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Original Research

Metabotropic Glutamate Receptor 8 Suppresses M1 Polarization in Microglia by Alleviating Endoplasmic Reticulum Stress and Mitochondrial Dysfunction

Yangzhi Xie[1,†], Liang Chen[1,†], Jiacheng Chen[2], Yan Luo[1], Zhe Peng[1], Hao Zhang[1], Zhao Pan[1], Yongjun Chen[1,*]

[1]Department of Neurology, The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, 421001 Hengyang, Hunan, China
[2]Department of Intensive Care Unit, The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, 421001 Hengyang, Hunan, China
*Correspondence: chenyj-usc@foxmail.com (Yongjun Chen)
[†]These authors contributed equally.
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Abstract

Background: Microglia-mediated neuroinflammation is a hallmark of neurodegeneration. Metabotropic glutamate receptor 8 (GRM8) has been reported to promote neuronal survival in neurodegenerative diseases, yet the effect of GRM8 on neuroinflammation is still unclear. Calcium overload-induced endoplasmic reticulum (ER)-mitochondrial miscommunication has been reported to trigger neuroinflammation in the brain. The aim of this study was to investigate putative anti-inflammatory effects of GRM8 in microglia, specifically focusing on its role in calcium overload-induced ER stress and mitochondrial dysfunction. **Methods**: BV2 microglial cells were pretreated with GRM8 agonist prior to lipopolysaccharide administration. Pro-inflammatory cytokine levels and the microglial polarization state in BV2 cells were then quantified. Cellular apoptosis and the viability of neuron-like PC12 cells co-cultured with BV2 cells were examined using flow cytometry and a Cell Counting Kit-8, respectively. The concentration of cAMP, inositol-1,4,5-triphosphate receptor (IP3R)-dependent calcium release, ER Ca^{2+} concentration, mitochondrial function as reflected by reactive oxygen species levels, ATP production, mitochondrial membrane potential, expression of ER stress-sensing protein, and phosphorylation of the nuclear factor kappa B (NF-κB) p65 subunit were also quantified in BV2 cells. **Results**: GRM8 activation inhibited pro-inflammatory cytokine release and shifted microglia polarization towards an anti-inflammatory-like phenotype in BV2 cells, as well as promoting neuron-like PC12 cell survival when co-cultured with BV2 cells. Mechanistically, microglial GRM8 activation significantly inhibited cAMP production, thereby desensitizing the IP3R located within the ER. This process markedly limited IP3R-dependent calcium release, thus restoring mitochondrial function while inhibiting ER stress and subsequently deactivating NF-κB signaling. **Conclusions**: Our results indicate that GRM8 activation can protect against microglia-mediated neuroinflammation by attenuating ER stress and mitochondrial dysfunction, and that IP3R-mediated calcium signaling may play a vital role in this process. GRM8 may thus be a potential target for limiting neuroinflammation.

Keywords: metabotropic glutamate receptor 8; endoplasmic reticulum stress; mitochondrial function; neuroinflammation; M1/M2 polarization

1. Introduction

Microglia are immune cells that are abundant in the central nervous system (CNS) and play a pivotal role in immune surveillance, as well as sustaining and nourishing neurons [1]. The interplay between microglia and neurons is critical for synaptic remodeling and pruning in physiological conditions [2]. Microglia are divided into the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype. These cells can transition from a resting state into an activated state in various pathological conditions such as neuronal injury, aging, oxidative stress, and misfolded protein aggregation [3]. Persistent activation of microglia accompanied by excessive pro-inflammatory cytokine release are common events in primary neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and epilepsy, as well as secondary neurodegeneration such as brain trauma, multiple sclerosis, stroke, and spinal cord injury caused by primary inflammation [4]. Disease-associated microglia and excessive microglial inflammation are thought to trigger irreversible brain damage. Several registered clinical drug trials have focused on attenuating microglial inflammation in neurodegenerative diseases, and some trials have shown significant amelioration of pathological and clinical manifestations [5]. Thus, the control of microglia-mediated neuroinflammation may be a feasible strategy for limiting neurodegeneration.

Almost 30% of the proteome undergoes folding and maturation within the endoplasmic reticulum (ER). Calcium signaling is crucial in the protein folding process.

As the major organelle for calcium storage, calcium depletion within the ER may disturb the protein folding process, leading to a condition termed ER stress [6]. In addition, ER-to-mitochondrial Ca^{2+} transfer via mitochondria-associated membranes is critical for oxidative phosphorylation and ATP generation. However, calcium depletion in the ER may lead to mitochondrial Ca^{2+} overload, resulting in mitochondrial dysfunction and the generation of reactive oxygen species (ROS) [7]. ROS-mediated oxidative stress may further aggravate ER stress [8]. Sustained ER stress is thought to promote the progression of neurodegeneration due to its potential to trigger downstream inflammatory signaling [9]. A previous study indicated that a treatment strategy aimed at modulating calcium homeostasis in microglia may alleviate the ER stress-associated inflammatory response [10]. Therefore, the control of Ca^{2+} within the ER is considered to play a vital role in the ER-mitochondrion connection, as well as associated inflammatory responses.

Metabotropic glutamate receptors (GRMs) are widely distributed in the CNS and are activated by glutamate. GRMs are classified into three groups according to their sequence homology and physiological function. Amongst these, group III GRMs (GRM4, 6, 7, 8) are reported to restrain glutamate release, limit neurons from excitotoxicity, and promote neuronal survival [11]. GRMs are members of the G protein-coupled receptor family. Following extracellular ligand binding, GRMs may facilitate intracellular G-protein ($G\alpha$-$\beta\gamma$) uncoupling. $G\alpha$-mediated intracellular second messenger production and $\beta\gamma$-mediated ion channel conformational changes can induce a series of cellular responses. While eight subtypes of GRM have been identified, the neuroprotective effects of GRM8 have been documented in several animal models of neurodegeneration [12–14]. However, the mechanism that controls this effect has not been fully elucidated. A recent study using a mouse model of multiple sclerosis suggests that neuronal GRM8 activation strongly protects against neuronal degeneration, and that the mechanism involves modulating calcium homeostasis [15]. GRM8 is also expressed in microglia, but it is not yet known what role microglial GRM8 plays in modulating neuroinflammation.

In this study, we sought to explore the anti-inflammatory effects of GRM8 in BV2 microglial cells challenged with lipopolysaccharide (LPS). We hypothesize that the anti-inflammatory effects of microglial GRM8 may in part be due to its potential to mitigate calcium overload-induced ER stress and mitochondrial dysfunction.

2. Materials and Methods

2.1 Cell Culture and Treatment

BV2 microglial cells were purchased from Procell Life Science & Technology (Wuhan, Hubei, China) and were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Anaheim, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 1% streptomycin/penicillin. PC12 cells were grown in dulbecco's modified eagle medium (DMEM)-high glucose medium (Gibco) containing 10% FBS, 5% horse serum, and 1% streptomycin/penicillin. Both BV2 and PC12 cells were incubated at 37 °C in a 5% CO_2 incubator. A quantitative polymerase chain reaction mycoplasma detection kit was used for mycoplasma testing (#4460623; Thermo Fisher, Waltham, MA, USA), and no mycoplasma infection was found in either cell line. cell line has been validated by short tandem repeat authentication.

BV2 cells were pretreated with the GRM8 agonist AZ12216052 (1 µM; 1290628-31-7; MedChemExpress, Monmouth Junction, NJ, USA) or saline vehicle for 2 h. Afterwards, the cells were treated with saline control or LPS (1 µg/mL) for 12 h.

For the establishment of PC12 and BV2 cell co-culture conditions, PC12 was first incubated in a 24-well plate for 72 h. Following this, BV2 cells were transferred to a 0.4 µm pore-sized Transwell insert. PC12 and BV2 cells (BV2:PC12 = 1:2) were subsequently co-cultured for 48 h.

2.2 Immunofluorescence Microscopy

Cells were seeded on a coverslip and subsequently treated with 0.5% Triton X-100 for 20 min. After washing, cells were incubated overnight at 4 °C with primary antibody. The primary antibodies used were rabbit anti-GRM8 (1:100; PA5-33830; Invitrogen, Waltham, MA, USA) and rat anti-CD11b (5 µg/mL; ab8878; Abcam, Cambridge, UK). Cells were subsequently incubated with secondary antibodies in a dark room. The secondary antibodies used in this experiment were fluoresceine isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) H&L (1:800; ab6717; Abcam) and Cy5-conjugated goat anti-rat IgG Heavy&Light (H&L) (1:800; ab6565; Abcam). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and cells were visualized using a laser scanning confocal microscope (Sp8, Leica, Heidelberg, Germany).

2.3 Cell Apoptosis

PC12 apoptosis in the co-culture system was detected using flow cytometry by staining PC12 cells with Annexin and propidium iodide (PI) (AP101; Multi Sciences, Hangzhou, Zhejiang, China) according to the manufacturer's instructions. Preparations were analyzed on a CytoFLEX flow cytometer (Beckman, Pasadena, CA, USA).

2.4 Cell Viability

Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; C0039; Beyotime, Shanghai, China). Briefly, PC12 cells were inoculated into a 96-well plate and 10 µL of CCK-8 stock solution was added to each well and incubated for 4 h. Absorbance at 450 nm was measured using a Varioskan LUX microplate reader (Varioskan LUX,

Table 1. Quantitative RT-PCR primers used.

Target gene	Forward primer sequence (5′ > 3′)	Reverse primer sequence (5′ > 3′)
iNOS	GGTGAAGGGACTGAGCTGTT	ACGTTCGTTCTCTTGCA
TNF-α	CAGGCGGTGCCTATGTCTC	CCATTTGGGAACTTCTCATCCCTT
Arg-1	CACCTGAGCTTTGATGTCG	TGAAAGGAGCCCTGTCTTG
CD206	AAGGAAGGTTGGCATTTGT	CTTTCAGTCCTTTGCAAGC
GAPDH	GCCAAGGCTGTGGGCAAGGT	TCTCCAGGCGGCACGCAGA

RT-PCR, reverse transcription-polymerase chain reaction; iNOS, nitric oxide synthase; TNF-α, tumor necrosis factor-alpha; Arg-1, arginase 1; CD206, cluster of differentiation 206.

Thermo Fisher, Moorhead, MN, USA). Cell viability was calculated using the equation: [ODexperiemnt group – ODblank]/[ODControl group – ODblank] × 100%.

2.5 Quantitative RT-PCR Analysis

TRIZOL reagent (Sigma-Aldrich, St. Louis, MO, USA) was used to extract total RNA from BV2 cells according to the manufacturer's instructions. RNA was subsequently reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara, Kusatsu, Japan). Relative mRNA levels were measured using SYBR Premix EX Taq I (Takara) on a 7300 Plus Real-Time PCR System (Thermo Fisher). Each 20 µL reaction mixture contained 10 µL of 2× PCR master mix, 1 µL cDNA template, 5 pmol primer, and water. Thermocycling conditions were: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 60 s. The primers are shown in Table 1. mRNA expression was determined relative to *GAPDH* mRNA levels.

2.6 ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure cAMP level (Abcam) and pro-inflammatory cytokine levels for interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 (Beyotime) in BV2 cells according to the manufacturer's instructions.

2.7 IP3R Activity and Ca^{2+} Concentration in the ER

Inositol-1,4,5-triphosphate receptor (IP3R) is expressed at the surface of the ER and when bound by IP3, calcium is released from the ER into the cytoplasm. Mag-Fluo-AM (GMS10189; GENMED, Shanghai, China) is a fluorescent probe with low affinity for calcium that can be specifically captured by the ER. This property is exploited for the detection of alterations in the concentration of ionized calcium within the ER. In this way, Mag-Fluo-AM can be used as a surrogate to examine the transfer activity of IP3R. Data were obtained 48 h after LPS treatment.

An IP3R functional fluorescence detection kit (GMS10189; GENMED, Shanghai, China) was used to evaluate IP3R activity. All procedures strictly followed the manufacturer's instructions. In brief, Relative Fluorescence Units (RFUs) were detected using a fluorescence microplate reader (Varioskan LUX, Thermo Fisher, Moorhead, MN, USA). The induced calcium release rate was calculated using the following equation: $[(RFU_{ER\ Ca}{}^{2+} - RFU_{induced\ Ca}{}^{2+}\ release)]/[(RFU_{ER\ Ca}{}^{2+} - RFU_{complete\ Ca}{}^{2+}\ release)] \times 100\%$. In this assay system, the higher the induced calcium release rate, the greater the transport activity of IP3R.

The ER Ca^{2+} concentration was measured using an ER calcium concentration assay kit (GMS10267.1; GENMED) and by measuring the fluorescence intensity obtained using Mag-Fluo-AM and combining this with ER Ca^{2+} using a fluorescence microplate reader (Varioskan LUX, Thermo Fisher). The ER Ca^{2+} concentration (µM/100 µg) was calculated using the following equation: $[RFU_{sample} - RFU_{blank\ control}]/[RFU_{max\ control} - RFU_{sample}] \times 22$.

2.8 Measurement of Mitochondrial Membrane Potential

A commercially available kit (MAK159; Sigma-Aldrich) was used to measure mitochondrial membrane potential (MMP) according to the manufacturer's instructions. Cells were seeded into a 96-well plate and 25 µL of buffer A containing JC-10 was then added to each well and incubated for 45 min. The cells were then incubated with 25 µL of buffer B. Fluorescence measurements were made at an excitation wavelength of 540 nm and an emission wavelength of 490 nm in a microplate reader (Varioskan LUX, Thermo Fisher). The 540 nm/490 nm fluorescence intensity ratio indicates the MMP.

2.9 Measurement of ATP Levels

A luminescent ATP detection assay kit (ab113849; Abcam) was used to measure the ATP levels in BV2 cells. Briefly, detergent solution was added to cell cultures for cell lysis and ATP stabilization. Substrate solution was then added to each well and preparations were analyzed on a luminescence plate reader (Varioskan LUX, Thermo Fisher,) after incubating for 10 min in the dark.

2.10 Measurement of Intracellular and Mitochondrial ROS Levels

Cells were seeded into 96-well plates and stained with 2′,7′-dichlorofluorescein diacetate (ab113851; Abcam) to measure intracellular ROS levels and with MitoSox (MAK145; Sigma-Aldrich) to measure mitochondrial ROS levels. Fluorescence intensity was measured using a microplate reader (Varioskan LUX, Thermo Fisher).

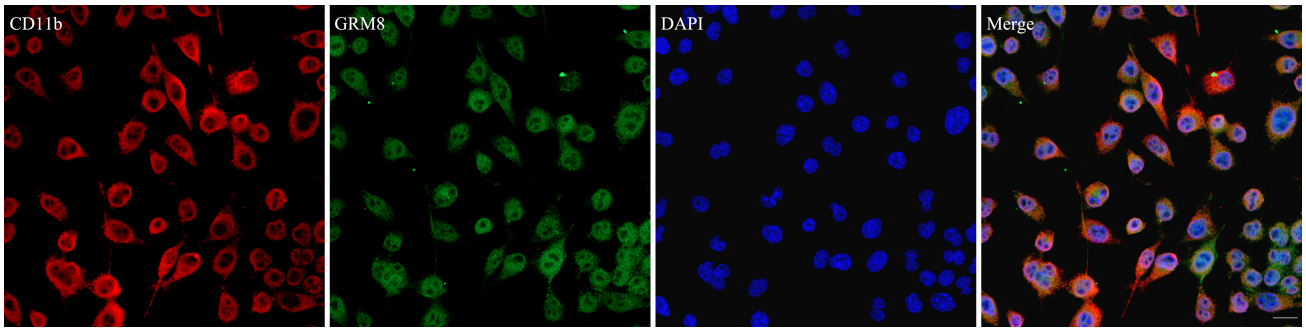

Fig. 1. Metabotropic glutamate receptor 8 (GRM8) and CD11b co-localize in BV2 cells. Red fluorescence indicates CD11b-positive cells, while green fluorescence indicates GRM8-positive cells. Nuclei were counter stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 20 µm.

2.11 Western Blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer supplemented with Phenylmethanesulfonyl fluoride (PMSF). The extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrotransferred onto polyvinylidene fluoride (PVDF) membranes. These were blocked for 1 h and then incubated separately with primary antibody against ATF6 (1:1000; #DF6009; Affinity, Cincinnati, OH, USA), IRE1α (1:1000; #3294; CST, Danvers, MA, USA), p-ERK (1:1000; AF5304; Affinity), phospho-p65 (1:800; #3033; CST), β-actin (1:1000; ET1701-80; HuaBio, Hangzhou, Zhejiang, China), or TATA-binding protein (TBP) (1:1000; HA500518; HuaBio). After several washes, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (1:1000; A0208; Beyotime). Protein bands were visualized using chemiluminescence and the Tanon-4200 gel imaging analysis system (Tanon, Shanghai, China).

2.12 Statistics

All values are expressed as the mean ± standard error of measurement (SEM). Data were analyzed using SPSS software (Version 22, IBM Corp., Armonk, NY, USA). Experiments with three or more groups were compared by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) *t*-test. The significance level was defined as $p < 0.05$.

3. Results

3.1 Co-Localization of GRM8 and CD11b

Immunofluorescence microscopy was used to examine the co-localization of GRM8 and CD11b, a marker for microglia. As shown in Fig. 1, GRM8 co-localized with CD11b, indicating that GRM8 is expressed in microglia.

3.2 GRM8 Activation Leads to the Switching of M1/M2 Polarization Phenotypes and Inhibition of Pro-Inflammatory Cytokine Release in Microglia Treated with LPS

Quantitative RT-PCR was used to measure relative mRNA levels of microglial M1/M2 markers. Compared with the controls, LPS markedly upregulated the expression of pro-inflammatory M1 phenotypic markers, including inducible nitric oxide synthase (*iNOS*) and *TNF-*α (*iNOS*, $p < 0.001$; *TNF-*α, $p < 0.001$; Fig. 2A). In contrast, microglial GRM8 activation significantly reduced the expression of M1 markers (*iNOS*, $p = 0.036$; *TNF-*α, $p = 0.003$; Fig. 2A) when the LPS and LPS+GRM8 groups were compared. LPS also significantly reduced the expression of anti-inflammatory M2 phenotype markers relative to controls, including arginase 1 (*Arg-1*) and cluster of differentiation 206 (*CD206*) (*Arg-1*, $p = 0.0012$; *CD206*, $p < 0.001$, Fig. 2B). Combined treatment of GRM8 agonist and LPS was found to increase the expression of M2 markers relative to LPS-only group (*Arg-1*, $p = 0.03$; *CD206*, $p = 0.51$; Fig. 2B).

We used ELISA to further evaluate pro-inflammatory cytokine levels in BV2 cells. GRM8 activation significantly inhibited the release of LPS-induced pro-inflammatory cytokines (TNF-α, $p = 0.014$; IL-1β, $p = 0.03$; IL-6, $p < 0.001$; Fig. 2C). These results suggest that microglial GRM8 activation shifts the M1/M2 polarization state and improves the inflammatory microenvironment in microglia challenged with LPS.

3.3 GRM8 Promotes Neuronal Survival in a Co-Culture Model

After incubating with LPS, GRM8, or LPS combined with a GRM8 agonist, BV2 cells were transferred to a co-culture system with PC12 cells and grown for 48 h (Fig. 3A). PC12 cells were then analyzed using flow cytometry (Fig. 3B) and CCK-8 (Fig. 3D) assays. The flow cytometry results showed that BV2 cells challenged with LPS significantly increased the apoptosis of PC12 cells in

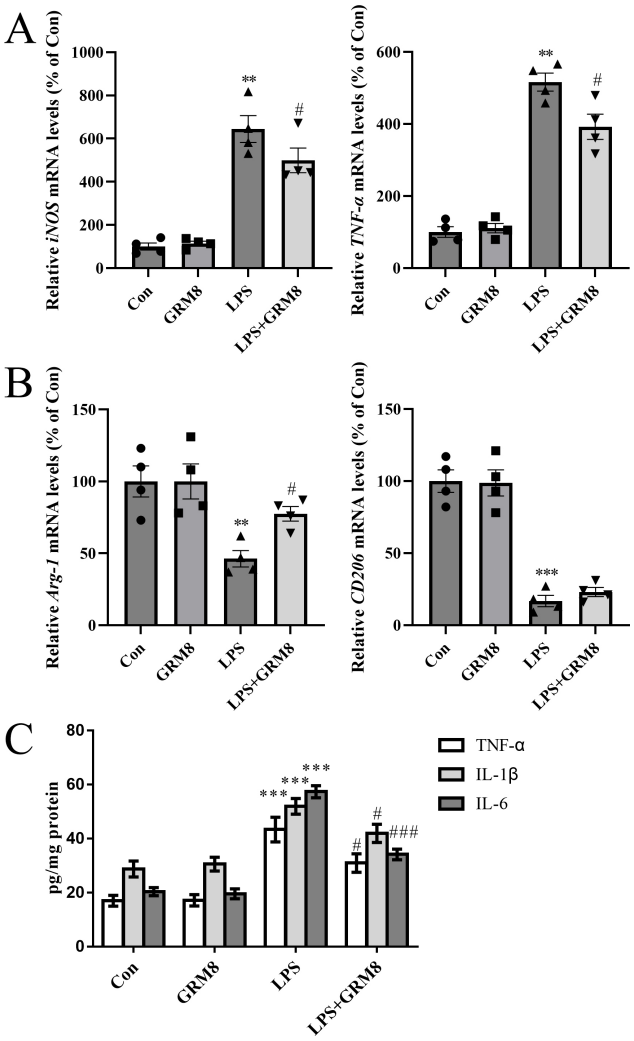

Fig. 2. GRM8 activation shifts M1/M2 polarization and inhibits the release of pro-inflammatory cytokines. (A) Relative mRNA expression of the pro-inflammatory M1 phenotype markers *iNOS* and *TNF-α*. n = 4 per group. (B) Relative mRNA expression of the anti-inflammatory M2 phenotype markers *Arg-1* and *CD206*. n = 4 per group. (C) Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of pro-inflammatory cytokines in BV2 cell extracts. n = 5 per group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control (Con) group. $\#p < 0.05$, $\#\#\#p < 0.001$ compared with the lipopolysaccharide (LPS) group.

the co-culture model system relative to controls ($p < 0.001$, Fig. 3C). However, activated GRM8 markedly attenuated LPS-induced apoptosis ($p = 0.002$, Fig. 3C).

In CCK-8 viability assays, LPS treatment markedly reduced the viability of PC12 cells relative to untreated controls ($p < 0.001$, Fig. 3D), while microglial GRM8 activation markedly increased cell viability following LPS challenge ($p = 0.022$, Fig. 3D). Taken together, these results indicate that microglial GRM8 activation can protect neurons from inflammation-induced death.

3.4 GRM8 Reduces IP3R-Dependent Calcium Release and cAMP Level in Microglia

To explore the mechanism controlling the anti-inflammatory response to GRM8 in microglia, the effects of GRM8 on IP3R-dependent calcium release were assessed. As shown in Fig. 4A, LPS treatment significantly increased calcium release from the ER to the cytoplasm relative to controls ($p < 0.001$, Fig. 4A). This subsequently depleted calcium storage levels within the ER ($p < 0.001$, Fig. 4B). Notably, GRM8 activation inhibited calcium release ($p = 0.004$, Fig. 4A) and maintained ER Ca^{2+} concentrations ($p = 0.011$, Fig. 4B) in BV2 cells challenged with LPS.

cAMP is a common second messenger that may facilitate calcium release by sensitizing IP3R. Therefore, we next measured cellular cAMP levels using ELISA. cAMP levels increased significantly following LPS stimulation ($p < 0.001$, Fig. 4C). However, GRM8 activation reduced cAMP levels in BV2 cells treated with LPS ($p = 0.002$, Fig. 4C). These results suggest that GRM8 modulates IP3R-dependent calcium homeostasis by regulating the cellular level of cAMP.

3.5 GRM8 Restores Mitochondrial Function and Inhibits the Release of ROS in Microglia

Since calcium overload can trigger mitochondrial dysfunction and promote excessive ROS production, ROS could also be responsible for increasing ER stress. We observed a marked decrease in MMP in BV2 cells treated with LPS relative to controls ($p < 0.001$, Fig. 5A), as well as lower ATP levels ($p < 0.001$, Fig. 5B). In contrast, GRM8 activation significantly increased both MMP ($p = 0.009$, Fig. 5A) and ATP levels ($p = 0.038$, Fig. 4B). LPS caused ROS accumulation in BV2 cells compared with untreated controls (intracellular ROS, $p < 0.001$; mitochondrial ROS, $p < 0.001$; Fig. 5C,D, respectively), whereas GRM8 activation reduced ROS accumulation (intracellular ROS, $p = 0.016$; mitochondrial ROS, $p < 0.001$; Fig. 5C,D, respectively). These findings indicate that microglial GRM8 activation restores toxin-induced mitochondrial dysfunction and inhibits the accumulation of ROS.

3.6 GRM8 Inhibits ER Stress and NF-κB Signaling in Microglia

Increased ER stress can activate inflammatory signaling pathways such as the canonical nuclear factor kappa B (NF-κB) pathway. Elevated expression of ER stress sensing proteins (ATF6, $p < 0.001$; IRE1α, $p < 0.001$; p-ERK, $p < 0.001$; Fig. 6A), and phosphorylation of the NF-κB subunit p65 ($p < 0.001$, Fig. 6B) were observed in BV2 cells treated with LPS. However, GRM8 activation markedly inhibited these markers of ER stress compared with treatment with LPS alone (ATF6, $p < 0.001$; IRE1α, $p < 0.001$; p-ERK, $p = 0.03$; Fig. 6A), as well as reducing p65 phosphorylation ($p = 0.01$, Fig. 6B). These results indicate that ER stress and NF-κB signaling are attenuated by the anti-inflammatory action of GRM8.

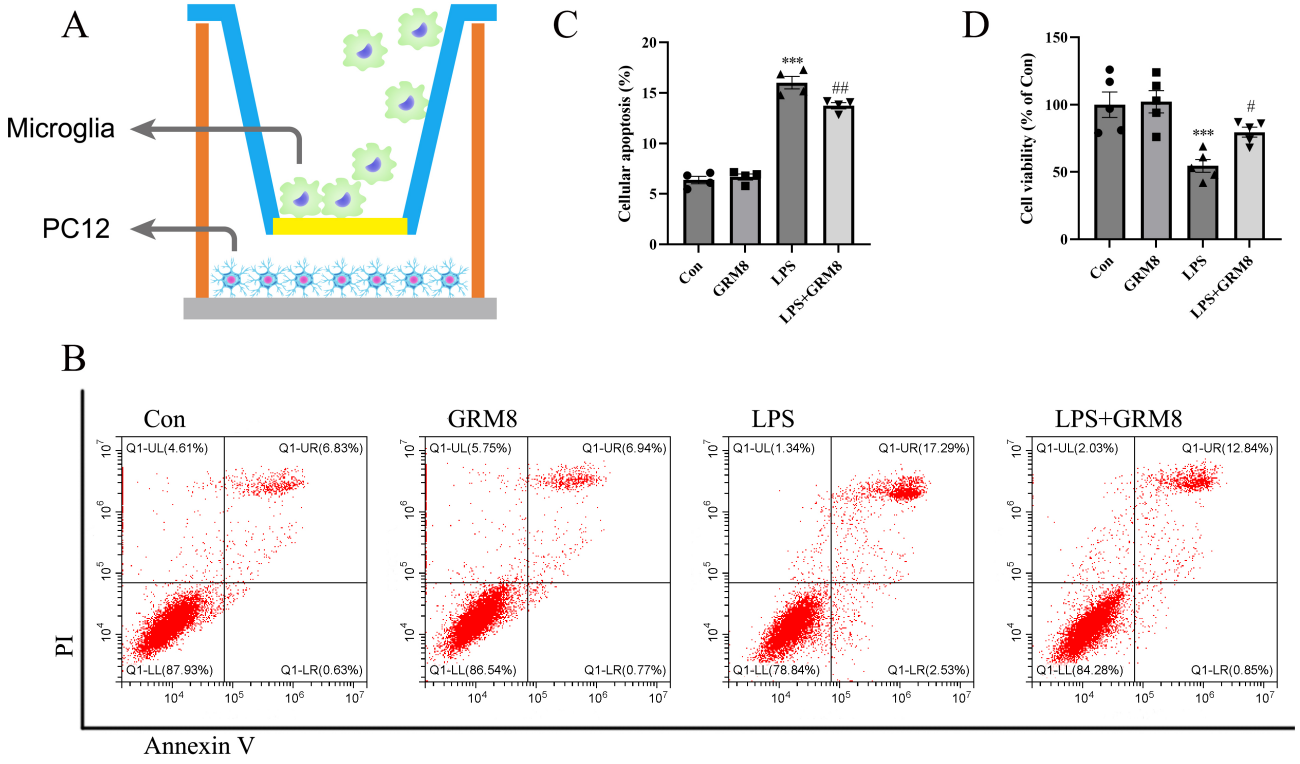

Fig. 3. Microglial GRM8 activation promotes neuronal survival in a co-culture model system. (A) Simplified schematic of the co-culture model. (B) Representative flow cytometry analyses showing apoptotic cell populations. (C) Bar graphs indicating mean numbers of apoptotic cells measured in the indicated experimental groups. n = 4 per group; dots show the individual assay values. (D) Cell viability examined by CCK-8 assays. n = 5 per group. ***$p < 0.001$ compared with the control (Con) group. #$p < 0.05$, ##$p < 0.01$ compared with the LPS group. CCK-8, Cell Counting Kit-8; PI, propidium iodide.

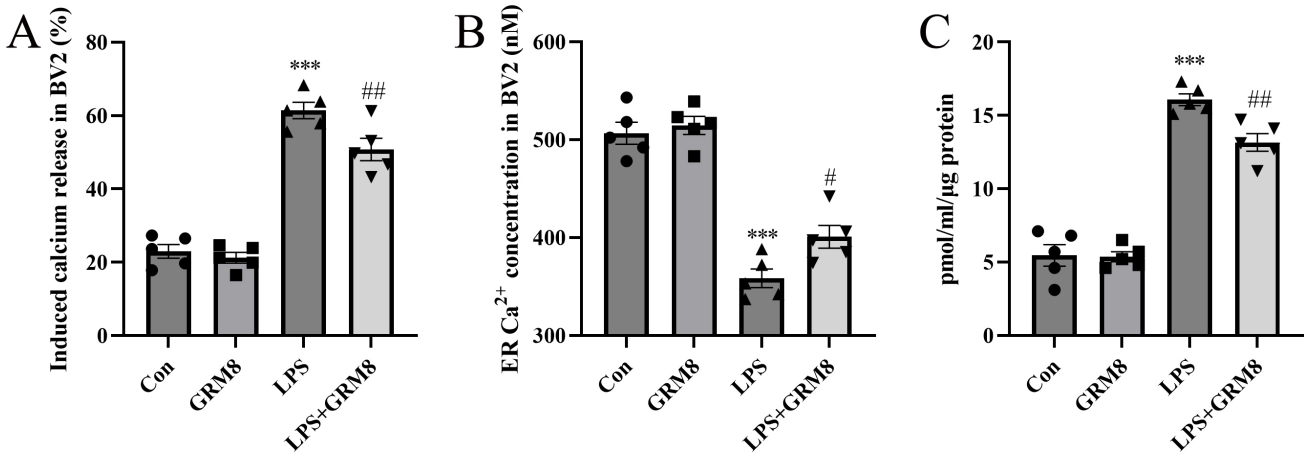

Fig. 4. Microglial GRM8 restores calcium homeostasis in the endoplasmic reticulum (ER) and reduces cAMP levels in BV2 cells. (A) Induced calcium release from the ER to the cytoplasm in BV2 cells. n = 5 per group. (B) ER Ca^{2+} concentration in BV2 cells. n = 5 per group. (C) cAMP levels in BV2 cells measured by ELISA. n = 5 per group. ***$p < 0.001$ compared with the control (Con) group. #$p < 0.05$, ##$p < 0.01$ compared with the LPS group.

4. Discussion

We studied the anti-inflammatory effects of GRM8 activation in BV2 cells challenged with LPS. GRM8 activation was found to ameliorate the pro-inflammatory response observed in LPS-treated BV2 cells, and to attenuate neuronal injury in a BV2/PC12 cell co-culture model. Mechanistically, microglial GRM8 activation inhibited LPS-induced excessive cAMP production, resulting in desensitization of IP3R and reduction of IP3R-dependent calcium release from the ER to the mitochondria. Calcium

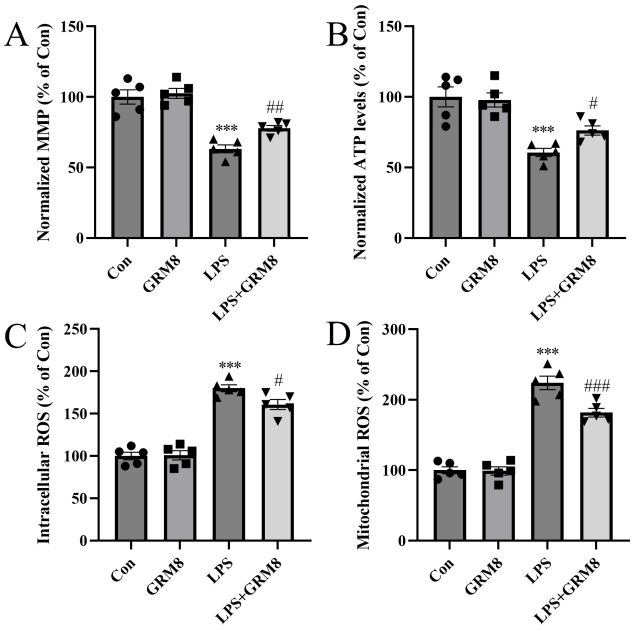

Fig. 5. Effects of GRM8 on mitochondrial function in BV2 cells challenged with LPS. (A) Relative mitochondrial membrane potential (MMP). (B) Relative ATP levels. (C) Intracellular reactive oxygen species (ROS) levels. (D) Mitochondrial ROS levels. n = 5 per group. ***$p < 0.001$ compared with the control (Con) group. #$p < 0.05$, #$p < 0.01$, ###$p < 0.001$ compared with the LPS group.

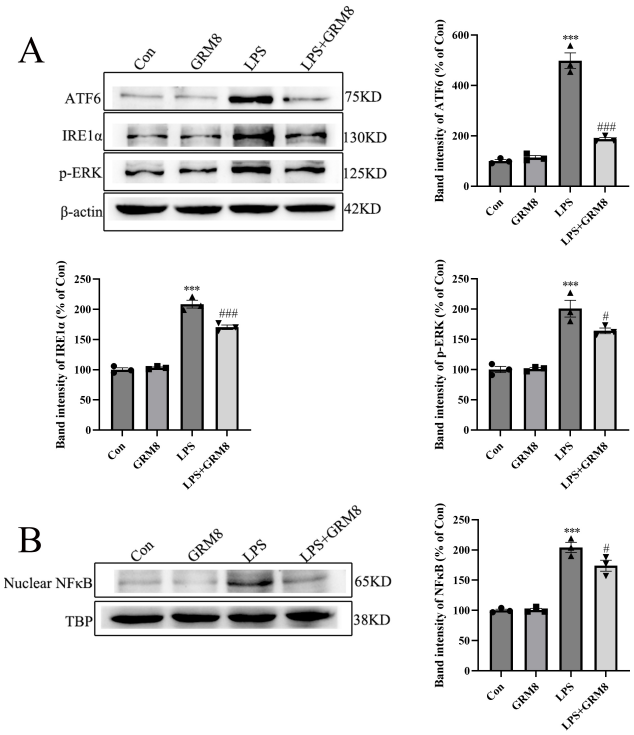

Fig. 6. GRM8 activation deactivates ER stress and NF-κB signaling. (A) Expression of ER stress sensing protein detected by western blotting. n = 3 per group. (B) Western blot analysis of NF-κB p65 subunit phosphorylation. n = 3 per group. ***$p <$ 0.001 compared with the control (Con) group. #$p < 0.05$, ###$p <$ 0.001 compared with the LPS group. TBP, TATA binding protein.

homeostasis is thought to inhibit ER stress by restoring the process of protein folding. In addition, the return of calcium homeostasis restores mitochondrial function and inhibits ROS generation. ROS is widely thought to play an essential role in mitochondrial-ER crosstalk. In accordance with this, reduced ROS levels also deactivated ER stress signaling. GRM8 activation was found to disrupt the canonical NF-κB inflammatory pathway, thus further inhibiting pro-inflammatory M1 polarization and alleviating neuronal death.

GRM8 is expressed extensively in the CNS, particularly in the substantia nigra, basal ganglia, thalamus, dorsal striatum, globus pallidus, subthalamic nucleus, and nucleus accumbens [16]. The neuroprotective effect of GRM8 has been confirmed in several animal models of neurological diseases, but a detailed mechanism of action has yet to be reported. GRM8 was initially thought to be expressed in the presynaptic membrane and to modulate excitatory neurotransmission [17]. However, GRM8 is not restricted to the control of synaptic transmission. By using immunofluorescent staining, we found that GRM8 was also expressed in microglia. Microglial inflammation can be a double-edged sword in neurodegeneration. Under physiological conditions, microglia function to clear debris and toxins from the CNS. Under pathological conditions, excessive microglial activation contributes to neuronal degeneration. To investigate a putative anti-inflammatory effect for GRM8, we established an LPS-induced, pro-inflammatory microglial cell model. Using this model, GRM8 activation was found to significantly reduce the release of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) and to promote a switch in microglial polarization from pro-inflammatory M1 to anti-inflammatory M2 phenotypes. We posit that these events result in amelioration of the pro-inflammatory niche and attenuation of neuronal injury. A prior study also demonstrated that type III GRM activation markedly reduced neuroinflammation in cultured primary microglia treated with LPS, Aβ25-35, or chromogranin A [18]. However, this earlier study did not address which type III GRM functions in the anti-inflammatory response, probably due to the lack of specific agonists. In the present study, we further elaborated that the anti-inflammatory potential of type III GRMs may be linked to GRM8.

Calcium is one of the most widely distributed biological second messengers, and hence calcium balance is crucial for several biological functions. The ER is a major site for calcium storage. Upon binding with IP3, the inositol triphosphate (IP3R) calcium channel located within the ER is opened and facilitates calcium release [19]. Ca^{2+} overload is thought to be central to the triggering of pathobiological changes observed in neurodegeneration, and Ca^{2+} channel blockers have been shown to restore behavioral

performance and neuronal survival [20,21]. Our results indicate that upon LPS challenge of microglia, significant calcium stores are released from the ER into the cytoplasm. This results in the depletion of calcium stores within the ER, yet we observed that GRM8 activation inhibits this calcium release. We suggest that the Ca^{2+}/cAMP signaling cascade may be involved in the anti-inflammatory effects linked to GRM8. cAMP is another versatile cellular second messenger generated from ATP through the action of adenylyl cyclase. cAMP upregulates protein kinase A (PKA) activity and also influences PKA-induced phosphorylation of IP3R. Such PKA-induced phosphorylation may facilitate the opening of IP3R and subsequent Ca^{2+} release from the ER [22]. Under physiologic conditions, the Ca^{2+}/cAMP interaction maintains a negative feedback loop, and chronic and sustained Ca^{2+}/cAMP signaling activation can trigger several detrimental responses. These responses include driving mitochondrial calcium overload, reducing neuronal firing, and aberrant activation of kinases leading to neuronal degeneration [23]. Thus, reduction of excessive cytoplasmic cAMP levels can desensitize IP3R and decrease subsequent Ca^{2+} release. LPS is reported to increase intracellular cAMP in several cell lines [24,25]. As an activator of toll-like receptor 4 (TLR4), LPS may activate TLR4-mediated cAMP signaling. We found that GRM8 is a potent negative regulator of cAMP levels. This might be explained by the fact that as a member of type III GRMs, GRM8 is coupled preferentially to Gαi/o, and Gαi/o can further inhibit the activity of adenylyl cyclase and limit cAMP production [26].

Our work suggests a vital role for GRM8 in modulating ER calcium homeostasis, based on its potential to inhibit cAMP production. Calcium sequestered in the ER is crucial for ER-mitochondrion interaction, as calcium released through IP3R is transferred to the intermembrane of the mitochondrion. Such calcium overload is widely believed to cause mitochondrial dysfunction. We evaluated mitochondrial function by quantifying MMP, the ATP level, and ROS production and found that GRM8 activation restored mitochondrial function in microglia following LPS challenge. The interplay between ROS and ER stress is complex in nature. Nevertheless, ROS, and particularly mitochondrial ROS, is thought to induce ER stress. Excessive ROS production may result in injury to ER Ca^{2+} channels, thereby exacerbating the depletion of ER calcium stores and promoting ER stress [27]. Following LPS treatment of BV2 cells in the present study, GRM8 agonist reduced ER stress-associated protein accumulation and NF-κB activation, as evidenced by p65 phosphorylation. This action reduced microglia-mediated neuroinflammation and promoted a switch in microglial polarization from the M1 to M2 phenotype. Prior studies also indicate that inhibition of the NF-κB pathway is beneficial in counteracting the pro-inflammatory immune response in microglial cells [28–30].

Based on evidence obtained from the use of knockout (KO) mice, a definitive role for GRM8 in neurological disease remains to be confirmed. GRM8 deletion has protective and detrimental effects in neurological disorders, as well as contradictory behavioral effects in KO mice [28–30]. It is worth noting that GRM4 may compensate for GRM8 deficiency, as a neuroprotective effect of GRM4 has been comprehensively reported [16]. Future studies should explore how GRM4 might influence GRM8-mediated anti-inflammatory potential. GRMs have also been shown to exhibit sex-related differences in brain function, stemming from circulatory estrogen levels. Estrogen receptors are coupled to GRMs, and upon binding with estrogen, intracellular G-protein signaling is engaged and ultimately affects cellular function [31]. However, whether estrogen receptors impact GRM8 function awaits further clarification.

5. Conclusions

The results of this study suggest that GRM8 strongly protects microglia from potentially harmful LPS-induced inflammatory responses in BV2 cells. It does this by reducing ER stress and improving mitochondrial function through mechanisms linked to IP3R-mediated calcium homeostasis. These findings indicate that targeting GRM8 may be a feasible therapeutic strategy for limiting neuroinflammation.

Availability of Data and Materials

The data used and analyzed during the current study are available on reasonable request.

Author Contributions

YX, LC, JC, YL and YC designed the research study. YX, ZPeng, HZ and LC performed the research. YX, ZPan and JC conducted experiments. YC analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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