NT-4 attenuates neuroinflammation via TrkB/PI3K/FoxO1 pathway after germinal matrix hemorrhage in neonatal rats

Tianyi Wang
Loma Linda University

Junyi Zhang
Loma Linda University

Peng Li
Loma Linda University

Yan Ding
Loma Linda University

Jiping Tang
Loma Linda University

Gang Chen
Soochow University

John Zhang (✉ johnzhang3910@yahoo.com)
Loma Linda University Medical Center   https://orcid.org/0000-0002-4319-4285

Abstract

Background: Neuroinflammation plays an important role in pathogenesis of germinal matrix hemorrhage (GMH). Neurotrophin-4 (NT-4) is a member of the neurotrophin family, and it interacts with the tyrosine kinase B (TrkB) receptor. It has been studied that NT-4 has neuroprotective effects following cerebral ischemia. We aimed to investigate the neuroprotective function of NT-4 and it's high affinity receptor TrkB as well as its downstream mediator phosphatidylinositol-3-kinases (PI3K)/protein kinase B (Akt)/Forkhead box protein O1 (FoxO1) following GMH in neonatal rats, with a specific focus on inflammation.

Methods: GMH was induced by intraparenchymal injection of bacterial collagenase (0.3U) in P7 rat pups. A total of 163 seven-day-old pups were used in this study. The recombinant human NT-4 was administered intranasally at 1 hour after the collagenase injection. The selective TrkB antagonist ANA-12, selective PI3K inhibitor LY294002 and FoxO1 activating CRISPR were administered intracerebroventricularly at 24 hours prior to NT-4 treatment to investigate the potential mechanism. Short-and-long-term neurobehavior assessments, immunofluorescence staining, Nissl's staining and Western blot were performed.

Results：The expression of p-TrkB increased after GMH with a peak at day3. The TrkB receptor was expressed by neurons, microglia, and astrocytes. The administration of rh-NT-4 increased phosphorylation of TrkB, expression of PI3K, phosphorylation of Akt and decreased FoxO1, IL-1beta and IL-6 levels. Selective inhibition of TrkB/PI3K/Akt signaling in microglia increased the expression levels of FoxO1 and pro-inflammatory cytokines. The use of FoxO1 activation CRISPR increased the expression of IL-6, suggesting that FoxO1 might potentially induce pro-inflammatory factors. These results demonstrated that PI3K/Akt/FoxO1 may be the downstream pathway of TrkB phosphorylation. The rat pups treated with rh-NT-4 performed better than untreated animals both in short-and-long-term behavior test.

Conclusion：These data showed that rh-NT-4 can reduce the expression of pro-inflammatory cytokines, improve neurological function, attenuate neuroinflammation and post-hemorrhagic hydrocephalus after GMH by promoting TrkB/PI3K/Akt/FoxO1 pathway. These results indicated that rh-NT-4 could be a promising therapeutic target to ameliorate neuroinflammation and hydrocephalus after GMH or other similar brain injuries.

Background

Germinal matrix hemorrhage (GMH) is the most common and devastating neurological injury of premature infants. It has been reported that GMH affects 7-30% of premature neonates, and depending on the grade of the hemorrhage, 25-80% of them will develop hydrocephalus[1, 2]. After the hemorrhage occurs, the inflammatory cascade will be activated to amplify the damage of brain tissues and it may lead to developmental delays, mental retardation, cerebral palsy, and hydrocephalus[3]. According to

previous studies, the suppression of pro-inflammatory factors could be beneficial in attenuating the GMH-induced brain injury[4]. Microglia, accounting for 10-15% of cells in the brain, function as the first and primary form of active immune cells in the central nervous system(CNS)[5]. Traditionally, microglia play a critical role in the immune response after GMH and are considered injurious in the post-hemorrhagic brain due to the production of inflammatory cytokines[4, 6]. Therefore, suppression of microglia activation could have a beneficial effect after GMH. TrkB is the high affinity receptor for several neurotrophins, such as BDNF, NT-4 and NT-3[7-9]. Its expression level is relatively high in the brain tissues. Previous studies shows that the activation of TrkB could attenuate the neurological deficits after hemorrhagic stroke[10, 11]. Neurotrophin-4(NT-4) is a neurotrophic factor that signals mainly through the tyrosine receptor kinase B (TrkB) [12]. It has been reported that the increase of NT-4 can decrease the infarct area after ischemic stroke[13-15]. However, the effect of NT-4 in GMH is still unclear. In this study, we are going to explore the anti-inflammation effect of NT-4/TrkB/PI3K/FoxO1 signaling pathway after GMH in neonatal rats[16-18].

Materials And Methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University. All studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and reported according to the ARRIVE guidelines. One hundred and sixty-three P7 Sprague–Dawley neonatal pups (weight = 12 – 14 g, Harlan, Livermore, CA) were randomly divided into Sham and GMH groups. All pups were kept in rooms with controlled temperature and 12-hour light/dark cycle, and given *ad libitum* access to food and water.

Experimental Design

Experiment 1.

To determine the time course of endogenous NT-4, TrkB and p-TrkB expressions after GMH, 36 7-day-old rat pups were randomly divided into six groups: Naive(n=6), 12 h after GMH(n=6), 1 day after GMH(n=6), 3 day after GMH(n=6), 5 day after GMH(n=6), 7 day after GMH(n=6), western blot analysis were used to detect their expression in the whole brain of each group.

Experiment 2.

The cellular localizaton of TrkB was evaluated by immunofluorescence staining to co-localize TrkB with ionized calciumbinding adaptor molecule 1(Iba-1), neuronal specific nuclear protein(NeuN), and glia fibrillary acidic protein(GFAP) respectively at day 3 after GMH(n=6). The expression exogenous NT-4 was

analyzed by western blot. In total 18 7-day-old rat pups were randomly divided into 3 groups(Sham, GMH + Vehicle, GMH + rh-NT-4). The whole brains were collected for western blot at 6 hours after GMH.

Experiment 3.

The outcome of rh-NT-4 treatment was assessed at the first 3 day and 21-28 days after GMH. The pups were randomly divided into 5 groups: Sham, GMH + Vehicle(5% DMSO), GMH + rh-NT-4(0.03mg/kg), GMH + rh-NT-4(0.1mg/kg), GMH + rh-NT-4(0.3mg/kg). Rh-NT-4 was dissolved in 5% DMSO and administrated in a total volume of 2ul intranasally at 1h, 25h and 49h post GMH. Short-term(Negative geotaxis and body righting reflex) behavior tests were performed to choose the best dosage of rh-NT-4 treatment for long-term outcome study. And then the sample capacity was added to 12 each group(Sham, GMH + Vehicle, GMH + rh-NT-4(best dosage)) for long-term(rotarod test, foot fault test and water maze) behavior test . The whole brains were collected for Nissl staining at 28th days after GMH.

Experiment 4.

To explore the underlying mechanisms of rh-NT-4 mediated anti-inflammatory effects after GMH, the selective TrkB antagonist ANA-12 was administrated intraperitoneally 1 hour before GMH induction, the specfic PI3K inhibitor LY-294002 was administrated intracerebroventricularly 1 hour before GMH induction, and the FoxO1 activate CRISPR was administrated intracerebroventricularly 24h before GMH induction, then followed with rh-NT-4(0.1mg/kg) treatment after GMH. Rat pups were divided into nine groups: Sham(n=6), GMH + Vehicle(n=6), GMH + rh-NT-4(n=6), GMH + rh-NT-4 + ANA-12(n=6), GMH + rh-NT-4 + ANA-12 Control(n=6), GMH + rh-NT-4 + LY294002(n=6), GMH + rh-NT-4 + LY294002 Control(n=6), GMH + rh-NT-4 + FoxO1 CRISPR(n=6), GMH + rh-NT-4 + CRISPR Control(n=6). The whole brains were collected on the 3rd days after GMH.

GMH model

The general procedure for inducing GMH in unsexed P7 rats using collagenase infusion was performed as previously described[19]. The rat pups were anesthetized with 3% isoflurane. The skin was incised on the longitudinal plane to expose the bregma. A burr hole (1 mm) was drilled on the skull (1.6 mm lateral, 1.5 mm anterior to the bregma), and a 27-gauge needle was inserted (2.7 mm deep from the dura) for collagenase (0.3 unites of clostridial collagenase VII-S, Sigma-Aldrich, MO) infusion (3μl/3min) using a 10μl Hamilton syringe (Hamilton Co, Reno, NV, USA) guided by a microinfusion pump (Harvard Apparatus, Holliston, MA). After the infusion, the needle was left at the place for 10 min to prevent "back leakage" and then was withdrawn at rate of 0.5 mm/min. Once the needle was removed, bone wax was used to seal the burr hole and the incision site was sutured. Animals were allowed to recover on a 37 °C heated blanket. Animals were put back with their dams after recovering from anesthesia.

Drug administration

Recombinant human Neurotrophin-4(Genscript) was dissolved in 5%DMSO. Pups were assigned to receive rh-NT-4 (0.03mg/kg/day, 0.1mg/kg/day or 0.3mg/kg/day) or 5%DMSO intranasally at one hour post GMH and then once a day for all studies. The dosage was based on previous studies [20]. ANA-12(Selleckchem) is a selective TrkB antagonist. It can bind directly and selectively to TrkB and inhibit the downstream signaling of TrkB. ANA-12(0.5mg/kg) was dissolved in 20% DMSO at 50mmol/L and administered intraperitoneally at 1h prior to GMH induction[21]. LY-294002 (Selleckchem) is the first synthetic molecule known to inhibit PI3K in previous studies. LY-294002 was dissolved in 25% DMSO at 50mmol/L and administrated intracerebroventricularly at 1h prior to GMH induction[22]. FoxO1 CRISPR (Santa Cruz Biotechnology) was suspended in the plasmid transfection medium and activated with transfection reagent, totaling 2ug per animal. The control scramble CRISPR followed the same steps and a total of 2ug per animal was given intracerebroventricularly [23, 24]. Animals were randomly divided into the following groups: sham group(n=6), GMH + Vehicle group(n=6, i.n.), GMH + rhNT-4 group(n=6, i.n.), GMH + rhNT-4 + ANA-12 group(n=6, i.c.v.), GMH + rhNT-4 + Vehicle1 group(n=6, i.p.), GMH + rhNT-4 + LY294002 group(n=6, i.p.), GMH + rhNT-4 + Vehicle2 group(n=6, i.c.v.), GMH + rhNT-4 + FoxO1 CRISPR group(n=6, i.c.v.), GMH + rhNT-4 + Control CRISPR group(n=6, i.c.v.). (Vehicle: 5%DMSO, intranasal administration; Vehicle 1: 20%DMSO, intraperitoneal injection; Vehicle 2: 25% DMSO, intracerebroventricular injection)

Immunofluorescence staining

Immunofluorescence staining was performed on fixed frozen brain sections as previously reported[25, 26]. 10 mm thickness slices were permeabilized with 0.3% Triton X-100 for 20 min at room temperature, then 5% normal donkey serum in PBS was used to block the slices for 2 h. After washing with PBS for three times (10 min each), sections were incubated with anti-TrkB (Abcam), anti-ionized calcium binding adapter molecule 1 (Iba-1, Abcam) at 4℃ overnight. After that, the slices were incubated 1h with FITC or Texas Red-conjugated secondary antibodies at room temperature. Then sections were washed again with PBS for three times (10 min each). Slides were covered with DAPI. The sections were imaged under fluorescence microscope (Leica DMi8, Leica Microsystems) equipped with LASX software.

Nissl staining

Nissl staining was performed and analyzed as in previous studies[25]. Coronal brain sections (20mm thick) were respectively dehydrated in 95% and 70% FLEX (ThermoFisher) for 1 min, then rinsed in tap water and distilled water for 10 seconds. Sections were stained with 0.5% cresyl violet (Sigma-Aldrich) for 1.5 min and washed in distilled water for 10 seconds followed by dehydration with 100% FLEX and

xylene for 1 min (two times, respectively). The permount was applied before the coverslip was placed. The sections were imaged by microscope (Olympus-BX51). Brain tissue loss and ventricular volume were measured and calculated with ImageJ[2, 27]. Volumes were calculated by the following formula: average (area of coronal section) × section interval × number of sections[28, 29]. Calculations were performed in a blinded fashion.

Western blot

Brain tissues were collected and stored in -80°C freezer after being perfused with cold PBS(0.1M, pH7.4) at 12 h, 1 day, 3 day, 5 day, 7 day for the time course study, at 3 day for the mechanistic study after GMH induction separately. Western blot was performed as previously described[30]. After sample preparation, 50 μg protein per sample was loaded onto an 10–12% SDS-PAGE gels, ran for 90 min at 100 V, and was transferred onto 0.2 mm or 0.45 mm nitrocellulose membranes at 100 V for 120 min (Bio-Rad). The membranes were blocked for 2 hours in 5% non-fat milk in Tris-buffered saline with 0.1% Tween20, followed by overnight incubation at 4°C with the following primary antibodies: anti-humanNT-4 (ThermoFisher, USA), anti-NT-4(Santa Cruz, USA), anti-TrkB (Abcam, USA), anti-pTrkB(ThermoFisher, USA), anti-PI3K(Abcam, USA), anti-Akt(Abcam, USA), anti-pAkt(Abcam, USA), anti-FoxO1(CST, USA), anti-IL6 (Santa Cruz, USA). The same membranes were probed with actin (Santa Cruz, USA) as internal loading controls. Appropriate secondary antibodies (Santa Cruz, USA) as internal loading controls. Appropriate secondary antibodies (Santa Cruz, USA) were incubated with membranes for 2 h at room temperature. Bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences, USA) and quantified through ImageJ 4.0 (Media Cybernetics).

Neurobehavioral test

Neurobehavioral tests were randomly performed by two blinded researchers in a unbiased setup according to previous studies [4, 19]. Short-term neurobehavioral tests, including righting reflex and negative geotaxis tests, were performed from 1d to 3d after GMH. Long-term neurological tests, including foot-fault, rotarod, and water maze, were performed from 21d to 28d after GMH.

Righting Reflex: Pups were placed in a supine position to record the duration of completely rollover onto four limbs. The maximum duration was 60 seconds per trial (3 trials/pup/day) and the average values were calculated. Negative geotaxis: The duration for the pups to rotate to turn 180° after being placed with the head downward on a 45° slope was recorded. The maximum duration was 60 seconds per trial (3 trials/pup/day) and the average values were calculated.

Rotarod test: Pups were placed on a rotarod (Columbus Instruments), and tested at a starting 5RPM or 10RPM with acceleration at 2RPM per 5 seconds. The latency to fall was recorded, and the maximum recording time was 60 seconds.

Foot-fault: Foot-fault was recorded as the number of missteps (inaccurately placed a fore- or hindlimb and fell through one of the openings in the grid) were recorded over 60 seconds.

Water maze test: Water maze test is a six-day test, including cued tests and hidden tests. The apparatus consisted of a metal pool (180 cm in diameter) and a small platform (11 cm in diameter) for the pups to climb onto. In the cued test, pups were manually guided to the platform if they had not found the platform, and the location of platform was changed every other trail. In hidden tests, the platform was removed from the pool, and the time spent in probe quadrant was recorded. Data were analyzed by a tracking software (Noldus Ethovision)

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). All the data values were presented as mean ± SD. One-way ANOVA with Dunnet's *post-hoc* test was used for multiple-comparison and two-tailed Student's t test was performed for two-group comparisons. Statistically significance was defined as p values <0.05.

Results

The expression of phosphoralyted TrkB increased after GMH.

Western blot was performed to test the protein expression of NT-4 and p-TrkB at 0 h, 12 h, 1 d, 3 d, 5 d and 7 d in the whole brain after GMH. The results showed that the expression of endogenous NT-4 significantly decreased at 12 h and 1 day, and then increased and maintained at a similar level after 3 days (p<0.05, Fig.1A). The expression of phosphorylated TrkB increased after GMH and peaked at 3 days (p<0.05, Fig.1B).

Intranasal administration of recombinant human NT-4 improved neurological functions during the first 3 days post GMH.

To investigate the effect of recombinant human NT-4 treatment, 3 doses (0.03, 0.1, 0.3 mg/kg) of rh-NT-4 with intranasal administration after GMH. According to the time course study, the expression of endogenous NT-4 decreased at 12 h and 1 day and then increased at 3 days after GMH. Thus, we used the exogenous rh-NT-4 to supplement the decreased endogenous NT-4 in the first three days. Therefore, different doses of rh-NT-4 were administered at 1 h, 25 h and 49 h after GMH. As shown in Figure 2, pups in the vehicle group performed worse than the sham group in the short-term neurobehavioral tests, namely negative geotaxis test and righting reflex test, during the first 3 days after GMH (Fig.2A,2B,2C).

The administration of rh-NT-4(0.1 mg/kg) significantly improved performance in these two neurobehavioral test compared with the vehicle group and low dose(0.03mg/kg) group. The high dose treatment (0.3 mg/kg) had similar therapeutic effect to the medium dose (0.1 mg/kg). Therefore, the medium dose of rh-NT-4 was chosen for long-term study and mechanism study.

Exogenous NT-4 expressed in brain tissue after intranasal administration.

To verify the intranasal administration did deliver the drug into the brain, we used Western blot to test the concentration of human NT-4 at 6 hours in Sham(n=6), GMH + Vehicle(n=6) and GMH + rh-NT-4(n=6) groups. As shown in the blot, the expression of human NT-4 significant increased in the treatment group compared with Sham and GMH + Vehicle groups (Fig.3A). Brain samples with schematic illustration showing the area in the perihematomal region (indicated by red square) were taken for immunofluorescence staining (Fig.3B). Double immunofluorescence staining was performed to detect the co-localization of TrkB with microglia (Iba-1), neurons (NeuN) and astrocytes (GFAP) on Day 3 after GMH (Fig.3C). These images showed that TrkB was primarily expressed on microglia and neurons.

Rh-NT-4 treatment ameliorated long-term neurological deficits after GMH.

To investigate the effects of rh-NT-4 treatment on the long-term neurological impairment induced by GMH, neurological functions were assessed by rotarod test, foot fault test and water maze at four weeks post GMH. Rh-NT-4 treatment significantly increased the falling latency at both 5 rpm and 10 rpm acceleration compared with the vehicle group (Fig.4B,4C). In the foot fault test, we found that vehicle animals had significantly more foot slips compared to sham animals, and the rh-NT-4 treatment significantly improved the performance (Fig.4A). In the water maze test, vehicle-treated animals spent more time finding the platform and had less time in the target quadrant compared with the sham group, suggesting that pups in the vehicle group had more severe cognitive impairment (Fig.4G,4H). rh-NT-4 treated animals performed significantly better than vehicle treated animals (Fig.4E,4F). Meanwhile, there is no significant difference in swimming velocity (Fig.4D) between these three groups, which means that it was primarily the spatial memory impairment, but not the slower velocity, that led to the longer escape latency.

Rh-NT-4 treatment improves long-term brain morphology.

Ventricular dilation is the major complication of GMH. Thus, we used Nissl staining to assess the brain morphology at 28 day post GMH to see whether the rh-NT-4 treatment attenuated the long-term ventricular dilation (Fig.5A). As shown in Figure 5, the ventricular volume significantly increased in the vehicle group compared with the sham group, and the rh-NT-4 treatment group attenuated ventricular dilation (Fig.5B). Cortical thickness (Fig.5C), white matter loss (Fig.5D) and gray matter loss (Fig.5E) were

presented as a ratio to the mean of the sham group. rh-NT-4 treatment significantly reduced these brain tissue losses compared with the vehicle group.

ANA-12(a selective TrkB antagonist), LY294002(a PI3K inhibitor) and FoxO1 Activation CRISPR reversed the protective effects of rh-NT-4 after GMH.

In this part, we used the IL-6 and IL-1 beta as the markers of inflammation. According to the outcome study, the medium dose of rh-NT-4 (0.1 mg/kg) was administered at 1h, 25h and 49h post GMH. The samples were collected on Day 3 post GMH. In the vehicle group, the phosphorylated TrkB increased and its downstream factors PI3K and phohphorylated Akt decreased. FoxO1 and IL-6 increased compared with the sham group. With the treatment of rh-NT-4, phosphorylated TrkB significantly increased compared with the vehicle group, and the expression of PI3K and phosphorylated Akt increased to the base level which is similar to the sham group. The downstream factors, such as FoxO1 and IL-6, also decreased compared with the vehicle group. Thus, the ultimate effect of rh-NT-4 treatment was suppressing the expression of inflammatory cytokines, such as IL-6 (Fig.6A,7A). With the co-administration with ANA-12, the expression of phosphorylated TrkB significantly decreased compared with the treatment group. However, its downstream factors were unchanged compared with the vehicle group (Fig.6B-H). In the LY294002 group, the elevated level of phosphorylated TrkB did not increase the expression of PI3K and phosphorylated Akt because of the inhibition of PI3K. Also, the downstream proteins FoxO1 and IL-6 were unchanged (Fig.6B-H). FoxO1 Activation CRISPR was also used to verify the pathway. Although rh-NT-4 treatment increased the expression of phosphorylated TrkB, PI3K and phosphorylated Akt, the FoxO1 expression was increased with the CRISPR (Fig.7B-H), abolishing the therapeutic effect of rh-NT-4.

Discussion

GMH is the most common and devastating neurological injury of premature infants between 24-32 weeks of gestation[31]. A previous publication indicated that P7 SD tats are comparable to 32 weeks of human gestational age. It is a devastating neonatal stroke characterized as neuroinflammation, hydrocephalus, primary and secondary brain injury, neurodevelopmental delay. And neuroinflammation is a trigger of secondary brain injury after GMH. Previous studies showed that NT-4 and BDNF are the high affinity ligands of tyrosine kinase B (TrkB), and the phosphorylation of TrkB by BDNF can attenuate brain injury in stroke[13, 14]. In the present study, we investigated the potential anti-inflammatory effects of NT-4 binding to TrkB and its underlying mechanism after collagenase-induced GMH in rat pups.The results showed that the activation of TrkB with its ligand NT-4 improved the neurobehavioral functions, decreased the expression of pro-inflammatory cytokines and improved the long-term brain morphology. Mechanistically, administration of rh-NT-4 was associated with the upregulation of phosphorylated TrkB, PI3K and phosphorylated Akt and downregulation of FoxO1, IL-1beta and IL-6 after GMH[32, 33].

In the first part of this study, we demonstrated that the expression of endogenous NT-4 was decreased after GMH induction, and then increased to normal level at 3 day. Phosphorylated TrkB increased in the early phase after GMH. We deduced that the endogenous NT-4 was initially consumed to phosphorylate TrkB to mitigate neuroinflammation after GMH. Furthermore, higher level of endogenous NT-4 kept phosphorylated TrkB at a high level in order to exert its anti-inflammatory function. Phosphorylation is considered a transient process, not a lasting process. So we try to complement the endogenous NT-4 with rh-NT-4 in the early phase after GMH to keep the high level of phosphorylated TrkB.

We did negative geotaxis test and righting reflex test during the first 3 days after GMH in our outcome study. As shown in Figure 2, the GMH + vehicle group demonstrated worse body reflex compared with the sham group. We gave 3 different doses of recombinant human NT-4 to choose the best dose for further studies. According to the data, the medium dose(0.1mg/kg/day) and high dose(0.3mg/kg/day) groups significantly improved the neurological outcomes after GMH, but the low dose(0.03mg/kg/day) group performed worse than these two groups. Based on the short-term behavioral tests, we chose medium dose(0.1mg/kg/day) for the long-term study and mechanism study.

Furthermore, we have also tested whether the recombinant human NT-4 was successfully delivered into the brain tissue via intranasal administration. Previous study showed that NT-4 levels in the brain and spinal cord were increased after intranasal administration, and it may have the potential neuroprotective effects when used in models of CNS injury and disease[20]. To verify this, we used the anti-human-NT-4 antibody which can not react with rats or mouse protein. The Western blot results showed that the expression of human-NT-4 significantly increased in medium dose(0.1mg/kg/day) group at 3 days after GMH compared with sham and GMH + vehicle groups. According to this, we ensured that the intranasal administration of rh-NT-4 was successful.

In the outcome study, we also did the immunofluorescence double staining of TrkB with neurons (NeuN), microglia (Iba-1) and astrocytes (GFAP) to investigate the receptor localization. As shown in the immunofluorescence images, the majority of TrkB co-localized with microglia and neurons, and the minority of TrkB co-localized with astrocytes. In this study, we aimed to focus on the TrkB function on microglia.

In the long-term behavioral study, we chose the medium dose (0.1mg/kg/day * 7 days) rh-NT-4 for the treatment group to assess its therapeutic effects on Day 28. In this part, we did foot fault test, rotarod test and water maze test to evaluate the differences between sham, GMH + vehicle and GMH + rh-NT-4 groups. The foot fault test and rotarod test were performed to test the motor function at 28 days post GMH. Morris water maze test was performed to test the cognitive impairment in memory function. These tests indicated that the GMH + vehicle group showed worse motor function and cognition, and that the rh-NT-4 treatment attenuated this impairment.

Ventricular dilation is a common long-term outcome of GMH. [19]Recent studies have demonstrated that the inflammatory response in the choroid plexus results in the post-hemorrhagic ventricular dilation[34]. As shown in the Nissl staining results, rh-NT-4 treatment not only reduced the ventricular expansion, but

also ameliorated gray matter loss, white matter loss and cortical thickness impairment. Based on these results, the activation of TrkB may ameliorate hydrocephalus and the mechanism might be due to the reduced microglia activation and pro-inflammatory cytokine generation. Collectively, TrkB activation on microglia may play an important role in the development of hydrocephalus, and the effect of NT-4 in reducing ventricular dilation may due to the suppression of pro-inflammatory cytokine release.

In this study, we focus on the effects of NT-4 mainly mediated by microglia signaling in GMH. Based on the mechanism study results, it is deducible that the PI3K/Akt/FoxO1 pathway, which was activated by TrkB activation, may be an important signaling pathway to suppress microglia from generating pro-inflammatory cytokines, such as IL-1beta and IL-6. Alternate pathways of NT-4 and TrkB were not evaluated in this study. Moreover, the immunofluorescence double staining indicated that the receptor TrkB was also expressed on neurons. Thus, we cannot eliminate the effects of NT-4 on neuron. As we all know, microglia are the resident macrophages of the brain and spinal cord, and they act as the first and main form of active immune defense in the CNS[35, 36]. Thus, it is deducible that microglial NT-4/TrkB signaling played a primary role in this neuroinflammation study. However, other effects of NT-4 directly on neurons are worthy of further investigation.

Previous studies indicated that NT-4 may be the preferable TrkB ligand as there would be less downregulation of TrkB with prolonged systemic treatments compared to BDNF[7, 37, 38]. However, these two ligands both have high affinity to TrkB. There might be different functions or effects between these two ligands, and this could be one of the weakness of this study. It would be more convincing if we compared the different therapeutic effects of these two ligands in the same pathway.

Conclusions

Our study demonstrated that recombinant human NT-4 reduced the expression of pro-inflammatory cytokines and attenuated neurological deficits in rat pups after GMH. The neuroprotective effects of rh-NT-4 are associated with TrkB/PI3K/Akt/FoxO1 pathway. These results indicated that NT-4 could be a promising therapeutic target to ameliorate neuroinflammation and hydrocephalus after GMH or other similar brain injury.

Abbreviations

Akt	Protein kinase B
BDNF	Brain Derived Neurotrophic Factor
CNS	Central nervous system
FoxO1	Forkhead box protein O1
GMH	Germinal matrix hemorrhage
GFAP	Glial fibrillary acidic protein
Iba-1	Ionized calcium-binding adaptor molecule
NT-4	Neurotrophin-4
NeuN	Neuronal specific nuclear protein
PI3K	phosphoinositide 3-kinase
TrkB	Tropomyosin receptor kinase B

Declarations

Ethics approval

Humans were not used in this study. All animals` experiments were approved by the Loma Linda University Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Availability of data and materials

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its supporting information files.

Funding

Authors contributions

Yan Ding and Peng Li worked on the experimental design. Tianyi Wang and Junyi Zhang conducted the experiments, analyzed the data, and drafted the manuscript. Flores Jerry and Yan Ding worked on the manuscript revision. John H Zhang, Gang Chen and Jiping Tang participated in the experimental design, data analysis and interpretation, and manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

No.

References

[1] P. Ballabh, Pathogenesis and prevention of intraventricular hemorrhage, Clin Perinatol, 41 (2014) 47-67.

[2] P. Ballabh, Intraventricular hemorrhage in premature infants: mechanism of disease, Pediatr Res, 67 (2010) 1-8.

[3] M. Heron, P.D. Sutton, J. Xu, S.J. Ventura, D.M. Strobino, B. Guyer, Annual summary of vital statistics: 2007, Pediatrics, 125 (2010) 4-15.

[4] Y. Zhang, N. Xu, Y. Ding, Y. Zhang, Q. Li, J. Flores, M. Haghighiabyaneh, D. Doycheva, J. Tang, J.H. Zhang, Chemerin suppresses neuroinflammation and improves neurological recovery via CaMKK2/AMPK/Nrf2 pathway after germinal matrix hemorrhage in neonatal rats, Brain Behav Immun, 70 (2018) 179-193.

[5] J. Aronowski, X. Zhao, Molecular pathophysiology of cerebral hemorrhage: secondary brain injury, Stroke, 42 (2011) 1781-1786.

[6] Y. Tao, L. Li, B. Jiang, Z. Feng, L. Yang, J. Tang, Q. Chen, J. Zhang, Q. Tan, H. Feng, Z. Chen, G. Zhu, Cannabinoid receptor-2 stimulation suppresses neuroinflammation by regulating microglial M1/M2 polarization through the cAMP/PKA pathway in an experimental GMH rat model, Brain Behav Immun, 58 (2016) 118-129.

[7] N. Khan, M.T. Smith, Neurotrophins and Neuropathic Pain: Role in Pathobiology, Molecules, 20 (2015) 10657-10688.

[8] S.K. Anand, A.C. Mondal, TrkB receptor antagonism inhibits stab injury induced proliferative response in adult zebrafish (Danio rerio) brain, Neurosci Lett, 672 (2018) 28-33.

[9] A. Esmaeili, G. Pakravan, K. Ghaedi, M. Noorbakhshnia, Alteration in messenger RNA neurotrophin 4 and tyrosine kinase receptors B expression levels following spinal cord injury, J Neurosurg Sci, 62 (2018) 146-152.

[10] J. Tang, Q. Hu, Y. Chen, F. Liu, Y. Zheng, J. Tang, J. Zhang, J.H. Zhang, Neuroprotective role of an N-acetyl serotonin derivative via activation of tropomyosin-related kinase receptor B after subarachnoid hemorrhage in a rat model, Neurobiol Dis, 78 (2015) 126-133.

[11] O. Lv, F. Zhou, Y. Zheng, Q. Li, J. Wang, Y. Zhu, Mild hypothermia protects against early brain injury in rats following subarachnoid hemorrhage via the TrkB/ERK/CREB signaling pathway, Mol Med Rep, 14 (2016) 3901-3907.

[12] S.D. Skaper, Neurotrophic Factors: An Overview, Methods Mol Biol, 1727 (2018) 1-17.

[13] M. Endres, G. Fan, L. Hirt, M. Fujii, K. Matsushita, X. Liu, R. Jaenisch, M.A. Moskowitz, Ischemic brain damage in mice after selectively modifying BDNF or NT4 gene expression, J Cereb Blood Flow Metab, 20 (2000) 139-144.

[14] M. Endres, G. Fan, L. Hirt, R. Jaenisch, Stroke damage in mice after knocking the neutrophin-4 gene into the brain-derived neurotrophic factor locus, J Cereb Blood Flow Metab, 23 (2003) 150-153.

[15] J.Y. Chung, M.W. Kim, M.S. Bang, M. Kim, Increased expression of neurotrophin 4 following focal cerebral ischemia in adult rat brain with treadmill exercise, PLoS One, 8 (2013) e52461.

[16] X.Y. Mao, H.H. Zhou, X. Li, Z.Q. Liu, Huperzine A Alleviates Oxidative Glutamate Toxicity in Hippocampal HT22 Cells via Activating BDNF/TrkB-Dependent PI3K/Akt/mTOR Signaling Pathway, Cell Mol Neurobiol, 36 (2016) 915-925.

[17] H. Dong, X. Zhang, X. Dai, S. Lu, B. Gui, W. Jin, S. Zhang, S. Zhang, Y. Qian, Lithium ameliorates lipopolysaccharide-induced microglial activation via inhibition of toll-like receptor 4 expression by activating the PI3K/Akt/FoxO1 pathway, J Neuroinflammation, 11 (2014) 140.

[18] P.V. Nerurkar, L.M. Johns, L.M. Buesa, G. Kipyakwai, E. Volper, R. Sato, P. Shah, D. Feher, P.G. Williams, V.R. Nerurkar, Momordica charantia (bitter melon) attenuates high-fat diet-associated oxidative stress and neuroinflammation, J Neuroinflammation, 8 (2011) 64.

[19] T. Lekic, A. Manaenko, W. Rolland, P.R. Krafft, R. Peters, R.E. Hartman, O. Altay, J. Tang, J.H. Zhang, Rodent neonatal germinal matrix hemorrhage mimics the human brain injury, neurological consequences, and post-hemorrhagic hydrocephalus, Exp Neurol, 236 (2012) 69-78.

[20] S.R. Alcala-Barraza, M.S. Lee, L.R. Hanson, A.A. McDonald, W.H. Frey, 2nd, L.K. McLoon, Intranasal delivery of neurotrophic factors BDNF, CNTF, EPO, and NT-4 to the CNS, J Drug Target, 18 (2010) 179-190.

[21] D.L. Fischer, C.J. Kemp, A. Cole-Strauss, N.K. Polinski, K.L. Paumier, J.W. Lipton, K. Steece-Collier, T.J. Collier, D.J. Buhlinger, C.E. Sortwell, Subthalamic Nucleus Deep Brain Stimulation Employs trkB Signaling for Neuroprotection and Functional Restoration, J Neurosci, 37 (2017) 6786-6796.

[22] Z. Xie, B. Enkhjargal, L. Wu, K. Zhou, C. Sun, X. Hu, V. Gospodarev, J. Tang, C. You, J.H. Zhang, Exendin-4 attenuates neuronal death via GLP-1R/PI3K/Akt pathway in early brain injury after subarachnoid hemorrhage in rats, Neuropharmacology, 128 (2018) 142-151.

[23] K. Suzuki, Y. Tsunekawa, R. Hernandez-Benitez, J. Wu, J. Zhu, E.J. Kim, F. Hatanaka, M. Yamamoto, T. Araoka, Z. Li, M. Kurita, T. Hishida, M. Li, E. Aizawa, S. Guo, S. Chen, A. Goebl, R.D. Soligalla, J. Qu, T. Jiang, X. Fu, M. Jafari, C.R. Esteban, W.T. Berggren, J. Lajara, E. Nunez-Delicado, P. Guillen, J.M. Campistol, F. Matsuzaki, G.H. Liu, P. Magistretti, K. Zhang, E.M. Callaway, K. Zhang, J.C. Belmonte, In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration, Nature, 540 (2016) 144-149.

[24] N. Matei, J. Camara, D. McBride, R. Camara, N. Xu, J. Tang, J.H. Zhang, Intranasal wnt3a Attenuates Neuronal Apoptosis through Frz1/PIWIL1a/FOXM1 Pathway in MCAO Rats, J Neurosci, 38 (2018) 6787-6801.

[25] Q. Chen, X. Shi, Q. Tan, Z. Feng, Y. Wang, Q. Yuan, Y. Tao, J. Zhang, L. Tan, G. Zhu, H. Feng, Z. Chen, Simvastatin Promotes Hematoma Absorption and Reduces Hydrocephalus Following Intraventricular Hemorrhage in Part by Upregulating CD36, Transl Stroke Res, 8 (2017) 362-373.

[26] S. Chen, L. Zhao, P. Sherchan, Y. Ding, J. Yu, D. Nowrangi, J. Tang, Y. Xia, J.H. Zhang, Activation of melanocortin receptor 4 with RO27-3225 attenuates neuroinflammation through AMPK/JNK/p38 MAPK pathway after intracerebral hemorrhage in mice, J Neuroinflammation, 15 (2018) 106.

[27] D. Klebe, P.R. Krafft, C. Hoffmann, T. Lekic, J.J. Flores, W. Rolland, J.H. Zhang, Acute and delayed deferoxamine treatment attenuates long-term sequelae after germinal matrix hemorrhage in neonatal rats, Stroke, 45 (2014) 2475-2479.

[28] J.J. Flores, D. Klebe, W.B. Rolland, T. Lekic, P.R. Krafft, J.H. Zhang, PPARgamma-induced upregulation of CD36 enhances hematoma resolution and attenuates long-term neurological deficits after germinal matrix hemorrhage in neonatal rats, Neurobiol Dis, 87 (2016) 124-133.

[29] C.L. MacLellan, G. Silasi, C.C. Poon, C.L. Edmundson, R. Buist, J. Peeling, F. Colbourne, Intracerebral hemorrhage models in rat: comparing collagenase to blood infusion, J Cereb Blood Flow Metab, 28 (2008) 516-525.

[30] X. Li, T. Wang, D. Zhang, H. Li, H. Shen, X. Ding, G. Chen, Andrographolide ameliorates intracerebral hemorrhage induced secondary brain injury by inhibiting neuroinflammation induction, Neuropharmacology, 141 (2018) 305-315.

[31] P. Georgiadis, H. Xu, C. Chua, F. Hu, L. Collins, C. Huynh, E.F. Lagamma, P. Ballabh, Characterization of acute brain injuries and neurobehavioral profiles in a rabbit model of germinal matrix hemorrhage, Stroke, 39 (2008) 3378-3388.

[32] M. Abbasi, V. Gupta, N. Chitranshi, Y. You, Y. Dheer, M. Mirzaei, S.L. Graham, Regulation of Brain-Derived Neurotrophic Factor and Growth Factor Signaling Pathways by Tyrosine Phosphatase Shp2 in the Retina: A Brief Review, Front Cell Neurosci, 12 (2018) 85.

[33] P. Yan, S. Tang, H. Zhang, Y. Guo, Z. Zeng, Q. Wen, Palmitic acid triggers cell apoptosis in RGC-5 retinal ganglion cells through the Akt/FoxO1 signaling pathway, Metab Brain Dis, 32 (2017) 453-460.

[34] J.K. Karimy, J. Zhang, D.B. Kurland, B.C. Theriault, D. Duran, J.A. Stokum, C.G. Furey, X. Zhou, M.S. Mansuri, J. Montejo, A. Vera, M.L. DiLuna, E. Delpire, S.L. Alper, M. Gunel, V. Gerzanich, R. Medzhitov, J.M. Simard, K.T. Kahle, Inflammation-dependent cerebrospinal fluid hypersecretion by the choroid plexus epithelium in posthemorrhagic hydrocephalus, Nat Med, 23 (2017) 997-1003.

[35] A.R. Najafi, J. Crapser, S. Jiang, W. Ng, A. Mortazavi, B.L. West, K.N. Green, A limited capacity for microglial repopulation in the adult brain, Glia, DOI 10.1002/glia.23477(2018).

[36] P.P. Lowe, B. Gyongyosi, A. Satishchandran, A. Iracheta-Vellve, Y. Cho, A. Ambade, G. Szabo, Reduced gut microbiome protects from alcohol-induced neuroinflammation and alters intestinal and brain inflammasome expression, J Neuroinflammation, 15 (2018) 298.

[37] M. Bothwell, NGF, BDNF, NT3, and NT4, Handb Exp Pharmacol, 220 (2014) 3-15.

[38] C.C. Proenca, M. Song, F.S. Lee, Differential effects of BDNF and neurotrophin 4 (NT4) on endocytic sorting of TrkB receptors, J Neurochem, 138 (2016) 397-406.
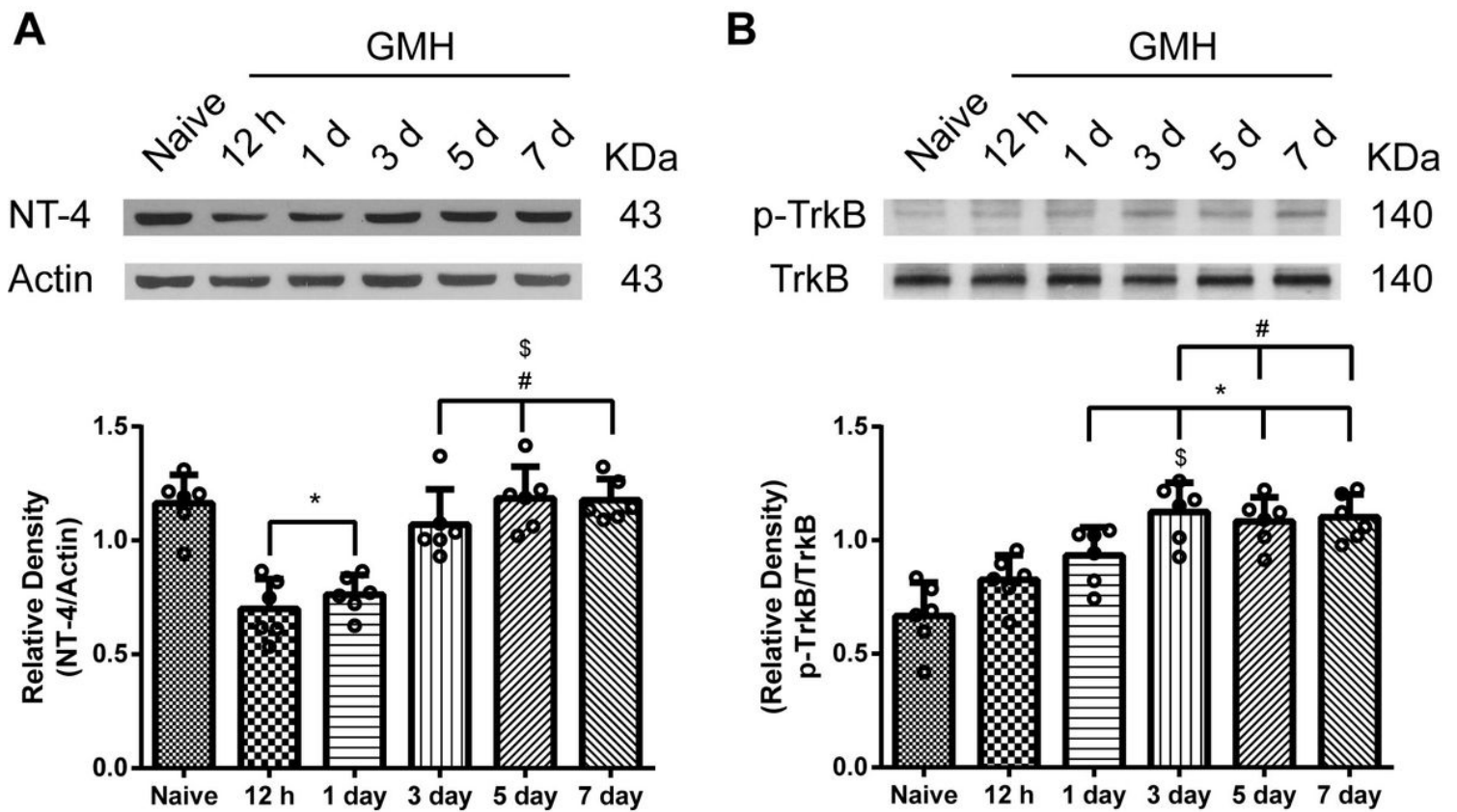
Figures

Figure 1

Expression profile of NT-4 and TrkB after GMH. A Representative western blot bands of time course and quantitative analyses of NT-4 expression after GMH. B Representative western blot bands of time course and quantitative analyses of phosphorylated TrkB expression after GMH. * P <0.05 vs Naive, # P<0.05 vs 12 h, $ P<0.05 vs 1 d, one-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD.
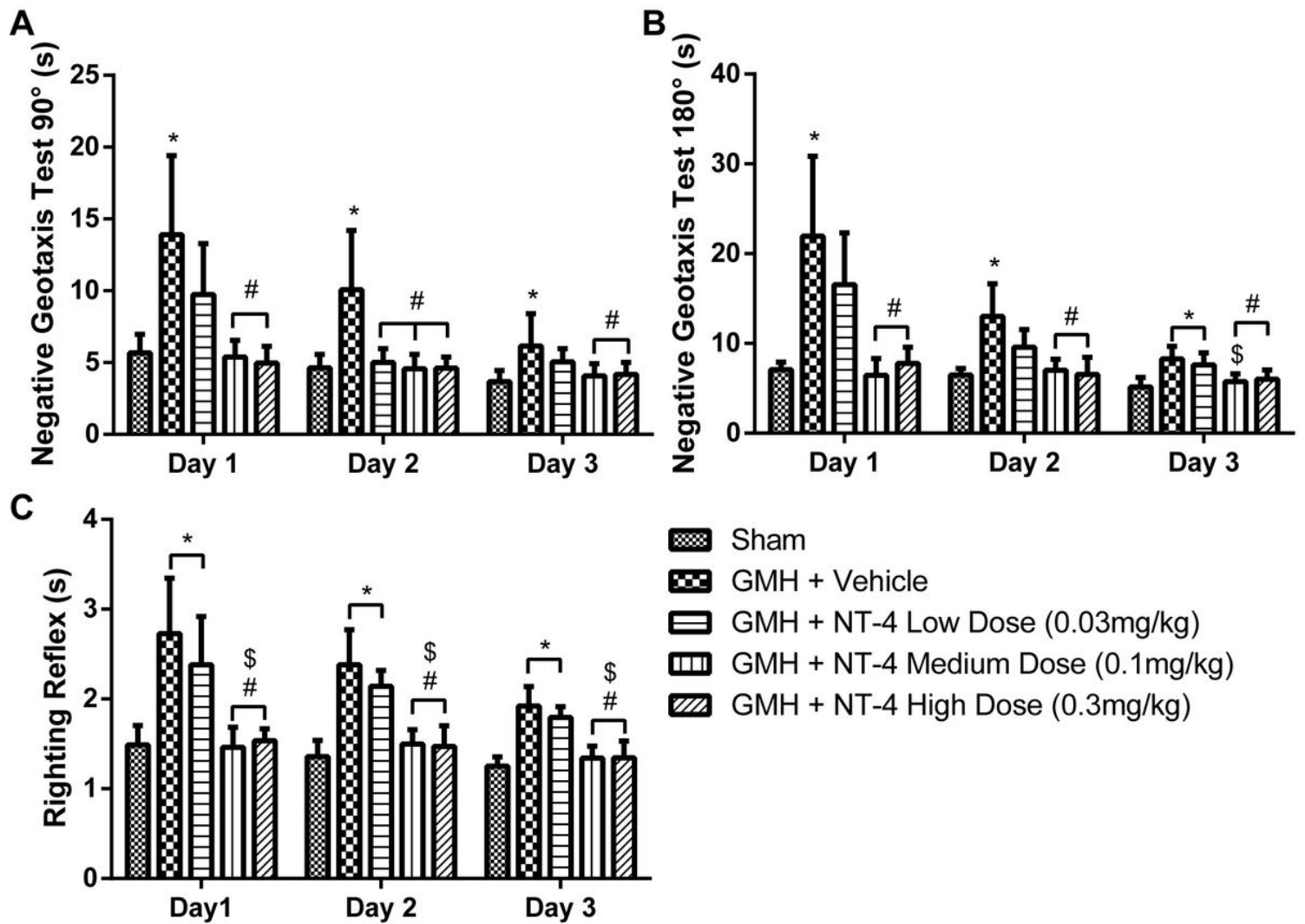
Figure 2

Intranasal administration of human recombinant NT-4 improved short-term function tests at 72 h post GMH. A, B Negative Geotaxis test and C Righting Reflex test showed that medium dose (0.1mg/kg) and high dose (0.3mg/kg) of rh-NT-4 significantly improved neurological function compared to vehicle treated pups at first 3 days after GMH. * P<0.05 vs Sham, # P<0.05 vs GMH + Vehicle, $ p<0.05 vs low dose (0.03mg/kg) group, one-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD.

Figure 3

The expression of recombinant human NT-4 in the brain and immunofluorescence double staining results. A The expression of recombinant human NT-4 in the Sham Group, Vehicle Group and NT-4 group. * P<0.05 vs Sham, # P<0.05 vs GMH + vehicle, One-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD. B Brain sample with schematic illustration showing the area in the perihematomal region (indicated by red square) from where the images were taken for immunofluorescence staining. C Immunofluorescence co-localization of TrkB with microglia (Iba-1), neuron (NeuN) and astrocyte (GFAP) on Day 3 after GMH. (N = 2. Scale bar=50µm.)
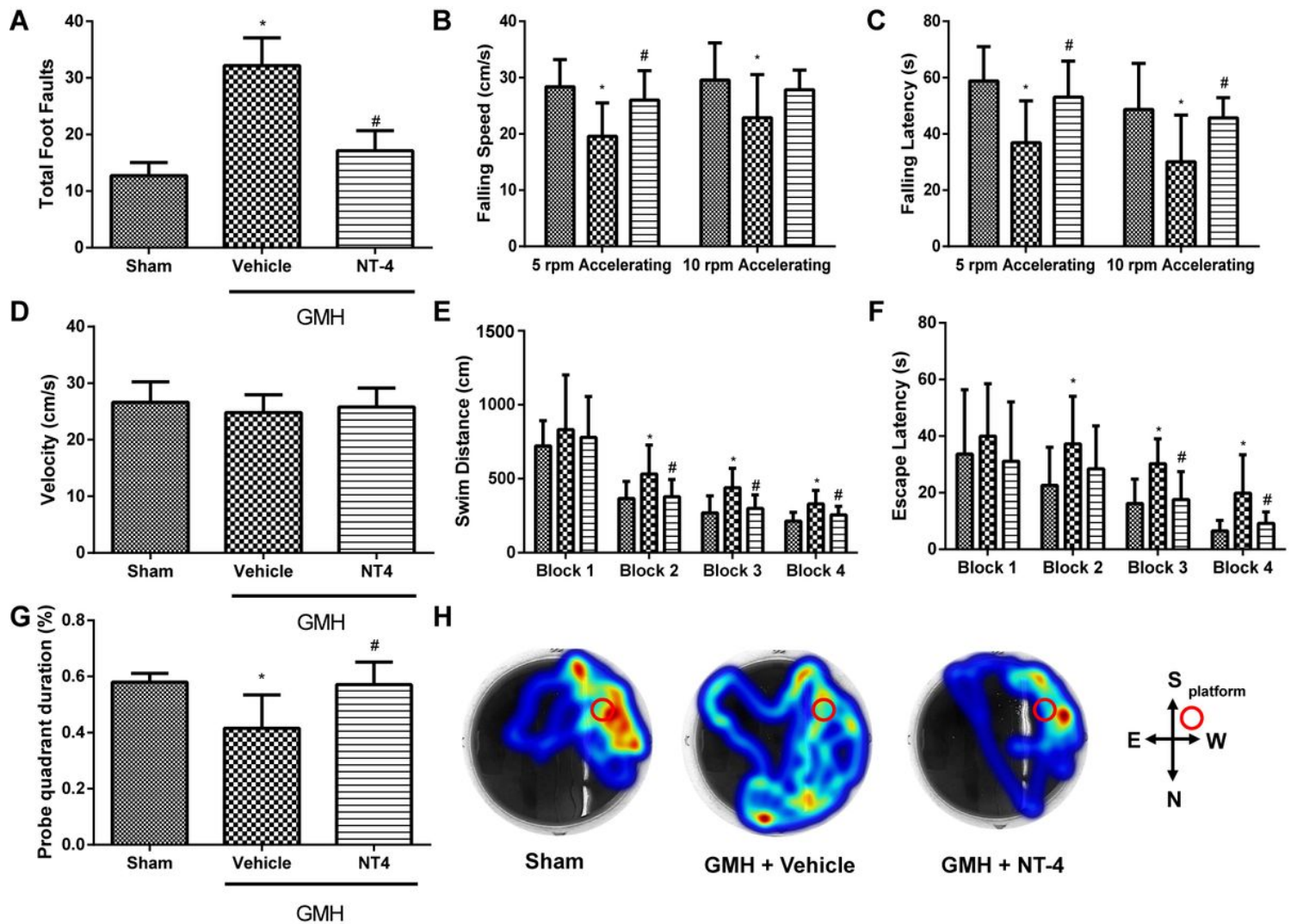
Figure 4

The effects of NT-4 (0.1mg/kg) on long-term neurobehavioral outcomes after GMH. A Foot fault test and B,C Rotarod test at 28 days after GMH. D Swim velocity, E swim distance and F Escape latency of Morris water maze on days 24 to 27 after GMH. G Probe quadrant duration of Morris water maze on day 28 after GMH. * P<0.05 vs Sham, # P<0.05 vs GMH + Vehicle, one-way ANOVA, Tukey's test, n=12/group. Data is presented as mean ± SD.
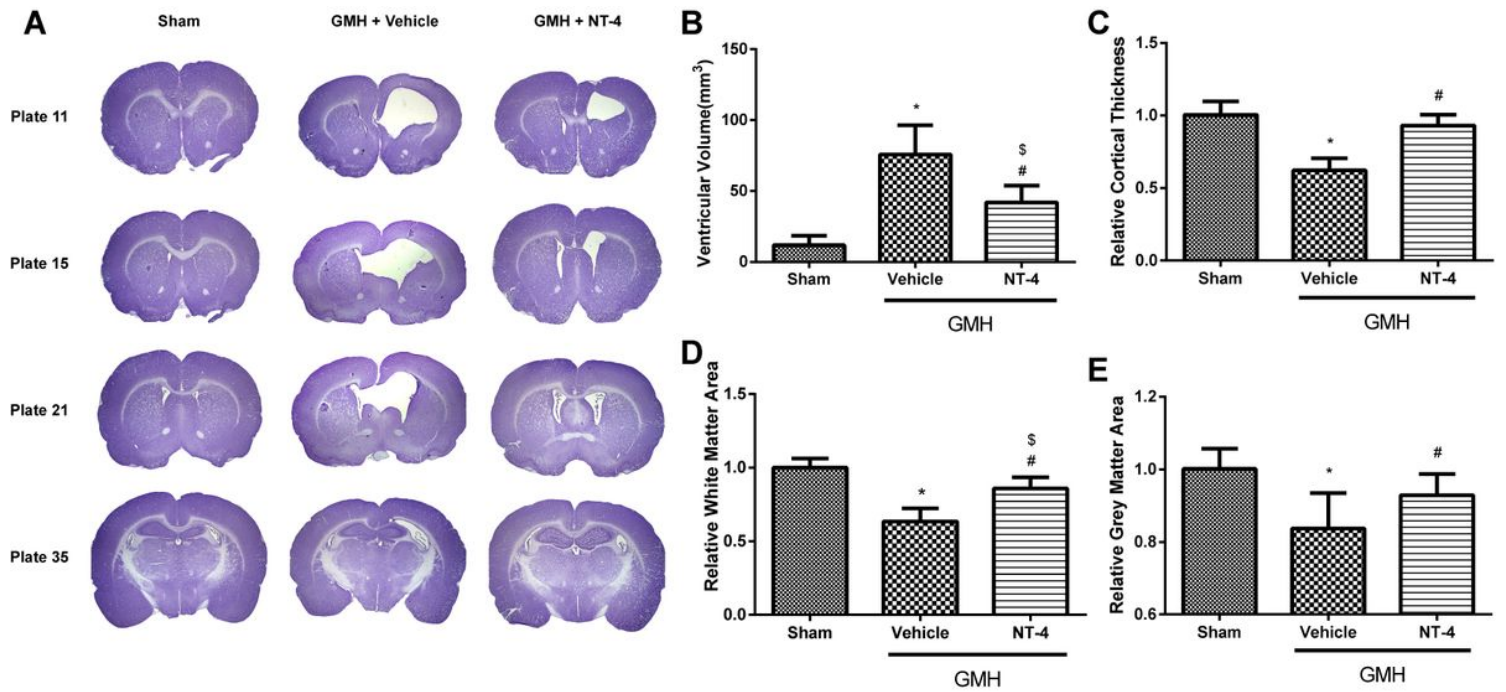
Figure 5

Nissl's staining results of sham, vehicle and NT-4 treatment groups at 28 days after GMH. A Nissl's staining pictures, B ventricular volume, C relative cortical thickness, D relative white matter area, E relative grey matter area. * P<0.05 vs Sham, # P<0.05 vs GMH + Vehicle, one-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD.
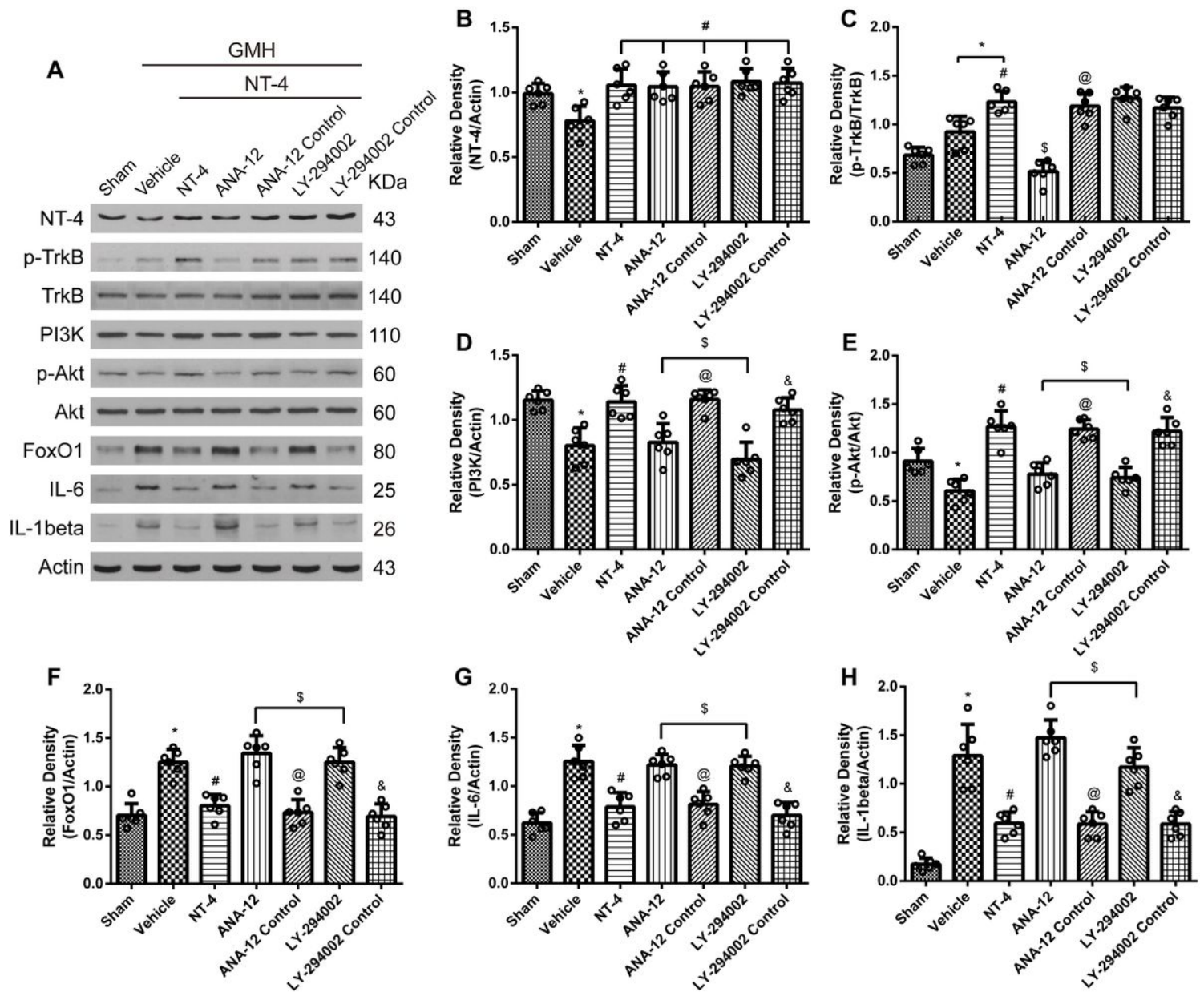
Figure 6

Selective inhibition of TrkB and PI3K in microglia abolished the effect of rh-NT-4 on inhibiting neuroinflammation at day 3 after GMH. A Representative images of Western blot bands. B-H Quantitative analyses of nt-4, phosphorylated TrkB, PI3K, phosphorylated Akt, FoxO1, IL-6, and IL-1β at day 3 after GMH. * $P<0.05$ vs Sham, # $P<0.05$ vs GMH + Vehicle, $ $P<0.05$ vs GMH + NT-4, @ $P<0.05$ vs GMH + ANA-12, & $P<0.05$ vs GMH + LY-294002. one-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD.
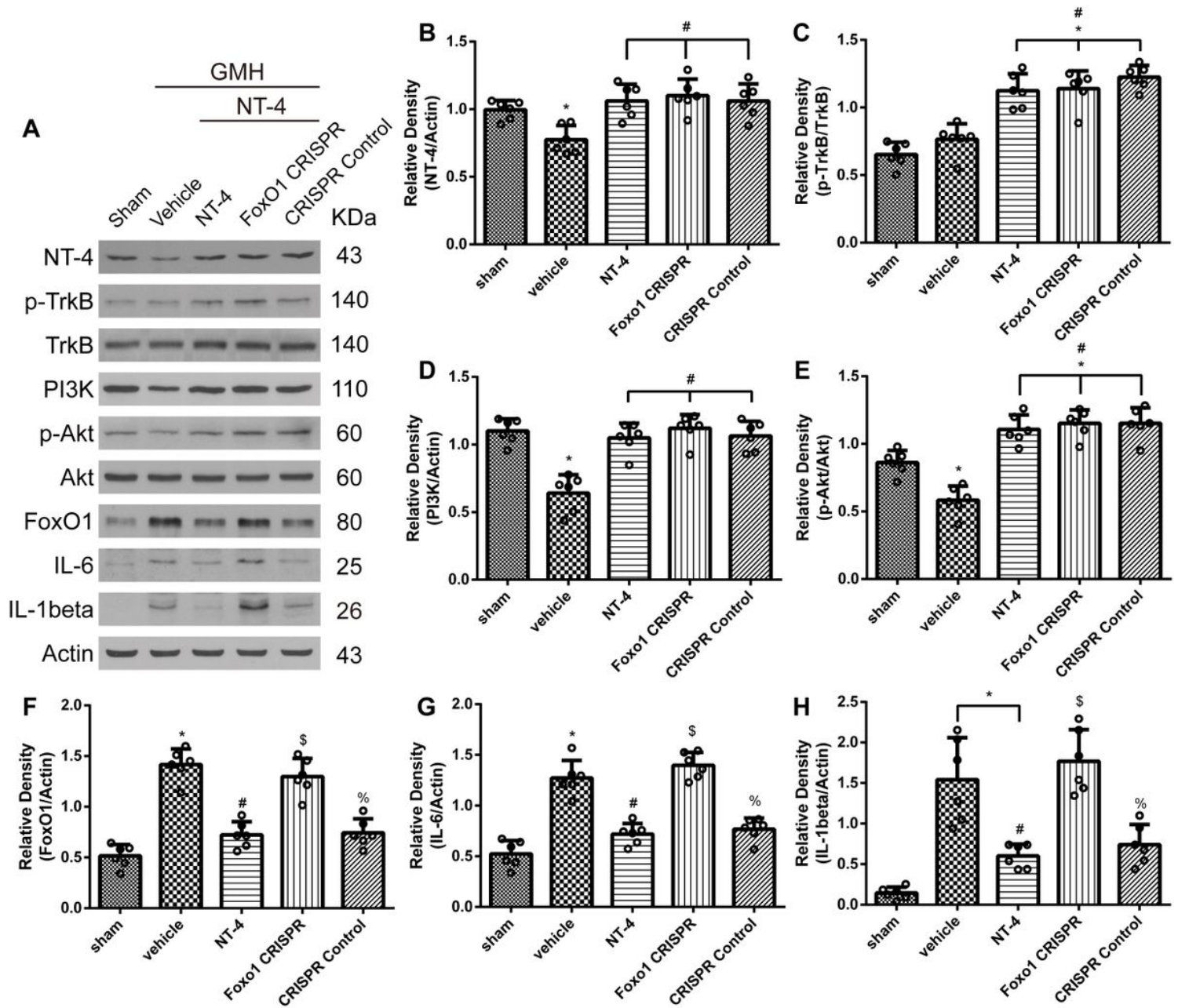
Figure 7

FoxO1 activate CRISPR in microglia abolished the effect of rh-NT-4 on inhibiting neuroinflammation at day 3 after GMH. A Representative images of Western blot bands. B-H Quantitative analyses of nt-4, phosphorylated TrkB, PI3K, phosphorylated Akt, FoxO1, IL-6, and IL-1β at day 3 after GMH. * P<0.05 vs Sham, # P<0.05 vs GMH + Vehicle, $ P<0.05 vs GMH + NT-4, % P<0.05 vs GMH + FoxO1 CRISPR. one-way ANOVA, Tukey's test, n=6/group. Data is presented as mean ± SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryWB.tif

- supplementaryFig1.tif
- supplementaryFig2.tif