Repeated Neonatal Sevoflurane Induced Neurocognitive Impairment Through NF-κB-Mediated Pyroptosis

Jing Dai  
Jiangsu University Affiliated Jintan Hospital

Xue Li  
Jiangsu University Affiliated Jintan Hospital

Cai Wang  
Jiangsu University Affiliated Jintan Hospital

Shuxin Gu  
Jiangsu University Affiliated Jintan Hospital

Lei Dai  
Jiangsu University Affiliated Jintan Hospital

Jingyun Zhang  
Jiangsu University Affiliated Jintan Hospital

Yunxia Fan (fanyinjia@aliyun.com)  
Jiangsu University  https://orcid.org/0000-0003-4488-2511

Jing Wu  
Zhengzhou University First Affiliated Hospital

Research

**Keywords:** NF-κB, Pyroptosis, Neuroinflammation, Neurocognition, General anesthesia

**DOI:** https://doi.org/10.21203/rs.3.rs-404817/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Exposure to general anesthesia (GA) during the postnatal period is associated with neuroinflammation and long-term neurocognitive impairment in preclinical and clinical settings. Pyroptosis, a novel type of programmed cell death that along with inflammation, plays an important role in the mechanism of diverse neurological diseases. Nevertheless, its role in GA-induced neuroinflammation and neurocognitive impairment in developing brain has not been investigated.

**Methods:** Rats at postnatal day 6 or primary hippocampal neurons at 9 days *in vitro*, received 3% sevoflurane for 2 hours daily for three consecutive days. A pharmacological inhibitor of nuclear factor (NF)-κB (BAY 11-7082) was administered to suppress NF-κB activation. Histological and biochemical analysis were performed to assess the pyroptosis and neuronal and synaptic damages both *in vivo* and *in vitro*. In addition, behavioral tests were performed to evaluate the neurocognitive ability in rats.

**Results:** Repeated sevoflurane exposures activated NF-κB-mediated pyroptosis and neuroinflammation in the hippocampus of developing rats, caused damages in neuronal morphology and synaptic integrity, and induced neurocognitive impairment in rats. BAY 11-7082, the inhibitor of NF-κB, suppressed the activation of pyroptosis, attenuated the neuronal and synaptic damages, and ameliorated the neurocognitive impairment induced by repeated sevoflurane in the developing rats.

**Conclusions:** These results demonstrated that repeated sevoflurane GA might induce neuroinflammation and cognitive impairment in developing rats via activation of NF-κB-mediated pyroptosis. Our findings characterize a novel role for pyroptosis as a potential therapeutic target in neuroinflammation to repeated neonatal GA.

Introduction

Each year around the world, increasing numbers of children and infants receive surgical and diagnostic procedures under general anesthesia (GA), often requiring repeated exposures. Accumulating evidence from animal and preclinical studies has demonstrated that general anesthetics (GAs) cause neuroinflammation in the developing brain, leading to neurodevelopmental deficits later in life [1-5]. Sevoflurane is the most common used inhalational GAs for pediatric patients, with excellent properties of respiratory tolerance, rapid onset, rapid offset, and hemodynamic stability [6]. It has been shown that sevoflurane induces activation of nuclear factor (NF)-κB, a master regulator of inflammation, increases inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 in the developing brain, resulting in long-term cognitive dysfunction in adulthood [2]. Despite of role of NF-κB in neuroinflammation, a large gap remains in understanding of the consequences of NF-κB activation in the developing brain after neonatal GAs exposures.

Pyroptosis is a novel inflammatory form of programmed cell death. This type of cell death can be triggered through the canonical nod-like receptor pyrin domain-containing 3 (NLRP3) inflammasome-caspase-1 pathway and non-canonical caspase-4/5/11 pathway [7-10]. More specifically, activated
inflammatory caspases cleave Gasdermin D (GSDMD) protein, the executor of pyroptosis, into two fragments (the N domain and C domain). As a result, the N-terminal fragment of GSDMD (GSDMD-N) forms nanoscopic pores on the cell membrane, leading to cell swelling and the release of proinflammatory materials [7-10]. Our previous study has demonstrated that canonical NLRP3 inflammasome-caspase-1 pyroptotic pathway was involved in isoflurane (a volatile anesthetic)-induced cognitive impairment in aged mice [11]. However, the link between GAs, pyroptosis and cognitive function remains largely unknown.

NF-κB is a nuclear transcription factor that participated in the control of a variety of cellular processes. Recent studies have discovered that the activation of the NF-κB family of transcription factors is a key step in the regulation of pyroptosis, through promoting the transcription and translation of pyroptosis-related proteins, including NLRP3, caspase-1 and caspase-11 [12-14]. Thus, the present study was set out to investigate whether GSDMD-induced pyroptosis mediated by inflammatory caspases is involved in the pathophysiology of neuroinflammation and cognitive deficits after repeated neonatal sevoflurane exposures in developing rats. In addition, BAY 11-7082, a selective NF-κB inhibitor [15, 16], was used to further investigate the link between sevoflurane GA and pyroptosis.

Materials And Methods

2.1. Animals

Sprague-Dawley rat pups at postnatal day (PND) 6 were used in the present study. All experimental procedures and protocols were reviewed and approved by the Animal Investigation Ethics Committee of Jiangsu University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. The pups were housed in a room maintained under constant environmental conditions (temperature 22-24 °C, a 12-h light/dark cycle, and 50 ± 10 % humidity) with their mothers till PND 20. At PND 21, the pups were weaned and housed 4-5 per cage in standard condition.

2.2. General anesthesia

Rat pups at PND 6 were randomly assigned to one of following four treatment protocols: control + vehicle (Con group), control + BAY 11-7082 (Con+BAY group), sevoflurane + vehicle (Sev group), and sevoflurane + BAY 11-7082 (Sev+BAY group). BAY 11-7082 (MilliporeSigma, USA) was first dissolved in a small amount of DMSO and then diluted by PBS (phosphate buffered saline) according to the previously published method [17, 18]. BAY 11-7082 (20 mg/kg) or PBS (vehicle) was intraperitoneally administered to the pups 30 min before gas inhalation [17, 18]. Sevoflurane anesthesia was induced by putting the rat pups in an anesthetizing chamber delivered with 3% sevoflurane for 2 h daily for three consecutive days [19, 20]. For control condition, 30% O₂ was delivered at the same flow rate. The composition of the chamber gas was continuously monitored using a DatexTM infrared analyzer (Capnomac, Finland). Rats were kept normothermic throughout the experiment. Six rat pups from each group were sacrificed.
immediately after 2 h gas inhalation at PND 8, and the brains were rapidly removed for histological and biochemical studies. Twelve rats from each group were used for behavioral studies at PND 40, 50 and 60.

2.3. Rat hippocampal neuronal culture and anesthetic exposure

Primary neuronal cultures were prepared from embryonic day 16-17 (E16-17) embryos of Sprague-Dawley rats as previously described [21]. Neurons were dissociated and seeded on poly-D-lysine-coated plates with neurobasal medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with B27 (Thermo Fisher Scientific, USA), GlutaMAX-I (Thermo Fisher Scientific, USA), 5% FBS (Invitrogen GIBCO Life Technologies, USA) and antibiotics. After 2 h incubation, primary cultures were maintained in neurobasal medium without FBS in 5% CO$_2$ incubator at 37 °C. Subsequently, half of the medium was replaced every 2 days.

After 9 days in vitro (DIV), the neurons were treated with 3% sevoflurane plus 5% CO$_2$ for 2 h daily for three consecutive days at 37 °C, whereas the control group was maintained in same amount of culture medium. BAY 11-7082 (5 μM) or equal volume of DMSO was added to the culture medium 30 min before GA exposure according to group assignment [22].

2.4. Cell viability assays

At DIV 11, neuronal cell viability was detected with the Cell Counting Kit-8 (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. Results were expressed as the percentage of reduction of absorbance at 450 nm by calibration with the absorbance of the control cells.

2.5. Measurement of mRNA Levels

The total RNA in the hippocampi of the rat was extracted by Trizol Reagent (Life Technologies, Inc., Grand Island, NY, USA). First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to synthesize cDNA. Real time-PCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA). The sequences of the primers were as follows: NLRP3, forward 5'-CGGTGACCTTGTGTGCTT-3' and reverse 5'-TCATGTCCTGAGCCATGGAAG-3'; Caspase-1, forward 5'-GAACAAAGAAGGTGGCGCAT-3' and reverse 5'-AGACGTGTACGAGTGCTTGC-3'; caspase-11, forward 5'-ATGTGGAGAAGGACTTCATTGC-3' and reverse 5'-AGATGACAAGAGCAGGCATGTA-3'; β-actin, forward 5'-TCAGCAAGCAGGACTTGC-3' and reverse 5'-GTGTAAAACGCAGCTCAGTAACA-3'. The expression of β-actin was used as the internal control to assess the expression of target genes.

2.6. Western blotting analysis

Protein quantification was performed using the Pierce BCA Protein Assay Kit (Beyotime Institute of Biotechnology), and 30-50 μg of total protein was dissolved by polyacrylamide gel electrophoresis (SDS-PAGE, 8%). Protein levels were determined via incubation with antibodies against NF-κB-p65 (1:500; Abcam, UK), IκBα (1:500; Abcam, UK), NLRP3 (1:500; Abcam, UK), caspase-1 (1:500; Santa Cruz Biotechnology, USA), caspase-11 (1:500; Santa Cruz Biotechnology, USA), IL-1β (1:500; Santa Cruz Biotechnology, USA), IL-1β (1:500; Santa Cruz Biotechnology, USA)
Biotechnology, USA), IL-18 (1:500; Abcam, UK), synapsin 1 (1:500; Millipore, USA), PSD-95 (1:500; Abcam, UK) GSDMD (1:500; Santa Cruz Biotechnology, USA), Lamin B (1:1000; Proteintech, USA), and GAPDH (1:500; Abcam, UK). The blots were imaged using ECL Plus western blotting detection reagents. Image J software was used to determine the average absorbance value of the corresponding bands.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β and IL-18 in the hippocampus were performed by ELISA kit following the manufacturer's instructions (Abcam, UK). Briefly, the supernatant of hippocampal tissue was added to 96-well plates coated with the indicated antibodies. After the reaction between the enzyme and substrate, the absorbances of the sample were assessed at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

2.8. Immunocytochemistry (ICC) staining

Immunofluorescence staining was performed as previously described. Briefly, neuronal coverslips were fixed with 4% PFA for 10 min and then permeabilized with blocking buffer comprising 5% goat serum, 1% bovine serum albumin (BSA) and 0.3% Triton X-100 at room temperature for 1 h. The samples were incubated overnight with primary antibodies against GSDMD (1:200, Santa Cruz Biotechnology, USA) and microtubule associated protein-2 (MAP2) (1:500, MilliporeSigma, USA) at 4 °C, followed by incubation with appropriate Alexa Fluor-488/594-conjugated secondary antibodies (Jackson ImmunoResearch, USA) and DAPI. Images were obtained with confocal microscopy (Fluoview FV 10i, Olympus, USA) and analyzed using FV10-ASW 2.1 Viewer software.

2.9. Immunohistochemical (IHC) staining

IHC was used to detect the immunoreactivity of GSDMD. The brain tissues were immediately perfused with 4% paraformaldehyde in PBS after removal and embedded in paraffin for sectioning. Brain sections (4 μm thickness) were incubated overnight at 4 °C with primary antibody against GSDMD (1:200, Santa Cruz Biotechnology, USA). The sections were then incubated with a secondary antibody labeled with horseradish peroxidase for 30 min at room temperature. Cells with brownish-yellow cytoplasm were counted as positive cells. For quantitative immunostaining, GSDMD-positive cells were observed under an inverted microscope, and the CA1 region was counted for all groups in ImageJ software.

2.10. Open field test

At PND 40, each rat (n = 12 for each group) was gently placed in the center of a black plastic chamber (100 cm × 100 cm × 40 cm) for 5 min. The exploratory behavior was automatically recorded by a video tracking system (XR-XZ301, Shanghai Soft Maze Information Technology Co., Ltd., China). The total distance and the amount of time traveled in the center area (50 cm × 50 cm) of the maze were measured. After each test, the arena was cleaned with 75% alcohol to avoid the presence of olfactory cues.

2.11. Morris water maze (MWM) test
The MWM test (XR-XM101; Shanghai Xinruan Information Technology Co., Ltd., China) was performed at PND 50. In the training phase, the rat was allowed to face to the pool wall in four random places (N, S, E, W) in the pool to find the fixed platform. The trial was terminated once the rat reached the platform. If the rat failed to reach the platform within 60 s, it would be guided to the platform and allowed to stay for 10 s, and then the latency was recorded for 60 s. In the probe test, single-probe trial was conducted with the original platform removed 24 h after the last training session. The rat was released at the opposite position of the platform and allowed to swim for 60 s in the pool.

2.12. Fear conditioning test

Fear conditioning tests (XR-XC404; Shanghai Softmaze Information Technology Company Limited, China) was performed at PND 60. Each rat was placed into a conditioning chamber and allowed to explore freely for 3 min. Then one tone-foot-shock pairing (tone, 30 s, 85 dB, 2 kHz; foot-shock, 2 s, 0.8 mA) was delivered. The rat then stayed in the chamber for another 30 s and was then returned to the home cage. The contextual fear conditioning test (a hippocampus-dependent task) was performed 24 h later by placing each rat back in the same test chamber for 5 min without any stimulation. Two hours later, the tone fear conditioning test (a hippocampus-independent task) was performed by placing each rat in a novel chamber with a different shape, color, and smell from the previous chamber, and the same tone was presented for 3 min without foot shock. Freezing behavior, defined as the absence of all visible movement except for respiration, was automatically recorded by the video tracking system.

2.13. Statistical analysis

Data are presented as the mean ± SEM and analyzed by the Graphpad Prism 8.0 software. The difference between the groups was determined by one-way analysis of variance followed by the Tukey's tests. Comparisons for the spatial training sessions of MWM were performed by repeated two-way ANOVA followed by LSD test. A \( p \) value <0.05 was regarded as statistical significance.

Results

3.1. Repeated sevoflurane induces activation of the NF-κB signaling in the hippocampus of neonatal rats

Sevoflurane has been shown to up-regulate the NF-κB signaling pathway in the hippocampus of neonatal rats [2, 5]. Herein, our results showed that the protein level of phosphorylation \( IκBα (p-IκBα) \) was significantly increased and the total level of \( IκBα \) was significantly decreased in the hippocampus of the developing rats after repeated sevoflurane exposure (Figure 1A). Moreover, the protein level of nuclear NF-κB p65 increased while the level of cytosolic NF-κB p65 reduced in the sevoflurane group compared with the control group (Figure 1B). BAY 11-7082, the most popular NF-κB activation inhibitor, can freely cross the blood-brain barrier [23]. We further investigated the effect of BAY 11-7082 on the expression of NF-κB and IκB. Notably, changes of the protein levels of NF-κB p65 and IκBα induced by sevoflurane GA were reversed by the administration of BAY 11-7082 (Figure 1). Our results indicate that repeated sevoflurane...
induces activation of NF-κB signaling in the hippocampus of neonatal rats and the activation is successfully inhibited by BAY 11-7082 administration.

3.2. Inhibiting NF-κB by BAY 11-7082 suppresses the activation of canonical and non-canonical inflammatory caspases in sevoflurane anesthesia rats

Pyroptosis can be triggered in the canonical (caspase-1-mediated) and non-canonical (caspase-11-mediated) inflammasome signaling pathways. Both processes are tightly controlled by the activation of NF-κB [12-14]. In the present study, we showed that the mRNA levels of NLRP3, caspase-1 and caspase-11 were obviously increased in the hippocampus of neonatal rats after repeated sevoflurane exposures (Figure 2A). In addition, the protein levels of NLRP3, Pro-caspase-1, Cleaved-caspase-1, Pro-caspase-11 and Cleaved-caspase-11 were significantly elevated in the sevoflurane group compared with the control group (Figure 2B and 2C). These changes are molecular characteristics of canonical and non-canonical pyroptotic pathways. Remarkably, in the rats that were pre-treated with BAY 11-7082, sevoflurane did not increase both the mRNA and protein levels of NLRP3, caspase-1 and caspase-11 when compared to those of control conditions (Figure 2), suggesting that inhibiting NF-κB activation by BAY 11-7082 suppressed both the canonical and non-canonical pyroptotic pathways after sevoflurane anesthesia exposures.

3.3. Inhibiting NF-κB by BAY 11-7082 attenuates sevoflurane-induced pyroptosis and neuroinflammation

The GSDMD is the substrate of active caspase-1 and caspase-11 and the executor of pyroptosis. Activated caspase-1 or caspase-11 cleaves the N- and C-terminals of GSDMD and triggers pyroptosis [7-10]. In vivo study, we showed that repeated sevoflurane exposures induced upregulations of GSDMD, GSDMD-N and inflammatory cytokines IL-1β and IL-18 (Figure 3A and 3B) in the hippocampus of developing rats. These findings were further confirmed with increased number of GSDMD-positive cells in CA1 area of brain sections in sevoflurane-treatment group (Figure 3C and 3D). In vitro study, western blot analysis from primary neuronal cultures showed that the protein levels of GSDMD and GSDMD-N were significantly increased in hippocampal neurons after repeated sevoflurane exposures (Figure 4A). Moreover, ICC staining showed that the intensity of GSDMD-immunofluorescence was elevated in the sevoflurane group (Figure 4B and 4C). Notably, BAY 11-7082 attenuated the cleavage of GSDMD and the release of inflammatory cytokines (Figure 3 and 4), suggesting that inhibiting NF-κB by BAY 11-7082 successfully attenuates pyroptosis and neuroinflammation induced by sevoflurane anesthesia.

3.4. BAY 11-7082 rescues sevoflurane-induced neuronal damage and synaptic dysfunction

Pyroptosis plays important roles in regulating neuronal cell death and in maintaining synaptic integrity and thereby modulating neural networks. To tested whether attenuating NF-κB-mediated pyroptosis could rescue sevoflurane-induced neuronal damage. Primary neurons were treated with BAY 11-7082, and MAP2 immunostaining was performed to examine neuronal morphology and neuronal outgrowth. We observed that neurons exposed to sevoflurane displayed impaired morphology and dystrophic neurites (Figure 5A). Remarkably, neurons pre-treated with BAY 11-7082 showed significantly improved
morphology compared with Sevoflurane group. The treatment did not alter neuron morphology in Con+BAY group, indicating that NF-κB inhibitors do not generally improve neuronal outgrowth and branching, but rather protect sevoflurane-mediated neurotoxicity. In addition, the cell viability assay showed that BAY 11-7082 treatment increased neuronal viability in the neurons exposed to sevoflurane compared to that of vehicle treatment (Figure 5B).

We further measured the protein levels of synapsin 1 and PSD-95, two important indicators of synaptic structure [24], in the hippocampus of developing rat. Our results showed that repeated sevoflurane exposures induced downregulation of synapsin 1 and PSD-95 and BAY 11-7082 pretreatment significantly attenuated the downregulation (Figure 5C), thus suggesting a protective effect of BAY 11-7082 toward synaptic integrity. Collectively, our results provide important evidence that BAY 11-7082 rescues sevoflurane-induced neuronal damage and synaptic dysfunction.

3.5. BAY 11-7082 ameliorates sevoflurane-induced neurocognitive deficits in adolescent rats

To further verify the role of NF-κB-mediated pyroptosis in sevoflurane-induced cognitive deficits, open field test, MWM test and fear conditioning test were performed at PND 40, 50 or 60, respectively. Open field test showed no difference among the four groups in the spontaneous locomotor activity as reflected by the total distance (Figure 6A) and the time spent in the center (Figure 6B), excluding the possibility that locomotor activity per se affected the results in MWM test and fear conditioning test.

The MWM test is a hippocampus-dependent memory test for assessing spatial learning and memory [25, 26]. We showed that BAY 11-7082 pretreatment successfully shortened the escape latency in training test (Figure 6C) and increased the target quadrant time (Figure 6D) and crossing platform times (Figures 6E) in probe trial in developing rats exposed to sevoflurane. The contextual fear conditioning test was used to evaluate the ability of hippocampus-dependent memory [27, 28]. We showed that BAY 11-7082 pretreatment ameliorated sevoflurane-induced reduction in percentage of freezing time (Figure 6F). There was no difference in the cued fear conditioning test results among the four groups (Figure 6G). Our results suggest that NF-κB-mediated pyroptosis may involve in pathogenesis of sevoflurane-induced neurocognitive deficits and BAY 11-7082 has a cognitive protective effect in adolescent rats after early exposure of sevoflurane.

Discussion

In the present study, we assessed whether NF-κB-mediated pyroptosis is involved in the pathophysiology of neuroinflammation and cognitive deficits after repeated neonatal sevoflurane exposures in developing rats. We found that repeated neonatal sevoflurane exposures upregulated the expression of NF-κB, NLRP3, caspase-1 and caspase-11, and induced neuroinflammation and pyroptosis in the hippocampus of developing mice. Remarkably, pretreatment with BAY 11-7082, a selective NF-κB inhibitor, inhibited the activation of NF-κB signaling and canonical and non-canonical pyroptotic pathways. Consequently, BAY 11-7082 rescued the hippocampal neuronal damage and synaptic dysfunction and improved the long-term cognitive function in adolescent rats after early exposure of sevoflurane. Collectively, our findings
suggested that NF-κB-mediated pyroptosis may play an important role in sevoflurane-induced cognitive deficits in developing brain, and NF-κB inhibition might be a potential target for ameliorating neuroinflammation and GAs-induced, developing-related neurocognitive dysfunction.

Neuroinflammation is an essential process in the pathophysiology of GAs-induced neuronal damage and cognitive deficits in developing brain [1-5]. Pyroptosis is a novel and unique type of inflammatory form of programmed cell death and GSDMD has recently been identified as the key effector in pyroptosis. Cleavage of GSDMD frees the GSDMD-N domain, which oligomerizes to form pores on the cell membrane. These pores lead to the increase of membrane permeability and the release of inflammatory cytokines, which trigger a cascade of inflammatory response [7-10]. Pyroptosis has been implicated in the pathogenesis of many inflammatory and non-inflammatory diseases [7-10]. Our previous study and reports from others have recently demonstrated that the GSDMD-induced pyroptosis is involved in isoflurane (a volatile anesthetic)-induced cognitive impairment in aged mice [11] and in ketamine (an intravenous anesthetic)-induced hippocampal neurotoxicity in mouse primary hippocampal neurons [29]. Here, our *in vitro* study showed that repeated neonatal sevoflurane exposures upregulated the expression of GSDMD and GSDMD-N in primary hippocampal neurons, induced impairment of neuronal morphology and network, and caused the decrease of neuronal viability. In addition, our *in vivo* study showed that sevoflurane upregulated the expression of GSDMD, GSDMD-N, IL-1β, IL-18 and synaptic synapsin 1 and PSD-95 in the hippocampus of developing rat. More importantly, sevoflurane caused long-term cognitive deficits in adolescent rats. Thus, we speculate that GSDMD-induced pyroptosis is involved in neuroinflammation and neurocognitive impairment after repeated neonatal sevoflurane exposures in developing brain.

GSDMD is the substrate for active caspase-1 and caspase-11. The canonical caspase-1 pathway can be triggered by the NLRP3 inflammasome that functions through an interaction with apoptosis-associated speck-like protein (ASC) and the subsequent recruitment of the precursor form of caspase-1 (Pro-caspase-1), leading to the cleavage of caspase-1 and the maturation of IL-1β and IL-18. The non-canonical caspase-11 inflammasome pathway is activated by lipopolysaccharide molecules in the cytoplasm of infected cells. Upon activation, cleaved caspase1/11 can directly mediate GSDMD cleavage and thus serve as important checkpoints in GSDMD-mediated pyroptosis [7-10]. It has been demonstrated that the levels of pyroptosis-related proteins, including NLRP3, Pro-caspase-1, Pro-caspase-11, Cleaved-caspase-1, and Cleaved-caspase-11 significantly increased after multiple doses of ketamine administration in mouse primary hippocampal neurons [29]. Our previous study also observed that the protein levels of NLRP3 and Cleaved-caspase-1 increased in the hippocampus of aged mice after isoflurane anesthesia [11]. In line with these studies, the present study data showed that the protein levels of NLRP3, Pro-caspase-1, Cleaved-caspase-1, Pro-caspase-11 and Cleaved-caspase-11 were significantly elevated in the hippocampus of neonatal rats after repeated sevoflurane exposures. Consistent results were also found in RT-PCR assay with increased mRNA levels. These results indicated that both the canonical and non-canonical pyroptotic pathways were activated in the developing brain after sevoflurane anesthesia.
The mechanisms underlying inflammatory caspases activation in pyroptosis are multifaceted. Recent studies showed that NF-κB is an essential transcription factor in pyroptosis. NF-κB is normally sequestered in the cytoplasm, bound to the regulatory protein IκB. Upon activation, IκB gets phosphorylated by the enzyme IκB kinase, which results in the release of the NF-κB. The liberated NF-κB then translocates to the nucleus and induces expression of target genes [30, 31], including NLRP3, caspase-1 and caspase-11, for the canonical and non-canonical inflammatory responses [12-14]. NF-κB signaling is considered a key factor in the regulation of neuroinflammation and is reported to be activated by inhalation anesthetics, such as isoflurane and sevoflurane [2, 5, 32, 33]. In this study, repeated sevoflurane exposures decreased IκBα and cytosolic NF-κB p65 and increased p- IκBα and nuclear NF-κB p65 in the hippocampus, suggesting the activation of NF-κB signaling after sevoflurane anesthesia. Furthermore, after NF-κB activation, NF-κB translocates into the nucleus and subsequently initiate the transcription and translation of NLRP3, caspase-1 and caspase-11 proteins. Therefore, our study suggested a correlation between NF-κB activation and pyroptosis in the developing brain after neonatal GAs exposures.

In order to further demonstrate the role of NF-κB-mediated pyroptosis in cognitive impairment induced by sevoflurane anesthesia, Bay 11-7082, a selective inhibitor of NF-κB, was selected for this study. Bay 11-7082 has been shown to irreversibly inhibit IκBα phosphorylation and NF-κB activation [15, 16]. It has been proven that Bay11-7802 could attenuate inflammation-induced memory injury in neurodegenerative diseases [34, 35]. Our results showed that Bay 11-7082 pretreatment attenuates sevoflurane-induced pyroptosis and neuroinflammation by inhibiting activation of canonical and non-canonical inflammatory caspases. Moreover, Bay 11-7082 protected against neuronal damage and synaptic dysfunction and attenuated cognitive impairment in developing rats after sevoflurane anesthesia. GSDMD is the executioner of pyroptosis and recent efforts are focusing on the development of inhibitors to interfere with the pore-forming function of GSDMD. In addition to inhibit NF-κB activation, Bay 11-7082 has been shown to potently inhibit GSDMD pore formation in liposomes and inammasome-mediated pyroptosis and IL-1β secretion in human and mouse cells [36, 37]. Thus, inhibition of GSDMD pore formation is supposed to be another protective effect of Bay 11-7082 in GA-induced cognitive deficits. Future studies are needed to confirm this hypothesis.

**Conclusion**

This study demonstrated, for the first time, that the NF-κB-mediated pyroptosis may be involved in neuroinflammation and cognitive impairment after repeated neonatal sevoflurane. When inhibiting the activation of NF-κB by Bay 11-7082, the pyroptosis mediated by canonical and non-canonical inflammatory caspases was significantly alleviated. Our study provides a promising strategy for the treatment of cognitive deficits in the developing brain involving pyroptosis.

**Abbreviations**
GA: general anesthesia; GAs: general anesthetics; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; NLRP3: nod-like receptor pyrin domain-containing 3; GSDMD: Gasdermin D; PND: postnatal day; DIV: days in vitro; ICC: immunocytochemistry; MWM: Morris water maze; ASC: apoptosis-associated speck-like protein.

Declarations

Availability of data and materials

All data generated in this study are included in this manuscript.

Acknowledgements

It has been shown as Funding.

Funding

This study was supported by the National Natural Science Foundation of China (grant 81901944), and the Science and Technology Plan Project of Changzhou (CJ20200001).

Contributions

YF and JW conceived and designed the experiments. JD, XL, CW, SG, LD, and JZ performed the experiments and analyzed the data. JD and YF wrote the manuscript. All authors discussed, edited, and approved the final version of the manuscript.

Ethics declarations

Ethics approval and consent to participate

All experimental procedures and protocols were reviewed and approved by the Animal Investigation Ethics Committee of Jiangsu University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, USA.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References
1. Shen X, Dong Y, Xu Z, Wang H, Miao C, Soriano SG, Sun D, Baxter MG, Zhang Y, Xie Z: Selective anesthesia-induced neuroinflammation in developing mouse brain and cognitive impairment. *Anesthesiology* 2013, **118**:502-515.

2. Shi Y, Wang G, Li J, Yu W: Hydrogen gas attenuates sevoflurane neurotoxicity through inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells signaling and proinflammatory cytokine release in neonatal rats. *Neuroreport* 2017, **28**:1170-1175.

3. Zhao S, Fan Z, Hu J, Zhu Y, Lin C, Shen T, Li Z, Li K, Liu Z, Chen Y, Zhang B: The differential effects of isoflurane and sevoflurane on neonatal mice. *Sci Rep* 2020, **10**:19345.

4. Wu L, Zhao H, Weng H, Ma D: Lasting effects of general anesthetics on the brain in the young and elderly: "mixed picture" of neurotoxicity, neuroprotection and cognitive impairment. *J Anesth* 2019, **33**:321-335.

5. Tian Y, Chen KY, Liu LD, Dong YX, Zhao P, Guo SB: Sevoflurane Exacerbates Cognitive Impairment Induced by Abeta 1-40 in Rats through Initiating Neurotoxicity, Neuroinflammation, and Neuronal Apoptosis in Rat Hippocampus. *Mediators Inflamm* 2018, **2018**:3802324.

6. Gibert S, Sabourdin N, Louvet N, Moutard ML, Piat V, Guye ML, Rigouzzo A, Constant I: Epileptogenic effect of sevoflurane: determination of the minimal alveolar concentration of sevoflurane associated with major epileptoid signs in children. *Anesthesiology* 2012, **117**:1253-1261.

7. Shi J, Gao W, Shao F: Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. *Trends Biochem Sci* 2017, **42**:245-254.

8. Man SM, Karki R, Kanneganti TD: Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev* 2017, **277**:61-75.

9. Voet S, Srinivasan S, Lamkanfi M, van Loo G: Inflammasomes in neuroinflammatory and neurodegenerative diseases. *EMBO Mol Med* 2019, **11**.

10. Wang K, Sun Q, Zhong X, Zeng M, Zeng H, Shi X, Li Z, Wang Y, Zhao Q, Shao F, Ding J: Structural Mechanism for GSDMD Targeting by Autoprocessed Caspases in Pyroptosis. *Cell* 2020, **180**:941-955 e920.

11. Fan Y, Du L, Fu Q, Zhou Z, Zhang J, Li G, Wu J: Inhibiting the NLRP3 Inflammasome With MCC950 Ameliorates Isoflurane-Induced Pyroptosis and Cognitive Impairment in Aged Mice. *Front Cell Neurosci* 2018, **12**:426.

12. He Y, Hara H, Nunez G: Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem Sci* 2016, **41**:1012-1021.

13. Lee DJ, Du F, Chen SW, Nakasaki M, Rana I, Shih VFS, Hoffmann A, Jamora C: Regulation and Function of the Caspase-1 in an Inflammatory Microenvironment. *J Invest Dermatol* 2015, **135**:2012-2020.

14. Ye L, Li G, Goebel A, Raju AV, Kong F, Lv Y, Li K, Zhu Y, Raja S, He P, et al: Caspase-11-mediated enteric neuronal pyroptosis underlies Western diet-induced colonic dysmotility. *J Clin Invest* 2020, **130**:3621-3636.
15. Kumar A, Negi G, Sharma SS: Suppression of NF-kappaB and NF-kappaB regulated oxidative stress and neuroinflammation by BAY 11-7082 (IkappaB phosphorylation inhibitor) in experimental diabetic neuropathy. Biochimie 2012, 94:1158-1165.

16. Fann DY, Lim YA, Cheng YL, Lok KZ, Chunduri P, Baik SH, Drummond GR, Dheen ST, Sobey CG, Jo DG, et al: Evidence that NF-kappaB and MAPK Signaling Promotes NLRP Inflammasome Activation in Neurons Following Ischemic Stroke. Mol Neurobiol 2018, 55:1082-1096.

17. Liu F, Liu TW, Kang J: The role of NF-kappaB-mediated JNK pathway in cognitive impairment in a rat model of sleep apnea. J Thorac Dis 2018, 10:6921-6931.

18. Jiang W, Li M, He F, Zhou S, Zhu L: Targeting the NLRP3 inflammasome to attenuate spinal cord injury in mice. J Neuroinflammation 2017, 14:207.

19. Tang XL, Wang X, Fang G, Zhao YL, Yan J, Zhou Z, Sun R, Luo AL, Li SY: Resveratrol ameliorates sevoflurane-induced cognitive impairment by activating the SIRT1/NF-kappaB pathway in neonatal mice. J Nutr Biochem 2021, 90:108579.

20. Wu J, Yang JJ, Cao Y, Li H, Zhao H, Yang S, Li K: Iron overload contributes to general anaesthesia-induced neurotoxicity and cognitive deficits. J Neuroinflammation 2020, 17:110.

21. Seibenhener ML, Wooten MW: Isolation and culture of hippocampal neurons from prenatal mice. J Vis Exp 2012.

22. Yang Y, Yang S, Guo J, Cui Y, Tang B, Li XJ, Li S: Synergistic Toxicity of Polyglutamine-Expanded TATA-Binding Protein in Glia and Neuronal Cells: Therapeutic Implications for Spinocerebellar Ataxia. J Neurosci 2017, 37:9101-9115.

23. Jayakumar AR, Tong XY, Ruiz-Cordero R, Bregy A, Bethea JR, Bramlett HM, Norenberg MD: Activation of NF-kappaB mediates astrocyte swelling and brain edema in traumatic brain injury. J Neurotrauma 2014, 31:1249-1257.

24. Mirza FJ, Zahid S: The Role of Synapsins in Neurological Disorders. Neurosci Bull 2018, 34:349-358.

25. Vorhees CV, Williams MT: Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 2006, 1:848-858.

26. Bromley-Brits K, Deng Y, Song W: Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. J Vis Exp 2011.

27. Frankland PW, Bontempi B, Talton LE, Kaczmarek L, Silva AJ: The involvement of the anterior cingulate cortex in remote contextual fear memory. Science 2004, 304:881-883.

28. Einarsson EO, Pors J, Nader K: Systems reconsolidation reveals a selective role for the anterior cingulate cortex in generalized contextual fear memory expression. Neuropsychopharmacology 2015, 40:480-487.

29. Ye Z, Li Q, Guo Q, Xiong Y, Guo D, Yang H, Shu Y: Ketamine induces hippocampal apoptosis through a mechanism associated with the caspase-1 dependent pyroptosis. Neuropharmacology 2018, 128:63-75.
30. Oeckinghaus A, Ghosh S: The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 2009, 1:a000034.

31. Scheidereit C: IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene* 2006, 25:6685-6705.

32. Cao Y, Li Z, Ma L, Ni C, Li L, Yang N, Shi C, Guo X: Isofluraneinduced postoperative cognitive dysfunction is mediated by hypoxia-inducible factor1alphadependent neuroinflammation in aged rats. *Mol Med Rep* 2018, 17:7730-7736.

33. Zhang L, Zhang J, Yang L, Dong Y, Zhang Y, Xie Z: Isoflurane and sevoflurane increase interleukin-6 levels through the nuclear factor-kappa B pathway in neuroglioma cells. *Br J Anaesth* 2013, 110 Suppl 1:i82-91.

34. Ruan Y, Qiu X, Lv YD, Dong D, Wu XJ, Zhu J, Zheng XY: Kainic acid induces production and aggregation of amyloid beta-protein and memory deficits by activating inflammasomes in NLRP3- and NF-kappaB-stimulated pathways. *Aging (Albany NY)* 2019, 11:3795-3810.

35. Zheng XY, Lv YD, Jin FY, Wu XJ, Zhu J, Ruan Y: Kainic acid hyperphosphorylates tau via inflammasome activation in MAPT transgenic mice. *Aging (Albany NY)* 2019, 11:10923-10938.

36. Pandeya A, Li L, Li Z, Wei Y: Gasdermin D (GSDMD) as a new target for the treatment of infection. *Medchemcomm* 2019, 10:660-667.

37. Hu JJ, Liu X, Xia S, Zhang Z, Zhang Y, Zhao J, Ruan J, Luo X, Lou X, Bai Y, et al: FDA-approved disulfiram inhibits pyroptosis by blocking gasdermin D pore formation. *Nat Immunol* 2020, 21:736-745.

**Figures**
Figure 1

BAY 11-7082 inhibits sevoflurane-induced NF-κB activation. Rat pups at PND 6 were randomly assigned to one of the following four treatment protocols: control + vehicle (Con group), control + BAY 11-7082 (Con+BAY group), sevoflurane + vehicle (Sev group), and sevoflurane + BAY 11-7082 (Sev+BAY group). BAY 11-7082 (20 mg/kg) or PBS (vehicle) was intraperitoneally administered to the pups 30 min before gas inhalation. Sevoflurane anesthesia was induced by putting the rat pups in an anesthetizing chamber delivered with 3% sevoflurane for 2 h daily for three consecutive days. For control condition, 30% O2 was delivered at the same flow rate. (A) and (B) Representative western blotting and quantitative analysis of protein levels of IκBα, p-IκBα, cytoplasmic NF-κB and nuclear NF-κB from fresh hippocampal tissue homogenates obtained on PND 8. Values are presented as mean ± SEM (n = 6 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.
Figure 2

BAY 11-7082 suppresses sevoflurane-induced activation of canonical and non-canonical inflammatory caspases. (A) Representative real-time PCR analysis of mRNA levels of NLRP3, caspase-1 and caspase-11 in the hippocampus in developing rats. (B) Representative western blotting and quantitative analysis of protein levels of NLRP3, Pro-caspase-1, and cleaved caspase-1 in the hippocampus in developing rats. (C) Representative western blotting and quantitative analysis of protein levels of Pro-caspase-11 and cleaved caspase-11 in the hippocampus in developing rats. Values are presented as mean ± SEM (n = 6 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.
Figure 3

BAY 11-7082 attenuates sevoflurane-induced pyroptosis and neuroinflammation in the rat hippocampus. (A) Representative western blotting and quantitative analysis of protein levels of GSDMD, GSDMD-N, IL-1β and IL-18 in the hippocampus in developing rats. (B) ELISA analysis of IL-1β and IL-18 levels. (C) Representative images of GSDMD staining in the hippocampal CA1 region. Scale bar = 50 μm for all photographs. (D) Representative the number of GSDMD-positive cells in the CA1 region of the hippocampus. Values are presented as mean ± SEM (n = 6 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.
Figure 4

BAY 11-7082 attenuates sevoflurane-induced pyroptosis in primary hippocampal neurons. (A) Representative western blotting and quantitative analysis of protein levels of GSDMD and GSDMD-N from primary hippocampal neuron homogenates obtained on DIV 11. (B) Representative immunofluorescence staining images of GSDMD, MAP2 and DAPI in the primary hippocampal neurons. Scale bars = 50 μm. (C) Quantitative analysis of intensity of GSDMD-immunofluorescence in the primary hippocampal neurons. Values are presented as mean ± SEM (n = 6 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.
Figure 5

BAY 11-7082 rescues sevoflurane-induced neuronal damage and synaptic dysfunction. (A) Representative immunofluorescence images of MAP2 staining in the primary hippocampal neurons. Scale bar = 25 mm for all photographs. (B) Cell viability of cultured hippocampal neurons on DIV 11. (C) Representative western blotting and quantitative analysis of protein levels of synapsin 1 and PSD-95 in the hippocampus in developing rats. Values are presented as mean ± SEM (n = 6 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.
Figure 6

BAY 11-7082 ameliorates sevoflurane-induced cognitive deficits in adolescent rats. (A) Total distance traveled and (B) time spent in the center in open field tests. The open field tests in developing rats were performed at PND 40. (C) Escape latency during the spatial training of MWM for 5 consecutive days. The MWM tests were performed at PND 50. (D) Time spent in the target quadrant and (E) crossing platform times in the probe trial of MWM. (F) Freezing time to context and (G) freezing time to tone in the fear conditioning tests. The fear conditioning tests were performed at PND 60. Values are presented as mean ± SEM (n = 12 rats/group). *p < 0.05 versus the Con group; #p < 0.05 versus the Sev group.