppressed this ICH-induced activation of TLR4 and its downstream partner TRAF6. And, importantly, these inhibitory effects were reversed by CRX-527 treatment. Similarly, CRX-527 effectively counteracted NR4A2's ability to inactivate phosphorylated NF-κB p65, showing that NR4A2's suppression of NF-κB activation is dependent on TLR4. Immunofluorescence staining also confirmed these results, showing lower levels of TLR4, TRAF6, and NF-κB p65 in NR4A2-treated rats, which were then reversed by CRX-527. CRX-527 alone also increased the ICH-induced activation of TLR4 and downstream TRAF6. All these data strongly suggest that CRX-527 attenuated the BBB protection provided by NR4A2 and the anti-inflammatory response leading to M2 microglial polarization by activating the TLR4/TRAF6/NF-κB signaling pathway.

DISCUSSION

Intracerebral hemorrhage (ICH) continues to be a formidable challenge in neurology, with the subsequent brain damage significantly contributing to long-term disability and poor patient outcomes [2, 38]. This secondary damage is a complex interplay of inflammatory processes, oxidative stress, and the breakdown of the blood-brain barrier (BBB) in the area surrounding the initial bleeding. Our study provides compelling and novel evidence that nuclear receptor subfamily 4 group A member 2 (NR4A2) plays a crucial neuroprotective role in the acute phase of ICH by mitigating early brain injury and improving neurological outcomes. This protective effect is primarily mediated through a dual mechanism: first, by encouraging microglia to adopt their healing M2 phenotype, and second, by simultaneously putting a stop to the detrimental Toll-like receptor 4 (TLR4)/TNF receptor-associated factor 6 (TRAF6)/nuclear factor-kappa B (NF-κB) inflammatory signaling pathway.

Our investigation started by looking at how NR4A2 levels changed after ICH. We observed a significant drop in NR4A2 expression in the early stages of ICH, both in the brain tissue around the hematoma and in our lab-based cell models. This reduction in NR4A2 levels suggests a potential weakness that could exacerbate the pathological cascade after hemorrhage. Interestingly, we found NR4A2 predominantly in microglia, endothelial cells (which line blood vessels), and astrocytes (brain support cells), highlighting its widespread involvement in key cellular components of the neurovascular unit. These data suggest a possible link between decreased levels of NR4A2 after ICH and the development of neurovascular conditions. Further research showed that increasing NR4A2 levels could significantly reduce the size of the hematoma, improve nerve function, and lessen neurological deficits after ICH. This is a critical finding, as hematoma expansion and neurological deterioration are major determinants of poor prognosis in ICH.

The area around the hematoma after ICH is characterized by active bleeding and swelling. The main reasons for this are direct physical damage to the BBB and indirect inflammatory harm, which allows blood to seep into the central brain tissue, causing the hematoma to grow and brain injury to worsen. We then looked at how NR4A2 affects BBB integrity. Our findings demonstrated that NR4A2 significantly prevented the leakage of FITC-dextran (a tracer molecule), IgG (a large protein), and Evans Blue dye, all of which are signs of increased BBB permeability. Furthermore, NR4A2 preserved the microscopic health of endothelial cells and reduced the loss of crucial BBB tight junction proteins, like ZO-1 and claudin-5, as seen with immunofluorescence and transmission electron microscopy. These BBB-protective effects of NR4A2 happened alongside a smaller hematoma volume and less pronounced neurological impairments, underscoring the vital connection between a healthy BBB and a better outcome in ICH. This suggests that NR4A2 directly contributes to the maintenance of the physical barrier, reducing brain swelling and secondary injury.

Inflammation within the brain's blood vessels is closely linked to how ICH progresses. The increased "leakiness" of the BBB in the perihematomal area in early ICH patients allows large amounts of immune-associated factors to get into the core brain [38], which then further aggravates

neurovascular injury [39]. Interestingly, in this study, our RNA sequencing of brain tissue from rats (both control and NR4A2-overexpressing) showed that the genes affected by NR4A2 overexpression were primarily involved in inflammation and related pathways, further solidifying the role of NR4A2 in modulating the inflammatory response. Moreover, in vivo data revealed increased activation of microglia and astrocytes in the perihematomal area after ICH; however, NR4A2 overexpression was able to suppress this activation. Notably, we also found that NR4A2 treatment effectively stopped the infiltration of neutrophils (a type of white blood cell) and reduced the build-up of adhesion molecules (P-selectin, ICAM-1, VCAM-1, and chemokines like CXCL1, CCL2, CCR1). This suggests that NR4A2 calms neuroinflammation around the hematoma by preventing these peripheral immune cells from entering the brain.

Microglia are the brain's primary defense cells among its innate immune system, possessing powerful sensing and housekeeping functions that are essential for maintaining the brain's delicate environment [40]. It's been reported that microglia, which significantly contribute to the progression of neuroinflammation, can be rapidly activated and then polarized (change their functional state) during hemorrhage [9, 14]. Among the microglial types, M1 microglia typically secrete pro-inflammatory factors (e.g., IL-6, TNF-alpha, iNOS, CD86), while M2 microglia generally produce anti-inflammatory factors (e.g., IL-10, TGF-beta) and help with tissue repair (e.g., Arg-1, CD206). Therefore, helping microglia transform into the M2 phenotype during the early stages of ICH is crucial for improving the prognosis of ICH patients. In our study, we looked at how NR4A2 affects microglial M1/M2 polarization. The results demonstrated that increasing NR4A2 significantly decreased the expression of M1 microglial markers (CD86 and iNOS) in the perihematomal tissue of ICH rats and boosted the expression of M2 microglial markers (CD206 and Arg-1). Furthermore, treating cells with hemin/hypoxia can trigger microglial phagocytosis, increasing their movement and causing neuroinflammation. Our findings indicated that increasing NR4A2 reduced the sticking of THP-1 cells to the endothelial barrier and then guided M1-type microglia to polarize into M2-type microglia in an in vitro ICH model, thereby fighting inflammation. Based on all this data, our results confirmed that NR4A2's anti-neuroinflammatory effect after ICH likely depends on its ability to promote microglial M2 polarization. This shift towards a healing phenotype is a cornerstone of NR4A2's brain-protective action.

The TLR signaling pathway is widely recognized for its role in controlling inflammatory reactions [41] and maintaining BBB integrity in both hemorrhagic [42] and neurodegenerative diseases [21, 22]. Toll-like receptor 4 (TLR4), a key member of the TLR family, gets activated when exposed to bleeding stimuli and plays a role in regulating microglia and inflammation [43, 44]. In the brains of ICH patients, glial cells surrounding hematomas show activation of NF-κB. Stress responses, like inflammation and oxidative stress, can speed up microglial activation and regulate a process called ubiquitination of downstream TRAF6 via the TLR2 and TLR4 receptors [45]. This then helps NF-κB move into the cell's nucleus, leading to the production of inflammatory factors such as IL-1β, IL-6, and TNF-alpha, ultimately causing neuroinflammation. In ICH, this process contributes to larger hematomas, damages brain microvessels, and subsequently hampers recovery. Mechanistically, our co-IP, luciferase, and ChIP assays showed that NR4A2 could directly interact with TLR4 after ICH, providing new and exciting insight into how NR4A2 and TLR4 regulate each other, suggesting that NR4A2 might directly interfere with TLR4-mediated signaling.

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DISCUSSION

Intracerebral hemorrhage (ICH) continues to be a formidable challenge in neurology, with the subsequent brain damage significantly contributing to long-term disability and poor patient outcomes [2, 38]. This secondary damage is a complex interplay of inflammatory processes, oxidative stress, and the breakdown of the blood-brain barrier (BBB) in the area surrounding the initial bleeding. Our study provides compelling and novel evidence that nuclear receptor subfamily 4 group A member 2 (NR4A2) plays a crucial neuroprotective role in the acute phase of ICH by mitigating early brain injury and improving neurological outcomes. This protective effect is primarily mediated through a dual mechanism: first, by encouraging microglia to adopt their healing M2 phenotype, and second, by simultaneously putting a stop

to the detrimental Toll-like receptor 4 (TLR4)/TNF receptor-associated factor 6 (TRAF6)/nuclear factor-kappa B (NF-κB) inflammatory signaling pathway.

Our investigation started by looking at how NR4A2 levels changed after ICH. We observed a significant drop in NR4A2 expression in the early stages of ICH, both in the brain tissue around the hematoma and in our lab-based cell models. This reduction in NR4A2 levels suggests a potential weakness that could exacerbate the pathological cascade after hemorrhage. Interestingly, we found NR4A2 predominantly in microglia, endothelial cells (which line blood vessels), and astrocytes (brain support cells), highlighting its widespread involvement in key cellular components of the neurovascular unit. These data suggest a possible link between decreased levels of NR4A2 after ICH and the development of neurovascular conditions. Further research showed that increasing NR4A2 levels could significantly reduce the size of the hematoma, improve nerve function, and lessen neurological deficits after ICH. This is a critical finding, as hematoma expansion and neurological deterioration are major determinants of poor prognosis in ICH.

The area around the hematoma after ICH is characterized by active bleeding and swelling. The main reasons for this are direct physical damage to the BBB and indirect inflammatory harm, which allows blood to seep into the central brain tissue, causing the hematoma to grow and brain injury to worsen. We then looked at how NR4A2 affects BBB integrity. Our findings demonstrated that NR4A2 significantly prevented the leakage of FITC-dextran (a tracer molecule), IgG (a large protein), and Evans Blue dye, all of which are signs of increased BBB permeability. Furthermore, NR4A2 preserved the microscopic health of endothelial cells and reduced the loss of crucial BBB tight junction proteins, like ZO-1 and claudin-5, as seen with immunofluorescence and transmission electron microscopy. These BBB-protective effects of NR4A2 happened alongside a smaller hematoma volume and less pronounced neurological impairments, underscoring the vital connection between a healthy BBB and a better outcome in ICH. This suggests that NR4A2 directly contributes to the maintenance of the physical barrier, reducing brain swelling and secondary injury.

Inflammation within the brain's blood vessels is closely linked to how ICH progresses. The increased "leakiness" of the BBB in the perihematomal area in early ICH patients allows large amounts of immune-associated factors to get into the core brain [38], which then further aggravates neurovascular injury [39]. Interestingly, in this study, our RNA sequencing of brain tissue from rats (both control and NR4A2-overexpressing) showed that the genes affected by NR4A2 overexpression were primarily involved in inflammation and related pathways, further solidifying the role of NR4A2 in modulating the inflammatory response. Moreover, in vivo data revealed increased activation of microglia and astrocytes in the perihematomal area after ICH; however, NR4A2 overexpression was able to suppress this activation. Notably, we also found that NR4A2 treatment effectively stopped the infiltration of neutrophils (a type of white blood cell) and reduced the build-up of adhesion molecules (P-selectin, ICAM-1, VCAM-1, and chemokines like CXCL1, CCL2, CCR1). This suggests that NR4A2 calms neuroinflammation around the hematoma by preventing these peripheral immune cells from entering the brain.

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CONCLUSION

In summary, our findings demonstrate that NR4A2 significantly lessens early brain injury after intracerebral hemorrhage by encouraging microglia to adopt their healing M2 phenotype and by putting a stop to the harmful TLR4/TRAF6/NF-κB inflammatory pathway. Specifically, in the context of ICH, NR4A2 expression is reduced, and restoring it helps to calm perihematomal inflammatory damage, thereby improving ICH outcomes by shifting microglia towards the anti-inflammatory M2 phenotype. Mechanistically, NR4A2 interacts with TLR4, suppressing the downstream TRAF6/NF-κB signaling pathway. This study offers crucial insights into the role of NR4A2 in mitigating early brain damage caused by ICH and reveals its new function in guiding microglial M2 polarization by inhibiting the TLR4-mediated TLR4/TRAF6/NF-κB pathway. We believe that NR4A2 holds great promise as a potential new treatment for acute hemorrhagic brain injuries in the foreseeable future.

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