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Pretreatment with volatile anesthetics, but not with the nonimmobilizer 1,2-dichlorohexafluorocyclobutane, reduced cell injury in rat cerebellar slices after an *in vitro* simulated ischemia

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Abstract

A prior exposure to the volatile anesthetic isoflurane has been shown to induce neuroprotection in rats. This phenomenon is called preconditioning. We designed this study to determine whether the potency of volatile anesthetics in inducing neuroprotection is related to their potency to induce anesthesia. Cerebellar slices of adult male Sprague-Dawley rats were exposed to various concentrations of isoflurane, halothane, sevoflurane, desflurane or the nonimmobilizer 1,2-dichlorohexafluorocyclobutane for 15 min, followed by a 15-min drug-free period, and then were subjected to oxygen-glucose deprivation for 10 min at 37°C. After a 5-hr recovery at 37°C, brain slices were used for quantification of cell injury by spectrophotometric measurement of formazan produced from 2,3,5-triphenyltetrazolium chloride. All four volatile anesthetics induced a concentration-dependent preconditioning effect. The EC50 for this effect induced by isoflurane, halothane, sevoflurane or desflurane was 221, 173, 184 and 929 μM, respectively. This EC50 was linearly correlated with the aqueous concentration of one minimum alveolar concentration. The volatile anesthetic preconditioning-induced neuroprotection was abolished by DL-threo-β-hydroxyaspartic acid, DL-threo-β-benzyloxyaspartate or dihydrokainate, glutamate transporter inhibitors. The volatile nonimmobilizer 1,2-dichlorohexafluorocyclobutane at any concentrations tested in the study did not induce a significant preconditioning effect. Isoflurane preconditioning did not change the oxygen-glucose deprivation-induced glutamate accumulation. These results suggest that the preconditioning-induced neuroprotection by volatile anesthetics is not agent-specific. Mechanisms that are involved in inducing anesthesia may contribute to the induction of preconditioning effect by volatile anesthetics. Modification of glutamate transporter activity may be one of such mechanisms to induce these protective effects.

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Keywords

glutamate transporters; neuroprotection; oxygen-glucose deprivation; preconditioning; volatile anesthetics

1. Introduction

Ischemic brain injury is the underlying pathophysiology of stroke and brain trauma that are leading causes of mortality and morbidity in the USA (Martin et al., 1999). Although intensive research has been performed for many years, clinically effective and safe interventions to reduce ischemic neuronal death have not been well established yet. Thus, finding such interventions is an urgent need in medical research.

The concept ischemic preconditioning was introduced about 21 years ago (Murry et al., 1986, Schurr et al., 1986). Ischemic preconditioning is a phenomenon in which brief episodes of sub-lethal ischemia induce a robust protection against the deleterious effects of subsequent, prolonged, lethal ischemia (Murry et al., 1986, Ferrari et al., 1999, Tomai et al., 1999). Subsequently, several preconditioning stimuli, in addition to short episodes of ischemia, also have been shown to be effective at providing neuroprotection. These stimuli include spreading depression, hypoxia, hypothermia, hyperthermia, long-term hyperbaric oxygen, toxins and cytokines (Page et al., 1989, Fu et al., 1994, Kobayashi et al., 1995, Wada et al., 1996, Nawashiro et al., 1997, Tasaki et al., 1997, Nishio, 1999). One of the drawbacks of most preconditioning stimuli (such as ischemic preconditioning) is that they also are capable of producing injury with only minor changes in their intensity or duration. Another limitation is that many preconditioning methods will be difficult to implement in the clinic due to the dangers (such as toxins and cytokines) or the complex biological effects (such as hyperthermia and hypothermia) of the stimuli (Nishio, 1999). Thus, identifying relatively safe drugs to precondition the central nervous system will be important for potential clinical use.

We and others have shown that the volatile anesthetics isoflurane and sevoflurane also can induce preconditioning effects in the brain (Kapinya et al., 2002, Zheng and Zuo, 2003, Kehl et al., 2004, Zhao and Zuo, 2004, Zheng and Zuo, 2004, Payne et al., 2005). Ischemic preconditioning-induced protection has two temporal phases: acute phase and delayed phase. The acute phase appears within minutes after a preconditioning stimulus, sustains for a few hours and usually is mediated by altered functions of proteins that already exist (Nandagopal et al., 2001). Delayed phase develops a few hours after a preconditioning event, can sustain for several days and often requires new protein synthesis (Nandagopal et al., 2001, Schulz, 2001). Volatile anesthetic preconditioning-induced neuroprotection also has the acute phase and the delayed phase (Zheng and Zuo, 2003, Zheng and Zuo, 2004).

It is not known, however, whether the potency of volatile anesthetics to induce preconditioning effects in the brain is related to their potencies to induce anesthesia. Three volatile anesthetics are commonly used in the USA: isoflurane, sevoflurane and desflurane. While halothane has a long history of use, halothane is no longer manufactured and sold. These anesthetics share many properties and effects, such as inducing anesthesia, reducing cerebral metabolic rate and having a cerebral vasodilating effect (Drummond and Patel, 2000). However, these drugs are significantly different in many aspects. For example, the order of cerebral vasodilating potency is halothane > isoflurane = sevoflurane = desflurane (Drummond and Patel, 2000). Patients under isoflurane anesthesia tolerate lower cerebral blood flow without EEG evidence of brain ischemia than do patients under halothane anesthesia (Michenfelder et al., 1987). However, animal data have not consistently shown that isoflurane provides better neuroprotection than other volatile anesthetics when they are applied during focal brain ischemia (Drummond and

Patel, 2000). We hypothesize that the commonly used volatile anesthetics, but not 1,2-dichlorohexafluorocyclobutane (F6), a nonimmobilizer that is structurally related to volatile anesthetics, can induce preconditioning effects in the brain. To test this hypothesis, we used rat cerebellar slices and simulated ischemia *in vitro* by oxygen-glucose deprivation (OGD) to determine the acute preconditioning effects of volatile reagents.

2. Results

The 2,3,5-triphenyltetrazolium chloride (TTC) staining method has been a useful technique to quantify infarct volume after focal brain ischemia. Staining the brain tissue with TTC and then measuring the extracted formazan by spectrophotometry has been used to quantify brain injury in rodents after brain ischemia in many publications (Preston and Webster, 2000, Jiang et al., 2005, Weaver et al., 2005). This method could be a relatively simple and objective approach to assess brain injury that is diffused in nature. We applied this method to quantify cell injury in the cerebellar slices after OGD. As shown in figure 1, the longer the OGD was, the greater the cell injury. The formazan production in brain slices exposed to a 10-min OGD was $59 \pm 19\%$ of control ($P < 0.001$). We used this duration of OGD for subsequent experiments.

Preconditioning by the four volatile anesthetics induced a concentration-dependent protection against OGD (Fig. 2). Notably, the injury of cerebellar slices exposed to $74\mu\text{M}$ halothane and then OGD was more severe than that of slices subjected to OGD only (Fig. 2A). The results of isoflurane treated slices had bigger standard deviations than those of slices treated with other volatile anesthetics (Fig. 2). The reasons for these two phenomena are not known. We performed the experiments in the same way. Low concentrations of halothane have not been shown to be detrimental to the brain after ischemic injury. Future studies are needed to confirm this phenomenon.

The EC₅₀ for the preconditioning-induced neuroprotection by isoflurane, halothane, sevoflurane and desflurane was 221, 173, 184 and $929\mu\text{M}$, respectively. Strikingly, this EC₅₀ was linearly related with the aqueous concentration of one minimum alveolar concentrations (MAC) (Fig. 3, $r = 0.988$ for the positive correlation, $P < 0.01$). However, caution needs to be exercised for this interpretation because 3 out of the 4 data points are close to each other and relationships other than linear correlation between the EC₅₀ and the MAC cannot be reasonably excluded unless more data points are added between the isoflurane and desflurane data points (Fig. 3). However, volatile anesthetics whose aqueous concentrations of MAC are between isoflurane and desflurane are not available yet (Campagna et al., 2003, Sonner et al., 2003). Interestingly, pretreatment of the brain slices with the nonimmobilizer F6 did not significantly reduce the injury caused by OGD (Fig. 4). We also failed to find a best-fit curve for the data to calculate an EC₅₀ by using the GraphPad Prism 4 program.

The protection induced by 2 MAC isoflurane, halothane or sevoflurane was abolished by 1 mM DL-threo- β -hydroxyaspartic acid (THA) (Fig. 5); whereas THA alone did not change the OGD-induced cell injury in the cerebellar slices ($51 \pm 3\%$ of control under OGD condition, $n = 4$ vs. $49 \pm 14\%$ of control under OGD + THA condition, $n = 6$, $P > 0.05$). Cerebellar slices pretreated with 2 MAC desflurane had reduced OGD-induced injury and slices pretreated with 1 mM THA and 2 MAC desflurane before the OGD suffered from the same degree of injury as slices subjected to the OGD only (Fig. 5A). These results suggest that THA also inhibits the desflurane preconditioning-induced neuroprotection. The protection induced by 2 MAC isoflurane also was inhibited by $200\mu\text{M}$ DL-threo- β -benzyloxyaspartate (TBOA) or $300\mu\text{M}$ dihydrokainate, although TBOA or dihydrokainate alone did not affect the OGD-induced cell injury (Fig. 5). THA and TBOA are two general glutamate transporter (also named excitatory amino acid transporter, EAAT) inhibitors (Swanson et al., 1997, Koch et al., 1999) and dihydrokainate is a relatively specific inhibitor for EAAT type 2 (Arriza et al., 1994). OGD

significantly increased the glutamate levels in the extracellular fluid. This glutamate accumulation was not reduced by preconditioning with various concentrations of isoflurane (Fig. 6).

3. Discussion

It has been shown that isoflurane and sevoflurane can induce a preconditioning effect in rat brain (Kapinya et al., 2002, Zheng and Zuo, 2003, Kehl et al., 2004, Zhao and Zuo, 2004, Zheng and Zuo, 2004, Payne et al., 2005). We found that isoflurane, halothane, sevoflurane and desflurane induced an acute preconditioning effect in rat cerebellar slices. These results suggest that induction of preconditioning effect is a shared feature among volatile anesthetics and may be mediated by common pathways activated by volatile anesthetics.

Since induction of anesthesia is a shared effect among anesthetics, we determined whether the potency of volatile anesthetics of inducing anesthesia is correlated with their potency of inducing the preconditioning effects in the brain. Our results showed a statistically significant linear correlation between these two potencies, suggesting shared mechanisms for volatile anesthetics in inducing anesthesia and preconditioning effects. Consistent with this notion, the nonimmobilizer F6 did not induce a preconditioning effect. F6 is a polyhalogenated cyclobutane that is structurally-related to volatile anesthetics. The predicted MAC of F6, based on the Meyer-Overton correlation, is 4.2% (Koblin et al., 1994). However, F6 does not induce anesthesia and can cause convulsion with an EC50 3.5% (Eger et al., 2001).

Anesthesia mechanisms have been studied for many decades. However, the mechanisms of anesthesia induction by volatile anesthetics are not well understood yet. A popular theory is that anesthesia is induced by inhibiting excitatory neurotransmission and/or by enhancing inhibitory neurotransmission (Nelson et al., 2002, Campagna et al., 2003). In searching for the mechanisms/molecules that may be involved in induction of both anesthesia and preconditioning effects, EAATs appear to be good candidates. EAATs are expressed in neurons and glial cells and transport glutamate from the extracellular space to intracellular compartments under physiological conditions (Danbolt, 2001). Thus, glutamate transporters play a critical role in securing a high signal-to-noise ratio in synaptic transmission and in preventing harmful receptor over-stimulation. It has been shown that inhibition of EAAT activity prolongs glutamate-induced excitatory postsynaptic current (Mennerick and Zorumski, 1994). Intrathecal injection of EAAT inhibitors increased the MAC for isoflurane in rats (Cechova and Zuo, 2006). Volatile anesthetics have been shown to increase EAAT activity (Do et al., 2002, Huang and Zuo, 2005, Huang et al., 2006). Thus, EAATs may be a site of action for the isoflurane-induced anesthesia. Interestingly, the EAAT inhibitor THA abolished isoflurane preconditioning-induced neuroprotection in rat cerebellar slices (Zheng and Zuo, 2003). In our current study, THA also inhibited halothane, sevoflurane or desflurane preconditioning-induced neuroprotection. The isoflurane preconditioning-induced neuroprotection was inhibited by TBOA and dihydrokainate, two non-transportable EAAT inhibitors (Koch et al., 1999). These results suggest the involvement of EAAT in the induction of the neuropreconditioning effects of volatile anesthetics. Thus, the available data suggest a critical role of EAATs in the induction of anesthesia and preconditioning effects by volatile anesthetics.

Ischemia-induced extracellular glutamate accumulation and the subsequent excitotoxicity contribute significantly to ischemic brain injury (Lipton, 1999). EAAT internalizes glutamate under physiological condition. Due to energy supply failure, EAAT reverses glutamate transport, meaning that EAAT transports glutamate from intracellular space to extracellular space, under ischemic conditions. This reversed transport has been shown to be one of the major mechanisms for ischemia-induced glutamate accumulation (Jabaudon et al., 2000, Rossi

et al., 2000). In line with these findings, one possible mechanism for volatile anesthetic preconditioning-induced neuroprotection could be reduction of the reversed transport and glutamate accumulation during ischemia. However, our results do not support this idea because isoflurane preconditioning did not change the OGD-induced extracellular glutamate accumulation in this study. In addition to the well-documented transporting function, activity of EAATs has been shown to lead to phosphorylation/activation of extracellular signal-regulated kinase (Abe and Saito, 2001), a protein kinase whose activation is shown to be involved in cell survival after ischemia (Hu et al., 2000). Thus, EAAT functions other than its transporting function may be involved in the volatile anesthetic preconditioning-induced neuroprotection.

Our study has limitations. We used a TTC conversion method to quantify the cell injury. It is not possible to determine what types of cells are injured by OGD and are protected by the preconditioning effects. However, since neurons are generally more sensitive to ischemic injury than glial cells, it is likely that our findings mainly reflect the changes of neurons in the cerebellar slices. We anesthetized rats with isoflurane before we removed their brains for slice preparation. The slices might have been preconditioned by isoflurane. However, as we showed before (Zheng and Zuo, 2003), isoflurane exposure for less than 5 min induced a minimal preconditioning effect. Since it took less than 2 min to anesthetize the rats by isoflurane, the preconditioning effect by this short isoflurane exposure should be minimal. In addition, all brain slices in various study groups were equally exposed to this isoflurane exposure.

In summary, we have shown that preconditioning with the volatile anesthetic isoflurane, sevoflurane, desflurane and halothane induced neuroprotection. The potency of this effect is linearly correlated with their anesthetic potency. The nonimmobilizer F6 did not induce a preconditioning effect. EAAT, probably via functions other than its transporting function, may mediate the volatile anesthetic preconditioning-induced neuroprotection.

4. Experimental Procedure

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia. All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

Isoflurane and desflurane were obtained from Ohmeda Inc. (Liberty Corner, NJ). Halothane was purchased from Halocarbon Laboratories (River Edge, NJ). Sevoflurane was obtained from Abbott International Ltd. (Abbott Park, IL). F6 was acquired from PCR (Gainesville, FL). Unless specified in the text, all other reagents were obtained from Sigma (St. Louis, MO).

4.1. Preparation of brain slices

Similar to the methods reported before (Li et al., 2002, Zheng and Zuo, 2003, Yuan et al., 2004), cerebellar brain slices were prepared from 2- to 3-month-old, 250 - 300 g, Sprague-Dawley male rats (Hilltop, Scottdale, PA). Rats were anesthetized with 3% isoflurane and then decapitated. Cerebellum was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 5% CO₂-95% O₂. The aCSF contained 116 mM NaCl, 26.2 mM NaHCO₃, 5.4 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, 0.9 mM NaH₂PO₄, and 5.6 mM glucose, pH 7.4. The cerebellum was then sectioned with a tissue slicer into 400µm transverse slices. After sectioning, slices were placed into cell strainers (70µm Nylon Sterile/gamma irradiated; Becton Dickinson Labware, Franklin Lakes, NJ). These strainers allowed free diffusion of gases and fluid to slices and avoided direct gas bubbling on slices. The slices remained in oxygenated aCSF at room temperature for about 1 h for recovery of the synaptic

function. Slices then were placed in oxygenated aCSF at 37°C for 15 min before they were subjected to various incubation conditions.

4.2. Pretreatment by volatile anesthetics or F6

aCSF (50 ml) was continuously pre-gassed with various concentrations of volatile anesthetics or F6 in the carrier gases 5% CO₂-95% O₂ for 30 min at 37°C. A single direction of gas delivery was used to deliver volatile anesthetics and F6. Care was taken to make sure the opening of the gas delivery tube was at the bottom of the solution. Cerebellar slices were transferred into the pre-gassed aCSF and the incubation was for 15 min at 37°C. During the incubation, the aCSF was continuously bubbled with the same pre-gases containing volatile anesthetics or F6. The slices then are transferred into a glass beaker containing 50 ml aCSF gassed with 5% CO₂-95% O₂ for 15 min at 37°C before they were subjected to a 10-min OGD. This protocol will reduce/eliminate the presence of volatile reagents during the OGD so that the preconditioning effects can be studied. The concentrations of volatile anesthetics studied were 0.4, 0.7, 1.0, 1.5, 2.0 and 3.0 MAC. One MAC for halothane, isoflurane, desflurane and sevoflurane was defined to be 0.7, 1.1, 6.0, and 1.9%, respectively (Orliaguet et al., 2001). They were delivered by agent-selective vaporizers and their concentrations in the gases were monitored by a Datex infrared analyzer (Capnomac, Helsinki, Finland). We aimed to deliver 0.8, 1.7, 2.9, 4.2, 6.3 and 7% F6 by an enflurane vaporizer because F6 and enflurane have a similar vapor pressure (182 and 184 mmHg, respectively, for F6 and enflurane) (Koblin et al., 1994; Eger, 2000). The concentrations of volatile anesthetics and F6 in the aCSF were determined by gas chromatography as we described previously (Zuo and Johns, 1995). The aqueous concentrations were approximately 184, 247, 194, and 472 μM, respectively, for 1 MAC halothane, isoflurane, sevoflurane and desflurane and 24 μM for 4.2% F6.

4.3. Pretreatment by glutamate transporters

To determine whether glutamate transporters are involved in volatile anesthetic preconditioning-induced neuroprotection, cerebellar slices were preincubated with 1 mM THA for 30 min at 37°C. Brain slices then were preconditioned with 2 MAC of halothane, isoflurane, sevoflurane or desflurane for 15 min in the presence of 1 mM THA. Additional experiments were performed by preincubating cerebellar slices with 200 μM TBOA or 300 μM dihydrokainate for 30 min at 37°C and then preconditioned the slices with 2 MAC of isoflurane for 15 min in the presence of 200 μM TBOA or 300 μM dihydrokainate. After a 15-min drug-free period, the brain slices were subjected to a 10-min OGD. Control experiments were performed by exposing cerebellar slices to 1 mM THA, 200 μM TBOA or 300 μM dihydrokainate for 45 min followed by a 15-min drug-free period before a 10-min OGD.

4.4. *In vitro* oxygen-glucose deprivation

Ischemia was simulated *in vitro* by OGD. As described previously (Popovic et al., 2000; Zheng and Zuo, 2003; Yuan et al., 2004), cerebellar slices were transferred into a glass beaker containing glucose-free aCSF (also containing 1 mM dithionite, an oxygen absorbent) bubbled with 5% CO₂ and 95% N₂ (30-min bubbling before the placement of brain slices was allowed to reduce the oxygen content in the solution). Under these conditions, the PO₂ in the aCSF was lower than 0.1 mmHg as measured by a Clark oxygen electrode (Cameron Instrument Co., Port Aransas, TX). The beaker containing the slices was immersed in a water bath to maintain the glucose-free aCSF temperature at 37 ± 0.2 °C. In the time-course experiments, the OGD was maintained for 5, 10, 15, 20, 25, and 30 min. In the anesthetic and F6 pretreatment experiments, the OGD was maintained for 10 min. After the OGD, slices were recovered in circulating oxygenated aCSF at 37 °C for 5 hr to allow cell injury and death that may not be evident immediately after the OGD episode to become apparent.

4.5. Quantification of cell injury

Cell injury after OGD was quantified by the conversion of TTC to formazan as described previously (Preston and Webster, 2000). TTC is normally colorless and can be reduced by succinate dehydrogenase in mitochondria of living cells to formazan that is red. TTC (2%) was freshly dissolved in solution containing: 140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 10 mM Hepes and 3 mM glucose. Slices were placed in 3 ml of fresh TTC solution contained in each well of 12-well plate for 80 minutes at $37 \pm 0.2^\circ\text{C}$. The TTC solution was removed and the slices were rinsed twice by 3 ml normal saline. The formazan in the brain slices was extracted by 2.5 ml 50:50 mixture of ethanol/dimethylsulfoxide. The 12-well plate was sealed and placed in dark for 24 hr. The red solvent extract (1000 μl) in duplicates was placed in cuvettes and the absorbance was read at 485 nm in a spectrophotometer. The absorbance of the duplicates was averaged to reflect the amount of formazan in the sample. Preliminary study in our laboratory showed that this method of extraction was complete because a longer extraction time (48 hr) did not increase the reading, a second extraction after the first extraction had a reading similar to the plain extraction solution and the brain slices were white after the extraction. The slices were then kept in a clean 12-well plate for 2-day and the dry weight of each slice was measured. The absorbance was normalized by the dry weight of the slice and the results under experimental conditions were then converted to the percentage of control slices included in each experiment. The final results were percentage of the control.

4.6. OGD-induced extracellular glutamate accumulation

Cerebellar slices were pretreated with or without 1, 2, 3, or 4% isoflurane for 15 min at 37°C . After a 15-min drug-free period, they were subjected to OGD for 15 min in a chamber containing 2 ml of glucose-free aCSF. The aCSF and the cerebellar slices were collected immediately after the OGD and were kept at -70°C until they were used to assay glutamate levels and protein contents, respectively.

4.7. Analysis of glutamate levels by high performance liquid chromatography (HPLC)

The glutamate concentrations in the aCSF samples were measured by HPLC (Shimadzu, Kyoto, Japan) using the electrochemical detector Coulochem III (ESA, Inc., Chelmsford, MA) after a precolumn derivatization with OPA/ β ME solution (1,2-phthalic dicarboxaldehyde, Acros, New Jersey / β -mercapto-ethanol, Sigma). The reversed-phase column Supelcosil LC-8-DB, 5 μm , 5 cm $\times$ 4.6 cm (Supelco Inc., Bellefonte, PA) was eluted isocratically with 0.1 M Na_2HPO_4 mobile phase in 35% methanol (pH = 6.4 adjusted with phosphoric acid) at 1 ml/min.

Standard stock solution of glutamate (1 mg/ml in 50% methanol, kept refrigerated) was diluted with 0.05 M HClO_4 (1:1) before analysis. Stock OPA/ β ME solution was prepared by dissolving 27 mg OPA in 1 ml of HPLC grade methanol and then adding 5 μl β ME and 9 ml of 0.1 M sodium tetraborate. The solution was kept in amber glass at room temperature for up to 5 days. On the day of analysis, 1 ml of OPA/ β ME stock solution was diluted with 3 ml of 0.1 M sodium tetraborate solution to prepare OPA/ β ME working solution. Using auto-injector precolumn derivatization program, 10 μl of samples and 5 μl of OPA/ β ME working solution were injected to column for analysis. Electrochemical detector with analytical cell model 5011 was set as follows: electrode 1: -0.40 volts, electrode 2: $+0.60$ volts, guard cell: $+0.65$ volts, filter: 5 second and current range: 200 nA. The aCSF samples were analyzed after the standard curve of glutamate has been established.

4.8. Statistical analysis

Each experimental condition was repeated at least 5 times with cerebellar slices from different rats. Results are means $\pm$ S.D. Statistical analysis for the comparisons between treatment groups

and the corresponding OGD group was performed by Student t-test or one way ANOVA followed by the Fisher's LSD test for *post hoc* comparison after confirmation of a normal distribution of the data. A $P < 0.05$ was accepted as significant. The EC50 for each agent to induce the preconditioning effect was calculated by using GraphPad Prism 4 (GraphPad Software, San Diego California). This calculation was performed by fitting the data to a sigmoidal dose-response curve using the principle of the smallest sum of squares by the program.

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Abbreviations

aCSF	artificial cerebrospinal fluid
EAAT	excitatory amino acid transporter
HPLC	high performance liquid chromatography
MAC	minimum alveolar concentrations
F6	1,2-dichlorohexafluorocyclobutane
OGD	oxygen-glucose deprivation
TBOA	DL-threo- β -benzyloxyaspartate

THADL-threo- β -hydroxyaspartic acid**TTC**

2,3,5-triphenyltetrazolium chloride

Figure 1. Time-course of oxygen-glucose deprivation (OGD)-induced cell injury

Cerebellar slices from adult male rats were subjected to various lengths of OGD. Cell injury was quantified by 2,3,5-triphenyltetrazolium chloride (TTC) conversion 5 hrs after OGD. Results are means $\pm$ S.D. (n = 6 – 8 per time point). * P < 0.001 compared with control.

Figure 2. Volatile anesthetic preconditioning-induced neuroprotection

Cerebellar slices were preconditioned with various concentrations of volatile anesthetics for 15 min before a 10-min oxygen-glucose deprivation (OGD). Cell injury was quantified by 2,3,5-triphenyltetrazolium chloride (TTC) conversion 5 hrs after OGD. Results are means $\pm$ S.D. (n = 5 – 8 per concentration). * P < 0.05 compared with OGD without anesthetic pretreatment.

Figure 3. Linear correlation between the EC50 for the neuroprotection induced by volatile anesthetic preconditioning and the aqueous concentration of one minimum alveolar concentration of isoflurane, sevoflurane, desflurane and halothane

The EC50 was derived from the data presented in figure 2. The $r = 0.988$ for the correlation ($P < 0.01$).

Figure 4. The effects of 1,2-dichlorohexafluorocyclobutane (F6) pretreatment on oxygen-glucose deprivation (OGD)-induced cell injury

Cerebellar slices were pretreated with various concentrations of F6 for 15 min before a 10-min OGD. Cell injury was quantified by 2,3,5-triphenyltetrazolium chloride (TTC) conversion 5 hrs after OGD. Results are means $\pm$ S.D. (n = 5 – 8 per concentration).

Figure 5. The inhibition of volatile anesthetic preconditioning-induced neuroprotection by glutamate transporter inhibitors

Cerebellar slices were pretreated with 1 mM DL-threo- β -hydroxyaspartic acid (THA), 200 μ M DL-threo- β -benzyloxyaspartate (TBOA) or 300 μ M dihydrokainate (DHK) for 30 min. The slices then were preconditioned with 2 minimum alveolar concentrations of volatile anesthetics in the presence or absence of 1 mM THA, 200 μ M TBOA or 300 μ M dihydrokainate for 15 min before a 10-min oxygen-glucose deprivation (OGD). Cell injury was quantified by 2,3,5-triphenyltetrazolium chloride (TTC) conversion 5 hrs after OGD. Results are means $\pm$ S.D. (n = 6 – 16 per condition). * P < 0.05 compared with OGD only. ^ P < 0.05 compared with the corresponding anesthetic preconditioning plus OGD.

Figure 6. The effects of isoflurane preconditioning on the oxygen-glucose deprivation (OGD)-induced extracellular glutamate accumulation

Cerebellar slices were preconditioned with various concentrations of isoflurane for 15 min before a 15-min OGD. The glutamate concentrations in the OGD solutions were assayed. Results are means $\pm$ S.D. (n = 5 per condition). * P < 0.05 compared with control slices.