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Critical role of P2X7 receptors in the neuroinflammation and cognitive dysfunction after surgery

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Abstract

Postoperative cognitive dysfunction worsens patient outcome after surgery. Neuroinflammation is a critical neuropathological process for it. We determined the role of P2X7 receptors, proteins that participate in inflammatory response, in the neuroinflammation induction after surgery, and whether the choice of volatile anesthetics affects its occurrence. Eight-week old C57BL/6J or P2X7 receptor knockout male mice were subjected to right carotid arterial exposure under anesthesia with 1.8% isoflurane, 2.5% sevoflurane or 10% desflurane. They were tested by Barnes maze and fear conditioning from 2 weeks after the surgery. Hippocampus was harvested 6 h, 24 h and 7 days after the surgery for immunohistochemical staining and Western blotting. Mice with surgery under anesthesia with isoflurane, sevoflurane or desflurane took longer than control mice to identify the target box 1 or 8 days after the training sessions in Barnes maze. Mice anesthetized by isoflurane or sevoflurane, but not by desflurane, had less freezing behavior than control mice in fear conditioning test. Mice with surgery and anesthesia had increased ionized calcium binding adapter molecule 1 and interleukin 1 β in the hippocampus but this increase was smaller in mice anesthetized with desflurane than mice anesthetized with isoflurane. Mice with surgery had increased P2X7 receptors and its downstream molecule caspase 1. Inhibition or knockout of P2X7 receptors attenuated surgery and anesthesia-induced neuroinflammation and cognitive impairment. We conclude that surgery under desflurane anesthesia may have reduced neuroinflammation and cognitive impairment compared with surgery under isoflurane anesthesia. P2X7 receptors may mediate the neuroinflammation and cognitive impairment after surgery.

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Keywords

learning and memory; neuroinflammation; P2X7 receptors; volatile anesthetics

1. Introduction

Postoperative cognitive dysfunction (POCD) is a recognized clinical syndrome (Baranov et al., 2009). It occurs after cardiac and non-cardiac surgery (Moller et al., 1998; Monk et al., 2008; Newman et al., 2001). POCD not only affects the daily life of patients but also is associated with increased mortality within one year after surgery (Monk et al., 2008; Steinmetz et al., 2009). Thus, it is urgently needed to identify the mechanisms and effective interventions for POCD.

We and others have shown that neuroinflammation may be a critical neuropathological process for POCD in rodents (Cao et al., 2012; Cibelli et al., 2010; Terrando et al., 2011; Zhang et al., 2014a; Zhang et al., 2014b). There is also evidence for the occurrence of neuroinflammation after surgery in human (Tang et al., 2011). However, the mechanisms for the induction of neuroinflammation after anesthesia and surgery are not fully understood.

P2X7 receptors have been shown to be involved in inflammatory responses in many organs under various conditions (Arulkumaran et al., 2011). They are purinergic receptors that are inotropic ligand-gated non-selective cation channels. ATP and its breakdown products are known ligands for P2X7 receptors (Burnstock, 2006). The activation of P2X7 receptors facilitates the formation of inflammasome, which activates one of its component precursor caspase 1. The P20 and P10 subunits of caspase 1 form active enzyme that then releases active interleukin (IL)-1 β and IL-18, important proinflammatory cytokines (Arulkumaran et al., 2011; Karmakar et al., 2015; Latz et al., 2013). It has been a consistent finding that activation of P2X7 receptors releases IL-1 β and IL-18 from microglia in the brain, although other inflammatory mediators, such as IL-6, may also be released. Thus, P2X7 receptors are intimately involved in neuroinflammation and inhibiting the activation of these receptors has been proposed as a therapeutic method for various diseases in the brain, such as stroke and neurodegenerative diseases, in which neuroinflammation plays a detrimental role (Bhattacharya and Biber, 2016). We and others have shown that IL-1 β plays a critical role in anesthesia and surgery-induced cognitive dysfunction (Cao et al., 2012; Cibelli et al., 2010; Lin and Zuo, 2011). Thus, we hypothesize that anesthesia and surgery activate P2X7 receptors to induce neuroinflammation and cognitive dysfunction. We tested this hypothesis by using a selective P2X7 antagonist, Brilliant Blue G (BBG) (Jiang et al., 2000), and P2X7 knockout mice in this study.

Most surgeries are performed under general anesthesia in the U.S.A. Our previous studies have shown that the volatile anesthetic isoflurane but not the intravenous anesthetic propofol induces neuroinflammation and cognitive dysfunction in rats (Cao et al., 2012; Zhang et al., 2014a). However, rats that had carotid arterial exposure under isoflurane or propofol anesthesia had a similar degree of neuroinflammation and cognitive dysfunction (Zhang et al., 2015), suggesting that choice of intravenous and volatile anesthetics may not affect the occurrence of POCD. Three volatile anesthetics, isoflurane, sevoflurane and desflurane, are

used clinically in the U.S.A. Desflurane may cause less neurotoxicity in the developing mouse brain (Zhang et al., 2012) and less disruption of intracellular calcium homeostasis than isoflurane (Yang et al., 2008). Although an early study showed that isoflurane and sevoflurane did not affect the activity of P2X7 receptors (Nakanishi et al., 2007), a recent study showed that isoflurane activated P2X7 receptors in rat hippocampal neurons (Liu et al., 2013). The effects of desflurane on P2X7 receptors have not been reported. Interestingly, it is not known whether the choice of volatile anesthetics affects the occurrence of neuroinflammation and cognitive dysfunction after surgery. Such information is very useful to improve patient outcome during the perioperative period. Thus, we compared the degree of neuroinflammation and cognitive dysfunction of mice under surgical level of isoflurane, sevoflurane or desflurane anesthesia for their surgery in this study.

2. Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23) revised in 2011.

2.1. Animal Groups

Eight-week old male C57BL/6J mice weighing 23 – 26 g from Charles River Laboratories International (Wilmington, MA) were randomly assigned to the following groups: (1) control, (2) surgery under anesthesia with 1.8% isoflurane, (3) surgery under anesthesia with 2.5% sevoflurane, and (4) surgery under anesthesia with 10% desflurane. Each group had 12 – 13 mice.

In the second experiment, the mice were randomly assigned to the following groups: (1) control, (2) Brilliant Blue G (BBG) injection, (3) surgery under anesthesia with 1.8% isoflurane plus saline injection, and (4) surgery under anesthesia with 1.8% isoflurane plus BBG injection. Each group had 12 mice. BBG and the saline were injected intraperitoneally.

In the third experiment, eight-week old male P2X7^{-/-} mice weighing 22 – 25 g from the Jackson Laboratory (Bar Harbor, Maine) were randomly assigned to two groups: (1) control and (2) surgery under anesthesia with 1.8% isoflurane. Each group had 8 mice.

Two weeks later, the learning and memory assessment of these mice was performed by Barnes maze and fear conditioning. Separate mice were sacrificed at 6 h, 24 h or 7 days after the surgery for ELISA, Western blotting and immunofluorescent staining.

2.2. Anesthesia and Surgery

The surgery was a right carotid arterial exposure (Fan et al., 2016). Briefly, mice were anesthetized by 1.8% isoflurane, 2.5% sevoflurane or 10% desflurane that were delivered by anesthetic-specific vaporizers. The concentration for each anesthetic was selected to be about 1.2 minimum alveolar concentrations (Ichinose et al., 1998; Sonner et al., 1999). The anesthetic concentrations were monitored with a Datex™ infrared analyzer (Capnomac, Helsinki, Finland). During the procedure, the mouse was kept spontaneous respiration. A

1.5-cm midline neck incision was made after the mouse was exposed to volatile anesthetics at least for 30 min. The soft tissues over the trachea were retracted gently. One centimeter long right common carotid artery was dissected carefully free from adjacent tissues without damaging vagus nerve. The wound was then irrigated and closed by using surgical suture. The surgical procedure was performed under sterile conditions and lasted around 15 min. After the surgery, all animals received a subcutaneous injection of 0.003 mg/kg bupivacaine. The total duration of anesthesia was 2 h, a clinically relevant duration of anesthesia. No response to toe pinching was observed during the whole course of anesthesia. During anesthesia, rectal temperature was monitored and maintained at 37°C with the aid of servo-controlled warming blanket (TCAT-2LV, Physitemp instruments, Clifton, NJ). Mouse's heart rate and pulse oxygen saturation were monitored continuously by MouseOX Murine Plus Oximeter System (Starr Life Sciences Corporation, Oakmont, PA). Animals in the control group of all three sets of experiments did not receive anesthesia, surgery or bupivacaine.

2.3. Drug Application

BBG (Sigma-Aldrich, St. Louis, MO) was prepared freshly each day by dissolving the powder in normal saline and injected intraperitoneally at 50 mg/kg/day for 7 days with the first dose at 15 min before surgery. Similar injections were performed in the BBG only group, except that no surgery and anesthesia were performed. The BBG dose was chosen based on a previous study (Kimble et al., 2012).

2.4. Barnes Maze

Two weeks after surgery, the animals were subjected to Barnes maze as we previously described (Fan et al., 2016) to test their spatial learning and memory. Animals were first placed in the middle of a circular platform with 20 equally spaced holes (SD Instruments, San Diego, CA). One of these holes was connected to a dark chamber called target box. Aversive noise (85 dB) and bright light (200 W) shed on the platform were used to encourage mice to find the target box. They had a spatial acquisition phase that lasted for 4 days with 3 min per trial, 4 trials per day and 15 min between each trial. Animals then went through the reference memory phase to test the short-term retention on day 5 and long-term retention on day 12. No test or handling was performed from day 5 to day 12. The latency to find the target box during each trial was recorded with the assistance of ANY-Maze video tracking system (SD Instruments, San Diego, CA).

2.5. Fear Conditioning

One day after Barnes maze test, mice were subjected to fear conditioning test as we previously described (Fan et al., 2016). Each mouse was placed into a test chamber wiped with 70% alcohol and exposed to 3 tone-foot shock pairings (tone: 2000 Hz, 85db, 30 s; foot shock: 0.7 mA, 2 s) with an interval 1 min in a relatively dark room. The mouse was removed from this test chamber 30 s after the conditioning stimuli. The animal was placed back to the same chamber without the tone and shock 24 h later for 6 min. The animal was placed 2 h later into another test chamber that had different context and smell from the first test chamber in a relatively light room. This second chamber was wiped with 1% acetic acid. Freezing behavior was recorded for 3 min without the tone stimulus. The tone was then turned on for 3 cycles, each cycle for 30 s followed by 1-min inter-cycle interval (4.5 min in

total). Animal behavior in these two chambers was video recorded. The freezing behavior in the 6 min in the first chamber (context-related) and 4.5 min in the second chamber (tone-related) was scored in a 6s interval by an observer who was blind to the group assignment.

2.6. Brain Tissue Harvest

Mice were deeply anesthetized with isoflurane for 2 min and perfused transcardially with saline at 6 h, 24 h or 7 days after anesthesia and surgery. Their hippocampus was dissected out immediately for Western blotting and ELISA assay. The cerebral hemisphere from Bregma -2 to -4 mm was used for immunofluorescent staining.

2.7. Western Blot Analysis

Hippocampal tissues were homogenized in RIPA buffer (catalog number: 89900; Thermo Scientific) containing protease inhibitor cocktail (catalog number: P2714; Sigma-Aldrich) on ice as we did before (Wang et al., 2015). Homogenates were centrifuged at 13,000 g at 4°C for 30 min. The supernatant was saved and its protein concentration was determined by Bradford assay. Twenty microgram proteins per lane were electrophoresed in a polyacrylamide gel and then blotted onto a poly-vinylidene difluoride membrane. After being blocked with Protein-Free T20 Blocking Buffer (catalog number: 37573; Thermo Scientific), membranes were incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-caspase-1 antibody (1:500; catalog number: sc-1218-R; Santa Cruz), rabbit polyclonal anti-P2X7 antibody (1:500; catalog number: APR-004, Alomone labs) and rabbit anti- β -actin antibody (1:4000; Cell Signaling Technology). A secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000; Pierce) was used. Protein bands were visualized by Genesnap version 7.08 and quantified by Genetools version 4.01. The relative protein expression was normalized to those of β -actin proteins from the same sample to control for errors in protein sample loading and transferring during Western analysis.

To determine whether the P2X7^{-/-} mice expressed P2X7 receptors, hippocampus and cerebral cortex of 8-week old male C57BL/6J and P2X7^{-/-} mice were homogenized in a lysis buffer (80 mM HEPES, 200 mM mannitol and 41 mM KOH, pH 7.4) containing 200 μ M phenylmethylsulfonyl fluoride, 0.5 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich). The tissue lysates were then incubated on ice for 30 min and centrifuged at 500 g for 5 min at 4°C. The supernatants were ultra-centrifuged at 100,000 g for 1 h at 4°C. The pellet was re-suspended in 200 μ l lysis buffer thoroughly with sonication. These samples at 20 μ g protein per lane were separated on 12% polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane. The membranes were incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-P2X7 antibody (1:200, catalogue number: sc-25698; Santa Cruz) and rabbit polyclonal anti-Na⁺/K⁺-ATPase antibody (1:1000, catalogue number: 3010; Cell signaling Technology). The relative protein expression of P2X7 receptors was normalized to that of Na⁺/K⁺-ATPase, a known membrane protein (Lee et al., 2001).

2.8. Immunofluorescent staining

The staining and quantification of the staining were performed as we have described before (Zhang et al., 2014a; Zhang et al., 2015). Briefly, mice were killed by deep isoflurane anesthesia and transcardially perfused with 4% paraformaldehyde at 6 h or 7 days after the surgery. Brains were harvested, fixed in 4% paraformaldehyde at 4°C for 24 hours and then embedded in paraffin. Coronal sections at 5 µm were mounted on slides. Antigen retrieval was performed in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 95 to 100°C for 20 minutes. The sections were then incubated with 5% normal donkey serum and 1% bovine serum albumin in Tris-buffered saline for 2 hours at room temperature. To stain ionized calcium binding adapter molecule 1 (Iba-1) for its quantification, the sections were incubated at 4°C overnight with rabbit polyclonal anti-Iba-1 antibody (1:500; Wako Chemicals) and then rinsed in Tris-buffered saline containing 0.25% triton-X 100. The donkey anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (1:200; Invitrogen) was applied for 1 h at room temperature in a dark room. To determine which cell type expressed P2X7, sections were incubated with goat polyclonal anti-Iba-1 antibody (1:200; Abcam) and then donkey anti-goat IgG antibody conjugated with Alexa Fluor 488 (1:200; Invitrogen), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:300; Chemicon) and then donkey anti-mouse IgG antibody conjugated with NL493 (1:200; R&D Systems), or the mouse monoclonal anti-neuronal nuclei (NeuN) antibody (1:250 Millipore) and then donkey anti-mouse IgG antibody conjugated with NL493. To stain P2X7 receptors, the rabbit anti-P2X7 antibody (1:200; Alomone labs) and the donkey anti-rabbit IgG antibody conjugated with NL557 (1:200; R&D Systems) were applied. Images were acquired with a fluorescence microscope with a charge-coupled device camera. A negative control omitting the incubation with the primary antibody was included in all experiments.

For quantification of Iba-1 staining, three independent microscopic fields in each section were randomly acquired in the DG and CA3 areas. Three sections per mouse were imaged. The number of pixels per image with intensity above a predetermined threshold level was considered to be positively stained areas. This measurement was performed by using the Image J 1.47n software. The degree of positive immunoreactivity was reflected by the percentage of the positively stained area in the total area of the image. All quantitative analyses were performed in a blinded fashion.

2.9. Quantification of IL-1β

Brain tissues were homogenized on ice in 20 mM Tris-HCl buffer (pH 7.3) containing protease inhibitors (10 mg/ml aprotinin, 5 mg/ml pepstatin, 5 mg/ml leupeptin, and 1 mM phenylmethanesulfonylfluoride). Homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was then ultra-centrifuged at 150,000 g for 2 h at 4°C. Bradford protein assay of the supernatant was performed for each sample. ELISA kit for measuring IL-1β (catalog number: MLB00C; R&D Systems) were used to quantify its content in the samples according to the manufacturer's instructions as we described before (Zhang et al., 2014a; Zhang et al., 2015). The quantity of IL-1β in each brain sample was standardized to the protein contents. The results from animals under various experimental conditions were then normalized by the mean values of the corresponding control animals in each ELISA assay.

2.10. Statistical analysis

Parametric data with normal distribution are presented as mean $\pm$ S.E.M. ($n = 5$). The other data are in box plot in the figures. One-way or two-way repeated measures analysis of variance followed by Tukey test was used to analyze the data from the training sessions of Barnes maze test within the same group or between groups, respectively. Other data were analyzed by one-way analysis of variance followed by the Tukey test with normally distributed data or by one-way analysis of variance on ranks followed by the Tukey test with non-normally distributed data. Differences were considered statistical significant at $P < 0.05$ based on two-tailed hypothesis testing. All statistical analyses were performed with SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

No animal died during the experiments. Data from all animals in the study were included for analysis.

3.1. Surgery under general anesthesia by different volatile anesthetics might induce different degrees of neuroinflammation and cognitive dysfunction

The time for the control mice and mice after surgery under isoflurane, sevoflurane or desflurane anesthesia to identify the target box in the Barnes maze test was decreased with the increased training sessions. The time for all four groups of mice to identify the target box on training day 3 and 4 were shorter than the time on training day 1 (Fig. 1A). Surgery under isoflurane anesthesia was not a significant factor to affect the time needed to identify the target box during the training sessions [$F(1,24) = 1.842$, $P = 0.187$]. However, mice that had surgery under isoflurane, sevoflurane or desflurane anesthesia took longer than control mice to find the target box on day 1 and day 8 after the training sessions in Barnes maze. There was no significant difference among the three groups of mice with surgery (Fig. 1B). Mice with surgery under isoflurane or sevoflurane anesthesia, but not under desflurane anesthesia, had less freezing behavior than control mice in the context-related fear conditioning, which tests hippocampus-dependent learning and memory (Kim and Fanselow, 1992). Mice with desflurane anesthesia also had more freezing behavior than mice with isoflurane anesthesia in the context-related fear conditioning. However, there was no difference in the freezing behavior of tone-related fear conditioning (Fig. 1C).

Surgery under isoflurane or desflurane anesthesia had increased expression of Iba-1 and IL-1 β in the hippocampus, although this increase was less in the mice under desflurane anesthesia than that in the mice anesthetized by isoflurane (Fig. 2).

3.2. Surgery under isoflurane anesthesia activated caspase 1

To determine which cell types expressed P2X7 receptors, hippocampus was harvested 6 h after the surgery. Positive staining for P2X7 receptors was co-localized with positive staining of Iba-1, a microglial marker, and was not co-localized with positive staining of GFAP and NeuN (Fig. 3A).

Since neuroinflammation appeared to be presented in mice subjected to surgery under general anesthesia with volatile anesthetics, we focused mechanistic studies on mice anesthetized with isoflurane because these mice had obvious neuroinflammation and impairment of learning and memory. P2X7 receptors, precursor caspase 1 and the P20 subunit of caspase 1 in these mice were increased 24 h after the surgery under isoflurane anesthesia (Fig. 3B).

3.3. P2X7 receptor inhibition attenuated anesthesia and surgery-induced caspase 1 activation, neuroinflammation and cognitive dysfunction

The time for control mice and mice treated with BBG, exposed to surgery under isoflurane anesthesia or subjected to surgery under isoflurane anesthesia plus BBG to identify the target box on day 3 and day 4 of training sessions was less than that on day 1 (Fig. 4A). Surgery was a significant factor to affect the time for the mice to find the target box during the training sessions [$F(1,21) = 50.58$, $P < 0.001$]. Although BBG was not a significant factor to affect the time to identify the target box under control condition [$F(1,21) = 0.0612$, $P = 0.807$], BBG significantly affect the time of mice exposed to anesthesia and surgery to identify the target box during the training sessions of Barnes maze [$F(1,22) = 49.225$, $P < 0.001$]. BBG also attenuated anesthesia and surgery-induced increase of time to identify the target box on day 1 and day 8 after the training sessions in the Barnes maze test (Fig. 4B). Similarly, BBG reversed anesthesia and surgery-induced decrease of freezing behavior in the fear conditioning (Fig. 4C).

Consistent with the learning and memory data, BBG attenuated the anesthesia and surgery-induced increase of Iba-1 in the hippocampus at 6 h and 7 days after the surgery (Fig. 5). IL-1 β concentrations in the hippocampus at 6 h after surgery were 2.7 ± 0.4 folds of those in the control group ($P < 0.001$, $n = 12$). This increase was reduced by BBG to 1.4 ± 0.3 folds of those in the control group ($P = 0.007$ compared with anesthesia plus surgery group, $n = 12$), although BBG did not affect IL-1 β concentrations of mice without anesthesia and surgery (0.8 ± 0.1 fold of those in the control group, $P > 0.05$, $n = 12$).

To determine whether the expression changes of P2X7 receptors and the associated proteins were long lasting, their expression was examined at 6 h and 7 days after the surgery. The expression of P2X7 receptors, precursor caspase 1 and P20 subunits was increased 6 h after the surgery. This increase lasted at least till 7 days after the surgery. However, the increase at 7 days after the surgery appeared to be less than that 6 h after the surgery. BBG attenuated these increases except for the increase of P2X7 receptors 6 h after the surgery (Fig. 6).

3.4. P2X7 knockout blocked anesthesia and surgery-induced neuroinflammation and cognitive dysfunction

To provide additional evidence for the involvement of P2X7 receptors in anesthesia and surgery-induced neuroinflammation and impairment of learning and memory, we used P2X7 receptor knockout mice. These mice indeed had their P2X7 receptor knocked out because there was no clear protein band corresponding to P2X7 receptor in the Western blot using cerebral cortical and hippocampal samples (Fig. 7A). In the Barnes maze test, the time for P2X7 receptor knockout mice to identify the target box was decreased with the increase of

training sessions. Surgery was not a significant factor to affect the time of these P2X7 receptor knockout mice to find the target box in the training sessions [$F(1,14) = 0.450$, $P = 0.513$]. Surgery also did not affect the time for them to identify the target box 1 or 8 days after the training sessions, the freezing behavior in the fear conditioning and the concentration of IL-1 β in the hippocampus (Fig. 7).

4. Discussion

Isoflurane, sevoflurane and desflurane are commonly used volatile anesthetics in the U.S.A. About 95% patients anesthetized with 1.2 minimum alveolar concentrations of volatile anesthetics will not respond to surgical stimulation. We used this concentration of isoflurane, sevoflurane and desflurane to anesthetize the mice (Cesarovic et al., 2010; Sonner et al., 1999). Our results showed that mice with surgery under anesthesia by these three anesthetics took longer than control mice to identify the target box in the memory test of the Barnes maze. Also, mice anesthetized with isoflurane and sevoflurane for the surgery had reduced context-related freezing behavior compared with control mice. These results suggest that surgery and anesthesia impair learning and memory, a finding that is consistent with previous studies (Cao et al., 2012; Cibelli et al., 2010; Terrando et al., 2011; Zhang et al., 2014a; Zhang et al., 2014b).

Our results showed that mice anesthetized with desflurane for surgery had more context-related freezing behavior than mice anesthetized with isoflurane. In addition, mice anesthetized with desflurane had reduced levels of Iba-1 and IL-1 β , indicators of inflammation (Wang et al., 2015; Wang et al., 2013), in the hippocampus compared with mice anesthetized with isoflurane. These results suggest that surgery under desflurane anesthesia may have reduced neuroinflammation and cognitive impairment and that desflurane anesthesia may be preferable in this regard. Consistent with our finding, desflurane anesthesia for 6 h caused less neurotoxicity in the developing mouse brain than isoflurane anesthesia. No surgery was performed on these mice (Zhang et al., 2012). Desflurane also caused less disruption of intracellular calcium homeostasis than isoflurane in cell cultures (Yang et al., 2008). Our previous studies have shown that a short exposure to isoflurane immediately before or after a damaging insult provides protection in rat brain and bovine pulmonary arterial endothelial cells. However, this protection is not induced by desflurane (Kim et al., 2009; Li and Zuo, 2009). These findings suggest that desflurane may behave differently from other currently used volatile anesthetics in inducing cell protection or injury.

We and others have shown the critical role of IL-1 β in anesthesia and surgery-induced cognitive dysfunction (Cao et al., 2012; Cibelli et al., 2010; Lin and Zuo, 2011). IL-1 β is stored intracellularly in the form of precursor protein. Cleavage is required to process the precursor into mature IL-1 β . Caspase 1 is a critical enzyme for this process. Signals through various plasma membrane proteins, such as P2X7 receptors, induce the formation of inflammasome. Caspase 1 is a component of the inflammasome and is activated in this multiple protein complex. The pre-IL-1 β can be located in proximity to the activated caspase 1 for processing (Arulkumaran et al., 2011; Karmakar et al., 2015; Latz et al., 2013). Our results suggest that this mechanism contributes to the neuroinflammation and cognitive

dysfunction after surgery because inhibition or knockout P2X7 receptors attenuated anesthesia and surgery-induced neuroinflammation and impairment of learning and memory. Also, anesthesia and surgery increased the precursor and active caspase 1 and this increase was attenuated by BBG, a selective P2X7 receptor antagonist (Jiang et al., 2000). These findings suggest that P2X7 receptors and caspase 1 may be targets for reducing neuroinflammation and cognitive impairment after surgery.

Our results suggest a rapid response of the brain P2X7 receptor signaling pathway to surgery and anesthesia because the active caspase 1 subunit P20, Iba-1 and IL-1 β in the hippocampus were increased in the hippocampus 6 h after the surgery. However, this activation is relatively short-lived because the degree of P20 increase was reduced 7 days after the surgery compared with that 6 h after the surgery. This feature is consistent with previous findings that neuroinflammation is short-lived after surgery (Cibelli et al., 2010; Zhang et al., 2014a; Zhang et al., 2014b). For example, the increase of Iba-1, a neuroinflammatory marker, was reduced 10 days after carotid arterial exposure in rats in our previous study (Zhang et al., 2014b) and 7 days after the carotid arterial exposure in mice in this study. Brain changes, such as reduced neurogenesis, may then occur to contribute to learning and memory impairment detected at a later phase (Fan et al., 2016).

The immunostaining of P2X7 receptors are co-localized with Iba-1, a microglial marker, but not with GFAP, an astrocytic marker, or NeuN, a neuronal marker (Zhang et al., 2014b), in the hippocampus. These results suggest that P2X7 receptors are expressed mostly in the microglia. This cell type distribution is similar to that reported in the spinal cord (Chu et al., 2010; Ying et al., 2014).

BBG is described as a selective P2X7 receptor antagonist (Jiang et al., 2000). Our results showed that increase of P2X7 receptors 7 days after the surgery was attenuated by BBG. Blocking the increased expression of P2X7 receptors by BBG has been reported in previous studies (Choi et al., 2007; Chu et al., 2010). However, the mechanisms for this effect are not clear.

Of note, the potential neurotoxicity of long duration anesthetic exposure has been studied extensively in the last decade (Cao et al., 2012; Lin and Zuo, 2011; Zuo, 2012). However, patients do not usually receive anesthesia without an accompanying surgical procedure. To simulate clinical situation, we subjected animals to surgery under general anesthesia and gave local anesthesia for pain control after surgery. Thus, the goal of our study was not to determine which perioperative factors, such as anesthesia or surgical procedure, are important for the neuroinflammation or impairment of learning and memory. We rather wanted to determine whether anesthetic choice would affect these detrimental effects on the brain under clinical situation and the role of P2X7 receptors in these effects. Thus, general anesthesia, surgery and local anesthesia were considered a combination that often occurs in patients having surgery. Our control mice were not exposed to any anesthesia or surgery. Thus, our study design is identical to many clinical studies in which a surgical patient group is compared with a control group (Moller et al., 1998; Monk et al., 2008; Newman et al., 2001). Anesthesia alone group was not included in this study because of the focus of our study.

We performed carotid arterial exposure that is a component of carotid endarterectomy, a common surgery performed in elderly patients. This surgery does not affect sensory and motor functions of extremities, which may be confounding factors in interpreting the results of cognitive tests.

Interestingly, the difference between control group and surgery group with isoflurane anesthesia in the performance of Barnes maze test was larger in the second experiment (data are presented in figure 4) than that in the first experiment (data are presented in figure 1). Although the experimental condition was designed to be identical, many factors, such as different sets of animals and subtle change in environment, can influence animal performance in learning and memory tests. Thus, it is important to assign the same set of animals to different groups of one experiment and assess their learning and memory at the same time as we did in this study.

Our findings may have significant implications. Our results suggest that surgery under desflurane anesthesia may have reduced neuroinflammation and cognitive impairment. If this finding is confirmed in human, desflurane anesthesia could be preferable in clinical practice to reduce the occurrence of POCD. Our study also showed that BBG reduced neuroinflammation and cognitive impairment after surgery. BBG is a commonly used food color reagent with minimal toxicity (Peng et al., 2009). BBG use could be a practical intervention for reducing POCD. Finally, our results suggest that agents specifically inhibiting P2X7 receptors and caspase 1 may be used to reduce cognitive dysfunction and neuroinflammation after surgery.

Our study has limitations. We compared the effects of one concentration of isoflurane, sevoflurane and desflurane on the occurrence of neuroinflammation and cognitive impairment. It is not known whether the different results of isoflurane vs. desflurane observed here exist in other concentrations of the drugs.

In summary, we have shown that surgery under desflurane anesthesia may have a reduced level of neuroinflammation and impairment of learning and memory. P2X7 receptors and the formation of caspase 1 containing inflammasome may contribute to the anesthesia and surgery-induced neuroinflammation and cognitive impairment.

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Highlights

- Anesthetic choice may affect the degree of neuroinflammation and cognitive dysfunction after surgery
- Surgery activates P2X7 receptors and its associated protein.
- P2X7 receptors mediate surgery-induced neuroinflammation and cognitive dysfunction

Fig. 1.

Surgery and anesthesia impaired the learning and memory. Mice were started to be tested by Barnes maze and fear conditioning 2 weeks after the surgery. A: training sessions of Barnes maze. Results are mean $\pm$ S.E.M. ($n = 12 - 13$). * $P < 0.05$ compared with the corresponding data on day 1. B: memory phase of Barnes maze. Results are in box plot format ($n = 12 - 13$). •: lowest or highest score (the score will not show up if it falls in the 95th percentile); between lines: 95th percentile of the data; inside boxes: 25th to 75th percentile including the median of the data. ^ $P < 0.05$ compared with control group. C: Fear conditioning. Results

are mean $\pm$ S.E.M. ($n = 12 - 13$). $^{\wedge} P < 0.05$ compared with control group; $\# P < 0.05$ compared with surgery plus isoflurane anesthesia.

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Fig. 2. Surgery and anesthesia induced neuroinflammation. Hippocampus was harvested 6 h after the surgery. A: representative images of Iba-1 staining. Scale bar = 100 μ m. B: quantitative data of Iba-1. C: IL-1 β concentrations in the hippocampus. Results are mean $\pm$ S.E.M. (n = 6). * P < 0.05 compared with control group; ^ P < 0.05 compared with surgery plus isoflurane anesthesia.

A

B

C

Fig. 3.

Surgery increased P2X7 receptor and caspase 1 expression. Hippocampus was harvested 6 h after the surgery for immunohistochemical staining or 24 h after the surgery for Western blotting. A: representative images of immunostaining. Scale bar = 100 μ m. B: representative images of Western blotting. C: quantitative data of Western blotting. Results are mean $\pm$ S.E.M. (n = 5 – 6). * P < 0.05 compared with control group.

Fig. 4.

Inhibition of P2X7 receptors attenuated surgery-induced learning and memory impairment. Mice were started to be tested by Barnes maze and fear conditioning 2 weeks after the surgery under isoflurane anesthesia. A: training sessions of Barnes maze. Results are mean $\pm$ S.E.M. (n = 12). * P < 0.05 compared with the corresponding data on day 1. B: memory phase of Barnes maze. Results are in box plot format (n = 12). •: lowest or highest score (the score will not show up if it falls in the 95th percentile); between lines: 95th percentile of the data; inside boxes: 25th to 75th percentile including the median of the data. ^ P < 0.05

compared with control group. # $P < 0.05$ compared with surgery plus isoflurane anesthesia group. C: Fear conditioning. Results are mean $\pm$ S.E.M. (n = 11 – 12). ^ $P < 0.05$ compared with control group; # $P < 0.05$ compared with surgery plus isoflurane anesthesia.

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Fig. 5.

Inhibition of P2X7 receptors attenuated surgery-induced neuroinflammation. Hippocampus was harvested 6 h or 7 days after the surgery. A: representative images of Iba-1 staining of hippocampus harvested 6 h after the surgery. Scale bar = 100 μ m. B: quantitative data of Iba-1 of hippocampus harvested 6 h after the surgery. C: representative images of Iba-1 staining of hippocampus harvested 7 days after the surgery. D: quantitative data of Iba-1 of hippocampus harvested 7 days after the surgery. Results are mean $\pm$ S.E.M. (n = 5 for panels B and D). * $P < 0.05$ compared with control group; ^ $P < 0.05$ compared with surgery plus isoflurane anesthesia group.

Fig. 6.

Inhibition of P2X7 receptors attenuated surgery-induced increase of P2X7 receptor and caspase 1 expression. Hippocampus was harvested 6 h or 7 days after the surgery under isoflurane anesthesia. A and B: representative images of Western blotting. C: quantitative data of P2X7 receptors. D: quantitative data of P20. E: quantitative data of precursor caspase 1. Results are mean $\pm$ S.E.M. (n = 6). * $P < 0.05$ compared with control group; ^ $P < 0.05$ compared with the corresponding surgery plus isoflurane anesthesia group; # $P < 0.05$ compared with the level of P20 at 6 h after surgery under isoflurane anesthesia.

Fig. 7.

P2X7 receptor knockout blocked surgery-induced neuroinflammation and cognitive impairment. Cerebral cortex and hippocampus of C57BL/6J mice or P2X7 receptor knockout mice were harvested for Western blotting of P2X7 receptors. Also, P2X7 receptor knockout mice were started to be tested by Barnes maze and fear conditioning 2 weeks after the surgery under isoflurane anesthesia. Mouse hippocampus was harvested 6 h after the surgery for measuring IL-1 β . A: Western blot images of P2X7 receptor. B: training sessions of Barnes maze. Results are mean $\pm$ S.E.M. (n = 8). * P < 0.05 compared with the corresponding data on day 1. C: memory phase of Barnes maze. Results are in box plot

format ($n = 8$). •: lowest or highest score (the score will not show up if it falls in the 95th percentile); between lines: 95th percentile of the data; inside boxes: 25th to 75th percentile including the median of the data. D: Fear conditioning. Results are mean $\pm$ S.E.M. ($n = 8$). E: IL-1 β concentrations in the hippocampus. Results are mean $\pm$ S.E.M. ($n = 5$).