

## ***Inhibition of Dopamine Transport in Rat Brain Synaptosomes by Volatile Anesthetics***

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**Background:** Volatile anesthetics may depress transmission by altering synaptic concentrations of neurotransmitter. Microdialysis studies have found an increase in brain extracellular dopamine concentration on exposure to volatile anesthetics. We investigated the possibility that synaptosomal dopamine transport is reversibly inhibited by the halothane and isoflurane.

**Methods:** Rat brain synaptosomes were incubated with 5 nM <sup>3</sup>H-DA and increasing concentrations of anesthetic in Teflon-sealed microvials. Cocaine (100 μM) was used to quantify non-specific binding/uptake. Uptake was stopped by vacuum filtration and washing; label incorporation into synaptosomes was determined by liquid scintillation counting. <sup>3</sup>H-DA release from preloaded synaptosomes also was studied in the presence of the anesthetic to allow distinction between uptake inhibition and release stimulation in the synaptosomes.

**Results:** Both halothane and isoflurane inhibited the specific <sup>3</sup>H-DA uptake in a concentration-dependent fashion with an IC<sub>50</sub> of 0.72 ± 0.14 mM for halothane and 2.24 ± 0.85 mM for isoflurane. No stereoselectivity of isoflurane's action on dopamine (DA) uptake was observed. The inhibition produced by halothane and isoflurane was kinetically characterized as noncompetitive, but full reversal was demonstrated after removal of the anesthetic from the incubation mixture. The anesthetics did not stimulate <sup>3</sup>H-DA release from preloaded synaptosomes.

**Conclusions:** These results demonstrate volatile anesthetic-induced inhibition of the dopamine transporter in this preparation of synaptosomes. The calculated IC<sub>50</sub>s suggest this inhibition occurs with clinically relevant concentrations of halothane but not with isoflurane. The results are consistent with and may explain the increase in extracellular dopamine concentrations demonstrated by microdialysis. (Key words: Anesthetics, local: cocaine. Anesthetics, volatile: halothane,

isoflurane. Brain: synaptosomes. Sympathetic nervous system, catecholamines: dopamine.)

AN important component of general anesthetic action appears to be an alteration of synaptic transmission.<sup>1-5</sup> Although synaptic transmission may be disrupted at multiple sites, both pre- and postsynaptically, one possible mechanism for an anesthetic-induced alteration of transmission is through a change in the concentration of neurotransmitter in the synaptic cleft. Consistent with this possibility are studies that demonstrate a decrease in neurotransmitter secretion produced by general anesthetics<sup>5,6</sup> and that show that changing the concentration of a single neurotransmitter in the central nervous system can modulate the requirements for other anesthetic drugs to produce surgical anesthesia. For example, systemic administration of α<sub>2</sub>-adrenergic drugs<sup>7</sup> or L-dopa<sup>8</sup> reduces the minimum alveolar concentration (MAC) of halothane required to produce surgical anesthesia by greater than 50%. Although changes in the synaptic concentrations of neurotransmitter may be produced by action of the anesthetic agent at a number of different synaptic sites, the neurotransmitter transporters (reuptake mechanism) are likely targets, due to, in part, the extreme hydrophobicity of this recently described family of transmembrane proteins.<sup>9</sup> In support of this possibility, recent studies have shown that the serotonin (5-hydroxytryptamine) transporter is reversibly inhibited by both halothane and isoflurane.<sup>10,11</sup> If unopposed by a decrease in secretion, the *in vivo* effect of transporter inhibition would be an increase in the extracellular concentration of neurotransmitter. Such increases in extracellular neurotransmitter have been observed in *in vivo* microdialysis studies in which rapid and large increases in extracellular dopamine were measured with the induction of halothane anesthesia.<sup>12,13</sup> We examined the hypothesis that the dopamine transporter is reversibly inhibited by clinically relevant concentrations of volatile anesthetics, both as a possible explanation for this increase in extracellular dopamine

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concentrations and as a potential component of general anesthetic action.

## Methods

### Materials

3,4,-[7-<sup>3</sup>H]-Dihydroxyphenylethylamine (dopamine, <sup>3</sup>H-DA), specific activity 24.1 Ci/mM, was purchased from DuPont-NEN (Boston, MA). Cocaine HCl was obtained from Astra Pharmaceutical (Westborough, MA), and other chemicals were purchased from Sigma (St. Louis, MO). Purified D(+) and L(-) optical isomers of isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether), as well as the commercially available racemic mixture, were obtained from Anaquest (Murray Hill, NJ). The racemic mixture of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from Halocarbon Laboratories (Hackensack, NJ).

### Tissue Preparation

Animal protocols were approved by the Institutional Animal Care and Use Committee. The whole brains (excluding the cerebellum and midbrain ~ 2 g) of adult male Sprague-Dawley rats were processed as follows: Brains were removed from anesthetized (50 mg/kg intraperitoneal pentobarbital) animals (generally two per preparation), washed, minced, and homogenized in 10 volumes of ice cold 10 mM Na<sub>2</sub>HPO<sub>4</sub>/0.25 M sucrose buffer, pH 7.4 using a Potter-Elvehjem glass homogenizer with Teflon pestle. The crude homogenate was centrifuged at 1,000 G for 10 min, and the supernatant fraction was recentrifuged at 30,000 G for 30 min. The final pellet containing crude synaptosomal membranes was resuspended in 20 volumes of 11 mM glucose/0.25 M sucrose buffer and used immediately for dopamine uptake assays. Protein concentration was determined by Coomassie blue assay as described by Bradford.<sup>14</sup>

### Dopamine Uptake Assay

<sup>3</sup>H-DA was prepared in a Krebs's phosphate buffer with the following composition (in mM): Na<sub>2</sub>HPO<sub>4</sub> 15.8, NaCl 122, KCl 4.8, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.3, glucose 11.0, ascorbate 1.0, and pargyline 0.2. The pH was adjusted to 7.4 at 22° C. About 200 µg of synaptosomal protein were incubated in this buffer, which also contained 5 nM <sup>3</sup>H-DA and increasing concentrations of the volatile anesthetics in Teflon-sealed microvials. Solutions of the anesthetics were prepared by adding 15 µl halothane or 30 µl isoflurane to 22-ml foil-sealed glass vials filled with 4° C Krebs phosphate buffer and intermittently vortexing for 1 h. This buffer (containing 6.4 mM halothane or 11.1 mM isoflurane) was added

to the microvials by injection from repeating gas tight Hamilton syringes (Reno, NV) into the mixture, and volumes were adjusted so that the final addition (<sup>3</sup>H-DA) completely filled the vial (600 µl). Uptake was initiated by adding <sup>3</sup>H-DA. After a 5-min incubation at 22° C, the mixture was vacuum-filtered through Whatman GF/B glass fiber filters (Maidstone, England) and washed with 10 ml of ice cold saline, and the retained radioactivity on the filters was determined by liquid scintillation. Cocaine (100 µM final concentration) was added to half of the vials to inhibit the Dopamine (DA) transporter<sup>15</sup> and allow determination of nonspecific binding/uptake. In addition, stereoselectivity of isoflurane's action on this transporter was examined by incubating the synaptosomes with 0.015–9.0-mM concentrations of both the purified D(+) and L(-) optical isomers of isoflurane (Anaquest). Finally, the ability of an injectable (nonvolatile) anesthetic, pentobarbital sodium, to influence <sup>3</sup>H-DA uptake was examined. This barbiturate was prepared (1.0 nM–1.00 mM final concentration) in the same Krebs phosphate buffer and was added as for the volatile anesthetic solutions. Volatile anesthetic concentrations were verified by extracting 100-µl aliquots of all stock and some assay samples with 2.0 ml hexane and determining anesthetic mass in the hexane by gas chromatography.

To determine whether any observed effects of the anesthetics were reversible, and not due to irreversible synaptosomal damage, <sup>3</sup>H-DA uptake was studied after removal of the anesthetic from the buffer/synaptosomal mixture by vacuum and stirring rapidly for 60 min. Aliquots were removed from an uptake mixture initially containing 3.0 mM halothane or 5.0 mM isoflurane, and total <sup>3</sup>H-DA uptake was determined as a function of the time that the mixture was exposed to vacuum and stirring. These total <sup>3</sup>H-DA uptake results were expressed as a percentages of total uptake determined in parallel mixtures also exposed to vacuum and stirring, but which did not initially contain anesthetics.

Kinetics of the anesthetic-induced inhibition were studied by incubating synaptosomal membranes as above but at 37° C for 3 min with varying concentrations of <sup>3</sup>H-DA (50–500 nM) in the presence and absence of 0.9 mM halothane or 1.5 mM isoflurane. As before, <sup>3</sup>H-DA uptake was terminated by the addition of 2° C saline and vacuum filtration.

### Dopamine Release Assay

Synaptosomes (~250 µg protein) were incubated with 10 nM <sup>3</sup>H-DA at 22° C for 15 min and then loaded onto GF/B filters. Each filter was then superfused in a

vacuum filtration chamber with 10 ml of Krebs' buffer at approximately 5 ml/min and then with an additional 20 ml of the same buffer containing one of the following four additions: 100  $\mu\text{M}$  imipramine, 1 mM halothane, 2 mM isoflurane, or no addition (controls). After the 20-ml superfusion, the filter was washed with 10 ml of 2° C saline and counted by liquid scintillation as above. Dissociations per minute (DPM) were normalized to protein, and the effect of the additions compared to controls (no additions).

#### Statistics and Analysis

The inhibition data from figures 1 and 2 were fit to logistic equations using nonlinear least squares regression to allow determination of the  $\text{IC}_{50}$  values. The kinetic parameters,  $V_{\text{max}}$  and  $K_m$ , were calculated using linear regression of double-reciprocal plots (Lineweaver-Burke). Statistical significance of individual points in the inhibition or release experiments was determined by a one-way analysis of variance and *post hoc* Scheffe's test. Student's *t*-test was used only to compare two means (kinetic parameters).

### Results

The two volatile anesthetic agents examined in this study inhibited specific  $^3\text{H}$ -DA uptake in a concentra-

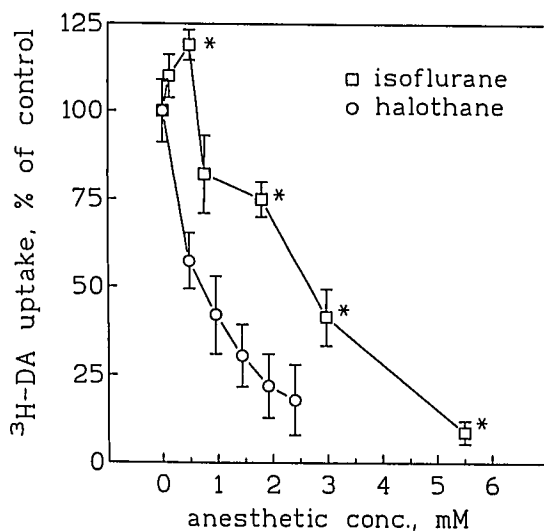


Fig. 1.  $^3\text{H}$ -DA uptake by rat brain synaptosomes in the presence of halothane and isoflurane. Points are mean  $\pm$  SD of two experiments with three replicates each. Values are the specific uptake normalized to that in the absence of anesthetic. \*  $P < 0.05$  by analysis of variance and *post hoc* Scheffe's test; all of the halothane points are significantly different than control values.  $\text{IC}_{50}$  values given in the text derive from a nonlinear least squares fit of these data to logistic equations. The fit was significant in both cases with an  $r^2$  of greater than 0.98.

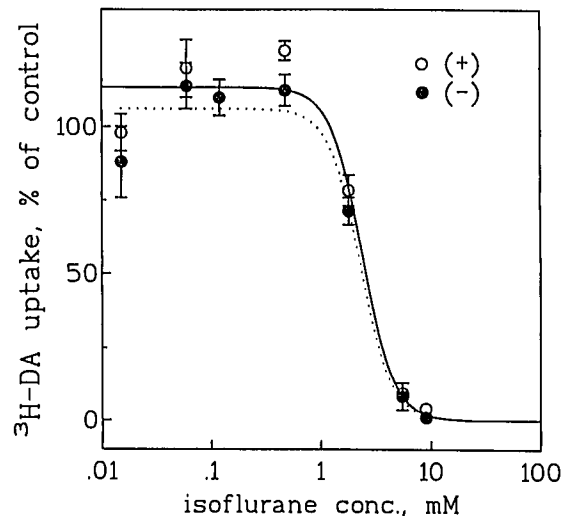


Fig. 2. The influence of the stereoisomers of isoflurane on  $^3\text{H}$ -DA uptake by rat brain synaptosomes. The inhibition of uptake by the D(+) isomer (O) is not statistically distinguishable from that of the L(-) isomer ( $\bullet$ ). Data are mean  $\pm$  SD and are normalized to control uptake as in figure 1. The curves are nonlinear least squares fit of the data to logistic equations ( $r^2 > 0.98$  for both). The calculated  $\text{IC}_{50}$  values for both isomers of about 2.1 mM agrees closely with that of the racemic mixture.

tion-dependent manner with an  $\text{IC}_{50}$  of  $0.72 \pm 0.13$  and  $2.24 \pm 0.85$  mM for halothane and isoflurane, respectively (fig. 1). The maximum inhibition was not reached with concentrations used in our experiments but appeared to be similar for the two agents. A small but consistent and significant increase ( $\sim 20\%$ ) in  $^3\text{H}$ -DA uptake was produced by low concentrations of isoflurane ( $\leq 0.7$  mM). There was no significant difference between the inhibition of  $^3\text{H}$ -DA uptake produced by the two optical isomers of isoflurane, as shown in figure 2, and these data are similar to those obtained with the racemic mixture. A wide concentration range of the injectable anesthetic pentobarbital sodium had no significant effect on synaptosomal  $^3\text{H}$ -DA uptake (data not shown).

Nonspecific uptake or binding of  $^3\text{H}$ -DA (in the presence of 100  $\mu\text{M}$  cocaine) by the control preparation of synaptosomes averaged between 10% and 20% of the total uptake. A small but significant increase in nonspecific  $^3\text{H}$ -DA uptake was noted with the highest concentrations of both volatile anesthetics (fig. 3). However, because of the small contribution to total uptake, this increase in nonspecific uptake could not explain the much larger decrease in total uptake induced by the anesthetics.

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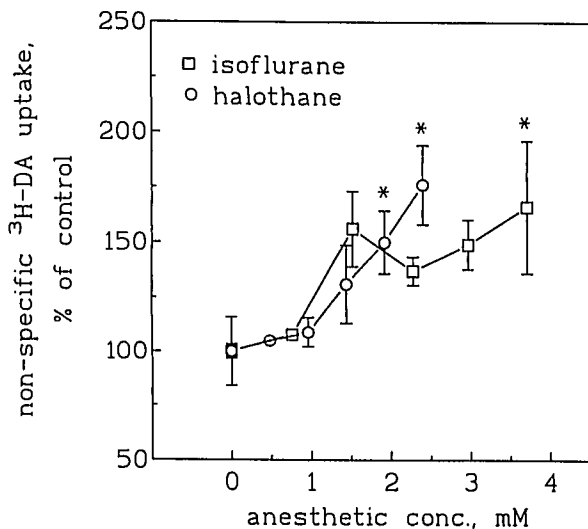


Fig. 3. Influence of increasing concentrations of isoflurane and halothane on nonspecific  $^3\text{H}$ -DA binding/uptake (uptake in the presence of cocaine). \*  $P < 0.05$  compared to control values (0.0 mM anesthetic) using analysis of variance and *post hoc* Scheffe's test.

The inhibition of  $^3\text{H}$ -DA uptake was reversed by removing halothane or isoflurane from the incubation mixture by vacuum and stirring. In both cases, anesthetic-exposed solutions had returned to greater than 90% of control activity after 60 min of off-gassing (halothane  $99 \pm 8$  and isoflurane  $94 \pm 4$  [mean % of control  $\pm$  SD]).

This preparation of synaptosomes demonstrated specific  $^3\text{H}$ -DA uptake characteristics that are consistent with those reported in the literature.<sup>16</sup> The kinetic parameters of  $^3\text{H}$ -DA uptake by synaptosomes were determined in the presence and absence of either 0.9 mM halothane or 1.5 mM isoflurane (fig. 4) and are shown

Table 1. Kinetic Parameters for Synaptosomal Dopamine Uptake

Condition	$K_m$ (nM)	$V_{max}$ (pmol/mg protein/3 min)
Control	$107 \pm 19$	$30.6 \pm 5.6$
Halothane	$97 \pm 19$	$14.5 \pm 3.2^*$
Isoflurane	$91 \pm 19$	$19.3 \pm 1.8^*$

Values are mean  $\pm$  SD.

\*  $P < 0.05$  by ANOVA and *post hoc* Scheffe's test.

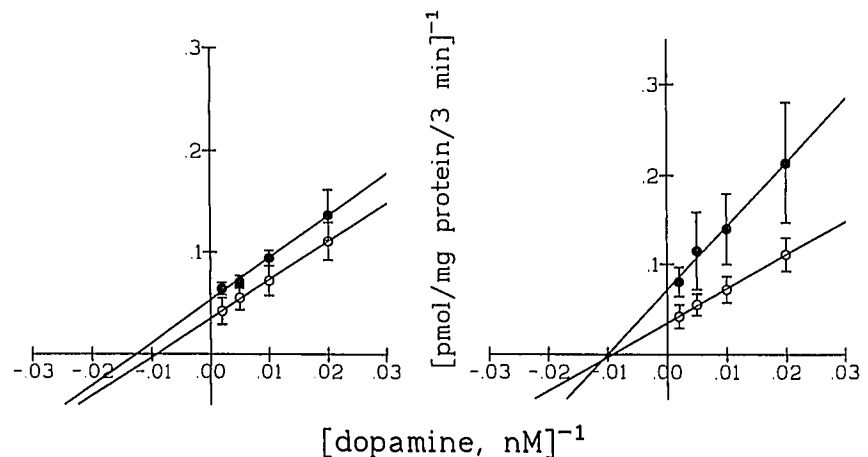
in table 1. Both anesthetics significantly reduced  $V_{max}$  from the control level. Changes in the apparent  $K_m$  for both anesthetics were small and not significantly different from control values. These data suggest non-competitive inhibition of  $^3\text{H}$ -DA uptake by both volatile anesthetics.

Synaptosomal  $^3\text{H}$ -DA uptake after the 15-min incubation averaged  $1.83 \pm 0.20$  pmol/mg protein and was not significantly reduced after the 20-ml superfusion (basal release; table 2). Superfusion with 1 mM halothane or 2 mM isoflurane did not alter the basal release of  $^3\text{H}$ -DA, whereas 100  $\mu\text{M}$  imipramine, a known dopamine-releasing drug,<sup>17</sup> caused a 21% decrease in  $^3\text{H}$ -DA retained by the preloaded synaptosomes.

## Discussion

The dopamine transporter is a recently isolated and sequenced member of the neurotransmitter transporter family.<sup>9</sup> Like that for norepinephrine and serotonin, the dopamine transporter is responsible for decreasing the synaptic concentration of dopamine, and thus terminating its action on the postsynaptic membrane. This

Fig. 4. Double reciprocal plots (Lineweaver-Burke) of experiments in which the concentration of substrate ( $^3\text{H}$ -DA) is increased from 50 to 500 nM in the presence (solid) or absence (open) of a fixed concentration of anesthetic (see Methods). (Left) Isoflurane data. (Right) Halothane data. Kinetic parameters for these plots are given in table 1.



**Table 2.** <sup>3</sup>H-DA Release from Preloaded Synaptosomes

Treatment	Retained <sup>3</sup> H-DA (pmol/mg protein ± SD)*
Start†	1.83 ± 0.20
Control‡	1.67 ± 0.14
Imipramine	1.45 ± 0.16§
Halothane	1.81 ± 0.20
Isoflurane	1.86 ± 0.18

\* N = 7, 2 preparations.

† Retained <sup>3</sup>H-DA after the 10-ml initial wash.

‡ Retained <sup>3</sup>H-DA after the 20-ml superfusion.

§ P < 0.05, vs "Start" by ANOVA and *post hoc* Scheffe's test.

transporter has been characterized extensively *in vitro* using the cocaine-inhibited uptake of <sup>3</sup>H-DA into synaptosomes and tissue slices.<sup>16</sup> The present study demonstrates that halothane and isoflurane reversibly inhibit <sup>3</sup>H-DA uptake by brain synaptosomes in concentration-dependent fashion, suggesting that these anesthetics inhibit dopamine transporter function. That this inhibition may be relevant to anesthetic action is suggested by an IC<sub>50</sub> for halothane, which is approximately within the clinical concentration range (0.7 mM is approximately 2 MAC). Although the IC<sub>50</sub> for isoflurane exceeds concentrations used clinically (2.2 mM isoflurane is about 6 MAC), the small increase in <sup>3</sup>H-DA uptake induced by 0.5 mM isoflurane lies within the clinical concentration range (about 2 MAC) and may have physiologic relevance. Isoflurane's biphasic effect on <sup>3</sup>H-DA uptake may reflect different sites of action on the transporter or competing sites on both the transporter and release systems. The difference between halothane and isoflurane emphasizes that subtle differences in action and site may exist for these small molecules. Further, inhibition of the dopamine transporter does not appear to be a universal feature of general anesthetics, because pentobarbital sodium, an intravenous general anesthetic, produced no detectable inhibition over a very wide concentration range, which includes the *in vivo* EC<sub>50</sub>.

Because our data demonstrate only a decreased net synaptosomal content of <sup>3</sup>H-DA in the presence of volatile anesthetics, enhanced basal secretion of dopamine could have caused the same result.<sup>17</sup> However, this is unlikely because halothane and isoflurane had no significant effect on release of <sup>3</sup>H-DA from preloaded synaptosomes in our studies and because both anesthetics increased the nonspecific uptake. Consistent with this conclusion are previous studies that report a reduction in neurotransmitter release on exposure to halothane.<sup>5,6</sup>

Thus, although the influence of general anesthetics on the dopamine release system (basal *vs.* stimulated) will require more detailed study, the most reasonable explanation for the current results is an inhibition of the dopamine transporter.

If this inhibition of dopamine transport is not matched by a similar decrease in the rate or quanta of secretion, then it is likely that halothane anesthesia will be accompanied by increased synaptic concentrations of dopamine in at least some regions of the brain. As discussed above, this is consistent with and may explain the increased extracellular dopamine concentrations observed in microdialysis studies.<sup>12,13</sup> It is not yet clear what the physiologic consequences of increased synaptic dopamine levels are in the intact system, especially since dopamine receptor subtypes mediate opposite influences on the postsynaptic neurons (D<sub>1</sub> increases and D<sub>2</sub> decreases cAMP content).<sup>18</sup> Now that these receptor subtypes have been identified clearly,<sup>9</sup> further experiments may be able to sort out the physiologic impact of increased synaptic dopamine concentrations produced by halothane. It is important to note, however, that increased striatal levels of dopamine produced through systemic application of exogenous L-dopa (as well as the "false" transmitter  $\alpha$ -methyl dopa<sup>19</sup>) significantly reduced the requirement for volatile anesthetics in the mouse,<sup>8</sup> suggesting a relevance of dopaminergic transmission in the mechanism of volatile anesthetic action.

The anesthetic-induced inhibition of the dopamine transporter was characterized as kinetically noncompetitive, consistent with studies of the 5-HT transporter.<sup>11,20</sup> Noncompetitive inhibition could be due to synaptosomal damage (increased permeability to dopamine) or an allosteric interaction of the anesthetics with the dopamine transporter. The first possibility is unlikely because of the small effect on nonspecific dopamine uptake and the finding that <sup>3</sup>H-DA uptake returned to control values after removing the anesthetic from the incubation mixture. Thus, the inhibition of the transporter by these anesthetics is explained most easily on the basis of allosteric interactions, either within the transporter or through a more indirect effect on lipid. The site of this allosteric inhibition is not apparent from our results and will await the ability to quantitatively examine volatile anesthetic binding to the transporter protein. Our results suggest, however, that if the site of volatile anesthetic action is directly on the transporter protein, it is unlikely to be a highly specific "receptor" site because of the lack of stereo-

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selectivity. The presence of stereoselectivity would have implicated a direct protein site of volatile anesthetic action, as was suggested in recent results on a potassium channel in *Lymnea stagnalis* using the optical isomers of isoflurane.<sup>21</sup> The lack of stereospecificity, however, in this dopamine uptake system does not necessarily commit isoflurane's action as occurring through an indirect effect on lipid (membrane); it only suggests that isoflurane is less confined in its site, whether protein or lipid. The present results do not allow assignment of the anesthetic's action to either a protein or lipid site of action.

These results may have implications to issues other than anesthetic mechanisms. It has been shown recently that cerebral ischemia and reperfusion are accompanied by a massive efflux of dopamine in specific areas of the brain, for example, the striatum.<sup>22,23</sup> It is not yet clear whether this efflux simply *reflects* the injury,<sup>24</sup> or whether the large pool of extracellular dopamine participates in the injury,<sup>25</sup> in an analogous fashion to the excitotoxicity produced by excess amino acid neurotransmitter.<sup>26</sup> If the high extracellular dopamine concentrations participate in the pathogenesis of neuronal injury, then an inhibition of reuptake mechanisms by volatile anesthetics may aggravate the injury. This may be especially important during the reperfusion phase, when normal operation of the transporter is required to rapidly lower the excessive extracellular dopamine concentrations.<sup>27</sup> It is important to point out, however, that many of these *in vivo* microdialysis studies of cerebral ischemia<sup>23,25,27</sup> have used halothane as an anesthetic. It is conceivable, therefore, that the ischemia-induced elevation in extracellular dopamine may have been less pronounced in the absence of the anesthetic. Regardless, the ultimate effect of volatile anesthetic-induced dopamine reuptake inhibition during cerebral ischemia in the intact system is difficult to predict; further studies will be required.

In summary, we have demonstrated reversible inhibition of the dopamine transporter by both halothane and isoflurane, but the potency suggests that inhibition would be observed only with clinical concentrations of halothane. Isoflurane's effect on net dopamine uptake by synaptosomes was biphasic and was not associated with stereoselectivity. Neither volatile anesthetic influenced basal release of dopamine from preloaded synaptosomes.

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