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Activation of metabotropic glutamate receptor 5 reduces the secondary brain injury after traumatic brain injury in rats

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ABSTRACT

A wealth of evidence has shown that microglia-associated neuro-inflammation is involved in the secondary brain injury contributed to the poor outcome after traumatic brain injury (TBI). *In vitro* studies were reported that activation of metabotropic glutamate receptor 5 (mGluR5) could inhibit the microglia-associated inflammation in response to lipopolysaccharide and our previous study indicated that mGluR5 was expressed in activated microglia following TBI. However, there is little known about whether mGluR5 activation can provide neuro-protection and reduce microglia-associated neuro-inflammation in rats after TBI. The goal of the present study was to investigate the effects of mGluR5 activation with selective agonist CHPG, on cerebral edema, neuronal degeneration, microglia activation and the releasing of pro-inflammatory cytokines, in a rat model of TBI. Rats were randomly distributed into various subgroups undergoing the sham surgery or TBI procedures, and 250 nmol of CHPG or equal volume vehicle was given through intracerebroventricular injection at 30 min post-TBI. All rats were sacrificed at 24 h after TBI for the further measurements. Our data indicated that post-TBI treatment with CHPG could significantly reduce the secondary brain injury characterized by the cerebral edema and neuronal degeneration, lead to the inhibition of microglia activation and decrease the expression of pro-inflammatory cytokines in both mRNA transcription and protein synthesis. These results provide the substantial evidence that activation of mGluR5 reduces the secondary brain injury after TBI, in part, through modulating microglia-associated neuro-inflammation.

1. Introduction

As a leading cause of death and morbidity worldwide, traumatic brain injury (TBI) has been a serious social and economic problem [1]. Unfortunately, although intensive researches have been carried out in the area of TBI, the therapeutic interventions remain limited [2]. After the irreversible primary brain injury caused by the direct physical disruption of brain structure, TBI is followed by potentially reversible secondary brain injury [3]. In the clinical setting, the secondary brain injury after TBI mainly manifests as cerebral edema and progressive neuronal degeneration, which requires hours to days to become fully manifested post-TBI [4]. Therefore, this time window provides opportunity for therapeutic intervention to interrupt the secondary brain injury.

It is well-known that the mechanisms underlying the secondary brain injury after TBI are complicated and neuro-inflammatory response plays a crucial role in the secondary brain injury [5]. Microglia, as the resident immune cells sensing threats in the brain, along with infiltrating macrophages/monocytes from the circulation, have been implicated as the predominant cell type governing the inflammation-mediated brain insults [6]. After TBI, microglia are robustly activated, change their phenotype and migrate to the injured site with the releasing of the pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), which contribute to the development of the cerebral edema and neuronal degeneration [7]. Although there is evidence that shows the inflammatory response is a double-edged sword, the general consensus is that the robust microglia activation and excessively releasing of pro-inflammatory cytokines in the initial post-TBI period is detrimental and that inhibiting acute surges of neuro-inflammation may reduce cerebral edema and neuronal damage and improve outcome [8].

In recent years, metabotropic glutamate receptors (mGluRs) have been considered as promising targets for neuro-protective drugs in a variety of acute and chronic neurodegenerative disorders [9]. mGluRs are G-protein-coupled receptors that include eight subtypes, which have been divided into three groups based

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on their sequence homology, signal transduction pathways and pharmacological profiles [10]. mGluR5 belongs to the group I mGluR that stimulates the phospholipase C, leading to phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization. Previous studies focused on the effects of mGluR5 in neurons showed that the selective mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) was neuro-protective when neurons were challenged with insults, such as staurosporine, etoposide and beta-amyloid [11,12]. Moreover, Evidence is emerging that mGluR5 activation modulates microglia function, resulting in predominantly anti-inflammatory and neuro-protective effects after insults *in vitro* [13,14]. However, the roles of mGluR5 in the *in vivo* study have received limited attention. More recently, we demonstrated that there was significant expression of mGluR5 in activated microglia surrounding the injured sites after TBI [15], while the expression of mGluR5 in the resting microglia is negligible in the intact brain. These findings promote us to further study the role of mGluR5 in the neuro-inflammation induced by the TBI.

In the present study, we examined the effects of mGluR5 activation, on cerebral edema, neuronal degeneration, microglia activation and the releasing of pro-inflammatory cytokines, in a rat model of TBI. Our hypothesis was that mGluR5 activation will reduce the secondary brain injury induced by TBI, in part, through modulating microglia-associated neuro-inflammation.

2. Materials and methods

2.1. Animal preparation

Male Sprague–Dawley rats weighting 250–300 g were provided by the Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were raised on a 12-h dark-light cycle circumstance with free access to food and water. The protocols including all surgical procedures and animal usages were approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

2.2. Experimental protocol

Animals were randomly distributed into four subgroups: (A) the sham group, (B) the sham + vehicle group, (C) the TBI + vehicle group, and (D) the TBI + CHPG group, with 30 rats each group. All rats were sacrificed under deep anesthesia at 24 h after TBI or sham-injury, since at this time point, the cerebral edema and neuronal damage has been shown to peak after moderate controlled cortical injury [16].

2.3. Traumatic brain injury

TBI was produced using the Feeney's weight-drop model with a minor modification in our laboratory [17]. After intraperitoneal (i.p.) anesthesia with sodium pentobarbital (50 mg/kg), rats were fixed on the stereotactic device. The scalp was cleaned with iodophor, and aseptic techniques were used throughout surgery. The scalp was opened, and a right parietal craniotomy (5 mm in diameter, 1.5 mm posterior and 2.5 mm lateral to the bregma) was performed by a dental drill. The dura was exposed and kept intact. Focal cortical contusion injury was induced by dropping a 40 g steel weight with a flat end from a height of 15 cm onto a pillar (4 mm in diameter) resting on the dura. The pillar was allowed to compress brain tissue for a maximum depth of 5 mm. Then the scalp was sutured. The body temperature was monitored and maintained at 37 ± 0.5 °C with heating pads and lamps. The animals returned to the feeding room after recovery from anesthesia. Sham-injured animals underwent procedures identical to those of the TBI animals, but did not receive the cortical contusion injury.

2.4. Drug administration

At 30 min after TBI or sham-injury, rats received a single intracerebroventricular injection of CHPG (Tocris Bioscience, UK) or equal volume vehicle. A 50 mM solution (saline with 1% dimethylsulfoxide) was injected into the left ventricle (coordinates from bregma = anteroposterior: −1.5 mm, lateral: 1.0 mm, ventral: −3.6 mm) using a 30 gauge needle attached to a Hamilton syringe at a rate of 0.5 μL/min, with a final volume of 5 μL, or 250 nmol of CHPG. Dosages and time of administration were based upon prior investigations in traumatic spinal cord injury and focal cerebral ischemia models [18,19].

2.5. Brain tissue preparation

After sacrificed under deep anesthesia, rats were treated according to requirements of further studies. For cerebral edema measurement, the brains were promptly removed, and the surrounding cerebral cortex of the contusional cortex (pericontusional cortex) was dissected from the region that was less than 3 mm from the margin of the contusion site on ice as described in our previous study [17]. For ELISA and RT-qPCR, rats were perfused transcardially with 250 ml of cold heparinized 0.9% saline only, and then the pericontusional cortex was removed rapidly and stored in liquid nitrogen immediately until use. For immunofluorescence and Fluoro-Jade C staining, rats were perfused transcardially with 250 ml of cold heparinized 0.9% saline and followed by 250 ml of cold 4% paraformaldehyde in phosphate-buffered saline (0.1 M, pH 7.4, PBS). Then pericontusional coronal slice of brain including the contusional cortex was dissected and immersed in 4% paraformaldehyde overnight for additional fixation, and was allowed to sink in 20%/30% sucrose solution subsequently, finally stored at −80 °C until use.

2.6. Cerebral edema measurement

Cerebral edema was determined by measuring brain water content (BWC) using the wet weight - dry weight technique, and the results are expressed as a percentage of the water content. In brief, when rats were sacrificed, the fresh pericontusional cortex samples were immediately harvested and weighed (wet weight), and then were placed in an incubator at 100 °C for 24 h. The samples were weighed once again to determine the dry weight. The BWC was calculated as follows: BWC = [(wet weight-dry weight)/wet weight] × 100%.

2.7. Immunofluorescence labeling activated macrophage/microglia

The brains were sectioned coronally with a cryostat at −24 °C (10 μm thick, 1:10 series, 100 μm between series) and stored at −20 °C for further staining. In the immunofluorescent staining, slides were washed with PBS three times for 10 min each. Then sections were incubated in blocking buffer for 30 min, followed by overnight incubation at 4 °C with primary antibody anti-ED1 (1:100, Millipore, USA). The next day, slides were washed with PBS three times for 10 min, followed by secondary antibody Alexa Flour 488-conjugated goat anti-mouse IgG incubation for 1 h at room temperature, and three washes with PBS. Finally, sections were counterstained with DAPI for 3 min and rinsed with PBS, and then sections were cover-slipped. As a negative control, alternative sections were incubated without primary antibodies.

2.8. Double labeling with Fluoro-Jade C staining and immunofluorescence

For the double labeling with Fluoro-Jade C (FJC) staining detecting the degenerative neurons and immunofluorescent marker (neuron-specific nuclear protein, NeuN), standard FJC staining protocol with a minor modification was used in this study [20]. In brief, sections were first incubated overnight at 4 °C with primary antibody anti-NeuN (1:100, Millipore, USA) as described previously, and followed by the Alexa Flour 594-conjugated goat anti-mouse IgG secondary antibody. Then the sections were immersed in a basic alcohol solution for 5 min. after rinsed in 70% ethanol and distilled water, the slides were then transferred to a 0.0001% solution of FJC (Chemicon, USA) for 10 min. The slides were then rinsed again, air-dried, cover-slipped and stored in dark at 4 °C. As a negative control, alternative sections were incubated without primary antibodies or FJC solution.

2.9. Fluorescent cell counting

Fluorescence microscopy images were captured by Olympus IX71 inverted microscope system and handled by Image-Pro Plus 6.0 software. Every section under analysis was at a minimum distance of 100 μm from the next and the microscopic examination of the stained sections was performed by the experienced pathologist in a blinded manner. To evaluate the differences in macrophage/microglia activation and neuronal degeneration, semi-quantitative methods were used to count the number of positive cells in the pericontusional cortex. For macrophage/microglia activation, the summed number of ED1-positive cells in six randomly selected non-overlapped high-power fields per section was counted in four sections per brain through the injury core. The mean number of ED1-positive cells in each section per brain was calculated and used to assess the extent of macrophage/microglia activation. For neuronal degeneration, six non-overlapped high-power fields were randomly selected in areas surrounding the contusion core. The respective number of NeuN-positive cells (A) and cells that were both FJC and NeuN positive (B) per high-power field per section was counted in four sections per brain through the injury core. The degree of neuronal degeneration was assessed by degenerative index that was the mean percentage in each high-power field per brain that was calculated as follows: the percentage = $(B/A) \times 100\%$.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The frozen cortex tissue was mechanically homogenized in 1 ml of lysate buffer, and centrifuged at 12,000*g* for 20 min at 4 °C. Then the supernatant was collected and total protein was determined using a bicinchoninic acid assay kit. The protein levels of inflammatory cytokines were quantified using specific ELISA kits for rat cytokines according to the manufacturers' instructions (TNF-α from Diaclone Research, France; IL-1β and IL-6 from BioSource Europe SA, Belgium). The cytokine contents were expressed as picograms per milligram of protein.

2.11. Quantitative real-time polymerase chain reaction (RT-qPCR)

The mRNA levels of inflammatory cytokines were determined by RT-qPCR. Total RNA was extracted from frozen pericontusional cortex samples using RNAiso Plus (TaKaRa Bio, China) according to the RNA extraction protocol. The concentration and purity of total RNA were determined by spectrophotometer analysis (OD260/280:1.8–2.2) and agarose gel electrophoresis. Then the total RNA was reverse-transcripted to cDNA immediately using PrimeScript™ RT reagent Kit (TaKaRa Bio, China). The primers were designed according to PubMed GenBank, and synthesized by Invitrogen Life Technologies (Shanghai, China). The primer sequences and PCR conditions were shown in Table 1. RT-qPCR analysis was performed by using the iQ5™ Real-Time PCR System (Bio-Rad, USA), applying real-time SYBR Green PCR technology. PCR reaction mixture was prepared according to the SYBR® Premix Ex Taq™ kit protocol (TaKaRa Bio, China). PCR amplification program consisted of three steps. Fluorescent readings were taken during the extension step of each cycle. Melting curve analysis was also performed to ensure the amplification of a single PCR product. All samples were analyzed in triplicate. Reaction without cDNA was used as no-template control, and no-reverse transcription controls were also set up to rule out genomic DNA contamination. β-actin was selected as an acceptable endogenous reference "housekeeping" gene. Relative change in mRNA expression of target cytokines was determined by the equation: fold change = $2^{-[\Delta\Delta Ct]}$, Ct value is the cycle number at which fluorescence signal crosses the threshold.

Table 1
Primer sequences and PCR conditions.

Gene name	Primer	sequences (5′-3′)	Annealing temperature	Amplicon length (bp)
IL-1β	Forward	CACCTCTCAAGCAGAGCACAG	58 °C	79
	Reverse	GGGTTCCATGGTGAAGTCAAC		
IL-6	Forward	TCCTACCCCAACTTCCAATGCTC	65 °C	79
	Reverse	TTGGATGGTCTTGGTCCTTAGCC		
TNF-α	Forward	AAATGGGCTCCCTCTCATCAGTTC	58 °C	111
	Reverse	TCTGCTTGGTGGTTTGCTACGAC		
β-actin	Forward	AAGTCCCTCACCCTCCCAAAAG	58 °C	97
	Reverse	AAGCAATGCTGTCACCTTCCC		

2.12. Statistical analysis

SPSS16.0 software was used for the statistical analysis (SPSS, Inc., Chicago, IL, USA). All data were presented as mean ± S.E.M. The data were subjected to one-way analysis of variance followed by Fisher's protected least significant difference test. Statistical significance was accepted with $P < 0.05$.

3. Results

3.1. CHPG treatment reduced cerebral edema after TBI

Considering that cerebral edema was of particular importance in the clinical setting, we investigated effects of CHPG treatment

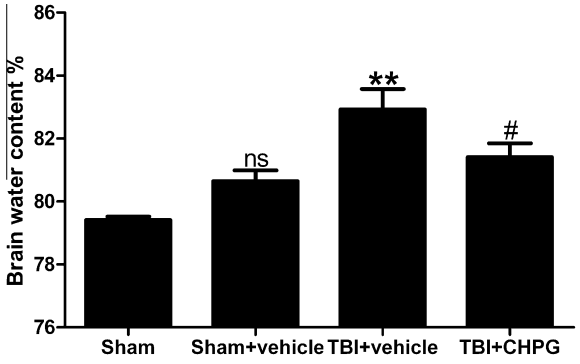

Fig. 1. Effects of CHPG treatment on the BWC ($n = 6$ each group). Compared with the sham group with vehicle treatment, TBI led to a significant increase in the BWC. The increase in the BWC induced by the TBI was significantly reduced by CHPG treatment. Data are presented as the mean ± S.E.M. $^{ns}P > 0.05$ versus sham group, $^{**}P < 0.01$ versus sham + vehicle group, $^{\#}P < 0.05$ versus TBI + vehicle group.

on the BWC at 24 h after TBI. The BWC of the sham-injured rats with vehicle treatment showed no significant difference compared with sham rats without vehicle treatment ($P > 0.05$; Fig. 1). At 24 h after injury, significant increase in BWC was detected in the pericontusional cortex when compared with those of the sham rats with vehicle treatment ($P < 0.01$; Fig. 1), which indicated cerebral edema. The increase in BWC induced by TBI was significantly attenuated in the TBI rats with CHPG treatment ($P < 0.05$; Fig. 1), suggesting the improvement of cerebral edema.

3.2. CHPG treatment attenuated the neuronal degeneration induced by TBI

To investigate the role of mGluR5 activation with CHPG on the neuronal degeneration after TBI, the Fluoro-Jade C staining that selectively stained degenerating neurons regardless of the mechanism of cell injury [20], combined with immunofluorescent labeling with NeuN to provide the general estimation of neurons in the pericontusional cortex, was performed in the present study. As shown in Fig. 2, non-injured neurons expressed NeuN, but were not stained with FJC while the injured neurons were both NeuN and FJC positive. A very small amount of injured neurons were found in the sham groups with or without vehicle treatment. At 24 h after injury, significant increase in degenerative index ($P < 0.01$; Fig. 2) was detected in the pericontusional cortex compared with the one in the sham rats with vehicle treatment, which indicated serious neuronal loss after TBI. When given 250 nmol of CHPG, rats showed remarkable improvement in the degenerative index ($P < 0.05$; Fig. 2) in comparison with the vehicle-treated TBI rats, demonstrating the less neuronal degeneration.

3.3. CHPG administration inhibited the neuro-inflammation after TBI

Currently, ED1, as a lysosomal protein, is recognized as the activated macrophage/microglia marker and generally used in the study of microglia after CNS insult [8]. Thus, to test the hypothesis

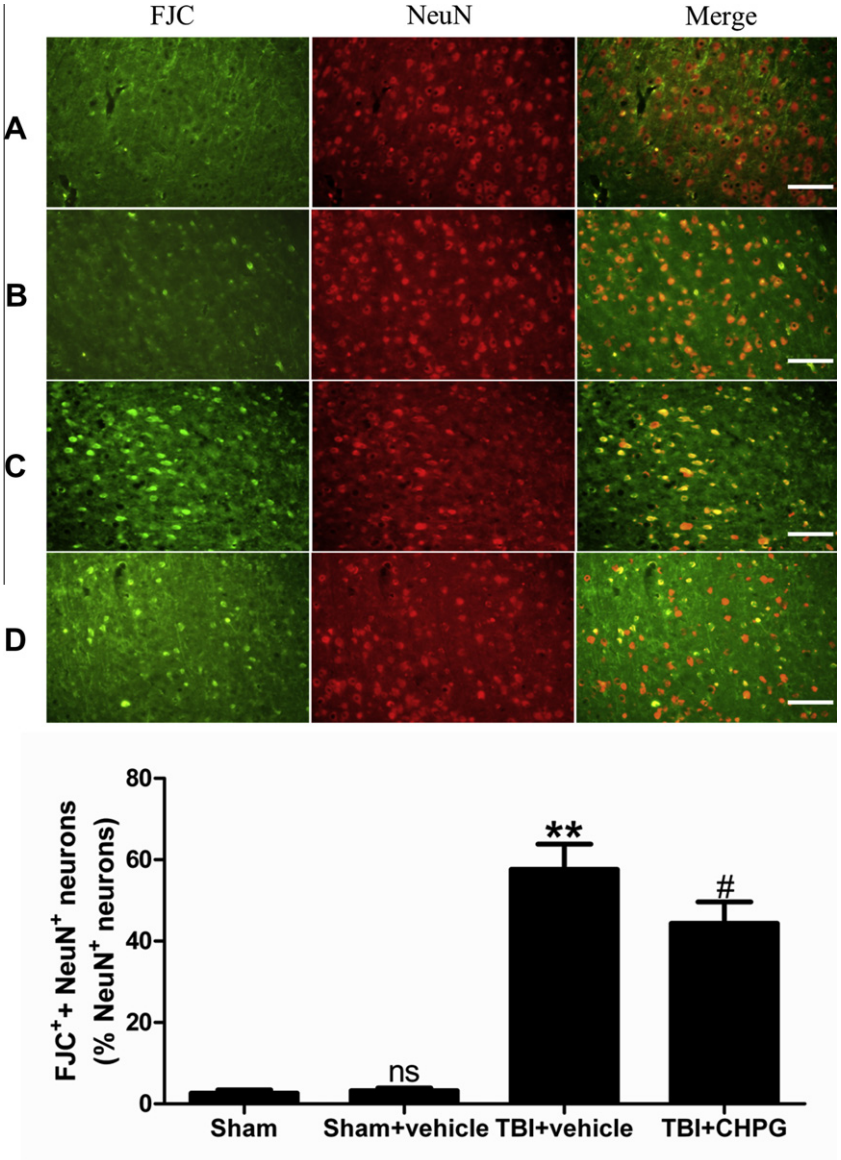

Fig. 2. Effects of CHPG treatment on the neuronal degeneration ($n = 6$ each group). Non-injured neurons expressed NeuN, but were not stained with FJC while the injured neurons were both NeuN and FJC positive. There were a very small amount of injured neurons in the sham groups with (B) or without vehicle treatment (A) whereas numerous degenerative neurons were detected in the vehicle-treated TBI rats (C). And rats with post-TBI CHPG treatment (D) showed fewer injured neurons and significantly reduced degenerative index in comparison with those of vehicle-treated TBI rats at 24 h after injury. Data are presented as the mean ± S.E.M. $^{ns}P > 0.05$ versus sham group, $^{**}P < 0.01$ versus sham + vehicle group, $^{\#}P < 0.05$ versus TBI + vehicle group. Scale bar: 50 μm.

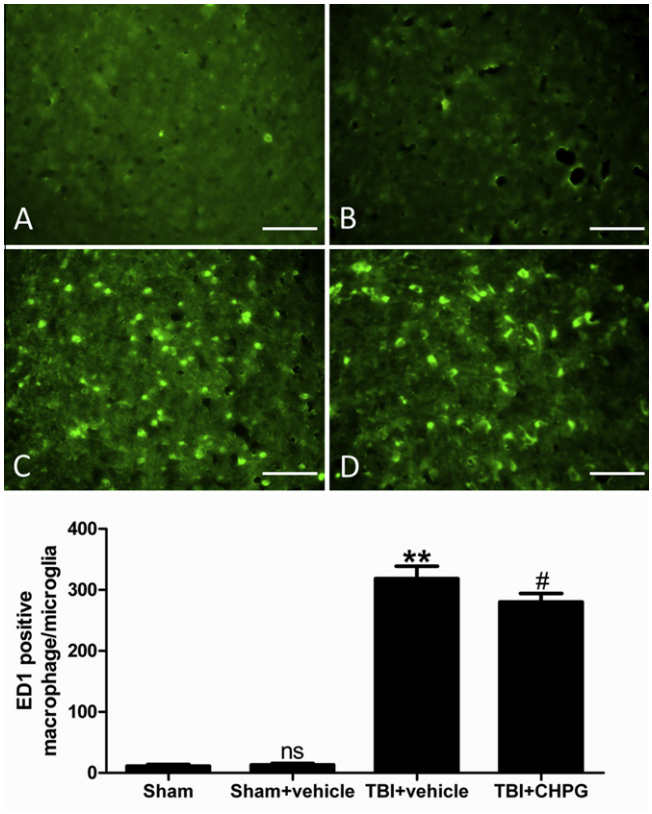

Fig. 3. Effects of CHPG treatment on the macrophage/microglia activation ($n = 6$ each group). There were a very small amount of ED1-positive cells in the sham groups with (B) or without vehicle treatment (A) whereas numerous ED1-positive cells were detected in the vehicle-treated TBI rats (C), indicating the excessive macrophage/microglia activation after injury. And rats with post-TBI CHPG treatment (D) showed fewer ED1-positive cells and significantly reduced macrophage/microglia activation in comparison with those of vehicle-treated TBI rats. Data are presented as the mean ± S.E.M. $^{ns}P > 0.05$ versus sham group, $^{**}P < 0.01$ versus sham + vehicle group, $^{\#}P < 0.05$ versus TBI + vehicle group. Scale bar: 50 μm.

that the effects of mGluR5 activation *in vivo* were mediated, in part, by the suppression of microglia-associated neuro-inflammation, we measured the expression of ED1 in pericontusional cortex at 24 h after injury. As shown in Fig. 3, there was a very small amount of ED1-positive cells in the sham groups with or without vehicle treatment while the number of ED1-positive cells was significantly increased in the vehicle-treated TBI rats compared with the vehicle-treated sham rats($P < 0.01$; Fig. 3), which demonstrated robust macrophage/microglia activation after injury. CHPG treatment could significantly reduce the number of ED1-positive cells ($P < 0.05$; Fig. 3) in comparison with the vehicle-treated TBI rats, suggesting the inhibition of macrophage/microglia activation.

To further investigate the effects of mGluR5 activation on the inflammation after TBI, we assessed the protein and mRNA levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. As shown in Fig. 4, the protein and mRNA levels of pro-inflammatory cytokines were similar in the sham rats with or without vehicle treatment ($P > 0.05$; Fig. 4). At 24 h after TBI, significant increase in the protein levels of IL-1β, IL-6 and TNF-α and the mRNA levels of IL-1β and TNF-α in the vehicle-treated TBI rats were detected in comparison with those of the sham rats with vehicle treatment ($P < 0.01$; Fig. 4). Post-TBI administration of CHPG led to significant decrease in the protein levels and mRNA levels of IL-1β, IL-6 and TNF-α in comparison with vehicle-treated TBI rats (Fig. 4) at 24 h after TBI. These results indicated CHPG could inhibit the induction of pro-inflammatory cytokines at both protein and mRNA levels.

4. Discussion

In the present study, we studied the effects of mGluR5 activation on secondary brain injury and neuro-inflammation after TBI in rats. The main findings are as follows: (1) mGluR5 activation could reduce the secondary brain injury characterized by the cerebral edema and neuronal degeneration; (2) mGluR5 activation resulted in the inhibition of macrophage/microglia activation and the decrease of pro-inflammatory cytokines in both mRNA transcription and protein synthesis. These findings provide the substantial evidence that mGluR5 activation reduces the secondary brain injury induced by TBI, in part, through modulating microglia-associated neuro-inflammation.

In recent years, considerable attention has been attached to the mGluR5 since it is considered as a promising target for neuroprotective drugs in a variety of acute and chronic neurodegenerative diseases. As previously reported [21], combining clinic-associated pathology assessment may contribute to the improved evaluation value of the novel neuroprotective drugs. The cerebral edema and neuronal degeneration investigated in the present study are

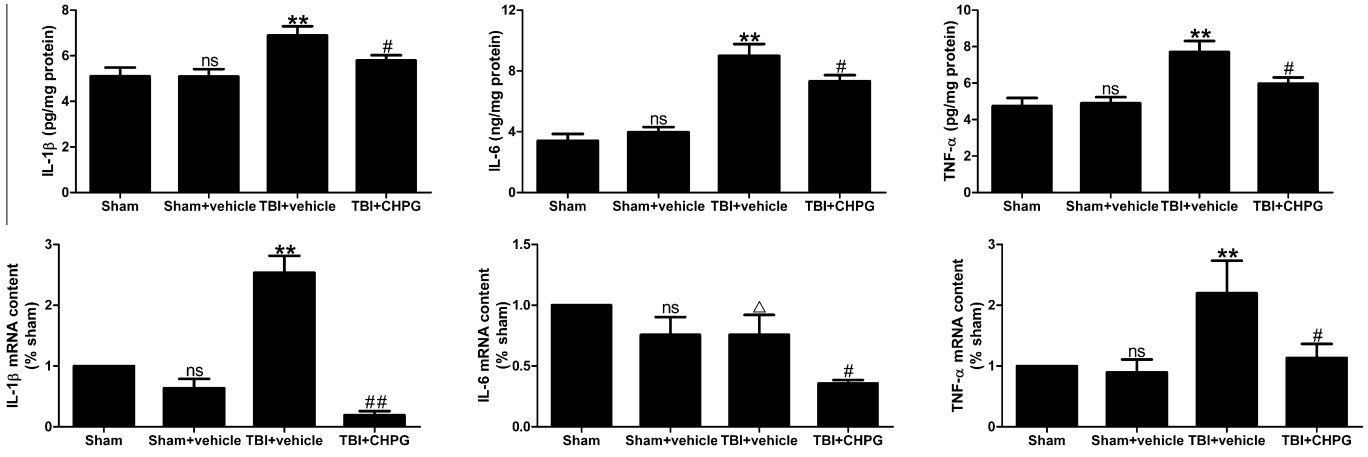

Fig. 4. Effects of CHPG treatment on the protein and mRNA levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α ($n = 6$ each group). There was no significant difference in the sham rats with or without vehicle treatment. TBI led to significant increase in the protein levels of IL-1β, IL-6 and TNF-α and the mRNA levels of IL-1β and TNF-α in comparison with the sham group with vehicle treatment. CHPG treatment could remarkably reduce the pro-inflammatory cytokines concentration in both protein and mRNA levels compared with vehicle treatment. β-actin was used as an internal control, and the mRNA data were normalized with β-actin and expressed as percentage of sham. Data are presented as the mean ± S.E.M. $^{ns}P > 0.05$ versus sham group, $^{\triangle}P > 0.05$ versus sham + vehicle group, $^{**}P < 0.01$ versus sham + vehicle group, $^{\#\#}P < 0.01$ versus TBI + vehicle group, $^{\#}P < 0.05$ versus TBI + vehicle group.

of particular importance in the clinical setting. Our data indicated CHPG administration could improve cerebral edema and neuronal degeneration after TBI, which were consistent with researches reporting that CHPG treatment showed neuroprotective effects in other CNS insults such as focal brain ischemia [18] and spinal cord injury [19]. However, it should be paid close attention that although some researches indicated mGluR5 antagonists MTEP or MPEP were neuroprotective, it reflected the direct actions at inhibiting the NMDA receptor signaling instead of the mGluR5 pathways [22]. To date, the mechanism of CHPG mediated these neuroprotective effects was still underscored.

It is clear that neuro-inflammation is involved in the secondary brain injury after TBI although its precise role is still under debate. As the primary mediator of the innate immune response in the CNS, microglia plays a key role in the inflammation induced by a variety of CNS insults [6]. In response to TBI, there is activation of resting microglia and macrophage infiltration from the circulation, along with their robust releasing of pro-inflammatory cytokines. Considerable evidence indicate that dramatic neuroinflammation in the acute post-TBI period leads to the cerebral edema and neuronal degeneration, and suppression of the acute surges of inflammation contributes to the improved function recovery [8,23,24].

Previously, our group found that activated macrophage/microglia following TBI expressed the mGluR5 [15]. Here we demonstrated that post-TBI treatment with selective mGluR5 agonist CHPG could significantly reduce macrophage/microglia activation characterized by the expression of ED1 and inhibit the releasing of pro-inflammatory cytokines. Moreover, similar anti-inflammatory effects were also reported in the microglia cultures in response to lipopolysaccharide, which showed that CHPG treatment could reduce the microglia activation as well as the production of nitric oxide and TNF-α [14]. And the neuronal death induced by the activated microglia in culture could be inhibited by mGluR5 activation [13]. Taken together, these *in vivo* and *in vitro* data indicate that the neuroprotective effects of mGluR5 activation after TBI are mediated, at least in part, through the inhibition of microglia-associated inflammation. However, the signals and mechanisms by which activation of mGluR5 inhibits microglia-associated inflammation need to be addressed in the future.

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