Isoflurane and Pentobarbital Reduce AMPA Toxicity
In Vivo in the Rat Cerebral Cortex

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Background: Isoflurane and pentobarbital can reduce a-aminoadroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor–mediated toxicity in vitro. However, their effect on AMPA toxicity in vivo is not known. The present study was undertaken to evaluate the effects of isoflurane and pentobarbital on the in vivo neurotoxicity produced by AMPA.

Methods: Wistar-Kyoto rats were allocated to one of seven groups (n = 8 per group): isoflurane 1 minimum alveolar concentration, isoflurane electroencephalogram burst suppression (EEG-BS), low-dose pentobarbital, pentobarbital EEG-BS, NBQX, conscious, and sham groups. AMPA 30 nm was injected into the cortex. An equivalent volume of cerebrospinal fluid was injected into the cortex in the sham group. In the NBQX group, 200 nm NBQX was injected into the cortex with the AMPA. In the isoflurane and pentobarbital groups, anesthesia was maintained for a period of 5 h. Animals in the conscious, NBQX, and sham groups were allowed to awaken immediately after the AMPA injection. Injury to the cortex was evaluated 48 h later.

Results: Isoflurane reduced AMPA-induced cortical injury (4.5 ± 1.9 mm³ and 1.7 ± 0.8 mm³ in the 1 minimum alveolar concentration and EEG-BS groups, respectively) in comparison to the conscious group (7.2 ± 0.8 mm³). Pentobarbital reduced cortical injury when administered in EEG-BS doses (2.2 ± 0.7 mm³) but not when administered in sedative doses (8.6 ± 0.9 mm³). NBQX reduced AMPA-induced cortical injury (1.2 ± 0.5 mm³).

Conclusions: Isoflurane and pentobarbital reduced cortical AMPA excitotoxicity. The magnitude of injury reduction was similar to that produced by NBQX when the anesthetics were administered in EEG-BS doses. These results are consistent with the previously demonstrated ability of isoflurane and pentobarbital to inhibit AMPA receptor responses. (Key words: Anesthetic; excitotoxicity, isoflurane; neurotoxicity.)

ANESTHETIC agents have been shown to reduce cerebral infarction caused by focal ischemia.1–4 Although the mechanism by which they reduce ischemic neuronal injury is not known, it is conceivable that anesthetic agents might suppress excitotoxic injury. Glutamate-mediated excitotoxic injury is thought to contribute to posts ischemic neuronal injury. Glutamate is released into the extracellular space in large quantities during cerebral ischemia.5 Stimulation of postsynaptic glutamate receptors leads to excessive calcium accumulation in neurons, ultimately leading to their death.6 One subtype of glutamate receptor that is thought to play a central role in excitotoxic injury is the a-aminoadroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor. Antagonists of AMPA receptors have been demonstrated to reduce ischemic neuronal injury in models of severe global7 and focal cerebral ischemia.8

Recent in vitro data have indicated that volatile anesthetics and barbiturates can reduce AMPA receptor-mediated responses by up to 60% in cortical slices.9 Given this degree of suppression of AMPA responses, it is conceivable that anesthetic agents might also reduce neuronal excitotoxicity produced in vitro by excessive AMPA receptor stimulation. However, the effect of isoflurane and barbiturates on AMPA-mediated excitotoxicity in vivo is not known. The present study was
therefore undertaken to evaluate the dose-dependent effects of isoflurane and pentobarbital on *in vivo* neurotoxicity produced by AMPA in rats. The effects of these two anesthetic agents were compared directly to those of NBQX, a specific antagonist of the AMPA receptor.

**Methods**

The study was approved by the Institutional Animal Care and Use Committee. All experimental procedures were performed in accordance with the guidelines established in *The PHS Guide for the Care and Use of Laboratory Animals*.

Wistar-Kyoto rats weighing 275–325 g were fasted overnight. Access to water was provided. The rats were anesthetized with an inspired concentration of 5% isoflurane. After induction of anesthesia, the rats’ tracheas were intubated, and their lungs were mechanically ventilated with an inspired gas mixture of 30% oxygen, 70% nitrogen. Ventilation parameters were adjusted to maintain normocapnia (arterial carbon dioxide partial pressure, 35–40 mmHg). The inspired concentration of isoflurane was reduced to 2.5%. The tail artery was cannulated with PE-50 tubing, and the mean arterial pressure and heart rate were measured continuously. The right external jugular vein was cannulated with PE-60 tubing. A maintenance infusion of normal saline, at a rate of 4 ml kg⁻¹ h⁻¹, was then initiated via the external jugular vein. A rectal temperature probe was inserted to a depth of 6 cm, and rectal temperature was monitored continuously thereafter.

The rat’s head was then secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The scalp was opened via a midline incision, and the calvariae were exposed. A needle thermistor was inserted between the left temporalis muscle and the cranium. Thereafter, the pericranial temperature was servo-controlled to 37°C with an overhead heat lamp. Platinum needle electrodes were inserted into the scalp in a biparietal configuration. A 4-mm-diameter cranectomy, centered 2.3 mm rostral and 6.0 mm lateral to the bregma, was performed. During the cranectomy, the skull was continuously irrigated with room temperature saline. Care was taken to avoid injury to the underlying dura. Under stereomicroscopic magnification, the dura mater was incised carefully with a scalpel blade and was opened. Injury to blood vessels in the immediate vicinity was avoided. The cranectomy site was then bathed in warmed artificial cerebrospinal fluid (155.0 mM Na⁺, 0.83 mM Mg²⁺, 2.9 mM K⁺, 132.76 mM Cl⁻, 1.1 mM Ca²⁺, pH 7.4). A 28-gauge plastic injection cannula (Plastic One, Roanoke, VA) was then inserted into the lateral parietal cortex (2.5 mm rostral and 6.0 mm lateral to the bregma) by micromanipulator to a depth of 1.5 mm. The animals were then left undisturbed for a period of 30 min. Arterial blood gas tensions, serum glucose, and hematocrit were measured during this equilibration period. All wound sites were infiltrated with 0.25% bupivacaine (total dose, 0.5 mg).

After the equilibration period, the animals were allocated randomly to one of seven experimental groups (n = 8 per group):

1. Isoflurane 1 minimum alveolar concentration (MAC): the end-tidal concentration of isoflurane was maintained at 1.2%.
2. Isoflurane electroencephalogram burst suppression (EEG-BS): the end-tidal concentration of isoflurane was adjusted to maintain BS of the EEG (2.2–2.3%).
3. Pentobarbital, EEG-BS: pentobarbital was administered as a bolus dose (50 mg/kg). An infusion of pentobarbital at a rate of 50 mg kg⁻¹ h⁻¹ was then initiated.
4. Pentobarbital, low dose: pentobarbital was administered as a bolus dose (16.7 mg/kg). An infusion of pentobarbital at a rate of 16.7 mg kg⁻¹ h⁻¹ was then initiated. This is similar to the approach used by Warner et al.²
5. NBQX: the end-tidal concentration of isoflurane was reduced to 0.8%.
6. Conscious: the end-tidal concentration of isoflurane was reduced to 0.8%.
7. Sham: the end-tidal concentration of isoflurane was reduced to 0.8%.

In the conscious group, 30 nmol AMPA (Sigma Chemical, St. Louis, MO), 5 μl of 6 mM AMPA in artificial cerebrospinal fluid solution, was injected into the lateral parietal cortex by an infusion pump at a rate of 1 μl min⁻¹. In pilot studies, this dose of AMPA consistently produced a cortical lesion but did not elicit seizure activity. The cannula was then removed, and the isoflurane was discontinued. Upon resumption of spontaneous ventilation, the trachea was extubated. The animal was transferred to an incubator through which oxygen was continuously flushed. Spontaneous motor activity was apparent within 15 min in all animals. In the two isoflurane groups, 30 nmol AMPA was injected as described. Isoflurane anesthesia, at either 1 MAC or EEG-BS doses, was maintained for a period of 5 h after injection. The animals were awakened and subsequently transferred to an.
Table 1. Physiologic Variables in the Seven Experimental Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Conscious</th>
<th>Isof 1 MAC</th>
<th>Isof BS</th>
<th>Pent LD</th>
<th>Pent BS</th>
<th>NBQX</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>108 ± 16</td>
<td>114 ± 6</td>
<td>107 ± 10</td>
<td>104 ± 11</td>
<td>106 ± 9</td>
<td>114 ± 8</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>104 ± 9</td>
<td>115 ± 9</td>
<td>108 ± 8</td>
<td>104 ± 12</td>
<td>106 ± 8</td>
<td>113 ± 6</td>
<td>102 ± 9</td>
</tr>
<tr>
<td>PaCO2 (mmHg)</td>
<td>353 ± 58</td>
<td>363 ± 23</td>
<td>352 ± 25</td>
<td>346 ± 29</td>
<td>337 ± 27</td>
<td>348 ± 21</td>
<td>340 ± 19</td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>338 ± 27</td>
<td>360 ± 24</td>
<td>349 ± 20</td>
<td>341 ± 11</td>
<td>337 ± 37</td>
<td>345 ± 21</td>
<td>340 ± 19</td>
</tr>
<tr>
<td>pH</td>
<td>36.6 ± 1.3</td>
<td>37.5 ± 4.3</td>
<td>36.5 ± 5.3</td>
<td>37.6 ± 1.4</td>
<td>38.0 ± 4.2</td>
<td>36.1 ± 4.2</td>
<td>38.7 ± 4.1</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>177 ± 13</td>
<td>163 ± 29</td>
<td>155 ± 16</td>
<td>157 ± 21</td>
<td>175 ± 14</td>
<td>185 ± 30</td>
<td>169 ± 15</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
<td>177 ± 13</td>
<td>166 ± 16</td>
<td>155 ± 16</td>
<td>161 ± 19</td>
<td>175 ± 14</td>
<td>178 ± 18</td>
<td>169 ± 15</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>89 ± 11</td>
<td>101 ± 9</td>
<td>95 ± 16</td>
<td>96 ± 13</td>
<td>99 ± 15</td>
<td>92 ± 14</td>
<td>92 ± 15</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45 ± 2</td>
<td>44 ± 2</td>
<td>43 ± 2</td>
<td>43 ± 2</td>
<td>43 ± 1</td>
<td>44 ± 2</td>
<td>42 ± 2</td>
</tr>
</tbody>
</table>

Isof = isoflurane; Pent = pentobarbital; LD = low dose; BS = burst suppression.

incubator for observation for another 2 h. In the two pentobarbital groups, 30 nmol AMPA was injected as described. Pentobarbital administration was continued for a period of 5 h after injection. The animals were then transferred to an incubator and observed for another 2 h. In the NBQX group, 200 nmol NBQX (Sigma Chemical) was administered simultaneously with 30 nmol AMPA (total volume, 5 μl) directly into the parietal cortex. Immediately upon completion of the injection, isoflurane was discontinued, and the animals were allowed to awaken. In the sham control group, 5 μl artificial cerebrospinal fluid was injected into the parietal cortex, after which the animals were awakened. All animals were observed for 2 h after resumption of spontaneous ventilation.

Two days after the intracortical AMPA injection, the animals were anesthetized with an intraperitoneal injection of 150 mg chloral hydrate (Sigma Chemical). The animals were killed by transcardiac perfusion with 200 ml heparinized saline followed by 200 ml buffered 4% paraformaldehyde. The animals were decapitated, and the brains were left in situ at a temperature of 4°C for 24 h. The brains were then prepared for histologic analysis. After dehydration in graded concentrations of ethanol and butanol, the brains were embedded in paraffin. Eight-micron-thick coronal sections, at intervals of 250 μm, were prepared and stained with hematoxylin and eosin.

Injury to the brain was evaluated by image analysis using NIH Image 1.60 software (National Institutes of Health, Bethesda, MD) and a Macintosh 8500 computer (Apple Computer, Cupertino, CA). The analysis was performed by two observers who had no knowledge of the experimental groups. Within each section, the area of the tissue infarction and the area of neuronal necrosis were measured. Tissue infarction was defined as an area of the brain characterized by the complete loss of both neurons and the neuropil. Neuronal necrosis was defined as the area of the brain surrounding the tissue infarction in which only complete neuronal loss, but not that of the neuropil, was observed. The volumes of infarction and neuronal necrosis were determined by integration in consecutive sections. Total lesion volume was calculated by the summation of the infarct volume and neuronal necrosis volume.

The physiologic variables were evaluated by analysis of variance (Statview 4.0; Abacus Concepts, Berkeley, CA). The volume of tissue injury was analyzed by single-factor analysis of variance. When the analysis of variance identified significant differences, post hoc Bonferroni–Dunn tests were used for intergroup comparisons. A P value < 0.05 was considered to be statistically significant. All data are presented as mean ± SD.

Results

A total of 56 animals was studied. All animals survived the experimental period, and no seizure activity was observed in any of them.

The physiologic variables are presented in Table 1. There were no differences in mean arterial pressure,
heart rate, arterial oxygen or carbon dioxide partial pressures, $p$H, hematocrit, and blood glucose among the seven groups.

Cortical AMPA injection in the conscious group resulted in a reproducible lesion that was restricted to the lateral parietal cortex. The lesion was composed of a small central area of infarction that was characterized by the loss of both the neurons and neuropil. The infarction was surrounded by a larger area in which neuronal necrosis was observed. Within this region, all neurons were injured, but the neuropil was intact. The boundary between normal brain and the brain tissue manifesting neuronal necrosis was sharp. A transitional zone containing various combinations of normal and injured neurons was not observed. Injury to subcortical structures was not evident.

**Infarction**

Compared with the awake group, isoflurane, in 1-MAC and EEG-BS doses, reduced the extent of infarction ($1.9 \pm 0.8$ vs $0.8 \pm 0.5$ and $0.5 \pm 0.4$ mm$^3$, respectively) (fig. 1). In doses that produced EEG-BS, the volume of infarction was not different from that in the NBQX ($0.3 \pm 0.2$ mm$^3$) and sham control ($0.3 \pm 0.2$ mm$^3$) groups. The volume of infarction in the low-dose pentoobarbital was similar to that in the conscious group ($1.9 \pm 0.8$ vs $3.1 \pm 0.5$ mm$^3$). However, in doses that produced EEG-BS, pentoobarbital reduced AMPA-induced infarction ($0.6 \pm 0.4$ mm$^3$). As expected, NBQX reduced AMPA-induced infarction. The injury in the sham control group was limited to a small infarction caused by the trauma of cannula insertion. Neuronal necrosis was not observed in this group.

**Neuronal Necrosis**

The region of the parietal cortex in which neuronal necrosis was evident was considerably larger than the area of infarction (fig. 2). Compared with the conscious group, isoflurane in 1-MAC and EEG-BS doses reduced the volume of neuronal necrosis ($5.2 \pm 1.0$ vs $3.7 \pm 1.4$ and $1.2 \pm 0.5$ mm$^3$, respectively). In doses that produced EEG-BS, the volume of neuronal necrosis was not different from that in the NBQX ($1.0 \pm 0.3$ mm$^3$) and sham control ($0.6 \pm 0.3$ mm$^3$) groups. In low doses, pentoobarbital did not reduce the volume of neuronal necrosis ($5.4 \pm 0.5$ mm$^3$). In EEG-BS doses, pentoobarbital reduced the volume of neuronal necrosis ($1.6 \pm 0.6$ mm$^3$) in comparison to the conscious group. However, the volume of neuronal necrosis in this group was greater than that in the isoflurane EEG-BS, NBQX, and sham control groups. As expected, NBQX reduced the volume of neuronal necrosis; in this group, the injury was similar to that in the control group. The total lesion sizes (infarction and neuronal necrosis) in each of the seven groups are presented in figure 3.

Histologic examination of brain structures remote from the site of AMPA injection was also performed. The striatum, hippocampus (entire rostro-caudal extent), and contralateral cortex did not manifest any injury.

**Discussion**

The injection of AMPA into the parietal cortex of the rat resulted in a reproducible lesion. The simultaneous injection of NBQX, an antagonist of AMPA receptor, inhibited the development of this lesion. Isoflurane also decreased the size of AMPA-induced lesion in a dose-
dependent manner. In 1-MAC doses, isoflurane reduced lesion size by approximately 40%. The reduction in injury at EEG-BS doses (70%) was similar to that produced by NBQX. By contrast, pentobarbital reduced AMPA-mediated cortical injury only when administered in doses that produced EEG-BS. At this level, the reduction in injury (65%) was similar to that produced by isoflurane (high dose) and NBQX. The injury in the sham control group was limited to a small infarction caused by the trauma of cannula insertion.

The ability of isoflurane to reduce AMPA excitotoxicity is consistent with its ability to antagonize the AMPA receptor both in vitro and in vivo. For example, isoflurane has been shown to reduce AMPA-mediated responses in mouse cortical slices. The degree of reduction was dose-dependent, with maximum doses producing a 60% reduction in AMPA responses. Enflurane, an ether whose effects are similar to those of isoflurane, can reduce AMPA-induced currents in Xenopus oocytes expressing human brain mRNA by approximately 33%. Halothane reduced AMPA-evoked responses in a dose-dependent manner in hippocampal slices. In vivo, the excitatory responses activated by stimulation of the thalamus-prefrontal cortex pathway are mediated primarily by AMPA receptors. Administration of halothane significantly reduced this response. Collectively, these data indicate that volatile anesthetics can reduce AMPA receptor-mediated responses. This ability may have contributed to the reduction in AMPA excitotoxicity that was observed in the present study.

Barbiturates have also been shown to antagonize AMPA-mediated responses. Sawada and Yamamoto demonstrated that pentobarbital significantly attenuated kainate-induced depolarization responses in the CA3 sector in a hippocampal slice model. Similarly, the data of Weight et al. indicate that barbiturates can antagonize kainate-activated currents in mammalian neurons. In mouse cortical slices, thiopental reduced AMPA-induced depolarizing responses by up to 55%. These data clearly indicate that barbiturates can antagonize the AMPA subtype of excitatory glutamate receptors. Antagonism at the AMPA receptor probably contributed to the reduction in AMPA excitotoxicity in the present study. The recent study by Zhu et al. is consistent with this notion. In a hippocampal slice model, these investigators showed clearly that thiopental can attenuate AMPA-mediated toxicity (as demonstrated by a greater preservation...
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tion of the CA1 population spike in response to the stimulation of Schaffer collaterals).

In addition to the attenuation of excitotoxicity, enhancement by isoflurane and pentobarbital of inhibitory responses mediated by γ-aminobutyric acid might have contributed to the reduction in AMPA-induced cortical lesions. The AMPA receptor, upon stimulation by glutamate, results in Na⁺ influx and rapid depolarization of the postsynaptic neuron.16 Hyperpolarization of neurons could conceivably retard this depolarization. In this regard, pentobarbital can not only increase but also prolong the hyperpolarizing action of γ-aminobutyric acid.17 This is probably mediated by the enhancement of Cl⁻ influx. This effect has also been demonstrated for volatile anesthetics. Isoflurane, enflurane, and halothane all have been shown to augment Cl⁻ currents in response to exogenous γ-aminobutyric acid application in rat hippocampal neurons.18 The relative contributions of direct antagonism of the AMPA receptors and the enhancement of inhibition to the reduction in AMPA excitotoxicity observed in the present experiment are not known.

The results of the present investigation are at a variance with those reported by Lees,19 who demonstrated that the injection of kainate into the hippocampus produced a direct hippocampal injury and also produced injury to remote sites of the brain (contralateral hippocampus). The latter injury was attributed to the development of seizures. Pentobarbital, but not halothane, reduced injury caused by hippocampal injection of kainate (which has AMPA agonist activity).19 In that study, halothane anesthesia was discontinued immediately after kainate injection. By contrast, isoflurane anesthesia was maintained for a period of 5 h after injection. Furthermore, seizure activity was not observed in any of the animals. These data indicate that maintenance of anesthesia for a prolonged period (the 5-h period used in the present study seems to be sufficient) may be a prerequisite for the attenuation of AMPA-induced excitotoxicity.

The results of the present investigation may provide further insight into the mechanism by which anesthetics reduce ischemic neuronal injury. Both isoflurane4 and pentobarbital10 have been shown to reduce infarct volumes in animals subjected to focal cerebral ischemia. During ischemia, uncontrolled release of glutamate results in excessive stimulation of postsynaptic glutamate receptors.3 This excitotoxicity is thought to play a major role in the initiation of neuronal death.20 We have shown previously that isoflurane can attenuate ischemia-induced glutamate release.21,22 In addition, we have also demonstrated that isoflurane can attenuate NMDA toxicity in an experimental paradigm similar to the one used in the present study.23 Halothane has also been shown to reduce NMDA toxicity in vitro.24 Those data, together with the results of this study, provide strong support for the premise that volatile agents reduce ischemic neuronal injury, in part by attenuating excitotoxicity. However, the observations of the present study are not in complete agreement with the results of investigations in which the neuroprotective effect of anesthetic agents was evaluated in animal models of focal ischemia. For example, the work of Warner et al.10 demonstrated clearly that the reduction in infarct volume in rats subjected to focal ischemia was similar whether pentobarbital was administered in EEG-BS doses or in doses approximately one third of the dose required to produce EEG-BS. A dose-related reduction in injury was not apparent. By contrast, a dose-related reduction in AMPA excitotoxicity was observed in the present investigation. The reduction in AMPA toxicity was not observed at the low doses but was observed in EEG-BS doses. This indicates that, although AMPA toxicity does contribute to the development of ischemic neuronal injury, other mechanisms might also play a significant role.

Finally, the rationale for the dose of AMPA that was chosen for cortical injection deserves further comment. Lees19 showed that intraparenchymal AMPA injection can produce seizure activity that is sustained for several hours after injection. This has the potential to not only augment the direct AMPA toxicity but also to produce neuronal injury in brain regions remote from the site of injection.19 Seizure activity would make it difficult to separate the contribution of direct AMPA excitotoxicity and seizure activity per se to the extent of injury. We performed preliminary investigations to determine the dose of AMPA that was sufficient to produce a cortical lesion without inciting seizures. The dose of AMPA that was injected (30 nmol) was selected on this basis. As a result, the cortical lesion observed was entirely a result of the direct excitotoxic effects of AMPA. This was confirmed by the lack of neuronal death in remote brain locations (e.g., hippocampus) in which injury would be expected to occur with prolonged seizure activity.

In summary, isoflurane reduced AMPA excitotoxicity in a dose-dependent manner. Pentobarbital reduced AMPA excitotoxicity in EEG-BS doses. The effect of the two anesthetics on AMPA-induced cortical injury, when administered in EEG-BS doses, was similar to that of NBQX, a specific antagonist of the AMPA receptor. The
results of the present study indicate clearly that anesthetic agents can attenuate excitotoxicity. Furthermore, they provide strong evidence in support of the premise that a reduction in excitotoxicity plays an important role in the mechanism by which anesthetics reduce ischemic cerebral injury.

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