A selective CB2R agonist (JWH133) restores neuronal circuit after Germinal

Matrix Hemorrhage in the preterm via CX3CR1⁺ microglia

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Abstract

Microglia play dual roles after germinal matrix hemorrhage, and the neurotrophic phenotype maybe neuroprotective. However, the phenotype transformation and the way by which neuron-microglia dialogue remain unclear. We raise the hypothesis that a cannabinoid receptor2 agonist (JWH133) accelerates the CX3CR1⁺ microglia secreting neurotrophic factors and restores damaged neuronal circuit. Here, we report a novel function of JWH133 in transforming the microglia CX3CR1 positive that secrete brain-derived neurotrophic factor (BDNF), which triggers neuron proliferation and neuronal restoration. Using a collagen VII-induced GMH model in rat pups postnatal day 7 (P7), we found that the drug showed robust activity in neuronal precursors. Moreover, the FA value of DTI in the internal zone revealed the positive effects of JWH133 on neural restoration. CX3CR1, a critical modulating molecule expressed in microglia, was upregulated after treatment with JWH133 and the corresponding shRNA (NM_133534.1) was used to silence the expression of CX3CR1. 3 days after treatment with JWH133, we detected reduced expression of biomarkers for neural progenitor cells (NPCs) in pups pre-injected in the lateral ventricular tissue with CX3CR1 shRNA, but not in pups injected with control shRNA. Overall, this study provides evidence that JWH133 promoted a neurotrophic phenotype of microglia (CX3CR1⁺ microglia), beyond merely alleviating microglial

proliferation and inflammation. Moreover, JWH133 restored impaired neuronal circuit, which represent a novel therapeutic strategy following GMH in clinic.

Key words: Neuron-microglia dialogue, germinal matrix hemorrhage, CB2R agonist, neural circuit restoration, CX3CR1⁺ microglia

Introduction

Germinal Matrix Hemorrhage (GMH) is a significant cause of neonatal stroke and death (Hamilton et al., 2013). In addition to hydrocephalus, the consequences of GMH-induced brain injury include lifelong cerebral palsy and mental retardation (Ballabh et al., 2004; Murphy et al., 2002). Previously, in a series of studies, we have established the neuroprotective effects of cannabinoid receptor II (CB2R), which may partially be related to the suppression of microglial proliferation (Tang et al., 2016a; Tang et al., 2015). Recently, we have found that after GMH, microglia may have a positive role in the restoration of neural circuits after the activation of CB2R (Tang et al., 2016b).

Microglia, a major immune cell type in the brain, has typically been studied in the contexts of infection, injury and intracerebral hemorrhage (Reshef et al., 2014). Under conditions of neural pathology, “activated” microglia change their morphology from a “resting” or “quiescent” appearance to an amoeboid shape, and they are a

major source of neuroinflammation (Kettenmann et al., 2011). Recently, the positive role of microglia after brain damage has received substantial attention. Increasing evidence indicates that ramified microglia play an important role in the subgranular zone (SGZ) of the adult hippocampus, where they modulate neurogenesis by pruning newborn cells soon after birth (Sierra et al., 2010). In general, microglia are classified as M1-type or M2-type activated macrophages; however, this classification is not sufficiently specific for use in detailed neuronal studies. Within the central nervous system (CNS), intercellular cross-talk is an important process in which the secretion of various factors plays critical roles. Evidence suggests that various cell surface proteins, such as CD200R, CD127, CD45 and CX3CR1, contribute to the resting phenotype of microglia (Saijo and Glass, 2011). By contrast, microglia that secrete IL-4, IL-6, TNF- α and TGF- β are classified as an activated phenotype, which may contribute to the neuroinflammation that occurs after brain injury.

It has previously been established that brain injury triggers many repair processes in the adult brain, including angiogenesis, oligodendrogenesis, neurogenesis and remyelination (Lo, 2010). How these processes are maintained and triggered remains to be elucidated. Although the roles of cell surface receptors on microglia have been extensively studied, their contribution in damaged CNS remains unclear. The G protein-coupled seven-transmembrane chemokine receptor CX3CR1 is

expressed on monocytes, macrophages, dendritic cells and microglia(Jung et al., 2000). The only known CX3CR1 ligand, CX3CL1, is expressed on various neural cells and astrocytes. Early studies have not reported a role of CX3CR1 in the healthy brain, but recent animal studies have suggested that CX3CR1 deficiency might play negative roles in adult neurogenesis and hippocampus circuit integrity (Bachstetter et al., 2011; Cardona et al., 2006). Several studies have reported divergent results on the role of CX3CR1 deficiency in animal models of neural disease. In AD, CX3CR1-knockout mice have been found to benefit from reduced neuronal loss and improved behavioral deficits (Fuhrmann et al., 2010; Lee et al., 2010). Moreover, Tang et al. have suggested that the inhibition of CX3CR1 signaling might function as a therapeutic modality in ischemic brain injury (Tang et al., 2014). However, the pruning effects of activated microglia on synapses and circuits in the healthy or immature brain should be noted. After a brain injury such as GMH, microglia are activated and become self-protective cells that endocytose lysed blood cells and even prune the newborn neural circuits, which may be beneficial to the injured CNS. CX3CR1 is a “resting” signaling receptor in microglia, and the overexpression of CX3CR1 may partially suppress the over-pruning of neural cells by microglia. Harrison et al. have shown that the binding of CX3CR1 and neuronal CX3CL1

appears to play a critical role in the interactions between microglia and neurons (Harrison et al., 1998).

In this study, we hypothesized that the activation of CB2R promotes a neurotrophic phenotype in microglia and up-regulates microglia-derived BDNF, which benefit damaged neural cells and neural circuits. Here, we used the collagenase VII-induced GMH model in rat pups at postnatal day 7 (P7) and lentivirus-delivered CX3CR1 shRNA to reveal the role of CX3CR1 in microglial neurotrophic phenotype transformation. To the end, we sought to explain how the activation of CB2R might affect microglia and to identify the key molecules involved, in hopes of identifying novel pathways and therapeutic targets for GMH and even intracerebral hemorrhage in a clinical setting.

Materials and methods

Animals

Pregnant S-D rats were obtained from Chongqing, China. After birth, each litter was adjusted within 48 h to contain only males and no more than eight rat pups. At postnatal day 4 (p4), some rats were randomly selected to be treated with lentivirus by stereotactic ventricular injection, and the others were used for the model of GMH at P7. Overall, 180 rat pups were used in this study. Each pup was maintained along

with its mother in a plastic cage at a temperature of $22\pm 1^{\circ}\text{C}$ and a controlled 12-h light/dark cycle, and was given free access to food and water. All experimental procedures were performed in accordance with the animal care guidelines approved by the Animal Ethics Committee of the Third Military Medical University. Special care was taken to minimize suffering and reduce the number of animals used to the minimum required to obtain adequate statistical power. Rat pups were decapitated in an appropriate and humane manner.

Surgery and preparation of viruses

For the GMH model, a 26-gauge needle attached to a 5- μl Hamilton syringe with 0.3 U clostridial collagenase VII-S (Sigma, St Louis, MO) was stereotactically implanted in the right germinal matrix zone of P7 pups, as previously described (Tang et al., 2016a). Some pups were randomly chosen at P4 for intraventricular injection of virus for shRNA expression or anti-CX3CR1 antibody. This method was adopted from Wang et al. with slight modifications (Wang et al., 2015). Generally, at P4 the selected pups were anesthetized with 3% isoflurane (in mixed air and oxygen). The coordinates were 1–1.5 mm rostral to the neonatal coronary suture, 1.0 mm on the right side of the sagittal suture and 2.5 mm ventral from the skull surface. At these coordinates, a 1 mm burr hole was made, and a 30-gauge brain-infusion cannula

(BRAIN INFUSION KIT 3, Alzet, NO.10341-14) was lowered 2.25 mm below the skull surface into the right lateral ventricle. A micro-osmotic pump (MODEL 1003D, Alzet, No. 10314-13) was implanted subcutaneously and attached with catheter tubing to the cannula. The minipumps were loaded with a solution of viruses for infection of the various shRNAs or with CX3CR1 blocking antibody (Abcam, Cambridge, UK, 1 µg per day) or vehicle (0.9% NaCl, pH 7.4). For the following 3 days, intraventricular injection was administered. In the present study, three plasmids were generated and compared in vivo. PCX3CR1-shRNA had an unrelated insert whose expression was controlled by a 8.35 kb CX3CR1 promoter; the target site was on the U6 promoter (Fig. S1). After comparison of the plasmids, shRNA2 was selected for use in further studies. The lentivirus was used to transfect these plasmids in 293FT cells, per the manufacturer's protocol (Invitrogen, Carlsbad, CA). The entire virus was purified, and the viral titer of each solution was $\sim 10^{10}$ transducing units/ml in phosphate buffered saline. Before use, viruses were subpackaged and stored at -80°C.

shRNA verification tests in vivo and invitro

A total of three plasmids were generated in the present study. Additionally, an empty pLNCX vector was used. Before shRNAs were administered in vitro by intraventricular injection, we examined the transfection efficiency in rat microglia in

vivo. Primary rat microglia were acquired as previously described. Oligo sequences used in the study were as follows: shRNA1 CX3CR1, 5'-AATTGGATCTAGAGAACTTT-3', shRNA2 CX3CR1, 5'-GTCCAAGAGCATCACTGACAT-3', shRNA3 CX3CR1, 5'-GGGACCATCTTCCTATCTATT-3' and empty primer. For transient transfections, CX3CR1 shRNA and empty vectors were packaged using Lipofectamine 2000 (Invitrogen) and plus reagent in OptiMEM (Invitrogen) according to the manufacturer's protocol. At 24 h post-transfection, cells were identified by immunofluorescence and western blotting (Fig. S2). Moreover, we tested the primers in vivo and familiar result was obtained (Fig. S3). After comparison and verification, Cx3cr1 shRNA2 with the best transfection efficiency was selected for intraventricular injection in P4 rat pups.

Study protocol and pharmacological treatments

JWH133 and SR144528 were purchased from Tocris Bioscience, and minocycline was purchased from Aladdin (Shanghai, China). The dosage and methods were as described previously. In the present study, pups received pharmacological treatment for 7 days after surgery for a long-term efficiency examination for up to 3 days if necessary.

First, 88 pups were randomly divided into the following groups: Sham, GMH+Vehi., GMH+JWH (JWH133) and GMH+JWH+SR (SR144528). The positive effects of JWH133 on neural circuit restoration were evaluated along with microglia-neural cell cross-talk.

Second, 48 pups were used to confirm the neuroprotective effects of microglia-derived BDNF in comparison with pups treated with minocycline (a microglia-selective inhibitor). During surgery, 6 pups died.

Third, pups were pre-treated by intraventricular injection. The groups of animals were as follows: GMH+Vehi., GMH+JWH, GMH+shRNA+JWH, GMH+empty shRNA+JWH and GMH+antibody. These studies were conducted to characterize the molecular mechanisms that were involved.

5-bromo-2-deoxyuridine treatment

BrdU (5-bromo-2-deoxyuridine), a marker of proliferating cells, was used to study neurogenesis as previously described, with slight modifications (Rizzi et al., 2011). At P10, pups received intraperitoneal injections of 15 mg/ml BrdU (Sigma) in 0.9% NaCl solution (100 µg/g body weight) at 2, 4 and 6 h before decapitation.

Immunohistochemistry and confocal microscopy

At the indicated time points, rat pups were anaesthetized by injection of pentobarbital (100 mg/kg; i.p.) and then were perfused intracardially with saline (0.9% NaCl) followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. The method for acquisition of brain tissue and the protocol for generating brain slices were as previously described (Guo et al., 2015). Free-floating brain sections were generally maintained at -20°C in anti-freeze solution. Generally, after being washed and blocked with 2% normal goat serum, sections were incubated with primary antibodies for 24 h at 4°C (Supplementary Table 1). Sections were washed and incubated with Alexa Fluor® 488 (1:400, Abcam, Cambridge, UK) and Cy3-labelled secondary antibody (1:400, Jackson Labs) for 1 h at room temperature. When necessary, DAPI (1:2000, Beyotime Biotechnology, China) was used to stain cell nuclei. For BrdU staining, approximately every four sections from the hippocampus were chosen for immunohistochemical analysis of BrdU expression. After being washed in PBS, sections were incubated in 0.1% Triton X-100 and then in 2N HCl for 20 min at 37°C to denature DNA and reveal BrdU sites, and this was followed by washing in pH 8.8 Tris-HCl to neutralize acids. Thereafter, sections were incubated with mouse anti-BrdU antibodies (1:200, Abcam, Cambridge, UK) at 4°C overnight. The following process for double-antibody labeling was similar to that described above. After staining, fluorescent images were obtained with a Zeiss confocal microscope (Zeiss,

LSM780, Germany) and processed with ZEN Widefield 2012 software (Zeiss).

Different magnification times were used, and images were obtained with a screen resolution of 1024×1024 pixels.

Western blotting

At indicated time points, rat pups were perfused intracardially with saline (0.9% NaCl), and this was followed by immediate removal of brain tissues. Hippocampal zones were dissected on ice. The process for sample preparation and protein electrophoresis was as previously described. Generally, proteins separated on a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) were blocked with 5% non-fat dry milk in TBST-0.1% Tween20 and then incubated with primary antibodies (Supplementary Table 1) for at least 18 h. After being washed in TBS-T, membranes were incubated with the appropriate secondary antibodies, which were either HRP-conjugated anti-rabbit or anti-goat (1:1000, Beyotime Biotechnology, China) diluted in TBS-T containing 0.5% BSA for 1 h at room temperature. Membranes were washed again three times in TBS-T, and immunoreactive bands were visualized with luminol enhancer solution (prod# 1856189, Thermo Scientific). Images were acquired using a ChemiDOCTMxrs+system (Bio-Rad) and processed

using Image Lab (Microsoft). Corresponding histograms were generated with GraphPad Prism6 software.

Analysis of gene expression by qRT-PCR

At the indicated time points, pups were anaesthetized with pentobarbital (100 mg/kg; i.p.) and perfused intracardially with saline. Dissected hippocampal tissues from pups were stored at -80°C , and total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. A260/A280 readings from a NanoDrop (ThermoScientific) spectrophotometer were used to determine the RNA quantity and quality. The reverse transcription (RT) process was performed as described previously (Tang et al., 2016a). All assays contained "no template" and "no RT" controls. TaqMan probes specific to the following proteins were used: CX3CR1 (NM_133534.1), CX3CL1 (NM_134455.1), MAP2 (NM_013066.1), BDNF (NM_001270630.1), Doublecortin (NM_053379.3), and SOX2 (NM_001109181.1). Gene expression was normalized to GAPDH expression levels and quantified with standard samples. Corresponding histograms were generated with GraphPad Prism6 software.

Magnetic resonance imaging and diffusion tensor imaging

A horizontal 7.0 Tesla MR scanner (Bruker BioSpin, USA) was used to generate in vivo images. The surface temperature of the cryoprobe was maintained at 37°C. During imaging, pups were anesthetized with pentobarbital (50 mg/kg; i.p.) along with oxygen mixed (1:3) via a vaporizer and then were positioned in an animal holder. T2 MRI images were acquired as previously described (Tang et al., 2016a). Generally, the fast spin echo sequence (TR/TE = 3000/45 ms) was used for all rats. The field of view was $3.5 \times 3.5 \text{ cm}^2$, and the matrix was 256×256 . In vivo, DTI (Diffusion tensor imaging) of pups' brains (n=8) was performed using a modified 3D diffusion-weighted gradient. The acquisition parameters were as follows: TE/TR = 33/500 ms; 2 signal averages, 30 diffusion directions, $b_0=0 \text{ s/mm}^2$, $b_1=500 \text{ s/mm}^2$, $b_2=1000 \text{ s/mm}^2$, $b_3=2000 \text{ s/mm}^2$. The field of view (FOV) was $16 \text{ mm} \times 13 \text{ mm} \times 15 \text{ mm}$, and the native imaging resolution was $0.125 \text{ mm} \times 0.125 \text{ mm} \times 0.125 \text{ mm}$. The total imaging time was within 1.5 h with unobstructed respiration and detection of the respiration rate. Images were post-processed with a Diffusion Toolkit and TrackVis, which can be downloaded at (<http://www.trackvis.org/dtk/>) and DTIstudio (www.mristudio.org/), to yield apparent diffusion coefficient (ADC) map and fractional anisotropy (FA) values. The ADC and FA maps were chosen in the study because they provide complementary contrasts that define different brain zones (from the ADC image) and internal white matter tracts (from the FA image). Moreover, we

reconstructed fiber tracking in the ROIs (regions of interest) to define the relationship between the fiber roadmap and damaged brain region. The FA value was focused on the region of corpus callosum (CC) zone and internal capsule (IC). A total of 17 pictures were acquired, which contained the hematoma zone and hippocampus; each was 0.7 mm.

Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 6 and SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) software packages. Data are reported as means with standard derivations (S.D.). Statistics were analyzed by using unpaired Student's *t*-test or one-way ANOVA followed by post-hoc Bonferroni correction. The Mann–Whitney U-test was used for comparisons between unrelated groups. A *P*-value <0.05 was considered to be statistically significant.

Results

JWH133 prompted microglia-neuron cross-talk and improved neurogenesis in the hippocampus after GMH

The ventricular zone (SVZ) and dentate gyrus (DG) in the hippocampus is the germinal zone of newborns, which activates neurogenesis. The proliferation of neural cells in these zones is pivotal in CNS diseases. In this study, we developed a protocol to measure the hippocampal proliferation of neural cells (Fig. 1A). We found an increased number of BrdU-positive cells in the cornu ammonis (CA1, Fig. 1B, C, $p<0.05$) and DG zone (Fig. 1D, E, $p<0.05$) after treatment with JWH133 for three days after GMH, as compared with vehicle treatment. Compared with the sham treatment, GMH induced a significant reduction in BrdU-positive cells in these zones, which may have been a consequence of direct hematoma damage, neuroinflammation, or iron overload and toxicity from red blood cell lysis. Neurons are mature cells in the CNS, and neural progenitor cells (NPCs) are neuron precursors. The NPCs express markers including Tuj-1 and neurogenic differentiation 1 (NeuroD1). However, Sex-determining region Y (SRY)-related HMG box 2 (SOX2) is a transcription factor that plays an important role in maintaining the NPC properties, including the capacity to proliferate and self-renew. We found that GMH, compared with the sham treatment, reduced the levels of mature neuronal cells in the hippocampus, but three-day treatment with JWH133 significantly increased NeuN (marker of mature neurons)-positive cells in the hippocampus (Fig. 1F). Moreover, after treatment with JWH133, more SOX2-positive cells were co-expressed Tuj-1 protein in the hippocampus (Fig.

1G), which may be in accordance with the finding that the overexpression of SOX2 represses neuronal differentiation. The results suggest that JWH133 may promote more rapid proliferation of NPCs, such as Tuj-1-positive cells.

CB2R activation restored a neural circuit by alleviating nervous fiber bundle damage around hematomas

High-resolution T₂-weighted images were acquired (Fig. 2A, T₂) in which the yellow ellipse represented the internal capsule (IC) as a region of interest (ROI). In T₂-weighted images, red stars represented hematoma zones. Color map images were acquired by combining primary eigenvector (V1) images and fractional anisotropy (FA) into RGB images (Fig. 2A, EDC). In the color map, red was assigned to the medial-lateral axis, green to the rostral-caudal axis, and blue to the dorsal-ventral axis. From days 28 to 32, 18 pups (n=6) received MRI-DTI scan and T1-weighted images, T₂-weighted images and fiber tracking in ROI were acquired (Fig. 2B). By fiber tracking, we observed a significant fracture of fiber bundles in areas of hematomas in pups treated with vehicle.

Nevertheless, the fiber bundles were much more intact around the hematomas in pups after 7 day treatment with JWH133 (Fig. 2B). Diffusion anisotropy of the corpus callosum (CC) and IC were quantitatively measured on the basis of the FA value. We

found a significant reduction in the FA values in vehicle-treated pups at IC compared with pups in the sham group (0.37 ± 0.04 vs. 0.59 ± 0.03 , $p<0.01$), and JWH133 increased this value to 0.55 ± 0.09 (Fig. 2C). Moreover, we found no significant differences in the FA values at CC in the different experimental groups.

The neurotrophic microglial phenotype plays a key role in JWH133-dependent neuroprotective effects but not the suppression of microglial proliferation

In the present study, we found that microglia closely interact with MAP2-positive neural cells in the cerebral cortex zone (Fig. 3A), subventricular (SVZ) zone (Fig. 3B) and dentate gyrus (DG) zone (Fig. 3C). Furthermore, we found an increased level of MAP2 protein in vehicle-treated pups 3 days after GMH ($n=6$, Fig. 3D, $p<0.05$) and a significant increase in the JWH133-treated groups ($p<0.05$). We unexpectedly found that microglial cells in vehicle-treated pups were larger and less ramified; hence, these cells were considered to represent an activated cytotoxic phenotype (Fig. 3E). JWH133 increased the levels of MAP2-positive neural cells in the CA1 zone in the same brain slices, as compared with the levels in vehicle-treated pups. We found a similar trend for Nestin expression by western blotting (Fig. 3F) and qRT-PCR (Fig. 3G). Mature neurons were significantly decreased after GMH and JWH133 protected pups from GMH-induced neuron cell loss (Fig. 3H, I, $p<0.05$, $n=6$). In addition, we

assessed the relationship between treatment time and the NPC neurotrophic effects of JWH133. A 7 day treatment with JWH133 induced significantly higher expression of Tuj-1 and Neuro D proteins than did a 3 day treatment (Fig. 3J).

Despite these findings, the “resting” phenotype induced by JWH133 needed to be better identified by using a specific biological marker. In a further study, we co-marked BDNF and microglia in brain slices (Fig. 4A) and used minocycline (a microglia-selective inhibitor) to test the sources of BDNF. We found that a 3 day treatment with JWH133, but not minocycline, increased BDNF protein expression after GMH, by using western blotting (Fig. 4B) and qRT-PCR (Fig. 4C, $p<0.05$). In minocycline group, the number of ramified microglia did not significantly increase (Fig. 4D). Double-labelling of BrdU and microglia revealed that JWH133, but not minocycline, increased BrdU-positive microglial cells (Fig. 4E, $p<0.05$).

Activation of CB2R accelerates CX3CR1 expression following GMH in rats

As described in many previous studies, CX3CR1 plays a pivotal role in maintaining microglia in a “resting” state. We co-stained CX3CL1 and GFAP (a biological marker of astrocytes, Fig. 5A) and neurons (Fig. 5B). Moreover, we observed CX3CR1 expression in microglia (Fig. 5C) and analyzed the protein expression 3 days after GMH. No differences were detected between the sham and GMH group, but at day 3,

we observed a significant increase in CX3CR1 protein in the JWH133-treated group compared with the vehicle-treated group (Fig. 5D). In addition, we observed a significant decrease in CX3CR1 mRNA transcript levels after GMH at day 3, followed by a less marked up-regulation at day 7. Interestingly, JWH133 up-regulated CX3CR1 mRNA transcript levels at days 1, 3 and 7 (Fig. 5E, n=6).

CX3CR1 plays a fundamental role in neurotrophic microglial phenotypic transformation and the process of JWH133-induced neurogenesis

To strengthen our hypothesis, we detected marker proteins of NPCs following pre-treatment of CX3CR1 shRNA silencing CX3CR1 mRNA before JWH133 administration (Fig. 6A). We found that CX3CR1 shRNA showed a stronger inhibition of JWH133-induced up-regulation of doublecortin protein expression (Fig 6B), Tuj-1 protein expression (Fig. 6C) and Neuro D protein expression (Fig 6D, $p<0.05$). Moreover, we detected a significant reduction in BrdU-positive neurons in the SVZ and DG zones in CX3CR1 shRNA pre-treated pups followed by a seven-day JWH133 administration (Fig. 6E, F). In Cx3cr1 shRNA pre-injected group, NeuN protein was significantly decreased than that of only JWH133 treated pups (Fig. 7A, $p<0.05$, n=6). In the present study, we classified microglia into the following three basic phenotypes: “resting” type (light Iba-1-positive and full of long branches),

“phagocytic” type (amoeboid cell type with a large cell body and a few short branches) and “neurotrophic” type (a large cell body with shorter branches than those of the “resting” type) (Fig. 7B). We co-labeled microglia with Iba-1- and Neuro D-positive cells in the hippocampus and Tuj-1 positive cells in the cortex of pups treated with JWH and pre-injected with CX3CR1 shRNA (Fig. 7C, D). Here, we found more “neurotrophic” type microglia and accordingly more Neuro D-positive positive cells in JWH133 treated pups (Fig. 7E, n=6). In addition, a significant increase of microglia and Tuj-1 positive cells were detected in cortex of JWH133 treated pups compared with pups pre-treated with CX3CR1 shRNA (Fig. 7F, G, n=6).

In the present study, we represented the evidence that activation of CB2R accelerated microglia-neuron cross-talk through CX3CR1/CX3CL1 and restored damaged neuronal circuit following GMH. Moreover, microglia-derived BDNF acted as a medium in the process (Fig. 8).

Discussion

Previously, we have established the neuroprotective effects of a selective CB2R agonist in preventing neuroinflammation in a collagenase VII-induced GMH model of p7 rat pups (Tang et al., 2015). Nevertheless, how CB2R activation affects this

neuronal circuit had never been studied. Here, we found that activation of CB2R may not markedly alleviate microglial proliferation but transform the microglial phenotype into a neurotrophic phenotype, thus promoting microglia-neuron cross-talk. In addition, we studied the cross-talk between microglia and neuronal cells treated with a selective CB2R agonist (JWH133) and elucidated the pivotal role of CX3CR1 in this process.

Many studies have focused on the activation of CB2R after cerebral stroke in adults and in premature brains (Zarruk et al., 2012) (Li et al., 2015; Tang et al., 2015). They have demonstrated the function of cognitive recovery by activation of CB2R after GMH, as well as CB2R's anti-inflammatory activity after acute brain injury. Recently, Bravo-Ferrer et al. studied the positive function of JWH133 on neurogenesis and found that inhibition of CB2R decreased neuroblast migration toward the boundary of infarct area in mice (Bravo-Ferrer et al., 2017). Nevertheless, few studies have elucidated the relationship between activation of CB2R and neurotrophic microglia after hemorrhagic stroke. Generally, microglia in the CNS can be defined as M1 and M2 types along with macrophages (Saijo and Glass, 2011). However, as a native and special CNS cell type, microglia have specific properties and functions. In vivo imaging in mice has demonstrated that microglia not only passively monitor neuronal activity but also actively respond to it (Tremblay et al.,

2010; Wake et al., 2009). Moreover, ramified microglia processes have been observed to enwrap the soma of highly activated neurons in zebrafish, thereby decreasing targeted neurons (Li et al., 2012). Thus, ramified microglia are not really “resting” but instead are highly active in a “rapid response and surveillance” state in which they monitor and restore neuronal circuits, even in a healthy CNS. In addition, Mills et al. have recently demonstrated that stimulus-induced microglial activation does not correspond to the M1 and M2 plasticity observed with macrophages (Mills, 2012). Consequently, microglia should not be classified by ambiguous criteria but factors that are secreted by microglia and how microglia affect the neighboring neuronal cells.

To define the phenotype of microglia induced by JWH133, we abandoned the original M1 and M2 classification and developed a molecular definition. It has previously been established that neurotrophin BDNF is a critical mediator of neuronal survival, differentiation and plasticity (Chao, 2003). Rauskolb et al. have demonstrated that the major source of BDNF in the adult brain comes from neurons (Rauskolb et al., 2010), but it can be still detected in oligodendrocytes, astrocytes and microglia (Dougherty et al., 2000). Moreover, Greenberg et al. have found that small changes in BDNF levels have significant neurological effects (Greenberg et al., 2009). Accordingly, Parkhurst et al. have demonstrated that microglia-derived BDNF plays an important role by regulating synapse formation in the healthy brain (Parkhurst et

al., 2013). In the present study, we detected a significant increase in BDNF protein levels in brain tissues of JWH133-treated pups after GMH but not in minocycline-treated groups. It can be speculated that minocycline reduces microglial proliferation and decreases microglia-derived BDNF, but JWH133 increases microglia-derived BDNF. Our findings are in strong agreement with those of a previous study reporting that pharmacological suppression of microglial activation significantly inhibits neurogenesis and oligodendrogenesis in the SVZ zone of the developing CNS (Shigemoto-Mogami et al., 2014). Even a controversial study has proposed that the suppression of microglial activation in the prenatal brain is correlated with an increase in neuron numbers and brain size (Amaral et al., 2008). A recent study has documented direct dialogue between microglia and neurons, but the key role regulating this cross-talk remains unclear (Biber et al., 2007; Gemma and Bachstetter, 2013). Some researchers have hypothesized that neurons may maintain microglia in a surveillant/ramified state via fractalkine/CX3CR1 signaling, as established by the experimental finding that fractalkine suppresses microglial activation by binding CX3CR1 only in microglia (Bhaskar et al., 2010; Lee et al., 2010). Conversely, microglia can regulate neurons in many ways. Previously, it has been shown that deletion or pharmacological inhibition of CX3CR1 reduces neurogenesis in adult rats (Bachstetter et al., 2011). Moreover, how CX3CR1 can influence neuronal cells or

circuits has not yet been explained clearly or in detail. To prove the hypothesis that CB2R activation induces up-regulation of CX3CR1, increases the release of microglia-derived BDNF to feed damaged neuronal cells, and consequently restores the neural circuit, we used a lentivirus containing a CX3CR1 shRNA expression vector to knock down CX3CR1 expression in the pups' microglia. It was strongly suspected that CX3CR1 plays a key role in the microglial neurotrophic phenotype transformation mediated by CB2R activation, which increases microglia-derived BDNF, thereby feeding damaged neuronal cells and restoring neural circuits after GMH.

In the present study, we used MRI-DTI to detect the effects of CB2R activation on white matter and neuronal circuits. As a novel investigation method, DTI has shown potential in studies of white matter injury and neurological deficits after brain injury (Fan et al., 2013; Kuzu et al., 2012). We acquired visible and qualitative images around the hematomas, although the pictures are not of a high resolution, and a several technical mistakes may have occurred. Nevertheless, we established that a CB2R selective agonist protected or restored nervous fibers in the IC zone, but not in the CC zone, by evaluation of FA values, and our results were in accordance with those of several credible articles (Chaudhary et al., 2015; Zhang et al., 2016). Restoration of a damaged neural circuit after stroke may represent a novel research

target. Here, we present new evidence about the effects of CB2R activation after GMH and show that a selective CB2R agonist may promote the neurotrophic phenotypic transformation of microglia rather than alleviating microglial activation and proliferation.

Conclusions

Sequelae of GMH remain predominant therapeutic strategy targeting at post-hemorrhagic cerebral palsy and connective dysfunction. The present study provides evidence that CB2R activation stimulates CX3CR1 expression, which promotes transformation of microglia and increases microglia-derived BDNF, consequently restoring the GMH-induced neural circuit damage. As a potential field, Neuron-microglia dialogue may be a novel therapeutic strategy in neuroinflammation. This study may lead to the identification of novel research targets for microglia after cerebral stroke, increasing knowledge about the neuroprotective effects of CB2R selective agonists.

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Figure legends

Figure 1 JWH133 increased the number of neuronal cells in DG zone and CA1 zone (A) Experimental protocol for BrdU treatment. (B) Number of BrdU positive cells in CA1 zone in pups of different groups and (C) quantitative analysis of the BrdU positive cells in CA1 zone three days following JWH133 treatment. (D) Number of BrdU positive cells in DG zone in pups of different groups and (E) quantitative

analysis of the BrdU positive cells ($n=6$, $*p<0.05$, $**p<0.01$). (F) Staining of NeuN positive cells in DG zone in brain slices of groups as Sham, GMH+vehicle and GMH+JWH133 (bar=20 μ m). (G) Exhibition of double labeling of Tuj-1 and Sox2 positive cells in DG zone between vehicle and JWH133 treated GMH pups ($n=6$, bar=20 μ m).

Figure 2 CB2R selective agonist prompted the restoration of damaged neural circuit 28 days following GMH. (A) Shows coronal T2 weighted and direction-encoded color map (DEC) images of pups. In T2 weighted map, red star indicated to hydrocephalus region and the yellow circle indicated to region of interest (ROI) of internal capsule (IC). In DEC, region in dotted line indicated to the external capsule (EC) and that in white ellipse to corpus callosum (CC). The red-green-blue color scheme used in the DEC images is: red: left-right axis; blue: rostral-caudal axis; green: superior-inferior axis. (B) presents T1 weighted images, T2 weighted images, DEC images and fiber tracking in ROI of similar coronal brain sections in groups of sham, GMH+vehicle and GMH+JWH133. In images of fiber tracking, yellow arrow indicates to nervous fiber in damaged area. (C) ROI-based comparison of white matter diffusion properties in the in vivo brain of pups at day 28. The FA of CC and IC white matter tracks are presented as mean +standard deviation. $**p<0.01$ vs. sham, $\#p<0.05$ vs. GMH+vehicle.

Figure 3 Activation of CB2R transformed neurotrophic microglia feeding the damaged nervous fibers. Closely relationship by location of microglia (Iba-1 positive, green) and nervous fibers (MAP2 positive, red) were detected in cerebral cortex (A, CC zone), subventricular zone (B, SVZ zone) and Dentate Gyrus (C, DG zone). (D) Western blotting was used to detect MAP-2 protein of pups in groups of sham, GMH+vehicle and GMH+JWH133 and Actin was used as the reference (n=6, * $p<0.05$, # $p<0.05$). (E) Exhibition of double labeling on microglia and MAP-2 neuronal cells in CA1 zone of the pups in three groups, white arrow head referred to microglia in different phenotypes and white arrows indicated the number of MAP-2 positive neuronal cells. The bar was visible in the figure. (F, H) Exhibition of Nestin and Neu N expression by western in different groups and the quantification of the protein bands. (G, I) Qrt-PCR analysis on Nestin , Neu N mRNA in brain tissues of groups at 3 days after GMH and pharmacological prevention. (J) Comparison of Tuj-1 and Neuro D protein expression in brain tissues three days and seven days after GMH and pharmacological prevention by western blotting analysis and quantification (n=6). # $p<0.05$, ## $p<0.01$, Data are expressed as the mean $\pm$ SD.

Figure 4 Increased expression of microglia-derived BDNF in brain slices of pups treated by JWH133 but not by minocycline. (A) Representative confocal image of brain sections from healthy rat pups by triple-labeling of Iba-1 (red) and BDNF (green)

and NeuN (golden). White arrow indicates to microglia and yellow arrow to neurons. White bar=50μm and yellow bar=10μm. (B) Western blotting analysis and (C) qRT-PCR analysis of BDNF protein of pups brain in groups of sham, GMH +vehicle, GMH+JWH133 and GMH+JWH133+minocycline (n=6). In the quantitative analysis, tubulin was the reference. * $p<0.05$ vs. sham. ## $p<0.01$ vs. GMH+vehicle and & $p<0.05$ vs. gmh+JWH133. (D) Representative images of double-labeled microglia and BrdU positive cells and in CA1 zone. White arrows indicate BrdU positive microglia. Bar=50μm. (E) Average number of ramified microglia in CA1 zone in every 20 vision (n=6). (F) Average number of BrdU positive microglia in CA1 zone of brain slices in groups of GMH+JWH133 and GMH+JWH133+minocycline (n=6). Data are expressed as the mean $\pm$ SD.

Figure 5 JWH133 enhanced the up-regulation of CX3CR1 in microglia following GMH, which maybe the target of microglia phenotype transformation. (A) Co-location of CX3CL1 (green) and astrocytes (GFAP+, red) in SVZ. (B) Co-location of CX3CL1 (green) and neuron (Neu N, red) in cerebral cortex, white bar=50 mm. (C) Co-location of CX3CR1 (green) and microglia (Iba-1, red) in CA1 zone and the dotted box area was an amplified vision by confocal by 40×, yellow bar=50 mm. (D) Exhibition of CX3CR1 in group of sham, GMH+vehicle-treated and GMH+JWH133-treated pups one day after GMH and pharmacological prevention by western blotting

analysis and quantification of the protein bands. (E) qRT-PCR analysis on CX3CR1 mRNA expression at day1, day3 and day7 following GMH and treatment, $*p<0.05$ GMH+JWH vs. GMH+ Vehi., $**p<0.01$, $\#p<0.05$ GMH+Vehi. vs. Sham.

Figure 6 CX3CR1 play a pivotal role in JWH133 conducted microglia-neural cells cross-talk. (A) Exhibition of western blotting analysis on Doublecortin, Tuj-1 and Neuro-D protein. (B, C, D) Corresponding quantitative analysis on the bands of different proteins. (E) Proliferation of Neu N positive neural cells (Green) was detected by BrdU (Red) technique in subventriculr zone and (F) dentate gyrus zone. N. D= not detected, $**p<0.01$ and $*p<0.05$, $n=6$. Data are expressed as the mean $\pm$ SD.

Figure 7 JWH133 conducted the phenotype transformation of neurotrophic microglia via CX3CR1 pathway. (A) Three days after GMH and JWH133 administration, Neu N protein of brain tissue in group of JWH133 treatment, empty shRNA pre-injection and CX3CR1 shRNA pre-injection, $**p<0.01$, $n=6$. (B) The hypothesized microglial phenotypes of “Resting”, “Phagocytic” and “neurotrophic” state in developmental balance of different condition. (C) Exhibition of double-labeling on microglia (Iba-1, Green) and Neuro D positive cells (Red) in hippocampus and (D) Tuj-1 positive cells in cortex of pups treated by JWH133 and pups pre-injected with CX3CR1 shRNA, white bar=50 mm, red arrow referred to “Neurotrophic” state, blue to “Phagocytic” state and yellow to “Resting” state. (E) Average number of cell counting in each

20×field of 5 slices of hippocampus. (F) Average number of microglia in the cortex and (G) integrated density of Tuj-1 positive cells in role of interest (ROI), Data are expressed as the mean $\pm$ SD.* p <0.05, ** p <0.01.

Figure 8 CB2R activation up-regulated CX3CR1 expressions and enhanced the microglia-neuronal cells cross-talk. In microglia, CX3CR1 and CB2R are both the pivotal membrane receptors. CX3CR1 may be the trigger of microglia phenotype transformation from a “phagocytic” state to a “neurotrophic” state. After phenotype transformation, microglia can secret many neurotrophic factors feeding neuronal cells and even the damaged ones. BDNF/TrkB is one of the most accepted signal pathways conducting Neurogenesis.

C Association of FA value in different groups at location of CC (Corpus callosum) and IC (internal capsule)

Structures		
Group		
	CC	IC
Sham	0.50±0.02	0.59±0.03
GMH+Vehi.	0.48±0.07	0.37±0.04 ^{**}
GMH+JWH	0.46±0.04	0.55±0.09 [#]