Isoflurane-induced neuronal apoptosis in developing hippocampal neurons

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Abstract
We hypothesized that the P2X7 receptor may be the target of isoflurane, so we investigated the roles of the P2X7 receptor and inositol triphosphate receptor in calcium overload and neuronal apoptosis induced by isoflurane in cultured embryonic rat hippocampal neurons. Results showed that isoflurane induced widespread neuronal apoptosis and significantly increased cytoplasmic Ca²⁺. Blockade of P2X7 receptors or removal of extracellular Ca²⁺ combined with blockade of inositol triphosphate receptors completely inhibited apoptosis or increase in cytoplasmic Ca²⁺. Removal of extracellular Ca²⁺ or blockade of inositol triphosphate receptor alone could partly inhibit these effects of isoflurane. Isoflurane could directly activate P2X7-gated channels and induce inward currents, but did not affect the expression of P2X7 receptor protein in neurons. These findings indicate that the mechanism by which isoflurane induced neuronal apoptosis in rat developing brain was mediated by intracellular calcium overload, which was caused by P2X7 receptor mediated calcium influx and inositol triphosphate receptor mediated calcium release.

Key Words
neural regeneration; brain injury; isoflurane; P2X7 receptor; inositol triphosphate receptor; calcium homeostasis disturbance; neurodegenerative disease; apoptosis; developing brain; hippocampus; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) Many studies have focused on the neurotoxicity of isoflurane in recent years, but little is known about the mechanism involved in this neurotoxicity.
(2) This study focused on isoflurane-induced calcium overload to investigate the mechanism of isoflurane neurotoxicity.
(3) Results demonstrated that the neurotoxicity of isoflurane was mediated by the P2X7 receptor. Specifically, isoflurane induced neuronal apoptosis in developing rat brain by intracellular calcium overload caused by P2X7 receptor mediated calcium influx and inositol triphosphate receptor-mediated calcium release.

INTRODUCTION
The neurotoxicity of inhalation anesthetic agents in developing brain has been widely studied⁴, and isoflurane is a focus of study in recent years. Those previous studies only showed its neurotoxicity including neuronal apoptosis and subsequent potential cognitive dysfunction⁵, but little is known about the mechanism involved in the neurotoxicity. Disruption of cytoplasmic calcium homeostasis plays a key role in neuronal
Apoptosis. The intracellular calcium homeostasis is regulated by two factors: calcium influx and calcium release. Calcium influx is mainly mediated by voltage-gated or ligand-gated calcium channels, and calcium release mainly mediated by inositol triphosphate (IP3) receptor/ryanodine receptor in endoplasmic reticulum. It was found that voltage-gated calcium channels were not involved in isoflurane-induced neuronal apoptosis, but as IP3 receptors exist in the endoplasmic reticulum, isoflurane may not directly affect them. It remains unclear how isoflurane affects IP3 receptors and how P2X7-mediated calcium influx interacts with IP3-mediated calcium release.

Purinergic P2X7 receptors play a crucial role in cell apoptosis. Persistent activation of P2X7 receptors can cause cell apoptosis, which has led to the P2X7 receptor being called the “apoptotic receptor.” It has been found that P2X7 receptor protein was expressed in developing brain neurons, and 2’,3’-O-(4-benzoyl) benzoyl-ATP, the specific agonist of P2X7 receptors, can induce neuronal apoptosis when P2X7 receptors are persistently activated. Thus, isoflurane may directly activate the P2X7 receptor, which induces Ca\(^{2+}\) influx, and indirectly activates the IP3 receptor to induce Ca\(^{2+}\) release. These dual actions disrupt calcium homeostasis and neuronal apoptosis. The present study investigated the role of P2X7 receptor-mediated Ca\(^{2+}\) influx and IP3 receptor-mediated Ca\(^{2+}\) release in isoflurane-induced calcium overload and neuronal apoptosis in cultured embryonic rat hippocampal neurons.

RESULTS

Hippocampal neuronal apoptosis induced by 0.75% isoflurane
A total of 25 rats were used for the primary culture of hippocampal neurons. The neurons were treated with Hank’s balanced salt solution aerated with 21% O\(_2\)/5% CO\(_2\)/73.25% N\(_2\) containing 0.75% isoflurane for 8 hours (with no isoflurane as control and the final concentration of N\(_2\) was 74%), brilliant blue G, the P2X7 receptor antagonist, was added to the Hank’s balanced salt solution (at a final concentration of 1 μM) to study the role of the P2X7 receptor in neuronal apoptosis. To observe the effect of Ca\(^{2+}\) on neuronal apoptosis induced by isoflurane, Ca\(^{2+}\) was removed from the Hank’s balanced salt solution. Xestospongion C (IP3 receptor antagonist; final concentration of 1 μM) was added to the Hank’s balanced salt solution with or without Ca\(^{2+}\), and the experiments mentioned above were repeated. The percentage of neuronal apoptosis increased significantly compared with control (\(P < 0.01\)). Xestospongion C added into the extracellular fluid, or Ca\(^{2+}\) removed from extracellular fluid, could partly reduce neuronal apoptosis (\(P < 0.01\), vs. isoflurane + 1.8 mM Ca\(^{2+}\); \(P < 0.01\), vs. control), but brilliant blue G or Xestospongion C added to the extracellular fluid with Ca\(^{2+}\) removed completely inhibited neuronal apoptosis induced by isoflurane (\(P > 0.05\), vs. control). Brilliant blue G or Xestospongion C alone had no effect on neuronal apoptosis (Figure 1).

(A) Scatterplot of apoptosis induced by 0.75% Iso. (a) Control; (b) Iso + 1.8 mM [Ca\(^{2+}\)]; (c) Iso + 1 μM brilliant blue G (BBG); (d) Iso + 0 mM [Ca\(^{2+}\)]; (e) Iso + 1 μM Xestospongion C (Xc); (f) Iso + 0 mM [Ca\(^{2+}\)] + 1 μM Xc; (g) 1 μM BBG; (h) 1 μM Xc.

Right upper quadrant represents the area with necrotic neurons; right lower quadrant represents the area with apoptotic neurons; left lower quadrant represents the area with normal neurons.

(B) The percentage of neuronal apoptosis in all groups (mean ± SD, n = 6, one-way analysis of variance followed by least squares analysis). \(P < 0.01\), vs. control; \(P < 0.01\), vs. Iso + 1.8 mM Ca\(^{2+}\).

I: Control; II: Iso + 1.8 mM [Ca\(^{2+}\)]; III: Iso + 1 μM BBG; IV: Iso + 0 mM [Ca\(^{2+}\)]; V: Iso + 1 μM Xc; VI: Iso + 0 mM [Ca\(^{2+}\)] + 1 μM Xc; VII: 1 μM BBG; VIII: 1 μM Xc.
Changes of intracellular calcium concentration induced by 0.75% isoflurane in hippocampal neurons

The neurons were washed with Hank’s balanced salt solution three times, incubated with Hank’s balanced salt solution containing 2.5 μM Fura-2/AM for 40 minutes, then placed in a sealed chamber at 37°C, and infused with Hank’s balanced salt solution for 10 minutes. The intracellular calcium was measured as baseline, then the neurons were infused with Hank’s balanced salt solution (aerated with 0.75% isoflurane for 8 hours before the experiment) for another 10 minutes, and intracellular calcium was measured throughout this period (Figure 2A).

Brilliant blue G was added to the Hank’s balanced salt solution (final concentration 1 μM) to study the role of the P2X7 receptor in intracellular calcium changes. To observe the effect of Ca^{2+} influx on calcium overload induced by isoflurane, Ca^{2+} was removed from the Hank’s balanced salt solution (CaCl₂ was replaced with isotonic NaCl). Xestospongin C (final concentration 1 μM) was added to the Hank’s balanced salt solution with or without Ca^{2+}.

After the neurons were exposed to the Hank’s balanced salt solution aerated with 0.75% isoflurane with 1.8 mM Ca^{2+} extracellularly, the intracellular calcium concentration significantly increased (P < 0.01, vs. control). When 1 μM brilliant blue G was added to the Hank’s balanced salt solution, there was no change in intracellular calcium concentration induced by isoflurane compared with control (P > 0.05), but Xestospongin C partly reduced the increased intracellular calcium concentration induced by isoflurane (P < 0.01, vs. control; P < 0.01, vs. isoflurane + 1.8 mM [Ca^{2+}]o).

When calcium was removed from the Hank’s balanced salt solution, intracellular calcium concentration still increased by isoflurane (P < 0.01, vs. control), but did not increase as much with 1.8 mM Ca^{2+} extracellularly (P < 0.01, vs. isoflurane + 1.8 mM [Ca^{2+}]o). The increase of intracellular calcium induced by isoflurane could be inhibited completely when brilliant blue G or Xestospongin C was added to the Hank’s balanced salt solution without Ca^{2+}; but brilliant blue G or Xestospongin C alone had no effect on the intracellular calcium concentration (Figure 2B).

Effect of isoflurane on P2X7-gated currents in hippocampal neurons

Fifteen neurons were recorded with each treatment. 2’,3’-O-(4-benzoyl)benzoyl-adenosine triphosphate (100 μM) induced inward currents, which was inhibited by brilliant blue G. Isoflurane at a concentration of 0.75% dissolved in the extracellular fluid induced inward currents, that were inhibited by brilliant blue G completely, which confirmed that the inward currents induced by isoflurane were mediated by the P2X7 receptor (Figure 3).

Effect of isoflurane on P2X7 receptor protein expression in hippocampal neurons

P2X7 receptor protein expression was determined using western blotting analysis (Figure 4A). Neurons under normal conditions served as control. After neurons were treated with 0.75% isoflurane for 8 hours, the expression
of P2X7 receptor protein in neurons remained unchanged compared with control (P > 0.05; Figure 4B).

DISCUSSION

Isoflurane in clinically related concentrations has been used to study its neurotoxicity in developing brain in previous in vivo or in vitro experiments. In most of the in vivo studies, the animals were exposed to 0.75% isoflurane, which did not affect the respiration, hemodynamics, brain perfusion and blood glucose level[5-8, 16-19]. Thus, the present study investigated the mechanism of the neurotoxicity induced by 0.75% isoflurane.

In this study, 0.75% isoflurane could induce widespread hippocampal neuronal apoptosis in developing brain. This isoflurane-induced apoptosis was completely inhibited by brilliant blue G, a specific P2X7 receptor antagonist[20], which suggested that the neurotoxicity of isoflurane was mediated by P2X7 receptor. However, it remains controversial whether P2X7 receptors are expressed in neurons[21]. It was reported that P2X7 receptor protein could be expressed in cultured developing hippocampal neurons, and the P2X7 receptor specific agonist, B2ATP, could directly induce neuronal apoptosis[15]. Results of this study indicated that isoflurane at clinically relevant concentrations could directly activate P2X7 receptors in developing hippocampal neurons, but had no effect on the expression of P2X7 receptor protein. P2X7 receptor protein was found to be expressed in developing brain but not in adult brain in western blots[15]. Isoflurane had no effect on the expression of the P2X7 receptor in embryonic rat hippocampal neurons, possibly because the expression of P2X7 receptor protein is at peak levels during this period, and isoflurane can facilitate the function of P2X7 receptor by activating it. Also, isoflurane had no effect on P2X7 receptors in microglia cells[22], which was different from the results in this study. It is likely that the cell types are different, or P2X7 receptors in neurons of developing brain are more sensitive to isoflurane. The electrophysiological experiments were performed at room temperature in this study, because neurons could not maintain excitability, and the ligand-gated currents were not stable above room temperature, based on previous studies and our preliminary experiments[23].

Cytoplasmic calcium homeostasis disturbance plays a key role in neuronal apoptosis induced by general anesthetics[6]. It was found that L type calcium channels were not involved in isoflurane-induced neural calcium
overload, and Ca\(^{2+}\) release mediated by IP3 receptor were only partly involved in it\(^{[12]}\). Ca\(^{2+}\) influx occurs when P2X7 receptor-gated ion channels open\(^{[13]}\). This study was the first to show that calcium overload induced by isoflurane was mediated by P2X7 receptor mediated Ca\(^{2+}\) influx and IP3 receptor mediated Ca\(^{2+}\) release. Ca\(^{2+}\) influx resulted from the direct activation of the P2X7 receptor by isoflurane. Also, the effect of isoflurane on the IP3 receptor was mediated by the P2X7 receptor, which did not affect Ca\(^{2+}\) influx. Further studies are needed to investigate how the IP3 receptor is activated after the P2X7 receptor is activated by isoflurane.

Results of this study showed that cytoplasmic calcium homeostasis disturbance induced by isoflurane leads to neuronal apoptosis. The possible mechanisms for calcium homeostasis disturbance induced apoptosis may be: activating apoptotic-related enzymes, such as calpain; causing an overload of mitochondrial Ca\(^{2+}\), resulting in the collapse of mitochondrial membrane potential and release of cytochrome C from the mitochondria into the cytosolic space\(^{[24]}\), with subsequent caspase-3 activation.

Nearly 70% of the neurons undergo physiologic apoptosis during the development of the brain to adapt to brain functions\(^{[25]}\). It is unclear whether neuronal apoptosis induced by isoflurane was the same as physiologic apoptosis. Isoflurane induces widespread apoptosis in the developing brain, but it is still controversial whether isoflurane can induce cognitive dysfunction later on\(^{[5-8, 26]}\). Thus, the relationship between neuronal apoptosis and subsequent cognitive dysfunction needs further research in the future.

In conclusion, isoflurane at clinically related concentrations can directly activate P2X7 receptors in developing hippocampal neurons, and cause cytoplasmic calcium overload by P2X7 receptor-mediated Ca\(^{2+}\) influx and IP3 receptor activation induced Ca\(^{2+}\) release, eventually inducing neuronal apoptosis.

MATERIALS AND METHODS

Design
A molecular biology, electrophysiology, and cellular study.

Time and setting
The experiment was conducted at Central Laboratory of Xuzhou Medical College, China from October 2010 to November 2011.

Materials
Embryonic Sprague-Dawley rats of 18 days old, weighing 3.6–4.0 g, were provided by the Laboratory Animal Center of Xuzhou Medical College (SPF; license No. SYXK (Su) 2003-0038). Protocols were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by Ministry of Science and Technology of China\(^{[27]}\).

Methods
Culture of hippocampal neurons
Rats were decapitated. The hippocampus was dissociated, cut into pieces and treated with 0.25% trypsin at 37°C for 20 minutes. The culture of neurons was performed according to previously described methods\(^{[29]}\). Briefly, the suspension of hippocampal tissue was incubated in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (Gibco, Carlsbad, CA, USA) with fetal bovine serum (Gibco), then gently triturated, filtered through a steel net, and centrifuged at 900 r/min for 5 minutes. The precipitation was suspended with Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12, and plated in the culture dish coated with 0.1% poly-lysine at a cell density of 1 × 10\(^6\)/mL, then placed in the 95%O\(_2\)/5%CO\(_2\) incubator at 37°C. Arabinosylcytosine (Sigma, St. Louis, MO, USA) at 5 μM was added to the medium to inhibit glial cell growth. The culture was refreshed with normal medium every 2–3 days until the experiments were performed 12 days later. Under such conditions, more than 95% neurons were achieved, as characterized by neuron specific enolase antibody staining.

Flow cytometry for detection of neuronal apoptosis
The coverslips with hippocampal neurons were placed in a sealed chamber at 37°C, incubated with Hank’s balanced salt solution for 8 hours, which was aerated with 21%O\(_2\)/5%CO\(_2)/N\(_2\) containing 0.75% isoflurane (Baxter International Inc., Shanghai, China). A gas monitor (Capnomac Ultima, Datex-Ohmeda GE Healthcare, Madison, WI, USA) was used to adjust the isoflurane concentration in the sealed container. Flow cytometry (Becton, and Dickinson, Franklin Lakes, NJ, USA) with the Annexin V/FITC kit (Sigma) was used to measure neuronal apoptosis\(^{[29]}\). In brief, 1 × 10\(^6\) neurons were collected and washed with PBS buffer twice. Annexin V/FITC was then added, and neurons were incubated at room temperature for 10 minutes. Propidium iodide (Sigma) was added (final concentration
1 mg/L). 1 × 10^3 neurons were counted for each sample by flow cytometry, and the percentage of apoptotic cells was detected.

Brilliant blue G was added to the Hank’s balanced salt solution at a final concentration of 1 μM to study the role of the P2X7 receptor in neuronal apoptosis. To observe the effect of Ca^{2+} on neuronal apoptosis induced by isoflurane, Ca^{2+} was removed from the Hank’s balanced salt solution (CaCl_2 was replaced with isotonic NaCl). Xestospongin C (final concentration 1 μM) was added to the Hank’s balanced salt solution with or without Ca^{2+}, and the experiments mentioned above were repeated. Six coverslips with cultured neurons were included in each group.

**Intracellular calcium measurement in hippocampal neurons**

The coverslips with cultured hippocampal neurons were washed with Hank’s balanced salt solution three times, incubated with Hank’s balanced salt solution containing 2.5 μM Fura-2/AM (Sigma) for 40 minutes, placed in a sealed chamber at 37°C, infused with Hank’s balanced salt solution for 10 minutes, then infused with Hank’s balanced salt solution which was aerated with 0.75% isoflurane for 8 hours before the experiment. The intracellular calcium concentrations were measured using a fluorescence spectrophotometer (F2500; Hitachi, Tokyo, Japan). In brief, intracellular calcium concentration was determined according to the following equation: [Ca^{2+}] = K_d (sf/sb) × [(R−R_{min})/(R_{max}−R)], in which K_d is 224 nM, sf2 and sb2 represent the fluorescence at zero calcium level, or maximal calcium level at 380 nm of excitation wavelength; R represents the fluorescence at resting state; R_{min} represents the ratio of fluorescence after application of ethylene glycol bis-(2-aminoethyl)iminodiethylene-tetraacetic acid 5 mM, phosphocreatine 5, creatine triphosphate 2, glucose 10, TTX 0.001, adjusted to pH 7.4 with 0.1 mM KOH. The external solution consisted of (in mM): NaCl 145, KCl 5, ethylene glycol bis-(2-aminoethyl)tetraacetic acid 10, CaCl_2 2, glucose 10, TTX 0.001, adjusted to pH 7.4 with 0.1 mM NaOH. Patch clamp recordings were obtained from an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany) that was monitored with an IBM personal computer running pulse 8.02 software. The patch clamp recordings electrodes were pulled from thin-walled borosilicate glass using a two-stage process from a vertical puller (PIP5, HEKA) to an electrode resistance of 3–6 M in the water bath. When the neuron membrane was ruptured and sealed, series resistance (6–10 M) was compensated by 80% and monitored continually. The neural membrane potential was held at ~60 mV. Data were filtered at 10 kHz and digitized at 25 kHz with pulse 8.02 software, and stored on the hard disk of the computer.

Brilliant blue G and 2’, 3’-O-(4-benzoyl)benzoyl-adenosine triphosphate were prepared on the day of the experiment. The Hank’s balanced salt solution was aerated with 0.75% isoflurane for 8 hours before the experiment, with drug being applied by a RSC-200 system (Warner Instrument Co, Hamden, CT, USA). 100 μM of 2’,3’-O-(4-benzoyl) benzoyl-adenosine triphosphate was applied to the neurons for 5 seconds, and the currents were recorded. Isoflurane (0.75% dissolved in Hank’s balanced salt solution) was applied to the neurons for 5 seconds, the currents were recorded. Then 1 μM brilliant blue G was applied for 4 minutes, and 0.75% isoflurane dissolved in Hank’s balanced salt solution was applied to confirm the inward currents were mediated by P2X7 receptors. Fifteen neurons were recorded with each treatment.

**Western blot analysis of P2X7 receptor protein**

After neurons were treated with 0.75% isoflurane for 8 hours, P2X7 receptor protein expression was determined using western blotting [30]. Neurons under normal conditions served as control. In brief, neurons were pelleted in PBS, and the pellet was used for

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*Measurement of P2X7-gated currents in neurons*

The cultured hippocampal neurons were plated on the coverslips 1 day before the experiment. Whole-cell patch clamp recordings were performed under an inverted microscope (Olympus IX70, Tokyo, Japan) at room temperature. The internal solution consisted of (in mM): KCl 10, MgCl_2 3, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 15, K-adenosine triphosphate 2, ethylene glycol bis(2-aminoethyl) tetraacetic acid 5, phosphocreatine 15, creatine phosphokinase 50 U/mL, adjusted to pH 7.3 with 0.1 mM KOH. The external solution consisted of (in mM): NaCl 145, KCl 5, ethylene glycol bis-(2-aminoethyl) tetraacetic acid 10, CaCl_2 2, glucose 10, TTX 0.001, adjusted to pH 7.4 with 0.1 mM NaOH. Patch clamp recordings were obtained from an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany) that was monitored with an IBM personal computer running pulse 8.02 software. The patch clamp recordings electrodes were pulled from thin-walled borosilicate glass using a two-stage process from a vertical puller (PIP5, HEKA) to an electrode resistance of 3–6 M in the water bath. When the neuron membrane was ruptured and sealed, series resistance (6–10 M) was compensated by 80% and monitored continually. The neural membrane potential was held at ~60 mV. Data were filtered at 10 kHz and digitized at 25 kHz with pulse 8.02 software, and stored on the hard disk of the computer.

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**Western blot analysis of P2X7 receptor protein**

After neurons were treated with 0.75% isoflurane for 8 hours, P2X7 receptor protein expression was determined using western blotting [30]. Neurons under normal conditions served as control. In brief, neurons were pelleted in PBS, and the pellet was used for
protein extraction with cell lysis buffer (Cell Signaling Technologies, Beverly, MA) containing 20 mM Tris•HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂β-glycerophosphate, 1 mM ethylene diamine tetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1 mM phenylmethanesulfonfyl fluoride, with 1% (v/v) Triton X-100 and 1 µg/mL leupeptin. Samples were subjected to 7.5% tricine-sodium dodecyl sulfate gel electrophoresis and transferred to nitrocellulose membranes. The membranes were sequentially probed with a rabbit anti-mouse polyclonal antibody to P2X7 receptor (1:1 000; Bio-Rad, Hercules, CA, USA) at 4°C for 24 hours. Membranes were incubated for 1 hour with goat anti-rabbit IgG (1:400; Bio-Rad) and then rinsed with PBS. Similarly, membranes were incubated with polyclonal rabbit anti-mouse antibody to β-actin (Alpha Diagnostic International, San Antonio, TX, USA) as a control.

A Kodak Bio-Max MS imaging system (Sigma) was used for imaging and analyzing the immunoblots. The expression of P2X7 receptor protein was expressed as the ratio of the absorbance value between the P2X7 receptor protein and β-actin. The experiments were repeated six times with each treatment.

**Statistical analysis**

Data were expressed as mean ± SD, and were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). One-way analysis of variance followed by least squares analysis was performed to compare the differences in percentage of apoptotic neurons or in intracellular calcium concentrations. The student’s t test was used to compare the differences in P2X7 receptor protein expression. A value of P < 0.05 was considered statistically significant.

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**Author contributions:** Hongliang Liu conducted the experiments, and was involved in study design, data analysis, and manuscript drafting. Tijun Dai participated in study design and data analysis. Weitao Guo was involved in study design, and research performance. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** All animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical College, China.

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