Inhibition of glutamate transporters increases the minimum alveolar concentration for isoflurane in rats

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Background. Glutamate transporters [also named excitatory amino acid transporters (EAATs)] bind and take up extracellular glutamate, a major excitatory neurotransmitter, and can regulate glutamatergic neurotransmission in synapses. As anaesthesia is proposed to be induced by enhancing inhibitory neurotransmission, inhibiting excitatory neurotransmission, or both we hypothesize that inhibition of EAAT activity can increase the anaesthetic requirement.

Methods. The minimum alveolar concentration (MAC, the anaesthetic concentration required to suppress movement in response to noxious stimulation in 50% of subjects) for isoflurane was determined in adult male Sprague–Dawley rats after intrathecal administration of EAAT inhibitors.

Results. Application of DL-threo-β-benzyloxyaspartate, a selective EAAT inhibitor, dose- and time-dependently increased the MAC for isoflurane. The MAC was 109 (1)% and 116 (4)% of the baseline, respectively, for 0.2 and 0.4 μmol of DL-threo-β-benzyloxyaspartate 15 min after the injection of the drug (n=5, P<0.05 compared with the baseline MAC). Intrathecal injection of dihydrokainate, a selective inhibitor of EAAT type 2, also increased the MAC for isoflurane.

Conclusions. These results suggest that EAAT in the spinal cord can regulate the requirement of isoflurane to induce immobility. EAAT2 may be involved in this effect.

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Glutamate is a major excitatory neurotransmitter. Unlike the situation with many other neurotransmitters such as acetylcholine, no extracellular enzyme is known to metabolize glutamate. Thus, glutamate in the synaptic cleft can be cleared in two ways: diffusion and uptake into cells by glutamate transporters [also called excitatory amino acid transporters (EAATs)].1,2 Five EAATs have been characterized so far and are expressed in the central nervous system (CNS): EAAT1–5. EAAT1 and EAAT2 are primarily expressed by astroglial cells; EAAT3 and EAAT4 are mainly expressed in neurons; and EAAT5 is found in glial cells and neurons of the retina.2,3

EAATs, by binding and internalizing glutamate, can regulate glutamate concentrations in the local environment and may affect activation of glutamate receptors.4–6 It has been shown that inhibition of EAAT activity in brain slices and neuronal cultures increases the amplitude of excitatory postsynaptic current (EPSC) and prolongs EPSC decay.7 Thus, EAATs play a critical role in securing a high signal-to-noise ratio in synaptic transmission and in preventing harmful receptor over-stimulation under physiological conditions.

Although the mechanisms of anaesthesia for inhalational anaesthetics are not well understood, it is commonly accepted that anaesthesia induced by these drugs may be caused by enhancing inhibitory neurotransmission, inhibiting excitatory neurotransmission, or both.8 It has been shown that inhibition of glutamatergic neurotransmission by glutamate receptor inhibitors decreases volatile anaesthetic requirement as measured by the minimum alveolar concentration (MAC, the anaesthetic concentration required to suppress movement in response to noxious stimulation in 50% of subjects).8 As EAATs can regulate glutamatergic neurotransmission, we hypothesize that inhibition of EAATs increases anaesthetic requirements.

Materials and methods

This protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia. The animal experiments were carried out in accordance with the National Institute of Health Guide for the Care of Laboratory Animals (NIH Publications No. 80-23, revised
Measurement of MAC
Male Sprague–Dawley rats weighing 200–250 g were anaesthetized with isoflurane 2.5% (Abbott Laboratories, Chicago, IL, USA) in oxygen for 3–5 min. The trachea was intubated with a 16-gauge polyethylene catheter (Johnson and Johnson Medical Inc., Arlington, TX, USA). The isoflurane concentration was decreased to 1.5% and ventilation was controlled with a Harvard animal respirator. Isoflurane was delivered by an isoflurane-specific vaporizer. Cannulation of a femoral artery and vein with 24-gauge polyethylene catheters was performed. Alveolar isoflurane concentration and end-tidal CO$_2$ measurements were obtained using a Datex Engstrom Capomac gas monitor (Helsinki, Finland) that had been calibrated with gas standards. Heart rate and systolic and diastolic blood pressures were monitored and recorded using an AD Instruments MacLab (Mountain View, CA, USA) data recording system. Body temperature was measured using a FHC temperature controller (FHC, Bowdoinham, ME, USA) and maintained at normothermia using a heating blanket and warming lights.

MAC was measured according to our methods previously described. Briefly, an 8-inch haemostat (Biomedical Research Instruments, Inc., Rockville, MD, USA) was clamped to the first ratchet lock on the tail for 1 min. The next stimulation site on the tail is always proximal to the previous test site. A test was considered positive if there was gross movement of the head, extremities or body. Grimacing, swallowing, chewing and tail flick were taken as a negative test. The isoflurane concentrations were decreased in steps of 0.1% until the negative test became a positive test. The isoflurane concentrations were determined at three time points for each rat: MAC1 was before drug administration, MAC2 was started 15 min after the administration of the drug, and MAC3 was started 60 min after MAC2 determination.

Data analysis
Data are presented as means (SD). Comparison of the data at different time points within the same group of animals was performed by analysis of variance for repeated measures followed by the Student–Newman–Keuls test. To compare the effects of different doses of DL-TBOA on MAC, the MAC data after the application of DL-TBOA were normalized to the baseline/control data of the corresponding rat and were presented as percentage change over control. Statistical analysis for this comparison was performed by $t$-test. A $P$-value $<0.05$ was considered significant.

Results
The MAC of isoflurane in adult male rats before the administration of EAAT inhibitors was $\sim 1.14\%$ (the mean value of all 22 rats tested in this study). This value is similar to the MAC of isoflurane previously reported. As MAC is a measure of anaesthetic effect on immobility and the spinal cord has been found to be the primary site mediating immobility, we administered EAAT inhibitors intrathecally. By this route, 0.2 and 0.4 $\mu$mol DL-TBOA significantly increased the MAC of isoflurane 15 min after drug administration (Table 1). The increase in isoflurane MAC by 0.4 $\mu$mol of DL-TBOA was greater than that by 0.2 $\mu$mol DL-TBOA [116 (4)\% and 109 (1)\%, respectively, of the corresponding baseline MAC, $P<0.05$, $N=5$ for each group]. MAC returned to baseline 60 min after MAC2 was determined in rats injected with either 0.2 or 0.4 $\mu$mol of DL-TBOA (Table 1). Intrathecal injection of 10 $\mu$l of dimethyl sulfoxide (used to dissolve DL-TBOA) did not affect the MAC of isoflurane (Table 1). The MAC values in animals that received no intrathecal injection did not change over time (Table 1). These results suggest that inhibition of EAATs in the spinal cord increases the threshold for

<table>
<thead>
<tr>
<th>Intrathecal injection</th>
<th>MAC1</th>
<th>MAC2</th>
<th>MAC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-TBOA (0.2 $\mu$mol)</td>
<td>1.09 (0.09)</td>
<td>1.19 (0.09)*</td>
<td>1.11 (0.09)</td>
</tr>
<tr>
<td>DL-TBOA (0.4 $\mu$mol)</td>
<td>1.17 (0.04)</td>
<td>1.35 (0.00)*</td>
<td>1.25 (0.07)</td>
</tr>
<tr>
<td>DHK (0.45 $\mu$mol)</td>
<td>1.19 (0.05)</td>
<td>1.31 (0.05)*</td>
<td></td>
</tr>
<tr>
<td>DMSO (10 $\mu$l)</td>
<td>1.12 (0.06)</td>
<td>1.12 (0.06)</td>
<td></td>
</tr>
<tr>
<td>No intrathecal injection</td>
<td>1.10 (0.13)</td>
<td>1.10 (0.13)</td>
<td>1.08 (0.10)</td>
</tr>
</tbody>
</table>
may be involved in this effect because intrathecal injection of DHK, a selective EAAT2 inhibitor, also increased the MAC for isoflurane. The involvement of other EAATs in this effect has not been studied because of the lack of selective inhibitors.

It has been proposed that the components of the anaesthetic state should at least include amnesia/unconsciousness, immobilization and analgesia. MAC is used to measure anaesthetic potency for immobilization. By delivering anaesthetics separately to spinal cord or brain, Antognini and Schwartz\textsuperscript{14} have provided data to suggest that the spinal cord is the primary site required to mediate immobilization. In our study, intrathecal injection of DL-TBOA at the L4-5 level dose- and time-dependently increased the MAC for isoflurane. It has been shown that the drug may remain in the spinal cord and does not diffuse to the brain when applied by intrathecal injection.\textsuperscript{10} Thus, our results are consistent with the idea that the spinal cord is the primary site for mediating immobilization.

We observed a relatively small but reproducible increase of isoflurane MAC after application of EAAT inhibitors. It is not known whether greater increases in MAC value would have been seen if larger doses of EAAT inhibitors were used. However, the small magnitude of change in MAC may be expected because the regulation of glutamate neurotransmission by EAAT was mediated by an indirect action, glutamate uptake, and many compensatory mechanisms such as glutamate receptor desensitization and passive diffusion of glutamate away from synapses may take place.

Our findings may be significant. The involvement of EAATs in the regulation of glutamate neurotransmission has been shown in in vitro studies\textsuperscript{4–7} and there is very little evidence from in vivo studies. Our results suggest that inhibition of EAATs can increase glutamate concentrations in the synapses, which then increases glutamate neurotransmission, in intact rats. The involvement of glutamate neurotransmission in anaesthesia has been mainly based on the evidence that glutamate receptor antagonists can decrease the requirement for anaesthetics and that anaesthetics can modulate glutamate receptor activation.\textsuperscript{8,15} Our results provide additional evidence for this involvement. Finally, our results, along with the results showing that volatile anaesthetics enhance EAAT activity,\textsuperscript{16–18} suggest that EAATs may be a pharmacological target for volatile anaesthetics in the CNS.

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References

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**Table 2** Haemodynamic data recorded during the measurements of MAC. Full drug dose was administered intrathecally as one bolus. The time to determine MAC1, MAC2 and MAC3 was before, at 15 min after the injection and 60 min after the MAC2 determination, respectively. In the no intrathecal injection group, measurements were performed at the corresponding time points. Results are means (SD) (n=3–5). *P<0.05 compared with the value at measuring MAC1. MAC, minimum alveolar concentration; \textit{D}-TBOA, \textit{D}-threo-\textit{β}-benzylxoxaspartate; DHK, dihydrokainate; DMSO, dimethyl sulfoxide.

<table>
<thead>
<tr>
<th>Intrathecal injection</th>
<th>Mean arterial blood pressure (mm Hg) at MAC1</th>
<th>Mean arterial blood pressure (mm Hg) at MAC2</th>
<th>Mean arterial blood pressure (mm Hg) at MAC3</th>
<th>Heart rate (beats min(^{-1})) at MAC1</th>
<th>Heart rate (beats min(^{-1})) at MAC2</th>
<th>Heart rate (beats min(^{-1})) at MAC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{D}-TBOA (0.2 \text{μmol})</td>
<td>118 (9)</td>
<td>110 (18)</td>
<td>107 (18)</td>
<td>422 (68)</td>
<td>424 (23)</td>
<td>417 (12)</td>
</tr>
<tr>
<td>\textit{D}-TBOA (0.4 \text{μmol})</td>
<td>117 (12)</td>
<td>91 (13)*</td>
<td>89 (12)*</td>
<td>352 (24)</td>
<td>333 (23)</td>
<td>335 (27)</td>
</tr>
<tr>
<td>DHK (0.45 \text{μmol})</td>
<td>120 (12)</td>
<td>111 (21)</td>
<td>65 (26)</td>
<td>388 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO (10 \text{μl})</td>
<td>110 (4)</td>
<td>112 (3)</td>
<td>63 (21)</td>
<td>406 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No intrathecal injection</td>
<td>104 (4)</td>
<td>102 (12)</td>
<td>104 (9)</td>
<td>409 (29)</td>
<td>403 (15)</td>
<td>385 (24)</td>
</tr>
</tbody>
</table>
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