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Effects of Isoflurane and Propofol on Glutamate and GABA Transporters in Isolated Cortical Nerve Terminals

Robert I. Westphalen, Ph.D., Hugh C. Hemmings, Jr., M.D., Ph.D.†*

Background: **Depression of glutamate-mediated excitatory transmission and potentiation of γ-aminobutyric acid (GABA)–mediated inhibitory transmission appear to be primary mechanisms by which general anesthetics produce anesthesia. Since effects on transmitter transport have been implicated in anesthetic actions, the authors examined the sensitivity of presynaptic glutamate and GABA transporters to the effects of a representative volatile (isoflurane) and a representative intravenous (propofol) anesthetic.**

Methods: **A dual-isotope (L-[^{3}H]glutamate and [^{14}C]GABA) approach allowed simultaneous comparisons of anesthetic effects on three independent assays of glutamate and GABA transporters in adult rat cerebral cortex: transmitter uptake into isolated nerve terminals (synaptosomes), transmitter binding to lysed and washed synaptosomes (synaptic membranes), and carrier-mediated release (reverse transport) of transmitter from preloaded synaptosomes using a modified superfusion system.**

Results: **Isoflurane produced small but statistically significant inhibition of L-[^{3}H]glutamate and [^{14}C]GABA uptake, while propofol had no effect. Inhibition of uptake by isoflurane was noncompetitive, an outcome that was mimicked by indirectly affecting transporter function through synaptosomal depolarization. Neither isoflurane nor propofol affected L-[^{3}H]glutamate or [^{14}C]GABA binding to synaptic membranes or Ca^{2+}-independent carrier-mediated L-[^{3}H]glutamate or [^{14}C]GABA release (reverse transport).**

Conclusions: **These findings suggest that isoflurane and propofol at clinical concentrations do not affect excitatory glutamatergic transmission or inhibitory GABAergic transmission directly *via* effects on their presynaptic neuronal transporters.**

GENERAL anesthetics have profound effects on synaptic transmission in the central nervous system.[1] Recent work has focused on assessing the role of presynaptic *versus* postsynaptic actions in the depression of excitatory transmission and potentiation of inhibitory transmission by anesthetics. Potentiation of postsynaptic γ-aminobutyric acid $(GABA)_A$ receptors has been described for many anesthetics.[2] In contrast, the presynaptic mechanisms underlying anesthetic actions on glutamatergic and GABAergic synaptic transmission are less well characterized.

The concentration and rate of clearance of transmitters from the synaptic cleft are important determinants of synaptic function. The presynaptic processes of transmitter release and reuptake are therefore critical in modulating neurotransmission. Clearance of glutamate and GABA relies on passive diffusion and reuptake into glia and neurons by transporters rather than on enzymatic inactivation.[3,4] A role for transporters in controlling the amplitude of excitatory postsynaptic currents has been demonstrated in cultured rat hippocampal neurons.[5,6] Similarly, GABA uptake blockade prolongs inhibitory postsynaptic currents and inhibitory postsynaptic potentials in rat hippocampal slices.[7]

The results of previous studies of the effects of general anesthetics on amino acid neurotransmitter systems are inconsistent. Evidence exists for inhibition of GABA uptake[8–11] and potentiation of glutamate uptake[12,13] by anesthetics. However, anesthetics have also been reported to inhibit glutamate uptake[10,11] or have no effect on either glutamate or GABA uptake.[8,9,11,13–19] In an attempt to clarify the effects of general anesthetics on amino acid transporters, we investigated the effects of the widely used volatile anesthetic isoflurane and intravenous anesthetic propofol on neuronal glutamate and GABA transporters in isolated nerve terminals from rat cerebral cortex simultaneously using double-isotope methods. Three independent assays were used to assess anesthetic interactions with transporters: Na^+-dependent uptake into isolated nerve terminals (synaptosomes), Na^+-dependent transmitter binding to synaptic membranes, and carrier-mediated transmitter release (reverse transport).

Materials and Methods

Drugs

Isoflurane was obtained from Abbott Laboratories (North Chicago, IL), and propofol was obtained from Aldrich Chemical (St. Louis, MO). Dihydrokainic acid (DHK) and SKF89776A were from Tocris (Bristol, United Kingdom); nipecotic acid, NO-711, tetrodotoxin, and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (t-PDC) were from Sigma Chemical Co. (St. Louis, MO); L-[^{3}H]glutamate was from Amersham Radiochemical Center (42 Ci/mmol; Buckinghamshire, United Kingdom); and [^{14}C]GABA was from PerkinElmer Inc. (0.24 Ci/mmol; Boston, MA).

Synaptosome and Synaptic Membrane Preparation

Experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and

* Postdoctoral Associate, † Professor and Vice Chair of Research, Professor of Pharmacology.

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Address reprint requests to Dr. Hemmings: Box 50, LC-203A, Weill Medical College of Cornell University, 525 East 68th Street, New York, New York 10021. Address electronic mail to: hchemmi@med.cornell.edu.

Use of Laboratory Animals and approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee (New York, New York). Male Sprague-Dawley rats (weight, 250–350 g) were anesthetized with 80% CO_2–20% O_2 and sacrificed by cervical dislocation. The whole brain was rapidly removed and placed on ice. The cerebral cortex was dissected out and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a motor-driven (500 rpm) Teflon–glass homogenizer (Thomas Scientific, Swedesboro, NJ), and the homogenate was centrifuged for 2 min at 4,000*g*. Crude synaptosomes (supernatant) were demyelinated by centrifugation though 0.8 M sucrose (10 ml S1 layered onto 10 ml sucrose, 0.8 M) for 30 min at 36,000*g*.[20] The pellet was either resuspended in ice-cold 0.32 M sucrose for use within 5 h (for intact synaptosomes that sequester transmitter by active transport) or lysed in 10 volumes of ice-cold 5 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl, pH 7.4 (for synaptic membranes that contain neuronal transporters with functional transmitter binding sites). Synaptosomes resuspended in hypoosmotic buffer were allowed to lyse for 30 min on ice before centrifugation at 20,000*g* for 15 min. The resulting pellet was resuspended in two volumes of 5 mM Tris-HCl, pH 7.4, and frozen at −70°C until use (within 6 weeks). Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.[21]

Glutamate and GABA Uptake

Na^+-dependent uptake of glutamate and GABA was determined by incubating synaptosomes (40–90 μg protein) with 200 nM L-[^{3}H]glutamate and 50 nM [^{14}C]GABA with a range of concentrations of propofol (0.3–100 μM in 5-ml glass tubes), isoflurane (0.2–1.7 mM in closed 1.5 ml polyethylene microcentrifuge tubes; final concentrations determined by gas chromatography), tetrodotoxin (0.5–2 μM), unlabeled L-glutamate (0.3–1,000 μM), and/or unlabeled GABA (0.1–300 μM). Incubation was for 1.5–2 min at 37°C in 0.5 ml Krebs–HEPES buffer (composition: 140 mM NaCl, 5 mM KCl, 20 mM HEPES–NaOH, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 5 mM $NaHCO_3$, and 10 mM D-glucose, pH 7.4). Isoflurane was added to the assay tubes by appropriate dilution of saturated solutions of isoflurane (in assay buffer; 10–12 mM) prepared 12–24 h prior to experimentation. Propofol was added from concentrated solutions prepared in dimethyl sulfoxide and then diluted in assay buffer in glass tubes prior to addition to assay tubes (maximum dimethyl sulfoxide concentration < 0.5% [v/v] in assay). In some experiments, NaCl was replaced with choline chloride (ChCl) to determine the level of Na^+-dependent uptake, glutamate (t-PDC) and GABA (nipecotic acid and NO-711) uptake blockers were used to inhibit uptake directly, or the Na^+ channel blocker tetrodotoxin was used to alter the Na^+ gradient that can influence amino acid uptake. Synaptosomes (containing transported radiolabeled transmitter) were separated from the external medium by vacuum filtration through Whatman GF/C glass fiber filters (Maidstone, United Kingdom) and washed twice with 5 ml ice-cold NaCl, 0.9%. Filter radioactivity was quantified by liquid scintillation spectrometry using BioSafe II scintillation cocktail (RPI, Mt. Prospect, IL) and a liquid scintillation spectrometer (Beckman Coulter LS 6000IC, Fullerton, CA) with dual-isotope quench correction.

Glutamate and GABA Transporter Binding

Na^+-dependent binding of L-[^{3}H]glutamate and [^{14}C]GABA to their transporters on synaptic membranes was determined using a modification of previous methods.[22–25] Synaptic membrane preparations were thawed, washed with 2 × 20 volumes of 5 mM Tris-HCl, pH 7.4, to remove endogenous transmitter, and incubated (17–45 μg protein) with 200 nM L-[^{3}H]glutamate and 250 nM [^{14}C]GABA in 0.5 ml assay buffer (120 mM NaCl and 50 mM Tris-HCl, pH 7.4) in 1.5-ml polyethylene microcentrifuge tubes. In some experiments, NaCl was replaced with ChCl to determine the level of Na^+-dependent binding. Binding was measured at 20–22°C[24] in the presence of a range of unlabeled L-glutamate (0.01–100 μM) and GABA (0.01–100 μM). Drugs listed above (Methods: Glutamate and GABA Uptake section) were added in a parallel set of tubes. After a 20-min incubation, which allowed binding to reach equilibrium (data not shown), radioactivity bound to synaptic membranes was separated from supernatant (free radioactivity) by centrifugation (5 min at 15,000*g*, 4°C). The surface of the resulting pellet and the inside of the tube were washed with 2 × 1.8 ml ice-cold assay buffer, and the residue was analyzed for radioactivity as above (Methods: Glutamate and GABA Uptake section). Separate experiments showed no significant change in isoflurane concentration during the incubation and centrifugation steps of the assay (data not shown).

Glutamate and GABA Release

Demyelinated rat cerebrocortical synaptosomes were preloaded with 8 nM L-[^{3}H]glutamate and 440 nM [^{14}C]GABA for 15 min at 30°C in Krebs–HEPES buffer, centrifuged (10 min at 20,000*g*, at 4°C), and resuspended in ice-cold 0.32 M sucrose. Synaptosomes were confined by Whatman GF/B glass fiber filter disks and superfused at 0.5 ml/min with Ca^{2+}-free Krebs–HEPES buffer containing 0.1 mM EGTA (initially bubbled with 95% O_2–5% CO_2 for 10 min) at 36°C using a customized Brandel SF12 superfusion apparatus (Gaithersburg, MD). Release was induced by pulses of 30 mM K^+ (with KCl replacing equivalent NaCl) for 3 min, and fractions were collected every minute. At the end of each experiment, synaptosomes were lysed with 0.2 M perchloric acid, and the radioactivity in the synaptosomes and fractions was

determined as described above (Methods: Glutamate and GABA Uptake section). Release was expressed as a fraction of synaptosomal content of transmitter prior to each fraction collected (fractional release). Release pulse magnitude was determined by subtracting baseline release from cumulative fractional release during stimulation. Isoflurane or propofol were introduced 3 min prior to and during stimulation through polytetrafluoroethylene tubing from glass syringes (closed system) or glass tubes (open system), respectively. Blockers of glutamate (DHK)[26,27] and GABA (SKF89776A)[28,29] uptake were introduced using the closed-system procedure. These inhibitors do not stimulate release because they are not substrates for their respective transporters,[26,30] and they do not promote heteroexchange,[26,31] potentiate depolarization (as does NO-711),[32] or activate $GABA_A$-like ion channels (as does nipecotic acid).[33,34] In parallel experiments, isoflurane and propofol concentrations in the superfusate were determined by gas chromatography[35] or high-performance liquid chromatography,[36] respectively.

Data Analysis

Maximum binding (B_{max}) and apparent affinity (K_d) of L-[^{3}H]glutamate or [^{14}C]GABA binding to rat cortical synaptic membranes were determined from radioligand binding assays using equations 1 and 2:

$$B_{max} = \left[\frac{K_d L}{[L]} + 1\right] \times B \quad (1)$$

$$K_d I = \frac{IC_{50}}{1 + \frac{[L]}{K_d L}} \quad (2)$$

A constant radioligand concentration ([L]) was displaced by a range of concentrations of the unlabeled amino acid equivalent ([I]). The displacement data established B (amount of radioligand bound at concentration L in the absence of displacer) and IC_{50} (relative to [L]), which were estimated from linear curves created by plotting logit-converted B against log [I] and fitted using a least-squares curve fitting program (MKMODEL; Biosoft, Cambridge, United Kingdom). The program established the binding parameters B_{max} (maximum binding at ~[L]), K_dL (dissociation constant of L), and K_dI (dissociation constant of displacer) according to equations 1 and 2,[37] where K_dL was fixed to equal K_dI (radioligand = displacer).

Curve fitting used a one-site model, thereby analyzing only the parameters of the high-affinity binding site as the influence of the low-affinity binding site was removed by incorporating it into nonspecific binding. Any apparent experimental effect on B_{max} or K_d was reanalyzed with K_d or B_{max}, respectively, fixed to the control value. This procedure eliminated potential influences of each parameter on the other due to their mathematical interrelation.[38] Kinetic parameters (V_{max} and K_m) for accumulation of L-[^{3}H]glutamate or [^{14}C]GABA into rat cortical synaptosomes were estimated by the same method.

Inhibition data were analyzed using Prism version 3.02 (GraphPad Software, San Diego, CA) to estimate E_{max}, IC_{50}, and Hill slope. Unpaired *t* tests were used to test for significant differences between control K_d and K_m values and between control and experimental data from carrier-mediated transport experiments. Uptake inhibitors and binding displacers were tested for significant effects using one-way analysis of variance followed by the Dunnett multiple comparison test. The V_{max} or K_m values determined from uptake kinetic experiments, performed in the presence or absence of isoflurane, were tested for significant differences using paired *t* tests. Since the means of data sets in this study were obtained from *in vitro* studies, which display experimental rather than biologic variance, the SEM is reported.

Results

Glutamate and GABA Uptake

Uptake of L-[^{3}H]glutamate and [^{14}C]GABA into rat cortical synaptosomes was largely Na^+-dependent; uptake in the absence of Na^+ was 16 ± 5 and 5 ± 0.4% of total uptake in the presence of Na^+. These values are consistent with maximum inhibition of L-[^{3}H]glutamate by 1 mM unlabeled L-glutamate (93 ± 2% of total uptake) or [^{14}C]GABA uptake by 300 μM unlabeled GABA (94 ± 1% of total uptake) in the presence of Na^+ (data not shown).

Kinetic parameters for L-[^{3}H]glutamate and [^{14}C]GABA uptake (table 1) are consistent with previous reports of high-affinity excitatory amino acid transport[4,26,39–42] and GABA transport[43] in isolated nerve terminals. The transport assay parameters of V_{max} and K_m determined in the presence of both inhibiting amino acids did not differ significantly from those determined in parallel assays using each amino acid alone (table 1). This verified the accuracy of the dual-label assay in detecting changes in the transport of one amino acid independently of the other. Na^+-dependent L-[^{3}H]glutamate uptake was inhibited by increasing concentrations of unlabeled L-glutamate, DHK, and isoflurane but not by unlabeled GABA, NO-711, or propofol (fig. 1A). [^{14}C]GABA uptake was inhibited by unlabeled GABA, NO-711, SKF89976A, or isoflurane but not by unlabeled L-glutamate, DHK, or propofol (fig. 1B). Although isoflurane-induced [^{14}C]GABA uptake inhibition was not statistically significant, the data fitted to a sigmoidal inhibition curve. This effect was found to be significant by kinetic analysis using varying ligand concentrations (see below; Results: Glutamate and GABA Uptake section). Tetrodotoxin enhanced both L-[^{3}H]glutamate and [^{14}C]GABA uptake

Table 1. Kinetic Parameters of L-[^{3}H]Glutamate and [^{14}C]GABA Uptake and Binding

	Kinetic Parameters			
	Uptake		Binding	
Amino Acid	V_{max}, nmol · min^{-1} · mg^{-1}	K_m, μM	B_{max}, nmol · min^{-1} · mg^{-1}	K_d, μM
Glutamate	2.5 ± 0.4	14 ± 1.4	3.2 ± 0.6	2.3 ± 0.5*
	(116 ± 11%)	(100 ± 21%)	(100 ± 0.9%)	(95 ± 5%)
GABA	1.2 ± 0.2	4.6 ± 0.5	0.2 ± 0.1	1.4 ± 0.2*
	(94 ± 5%)	(96 ± 6%)	(103 ± 2%)	(87 ± 13%)

Results obtained from least-squares curve fitting analysis of uptake and binding data using a dual-label approach, in which single and dual inhibitor/displacer assays were performed in parallel. Parameters maximum velocity (V_{max}; nmol · min^{-1} · mg protein^{-1}), Michaelis-Menten constant (K_m; μM), maximum binding (B_{max}; nmol · min^{-1} · mg protein^{-1}), and dissociation constant (K_d; μM) are reported as mean ± SEM (n = 9–16). Differences between K_m and K_d values were analyzed using the *t* test analysis. Results are compared to those obtained using a single inhibitor/displacer (in parentheses), presented as a percentage of intraassay (paired) value from "single" assay and tested using paired *t* test analysis (n = 3–5).

* $P < 0.001$.

GABA = γ-aminobutyric acid.

(figs. 1A and B). This may result from an increase in the $[Na^+]_o/[Na^+]_i$ ratio, which is a factor in driving the uptake of transmitters coupled to the cotransport of Na^+.[29,44–47]

Inhibition by isoflurane of L-[^{3}H]glutamate uptake (IC_{50} = 0.74 ± 0.03 mM; I_{max} = 40 ± 2%) and [^{14}C]GABA uptake (IC_{50} = 0.78 ± 0.02 mM; I_{max} = 17 ± 2%) occurred at concentrations twofold higher than the clinically effective concentration (minimum alveolar concentration [MAC] = 0.35 mM in rat).[48] L-[^{3}H]glutamate uptake inhibition data yielded a Hill slope of −5 for isoflurane, compared to −1 for L-glutamate. Similarly, [^{14}C]GABA uptake inhibited by isoflurane or GABA produced Hill slope values of −8 and −1, respectively. This indicates a qualitative difference between the inhibition of uptake mediated by isoflurane and substrate.

Kinetic analysis was consistent with noncompetitive inhibition by isoflurane (0.7 mM) of L-[^{3}H]glutamate (fig. 2A) and [^{14}C]GABA (fig. 2B) uptake; this is demonstrated by a reduced V_{max} (y-intercept) with an unchanged K_m (slope). In contrast to the linear regression approach used in Eadie-Hofstee analysis, nonlinear curve fitting yields improved estimates of the parameters and avoids the inaccuracies of graphical methods.[49,50] Analysis by least-squares curve fitting confirmed that uptake inhibition by isoflurane was associated with significant reduction in V_{max}, with unchanged K_m (table 2). Uptake of both glutamate and, to a lesser degree, GABA was inhibited significantly by 0.7 mM isoflurane (table 2). In the concentration–effect assays (fig. 1), only inhibition of glutamate uptake was statistically significant, probably due to greater variance within these data and the smaller effect on [^{14}C]GABA uptake compared with L-[^{3}H]glutamate uptake.

Since a change in K_m can influence V_{max} because of their mathematical interrelation, V_{max} was reanalyzed with K_m held constant at the control value. This showed that the noncompetitive inhibition of L-[^{3}H]glutamate or [^{14}C]GABA uptake by isoflurane was not due to changes in K_m (table 2). Partial depolarization by in-

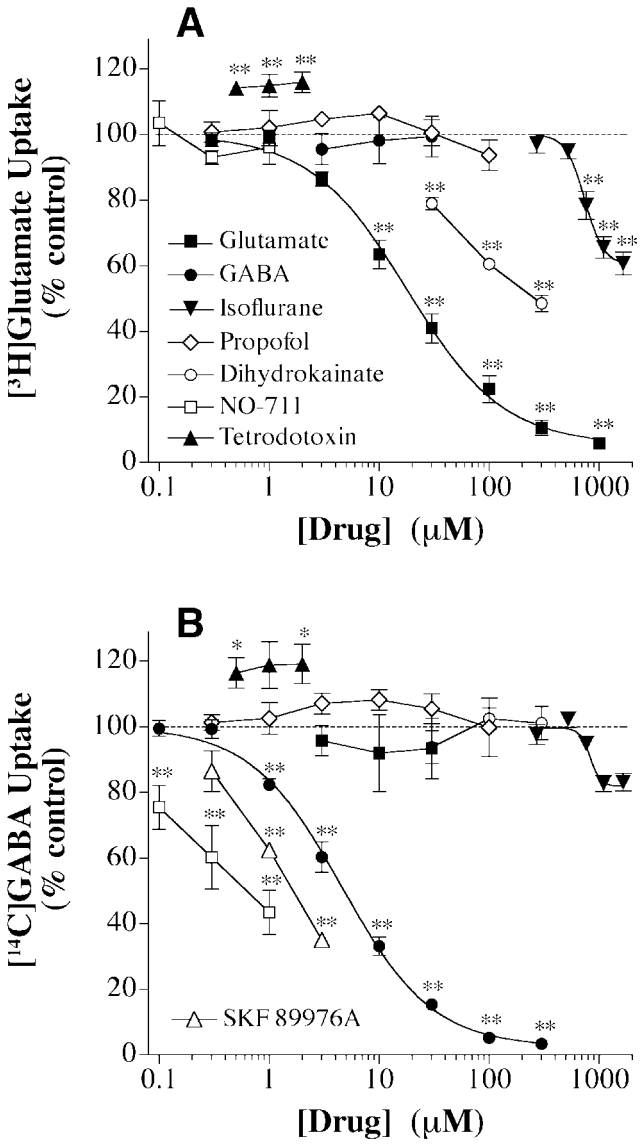

Fig. 1. Effects of anesthetics and other drugs on the uptake of L-[^{3}H]glutamate (*A*) and [^{14}C]GABA (*B*) into rat cortical synaptosomes. *$P < 0.05$, **$P < 0.01$ *versus* control (n = 4–7).

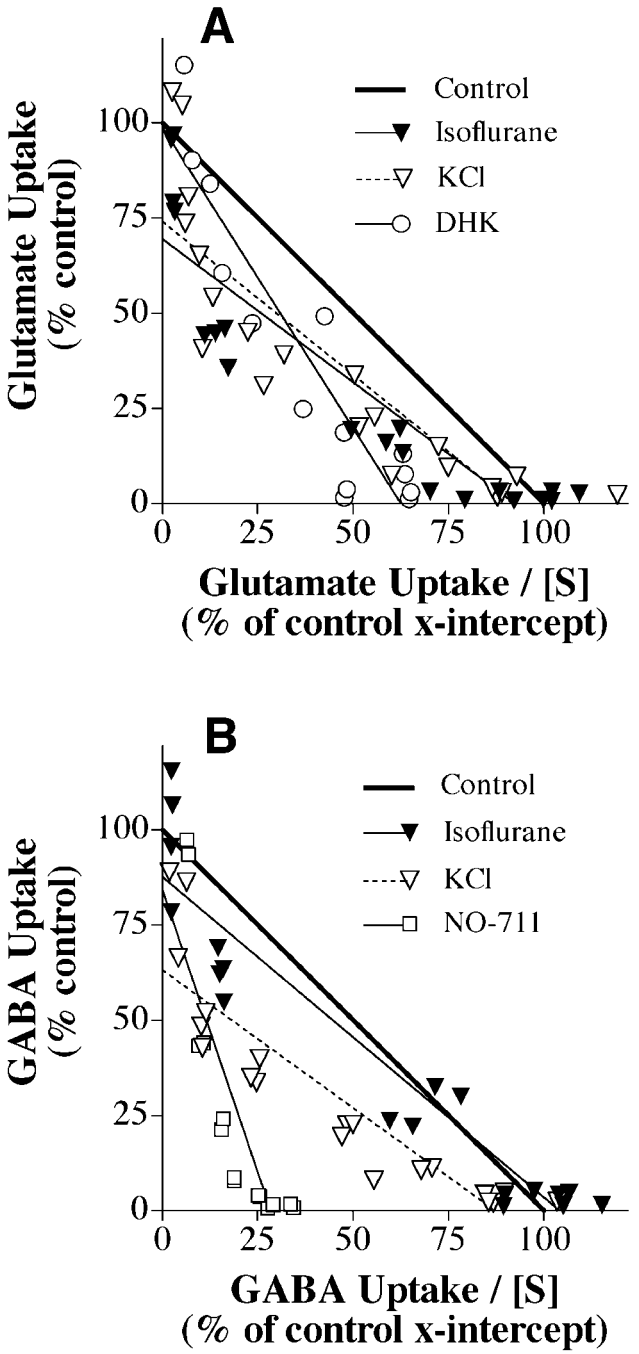

Fig. 2. Eadie-Hofstee plots of synaptosomal uptake of L-[^{3}H]glutamate (*A*) and [^{14}C]GABA (*B*) expressed as percentage of control axis intercepts and fitted by linear regression. L-[^{3}H]glutamate uptake was inhibited noncompetitively by 0.7 mM isoflurane or 15 mM KCl and inhibited competitively by 50 μM DHK. Control: V_{max} = 1.8 ± 0.5 nmol · min^{-1} · mg protein^{-1}; K_m = 26 ± 4 μM. [^{14}C]GABA uptake was inhibited noncompetitively by 0.7 mM isoflurane or 15 mM KCl and inhibited competitively by 0.2 μM NO-711. Control: V_{max} = 0.7 ± 0.3 nmol · min^{-1} · mg protein^{-1}; K_m = 7 ± 0.7 μM (n = 3–5).

creasing extracellular K^+ also produced noncompetitive inhibition (figs. 2A and B). In comparison, DHK (fig. 2A) or NO-711 (fig. 2B) competitively and selectively inhibited L-[^{3}H]glutamate or [^{14}C]GABA uptake, respectively.

Glutamate and GABA Transporter Binding

Maximal inhibition of L-[^{3}H]glutamate and [^{14}C]GABA binding by L-glutamate or GABA, respectively, matched the level of Na^+-dependent binding (fig. 3). Conversely, Na^+-independent binding equaled nonspecific binding (fig. 3). [^{14}C]GABA binding showed more nonspecific binding compared to L-[^{3}H]glutamate binding.

Na^+-dependent L-[^{3}H]glutamate and [^{14}C]GABA binding to cortical synaptic membranes was displaced by their respective unlabeled amino acids with kinetic parameters (table 1) consistent with published values.[25,41] Using a paired *t* test analysis, B_{max} and K_d values determined in dual-label, dual-inhibitor assays did not differ significantly from those determined from dual-label, single-inhibitor assays performed in parallel (table 1).

L-glutamate and t-PDC but not GABA, propofol, isoflurane, or tetrodotoxin displaced L-[^{3}H]glutamate binding (fig. 4A). [^{14}C]GABA binding was displaced partially by unlabeled GABA and nipecotate but not by glutamate, t-PDC, propofol, isoflurane, or tetrodotoxin (fig. 4B).

Glutamate and GABA Reverse Transport

In the absence of Ca^{2+}, K^+-induced release of L-[^{3}H]glutamate and [^{14}C]GABA from preloaded synaptosomes occurs primarily by carrier-mediated reverse transport of cytoplasmic transmitter.[51] Addition of Ca^{2+} (2 mM) triggers vesicular release,[52] such that total release increased significantly (fig. 5). As positive controls, 50 μM DHK and 0.5 μM SKF89976A selectively inhibited Ca^{2+}-independent release of L-[^{3}H]glutamate and [^{14}C]GABA, respectively (fig. 5). Ca^{2+}-independent release of L-[^{3}H]glutamate and [^{14}C]GABA induced by K^+ was unaffected by 1 mM isoflurane or 15 μM propofol (fig. 5).

Discussion

Changes in transporter uptake activity influence transmitter levels in the synaptic cleft and thereby affect synaptic transmission.[6,45] Since general anesthetic action is associated with prominent depressant effects on excitatory transmission and enhancement of inhibitory neurotransmission,[1] we investigated the actions of representative anesthetics on presynaptic transporter-mediated uptake of the major excitatory (glutamate) and inhibitory (GABA) amino acid neurotransmitters by their native neuronal transporters.

The results of previous studies of the presynaptic actions of general anesthetics on amino acid neurotransmitter uptake have been inconsistent. Evidence has been provided for inhibition of GABA uptake by intravenous[8,9] but not by volatile[8,13,15,19] anesthetics. Volatile anesthetics have been reported to potentiate,[12,13] inhibit,[10,11] or have no effect on[14–18] glutamate uptake. Intravenous anesthetics have been reported to inhibit[53] or have no effect on[11,14,16,17] glutamate uptake.

Table 2. Kinetic Parameters of l-[^{3}H]Glutamate and [^{14}C]GABA Uptake

	Kinetic Parameters			
	Eadie–Hofstee Linear Fit		Least-Squares Curve Fit	
	V_{max}, nmol · min^{-1} · mg^{-1}	K_m, μM	V_{max}, nmol · min^{-1} · mg^{-1}	K_m, μM
[^{3}H]Glutamate				
Control	3.0 ± 0.2	32 ± 3	4.1 ± 0.2	18 ± 2.5
0.7 mm isoflurane	2.1 ± 0.1†	26 ± 1	3.4 ± 0.3* (3.4 ± 0.2*)	17 ± 0.9
[^{14}C]GABA				
Control	0.94 ± 0.06	7.2 ± 0.4	1.15 ± 0.02	4.8 ± 0.7
0.7 mm isoflurane	0.83 ± 0.04	6.1 ± 0.3	1.06 ± 0.02* (1.05 ± 0.02*)	5.9 ± 0.5

The results from two methods of analysis are shown. Analysis using least-squares curve fit included the determination of maximum velocity (V_{max}) in the presence of isoflurane while fixing the Michaelis-Menten constant (K_m) to control values (results in parentheses). Statistical significance between control and isoflurane parameters of V_{max} (nmol · min^{-1} · mg protein^{-1}) and K_m (μM) was tested by paired *t* tests.

* $P < 0.05$]; † $P < 0.02$ *versus* control (n = 4).

These conflicting results may be a consequence of varying experimental approaches. Isolated nerve terminals,[8,11,12,17–19] rather than intact brain tissue preparations,[10,14,16] provide a superior experimental system for investigating presynaptic mechanisms of general anesthetic effects on synaptic transmission in isolation of indirect effects inherent in intact neural networks. Use of a dual-label experimental approach to compare effects under identical conditions and three independent assays of presynaptic transporters confers significant advantages in determining whether anesthetics directly affect glutamate or GABA uptake. An important consideration in transmitter uptake assays is incubation time. In this study, uptake was linear with time up to 1.5–2 min at 37°C (data not shown), which is consistent with other studies.[17] Pharmacological elimination of transporters on otherwise intact synaptosomes yields initial uptake rates that are proportional to transporter density but that are dissociated from transporter density when the plateau phase is reached.[54] Use of incubation times within the linear phase of initial uptake rate[17] yield more accurate transporter activity data compared to studies that introduce[8,11–13,19] or use[18] the plateau phase.

Apart from the known blockers of Na^+-dependent uptake of L-[^{3}H]glutamate (DHK) and [^{14}C]GABA (NO-711 and SKF8977A), only isoflurane produced significant inhibition. However, the efficacy of isoflurane was low (40% and 17% inhibition at 1.2 mM, respectively), consistent with relatively small and probably pharmacologically insignificant effects at clinical concentrations. Propofol had no significant effects at 15 μM, compared to an approximate free EC_{50} for anesthesia of 2.2 μM.[55] Kinetic analysis revealed that uptake inhibition was noncompetitive, suggesting that isoflurane did not compete with substrate. Moreover, isoflurane did not affect L-[^{3}H]glutamate or [^{14}C]GABA binding to transporters in synaptic membranes, further supporting the notion that the anesthetic did not affect substrate binding. Noncompetitive uptake inhibition was also produced by partial depolarization, a manipulation that indirectly affects membrane potential–dependent uptake of glutamate and GABA.[46] Inhibition of uptake with unaltered presynaptic transporter binding is consistent with an indirect effect of isoflurane on transporter function, possibly due to anesthetic-induced depolarization of glutamatergic and GABAergic nerve terminals.

In the presence of Na^+, L-[^{3}H]glutamate[22] and [^{14}C]GABA[25] binding to synaptic membranes is primarily to their respective transporters, although it has been argued that Na^+-dependent "binding" of excitatory amino acids is largely due to uptake into membrane-bound saccules.[56] Nevertheless, the binding assay, unlike the uptake assay, was not influenced by conditions that alter membrane potential and/or ion fluxes, as exemplified by the differing effects of tetrodotoxin on each assay. Also, the significant difference between K_d and K_m for glutamate ($P < 0.01$) and GABA ($P < 0.001$) reflect a clear distinction between the dynamics of transmitter uptake and binding.

Depolarization in the absence of extracellular Ca^{2+} induces reverse operation of presynaptic glutamate[27,51] and GABA[29,31,33] transporters. This nonvesicular release of cytoplasmic transmitter is Na^+-dependent and is blocked by transporter antagonists. Concentrations of DHK (50 μM) and SKF89976A (0.5 μM) that blocked the uptake of their respective amino acid to approximately the same extent as 1 mM isoflurane significantly inhibited Ca^{2+}-independent release of L-[^{3}H]glutamate or [^{14}C]GABA, respectively. This demonstrates that partial blockade of glutamate or GABA uptake significantly inhibits carrier-mediated transmitter release. However, neither isoflurane (1 mM) nor propofol (15 μM) affected Ca^{2+}-independent carrier-mediated release. Previous studies also found minimal effects of anesthetics on Ca^{2+}-independent glutamate release.[57] Our results further support the suggestion that direct presynaptic

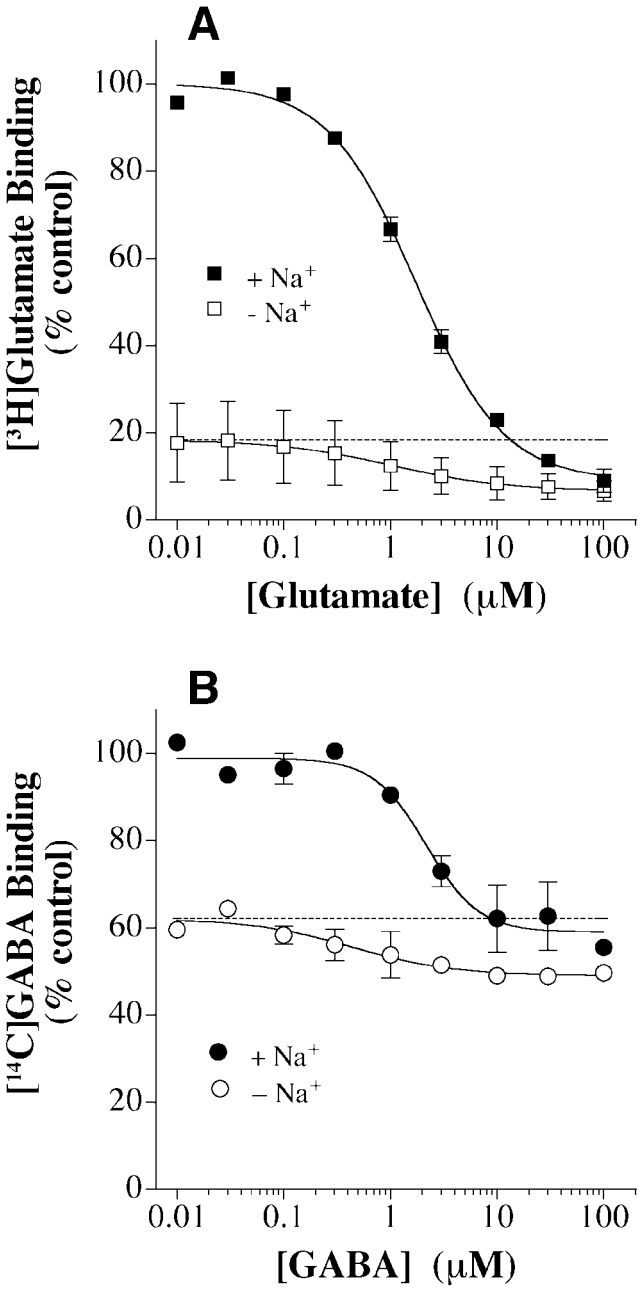

Fig. 3. Binding of L-[^{3}H]glutamate (*A*) and [^{14}C]GABA (*B*) to rat cortical synaptic membranes expressed as percentage of +Na^+ control. Binding was displaced by varying concentrations of unlabeled amino acids in the presence (120 mM NaCl) or absence (120 mM ChCl) of Na^+. Control binding in the absence of Na^+ (----) was not significantly displaced by unlabeled amino acids (n = 4).

transporter modulation is not involved in the actions of these anesthetics.

Currently, five mammalian glutamate (EAAT1-5)[58] and four GABA (GAT1-3 and betaine GAT [BGT]-1)[3] transporters have been described. These transporter subtypes have distinct cellular (neurons, glia) and regional (cortex, cerebellum, *etc.*) distributions. By using the radiolabeled substrates L-[^{3}H]glutamate and [^{14}C]GABA, all associated transporter subtypes are utilized in uptake, binding, and release assays of transporter function. The homogenization procedure used to isolate nerve endings also produces glial fragments with densities and sedimentation properties similar to synaptosomes.[59] The steps taken to enrich synaptosomes do not altogether remove glial membranes from the preparation.[20] Nevertheless, glial membranes are incapable of sequestering radiolabeled substrates to the same extent as active transport, rendering synaptosomal uptake an exclusive measure of neuronal transporter activity. However, the binding sites available to L-[^{3}H]glutamate and [^{14}C]GABA in synaptic membrane preparations, although mainly neuronal, contain both neuronal and glial amino acid

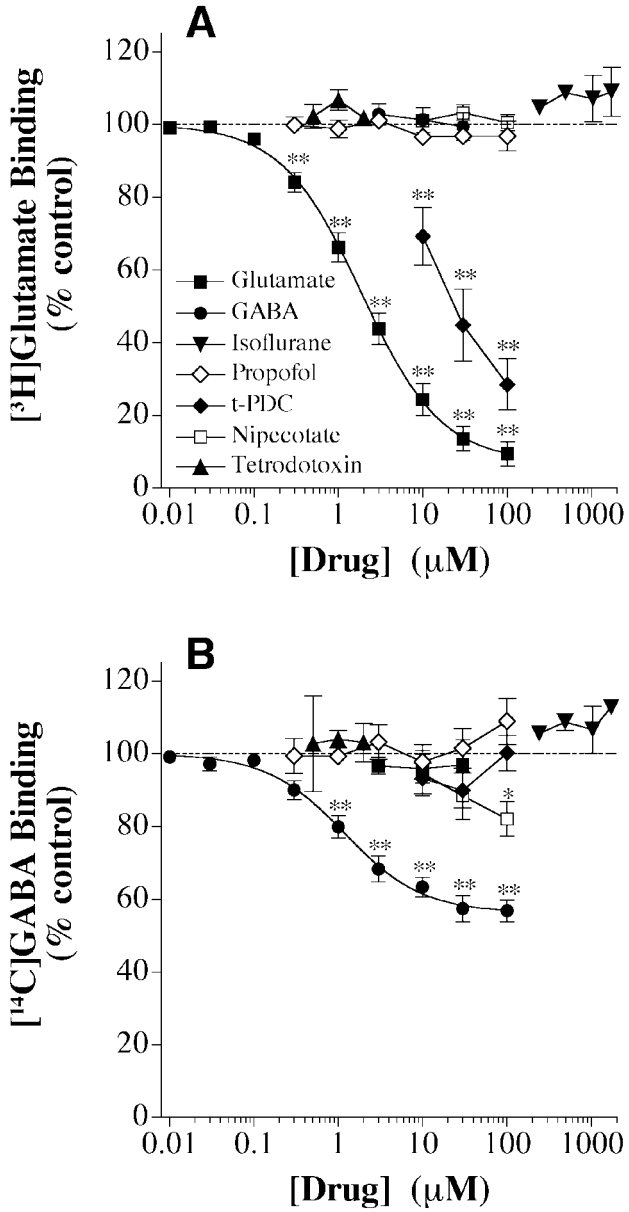

Fig. 4. Effects of anesthetics and other drugs on the binding of L-[^{3}H]glutamate (*A*) and [^{14}C]GABA (*B*) to rat cortical synaptic membranes. $^*P < 0.05$, $^{**}P < 0.01$ *versus* control (n = 3–7).

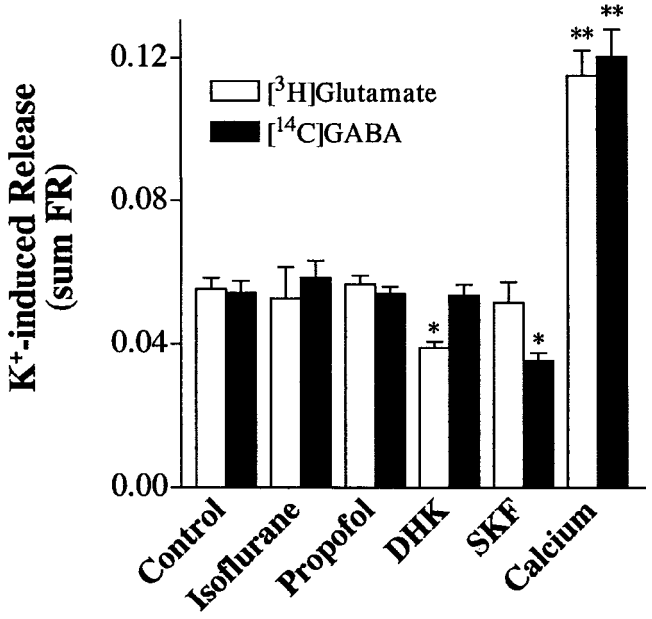

Fig. 5. Carrier-mediated release of L-[^{3}H]glutamate and [^{14}C]GABA from superfused synaptosomes. Release was induced by depolarization with a 3-min pulse of 30 mM KCl in the absence of extracellular Ca^{2+}. Calcium-independent release of L-[^{3}H]glutamate and [^{14}C]GABA was performed in the absence (control) and presence of 1 mM isoflurane, 15 μM propofol, 50 μM DHK, or 0.5 μM SKF89776A (SKF). *P < 0.05, **P < 0.01 *versus* control (n = 4–27).

transporter subtypes. The radiolabeled amino acids are common substrates for their respective transporter subtypes and are therefore available for displacement by potentially selective and nonselective competitors. Since direct effects of anesthetics were not found for any parameter of transporter function in cerebral cortex, investigation of possible regional variations in effects was not undertaken.

Given the lack of direct effects of isoflurane and propofol on transporter function observed in this study, it is unlikely that the presynaptic effects of these anesthetics on glutamate release from rat cerebrocortical synaptosomes[57] are due to effects on transport, as suggested previously.[12] This does not rule out a possible role for anesthetic actions on transport in glia[13] in mediating the effects of anesthetics on transmitter efflux from brain slices,[10,12] which is the sum of multiple direct and indirect synaptic and nonsynaptic actions.[60]

A direct transporter interaction cannot be inferred merely from inhibition of transport. In this study, transport inhibition was demonstrated both directly (by substrate and nonsubstrate competition) and indirectly (by partial depolarization), hindering an accurate determination of the site of action from a finding of transport inhibition alone. Isoflurane inhibited glutamate and GABA uptake by a noncompetitive mechanism, as did partial depolarization, which suggests an indirect effect by isoflurane on ion gradients that power glutamate uptake. The consequences of transporter inhibition for synaptic transmission are difficult to infer since alterations in transporter function may not correlate directly with strength of synaptic transmission. The effects of transporter activity on low-frequency excitatory postsynaptic potentials or inhibitory postsynaptic potentials depend on the density, location, and affinity of the carriers, as well as factors related to the sites and amount of transmitter release.[61] For example, glutamate pyruvate transaminase used to mimic increased glutamate uptake and reduce the amount of glutamate within the synaptic cleft increased synaptic strength.[62]

In summary, neither isoflurane nor propofol, at clinically relevant concentrations, had substantial effects on synaptosomal uptake, synaptic membrane binding, or Ca^{2+}-independent reverse transport of L-[^{3}H]glutamate or [^{14}C]GABA. Thus, it is unlikely that presynaptic neuronal transporters of the principle excitatory and inhibitory central nervous system transmitters represent important anesthetic targets.

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