Biochemical Pharmacology, Vol. 40, No. 2, pp. 187–192, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EFFECTS OF ENFLURANE ON 5-HYDROXYTRYPTAMINE TRANSPORT IN SYNAPTOSOMES FROM RAT BRAIN*

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(*Received* 5 *June* 1989; *accepted* 18 *October* 1989)

Abstract—The administration of volatile anesthetics to laboratory animals has been reported to alter brain 5-hydroxytryptamine (serotonin, 5-HT) homeostasis. To examine a potential anesthetic action that could account for these observations, the effect of enflurane on 5-HT accumulation by rat brain synaptosomes was examined. Established techniques were used to prepare synaptosomes and perform uptake assays using [^{3}H]5-hydroxytryptamine as substrate. Exposure of the synaptosomes to enflurane resulted in a concentration-dependent inhibition of serotonin uptake; the apparent I_{50} was 1.4 ± 0.3 mM enflurane. Maximum inhibition was observed between enflurane concentrations of 2.6 and 4.3 mM, which inhibited uptake between 62 and 70%. The inhibition was rapid and reversible, and kinetic analysis of the inhibition was consistent with competitive inhibition by enflurane of 5-HT uptake with an apparent K_I of 1.61 ± 0.07 mM. In summary, exposure of synaptosomes to clinically relevant concentrations of enflurane resulted in a rapid, concentration-dependent, and reversible inhibition of 5-HT accumulation. These observations could represent a molecular interaction contributing to the anesthetic properties of enflurane and other volatile anesthetics.

5-Hydroxytryptamine (serotonin, 5-HT) is a neuroregulatory amine that modulates sleep, pain perception, and cardiovascular function [1–4]. Substantial evidence supports the premise that enhanced central nervous system serotonergic activity elevates the threshold to noxious stimuli [3, 5–7]. Furthermore, these monoaminergic pathways appear to be important in the mediation of opioid analgesia [3, 8, 9].

The administration of both intravenous and inhalational anesthetic compounds alters brain serotonin content [10, 11]. In an early report by Rosenberg and Klinge [12], rat brain turnover of 5-HT was found to be altered by administration of enflurane. More recently, Althaus *et al.* [13] observed that during enflurane anesthesia, arterial blood pressure, heart rate, and plasma norepinephrine are higher in rats whose central serotonin stores have been depleted. This finding implicated the involvement of serotonergic mechanisms in cardiovascular homeostasis during enflurane anesthesia. These effects of enflurane were suggested to be localized to a step in neuronal 5-HT metabolism.

We have demonstrated previously that volatile anesthetics inhibit uptake of serotonin in rat brain synaptosomes [14]. Inhibition of 5-HT uptake by enflurane could account for observations by previous investigators that noted changes in brain 5-HT metabolism following anesthetic administration. Accordingly, the present study characterizes the effects of enflurane on 5-HT uptake by rat brain synaptosomes.

* This work was supported by a 1988 B. B. Sankey Anesthesia Advancement Award from the International Anesthesia Research Society and a Biomedical Research Support Grant from the Medical College of Georgia and was presented in part at the 1989 Meeting of the International Anesthesia Research Society.

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METHODS

Tissue preparation. Synaptosomes were prepared as described previously with slight modifications [14]. Brains (excluding cerebellum) from decapitated rats were suspended in 10 mL/g tissue of 0.32 M sucrose, 20 mM Tris (pH 7.4; 2–4°) containing 10 μM iproniazid, an irreversible inhibitor of monoamine oxidase. Following homogenization and differential centrifugation, the synaptosomal pellet was resuspended in 0.32 M sucrose–Tris, at approximately 1 mL sucrose/g original starting material, with the protein concentration determined by the method of Smith *et al.* [15].

Uptake assays. Serotonin uptake was measured as described previously [14], except that the final ionic concentrations in the reaction medium were as follows (mM): NaCl, 140; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 2.5; glucose, 10; ascorbate, 1; and Tris base, 20. The solution was buffered to a pH of 7.4 at 37° with HCl. All additions to the reaction medium, except for the synaptosomes, were prepared in the Tris–Krebs buffer. Synaptosomes were pre-equilibrated at 37° for 5 min prior to initiation of uptake by addition of [^{3}H]5-hydroxytryptamine (10.3 Ci/mmol; New England Nuclear). Non-specific uptake was determined in parallel experiments using a buffer containing ion equivalents of lithium substituted for sodium. In kinetic experiments, the serotonin concentration was varied between 2.96 and 189 nM. All other uptake assays were performed in the presence of serotonin (5–50 nM). All reactions were terminated at the indicated times by dilution of the mixture with ice-cold Tris–Krebs buffer (2.5 mL) followed by vacuum filtration. Filters were washed with additional buffer and dissolved in scintillation vials containing 1 mL of

2-methoxyethanol. Tritiated 5-HT levels were then determined by scintillation spectrometry.

Enflurane (Ethrane; Anaquest) was added to the reaction mixture in aliquots from a stock of Tris–Krebs buffer in which the anesthetic had been dissolved to approximately 10 mM. Uptake measured in the presence of the volatile drug was performed in capped reaction tubes. The actual enflurane concentrations were measured by gas–liquid chromatography of 1-μL buffer samples from sham test tubes run in parallel with reaction tubes.

Reversibility experiments were performed by incubating the synaptosomes with enflurane in uncapped reaction tubes allowing escape of the anesthetic. The application of low flow vacuum aeration through a manifold placed at the top of each tube (approximately 5 cm above the reaction medium) was undertaken to facilitate removal of the drug. Pilot studies have shown that vacuum aeration of the reaction tubes in this manner results in a loss of the volatile drug, such that by 40 min the reaction mixture is virtually free of the drug. Control assays were run under identical conditions except that the reaction medium did not contain the anesthetic. Synaptosomal 5-HT content of control and vacuum-aerated halothane tubes was compared with that obtained from enflurane-containing test tubes which were sealed throughout the duration of the experiment. Reactions were initiated by the addition of [^{3}H]5-HT (25 nM) to the uptake medium, and uptake was terminated as described above at designated time points (2–40 min).

Statistical methods. Synaptosomal uptake of serotonin conforms to Michaelis–Menten kinetic principles. Kinetic constants V_{max}, K_m, and K_I were calculated from a linear transformation of the Michaelis–Menten equation and by Lineweaver–Burk analysis using linear, least squares programs (Sigmaplot, Jandel Scientific, Sausalito, CA; or PHARM/PCS, MicroComputer Specialists, Philadelphia, PA).

Statistical differences between means were determined by analysis of variance. Differences between mean values in control and experimental groups were determined by Dunnett's test. Statistical significance was established at $P < 0.05$.

RESULTS

Exposure of synaptosomes to enflurane resulted in a concentration-dependent decrease in the high-affinity uptake rate of serotonin accumulation (Fig. 1). Statistically significant inhibition was observed in the presence of 0.82 mM enflurane, and within the concentration range examined, maximal inhibition was observed to occur between 2.6 and 4.3 mM enflurane, which inhibited uptake by 62 ± 5 and 70 ± 4% respectively. Non-specific uptake of 5-HT was not affected by any concentration of enflurane studied. Specific uptake inhibition by an approximate EC_{50} (concentration which caused 50% inhibition of control uptake; 2.55 ± 0.12 mM) was rapid and reached apparent steady state by 20 sec of exposure of the anesthetic to the synaptosomes (the shortest period tested; Fig. 2).

The effect of enflurane (1.7 ± 0.18 mM) on 5-HT

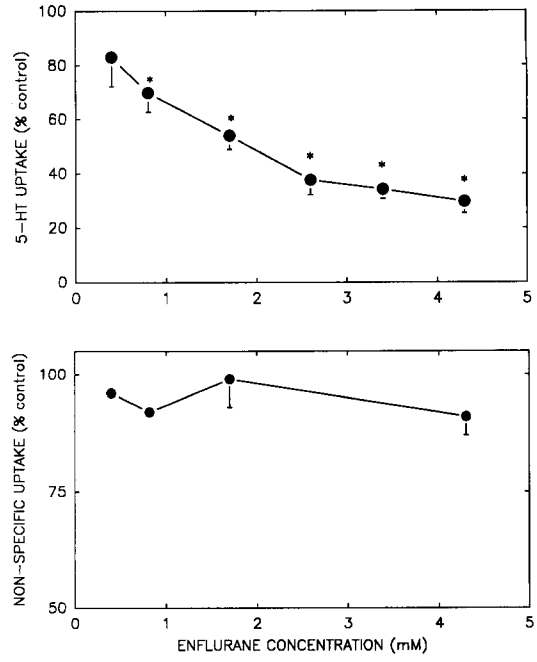

Fig. 1. Effects of enflurane on synaptosomal 5-HT uptake. The effects of enflurane (0.39 to 4.3 mM) on specific (top panel) and non-specific (bottom panel) [^{3}H]5-HT uptake are presented as percent of control uptake in the absence of the anesthetic. The [^{3}H]5-HT concentration was 5 nM and control rates of specific uptake equalled 1.01 ± 0.09 pmol/mg for the 2-min reaction. Non-specific rates of uptake were determined in buffer with equimolar lithium substituted for sodium and were 0.28 ± 0.01 pmol/mg in the absence of the anesthetic. Data are mean values ± SE where N = 3–6. An asterisk (*) = $P < 0.01$.

uptake as a function of serotonin concentration is presented in Fig. 3. Again specific uptake was inhibited at all substrate concentrations, whereas non-specific uptake was unaffected. Lineweaver–Burk plots of the same data are consistent with competitive inhibition of 5-HT uptake by enflurane (Fig. 3). As summarized in Table 1, increasing concentrations of enflurane resulted in a concentration-dependent increase of the apparent K_m without an effect on V_{max} values, supporting the observation that enflurane is competitive with 5-HT for the uptake process. The apparent K_I, calculated from kinetic experiments (N = 5), was 1.61 ± 0.07 mM which approximates the apparent I_{50} (enflurane concentration which produces 50% of maximal inhibition) of 1.4 ± 0.3 mM, as determined by probit analysis.

The competitive nature of the observed inhibition by enflurane indicated that irreversible changes in either synaptosomal structure or function did not occur. This was confirmed in synaptosomal experiments in which non-specific uptake measured in lithium-substituted buffer was not altered by exposure of the synaptosomes to the anesthetic (Figs. 1 and 3), indicating that no significant changes in membrane permeability were effected by enflurane. To further support these observations, studies were performed to examine the reversibility of the inhibition by enflurane (Fig. 4). Vacuum aeration of sham reaction tubes resulted in a decrease in the

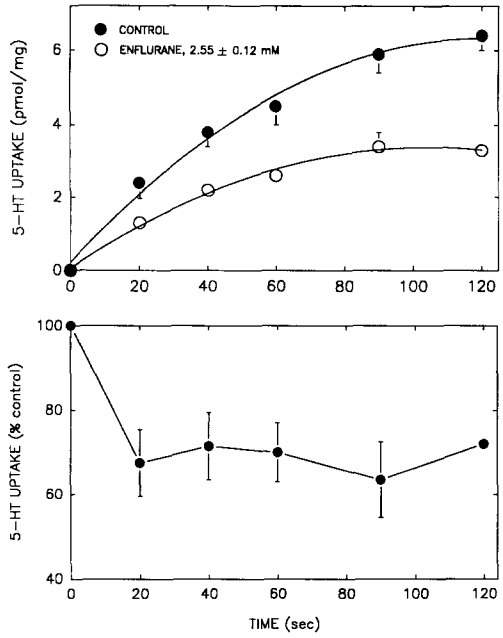

Fig. 2. Time course of 5-HT uptake inhibition by enflurane. 5-HT uptake was initiated by the addition of synaptosomes to buffer containing [^{3}H]5-HT (25 nM) in the absence (control) or presence of enflurane (2.55 ± 0.12 mM). Uptake was terminated at specified times by dilution of the reaction mixture with ice-cold buffer followed by vacuum filtration. The top panel represents absolute total uptake values (means ± SD) from a representative experiment performed in quadruplicate that was repeated once. The bottom panel presents data from the two separate experiments as percent of control uptake for each time point. Data are mean values ± SD where N = 2 experiments each performed in quadruplicate.

anesthetic concentration from 2.55 ± 0.12 mM at 2 min to 0.59 ± 0.2 mM at 40 min. During vacuum removal of the anesthetic, rates of uptake of serotonin returned to levels not different from control (Fig. 4). The inhibition of total uptake by enflurane (2.55 mM) at the 2-min reaction time was 53.0 ± 7% of control. As the anesthetic was removed from the reaction mixture, rates of uptake progressively increased relative to control values and were 96 ± 1.5% of control by 40 min. Rates of 5-HT accumulation measured in capped tubes (non-aerated) containing the anesthetic remained depressed and were not different at 40 min when compared to 2-min values (54.0 ± 4.5%).

DISCUSSION

The present results demonstrate that the volatile anesthetic agent enflurane inhibited sodium-dependent, high-affinity serotonin uptake by rat brain synaptosomes. The inhibition was concentration-dependent, rapid in onset, and readily reversible. Kinetic analysis indicated the inhibition by enflurane to be competitive with 5-HT.

The specificity of enflurane's inhibition of transport for 5-HT versus other biogenic amines is unclear. However, we have observed that a diversity of anesthetics inhibit 5-HT uptake. For example, the injectable anesthetic, ketamine, and other volatile anesthetics inhibit synaptosomal 5-HT transport at clinically relevant concentrations [14].

Following 5-HT release, the rate-limiting step for inactivation is removal of synaptic transmitter by a high-affinity substrate specific transport site. By not allowing neuronal uptake and subsequent intracellular deamination by monoamine oxidase, it is anticipated that serotonin uptake inhibition would elevate measured brain content of the parent amine. This would be consistent with the increase in central nervous system serotonin observed by Rosenberg and Klinge [12] following enflurane administration *in vivo*. In their study, brain concentrations of serotonin were increased above control values at certain periods following anesthetic exposure.

Synaptosomal uptake is driven by the transmembrane sodium gradient. Sodium is co-transported with the amine across the neuronal membrane and released in the sodium-poor cytoplasmic environment [16]. Enflurane could interfere with the uptake process at any one of several steps. As suggested by recent studies using halothane, it is possible that the anesthetic competes with a substrate for binding to the uptake recognition site [17]. The anesthetic molecule could interact with hydrophobic regions of receptor/uptake recognition sites resulting in the ability of the substrate to bind to the uptake transporter. Another possibility is that enflurane allosterically inhibits uptake by binding to a site near, yet distinct from the neurolemmal transporter. The tricyclic antidepressant imipramine, a non-specific inhibitor of serotonin uptake, appears to exert 5-HT uptake inhibition by a similar mechanism [18].

Crucial to 5-HT uptake are metabolic energy stores and transmembrane ion gradients. It is unlikely that depletion of cellular energy stores or inhibition of the sodium pump by the anesthetic would have produced the present results. While membrane Na^+,K^+-ATPase (sodium pump) inhibition by volatile anesthetics occurs rapidly [19], it is unlikely that dissipation of the sodium gradient and resultant inhibition of 5-HT uptake would occur in such a short time (2 min). Previous reports have demonstrated that the sodium pump ATPase must be inhibited for several minutes before decreases in synaptosomal accumulation of 5-HT are observed [20, 21]. In contrast, the uptake inhibition effected by enflurane was established after 20 sec of exposure. Furthermore, drugs which inhibit Na^+,K^+-ATPase, such as ouabain, inhibit 5-HT uptake in a non-competitive manner [18].

Substantial pharmacologic data support the idea that central serotonergic activity governs cardiovascular function by modulating peripheral sympathetic outflow [4]. In one report, the intrathecal administration of serotonin in rats resulted in dose-dependent decreases in arterial blood pressure [22]. In another study of rats, intrathecal serotonin resulted in a dose-dependent increase in blood pressure [23]. The observations by Althaus *et al.* [13] indicate an important interaction by enflurane and serotonergic mechanisms that contributes to the cardiovascular depression observed during the *in vivo* use of enflurane. Depletion of central serotonin in normotensive rats, while having no effect on control cardiovascular parameters, resulted in significant

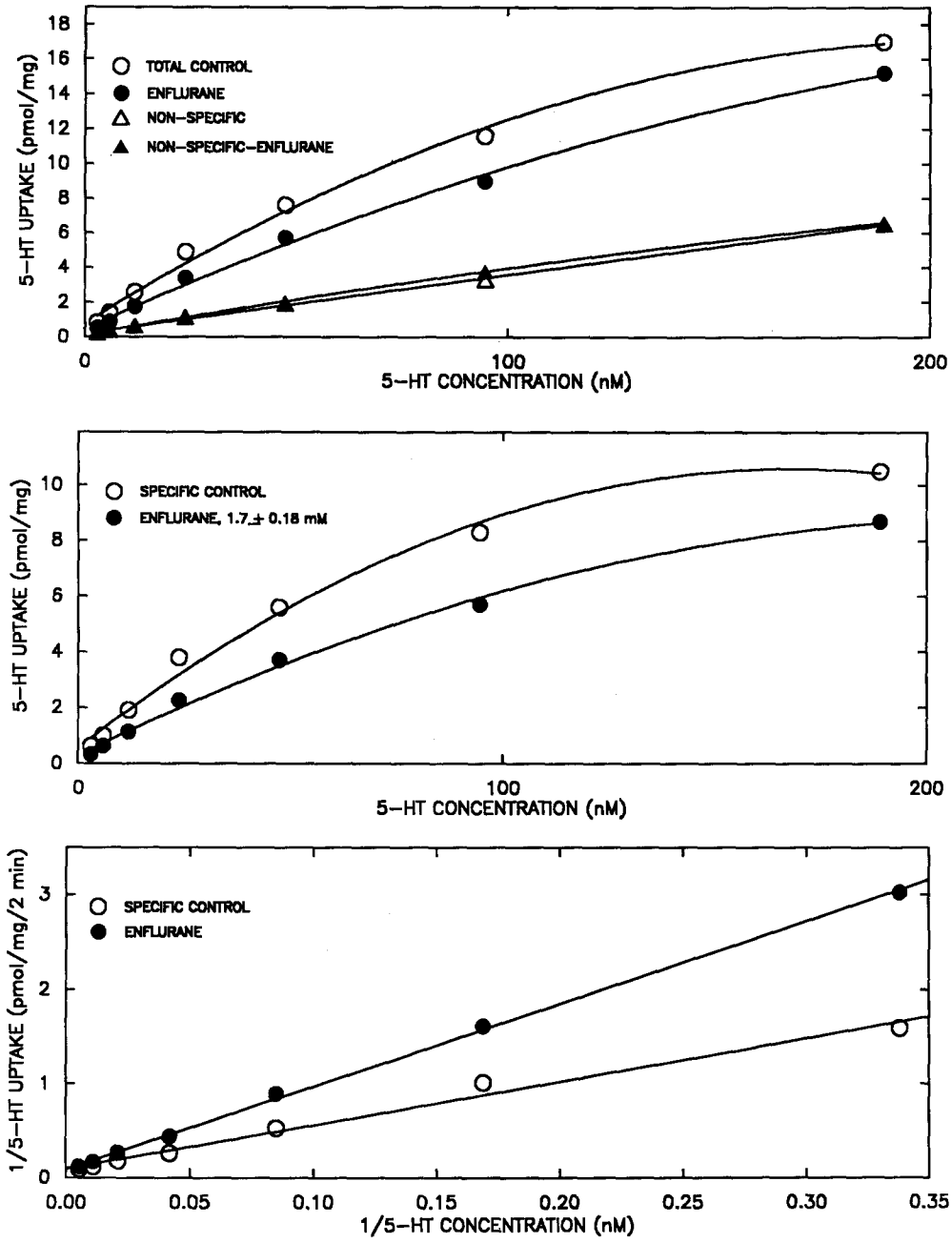

Fig. 3. Kinetic analysis of 5-HT uptake inhibition by enflurane. Top: Total and non-specific uptake of 5-HT was measured over 2 min as a function of [^{3}H]5-HT concentration (2.96 to 189 nM) in the absence and presence of enflurane (1.7 ± 0.18 mM). Data are mean values from a representative of 5 experiments each performed in triplicate. Middle: Specific uptake values determined in the presence and absence of enflurane (1.7 ± 0.18 mM) at the indicated [^{3}H]5-HT concentrations. Data are mean values of a representative experiment. Bottom: The data from the middle figure is presented as a double-reciprocal (Lineweaver–Burk) plot.

elevations of blood pressure, heart rate, and plasma norepinephrine concentration following the institution of general anesthesia with enflurane when compared to animals with normal serotonin stores. It is appreciated that serotonergic pathways mediate both pressor and depressor effects and that regional effects by anesthetic compounds in discrete serotonergic nuclei could exert diverse actions on descending serotonergic modulation of cardiovascular function. The current experiments, however, provide evidence of a potential mechanism by which enflurane could influence cardiovascular function during the clinical use of the drug.

It is also possible that the enhancement of serotonergic neurotransmission involved in the analgesic properties of certain drugs is localized to discrete brain regions and that observed inhibition of uptake in whole brain preparations does not accu-

Table 1. Kinetic analysis of the 5-HT uptake inhibition by enflurane

Experimental condition	V_{max} (pmol/mg protein/2 min)	K_m (nM)
Control	11.9 ± 0.78	68 ± 6
Enflurane		
1.7 ± 0.18 mM	12.6 ± 0.75	130 ± 10*
3.4 ± 0.27 mM	14.1 ± 1.50	220 ± 20†

Data are means ± SE of 4–5 experiments, each performed in triplicate.
* $P < 0.05$ versus control.
† $P < 0.01$ versus control.

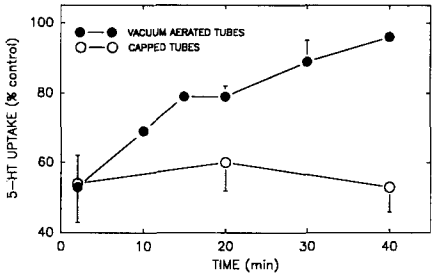

Fig. 4. Reversibility of the 5-HT uptake inhibition by enflurane. Synaptosomes were incubated in the presence and absence of enflurane (2.55 ± 0.12 mM) and [^{3}H]5-HT (25 nM). Reactions including the anesthetic were carried out in capped tubes, and tubes that were vacuum aerated. Reactions were terminated at specified times, and synaptosomal content of [^{3}H]5-HT was determined. Data represent experimental 5-HT content expressed as a percent of that measured in reactions not containing the anesthetic for that time point. Control 5-HT uptake values were 4.7 ± 0.4 and 15.6 ± 0.01 pmol/mg protein at 2 and 40 min respectively. Data are means ± SD where N = 2 experiments each performed in quadruplicate.

rately reflect regional interactions. Consistent with this are the observations by Roizen *et al.* [10] who described regional elevations of brain serotonin content following halothane anesthesia in rats. While a cause-and-effect relationship is not established, focal rather than global changes in serotonergic neurotransmission may be most important in effecting functional correlates in the intact animal.

The relationship between effective anesthetic levels *in vivo* and relevant concentrations of enflurane used in the present *in vitro* study can be derived from calculations involving the molecular weight, specific gravity, and available partition coefficient data for enflurane. At 2.2% (v/v), the minimum alveolar concentration (MAC) for rats [24], approximately 159 mg enflurane equilibrates with 1 L air at 37° and one atmosphere pressure. Division of this value by the molecular weight of enflurane (184.5) yields a molar gas concentration of 0.86 mM. Since the Krebs solution/air–gas partition coefficient for enflurane is 0.74 [25], the approximate concentration in Krebs solution equilibrated with 2.2% (v/v) gas at 37° would be 0.64 mM. Since MAC represents the ED_{50} for surgical anesthesia [26] and because anesthetic requirements vary due to inherent biological variation and type of surgical procedure, clinically relevant *in vitro* concentrations may be considered to range up to 2.5 times MAC [26, 27]. Between these *in vitro* concentrations of enflurane (0.64 to 1.6 mM), 5-HT accumulation was inhibited by nearly 50%. If one applies these calculations for human MAC (1.68%, v/v), equivalent gas and Krebs phase concentrations are 0.66 and 0.49 mM respectively. Thus, if the present observations apply to effects of enflurane anesthesia in humans, brain 5-HT uptake could be inhibited significantly.

In summary, the current experiments characterized the effects of enflurane on 5-HT uptake in rat brain synaptosomes. The anesthetic inhibited uptake of the amine in a concentration-dependent manner that was rapid and reversible. These observations suggest a mechanism by which enflurane may produce anesthetic and analgesic responses and affect cardiovascular homeostasis during clinical use.

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