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NMDA receptor blockade attenuates Japanese encephalitis virus infection-induced microglia activation

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Abstract

Neurodegeneration and neuroin□ammation are key components in the pathogenesis of Japanese Encephalitis caused by Japanese Encephalitis Virus (JEV) infection. The N-methyl-D-aspartate (NMDA)-type glutamate receptor displays excitatory neurotoxic and pro-in□ammatory properties in a cell context-dependent manner. Herein, potential roles of the NMDA receptor in excitatory neurotoxicity and neuroin□ammation and e□ects of NMDA receptor blockade against JEV pathogenesis were investigated in rat microglia, neuron/glia, neuron cultures, and C57BL/6 mice. In microglia, JEV infection induced glutamate release and activated post-receptor NMDA signaling, leading to activation of Ca^{2+} mobilization and Calcium/Calmodulin-dependent Protein Kinase II (CaMKII), accompanied by pro-in□ammatory NF-κB and AP-1 activation and cytokine expression. Additionally, increased Dynamin-Related Protein-1 protein phosphorylation, NAPDH Oxidase-2/4 expression, free radical generation, and Endoplasmic Reticulum stress paralleled with the reactive changes of microglia after JEV infection. JEV infection-induced biochemical and molecular changes contributed to microglia reactivity and pro-in□ammatory cytokine expression. NMDA receptor antagonists MK801 and memantine alleviated intracellular signaling and pro-in□ammatory cytokine expression in JEV-infected microglia. JEV infection induced neuronal cell death in neuron/glia culture associated with the concurrent production of pro-in□ammatory cytokines. Conditioned media of JEV-infected microglia compromised neuron viability in neuron culture. JEV infection-associated neuronal cell death was alleviated by MK801 and memantine. Activation of NMDA receptor-related in□ammatory changes, microglia activation, and neurodegeneration as well as reversal e□ects of memantine were revealed in the brains of JEV-infected mice. The current □ndings highlight a crucial role of the glutamate/NMDA receptor axis in linking excitotoxicity and neuroin□ammation during the course of JEV pathogenesis, and proposes the anti-in□ammatory and neuroprotective potential of NMDA receptor blockade.

Keywords Japanese encephalitis virus, Microglia polarization, Neuroin□ammation, NMDA receptor

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Introduction

Japanese EncephalitisVirus (JEV) is a mosquito-borne neurotropic virus belonging to the *Flaviviridae* family. Its family members include the Zika Virus, West Nile Virus, Hepatitis C Virus, St. Louis Encephalitis Virus, and Yellow Fever Virus [1]. Its tra cking from the periphery to the Central Nervous System (CNS) and viral ampli cation in the brain parenchyma both play determinant roles in JEV pathogenesis involving stepwise communication between the host and viral factors. JEV illness is called Japanese Encephalitis (JE), with the associated pathological hallmark characterized by neuronal cell death and in ammation which have close links to JEV pathogenesis. Along with experiencing fatal neuroinfection, survivors of clinical JE cases may su er from persistent neurological sequelae [2–4]. Appropriate immune responses help the host defense viral infection and promote neuronal survival, while aberrant immune responses exacerbate neuronal deterioration. Sustained microglia activation and in ammatory cytokine over-production are deteriorating components of JEV pathogenesis. Interventions aimed at suppressing aberrant immune responses by amelioration of microglia activation and/or in ammatory cytokine over-production are found to have bene cial outcomes in neuronal survival and neurobehavioral functions [5–7]. erefore, in ammation and neuronal cell death are key components to the development of JE and microglia represent targets crucial to therapeutic treatment against JE and its associated complications.

Neurons are the major target of JEV infection, with neuronal cell death being a key component of JEV infection-induced neurodegeneration and neurobehavioral abnormality. Several types of programmed cell death, including apoptosis, autophagy, and necroptosis are found in neurons upon JEV infection [8–10]. Along with direct neurotoxicity, bystander damage represents an alternative mechanism attributing to JEV infection-induced neuronal cell death. Severe JE-associated neuronal cell death is accompanied by immune cell accumulation and activation, as well as in ammatory cytokine over-production. Speci cally, in vitro cell study demonstrates that JEV-infected microglia release neurotoxic molecules and induce neuronal cell death [7, 9, 11]. ese ndings indicate that JEV infection causes neuronal cell death, with the compromise of neurons possibly coming from consequences of direct neurotoxicity and/or secondary JEV-infected microglia.

Glutamate is a physiological excitatory neurotransmitter in the nervous system. When seen in excess, glutamate turns into neurotoxic molecules and causes neuronal cell death mainly through the N-methyl-D-aspartate (NMDA) receptor [12]. Glutamate neurotoxicity has been implicated not only in a plethora of neurological disorders but also in virus infection-associated neuronal injury [13, 14]. Beyond being a neurotoxic molecule, glutamate displays additional pharmacological and biochemical functions through its communication with in ammatory responses. In ammatory stimuli and pro-in ammatory cytokines cause elevation in intracellular glutamate synthesis and extracellular glutamate release [15, 16]. Alternatively, glutamate provides dual e ects on pro-in ammatory cytokine expression through its actions on ionotropic and metabotropic glutamate receptors [17–19]. ese phenomena highlight the role of the interplay between neuronal cell death and in ammation, while underscoring their importance as preventive and therapeutic targets for the control of neurological disorders, including viral encephalitis. Critical to this interplay is that glutamate could act as a key integrator in linking neuronal cell death and in ammation.

Glutamate neurotoxicity has been demonstrated in rodent models of JE. Glutamate levels and NMDA receptor activity were both elevated in the brains of JEV-infected mice and rats. NMDA receptor antagonists, MK801 and memantine, alleviated neuronal cell death and in ammatory responses triggered by JEV infection, while having little e ect on viral ampli cation [4, 20, 21]. Using rat brain primary cultures, our previous study revealed the neuroprotective e ects of MK801 against JEV infection-induced neurotoxicity, while also demonstrating the active role microglia play in JEV infection-accompanied glutamate release, pro-in ammatory cytokine expression, and neuronal cell death [15]. Although whether microglia possess NMDA receptor-dependent currents in vivo is of controversy [22], microglia express functional glutamate NMDA receptors and the NMDA receptor blockade is reported to have inhibitory e ects on microglia activation and pro-in ammatory cytokine expression [23–25]. Along with glutamate, endogenous mimicking ligands and the Toll-Like Receptor 4 (TLR4) have been linked to NMDA receptor signaling leading to microglia activation [26]. JEV infection-induced neuronal cell death and in ammation correlated well with activation of the NMDA receptor axis [4, 20, 21], the speci c involvement of the NMDA receptor signaling in JEV infection-induced in ammation has not yet been completely investigated. Microglia are highly dynamic and plastic cells that display multivariate morphological, genetic, metabolic, and functional states in both normal physiology and disease pathophysiology [9, 11, 17]. To extend the scope of JEV pathogenesis study centered on in ammation, this present study investigated the alteration of glutamate/NMDA receptor signaling in infected cells, while also attempting to elucidate change in microglia states reactive to JEV infection by NMDA receptor blockade via microglia culture and infected mice.

Materials and methods

Cell cultures

For preparation of the primary cultures, cerebral cortices were collected from postnatal Day 1 Sprague-Dawley rats, which then proceeded to tissue trituration and cell isolation according to our previously reported methods [11]. e experimental protocols surrounding animal studies were reviewed and approved by e Animal Experimental Committee of Taichung Veterans General Hospital (IACUC approval code: La-1061509). e dissociated cells were then transferred to poly-D-lysine-coated (30 μg/mL) dishes/plates for cultivation. Neuron/glia cultures consisting of 30–40% neurons, 40–50% astrocytes, and 10–15% microglia were prepared by maintaining the cells in a Minimum Essential Medium, supplemented with 10% Fetal Bovine Serum (FBS) and 10% Horse Serum for 10–12 days. Neurobasal Medium supplemented with B27 and cytosine arabinoside (10 μM) was used to culture the neurons. Neuron cultures (more than 95% purity) were ready for use in the experiments after 10–12 days in vitro. Cultivation of the cells for 2 weeks was performed in Dulbecco's Modi ed Eagle Medium (DMEM)/F12 containing 10% FBS enriched astrocytes and microglia. Microglia (more than 90% purity) were separated from astrocytes by shaking and centrifugation.

e detached microglia were seeded onto poly-D-lysine-coated (30 μg/mL) dishes/plates and then maintained in DMEM/F12 containing 10% FBS until analyses could be performed. One batch of cell preparation was referred to one biological replicate. Usually, three to four biological replicates were included for each experiment.

Virus

e propagation of JEV strain NT113 was performed in C6/36 cells (BCRC-60114, Bioresource Collection and Research Center, Hsinchu, Taiwan), with viral titers counted using Baby Hamster Kidney cells (BHK21, BCRC-60041, Bioresource Collection and Research Center, Hsinchu, Taiwan) [11]. A mock virus was prepared from C6/36 cells without JEV infection, in parallel with propagation of JEV virus stock. For virus inactivation, virus stocks were manipulated with UV exposure (254 nm exposure for 30 min, JEV/UV-inactivated) or boiling (94 °C incubation for 15 min, JEV/Heat-inactivated). To infect the primary cultures, cells were inoculated with JEV stock or manipulated JEV at a Multiplicity of Infection (MOI) 5 for 1 h at 37 °C. After gentle washing with Phosphate-Bu ered Saline (PBS) to remove the unbound viruses, a fresh DMEM/F12 containing 2% FBS was added for further incubation. For treatment, pharmacological agents were added with the refreshing medium.

Cytotoxicity assessment

Cytotoxicity was assessed using the Pierce™ Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (ermo Fisher Scienti c, Waltham, MA, USA), according to the manufacturer's instructions.

Immunocytochemical staining

Cells were sequentially treated with 4% paraformaldehyde, 0.1% Triton X-100, and 5% nonfat milk for xation, permeabilization, and blocking, respectively. To visualize the neurons, astrocytes and microglia, cells were incubated with each corresponding recognizing antibody, Microtubule-Associated Protein 2 (MAP-2, 1:500, sc-390543), Glial Fibrillary Acidic Protein (GFAP, 1:500, sc-33673) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Cluster of Di erentiation 68 (CD68, 1:500, ab125212) (Abcam, Cambridge, UK), followed by diaminobenzidine color development. Under a light microscope, viable and MAP-2 immunoreactive neurons were de ned by a visible cell body having a length of neurite process greater than two cell bodies. For quantitation, the numbers of positive cells were counted in three randomly selected optical elds (100X magni cation) in the well of a 24-well plate, with two wells being included. Data from three di erent batches of cell preparation were combined for quantitative analyses.

Enzyme-linked immunosorbent assay (ELISA)

e levels of Nitric Oxide (NO, nitrite/nitrate), Tumor Necrosis Factor-α (TNF-α), and Interleukin-1β (IL-1β) in the cell supernatants were measured using a Griess Reagent Kit (ermo Fisher Scienti c, Waltham, MA, USA) and the corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA).

Measurement of reactive oxygen species (ROS)

To measure intracellular ROS, cells were incubated with the cell permeable uorogenic dye 2',7'-Dichloro uorescein Diacetate (DCFDA) (ermo Fisher Scienti c, Waltham, MA, USA). e uorescence signals of reacted DCFDA were measured using a uorometer with excitation/emission at 485 nm/535 nm.

RNA isolation and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Cellular RNA extraction andcomplementary DNA synthesis were completed using conventional procedures. Quantitative real-time PCR was performed on ABI StepOne™ (Applied Biosystems, Foster City, CA, USA), withthe data calculated based on the ΔΔCT method according to our previously reported methods [11]. e oligonucleotides used for ampli cation were: iNOS, 5'-ACAACGTGGAGAAAACCCCAGGTG and 5'-ACAGCTCCGGGCATCGAAGACC; TNF-α, 5'-CCCTCACACTC

AGATCATCTTCTCAA and 5'-TCTAAGTACTTGGGC AGGTTGACCTC; IL-1β, 5'-CACCTCTCAAGCAGAG CACAG and 5'-GGGTTCCATGGTGAAGTCAAC; JEV, 5'-AGAGCACCAAGGGAATGAAATAGTand 5'-AATA GGTTGTAGTTGGGCACTCTG; and β-actin, 5'-AAGT CCCTCACCCTCCCAAAAG and 5'-AAGCAATGCTG TCACCTTCCC.

Measurement of glutamate

e levels of glutamate in the cell supernatants were measured using the High Performance Liquid Chromatography (HPLC) technique, with chromatographic data processed using HP Chem Station (Hewlett Packard, Palo Alto, CA, USA), according to our previously reported methods [15].

Measurement of cytosolic Ca2+ levels

e levels of cytosolic Ca^{2+} were measured using Fluo-4 AM according to our previously reported methods with modi cations [15]. e uorescence signals were measured in a uorometer with excitation wavelength at 485 nm and the emission wavelength at 510 nm.

Western blot analysis

Conventional protein extraction, separation, and SDS-PAGE were performed according to our previously reported methods [11]. e targeted proteins in the membranes were recognized by sequential incubation with primary antibodies, horseradish peroxidase-labeled IgG and enhanced chemiluminescence Western blotting reagents. e chemiluminescence on the membranes were visualized using the G: BOX mini Multi uorescence and chemiluminescence imaging system (Syngene, Frederick, MD, USA), and then quanti ed by Image J software (National Institute of Health, Bethesda, MD, USA). Targets of the used antibodies included JEV nonstructural protein 1 (NS1, 1:2000, ab41651), CD68 (1:1000, ab125212), phospho-IRE1 (Serine-724, 1:500, ab48187) (Abcam, Cambridge, UK), c-Jun (1:1000, sc-74543), MAP-2 (1:1000, sc-390543), GFAP (1:1500, sc-33673), Calcium/Calmodulin-dependent Protein Kinase II (CaMKII, 1:1000, sc-5306), NF-κB p65 (1:1000, sc-514451), phospho-NF-κB p65 (Serine-536, 1:500, sc-136548), c-Fos (1:1000, sc-8047), Extracellular Signal-Regulated Kinase (ERK, 1:1000, sc514302), c-Jun N-terminal Kinase (JNK, 1:1000, sc-7345), phospho-p38 (Tyrosine-182, 1:500, sc-166182), Interferon Regulatory Factor 5 (IRF5, 1:1000, sc-390364), P2X purinoceptor 7 (P2×7R, 1:1000, sc-514962), Dynamin-Related Protein 1 (Drp1, 1:1000, sc-271583), NADPH Oxidase 2 (NOX2, 1:1000, sc-130543), Protein Kinase RNA-like Endoplasmic Reticulum Kinase (PERK, 1:1000, sc-377400), eIF2α (1:1000, sc-133132), IRE1 (1:1000, sc-390960), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH, 1:3000, sc-32233) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Transforming Growth Factor β-Activated Kinase-1 (TAK1, 1:1000, 12330-2-AP), NOX4 (1:1000, 14347-1-AP) (Proteintech, Rosemont, IL, USA), phospho-c-Jun (Serine-63, 1:500, PA5-17890), phospho-TAK1 (Serine-192, 1:500, BS-5435R), phospho-Drp1 (Serine-616, 1:500, PA5-64821), phospho-PERK (reonine-982, 1:500, PA5-40294) (ermoFisher Scienti c, Waltham, MA, USA), NMDA GluN1 receptor (1:1000, #5704), phospho-GluN1 (Serine-896, 1:500, #3384), NMDA GluN2A receptor (1:1000, #4205), phospho-GluN2A (Tyrosine-1246, 1:500, #4206), NMDA GluN2B receptor (1:1000, #4207), phospho-GluN2B (Serine-1303, 1:500, #71335), phospho-eIF2α (Serine-51, 1:500, #3597), phospho-CaMKII (reonine-286, 1:500, #12716), phospho-ERK (reonine-202/Tyrosine-204, 1:500, #9101), phospho-JNK (reonine-183/Tyrosine-185, 1:500, #9255), p38(1:1000, #9212) (Cell Signaling Technology, Danvers, MA, USA).

Preparation of nuclear extracts and Electrophoretic mobility Shift Assay (EMSA)

An NE-PER Nuclear and Cytoplasmic Extraction Kit (ermoFisher Scienti c, Waltham, MA, USA) and an EMSA kit (LightShift™ Chemiluminescent EMSA Kit, ermoFisher Scienti c, Waltham, MA, USA) were used to extract nuclear proteins and evaluate transcription factor DNA binding activity, respectively, according to the manufacturer's instructions. As with our previous studies [11], nuclear extracts (5 μg) were reacted with either AP-1 oligonucleotide (5'-CGCTTGATGAGTCAGCCG GAA) or NF-κB oligonucleotide (5'-AGTTGAGGGGA CTTTCCCAGGC), with the complexes visualized using enhanced chemiluminescence Western blotting reagents. e chemiluminescence on the membranes were visualized using a G: BOX mini Multi uorescence and chemiluminescence imaging system (Syngene, Frederick, MD, USA), and quanti ed by Image J software (National Institute of Health, Bethesda, MD, USA).

Caspase 3 activity assay

Proteins were extracted from neuron/glia cultures and caspase 3 activity was measured using a commercially available kit (Fluorometric Assay Kit, BioVision, Mountain View, CA, USA), according to the manufacturer's instructions.

Animals

Adult male C57BL/6 mice (8 weeks old) were purchased from BioLASCO (Taipei, Taiwan). e experimental protocols surrounding animal studies were reviewed and approved by e Animal Experimental Committee of Taichung Veterans General Hospital (IACUC approval code: La-1061509). Eighteen mice were divided into

three groups receiving either PBS (*n*

Fig. 1 JEV infection activated NMDA receptor signaling in microglia. (A) Cultured microglia were infected with mock or JEV for 24 h. Microglia were examined by immunocytochemical staining with antibody recognizing CD68. Representative photomicrograph is shown. Bar, 50 μm. Microglia cultures were infected with mock or JEV over time. The supernatants were collected and subjected to HPLC for the measurement of glutamate concentration at the indicated times (B). The changes in cytosolic Ca^{2+} concentration were determined by Fluo-4 AM uorescence (arbitrary unit) measurements at the indicated times (C). Microglia cultures were infected with mock, JEV, JEV/UV-inactivated, or JEV/Heat-inactivatedfor 12 h. The supernatants were collected and subjected to HPLC for the measurement of glutamate concentration (D). The changes in cytosolic Ca^{2+} concentration were determined by Fluo-4 AM uorescence (arbitrary unit) measurements (E).(F) Microglia cultures were infected with mock or JEV in the absence or presence of various concentrations of MK801 (0–40 μM) or memantine (0–10 μM) for 12 h.The changes in cytosolic Ca^{2+} concentration were determined by Fluo-4 AM uorescence (arbitrary unit) measurements. (G) Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 8 h. Total cellular proteins were extracted and subjected to Western blot analysis with the indicated antibodies. One representative blot of three independent culture batches and the quantitative results of relative protein contents (arbitrary unit) are shown. *$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 4$

NF-κB and AP-1 for activation in microglia were assessed using EMSA and Western blotting, respectively. JEV infection caused an increase in the DNA binding activity of NF-κB and AP-1 (Fig. 3A) and upstream regulatory molecules of NF-κB and AP-1, including the protein phosphorylation of TAK1, ERK, JNK, p38, p65 and c-Jun, and the protein expression of c-Fos (Fig. 3B). JEV infection-increased DNA binding activity (Fig. 3A), protein phosphorylation, and protein expression (Fig. 3B) were all decreased by MK801 and memantine. e purinergic receptor P2×7 (P2×7R) is an ATP-gated non-selective cation channel that is highly expressed in microglia and can mediate oxidative stress and in ammatory responses. IRF5, a member of the intracellular mediators for type I interferon, is a transcription factor. Its expression in microglia participates in in ammatory responses [11]. In JEV-infected microglia, there were elevated expression of IRF5 and P2×7R. Protein levels of IRF5 and P2×7R

Fig. 2 MK801 and memantine alleviated cytokine expression in microglia. (A) Microglia cultures were infected with mock, JEV, JEV/UV-inactivated, or JEV/Heat-inactivated for 24 h. The supernatants were collected and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β. Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) over time. The supernatants were collected and subjected to the measurement of NO, TNF-α, IL-1β (24 h), and glutamate (12 h) (B). Total cellular RNAs were extracted (8 h) and subjected to quantitative RT-PCR for the measurement of iNOS, TNF-α, and IL-1β mRNA. Quantitative results of the relative mRNA levels (arbitrary unit) are shown (C). (D) Microglia cultures were infected with mock or JEV in the absence or presence of glutamate (500 μM) for 24 h. The supernatants were collected and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β. *$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 4$

in JEV-infected microglia were reduced by MK801 and memantine (Fig. 3B). Current ndings imply that the anti-in ammatory e ects of NMDA receptor blockade against JEV infection may come from the inhibition of the axis of NF-κB and AP-1 signaling as well as suppression of microglia reactive to JEV infection.

NMDA receptor antagonists alleviated JEV infection-activated free radical generation in microglia

e NOX-mediated free radical generation can come from the signals of the NMDA receptors and contribute to pro-in ammatory activation involving mitochondrial dysfunction. Dynamin-associated GTPase Drp1 mediates mitochondrial fragmentation and involves in in ammatory responses and microglia activation [29–31]. Using the redox-sensitive uorogenic probe, the levels of DCFDA uorescence (Fig. 4A) increased in microglia after JEV infection. At the protein levels, JEV infection increased NOX2 and NOX4 protein expression as well as mitochondrial ssion-related Drp1 protein phosphorylation in microglia (Fig. 4B). MK801 and memantine alleviated JEV infection-induced changes in uorescence (Fig. 4A) and protein levels (Fig. 4B). To further examine the biological implications of free radical generation and Drp1 activation, e ects of the corresponding antioxidant N-Acetyl Cysteine (NAC) and Drp1 inhibitor Mdivi-1 were evaluated. NAC and Mdivi-1 alleviated JEV infection-induced increases of DCFDA uorescence (Fig. 4C) and expressions of NO, TNF-α, and IL-1β (Fig. 4D). us, JEV infection increases NOX expression,

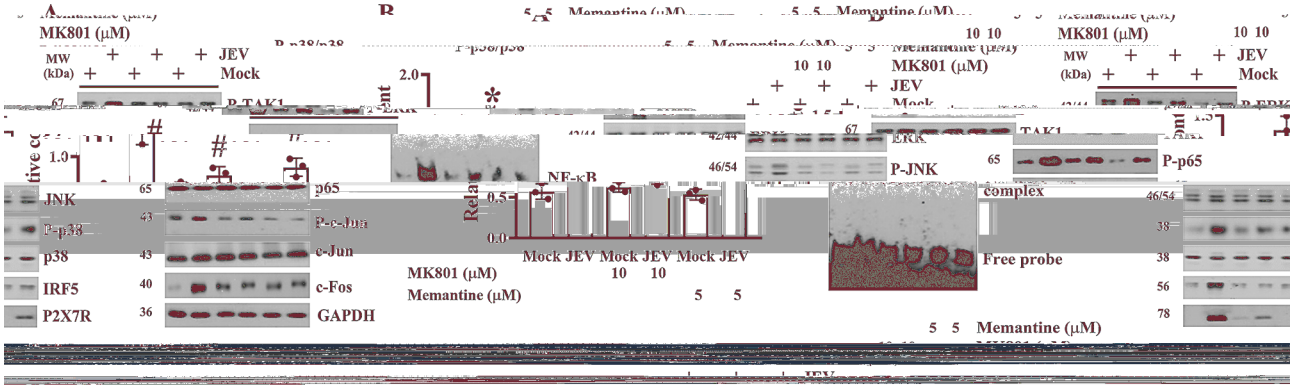

Fig. 3 MK801 and memantine alleviated NF-κB and AP-1 activation in microglia. Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 8 h. Nuclear proteins were extracted and subjected to EMSA for the measurement of NF-κB and AP-1 DNA binding activity. One representative blot of three independent culture batches and the quantitative results (arbitrary unit) are shown (A). Total cellular proteins were extracted and subjected to Western blot analysis with indicated antibodies. One representative blot of three independent culture batches and the quantitative results of relative protein contents (arbitrary unit) are shown (B). *$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 3$

induces intracellular free radical generation, and activates mitochondrial Drp1, contributing to pro-in ammatory cytokine expression. e aforementioned changes are alleviated by MK801 and memantine.

NMDA receptor antagonists alleviated JEV infection-activated endoplasmic reticulum (ER) stress in microglia

NMDA receptor signaling possesses the ability to induce Unfolded Protein Response (UPS) and ER stress, resulting in pro-in ammatory activation [30, 32]. Parameters of ER stress and potential contribution to JEV infection-induced changes in microglia were then assessed. Upon JEV infection, microglia increased protein phosphorylation in PERK, eIF2α, and IRE1. In the presence of MK801 and memantine, there was a decline in protein phosphorylation (Fig. 5A). ER stress inhibitor, Salubrinal, had an alleviative e ect on JEV infection-induced increases of DCFDA uorescence (Fig. 5C) and expressions of NO, TNF-α, and IL-1β (Fig. 5D). ese ndings reveal the role NMDA receptor signaling plays in JEV infection-induced ER stress and its accompanied pro-in ammatory e ects.

Crosstalk among intracellular signaling in microglia

e aforementioned ndings indicate the active role which NMDA receptor signaling plays in linking JEV infection and cytokine expression; probably involving CaMKII, oxidative stress, and ER stress. e central role and importance of CaMKII were next assessed using its pharmacological inhibitor KN93 in microglia. CaMKII inhibitor KN93 decreased NO, TNF-α, and IL-1β production in JEV-infected microglia (Fig. 6A). eir alleviation in cytokine expression paralleled with changes in DCFDA uorescence (Fig. 6B), the protein expression of c-Fos, IRF5, P2×7R, NOX2, and NOX4, the protein phosphorylation of TAK1, ERK, JNK, p38, p65, c-Jun, Drp1, PERK, eIF2α, and IRE1 (Fig. 6C), as well as the DNA binding activity of NF-κB and AP-1 (Fig. 6D).

ese ndings suggest that the Ca^{2+}-activated CaMKII axis is complicated and plays a central role in linking the

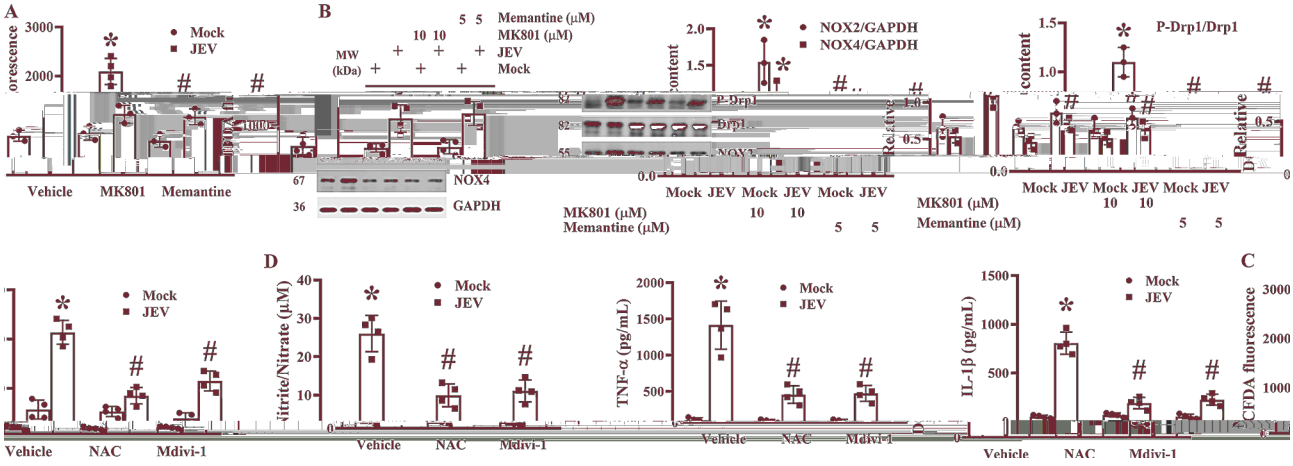

Fig. 4 MK801 and memantine alleviated free radical generation in microglia. Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 8 h. The levels of intracellular free radicals were measured using the uorogenic probe DCFDA (arbitrary unit) (A). Total cellular proteins were extracted and subjected to Western blot analysis with the indicated antibodies. One representative blot of three independent culture batches and the quantitative results of relative protein contents (arbitrary unit) are shown (B). Microglia cultures were infected with mock or JEV in the absence or presence of NAC (500 μM) or Mdivi-1(50μM) over time.The levels of intracellular free radicals (8 h) were measured using the uorogenic probe DCFDA (arbitrary unit) (C). The supernatants were collected (24 h) and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β (D). *$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 4$

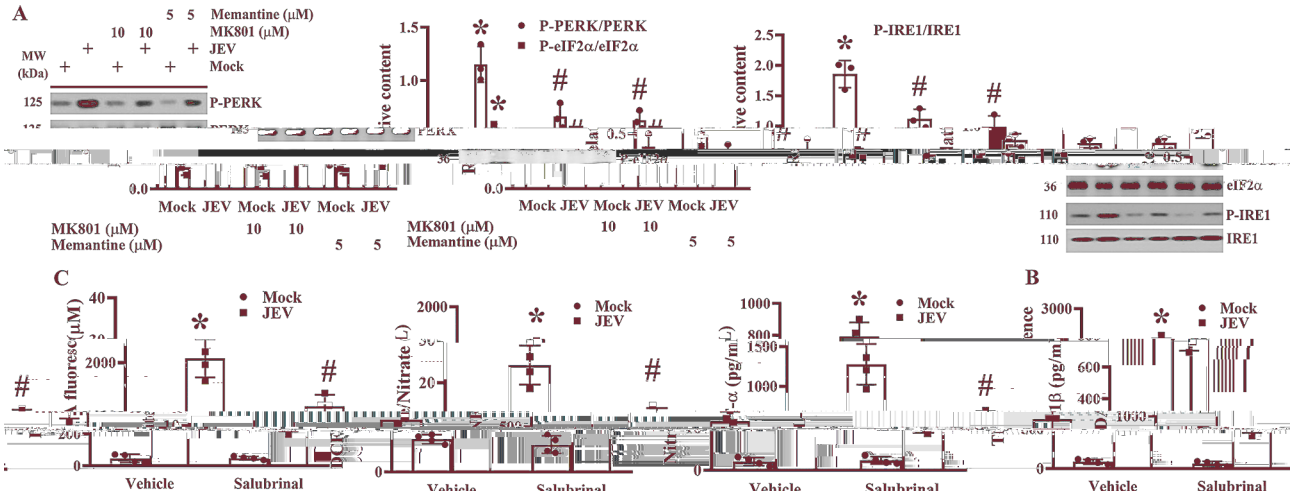

Fig. 5 MK801 and memantine alleviated ER stress in microglia. (A) Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 8 h. Total cellular proteins were extracted and subjected to Western blot analysis with indicated antibodies. One representative blot of three independent culture batches and the quantitative results of relative protein contents (arbitrary unit) are shown. Microglia cultures were infected with mock or JEV in the absence or presence of Salubrinal (10 μM) over time.The levels of intracellular free radicals (8 h) were measured using the uorogenic probe DCFDA (arbitrary unit) (B). The supernatants were collected (24 h) and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β (C).*$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 4$

glutamate/NMDA receptor and NF-κB/AP-1 pro-in ammatory program in JEV-infected microglia.

NMDA receptor antagonists protected against JEV infection-induced neuronal cell death and cytokine expression in neuron/glia and neuron

To implicate the consequences of MK801- and memantine-inhibited microglia activation in neuronal cell death, neuron/glia cultures consisting of neurons, astrocytes, and microglia were prepared for investigation. JEV infection increased CD68 immunoreactivity (Fig. 7A and B), CD68 protein content (Fig. 8A), and NO, TNF-α, and IL-1β production in neuron/glia cultures (Fig. 8B). As with microglia, MK801 and memantine alleviated expression in all pro-in ammatory parameters as well (Figs. 7A and B and 8A, and 8B). e degeneration of MAP-2 immunoreactive neurons (Fig. 7A and B) and reduction of MAP-2 protein content (Fig. 8A) in JEV-infected neuron/glia cultures were improved by MK801 and memantine. In contrast, the immunoreactivity of astrocytic

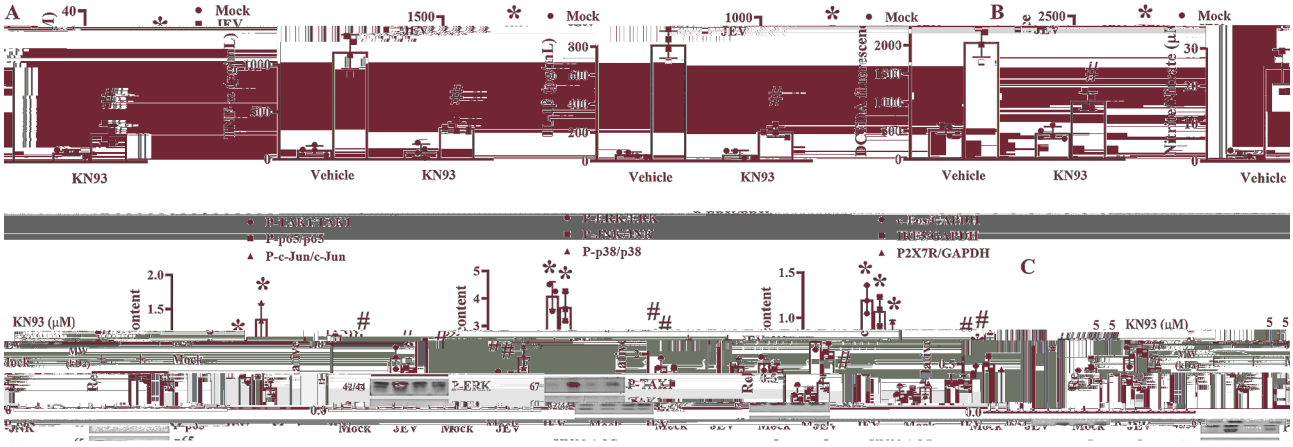

Fig. 6 E□ects on intracellular signaling cascades in microglia. Microglia cultures were infected with mock or JEV in the absence or presence of KN93 (5μM) over time. The supernatants (24 h) were collected and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β (A). The levels of intracellular free radicals (8 h) were measured using the □uorogenic probe DCFDA (arbitrary unit) (B). Total cellular proteins (8 h) were extracted and subjected to Western blot analysis with indicated antibodies. One representative blot of three independent culture batches and the quantitative results of relative protein contents (arbitrary unit) are shown (C). Nuclear proteins (8 h) were extracted and subjected to EMSA for the measurement of NF-κB and AP-1 DNA binding activity. One representative blot of three independent culture batches and the quantitative results (arbitrary unit) are shown (D). *$p < 0.05$ vs. Mock control and #$p < 0.05$ vs. JEV control, $n = 4$

GFAP (Fig. 7A and B) and GFAP protein content (Fig. 8A) were not markedly altered by either JEV infection, MK801, or memantine. JEV infection caused an elevation of apoptosis-related caspase 3 activity in neuron/glia cultures. MK801 and memantine alleviated the elevation of caspase 3 activity (Fig. 7C). Wild type JEV infection caused cell damage as evidenced by LDH e ux and the cytotoxicity was not seen in neuron/glia cultures infected with UV-inactivated and Heat-inactivated JEV (Fig. 8C).

e LDH e ux in JEV-infected neuron/glia cultures was improved by MK801 and memantine (Fig. 8D). Cultured neurons were modeled for further neurotoxic study (Fig. 9A). e conditioned media of JEV-infected microglia caused elevated LDH e ux in neuron cultures, while the conditioned media of MK801- and memantine-treated microglia upon JEV infection decreased ability in causing LDH e ux in neuron cultures (Fig. 9B). Additionally, the presence of MK801 and memantine in neuron cultures alleviated JEV-infected microglia conditioned media-induced LDH e ux (Fig. 9C). ese ndings show that JEV infection causes neuronal cell death and microglial activation, and that MK801 and memantine o er anti-in ammatory and neuroprotective e ects.

Memantine protected against JEV infection-induced changes in the brains of mice

To further verify the in vitro ndings, a C57BL/6 mouse model of JEV infection was produced for in vivo investigation [27]. It is reported that severe adverse e ects of MK801 preclude its clinical application, while memantine is well tolerated in clinical use [33]. erefore, memantine represents a preferable candidate of NMDA receptor antagonists for in vivo study. At day 7 post-infection, JEV-infected mice showed abnormal gait,

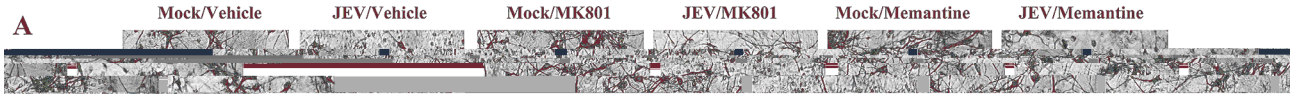

Fig. 7 MK801 and memantine alleviated neuronal cell death in Neuron/glia. Neuron/glia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 48 h. Cells were subjected to immunocytochemical staining with antibodies recognizing MAP-2, GFAP, and CD68. Representative photomicrographs are shown. Bar, 60 μm (A). The numbers of viable MAP-2 immunoreactive neurons, GFAP immunoreactive astrocytes, and CD68 immunoreactive microgliaare depicted (B). Total cellular proteins were isolated and subjected to the measurement of caspase 3 activity (arbitrary unit) (C). *$p < 0.05$ vs. mock control and #$p < 0.05$ vs. JEV control, $n = 4$

sti ness, and paralysis without death. We found that JEV infection caused neuronal cell degeneration and disrupted morphological integrity in the brains of mice, as evidenced by MAP-2 immuno uorescence in the hippocampal regions. Memantine alleviated JEV infection-induced neuronal cell degeneration (Fig. 10A). Upon JEV infection, Iba1-labelled microglia became reactive and the alteration was alleviated by memantine (Fig. 10A). However, GFAP immuno uorescence astrocytes were not remarkably altered by JEV infection or memantine (Fig. 10A). To substantiate histological ndings, quantitative measurement of corresponding MAP-2, CD68, and GFAP in protein content revealed the same changes as those in immuno uorescence study (Fig. 10B). In JEV-infected brains, contents of JEV genomic RNA (Fig. 10C) and JEV NS1 protein (Fig. 10D) in memantine group slightly decreased. However, the di erence failed to reach statistical signi cance. e ndings indicate that JEV infection induces neuronal cell degeneration and microglia activation in brains of mice and that memantine o ers protective e ects.

Memantine alleviated JEV infection-induced in□ammatory changes in the brains of mice

Data of in vitro microglia study revealed an active role of the NMDA receptor signaling in in ammatory responses caused by JEV infection. us, parameters of the identi ed in ammatory axis were determined in the brains of JEV-infected mice. Upon JEV infection, protein phosphorylation in GluN1, GluN2A, GluN2B, and CaMKII increased and the increments were alleviated by memantine (Fig. 11A). Double immuno uorescence assay in the brains of hippocampal regions highlighted that some signals of P-GluN2A uorescence were colocalized with signals of Iba1 uorescence (Fig. 11B), indicating an activation of the NMDA receptor signaling in microglia after JEV infection. e results were consistent with the in vitro microglia ndings, with brains from JEV-infected mice showing increased mRNA contents of iNOS, TNF-α, and IL-1β (Fig. 12A), DNA binding activity of NF-κB and AP-1 (Fig. 12B), protein contents of NOX2, NOX4, c-Fos, IRF5, and P2×7R, and protein phosphorylation of ERK, JNK, p38, PERK, eIF2α, IRE1, TAK1, p65, c-Jun, and Drp1 (Fig. 12C). Memantine alleviated those changes (Fig. 12C). e ndings further con rm the activation of a NMDA receptor-related in ammatory response and

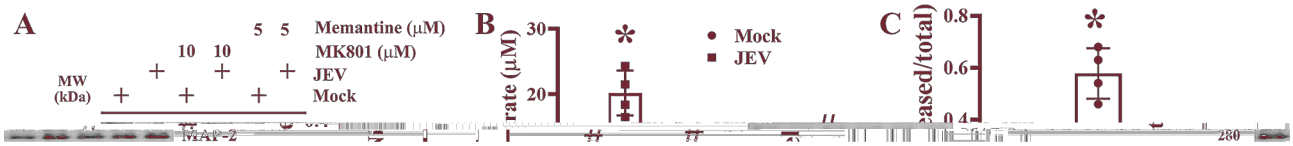

Fig. 8 MK801 and memantine alleviated cytokine expression in Neuron/glia. Neuron/glia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 48 h.Total cellular proteins were extracted and subjected to Western blot analysis with indicated antibodies. One representative blot of three independent culture batches and the relative protein contents (arbitrary unit) are shown (A).The supernatants were collected and subjected to ELISA for the measurement of NO, TNF-α, and IL-1β (B). (C) Neuron/glia cultures were infected with mock, JEV, JEV/UV-inactivated, or JEV/Heat-inactivatedfor 48 h. Cell damage was measured by LDH e□ux assay. (D) Neuron/glia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 48 h. Cell damage was measured by LDH e□ux assay. *$p < 0.05$ vs. mock control and #$p < 0.05$ vs. JEV control, $n = 4$

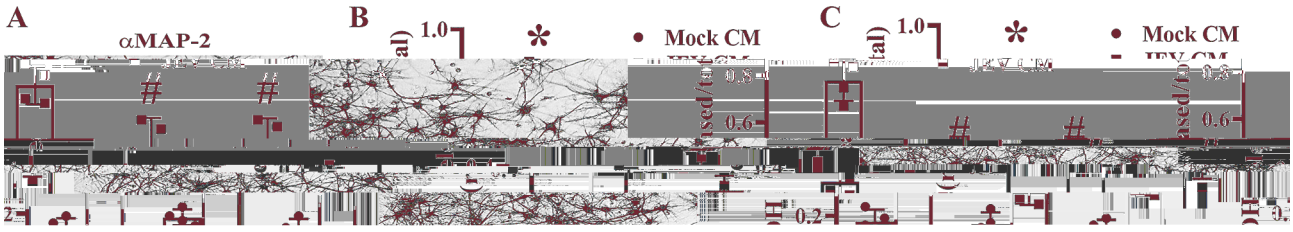

Fig. 9 MK801 and memantine alleviated neuronal cell death in neurons. (A) Cultured neurons were examined by immunocytochemical staining with antibody recognizing MAP-2. Representative photomicrograph is shown. Bar, 60 μm. (B) Microglia cultures were infected with mock or JEV in the absence or presence of MK801 (10 μM) or memantine (5 μM) for 48 h. The supernatants were collected and subjected to UV-inactivation. The resultant media (Mock CM and JEV CM) were mixed with an equal volume of fresh medium and then added to the cultured neurons for 24 h. Cell damage was measured by LDH e□ux assay. (C) Additionally, the manipulated media form mock- (Mock CM) and JEV-infected microglia (JEV CM) were added to cultured neurons in the presence of MK801 (10 μM) or memantine (5 μM) for 24 h. Cell damage was measured by LDH e□ux assay. *$p < 0.05$ vs. mock control and #$p < 0.05$ vs. JEV control, $n = 4$

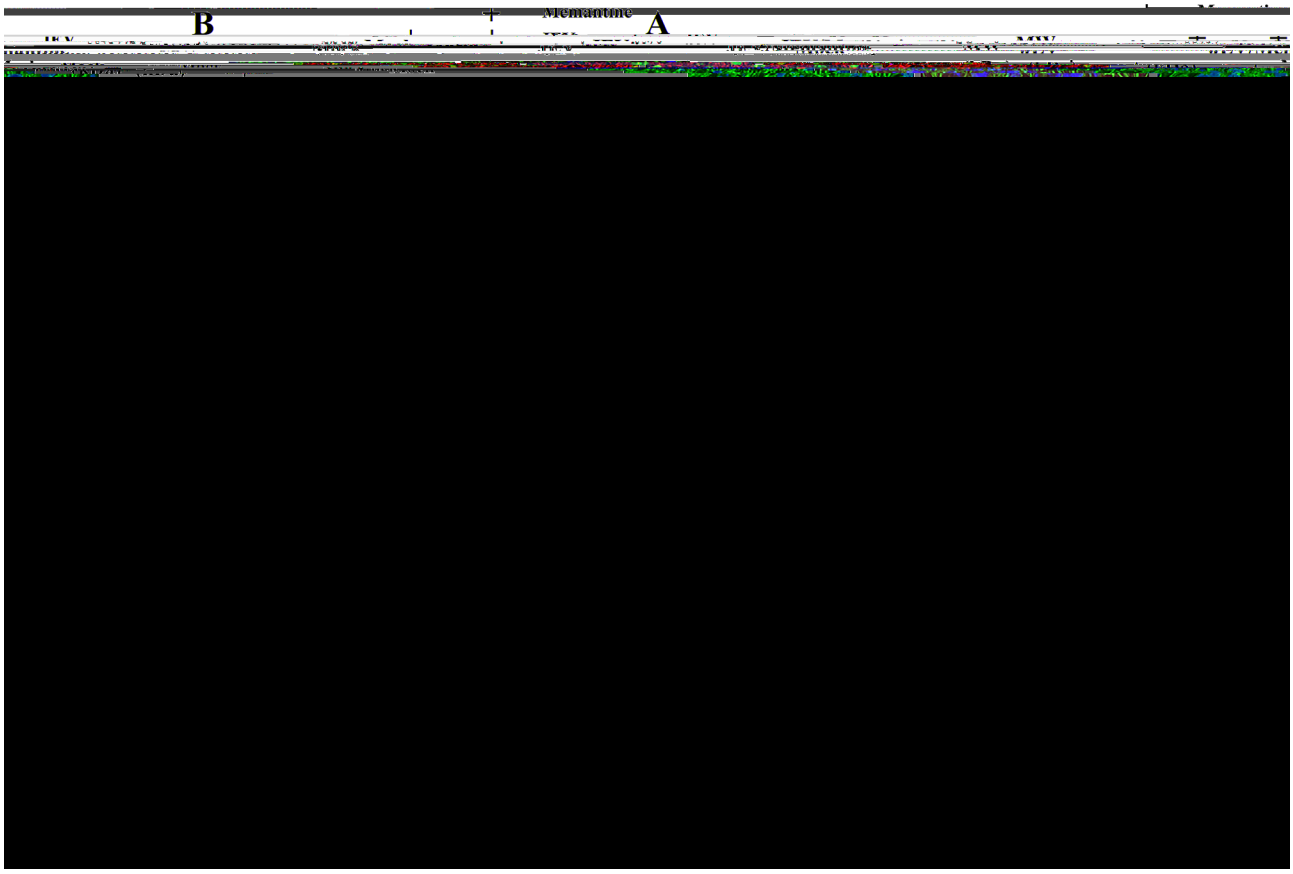

Fig. 10 Memantine alleviated cell alterations in the brains of JEV-infected mice. Eight-week-old male C57BL/6 mice were infected with mock or JEV through intraperitoneal injection. Mice were access to drinking water containing vehicle or memantine (20 mg/kg) starting from 1 day before infection and lasting for 8 days. On post-viral infection day 7, mice were euthanized and the brains were isolated. (A) The frozen sections of brain tissues ($n = 3$) were subjected to immunohistochemistry with uorescent labeling for the detection of MAP-2-immunoreactive (FITC), Iba1-immunoreactive (FITC), and GFAP-immunoreactive (FITC) cells. The cell nuclei were counterstained with DAPI. Representative photomicrographs of hippocampal regions are shown. Scale bar, 50 μm. (B) Total proteins were extracted from prefrontal cortices and hippocampus ($n = 3$) and subjected to Western blot analysis with indicated antibodies. One representative blot and the relative protein contents (arbitrary unit) are shown. (C) Total RNAs ($n = 3$) were isolated from prefrontalcortices and hippocampus and subjected to quantitative RT-PCR for the measurement of JEV RNA (arbitrary unit). (D) Total proteins were extracted from prefrontal cortices and hippocampus ($n = 3$) and subjected to Western blot analysis with indicated antibodies. One representative blot and the relative protein contents (arbitrary unit) are shown. *$p < 0.05$ vs. mockvehicle and #$p < 0.05$ vs. JEVvehicle, $n = 3$

an alleviative e ect of memantine in the brains of JEV-infected mice.

Discussion

Activation of the neuronal NMDA receptor and the neuroprotective e ects of NMDA receptor blockade have been demonstrated in rodent models of JE [4, 20, 21]. Using microglia cultures, this study demonstrated that JEV infection caused activation of the NMDA receptor, together with elevated signals of glutamate release, Ca^{2+} mobilization, CaMKII, MAPKs, NOXs, ER stress, Drp1, and NF-κB/AP-1. Data of molecular, biochemical, and pharmacological studies highlighted a crucial role of CaMKII in transducing the NMDA receptor signaling in JEV-infected microglia. CaMKII can communicate with regulators of NF-κB/AP-1 to induce microglia M1 polarization and pro-in ammatory cytokine expression involving phosphorylatory modi cation, ER stress, and mitochondrial dysfunction. Pro-in ammatory cytokines released from JEV-infected microglia and NMDA receptor activation provoke neurotoxic potential in cultured neurons and neuron/glia. NMDA receptor antagonists, MK801 and memantine, have anti-in ammatory and neuroprotective e ects in JEV-infected cells (Fig. 13). Activation of NMDA receptor-related in ammatory changes, microglia activation, and neurodegeneration as well as reversal e ects of memantine are revealed in the brains of JEV-infected mice, as well. Although activation of the NMDA receptor, CaMKII, and NF-κB/AP-1 has been implicated in the degeneration of neurons [34], currently, their changes in JEV-infected neurons were not investigated. e cell and rodent studies presented here further extend earlier ndings that showed JEV infection induces NMDA receptor activation in microglia as well,

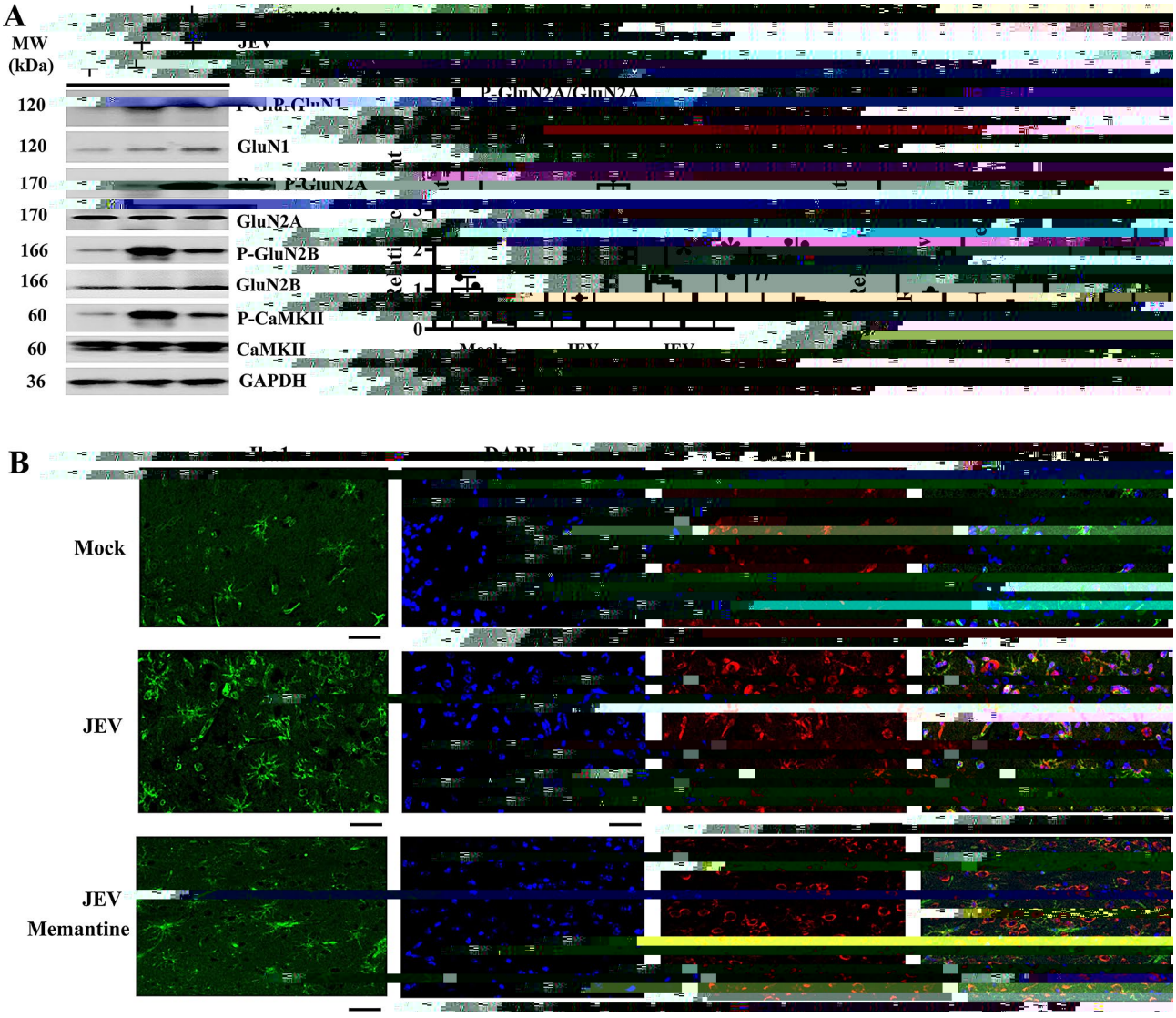

Fig. 11 Memantine alleviated NMDA receptor signaling in the brains of JEV-infected mice. Eight-week-old male C57BL/6 mice were infected with mock or JEV through intraperitoneal injection. Mice were access to drinking water containing vehicle or memantine (20 mg/kg) starting from 1 day before infection and lasting for 8 days. On post-viral infection day 7, mice were euthanized and the brains were isolated. (A) Total proteins were extracted from prefrontal cortices and hippocampus ($n = 3$) and subjected to Western blot analysis with indicated antibodies. One representative blot and the relative protein contents (arbitrary unit) are shown. (B) The frozen sections of brain tissues ($n = 3$) were subjected to immunohistochemistry with uorescent labeling for the detection of Iba1-immunoreactive (FITC) and P-GluN2A-immunoreactive (Rhodamine) cells. The cell nuclei were counterstained with DAPI. Representative photomicrographs of hippocampal regions and merged images are shown. Scale bar, 50 μm. *$p < 0.05$ vs. mockvehicle and #$p < 0.05$ vs. JEVvehicle, $n = 3$

while providing new insight into the anti-in ammatory and neuroprotective potential of NMDA receptor blockade in combating viral pathogenesis, such as that seen in JE.

Most cells express glutamate ionotropic receptors, metabotropic receptors, and transporters, including neurons, astrocytes, and microglia [18, 35, 36]. Microglia represent an emerging source of extracellular glutamate release through the transporter e ux system upon in ammatory stimulation [15, 35, 36]. Both glutamate release and neuronal NMDA receptor activation have been implicated in JE-associated neuronal cell death and neuroin ammation [4, 20, 21]. Previously, we demonstrated that JEV infection caused neuronal cell death and a protective e ect of MK801, implying a neurotoxic involvement of glutamate [15]. ere are both multiple and interrelated mechanisms which cause neuronal cell death and contribute to JEV pathogenesis. Besides well-known neurotoxicity of abnormal neuronal NMDA receptor activation, in the present study, we further demonstrated the activation of the NMDA receptor in microglia upon JEV infection, and provided additional

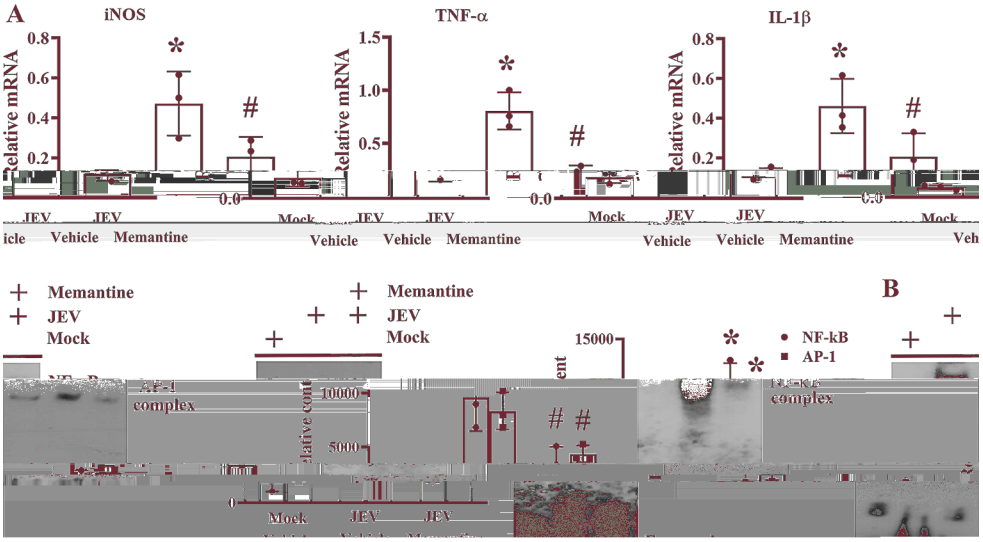

Fig. 12 Memantine alleviated signaling alterations in the brains of JEV-infected mice. Eight-week-old male C57BL/6 mice were infected with mock or JEV through intraperitoneal injection. Mice were access to drinking water containing vehicle or memantine (20 mg/kg) starting from 1 day before infection and lasting for 8 days. On post-viral infection day 7, mice were euthanized and the brains were isolated. (A) Total RNAs ($n = 3$) were isolated from prefrontalcortices and hippocampus and subjectedto quantitative RT-PCR for the measurement of iNOS, TNF-α, and IL-1β mRNA (arbitrary unit). (B) Nuclear proteins ($n = 3$) were extracted from prefrontalcortices and hippocampus and subjected to EMSA for the measurement of NF-κB and AP-1 DNA binding activity. One representative blot and the quantitative results (arbitrary unit) are shown. (C) Total proteins were extracted from prefrontal cortices and hippocampus ($n = 3$) and subjected to Western blot analysis with indicated antibodies. One representative blot and the relative protein contents (arbitrary unit) are shown. *$p < 0.05$ vs. mockvehicle and #$p < 0.05$ vs. JEVvehicle, $n = 3$

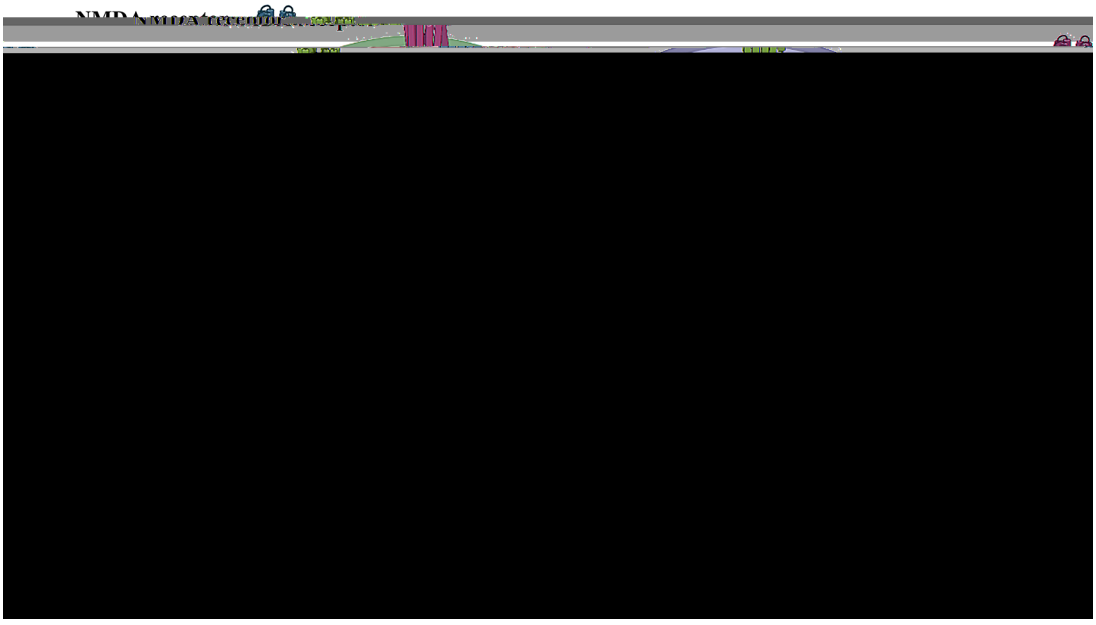

Fig. 13 A possible schema of actions of NMDA receptor elicited by JEV infection is proposed. This schematic diagram indicates the cellular molecules, signaling molecules, and cascades used in mediating microglia M1 polarization and pro-in□ammatory cytokine expression as well as neuronal cell death after JEV infection. JEV infection-activated microglia have ability to cause neuron toxicity. NMDA receptor coordinates cellular changes in neuron and microglia after JEV infection and NMDA receptor antagonists, MK801 and memantine, have alleviative e□ects. Some additional signaling molecules and cascades have been omitted for the sake of clarity

evidence implicating the role the dysregulated glutamate/NMDA receptor axis plays in JE-associated neuronal cell death and neuroin ammation.

e interplay between excitotoxicity and neuroin ammation is a key component to the development of neurodegeneration. Injection of NMDA in rat brains reveals an induction of excitotoxic neuronal damage and neuroin ammation [37]. Evidence indicates an emerging role where pro-in ammatory cytokines and TNF-α and IL-1β represent dominant candidates as linking molecules. TNF-α and IL-1β activate neuronal glutaminase expression and glutamate extracellular release, leading to glutamate production and glutamate-mediated neurotoxicity [16]. TNF-α potentiates glutamate-mediated neurotoxicity through the inhibition of glutamate uptake on astrocytes and the promotion of surface expression in the neuronal NMDA receptor [38]. A vicious crosstalk between glutamate and pro-in ammatory cytokines in JEV-infected microglia has been demonstrated in both the current study and our previously reported works. Upon JEV infection, microglia-released TNF-α increased both microglia glutaminase expression and glutamate production, resulting in glutamate extracellular release [15]. We found that activation of the NMDA receptor and accompanied Ca^{2+} mobilization led to both microglia activation and pro-in ammatory cytokine production by cultured microglia infected with JEV. e exogenous addition of glutamate augmented both JEV infection-induced microglia activation and pro-in ammatory cytokine production. Additionally, we further provided evidence to demonstrate the substantial roles excitotoxicity and neuroin ammation play in JEV pathogenesis. erefore, our ndings revealed the crucial role microglia plays in the ampli cation of driving molecules critical to excitotoxicity and neuroin ammation.

Unlike neurons, microglia are tolerated by glutamate excitotoxicity. On the contrary, microglia switch the glutamate/NMDA receptor signals to promote cell proliferation and initiate an in ammatory program. In addition to endotoxins and neurotoxic compounds, viral infections and viral proteins also orchestrate a pro-in ammatory state of microglia througha NMDA receptor mechanism involving the Ca^{2+} current, CaMKII, Mitogen-Activated Protein Kinases (MAPKs), TAK1 and NF-κB/AP-1 [24, 25, 39–41]. Upon JEV infection, elevation in the NMDA receptor component protein phosphorylation, labile free Ca^{2+}, CaMKII phosphorylation, TAK1 phosphorylation, ERK phosphorylation, JNK phosphorylation, p38 phosphorylation, NF-κB regulators and AP-1 regulators paralleled with microglia reactivity and pro-in ammatory cytokine expression, with those accompanied changes ameliorated by MK801 and memantine. erefore, JEV infection shifts microglia to a pro-in ammatory state, at least in part, through a typical NMDA receptor/CaMKII/TAK1/MAPKs/NF-κB, AP-1 axis.

Transcription factors, such as NF-κB and AP-1, determine transcriptional programs for orchestrating the pro-in ammatory state of microglia and governing

pro-in ammatory cytokine expression [11, 15, 25].

ere are additional intracellular signaling cascades lying downstream from the axis of NMDA receptor/Ca^{2+}/CaMKII, where ER stress, mitochondrial dysfunction, and NOXs are commonly investigated [29–32]. Microglial NOXs, particularly NOX2 and NOX4, have been implicated in the generation of free radicals, closely linking to ER stress, mitochondrial Drp1 activation, and redox-sensitive MAPKs, NF-κB, and AP-1 [31, 42–44]. An adequate ER stress will alert the cells to take on a defensive response for adaptation, while sustained or uncontrolled ER stress delivers signals to e ectors, which has an impact on diverse cell functions. Regarding microglial activation, ER stress-accompanied JNK and p38 have substantial roles in pro-in ammatory transcriptional programs and in ammasome assembly/activation [32, 45, 46]. NOX inhibitors have inhibitory e ects on Matrix Metalloprotease-9 (MMP-9) expression through JEV-infected astrocytes [47]. e induction and role of ER stress are characterized and demonstrated in JEV infection-induced neuronal cell apoptosis and autophagy [10, 48]. Using cultured rat microglia, increases in intracellular free radical generation, NOX2/NOX4 protein expression, and ER stress marker expression were revealed after JEV infection. Parallel elevations in JEV-infected microglia occurred in redox-sensitive MAPKs, NF-κB, and AP-1 signaling, along with pro-in ammatory cytokine expression. eir crosstalk and speci c contribution to JEV infection-acquired pro-in ammatory state of microglia and pro-in ammatory cytokine expression were further demonstrated using corresponding pharmacological inhibitors. Although a complicated and mutually interactive intracellular signaling cascade has been drawn in this study, the exact step-by-step crosstalk was not fully investigated in detail due to a lack of temporal study.

JE-associated neuronal cell death is a complicated mechanism involving diverse alterations, including apoptosis, autophagy, necroptosis, ER stress, oxidative stress, Receptor Interacting Serine/ reonine-Protein Kinase 3 (RIPK3), Mixed-Lineage Kinase Domain-Like Protein (MLKL), NMDA and Caveolin-1 [5, 8–10, 21, 47, 48]. e protective potential of NMDA receptor antagonists has been demonstrated in rodent models of JE [4, 20, 21]. Tethering with our previous report [15], the neuroprotective e ects of NMDA receptor blockade were further demonstrated in cultured neurons and neuron/glia by challenging with either JEV infection or conditioned media of JEV-infected microglia. ere are studies showing microglial activation upon JEV infection through numerous mechanisms, including TLRs, MLKL, impaired autophagy ux, CLEC5A and Heat Shock Protein (HSP) [8, 9, 11, 50, 51]. Herein, we have added the NMDA receptor axis to the list of mechanisms contributing to JEV infection-induced microglial activation. Like other stressed conditions [24, 25, 39–41], the activation of NMDA receptor signaling through component protein phosphorylatory mechanisms and their associated Ca^{2+} current and CaMKII was demonstrated in JEV-infected microglia with concurrent glutamate extracellular release. Along with glutamate and endogenous mimicking ligands, Src, Protein Kinase A (PKA), PKC, ERK and TLR4 protein interaction all play positive roles in the NMDA receptor subunit components phosphorylation and activation [26, 52]. e activation of TLR4, Src, ERK, PKA and PKC has been reported in JEV-infected cells, particularly in microglia [2, 11, 15, 27, 53]. erefore, JEV infection-activated microglial NMDA receptor signaling could be mediated through multifactorial mechanisms via ligand engagement and/or ligand-independent subunit component phosphorylation. Although phosphorylation and activation of the NMDA receptor had been revealed in JEV-infected microglia, neuron/glia, and mice brains, the detailed mechanisms underlying the NMDA receptor activation in JEV-infected cells are yet to be identi ed.

e NMDA receptor-controlled in ammatory responses, neuronal cell death, and lethality had been demonstrated in mouse model of JEV infection. NMDA receptor blockade abrogated JEV infection-induced neuronal cell death and glial activation in mice brain tissues and delayed the death of mice, while having little e ect on viral ampli cation [4, 21]. Similar post-infection nd-ings were seen in our JEV-infected mice treated with memantine. e results of molecular and biochemical studies in brain tissues were consistent with the in vitro nd-ings, con rming the activation of a NMDA receptor-related in ammatory response and an alleviative e ect of memantine. Importantly, data of double immuno uorescence staining centered on Iba1 and P-GluN2A suggested an activation of the NMDA receptor component in microglia in responding to JEV infection. e expression of NMDA receptor components in lectin positive amoeboid microglia was also revealed in rat brain tissues following hypoxic exposure [18]. Direct inhibition of viral ampli cation or targeting virus-induced signaling changes represents druggable options for the treatment of viral diseases. Current in vitro and in vivo nd-ings highlighted an active role of the NMDA receptor signaling in JEV infection-induced microglia activation and in ammation and revealed that NMDA receptor blockade provided protection against neuroin ammation and neurodegeneration during the JEV infection. Since the pathogenic role of the neuronal NMDA receptor in JE was reported [20] and the colocalization of P-GluN2A immunoreactivity with additional cells were not examined in the current study, the detailed action mechanisms

and cell types involved in the protective e ects of the NMDA receptor blockade required further investigation.

Although we provided interesting ndings, current study was still being su ered from some limitations. First, activation and functional execution of the NMDA receptor have been demonstrated in cultured microglia and microglia cell lines [25, 54,

Author contributions
C.-Y.C. and C.-J.C. conceived and designed the experiments; C.-Y.C., C.-C.W., C.-Y.T., J.-R.L., Y.-F.C., W.-Y.C., Y.-H.K., and S.-L.L. performed the experiments and analyzed the data; C.-Y.C. wrote the paper; C.-J.C. edited the paper.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent participate
The experimental protocols surrounding animal studies were reviewed and approved by The Animal Experimental Committee of Taichung Veterans General Hospital (IACUC approval code: La-1061509).

Consent for publication
Not applicable.

Con□ict of interest
The authors declare no con□icts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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References

1. Misra UK, Kalita J. Overview: Japanese encephalitis. Prog Neurobiol. 2010;91:108–20.
2. Ashraf U, Ding Z, Deng S, Ye J, Cao S, Chen Z. Pathogenicity and virulence of Japanese encephalitis virus: Neuroin□ammation and neuronal cell damage. Virulence. 2021;12:968–80.
3. Chauhan PS, Khanna VK, Kalita J, Misra UK. Japanese encephalitis virus infection results in transient dysfunction of memory learning and cholinesterase inhibition. Mol Neurobiol. 2017;54:4705–15.
4. Sun L, Zhou M, Liu C, Tang Y, Xiao K, Dai J, Gao Z, Siew L, Cao G, Wu X, Li L, Zhang R. Memantine can relieve the neuronal impairment caused by neurotropic virus infection. J Med Virol. 2019;91:935–40.
5. Bian P, Ye C, Zheng X, Luo C, Yang J, Li M, Wang Y, Yang J, Zhou Y, Zhang F, Lian J, Zhang Y, Jia Z, Lei Y. RIPK3 promotes JEV replication in neurons via downregulation of IFI44L. Front Microbiol. 2020;11:368.
6. Kumar S, Maurya VK, Kabir R, Nayak D, Khurana A, Manchanda RK, Gadugu S, Shanker K, Saxena SK. Antiviral activity of belladonna during Japanese encephalitis virus infection via inhibition of microglia activation and in□ammation leading to neuronal cell survival. ACS Chem Neurosci. 2020;11:3683–96.
7. Mukherjee S, Akbar I, Kumari B, Vrati S, Basu A, Banerjee A. Japanese Encephalitis Virus-induced let-7a/b interacted with the NOTCH-TLR7 pathway in microglia and facilitated neuronal death via caspase activation. J Neurochem. 2019;149:518–34.
8. Bian P, Zheng X, Wei L, Ye C, Fan H, Cai Y, Zhang Y, Zhang F, Jia Z, Lei Y. MLKL mediated necroptosis accelerates JEV-induced neuroin□ammation in mice. Front Microbiol. 2017;8:303.
9. Kumar A, Kalita J, Sinha RA, Singh GBA, Shukla M, Tiwari S, Dhole TN, Misra UK. Impaired autophagy □ux is associated with proin□ammatory microglia activation following Japanese encephalitis virus infection. Neurochem Res. 2020;45:2184–95.
10. Wang Q, Xin X, Wang T, Wan J, Ou Y, Yang Z, Yu Q, Zhu L, Guo Y, Wu Y, Ding Z, Zhang Y, Pan Z, Tang Y, Li S, Kong L. Japanese encephalitis virus induces apoptosis and encephalitis by activating the PERK pathway. J Virol. 2019;93:e00887–19.
11. Chang CY, Wu CC, Wang JD, Li JR, Wang YY, Lin SY, Chen WY, Liao SL, Chen CJ. DHA attenuated Japanese encephalitis virus infection-induced neuroin□ammation and neuronal cell death in cultured rat neuron/glia. Brain Behav Immun. 2021;93:194–205.
12. Simões AP, Silva CG, Marques JM, Pochmann D, Porciúncula LO, Ferreira S, Oses JP, Beleza RO, Real JI, Köfalvi A, Bahr BA, Lerma J, Cunha RA, Rodrigues RJ. Glutamate-induced and NMDA receptor-mediated neurodegeneration entails P2Y1 receptor activation. Cell Death Dis. 2018;9:297.
13. Nargi-Aizenman JL, Havert MB, Zhang M, Irani DN, Rothstein JD, Gri□n DE. Glutamate receptor antagonists protect from virus-induced neural degeneration. Ann Neurol. 2004;55:541–9.
14. Tian C, Sun L, Jia B, Ma K, Curthoys N, Ding J, Zheng J. Mitochondrial glutaminase release contributes to glutamate-mediated neurotoxicity during human immunode□ciency virus-1 infection. J Neuroimmune Pharmacol. 2012;7:619–28.
15. Chen CJ, Ou YC, Chang CY, Pan HC, Liao SL, Chen SY, Raung SL, Lai CY. Glutamate released by Japanese encephalitis virus-infected microglia involves TNF-alpha signaling and contributes to neuronal death. Glia. 2012;60:487–501.
16. Ye L, Huang Y, Zhao L, Li Y, Sun L, Zhou Y, Qian G, Zheng JC. IL-1beta and TNF-alpha induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. J Neurochem. 2013;125:897–908.
17. Liu F, Zhou R, Yan H, Yin H, Wu X, Tan Y, Li L. Metabotropic glutamate receptor 5 modulates calcium oscillation and innate immune response induced by lipopolysaccharide in microglial cell. Neuroscience. 2014;281:24–34.
18. Murugan M, Sivakumar V, Lu J, Ling EA, Kaur C. Expression of N-methyl D-aspartate receptor subunits in amoeboid microglia mediates production of nitric oxide via NF-kappaB signaling pathway and oligodendrocyte cell death in hypoxic postnatal rats. Glia. 2011;59:521–39.
19. Zhang YN, Fan JK, Gu L, Yang HM, Zhan SQ, Zhang H. Metabotropic glutamate receptor 5 inhibits alpha-synuclein-induced microglia in□ammation to protect from neurotoxicity in Parkinson's disease. J Neuroin□ammation. 2021;18:23.
20. Chauhan PS, Misra UK, Kalita J. A study of glutamate levels, NR1, NR2A, NR2B receptors and oxidative stress in rat model of Japanese encephalitis. Physiol Behav. 2017;171:256–67.
21. Chen Z, Wang X, Ashraf U, Zheng B, Ye J, Zhou D, Zhang H, Song Y, Chen H, Zhao S, Cao S. Activation of neuronal N-methyl-D-aspartate receptor plays a pivotal role in Japanese encephalitis virus-induced neuronal cell damage. J Neuroin□ammation. 2018;15:238.
22. Wendt S, Wogram E, Korvers L, Kettenmann H. Experimental cortical spreading depression induces NMDA receptor dependent potassium currents in microglia. J Neurosci. 2016;36:6165–74.
23. Chen Y, Shi Y, Wang G, Li Y, Cheng L, Wang Y. Memantine selectively prevented the induction of dynamic allodynia by blocking Kir2.1 channel and inhibiting the activation of microglia in spinal dorsal horn of mice in spared nerve injury model. Mol Pain. 2019;15:1744806919838947.

24. Raghunatha P, Vosoughi A, Kauppinen TM, Jackson MF. Microglial NMDA receptors drive pro-in□ammatory responses via PARP-1/TRMP2 signaling. Glia. 2020;68:1421–34.
25. Wu Q, Zhao Y, Chen X, Zhu M, Miao C. Propofol attenuates BV2 microglia in□ammation via NMDA receptor inhibition. Can J Physiol Pharmacol. 2018;96:241–8.
26. Cui J, Yu S, Li Y, Li P, Liu F. Direct binding of toll-like receptor 4 to ionotropic glutamate receptor N-methyl-D-aspartate subunit 1 induced by lipopolysaccharide in microglial cells N9 and EOC 20. Int J Mol Med. 2018;41:1323–30.
27. Chang CY, Li JR, Ou YC, Lin SY, Wang YY, Chen WY, Hu YH, Lai CY, Chang CJ, Chen CJ. Interplay of in□ammatory gene expression in pericytes following Japanese encephalitis virus infection. Brain Behav Immun. 2017;66:230–43.
28. Lu Y, Ding X, Wu X, Huang S. Ketamine inhibits LPS-mediated BV2 microglial in□ammation via NMDA receptor blockage. Fundam Clin Pharmacol. 2020;34:229–37.
29. Bhat SA, Sood A, Shukla R, Hanif K. AT2R activation prevents microglia pro-in□ammatory activation in a NOX-dependent manner: inhibition of PKC activation and p47(phox) phosphorylation by PP2A. Mol Neurobiol. 2019;56:3005–23.
30. Costa RO, Lacor PN, Ferreira IL, Resende R, Auberson YP, Klein WL, Oliveira CR, Rego AC, Pereira CM. Endoplasmic reticulum stress occurs downstream of GluN2B subunit of N-methyl-d-aspartate receptor in mature hippocampal cultures treated with amyloid-beta oligomers. Aging Cell. 2012;11:823–33.
31. Wang J, Ma MW, Dhandapani KM, Brann DW. Regulatory role of NADPH oxidase 2 in the polarization dynamics and neurotoxicity of microglia/macrophages after traumatic brain injury. Free Radic Biol Med. 2017;113:119–31.
32. Molagoda IMN, Lee KT, Choi YH, Jayasingha JACC, Kim GY. Anthocyanins from Hibiscus syriacus L. inhibit NLRP3 in□ammasome in BV2 microglia cells by alleviating NF-kappaB- and ER stress-induced ca(2+) accumulation and mitochondrial ROS production. Oxid Med Cell Longev. 2021;2021:1246491.
33. Song X, Jensen MØ, Jogini V, Stein RA, Lee CH, Mchaourab HS, Shaw DE, Gouaux E. Mechanism of NMDA receptor channel block by MK-801 and memantine. Nature. 2018;556:515–9.
34. Schwamborn R, Düssmann H, König HG, Prehn JHM. Time-lapse imaging of p65 and IkappaB alpha translocation kinetics following ca(2+)-induced neuronal injury reveals biphasic translocation kinetics in surviving neurons. Mol Cell Neurosci. 2017;80:148–58.
35. Mahmound S, Gharagozloo M, Simard C, Gris D. Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. Cells. 2019;8:184.
36. Danbolt NC, Furness DN, Zhou Y. Neuronal vs glial glutamate uptake: resolving the conundrum. Neurochem Int. 2016;98:29–45.
37. Chang YC, Kim HW, Rapoport SI, Rao JS. Chronic NMDA administration increases neuroin□ammatory markers in rat frontal cortex: cross-talk between excitotoxicity and neuroin□ammation. Neurochem Res. 2008;33:2318–23.
38. Olmos G, Lladó J. Tumor necrosis factor alpha: a link between neuroin□ammation and excitotoxicity. Mediators In□amm. 2014;2014:861231.
39. Li L, Wu Y, Bai Z, Hu Y, Li W. Blockade of NMDA receptors decreased spinal microglia activation in bee venom induced acute in□ammatory pain in rats. Neurol Res. 2017;39:271–80.
40. Nair A, Bonneau RH. Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. J Neuroimmunol. 2006;171:72–85.
41. Zhang X, Green MV, Thayer SA. HIV gp120-induced neuroin□ammation potentiates NMDA receptors to overcome basal suppression of inhibitory synapses by p38 MAPK. J Neurochem. 2019;148:499–515.
42. Liao K, Guo M, Niu F, Yang L, Callen SE, Buch S. Cocaine-mediated induction of microglial activation involves the ER stress-TLR2 axis. J Neuroin□ammation. 2016;13:33.
43. Park J, Choi H, Kim B, Chae U, Lee DG, Lee SR, Lee S, Lee HS, Lee DS. Peroxiredoxin 5 (Prx5) decreases LPS-induced microglial activation through regulation of ca. Free Radic Biol Med. 2016;99:392–404.
44. Park J, Min JS, Kim B, Chae UB, Yun JW, Choi MS, Kong IK, Chang KT, Lee DS. Mitochondrial ROS govern the LPS-induced pro-in□ammatory response in microglia cells by regulating MAPK and NF-kappaB pathways. Neurosci Lett. 2015;584:191–6.
45. Huang X, Chen Y, Zhang H, Ma Q, Zhang YW, Xu H. Salubrinal attenuates beta-amyloid-induced neuronal death and microglial activation by inhibition of the NF-kappaB pathway. Neurobiol Aging. 2012;33:e10079–17.
46. Ismael S, Wajidunnisa, Sakata K, McDonald MP, Liao FF, Ishrat T. ER stress associated TXNIP-NLRP3 in□ammasome activation in hippocampus of human Alzheimer's disease. Neurochem Int. 2021;148:105104.
47. Tung WH, Tsai HW, Lee IT, Hsieh HL, Chen WJ, Chen YL, Yang CM. Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF-kappaB signalling dependent on MAPKs and reactive oxygen species. Bri J Pharmacol. 2010;161:1566–83.
48. Sharma M, Bhattacharyya S, Sharma KB, Chauhan S, Asthana S, Abdin MZ, Vrati S, Kalia M. Japanese encephalitis virus activates autophagy through XBP1 and ATF6 ER stress sensors in neuronal cells. J Gen Virol. 2017;98:1027–39.
49. Xu Q, Cao M, Song H, Chen S, Qian X, Zhao P, Ren H, Tang H, Wang Y, Wei Y, Zhu Y, Qi Z. Caveolin-1-mediated Japanese encephalitis virus entry requires a two-step regulation of actin reorganization. Future Microbiol. 2016;11:1227–48.
50. Chen ST, Liu RS, Wu MF, Lin YL, Chen SY, Tan DT, Chou TY, Tsai IS, Li L, Hsieh SL. CLEC5A regulates Japanese encephalitis virus-induced neuroin□ammation and lethality. PLOS Pathog. 2012;8:e1002655.
51. Swaroop S, Mahadevan A, ShankarSK, Adlakha YK, Basu A. HSP60 critically regulates endogenous IL-1β production in activated microglia by stimulating NLRP3 in□ammasome pathway. J Neuroin□ammation. 2018;15:177.
52. Wang JQ, Guo ML, Jin DZ, Xue B, Fibuch EE, Mao LM. Roles of subunit phosphorylation in regulating glutamate receptor function. Eur J Pharmacol. 2014;728:183–7.
53. Chen CJ, Ou YC, Chang CY, Pan HC, Lin SY, Liao SL, Raung SL, Chen SY, Chang CJ. Src signaling involvement in Japanese encephalitis virus-induced cytokine production in microglia. Neurochem Int. 2011;58:924–33.
54. Jekabsone A, Jankeviciute S, Pampuscenko K, Borutaite V, Morkuniene R. The role of intracellular ca. Mitochondrial ROS Small Aβ1-42 oligomer-induced Microglial Death Int J Molr Sci. 2023;24:12315.
55. Wu CC, Tzeng CY, Chang CY, Wang JD, Chen YF, Chen WY, Kuan YH, Liao SL, Wang WY, Chen CJ. NMDA receptor inhibitor MK801 alleviated pro-in□ammatory polarization of BV-2 microglia cells. Eur J Pharmacol. 2023;955:175927.
56. Chisari M, Barraco M, Bucolo C, Ciranna L, Sortino MA. Purinergic ionotropic P2X7 and metabotropic glutamate mGlu5 receptors crosstalk in□uences pro-in□ammatory conditions in microglia. Eur J Pharmacol. 2023;938:175389.
57. Tai SH, Lee WT, Lee AC, Lin YW, Hung HY, Huangm SY, Wu TS, Lee EJ. Therapeutic window for YC-1 following glutamate-induced neuronal damage and transient focal cerebral ischemia. Mol Med Rep. 2018;17:6490–6.

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