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Chemerin suppresses neuroinflammation and improves neurological recovery via CaMKK2/AMPK/Nrf2 pathway after germinal matrix hemorrhage in neonatal rats

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Abstract

Chemerin, an adipokine, has been reported to reduce the production of pro-inflammatory cytokines and neutrophil infiltration. This study investigated the role of Chemerin and its natural receptor, ChemR23, as well as its downstream mediator calmodulin-dependent protein kinase kinase 2 (CAMKK2)/adenosine monophosphate-activated protein kinase (AMPK)/Nuclear factor erythroid 2-related factor 2 (Nrf2) following germinal matrix hemorrhage (GMH) in neonatal rats, with a specific focus on inflammation. GMH was induced by intraparenchymal injection of bacterial collagenase (0.3U) in P7 rat pups. The results demonstrated that human recombinant Chemerin (rh-Chemerin) improved neurological and morphological outcomes after GMH. Rh-Chemerin promoted accumulation and proliferation of M2 microglia in periventricular regions at 72 hours. Rh-Chemerin increased phosphorylation of CAMKK2, AMPK and expression of Nrf2, and decreased IL-1 β , IL-6 and TNF- α levels. Selective inhibition of ChemR23/CAMKK2/AMPK signaling in microglia via intracerebroventricular delivery of liposome-encapsulated specific ChemR23 (Lipo- α -NETA), CAMKK2 (Lipo-STO-609) and AMPK (Lipo-Dorsomorphin) inhibitor increased the expression levels of IL-1 β , IL-6 and TNF- α , demonstrating that ChemR23/CAMKK2/AMPK signaling in microglia suppressed inflammatory

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Author Contributions

The conception and design of this paper were made by YXZ, NX, YD, YTZ, JT, and JHZ. YXZ, NX, YTZ, QL, JF, MA, and DMD collected and analyzed the data. Drafting the article was done by YXZ. Critically revising the article was done by all the authors (YXZ, NX, YD, YTZ, QL, JF, MA, DMD, JT, and JHZ). Approval of the final version of the manuscript on behalf of all authors was done by YXZ and JHZ.

Potential Conflicts of Interest

None.

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response after GMH. Cumulatively, these data showed that rh-Chemerin ameliorated GMH-induced inflammatory response by promoting ChemR23/CAMKK2/AMPK/Nrf2 pathway, and M2 microglia may be a major mediator of this effect. Thus, rh-Chemerin can serve as a potential agent to reduce the inflammatory response following GMH.

Keywords

Chemerin; Microglia; Inflammation; Germinal matrix hemorrhage; Neonate

1. Introduction

Germinal matrix hemorrhage (GMH) is a common cerebrovascular event that affects up to 20% of premature infants, and leads to severe long-term neurological and cognitive deficits, including cerebral palsy and mental retardation (Ballabh, 2014). After the hemorrhage occurs, the damage of brain tissue is amplified by the activation of inflammatory cascades, which are responsible for the further neurodegeneration (Brown and Neher, 2010; Chen et al., 2015; Tao et al., 2016; Zhou et al., 2014). Given the extended inflammation-induced tissue destruction, the suppression of inflammatory response could be a particularly important intervention to limit GMH-induced brain injury.

Microglia play critical roles in the immune response after GMH (Aronowski and Zhao, 2011). Traditionally, microglia was considered injurious in the past-hemorrhagic brain due to the production of inflammatory cytokines (Lan et al., 2017). However, emerging data show that microglia have a beneficial role in neonatal stroke and that depletion of microglia exacerbates neuroinflammation and brain injury in neonatal ischemic stroke (Chip et al., 2017; Fernandez-Lopez et al., 2016). Furthermore, preclinical studies show that regulation of the immune response after GMH involves an M1 to M2 phenotype transformation in microglia, and that promoting M2 polarization inhibits the expression of pro-inflammatory cytokines (Tao et al., 2016; Xu et al., 2015).

Chemerin is synthesized as a 163-amino acid precursor, and released by several tissues, including immune cells, liver, and spleen (Mariani and Roncucci, 2015). This precursor is low biological activity, which needs further processing at the C terminus to be the active form (Kennedy and Davenport, 2018). During the tissue clearance in the terminal phases of acute response, serine or cysteine proteases released by both macrophages and apoptotic cells cleaved this precursor to generate anti-inflammatory and prophagocytic peptides (Mariani and Roncucci, 2015). Chemerin or Chemerin derived peptide has been demonstrated to inhibit the production of inflammatory cytokines in macrophages, reduce neutrophil recruitment and promote phagocytosis of apoptotic cells (Cash et al., 2013; Cash et al., 2010; Cash et al., 2008; Lin et al., 2017). Neutralization of endogenous chemerin exacerbates peritonitis, indicating the potent anti-inflammatory effect of endogenous Chemerin (Cash et al., 2008; Graham et al., 2009). Currently, three proteins have been identified as Chemerin receptors: the natural receptor, Chemerin Receptor 23 (ChemR23), as well as two other receptors, chemokine CC motif receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1) (Mariani and Roncucci, 2015). Of note, Chemerin was unable to

suppress proinflammatory cytokines and neutrophil infiltration in ChemR23(−/−) mice, suggesting that Chemerin exerts its anti-inflammatory effect primarily through ChemR23 (Cash et al., 2010; Cash et al., 2008). However, the effect of GMH on Chemerin/ChemR23 signaling in neonates and therapeutic benefits of Chemerin in an animal model of GMH remained unexplored.

The anti-inflammatory effects of Chemerin/ChemR23 signaling in brain hemorrhage are far from thoroughly understood. *In vitro* studies show that Chemerin potentiated the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) in endothelial cells and granulosa cells (Reverchon et al., 2012; Shen et al., 2013). Moreover, activation of calmodulin-dependent protein kinase kinase 2 (CAMKK2) reduced the transcription of pro-inflammatory cytokines and promoted the M2 polarization through the phosphorylation of AMPK in LPS-stimulated BV2 and primary microglial cells (Xu et al., 2015). Recent studies also revealed that nuclear factor erythroid 2-related factor 2 (Nrf2), one of the major downstream mediators of AMPK, contributed to hematoma clearance in brain hemorrhage (Zhao et al., 2015), suggesting that Nrf2 may modulate microglial polarization and function in hemorrhagic stroke (Lan et al., 2017).

Based on the above-mentioned evidence, we hypothesized that rh-Chemerin treatment would reduce pro-inflammatory cytokines, promote M2-like microglia polarization and alleviate neurological deficits in neonatal rat models of GMH and that these beneficial effects might be mediated by CAMKK2/AMPK/Nrf2 signaling. We also postulated that rh-Chemerin would reduce ventricular dilation by suppressing neutrophils infiltration in choroid plexus of the ventricle.

2. Methods

2.1 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 complied with the ARRIVE guidelines. Two hundred and fifty-five P7 Sprague–Dawley neonatal pups (weight=12–14g, Harlan, Livermore, CA) were randomly subjected to either Sham (n=32) or GMH (n=223) group. All pups were kept in rooms with controlled temperature and 12-hour light/dark cycle, and given *ad libitum* access to food and water.

2.2 Germinal matrix hemorrhage (GMH) model

The general procedure for inducing GMH in unsexed P7 rats using collagenase infusion was performed as previously described (Lekic et al., 2015). In brief, pups were anesthetized with isoflurane (3.0% induction, 1.0–1.5% maintenance) on a stereotaxic frame. The skin was incised on the longitudinal plane to expose the bregma. A burr hole (1mm) was drilled on the skull (1.6mm lateral, 1.5mm anterior to the bregma), and a 27-gauge needle was inserted (2.7mm 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). The core temperature was maintained at 37°C. After infusion, the needle was left in place for additional 10 minutes to prevent possible leakage and then was withdrawn at rate of 0.5mm/min. After infusion, the pups were then placed back to the dams until sacrifice.

2.3 Drug administration

Recombinant human Chemerin protein (rh-Chemerin, Abcam) was dissolved in saline as described previously (Shi et al., 2017). Pups were randomly assigned to receive rh-Chemerin (3µg/kg/day, 9µg/kg/day or 27µg/kg/day) or saline via intraperitoneal or intranasal administration at 1h post-GMH and then once daily for 3 days (short-term study) or 7 days (long-term study). The dosage and treatment regimen were based on previous studies (Brunetti et al., 2011; Flores et al., 2016; Shi et al., 2017; Zhao et al., 2014).

2.3.1 Intranasal administration of recombinant Chemerin—Intranasal administration was performed as previously described (Doyle et al., 2014; Rodriguez-Frutos et al., 2016; Topkuru et al., 2013). Under anesthesia, pups were administered phosphate buffered saline (PBS) or recombinant Chemerin dissolved in PBS at 2µl per drop every 2 minutes. A total volume of 6µl was delivered into the bilateral nares (alternating nostrils at one time).

2.3.2 In vivo RNAi—Rat-derived ChemR23 siRNA (0.5 nmol/2µl, Life Technologies) or scramble siRNA (2µl, Life Technologies) was delivered via intracerebroventricular injection at 24h prior to GMH induction (1.5mm anterior, 1.5mm lateral to the bregma and 1.7mm deep on the ipsilateral ventricle) (Chen et al., 2017).

2.3.3 Liposomes administration—Liposomes (FormuMax) that contain a lipid fluorescent dye, Fluorescein DHPE (Lipo-DHPE), Alpha-NETA (ChemR23 specific inhibitor, Lipo-Alpha-NETA, Santa Cruz Biotechnology) (Graham et al., 2014), STO-609 (CAMKK2 specific inhibitor, Lipo-STO-609, Santa Cruz Biotechnology) (Cary et al., 2013) or Dorsomorphin (AMPK specific inhibitor, Lipo-Dorsomorphin, Santa Cruz Biotechnology) (Shi et al., 2017) were prepared according to the manufacturer's protocol. Intra-liposomal Alpha-NETA, STO-609 or Dorsomorphin concentration determined by microplate reader system (400nm, SpectraMax i3x, Molecular Devices), was 6 µg/µl 1µg/g (2µl) rat was intracerebroventricularly administered into contralateral ventricle of P6 rats of either sex.

2.4 Histological analysis

Pups were deeply anesthetized with isoflurane (5%), then transcardially perfused with ice-cold PBS followed by 10% formalin. Brains were post-fixed in 10% formalin overnight at 4°C, cryoprotected in 30% sucrose in PBS at 4°C for 72h, snap frozen in liquid nitrogen and cut on cryostat (Leica CM3050S-3-1-1, Bannockburn).

2.4.1 BrdU labeling and immunofluorescence staining—To assess cell proliferation, pups were intraperitoneally (i.p.) administered Bromo-2-deoxyuridine (BrdU; Millipore) at 50 mg/kg of body weight twice daily (Chip et al., 2017). The injections were at

an interval of approximately 12 h at 24 h, and 48 h, and one injection at 4 h before sacrifice at 72 h after GMH.

Immunofluorescence staining was performed on fixed frozen brain sections as previously reported (Chen et al., 2017). 8µm thickness slices were permeabilized with 0.3% Triton X-100 for 20min at room temperature, then blocked in 5% normal donkey serum in PBS for 2h. After washing with PBS for three times (10 min each), sections were incubated with anti-Chemerin (Abcam), anti-ChemR23 (Abcam), anti-ionized calcium binding adapter molecule 1 (Iba-1, Abcam), anti-CD11b/c (Abcam), anti-mannose receptor (Abcam), anti-Ki67 (Abcam), anti-BrdU (Sigma), anti-myeloperoxidase (MPO, Santa Cruz Biotechnology), anti-glial fibrillary acidic protein (GFAP, Abcam), or anti-neuronal nuclei (NeuN, Abcam) at 4 °C overnight following 1h incubation with FITC or Texas Red-conjugated secondary antibodies (Jackson Immuno Research) at room temperature. Then wash again with PBS for three times (10min each). Finally, slides were covered with DAPI (Vector Laboratories). The sections were imaged under fluorescent microscope (Leica DMI8, Leica Microsystems) equipped with LASX software. The number of Iba1+, CD11b/c and Ki67+ cells per field of view was quantified manually in the periventricular region of sham group, vehicle and rh-Chemerin treated pups with GMH. Six sections per pup (each section with 3 images) over a microscopic field of 20× were averaged and expressed as cells/field of view (FOV), as described previously (Flores et al., 2016). Quantifications were performed in a blinded fashion.

2.4.2 Nissl staining—Nissl staining was performed and analyzed as reported previously (Chen et al., 2017). Coronal brain sections (16µm thick) were respectively dehydrated in 95% and 70% ethanol for 2 min, then rinsed in tap water and distilled water for 10s. Sections were stained with 0.5% cresyl violet (Sigma-Aldrich) for 2 min and washed in distilled water for 10s followed by dehydration with 100% ethanol and xylene for 2 min (two times, respectively) before a coverslip with permount was placed. The sections were imaged by microscope (Olympus-BX51). Brain tissue loss and ventricular dilation were measured and calculated with ImageJ 4.0 (Media Cybernetics) (Ballabh, 2010; Dixon et al., 2016). Calculations were performed in a blinded fashion.

2.5 Western blot analysis

Western blot was performed as previously described (Wan et al., 2016). After sample preparation, 50µg protein per sample was loaded onto an 10–12% SDS-PAGE gels, ran for 90min at 100V, and was transferred onto 0.2µm or 0.45µm nitrocellulose membranes at 100V for 120 min (Bio-Rad). The membranes were blocked for 2h 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-human Chemerin (Abcam), anti-Chemerin (Abcam), anti-ChemR23 (Abcam), anti-GPR1 (Abcam), anti-CCRL2 (Abcam), anti-CAMKK2 (Abcam), anti-Phospho-CaMKK2 (Ser511) (Cell Signaling Technology), anti-Phospho-AMPKα (Thr172) (Cell Signaling Technology), anti-AMPKα (Cell Signaling Technology), anti-Nrf2 (Abcam), anti-IL1 beta (Abcam), anti-IL6 (Abcam), anti-TNF-alpha (Abcam). The same membranes were probed with actin (Santa Cruz Biotechnology) as internal loading controls. Appropriate secondary antibodies (Santa Cruz Biotechnology) were incubated with

membranes for 2h at room temperature. Bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences) and quantified through ImageJ 4.0 (Media Cybernetics).

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the levels of gene expressions, total ribonucleic acid (RNA) was extracted from 30mg of brain tissue using the RNeasy kit (Qiagen) (Enkhjargal et al., 2017). cDNA synthesis was carried out using GoScript Reverse Transcriptase (Promega) following standard protocol; 1mg of total RNA was used for cDNA synthesis. qRT-PCR was performed using synthetic primers (Suppl. Table 1) and SYBR Green detection reagent in Bio-Rad iQ5 system (Hercules). After incubation at 50°C for 2min and 94°C for 5min, samples were subjected to 40 cycles of 94°C for 30s, 58°C for 30s, 72°C for 90s, 72°C for 15 min, followed by a melting curve analysis (60–90°C with a heating rate of 0.2C and continuous fluorescence measurement) (Enkhjargal et al., 2017).

2.7 Neurobehavioral tests

Neurobehavioral tests were performed by two blinded researchers in a random and unbiased setup, as previously reported (Klebe et al., 2014; Manaenko et al., 2014). Short-term neurological tests, including righting reflex and negative geotaxis tests, were performed from 1d to 3d after GMH (Manaenko et al., 2014). Long-term neurological tests, including water maze, rotarod, and foot-fault, were performed from 21d to 28d after GMH (Klebe et al., 2014; Manaenko et al., 2014).

2.7.1 Righting reflex—The duration for the pups to completely rollover onto four limbs after being placed in supine position was recorded. The maximum duration was 60s per trial (3 trials/pup/day). The average values of all three trials were calculated.

2.7.2 Negative geotaxis—Pups were placed head downward onto a 45° slope, and the duration for the pups to rotate to turn 180° was recorded. The maximum recording time was 20s (3 trials/pup/day). The average value of all three trials were calculated.

2.7.3 Water maze test—In this four-day test, both of cued and hidden tests lasted maximum 60 s. The apparatus consisted of a metal pool (110cm in diameter) and a small platform (11cm in diameter) for the pups to climb onto, and swim distance, latency, and velocity were digitally recorded and analyzed by a tracking software (Noldus Ethovision). 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 submerged 1cm below the water, and the time spent in probe quadrant was recorded.

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

2.7.5 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 s.

2.7 Statistical analysis

All the data were presented as mean $\pm$ SD. A power analysis (using G*Power3) suggests that a power of 0.8, and an alpha of 0.05 for detecting a medium effect size (0.3) would require a sample size of 6 to 8 per group. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). One-way ANOVA with Dunnett'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 less than 0.05.

3. Results

3.1 Endogenous Chemerin and ChemR23 were upregulated after GMH

First, we examined whether endogenous Chemerin and ChemR23 expression would change in response to GMH. We evaluated Chemerin and ChemR23 expression by western blot analysis and qRT-PCR in the pups with (3h, 6h, 12h, 1d, 3d, 5d, and 7 d after GMH) and without GMH. The protein (Fig. 1A) and mRNA levels (Suppl. Fig. 1C) of endogenous Chemerin was significantly increased at 24 h, and continuously upregulated till 7 days (Fig. 1A and Suppl. Fig. 1C) after GMH compared to pups without GMH. Accordingly, ChemR23 was also significantly increased from 3 days to 7 days relative to pups without GMH (Fig. 1A and Suppl. Fig. 1D).

Given that there were two other receptors of Chemerin, we then evaluated the expression of GPR1 and CCRL2 in pups with and without GMH. No changes were observed in expression of GPR1 and CCRL2 after GMH (Fig. 1A), indicating that these two receptors might have relatively weak internal signaling than ChemR23 after GMH. Therefore, ChemR23 was selected as the main receptor for Chemerin after GMH.

Furthermore, double immunofluorescence staining showed that Chemerin and ChemR23 were abundantly expressed in microglia (Fig. 1B), neurons (Suppl. Fig. 1A) and astrocytes (Suppl. Fig. 1B) surrounding the lateral ventricle of pups with GMH. By contrast, Chemerin and ChemR23 seemed to have weaker expression levels in the microglia (Fig. 1B), neurons (Suppl. Fig. 1A) and astrocytes (Suppl. Fig. 1B) around the periventricular area of pups without GMH. Chemerin and ChemR23 immunoreactivity was weak in the cortex of pups both with and without GMH (data not shown).

3.2 Intranasal administration of human recombinant Chemerin improved short-term neurological outcomes at 72h post GMH

To investigate the translational treatment regimen of Chemerin, three doses (3, 9 and 27 μ g/kg) with different routes of administration (intranasal and intraperitoneal injection) were performed 1 hour after GMH. Vehicle-treated pups spent more time flipping to the prone position (Fig. 2A, B) and rotating to head upward position (Fig. 2C, D) compared to the sham group at 1d and 2d after GMH. All three doses of rh-Chemerin treated groups showed a significantly improved short-term neurological function (Fig. 2A–D). Among these three groups, medium dose of rh-Chemerin treated pups displayed the best performance in both of body righting and negative geotaxis, and was comparable to sham group as early as 2d after GMH (Fig. 2A–D).

Given human recombinant Chemerin was applied for treatment, anti-human Chemerin antibody was used to evaluate the level of exogenous rh-Chemerin in the brain. Despite the same amount of chemerin delivered, intranasal administration delivered significantly more rh-Chemerin into the brain tissue compared to intraperitoneal administration (Fig. 2E). No abnormal behavior was observed due to rh-Chemerin administration. Collectively, 9µg/kg of rh-Chemerin with intranasal administration was used for all following experiments.

3.3 Rh-Chemerin treatment ameliorated long-term neurological deficits after GMH

To investigate the effects of rh-Chemerin treatment on the long-term neurological impairments induced by GMH, neurological functions were assessed by water maze, foot-fault, and rotarod at four weeks post GMH. In the water maze test, vehicle-treated animals traveled significantly longer in one minute (Fig. 3A), spent more time finding the platform and had less time in the defined quadrant compared to sham (Fig. 3B), which meant vehicle-treated animals had cognitive impairment in memorizing the platform location compared to sham animals. In contrast, rh-Chemerin-treated animals performed significantly better than vehicle-treated animals (Fig. 3A–C). Meanwhile, there was no significant difference in velocity amongst the three groups, indicating that it is the spatial memory loss, not the slower velocity, that led to the longer escape latency (Fig. 3D). In the foot fault test, we noted that vehicle controls displayed significantly more foot slips on left side compared to the sham group, and that the performance was significantly better in rh-Chemerin-treated group compared to the vehicle controls (Fig. 3E). Moreover, rh-Chemerin treatment significantly reduced the falling latency at both of the 5rpm and 10rpm acceleration compared to vehicle controls (Fig. 3F). As for the growth profile, GMH significantly slowed normal growth from 14d to 28d after hemorrhage, as demonstrated by decreased body weight in the vehicle group compared to the sham group. However, rh-Chemerin restored normal body weight (Fig. 3G).

3.4 rh-Chemerin treatment attenuated elevation of IL-1 beta, IL-6 and TNF alpha after GMH

In vivo studies suggested that Chemerin treatment inhibited the production of inflammatory cytokines and that this inhibitory effect mainly depended on ChemR23 (Cash et al., 2010; Cash et al., 2008; Lin et al., 2017; Luangsay et al., 2009). We postulated that treatment with rh-Chemerin would attenuate the elevation of IL-1 beta, IL-6 and TNF alpha via ChemR23 after GMH. To this end, we compared five sets of pups at 72 h after GMH: (a) sham; (b) GMH + vehicle; (c) GMH + rh-Chemerin; (d) GMH + rh-Chemerin + scramble siRNA; (e) GMH + rh-Chemerin + ChemR23 siRNA. ChemR23 expression was significantly decreased after the administration of ChemR23 siRNA at 72h after GMH compared to rh-Chemerin-treated pups and scramble siRNA group (Fig. 4A, B). GMH induced IL-1 beta, IL-6, and TNF-alpha in vehicle-treated pups, whereas the levels of IL-1beta, IL-6 and TNF alpha were significantly reduced in rh-Chemerin-treated pups (Fig. 4A, C–E). However, knockdown of ChemR23 significantly reversed the inhibitory effect of rh-Chemerin on the expression of IL-1 beta, IL6 and TNF-alpha relative to rh-Chemerin-treated and scramble siRNA groups (Fig. 4A, C–E), indicating that rh-Chemerin suppressed expression of pro-inflammatory cytokines via ChemR23.

3.5 Rh-Chemerin administration promoted accumulation and proliferation of M2 microglia in the periventricular area after GMH

To determine whether rh-Chemerin treatment changes the microglial response after GMH, we used Iba1 as a pan microglia marker and CD11b/c as an activated microglia marker in the periventricular area. Compared to the sham group, the number of Iba1+ (Fig. 5A, Suppl. Fig. 2A) and CD11b/c+ (Fig. 5E, F) microglia increased significantly in both vehicle and rh-Chemerin treated pups 72h post-GMH. In addition, the number of activated microglia was significantly more in rh-Chemerin group compared to vehicle group (Fig. 5E, F).

Given that microglial proliferation has protective effects in adult ischemic stroke (Lalancette-Hebert et al., 2007), we evaluated Ki67+/Iba1+ microglia, as well as BrdU+/Iba1+ microglia in the three sets of pups at 72h after GMH. We found that the number of Ki67+/Iba1+ and BrdU+/Iba1+ microglia were significantly increased in pups with rh-Chemerin compared to the vehicle group (Fig. 5A, B, Suppl. Fig. 2B). However, there was a slight increase in the number of Ki67+/Iba1+ and BrdU+/Iba1+ cells in the vehicle group compared to sham group, which was not reach statistically difference (Fig. 5B and Suppl. Fig. 2A, B).

Alternative polarization of microglia plays a beneficial role in attenuating inflammation after brain hemorrhage (Chip et al., 2017; Tao et al., 2016; Xu et al., 2015), we then examined if rh-Chemerin treatment upregulated the markers of alternatively activated microglia. No CD206+ microglia were detected in the sham group, and vehicle-treated pups showed a slight tendency towards having more CD206+ microglia (Fig. 5G, H). By contrast, rh-Chemerin treatment significantly increased CD206+ microglia compared to sham and vehicle groups (Fig. 5G, H), indicating that the activated and proliferating microglia in the periventricular region of rh-Chemerin-treated pups might be predominantly CD206+ microglia. Rh-Chemerin also markedly increasing the anti-inflammatory mediator, Arginase-1, expression at 72h after GMH (Fig. 5J). Furthermore, ChemR23 was co-located in CD206+ microglia in the periventricular area in pups after GMH (Fig. 5I).

3.6 Rh-Chemerin treatment reduced ventricular dilation by suppressing neutrophil infiltration in the choroid plexus

As ventricular dilation is the major complication of GMH (Feng et al., 2017), we assessed whether this could be alleviated by rh-Chemerin treatment. rh-Chemerin reduced the severity of ventriculomegaly 28 days after GMH (Fig. 6C). Compared to sham animals, a significant ventricular dilation occurred in vehicle-treated pups (Fig. 6C). At the same time, the ventricular volume was significantly reduced in rh-Chemerin-treated pups compared to the vehicle group (Fig. 6D). White matter volume was markedly reduced in vehicle treated pups, while it was significantly restored in rh-Chemerin treated pups (Suppl. Fig. 3A). Cortical thickness was significantly decreased in the vehicle-treated pups compared to the sham group (Suppl. Fig. 3B) and rh-Chemerin treated pups had significantly less cortical loss (Suppl. Fig. 3B).

Since ventriculomegaly could be associated with cerebrospinal fluid (CSF) overproduction after brain hemorrhage (Shooman et al., 2009), we next evaluated the effects of rh-Chemerin

treatment on microglial accumulation and neutrophil infiltration in the choroid plexus, the primary site of CSF production. We found that MPO immunoreactivity was significantly increased in pups subjected to GMH relative to sham group at 3d, 14d, and 28d post GMH (Fig. 6A, E). rh-Chemerin treatment significantly reduced the MPO immunoreactivity in choroid plexus at 3d, 14d, and 28d post GMH (Fig. 6A, E). There was no evident difference in the overall number of Iba1+ cells between different experimental groups at 3d, 14d, and 28d post GMH (Fig. 6B, F). These data indicated that GMH elicited neutrophil infiltration other than microglia accumulation into the choroid plexus.

3.7 CAMKK2/AMPK/Nrf2 pathways were elevated after GMH and knockdown of ChemR23 reduced CAMKK2/AMPK/Nrf2 levels

In vivo and *in vitro* studies show that CAMKK2/AMPK/Nrf2 signaling plays multiple roles in various models, including the regulation of microglia/macrophage-related inflammation (Kobayashi et al., 2016; Reverchon et al., 2012; Shen et al., 2013; Xu et al., 2015). We investigated whether CAMKK2/AMPK/Nrf2 signaling was the potential pathway of Chemerin/ChemR23 in regulating GMH-induced inflammation. Similar to the increasing pattern of Chemerin and ChemR23, the ratio of p-CAMKK2 to CAMKK2 (Fig. 7A, B) and p-AMPK to AMPK (Fig. 7A, C), as well as the expression of Nrf2 (Fig. 7A, D), were significantly increased, and continuously increased up to 7 days after GMH compared to sham group. We further tested whether rh-Chemerin treatment affected CAMKK2/AMPK/Nrf2 expression and CAMKK2/AMPK phosphorylation. We compared five sets of pups at 72 h after GMH: (a) sham; (b) GMH + vehicle; (c) GMH + rh-Chemerin; (d) GMH + rh-Chemerin + scramble siRNA; (e) GMH + rh-Chemerin + ChemR23 siRNA. GMH significantly increased the phosphorylation of CAMKK2 (Fig. 7E, F) and AMPK (Fig. 7E, G), as well as the expression of Nrf2 (Fig. 7E, H), compared to the sham group. In addition, rh-Chemerin treatment further increased the phosphorylation of CAMKK2 and AMPK, and Nrf2 expression compared to vehicle-treated pups. However, ChemR23 knockdown significantly suppressed the phosphorylation of CAMKK2 and AMPK, as well as the expression of Nrf2 compared to rh-Chemerin-treated and scramble siRNA groups (Fig. 7E–H). Together, these results indicated that CAMKK2/MPK/Nrf2 pathway were the potential downstream mediators of Chemerin/ChemR23 signaling after GMH.

3.8 Selective inhibition of ChemR23/CAMKK2/AMPK signaling in activated microglial cells abolished the effect of rh-Chemerin on inhibiting neuroinflammation after GMH

To directly test whether microglial ChemR23/CAMKK2/AMPK signaling protected neonatal brain from GMH, we delivered specific ChemR23/CAMKK2/AMPK inhibitors into microglial cells via intracerebroventricular injection of Lipo-DHPE, Lipo-Alpha-NETA (1µg/g, 2µl) (Graham et al., 2014), Lipo-STO-609 (1µg/g, 2µl) (Cary et al., 2013), Lipo-Dorsomorphin (1µg/g, 2µl) (Shi et al., 2017) 24h before GMH induction. Fluorescently labeled liposomes were engulfed almost exclusively in activated Iba1+ microglia (Fig. 8A, arrows) rather than in GFAP+ and NeuN+ cells at 72h after GMH (Fig. 8A, arrowheads), and no labeled liposomes were observed in the sham group (data not shown).

The addition of Lipo-Alpha-NETA, Lipo-STO-609 or Lipo-Dorsomorphin to rh-Chemerin did not affect ChemR23 expression (Fig. 8B, C). The phosphorylation of CAMKK2 was

significantly reduced in Lipo-Alpha-NETA and Lipo-STO-609 treated pups compared to rh-Chemerin with or without Lipo-PBS treated pups (Fig. 8B, D). In addition, Lipo-STO-609 inhibited phosphorylation of AMPK. However, Lipo-dorsomorphin had no effect on phosphorylation of CAMKK2, which indicated AMPK as a downstream kinase of CAMKK2 after GMH (Fig. 8B, D, E). Administration of Lipo-Dorsomorphin significantly reduced the phosphorylation of AMPK and expression of Nrf2 (Fig. 8B, E, F). Administration of all three liposome-encapsulated inhibitors significantly increased the expression of IL-1 beta, IL-6 and TNF alpha compared to rh-Chemerin with or without Lipo-PBS treated pups (Fig. 8B, G–I). Collectively, ChemR23/CAMKK2/AMPK/Nrf2 inhibition in functional microglial cells restored the inflammation after GMH, indicating a key role of microglial ChemR23/CAMKK2/AMPK/Nrf2 signaling in protecting neonatal brain from hemorrhage-induced inflammation.

4. Discussion

GMH is a devastating event in premature infants (Ballabh, 2010). Mounting evidence suggests that inflammation contributes to the progression of GMH-induced brain injury (Georgiadis et al., 2008). In the present study, we explored the anti-inflammatory roles of Chemerin in GMH rat pups and elucidated the potential mechanisms involved. We observed rh-Chemerin could exert a neuroprotective effect on GMH-induced inflammation by improving neurological outcomes, decreasing expressions of pro-inflammatory cytokines and promoting accumulation and proliferation of M2 microglia. Furthermore, rh-Chemerin could promote the phosphorylation of CAMKK2 and AMPK, and expression of Nrf2 via activation of ChemR23. Pharmacological inhibition of ChemR23/CAMKK2/AMPK signaling selectively in activated microglia after GMH could abolish the anti-inflammatory effects of rh-Chemerin. These results are consistent with the hypothesis that rh-Chemerin could alleviate neuroinflammation through the CAMKK2/AMPK/Nrf2 signaling.

In the first part of this study, we demonstrated that the endogenous expression of Chemerin increased after GMH. Chemerin has been shown to function by binding to three receptors: ChemR23, CCRL2 and GPR1 (Bondue et al., 2011). Knockdown of ChemR23 or CCRL2 exacerbated the expression of proinflammatory mediators and neutrophil recruitment *in vivo* and *in vitro* (Cash et al., 2013; Cash et al., 2008; Mazzon et al., 2016). However, whether these three receptors would respond to GMH remains to be investigated. Therefore, we tested the temporal expression profile of endogenous ChemR23, CCRL2 and GPR1 following GMH. We observed that the expression of ChemR23, rather than CCRL2 and GPR1 was elevated after GMH. Thus, we chose ChemR23 for the following studies. We then detected the expression of Chemerin and ChemR23 in different cell types in the neonatal brain after GMH and found that Chemerin and ChemR23 were co-localized with microglia, astrocytes and neurons.

Then we explored the protective effects of rh-Chemerin in both of short-term and long-term neurological outcomes. Firstly, we administered three doses of rh-Chemerin at 1 h after GMH, and the medium dose conferred the optimal effects in negative geotaxis and righting reflex at 24 h after GMH. We then compared the two delivery routes of rh-Chemerin, and found that intranasal delivery was more effective than intraperitoneal delivery. A previous

study showed that intranasal administration could significantly increase the uptake of neurotherapeutics within 1h and they remained at relatively high levels till 24h in the brain tissue when compared to intraperitoneal delivery (Chauhan and Chauhan, 2015). In addition, Chemerin exerted constrictive effect on the arterial system to increase blood pressure (Kennedy et al., 2016). To focus on the direct effect of Chemerin on the brain and avoid systemic side effects, the intranasal administration was used for following studies. Similar neurological improvement was observed in long-term experiments as assessed by water maze, foot-fault, and rotarod.

It has been known that GMH elicits pronounced production of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α (Tao et al., 2016). A previous study discovered that treatment with Chemerin derived peptide, Chemerin 15, could inhibit the production of IL-1 β , IL-6 and TNF- α in the zymosan-induced peritonitis mice model, which was entirely ChemR23 dependent (Cash et al., 2008). In this study, we showed that the expression of IL-1 β , IL-6 and TNF- α was increased 72 h after GMH and that management with rh-Chemerin could significantly suppress the expression of pro-inflammatory cytokines after GMH. Meanwhile, ChemR23 knockdown reversed the inhibitory effects of rh-Chemerin on the inflammatory response after GMH, indicating the potential anti-inflammatory effects of Chemerin/ChemR23 in the management of GMH.

Alternative polarization of microglia/macrophages into alternatively activated (M2) phenotype blunts the drastic increase in proinflammatory cytokines after GMH (Aronowski and Zhao, 2011; Klebe et al., 2015). Our current data showed that activated (CD11b/c+) microglia, as well as proliferating (Ki67/Iba1+, BrdU+/Iba1+) microglia, were upregulated in the treatment group. Of note, CD11b/c+ cells in the vehicle group dispersed in the perihematoma region, while these cells accumulated to the peri-hematoma region in rh-Chemerin-treated group. Although in colitis and experimental autoimmune encephalitis, chemerin has been shown to suppress M2 macrophage polarization and be a proinflammatory factor. However, in the current study, we showed that rh-Chemerin treatment could reduce the production of proinflammatory cytokines. Thus, we hypothesized that chemerin, in the context of GMH, might augment neuroprotective M2 microglia, instead of suppressing M2 polarization (Lin et al., 2014; Mazzon et al., 2016; Monnier et al., 2012). To further determine the nature of microglia in rh-Chemerin group, we used CD206 (an M2 marker) to fluorescently label the microglia in the perihematoma regions. The results showed that the significantly increased number of CD206+ cells in rh-Chemerin-treated GMH pups compared to other groups. Interestingly, the number of CD206+ cells slightly increased in vehicle-treated GMH pups compared to the sham group, indicating that hemorrhage may also slightly induce microglia to M2 phenotype. However, it was not sufficient to exert its neuroprotective role after GMH. Moreover, ChemR23 was abundantly expressed by CD206+ cells. Taken together, it is highly likely that the anti-inflammatory effects of Chemerin/ChemR23 rely on the accumulation and proliferation of M2 phenotype microglia.

We further investigated the molecular basis of Chemerin/ChemR23 signaling in resolution of inflammation following GMH. AMPK is expressed by various types of brain cells, including microglia, and activated when the intracellular ratio of AMP to ATP is imbalanced. Previous

studies have demonstrated that phosphorylated AMPK promotes M2 microglial polarization and reduces pro-inflammatory cytokines upon inflammatory stimuli (Sag et al., 2008; Xu et al., 2015). Nrf2, a downstream transcription factor of AMPK, has been reported to play an important role in suppressing inflammatory response of intracerebral hemorrhage (Kobayashi et al., 2016; Zhao et al., 2015). In this study, we found that the expression of phosphorylated AMPK and Nrf2 increased in parallel to Chemerin and ChemR23 at various time points. Moreover, our data were consistent with previous publications that Chemerin increased phosphorylation of AMPK and expression of Nrf2 (Reverchon et al., 2012; Shen et al., 2013), indicating that the activation of AMPK and Nrf2 may participate in Chemerin/ChemR23-mediated resolution of microglial inflammation. Considering the ChemR23 receptor is expressed on various brain cells and little is known about the ChemR23/AMPK/Nrf2 signaling in microglia in inflammation resolution *in vivo*, we used the Lipo-Alpha-NEtA (ChemR23 inhibitor) and Lipo-Dorsomorphin (AMPK inhibitor) to inhibit the AMPK and Nrf2 activation selectively in microglia. With the treatment of Lipo-Alpha-NEtA and Lipo-Dorsomorphin, AMPK activation and Nrf2 expression were completely reversed. By contrast, the expression of IL-1 β , IL-6 and TNF- α were significantly increased, suggesting that Chemerin alleviated GMH-induced inflammation may be primarily mediated through AMPK/Nrf2 signaling in microglia.

AMPK is activated by its upstream kinases, including LKB1 and CaMKK2 (Green et al., 2011; Lizcano et al., 2004). Previous studies showed that CaMKK2/AMPK pathway functionally blocked LPS-induced pro-inflammatory effects in BV2 cells and murine primary microglia by promoting M2 polarization. In our study, administration of Lipo-STO-609 (CaMKK2 inhibitor) significantly abolished the inhibitory effect of rh-Chemerin on microglial inflammation. Furthermore, targeted inhibition of ChemR23 in microglia with Lipo-Alpha-NEtA markedly downregulated the phosphorylation of CaMKK2 and AMPK, as well as the expression of Nrf2. While inhibiting the phosphorylation of CaMKK2 had no effect on the expression of ChemR23, and similar results were also found with AMPK inhibitor. All three inhibitors had markedly increased the expressions of pro-inflammatory cytokines. Taken together, these observations confirmed that rh-Chemerin exerted its anti-inflammatory effects primarily through the activation of ChemR23/CaMKK2/AMPK/Nrf2 signaling in microglia.

Ventricular dilation is a common long-term outcome of GMH (Ballabh, 2010). A recent study has demonstrated that the inflammatory response in the choroid plexus results in the formation of post-hemorrhagic ventricular dilation (Karimy et al., 2017). Consistent with the study on the rabbit pups with intraventricular hemorrhage (Georgiadis et al., 2008), MPO immunoreactivity in choroid plexus significantly increased at 7d, and remained high till 28d after GMH. Meanwhile, rh-Chemerin administration significantly reduced MPO immunoreactivity in choroid plexus at 7d, 14d, and 28d after GMH. However, microglial accumulation and morphological transformation in choroid plexus were comparable between pups with and without GMH at these three time points, indicating rh-Chemerin treatment ameliorated hydrocephalus and the mechanism might be due to reduced neutrophil infiltration other than reduced microglial accumulation on the choroid plexus. Collectively, this prolonged neutrophil infiltration may have a potential role in the development of

hydrocephalus, and the effect of rh-Chemerin in reducing ventricular dilation was, at least in part, via decreasing the infiltration of blood-derived immune cells.

In this study, we focused on effects of Chemerin mainly mediated by microglial signaling in GMH. The effects of Chemerin on neurons or astrocyte warrant further investigation. Alternate pathways of Chemerin and ChemR23 were not evaluated in this study. Previous studies showed that Chemerin modulated various downstream factors, including extracellular signal-regulated kinases 1/2 and mitogen-activated protein kinase (Kennedy and Davenport, 2018; Sell et al., 2009), that might contribute to the microglial proliferation after rh-Chemerin treatment. Moreover, the nuclear Nrf2 levels after Chemerin administration were not measured since Nrf2 exerts its function mainly in nucleus. We presumed that the increase in total Nrf2, as measured in the study, may reflect the nuclear Nrf2 activity.

In conclusion, we provided the first preclinical evidence that rh-Chemerin reduced expression of pro-inflammatory cytokines, promoted accumulation and proliferation of M2 microglia, and alleviated neurological deficits in rat pups with GMH. The neuroprotective effects of rh-Chemerin were associated with the ChemR23/CAMKK2/AMPK/Nrf2 pathway. These results supported the idea that rh-Chemerin could be a neuroprotective agent after GMH and that activation of the ChemR23/CAMKK2/AMPK/Nrf2 pathway could potentially ameliorate neuroinflammation after GMH or other similar brain injuries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Chemerin improves neurological and morphological outcomes after GMH.
2. Chemerin promotes accumulation and proliferation of M2 microglia.
3. Chemerin ameliorates neuroinflammation after GMH.
4. Activation of CAMKK2/AMPK/Nrf2 pathway reduces neuroinflammation.

Fig. 1. Endogenous Chemerin and ChemR23 were upregulated in the brain after GMH
(A) Western blot data showed that Chemerin expression levels significantly increased from 1d to 7d post GMH reaching peak at 7d post GMH. ChemR23 expression levels significantly increased from 3d to 7d, reached highest at the 7d after GMH. GPR1 and CCRL2 expression levels were comparable between pups with and without GMH at eight time points. Data are normalized to actin protein expression. Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, $N = 6/\text{group}$ (All sham samples in Western blot were from the same animals which were euthanized after short-term neurobehavioral tests). Representative images of immunofluorescence staining showing the co-localization of Chemerin and ChemR23 with microglia (**B**, Iba1, green) in the pups with or without GMH. Chemerin and ChemR23 immunoreactivities were greater on microglia in the periventricular area at 72h

after GMH. Arrows indicate Chemerin or ChemR23 colocalized with microglia (Arrowhead part was amplified) Scale bar = 50 μ m. N = 3/group.

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Fig. 2. Intranasal administration of human recombinant Chemerin improved short-term function tests at 72h post GMH

Righting reflex (A–B) and Geotaxis reflex (C–D) showed that medium dose (9 μ g/kg) of rh-Chemerin significantly improved neurological function compared to vehicle treated pups at 1d and 2d after GMH. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle, $N = 6–8/\text{group}$. (E) Intranasal administration delivered significantly more rh-Chemerin into brain tissue compared to intraperitoneal administration at 72h after GMH. Data are mean $\pm$ SD. ANOVA, Dunnett. * compared to GMH + rh-Chemerin (9 μ g/kg ip.), $N = 6–8/\text{group}$.

Fig. 3. rh-Chemerin administration restored long-term neurological function at four weeks post GMH

Water maze test (A–D) showed that rh-Chemerin treatment significantly improved spatial memory and learning as for the less swim distance to find the platform (A), more time in the defined quadrant (B) and less escape duration (C). Note that swim speed (D) had no statistically differences among three groups. Rh-Chemerin markedly improved GMH pups' motor function assessed by foot fault (E) and rotarod test (F). (G) Pups' body weight changes after GMH. Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle, $N = 8$ /group.

Fig. 4. rh-Chemerin treatment reduced expression of IL-1 beta, IL-6 and TNF alpha via ChemR23 at 72 h after GMH

(A) Representative image of Western blot data showing the expression of pro-inflammatory cytokines with or without ChemR23 siRNA. (B) Quantitative analysis of ChemR23 expression after siRNA knockdown showed that ChemR23 siRNA rather than scramble siRNA markedly reduced the expression of endogenous ChemR23 after GMH. (C–E) Western blot data quantification showed that the expression of IL-1 beta (C), IL-6 (D), and TNF alpha (E) were significantly increased after GMH. rh-Chemerin significantly reduced expressions of these three pro-inflammatory cytokines, while these effects were reversed when silencing ChemR23. (All samples of GMH + vehicle and GMH + rh-Chemerin in Western blot were from the same animals which were euthanized after short-term neurobehavioral tests). Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle, $N = 6-8/\text{group}$.

Fig. 5. rh-Chemerin promoted accumulation and proliferation of M2 microglia in the periventricular area 72 h post GMH
 (A–D) Representative images of immunofluorescence staining and quantification showing the accumulation and proliferation (Ki67+/BrdU+, red) (A, C) of Iba1+ (green) cells in the periventricular region. (E–H) Representative images of immunofluorescence staining and quantification showing the accumulation of activated microglia (CD11b/c+, green) (E, F) and M2 microglia (CD206+, green) (G, H) in the periventricular regions after GMH. Scale bar = 50µm. Dots in A–G represent data from individual pups. FOV = $2.3 \times 10^6 \mu\text{m}^3$. N = 6/group. Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle. (I) Representative images of immunofluorescence staining showing the co-localization of ChemR23 (red) and M2 microglia (CD206, green) in the periventricular regions after GMH. Arrows indicate ChemR23 colocalized with M2 microglia. Scale bar = 25µm. N = 3/group. (J) qRT-PCR results showed that rh-Chemerin significantly increased the mRNA level of Arginase-1 at 72h after GMH. N = 6/group. Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle.

Fig. 6. rh-Chemerin treatment improved long-term brain morphology, which may partially due to the suppression of neutrophils infiltration in choroid plexus after GMH

(A) Representative images of immunofluorescence staining showing co-localization of neutrophil marker myeloperoxidase (MPO) with DAPI in choroid plexus. rh-Chemerin treated group had significantly fewer MPO positively stained cells at 3d, 14d, 28d after GMH (E). (B) Representative images of immunofluorescence staining showing co-localization of Iba1 with DAPI in choroid plexus. Microglial accumulation was similar among sham, GMH + vehicle, and GMH + rh-Chemerin group at 3d, 14d, 28d after GMH (F). Scale bar = 50 μ m. N = 3/group. (C) Representative images of Nissl-stained brain sections showing the ventricular dilation and brain tissue damage at 4 weeks after GMH. Quantification showing rh-Chemerin significantly reduced the ventricular dilation (D) in pups with GMH. N = 8/group. (All samples in Nissl staining were from the same animals which were euthanized after long-term neurobehavioral tests). Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle.

Fig. 7. CAMKK2/AMPK/Nrf2 signaling is a potential pathway for rh-Chemerin-afforded anti-inflammation in GMH pups

(A) Representative images of Western blot data showing the endogenous expression of proteins of interest from 3h to 7d after GMH. (B–D) Western blot analysis showed that the phosphorylation of CAMKK2 (B), AMPK (C) and the expression of Nrf2 (D) significantly increased in a time-dependent manner post GMH. (E) Representative images of Western blot data showing the expression of proteins of interest with or without ChemR23 siRNA. (F–H) Western blot analysis showed that rh-Chemerin significantly increased the phosphorylation of CAMKK2 (F), AMPK (G) and the expression of Nrf2 (H) at 72h after GMH. ChemR23

siRNA but not scramble siRNA reversed the effect of rh-Chemerin on CAMKK2/AMPK/Nrf2 signaling. (All samples of GMH + rh-Chemerin in Western blot were from the same animals which were euthanized after short-term neurobehavioral tests). Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle, N = 6–8/group.

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Fig. 8. Selective inhibition of ChemR23/CAMKK2/AMPK signaling in activated microglial cells abolished the effect of rh-Chemerin on inhibiting neuroinflammation at 72h after GMH

(A) Representative images of immunofluorescence staining showing fluorescently labeled liposomes were engulfed almost exclusively in Iba1+ microglia (Arrows, red (a–c)) rather than in GFAP+ (Arrowheads, red (d)) and NeuN+ (Arrowheads, red (e)) cells at 72h after GMH. (B) Representative images of Western blot data showing the expression of pCAMKK2, pAMPK, and Nrf2, as well as IL-1beta, IL-6, and TNF alpha either with rh-Chemerin treatment alone, rh-Chemerin + Lipo-Alpha-NETA, rh-Chemerin + Lipo-STO-609 or rh-Chemerin + Lipo-Dorsomorphin. (C) Western blot analysis of ChemR23 showed that ChemR23 level does not change with Lipo-Alpha-NETA, Lipo-STO-609 or Lipo-Dorsomorphin intervention. (D) Western blot analysis of pCAMKK2 to CAMKK2 ratio showed that pCAMKK2 increased in the rh-Chemerin treatment group and decreased in Lipo-Alpha-NETA and Lipo-STO-609 groups. However, pCAMKK2 level does not change with Lipo-Dorsomorphin intervention. (E) Western blot analysis of pAMPK to AMPK ratio showed that pAMPK increased in the rh-Chemerin treatment group and decreased in Lipo-Alpha-NETA, Lipo-STO-609 and Lipo-Dorsomorphin groups. (F) Western blot analysis of Nrf2 showed that Nrf2 increased in the rh-Chemerin treatment group and decreased in Lipo-Alpha-NETA, Lipo-STO-609 and Lipo-Dorsomorphin groups. (G–I) Western blot data showed that Lipo-Alpha-NETA, Lipo-STO-609 and Lipo-Dorsomorphin reversed the inhibitory effects of rh-Chemerin on pro-inflammatory cytokines. (All samples of GMH + rh-Chemerin in Western blot were from the same animals which were euthanized after short-term neurobehavioral tests). Data are mean $\pm$ SD. ANOVA, Dunnett. * $p < 0.05$ compared to sham, # $p < 0.05$ compared to GMH + vehicle, N = 6–8/group.