IRAK-M ablation promotes status epilepticus-induced neuroinflammation via activating M1 microglia and impairing excitatory synaptic function

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Abstract

**Background:** Epilepsy is one of the most common neurological disorders. The proepileptic and antiepileptic roles of microglia have recently garnered significant attention. Interleukin-1 receptor-associated kinase (IRAK)-M, an important kinases in the innate immune response, is mainly expressed in microglia and acts as a negative regulator of the TLR4 signaling pathway that mediates the anti-inflammatory effect. However, whether IRAK-M exerts a protective role in epileptogenesis as well as the molecular and cellular mechanisms underlying these processes are yet to be elucidated.

**Methods:** An epilepsy mouse model induced by pilocarpine was used in this study. Real-time quantitative polymerase chain reaction and western blot analysis were used to analyze mRNA and protein expression levels, respectively. Whole-cell voltage-clamp recordings were employed to evaluate the glutamatergic synaptic transmission in hippocampal neurons. Immunofluorescence was utilized to show the glial cell activation and neuronal loss. Furthermore, the proportion of microglia was analyzed using flow cytometry.

**Results:** Seizure dynamics influenced the expression of IRAK-M. Its knockout dramatically exacerbated the seizures and the pathology in epilepsy and increased the N-methyl-d-aspartate receptor (NMDAR) expression, thereby enhancing glutamatergic synaptic transmission in hippocampal CA1 pyramidal neurons in mice. Furthermore, IRAK-M deficiency augmented hippocampal neuronal loss via a possible mechanism of NMDAR-mediated excitotoxicity. IRAK-M deletion promotes microglia toward the M1 phenotype, which resulted in high levels of proinflammatory cytokines and was accompanied by a visible increase in the expressions of key microglial polarization-related proteins, including p-STAT1, TRAF6, and SOCS1.

**Conclusions:** The findings demonstrate that IRAK-M dysfunction contributes to the progression of epilepsy by increasing M1 microglial polarization and glutamatergic synaptic transmission. This is possibly related to NMDARs, particularly Grin2A and Grin2B, which suggests that IRAK-M could serve as a novel therapeutic target for epilepsy.

Introduction

Epilepsy, defined as recurrent spontaneous seizures caused by the hyperexcitability and hypersynchrony of brain neurons, affects nearly 70 million people worldwide. The majority of antiepileptic medications target neuronal mechanisms involved in the inhibition of excitatory ion channels or the upregulation of inhibitory synaptic neurotransmission[3]. Despite the use of multiple medications, nearly one-third of patients with epilepsy are resistant to currently available drugs[4]. Recent studies indicate that non-neuronal cells, particularly microglia, play a key role in the pathophysiology of epilepsy[5], which makes it imperative to understand microglial pathogenesis in epilepsy.
Microglia, a type of glial cells, are resident immune cells of the central nervous system (CNS). Activated microglia are classified into two functional subtypes: M1 (classical) and M2 (substitutive) phenotypes\(^\text{[6]}\). M1 microglia release proinflammatory cytokines, such as interleukin-1 beta (IL-1\(\beta\)), IL-6, IL-12, tumor necrosis factor-alpha (TNF-\(\alpha\)), and interferon-gamma (IFN-\(\gamma\)), as well as neurotoxic chemicals, such as inducible nitric oxide synthase (iNOS), all of which induce neuroinflammation\(^\text{[7, 8]}\). In contrast, M2 phenotypes can release anti-inflammatory cytokines, such as IL-10, arginase-1 (Arg-1), and chitinase 3-like 3 (Ym-1)\(^\text{[9]}\), which respond in a manner that ameliorates inflammatory processes and potentially limits epileptogenesis\(^\text{[10]}\). Consequently, a therapeutic approach targeting activated microglia and inflammatory factors may be a promising antiepileptogenic strategy.

It is well-known that toll-like receptors (TLRs) are important mediators of innate immunity that play a critical role in microglial activation and act as signal transduction molecules in inflammatory responses. Of all TLRs, TLR4 is one of the most well-known and well-studied receptors and has been implicated in neuroinflammation that underlies the etiopathogenesis of epilepsy and convulsive disorders\(^\text{[11, 13]}\). TLR4 has been investigated as a biomarker for epilepsy. The activation of its signaling further promotes the M1 microglial polarization and releases proinflammatory cytokines, which may aggravate epilepsy. Interleukin-1 receptor-associated kinase (IRAK)-M, primarily expressed in microglia of the CNS, is a member of the IRAK family and functions as a negative regulator of TLR4 signaling\(^\text{[17]}\). IRAK-M prevents the dissociation of IRAK1–IRAK4 and interrupts the formation of the IRAK1–TRAF6 complex. These events subsequently disrupt TLR signaling and reduce the release of downstream proinflammatory cytokines, such as IL-1, IL-12, and TNF-\(\alpha\). Furthermore, IRAK-M plays a protective role in neurological diseases, such as ischemic neurovascular injuries and autoimmune encephalomyelitis\(^\text{[20]}\). However, whether IRAK-M exerts a protective role in epileptogenesis and the molecular and cellular mechanisms underlying these processes have not been adequately investigated.

In this study, the dynamic changes in IRAK-M in different spontaneous recurrent seizure periods were investigated, which suggested the positive association between IRAK-M and epilepsy. Moreover, transgenic knockout mice lacking IRAK-M developed more severe epilepsy after lithium–pilocarpine-induced status epilepticus (SE) than their wild-type (WT) littermates. In addition, whole-cell patch clamp recordings and real-time quantitative polymerase chain reaction (RT-qPCR) were performed to investigate the synaptic mechanism. The findings demonstrated that IRAK-M deficiency enhanced the presynaptic properties of glutamatergic synaptic transmission and postsynaptic strength via a mechanism involving the increased expressions of NMDAR2A and NMDAR2B in hippocampal CA1 pyramidal neurons. Furthermore, IRAK-M deletion promoted the polarization of M1 phenotypic microglia and the release of proinflammatory cytokines via intracellular signaling molecules (p-STAT1, TRAF6, and SOCS1), which ultimately led to the excessive production of proinflammatory cytokines. These results suggest that IRAK-M plays a pertinent role in epileptogenesis and exerts a promising therapeutic effect.

**Materials And Methods**
Animals

Male-specific pathogen-free (SPF) C57BL/6 mice (weight: 20–25 g, age: 6–8 weeks) were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). The IRAK-M\(^{-/-}\) mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Heterozygous (IRAK-M\(^{+/−}\)) mating pairs were maintained to produce wild-type (WT) and homozygous (IRAK-M\(^{-/-}\)) littermates for use in the study. Genotyping results for the IRAK-M\(^{-/-}\) mice were confirmed by 1% agarose protein gels (Supplementary Fig.1). The experimental study design is outlined in Figure 1.

Lithium-pilocarpine-induced SE model

SE was characterized by continuous limbic seizures, which were induced as reported elsewhere\[^{[21]}\]. Briefly, the mice were injected intraperitoneally (i.p.) with lithium chloride (127 mg/kg, Sigma–Aldrich, St. Louis, MO, USA). After 20 h, the mice received subcutaneous injections of pilocarpine hydrochloride (30 mg/kg, i.p., Sigma–Aldrich) to induce SE. To reduce the peripheral side-effects of pilocarpine, methyl scopolamine nitrate (1 mg/kg, i.p., Tokyo Chemical Industry, Tokyo, Japan) was administered 30 min before pilocarpine administration. Racine's scoring was performed to assess the severity of behavioral seizures, which ranged from 0 to 5 stages\[^{[22]}\]. Seizure latency was considered the time from pilocarpine injection to the first stage 3 seizure. SE was defined as a seizure of >4 stages that lasted for at least 30 min. Finally, the mice were intraperitoneally administered with diazepam (15 mg/kg, i.p., King York, Tianjin, China) after 2 h to stop the seizure, and the mice were kept warm. The mice recovering from SE received intraperitoneal injections of sodium chloride with glucose (5%, i.p., Kangjier, Zhejiang, China) once a day for energy supplementation. The mice recovering from SE were given a bolus of saline (5%, Kangjier) subcutaneously in the scruff to increase the survival chances. The mice in the control group were treated similarly, albeit they received an equal volume of saline instead of lithium-pilocarpine.

Monitoring for seizures

After injection with pilocarpine to induce SE, the seizures of the mice were recorded continuously for 2 h by using a camera equipped with infrared night vision (Xiaomi Technology, Shenzhen, China). For 28 days after SE, the behavior of the mice (n = 12) was videotaped for 24 h every day. The number of seizure episodes during 28 days and the behavioral score according to the Veliskova scoring system per seizure episode was recorded for every mouse. Finally, all video recordings were tallied by individuals who were independently blinded to this study.

Slice Preparation

The brain slices were prepared according to the procedures described previously\[^{[24, 25]}\]. Briefly, the experimental mice were deeply anesthetized with pentobarbital sodium (75 mg/kg, i.p.) and intracardially perfused with ice-cold oxygenated high-sucrose artificial cerebrospinal fluid (slice ACSF, in mM: 220 sucrose, 26 NaHCO\(_3\), 10 D-glucose, 2 KCl, 12 MgSO\(_4\), 1.3 NaH\(_2\)PO\(_4\), and 0.2 CaCl\(_2\)). The mice were sacrificed via decapitation, and their brains were rapidly removed and chilled in ice-cold oxygenated slice
ACSF. Multiple coronal slices (300-µm thick) containing the hippocampal CA1 were prepared using a
vibratome (Leica, VT1200S). The slices were transferred to a holding chamber containing continuously
oxygenated ACSF (recording ACSF, in mM: 124 NaCl, 3 KCl, 26 NaHCO₃, 10 D-glucose, 1 MgSO₄, 1.25
NaH₂PO₄, and 2 CaCl₂, at pH 7.4, 305 mOsm) at 34°C for 30 min and then kept at room temperature until
required. After an hour of the recovery period, the slices were transferred to the recording chamber that
was continuously perfused with oxygenated ACSF at the rate of 2–3 mL/min. Whole-cell patch-clamp
recordings were performed by using an upright microscope (ECLIPSE FN1, Nikon, Japan) equipped with a
40× water-immersion lens and infrared-sensitive camera (DAGE-MTI, IR-1000E). The patch pipettes were
fabricated from filamented borosilicate glass capillary tubes (inner diameter, 0.84 µm) with a horizontal
puller (Sutter Instruments, P-97). The recordings were obtained using a multiclamp 700B amplifier and
pClamp software (Molecular Devices). The data were low-pass filtered at 2 kHz and sampled at 10 kHz
with the Digidata 1550A device (Molecular Devices).

Electrophysiological Recordings

For spontaneous and miniature excitatory postsynaptic current (sEPSC and mEPSC, respectively)
recording, the neurons were held at a potential of −70 mV in the presence of 20 µM bicuculline (BMI, the
GABAA receptor antagonist) without or with 1 µM tetrodotoxin (TTX). The pipette resistance was typically
4–6 MΩ after it was filled with an internal solution containing (in mM) 130 K-gluconate, 20 KCl, 10
HEPES, 0.2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, and 10 NaCreatine (pH 7.3, 285 mOsm). To determine the
proportion of AMPA and NMDA components, the EPSCs were evoked at −70 mV (AMPA) and +40 mV
(NMDA) in the presence of 20 µM BMI. The internal solution contained (in mM): 110 Cs₂SO₄, 0.5 CaCl₂, 2
MgCl₂, 5 EGTA, 5 HEPES, 5 TEA, and 5 Mg-ATP (pH 7.3, 285 mOsm). The AMPA component was
measured as the EPSC peak amplitude, while the NMDA component was determined by measuring the
current amplitude at 50 ms after the EPSC onset (an average of 10 sweeps).

For each cell, the recordings were commenced after stabilization of the holding potential approximately
2–5 min after the break-in. Only the cells with a series resistance <30 MΩ and leak currents <100 pA were
included. The synaptic currents were recorded in the voltage-clamp mode and analyzed with the
Minianalysis (Synaptosoft Inc.) and Clampfit 10.7 (Molecular Devices).

Hematoxylin–Eosin (H&E) and Nissl Staining

The whole mouse brains were fixed in 4% paraformaldehyde (Leagene, Beijing, China) overnight at room
temperature and dehydrated in a gradient alcohol series, and then embedded in paraffin. The coronal
sections of the brain tissues were cut at a thickness of 5 µm for H&E staining (BaSO, Taiwan, China) or
Nissl staining (Beyotime, Jiangsu, China). Two regions in the ipsilateral hippocampus (CA1 and CA3)
were randomly selected in each mouse and photographed under a microscope (Olympus, Tokyo, Japan).
The cell counts were performed using the ImageJ software, and a total of 3 mice per group were used for
quantification (Supplementary Fig. 2).

Real-time quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted from the hippocampus with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The concentration and quality of the mRNA extracts were determined by measuring the 260/280 and 260/230 ratios using a DS-C spectrophotometer (DeNovix, DE, USA). Then, RNA (1 μg) of each sample was reverse transcribed to cDNA with the PrimeScript™ RT kit. (Takara Bio Inc., Shiga, Japan). Thereafter, RT-qPCR was performed with the SYBR® Premix Ex Taq™ (Takara Bio Inc.) using the LightCycler 96 instrument (Roche, Switzerland). Use the template (10 μL of the PCR mixture, 5 pmol of forward and reverse primers, 1 μL of the cDNA template, and an appropriate volume of water) in a 20-μL reaction volume. The PCR reactions are performed as follows: cycling was first performed at 95°C for 30 s with an initial DNA denaturation step, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Threshold cycle (CT) readings were collected. The relative expression of the target genes mRNA was calculated by the 2-ΔΔCT method,[26] and the mean value of the glycerol-3-phosphate dehydrogenase (GAPDH) served as an internal reference for normalization. Next, the expression level of mRNA was reported as the fold change when compared to the control group. The sequence of the primer pairs for the target gene is listed in Supplementary Table 1.

**Western blotting**

The hippocampal tissues were homogenized in the RIPA lysis buffer (Beyotime) containing phosphatase inhibitor (Sigma–Aldrich) and protease inhibitor (Sigma–Aldrich) on an ice bath for 30 min and centrifuged; the supernatant was aspirated for protein quantification and denaturation. The BCA Protein Assay Kit (Beyotime) was used to measure the protein concentration. Protein samples (approximately 40 μg) were separated by 7.5-15% PAGE Gel (EpiZyme, Shanghai, China) at 150 V, transferred onto a PVDF membrane (Millipore, MA, USA) at 375 mA for 120 min, and then blocked with 5% BSA (Biofroxx, Germany) for 2 h. The membranes were blotted overnight at 4°C with the primary antibodies, including anti-IRAK-M antibody (1:1000, Bioss, bs-16695R), anti-NMDAR2A antibody (1:1000, Bioss, bs-3507R), anti-NMDAR2B antibody (1:1000, Bioss, bs-0222R), anti-IRAK1 antibody (1:1000, ab238, Abcam, Temecula, CA, USA), anti-TRAF6 antibody (1:2000, Abcam, ab40675), anti-STAT1 antibody (1:1000, CST, 14994), anti-p-STAT1 antibody (1:1000, CST, 9167), anti-SOCS-1 (1:1000, Abcam, ab62584), and anti-GAPDH (1:50000, Proteintech, 60004-1-Ig). After the membranes were washed thrice with TBST, they were incubated with the corresponding IgG antibody for 2 h. Finally, the protein bands were visualized by using an ECL kit (Affinity, Cincinnati, OH, USA). Image J was performed to calculate the protein density.

**Immunofluorescence**

The brain slices were fixed in 4% paraformaldehyde (Leagene) for 24 h and then dehydrated in serial 15% and 30% sucrose solutions. The brain tissue was then sliced into 20-μm-thick coronal sections. After rinsing the brain sections with PBS for residual embedding medium (OCT), the sections were blocked with 5% goat serum (Beyotime) and 1% Triton-X 100 (Beyotime) for 1.5 h, followed by incubation with primary antibodies against NeuN (Rabbit, 1:200, Abcam) and Iba-1 (Rat, 1:500, Synaptic Systems, 234017) overnight at 4°C, followed by treatment with Alexa Fluor 594-conjugated IgG (1:200, Invitrogen) and Alexa Fluor 488-conjugated IgG (1:200, Invitrogen) secondary antibodies for 1 h at room temperature. Next, PBS
was used to rinse the sections thrice, followed by DAPI staining (Solarbio, Beijing, China) for 10 min. Subsequently, a confocal microscope (Nikon) was employed for observation. All fluorescent image data was quantified by using Image J. For microglia morphology data collection, the fluorescence photomicrographs were converted into representative binary and skeletonized images and then analyzed using the ImageJ plugins AnalyzeSkeleton (2D/3D) and FracLac.

**Flow Cytometry**

The hippocampal tissues were ground with a grinding rod, digested with the StemPro Accutase Cell Dissociation Reagent (Gibco, Spain), and then filtered through a 70-μm filter (BD Falcon, USA) to obtain a cell suspension, which was centrifuged at 400 × g for 5 min at 4°C. Pre-cooled PBS and the Debris Removal Solution (Miltenyi Biotec, Miltenyi) were used to remove myelin and impurities, followed by centrifugation at 4°C for 10 min at 3000 x g with full acceleration and full brake. Staining buffer (eBioscience, CA, USA) was used to resuspend the cells, at the density of 1 × 10⁷ cells/mL in each tube. The tube was incubated at RT for 30 min in the dark, followed by the addition of 1 μL/mL of Fixable Viability Dye (eFluorTM 450, eBioscience) to label the dead cells and 100 μL of IC Intracellular Fixation (eBioscience) to fix the cells. Next, 2 mL of 1X permeabilization buffer (eBioscience) was added to the cells, and the mixture was centrifuged for 10 min at 400 x g, and the supernatant was removed. The antibody mixture (2 μL CD11b, 2 μL CD45, 2 μL CD86, and 2 μL CD163) was added to the tubes (detailed information on the flow cytometry antibodies is available in Supplementary Table 2), and the tube contents were mixed and incubated for 20–60 min at 2–8°C away from direct light. All samples were read on the CytoFLEX flow cytometer (Beckman, USA) and analyzed with the CytExpert software (Beckman). Positive cell percentages were calculated.

**Statistical analysis**

All statistical analyses were performed using the SPSS 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA) by an investigator who was blinded to the experiment. Unless otherwise specified, all data were presented as the means ± standard error of the mean (SEM). The significance is denoted with an asterisk. All experiments were performed with at least 3 independent mice. First, the Shapiro–Wilk test was performed to assess the normality of data distributions. The data were compared using the Student’s t-test or one-way analysis of variance (ANOVA) with a non-parametric Kruskal–Wallis test, followed by Dunn’s multiple comparison test for post-hoc group comparisons, t-test, Mann–Whitney U-test, or Fisher’s least significant difference. Individual p-values are indicated in the figure legends, with p < 0.05 considered to indicate statistical significance.

**Results**

**Dynamic changes in IRAK-M at different time points after SE**

To investigate the role of IRAK-M in epileptogenesis and the dynamic expression of IRAK-M after SE, lithium–pilocarpine-induced epilepsy model was first established, and then the seizure frequency was
assessed at various time points. Increased seizure frequency was found in the SE mice that developed seizures during the 1–7-day and 21–28-day monitoring periods (Fig. 1B). Subsequently, IRAK-M mRNA and protein levels were measured in the hippocampus at appropriate time points (Fig. 1C–E), which revealed that the levels increased on d1, d7, and d28 and recovered to baseline values on d14. This observation corresponded to the dynamic changes in seizure frequency. These results suggest a strong link between the dynamic alterations in IRAK-M and epilepsy in these mice.

**IRAK-M deficiency exacerbated the seizures in the lithium–pilocarpine-induced epilepsy model**

To determine whether IRAK-M KO affected the seizures, IRAK-M−/− (Fig. 2A and B) and WT mice were treated with lithium chloride and pilocarpine to induce epilepsy and then continuously video monitored for 2 h. Racine’s seizure scoring signified slightly higher seizure severity in the IRAK-M−/− group compared with the WT group (Fig. 2C). IRAK-M−/− mice showed shorter latency and a higher mortality rate than the WT mice (Fig. 2D–E). At the chronic model stage, the mice developed chronic SRSs after SE induction[28, 29]. IRAK-M−/− mice experienced SRSs more frequently and for a longer period than the WT mice (Fig. 2F and G). These results allude that IRAK-M deficiency increases the severity of lithium–pilocarpine-induced seizures.

**IRAK-M deficiency enhanced the glutamatergic synaptic transmission of hippocampal CA1 pyramidal neurons**

Seizures have been reported to be associated with the neuronal hyperexcitability of glutamatergic synaptic transmission, thereby leading to hypersynchronous neuronal activity[30, 31]. To investigate the potential role of IRAK-M in glutamatergic synaptic transmission, electrophysiological recordings were performed in acute slices from adult IRAK-M−/− and their littermate WT mice. The data showed that the frequencies, but not the amplitudes, of sEPSCs and mEPSCs were increased in hippocampal CA1 pyramidal neurons of IRAK-M−/− mice compared with those in the WT group (Fig. 3A–F). Subsequently, NMDAR- and AMPAR-mediated EPSCs were measured, and the ratios of NMDAR- and AMPAR-dependent synaptic responses were calculated. The ratio of NMDAR to AMPAR responses (NMDA/AMPA ratio) within each cell was increased in IRAK-M−/− mice (Fig. 3G–H). To estimate the effects of IRAK-M deficiency on the expression levels of NMDAR and AMPAR subunits, RT-qPCR analysis was performed to detect the mRNA levels of NMDAR (Grin1, Grin2A, and Grin2B) and AMPAR (GluR1, GluR2, and GluR3) subunits in the hippocampus of WT and IRAK-M−/− mice. The mRNA levels of the Grin2A and Grin2B subunits in the hippocampus, but not those of Grin1, GluR1, GluR2, or GluR3, were found to be significantly elevated in IRAK-M−/− mice compared with WT mice (Fig. 3I). Collectively, these results indicate that IRAK-M deficiency enhances the presynaptic properties of glutamatergic synaptic transmission and postsynaptic strength via a mechanism that involves increased expressions of the NMDAR subunits NMDAR2A and NMDAR2B (but not NMDAR1) in hippocampal CA1 pyramidal neurons. This finding possibly explains the exacerbation of epileptic seizures in IRAK-M−/− mice.
IRAK-M deficiency increased hippocampal neuronal loss in the lithium–pilocarpine-induced epilepsy model

Excessive excitatory synaptic transmission during epileptogenesis facilitates glutamate excitotoxicity, which results in neuronal loss\[^{[32]}\]. To determine whether IRAK-M affected the loss of neurons in mice with epilepsy, H&E staining and NeuN immunostaining were performed to evaluate the pathological changes in the hippocampus. According to the results of the H&E staining, genetic deletion of IRAK-M significantly worsened the pathological changes, such as a shrunken cell body, condensed nuclei, and reduced or disappeared Nissl body in the CA1 and CA3 areas of the hippocampus in epileptic mice (Supplementary Fig. 2A). The survival of neurons in the CA1 and CA3 regions of the hippocampus were further assessed using NeuN immunostaining. Consistently, the results showed that the number of surviving neurons in the hippocampal CA1 and CA3 regions was significantly decreased in the pilocarpine-induced epileptic IRAK-M\(^{-/-}\) mice. (Fig. 4A–D; Supplementary Fig. 2A) Notably, no difference was observed in baseline neuronal survival in the hippocampal CA1 and CA3 regions between the KO and WT control groups. These results imply that the lack of IRAK-M accelerates seizure-induced hippocampal nerve damage.

IRAK-M deficiency increased the hippocampal microglial activation in the epilepsy model

Based on previous reports that microglial function was disrupted in epilepsy and that IRAK-M was expressed predominantly in microglia of the CNS\[^{[34, 35]}\], the impacts of the lithium–pilocarpine-induced epilepsy model on microglia were compared between the IRAK-M\(^{-/-}\) and WT mice. An increase in staining intensity and number of the microglial marker Iba1 was observed in hippocampal CA1 and CA3 regions of the pilocarpine-induced epileptic mice compared with that in the vehicle-treated group (Fig. 5A–D, G–H). In addition, the Iba1 intensity and number in the IRAK-M\(^{-/-}\) lithium–pilocarpine-induced epileptic mice were significantly higher than those in the WT epileptic mice. (Fig. 5A–D, G–H). Subsequently, the morphology of microglial cells was analyzed, which is known to correlate well with their activation status. A semiautomatic quantitative morphometric analysis was performed according to methods described in a previous study\[^{[27]}\]. The results revealed that the microglia displayed larger cell bodies, shorter processes, and fewer hippocampal branch points in the epileptic mice compared with those in the control mice (Fig. 5E and F for CA1, I and J for CA3); Furthermore, Iba-1 immunostaining revealed higher reactive microglia in CA3, but not in CA1, of IRAK-M\(^{-/-}\) epileptic mice. The only exception was the process length in the CA1 region. These findings imply that IRAK-M deficiency increases microglial activation, as assessed by the number and morphological changes in the microglia in epilepsy models.

IRAK-M ablation promoted M1 polarization and inhibited M2 polarization of microglia and caused a shift from a balanced cytokine response toward an inflammatory state in the epilepsy model

To assess whether IRAK-M affected the polarization of microglia in the hippocampus of lithium–pilocarpine-induced epileptic mice, flow cytometry was performed to distinguish microglia (CD11b+/CD45-) from other leukocytes (CD11b+/CD45+) and to quantify the expressions of M1 (CD86) and M2 (CD206) markers (Fig. 6A–E). The expression of the M1 microglial marker CD86 was significantly
higher in the epilepsy model than that in the control groups and even higher in the IRAK-M−/− epileptic mice. On the contrary, the expression of the M2 microglial marker CD206 was statistically lower in IRAK-M−/− epileptic mice. Furthermore, the IRAK-M-deficient microglia were polarized to an enhanced type 1 (M1) and secreted several proinflammatory cytokines, including IL-1β, iNOS, TNF-α, and IFN-γ (Fig. 6H). Conversely, the expressions of anti-inflammatory cytokines, such as IL-10, Arg1, and Ym-1, were significantly lower in the IRAK-M−/− epileptic mice compared with those in the WT mice (Fig. 6I).

Collectively, our data demonstrate that IRAK-M ablation promotes M1 polarization of microglia while suppressing M2 polarization, thereby resulting in a high inflammatory level in the hippocampus during epileptogenesis.

**IRAK-M knockout increased NMDA receptor expression and upregulated the STAT1-related signaling pathway**

To further explore the molecular mechanism underlying the exacerbation of seizures and microglial polarization in IRAK-M−/− mice, the protein expressions of NMDAR subunits and STAT1-related signaling pathway were assessed using western blot. Consistent with the above findings, the data revealed that the protein expressions of NMDAR2A and NMDAR2B were elevated in the epilepsy model. The expressions of NMDAR2A and NMDAR2B were even further elevated in IRAK-M KO epileptic mice compared with those in the WT epileptic group (Fig. 7A and B). Moreover, several molecular markers involved in modulating microglial M1/M2 polarization were investigated[20, 37, 38]. In the hippocampal tissues of WT epileptic mice, a significant increase in phosphorylated STAT1 (p-STAT1), IRAK1, TRAF6, and SOCS1 was observed (Fig. 7C and D); IRAK-M KO epileptic mice displayed an even greater elevation. Taken together, the results indicate that IRAK-M KO affects the polarization of M1 microglia by promoting STAT1-mediated upregulation of SOCS1 expression. Contrarily, IRAK-M KO enhanced the expressions of NMDAR subunits, which might be a factor in the aggravation of seizures caused by IRAK-M knockout.

**Discussion**

In this study, the dynamic expression of IRAK-M in mouse epilepsy models was first observed. Subsequently, IRAK-M deficiency was found to exacerbate the seizure behavior and pathology via a mechanism that involved the enhanced activity of glutamatergic synaptic transmission in hippocampal CA1 pyramidal neurons. Furthermore, IRAK-M deletion was observed to increase hippocampal neuronal loss via a possible mechanism of NMDAR-mediated excitotoxic. Moreover, IRAK-M deletion was noted to promote the transformation of microglia into M1 phenotype and produce high levels of proinflammatory cytokines combined with low levels of anti-inflammatory cytokines by regulating key expression proteins of microglial polarization, including p-STAT1, TRAF6, and SOCS1.

IRAK-M is known to be an important negative regulator of innate immunity. Recent studies have demonstrated significant associations between IRAK-M and certain neurological disorders, including stroke and multiple sclerosis, in which inflammation plays an important pathological role. However, little is known about the expression of IRAK-M in epileptogenesis or epilepsy. Our results showed that IRAK-M
levels increased on d1, d7, and d28 after SE and recovered to the baseline value on d14. In the acute phase, pilocarpine-treated mice exhibited SE and repetitive limbic seizures before d7\[^{39}\]. Afterward, the mice did not have any behavioral seizures, which usually occur within 2 weeks post-SE during the latent phase of the model. Finally, the mice progressed to the chronic phase, which was characterized by the presence of SRSs. These behavioral changes are analogous to the dynamic expression of IRAK-M. A possible hypothesis is that seizures activate TLR4 signaling, which results in high neuroinflammation that underlies the etiopathogenesis and convulsive disorders\[^{11,13}\]. As a negative regulator of the TLR4 signaling pathway, IRAK-M is a key mediator in the response to inflammatory processes and protects against seizures. Therefore, IRAK-M deletion increased seizure frequency and duration and also accelerated seizure onset, which indicates that it contributes to epileptogenesis and may serve as a potential therapeutic target.

Studies have shown that hippocampal sclerosis, a significant biomarker of epilepsy, is characterized histopathologically by prominent neuronal loss in the Cornu Ammonis (CA) regions. Consistent with our findings, epilepsy increased neuronal loss in the CA regions of epileptic mice, and IRAK-M deficiency further exacerbated neuronal loss. Previous studies have established that neuronal damage caused by seizure is mainly attributed to the excessive entry of Na\(^+\) and Ca\(^{2+}\) ions via glutamatergic neurotransmission into the neurons, which results in cell swelling and rupture, free radical production, proteolysis of cell and organelle membranes, and finally, cell death. Thus, the potential function of IRAK-M in excitatory glutamatergic synaptic transmission was focused on. The frequencies of sEPSCs and mEPSCs have been reported to be higher in epileptic pilocarpine-treated rats, and similarly, we found that these were increased in the hippocampal CA1 pyramidal neurons of IRAK-M KO mice compared with those in the WT group. Furthermore, basal synaptic transmission is predominantly mediated by the activation of fast excitatory ionotropic receptors (NMDAR/AMPAR). Overexpression of these receptors in the hippocampal formation has been widely documented both in the resected tissues of patients with temporal lobe epilepsy and in several epilepsy models. In the present study, the data showed that increased NMDA/AMPA ratio combined with elevated levels of the NMDR2A and NMDAR2B subunits in the hippocampus of IRAK-M KO mice may be a possible explanation for severe neuronal loss in IRAK-M KO epileptic mice.

IRAK-M is commonly believed to be mainly expressed in microglia in the CNS. However, how IRAK-M participates in the alteration of glutamatergic synaptic transmission in neurons remains unclear. Unbalanced microglial polarization or immune–inflammatory function is a frequent risk factor for the occurrence of seizures. During the progression of epilepsy, microglia undergo morphological and molecular activation within 3 h–3 d following drug delivery. Accordingly, an increased number of microglia, fewer endpoints, and shorter process length were observed after pilocarpine treatment, which displayed a slightly higher activation in IRAK-M KO epileptic mice. These results indicate IRAK-M affects microglial activation. During activation, microglia polarize toward M1/M2 phenotypes, which is associated with the release of various proinflammatory and anti-inflammatory mediators that may differentially contribute to the development of epilepsy. As a negative regulator of TLR signaling, IRAK-M
has been reported to inhibit M1 microglial polarization and promote M2 anti-inflammatory phenotype by inhibiting the STAT1 pathway\textsuperscript{[20,52]}. Furthermore, the suppressor of cytokine signaling 1 (SOCS1), primarily regulated by the activation of STAT1, has been shown to regulate M1 and M2 macrophage polarization. In this study, we found that IRAK-M deficiency shifts microglial polarization toward the M1 phenotype by activating the STAT1/SOCS1 pathway. Notably, the M1 phenotype is more likely associated with uncontrolled neuroinflammation often observed in epilepsy, whereas the M2 phenotype promotes inflammation resolution and tissue repair. The levels of proinflammatory cytokines IL-6, IL-1β, iNOS, TNF-α, and IFN-γ are elevated in patients with epilepsy. These mediators can also modulate neuronal excitability via inflammation-related interactions between microglia and neurons, which play a role in reducing the seizure threshold\textsuperscript{[58]}. For example, IL-1β can induce phosphorylation of the NMDAR2B subunit of the NMDA receptor, thereby facilitating NMDA-mediated Ca\textsuperscript{2+} influx into neurons. TNF-α increases the levels of glutamate in the brain and the functionality of the glutamatergic AMPA and NMDA receptors in the postsynaptic neuron, which leads to excitotoxicity\textsuperscript{[61,62]}. Our results revealed that IRAK-M KO enhanced the mRNA levels of proinflammatory cytokines secreted by M1, including IL-1β, iNOS, TNF-α, and IFN-γ, as well as the expressions of NMDA2R subunits. Taken together, our study may provide a mechanistic explanation that IRAK-M modulates the balance between proinflammatory and anti-inflammatory cytokines, thus resulting in NMDAR-mediated hyperexcitability in epilepsy.

In conclusion, our results indicate that IRAK-M dysfunction contributes to the progression of epilepsy by increasing M1 microglial polarization and glutamatergic synaptic transmission, which is possibly related to NMDARs, particularly Grin2A and Grin2B. To the best of our knowledge, this study is the first to provide evidence that IRAK-M modulates and impacts neuronal NMDAR function in epilepsy via M1/M2 microglial polarization. The findings of our mechanistic study are expected to facilitate the development of new antiepileptic drugs.

**Declarations**

**Ethics Approval**

All animal experimental protocols in this study were approved by the Southern Medical University Institutional Animal Care and Use Committee (permit number: L2020038) and conducted in accordance with the National Health and Medical Research Council animal ethics and ARRIVE guidelines. The experimental mice were housed in a controlled temperature (23–25°C) and humidity (45–55%) with a modified 12-h dark-light photophase (lights on from 07:00 AM to 07:00 PM) and free access to standard food and water.

**Consent to Participate**

This is an animal experiment, with no human participants.
Consent for Publication

All co-authors have explicitly acknowledged receipt of the material and consented to its publication.

Availability of data and materials

Data is contained within the article. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared that no conflict of interest exists.

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Authors’ Contributions

Wei Xie, Wei-Peng Li, Yue-Wen Ding, and Xiao-Shan Liang conceived and designed the experiments and revised the manuscript; Ting-Lin Qian, Yi-Fan Xiong, Xiao-Tao Liang, and Xiao-Yu Zhu performed the experiments; Yun-Lv Li, Jie-Li Zhou, and Le-Yi Tan assisted in some of the experimental work; Ting-Lin Qian and Wei-Peng Li analyzed data; The first draft of the manuscript was written by Xiao-Shan Liang, and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Figures
**Figure 1**

Dynamic changes in the interleukin-1 receptor-associated kinase (IRAK)-M at different post-SE time points.

(A) The experimental design for mice at different time points.

(B) The mean frequency (times/day) of seizures (n = 12).

(C–E) The relative mRNA and protein levels of IRAK-M were measured at d1, d7, d14, and d28 after SE. Marked upregulation was noted after d1, d7, and d28 of SE (n = 3–4). Con.: control group without treatment, Epi.: model induced by pilocarpine. The data are expressed as fold change versus WT group. *p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 2

IRAK-M deficiency promotes seizures induced by pilocarpine.

(A, B) Western blotting was performed to assess the knockout efficacy of IRAK-M. Representative immunoblots (A) and quantification (B) revealed the inhibition of the expression of IRAK-M protein. Data are presented as the fold change relative to the WT group (n = 4).

(C) Racine's scaling within 2 h of pilocarpine administration led to IRAK-M knockout increased seizure scores relative to that in the WT group (n = 12).

(D) The latency to the onset of over stage 3 seizures induced by pilocarpine indicates that IRAK-M knockout significantly shortened the latency (n=10).

(E) Mortality rate induced by pilocarpine in the WT group and IRAK-M-/- group (n=5).

(F) The frequency of spontaneous recurrent seizures (SRS) in the WT group and IRAK-M-/- group (n=10).

(G) SRS duration in the WT group and IRAK-M-/- group (n=10).

The data are presented as mean ± SEM. n.s: not statistically significant; *p <0.05; ** p <0.01; *** p <0.001.
Figure 3

Enhancement in glutamatergic synaptic transmission in hippocampal CA1 pyramidal neurons of IRAK-M-/- mice.

(A) Representative traces of sEPSCs recorded from hippocampus CA1 pyramidal neurons of WT (gray) and IRAK-M-/- (red) mice.
(B, C) Quantification of sEPSC frequency (B) and amplitude (C) recorded from hippocampus CA1 pyramidal neurons of WT and IRAK-M-/- mice.

(D) Representative traces of mEPSCs recorded from hippocampus CA1 pyramidal neurons of WT and IRAK-M-/- mice.

(E, F) Quantification of mEPSC frequency (E) and amplitude (F) recorded from hippocampus CA1 pyramidal neurons in the WT and IRAK-M-/- groups.

(G) Representative traces of evoked synaptic AMPA and NMDA currents were recorded at -70 and +40 mV, respectively.

(I) Representative mRNA levels measured by RT-qPCR analysis of the indicated genes in the hippocampus isolated from the WT and IRAK-M-/- groups. The data are presented as mean ± SEM. n.s: not statistically significant; *p <0.05; ** p <0.01; *** p <0.001.
Figure 4

**IRAK-M deletion increases hippocampal neuronal loss.**

(A, B) Representative images of immunofluorescent staining for NeuN (red) and DAPI (blue) in the hippocampal CA1 and CA3 regions. (Magnification: 200×, Scale bar = 100 μm).

(C, D) Quantitative analysis of NeuN-positive neurons in the hippocampal CA1 and CA3 regions. Con.: control group without treatment, Epi.: model induced by pilocarpine.

The data are presented as the mean ± SEM, n = 3 per group. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5

**IRAK-M deletion increased the activation of microglia in the hippocampal region.**

(A, B) Representative images of immunostaining for Iba1 (green) and DAPI (blue) in the hippocampal CA1 and CA3 regions (Magnification: 200×, Scale bar = 100 μm).

(C, D) Quantification of Iba1+ cell numbers and intensity in the hippocampal CA1 region. Intensity data are expressed as the fold change relative to the control group of WT mice.

(E, F) Semi-automatic quantification of microglia morphometry, including the total process length and the number of branchpoints of Iba1+ microglia in CA1.

(G, H) Quantification of Iba1+ cell numbers and intensity in the hippocampal CA3 region. Intensity data are expressed as fold change relative to that of the control group of WT mice.

(I, J) Semi-automatic quantification of microglia morphometry, including the total process length and the number of branchpoints of Iba1+ microglia in the CA3 region. Con.: control group without treatment, Epi.:
model induced by pilocarpine. The data are presented as the mean ± SEM, n = 3 per group. *p < 0.05, **p < 0.01, ***p < 0.001, n.s, nonsignificant.

**Figure 6**

IRAK-M ablation promoted an M2-to-M1 microglial phenotype shift and increased the inflammatory levels during epileptogenesis.

(A–D) Gating strategy for the identification of microglia from a CNS single-cell suspension.
(A) Singlets are gated on forward (FSC-A = size) and side scatters (SSC-A = internal structure) to exclude the cell debris and residual myelin.

(B) FSC-A and FSC-H plotting were performed to discriminate single cells from cell doublets/aggregates.

(C) PB450-marked dead cells were excluded.

(D) Microglia were identified by their relatively low CD45 expression and high CD11b expression.

(E) Representative scatter plots of M1 (CD86) and M2 (CD206) marker expression on the microglial cells, as determined by flow cytometry in a different group. (F) The proportions of M1 microglial cells within the CD86+ subsets.

(G) The proportions of M1 microglial cells within the CD163+ subsets.

(H) The mRNA levels of pro-inflammatory cytokines including IL-1β, iNOS, TNF-α, and IFN-γ in the hippocampus.

(I) The mRNA levels of anti-inflammatory cytokines including IL-10, Arg1, and Ym1 in the hippocampus. Con.: control group without treatment, Epi.: model induced by pilocarpine.

The data are presented as the mean ± SEM, n = 4 per group, *p < 0.05, **p < 0.01, ***p < 0.001, n.s, nonsignificant.
Figure 7

IRAK-M knockout increased the expression of NMDA receptors and upregulated the stat1-related signaling pathway.

(A-D) Representative Western blotting of NMDAR2A and NMDAR2B is shown. (E-F) Representative Western blotting of stat1, phospho-stat1 (p-stat1), and quantitative protein expression based on the intensity analysis of Western blotting.

(G-J) Representative Western blotting images and quantitation of IRAK1, TRAF6, and SOCS1 in IRAKM KO and WT mice. GAPDH was used as the loading control.

The data are presented as the mean ± SEM, n = 3-5 per group, *p< 0.05, **p <0.01, ***p< 0.001, n.s, nonsignificant.

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