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Volatile Anesthetic Effects on Glutamate *versus* GABA Release from Isolated Rat Cortical Nerve Terminals: 4-Aminopyridine Evoked Release*

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Volatile Anesthetics Inhibit Evoked Amino Acid Release

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BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

tetrakis(acetoxymethyl) ester; HEPES, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]; MAC, minimum alveolar concentration.

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Abstract

Inhibition of glutamatergic excitatory neurotransmission and potentiation of GABA-mediated inhibitory transmission are possible mechanisms involved in general anesthesia. We compared the effects of 3 volatile anesthetics (isoflurane, enflurane or halothane) on 4-aminopyridine (4AP)-evoked release of glutamate and GABA from isolated rat cerebrocortical nerve terminals (synaptosomes). Synaptosomes were prelabelled with L-[³H]glutamate and [¹⁴C]GABA, and release was evoked by superfusion with pulses of 1 mM 4AP in the absence or presence of 1.9 mM free Ca²⁺. All 3 volatile anesthetics inhibited Ca²⁺-dependent glutamate and GABA release: IC₅₀ values for glutamate were comparable to clinical concentrations (1-1.6 times MAC), while IC₅₀ values for GABA release exceeded clinical concentrations (> 2.2 times MAC). All 3 volatile anesthetics inhibited both Ca²⁺-independent and Ca²⁺-dependent 4AP-evoked glutamate release equipotently, whereas inhibition of Ca²⁺-dependent 4AP-evoked GABA release was less potent than inhibition of Ca²⁺-independent GABA release. Inhibition of Ca²⁺-independent 4AP-evoked glutamate release was more potent than of GABA release for isoflurane and enflurane, but equipotent for halothane. Tetrodotoxin inhibited both Ca²⁺-independent and Ca²⁺-dependent 4AP-evoked glutamate and GABA release equipotently, consistent with Na⁺ channel involvement. In contrast to tetrodotoxin, volatile anesthetics exhibited selective effects on 4AP-evoked glutamate vs. GABA release, consistent with distinct mechanisms of action. Preferential inhibition of Ca²⁺-dependent 4AP-evoked glutamate release vs. GABA release supports the hypothesis that reduced excitatory neurotransmission relative to inhibitory neurotransmission contributes to volatile anesthetic actions.

Introduction

Depression of neuronal activity by general anesthetics appears to involve inhibition of excitatory glutamatergic neurotransmission and enhancement of inhibitory γ -aminobutyric acid (GABA)ergic neurotransmission (Hemmings et al., 2005a). Positive modulation of postsynaptic GABA_A receptor function at GABAergic synapses is probably an important component of the depressant effects of volatile anesthetics (Jones et al., 1992; Franks and Lieb, 1994; MacIver, 1997). However, spinal GABA_A receptor potentiation does not explain the immobilizing action characteristic of volatile anesthetics (Zhang et al., 2004). Accumulating evidence indicates that volatile anesthetics affect multiple synaptic targets, both presynaptically and postsynaptically, that interact to produce their clinical effects (MacIver, 1997; Stucke et al., 2002; Hemmings et al., 2005a).

Action potential-evoked neurotransmitter release involves nerve terminal depolarization which triggers Ca²⁺ entry through voltage-gated Ca²⁺ channels coupled to synaptic vesicle exocytosis (Südhof, 2004). Basal neurotransmitter release includes spontaneous vesicular fusion and nonvesicular release via reverse transporter operation independent of Ca²⁺ entry. These pathways of release are mechanistically distinct, and are therefore differentially sensitive to presynaptic neuromodulators (Engelman and MacDermott, 2004). In the accompanying report, we show that volatile anesthetics produce significant transmitter-selective and agent-specific effects on basal glutamate and GABA release that result in a consistent net increase in inhibitory relative to excitatory basal transmitter release (Westphalen and Hemmings, 2005).

Neurotransmitter release from isolated nerve terminals can be evoked pharmacologically by 4-aminopyridine (4AP), a K⁺ channel blocker that induces release by mimicking action potential-evoked depolarizations (Tibbs et al., 1989). Inhibition of 4AP-evoked glutamate release by volatile anesthetics appears to involve suppression of presynaptic voltage-gated Na⁺ channels

coupled to transmitter release (Schlame and Hemmings, 1995; Ratnakumari et al., 2000; Westphalen and Hemmings, 2003b). However, Na⁺ channel block does not affect spontaneous transmitter release (Engelman and MacDermott, 2004), and anesthetics modulate basal release independent of their Na⁺ channel antagonism. Here we compare the effects of 3 widely used volatile anesthetics, the ethers isoflurane and enflurane and the alkane halothane, and the selective voltage-gated Na⁺ channel blocker tetrodotoxin on 4AP-evoked release of glutamate and GABA while compensating for their agent-specific effects on basal release (Westphalen and Hemmings, 2005).

Materials and Methods

Materials

Sources of all compounds are listed in companion paper (Westphalen and Hemmings, 2005).

Evoked Glutamate and GABA Release \pm Anesthetics

Experiments were done in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals as approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee. Synaptosomes were prepared from rat cerebral cortex and prelabelled with L-[^3H]glutamate and [^{14}C]GABA, whereupon simultaneous L-[^3H]glutamate and [^{14}C]GABA release was evoked by pulses of 1 mM 4-aminopyridine (4AP) as described in the companion paper (Westphalen and Hemmings, 2005). Using [^3H]H $_2\text{O}$ as a tracer, a 2 min pulse resulted in a peak of $70 \pm 4\%$ of the applied concentration to the perfusion chamber, suggesting a maximum concentration of 0.7 mM 4AP exposure to synaptosomes (data not shown). The accuracy of the dual radiolabel assay has been verified previously in detecting changes in the release of each amino acid independently of the other (Westphalen and Hemmings, 2003a).

Anesthetics at 0.1 to 8 times MAC (minimum alveolar concentration) were applied for 6 min (3 min prior to, during, and 1 min after stimulation with a 2 min pulse of 4AP) using a closed superfusion system (Westphalen and Hemming, 2005). This procedure insures the exposure to anesthetic throughout the 4AP-evoked release pulse (Westphalen and Hemmings, 2003b). After each experiment, anesthetic solutions were sampled and quantified by gas chromatography as previously described (Westphalen and Hemmings, 2005). In separate experiments, anesthetics

were continuously applied 12 min prior to stimulation by 4AP and remained present throughout the perfusion, with comparable effects on release.

Data Analysis

Release in each fraction was expressed as a fraction of synaptosomal content of labelled transmitter prior to each fraction collected (fractional release [FR]). The magnitude of the release pulse was determined by subtracting baseline release (average of basal release before and after pulse) from cumulative fractional release values for the release pulse (sum Δ FR; Garcia-Sanz et al., 2001). Sum Δ FR data for each experiment were normalized by the ratio of the individual assay control to the mean of all assay controls for release in the absence or presence of Ca^{2+} and for each anesthetic used prior to curve fitting.

Concentration-effect data for anesthetic effects on basal L-[^3H]glutamate and [^{14}C]GABA release were taken from Westphalen and Hemmings (2005). The basal effect on sum Δ FR for each anesthetic concentration was determined from the fitted equation, and was subtracted from the sum Δ FR values determined for 4AP-evoked release. Likewise, the derived Ca^{2+} -independent release values were subtracted from values obtained in the presence of Ca^{2+} at equivalent anesthetic concentrations to determine Ca^{2+} -dependent release. This subtraction method is standard procedure when baseline measurements of x -axis data cannot be accurately duplicated (Prism v.4.0; GraphPad Software, San Diego CA).

Data for anesthetic (0.1 – 8 times MAC) and tetrodotoxin (0.3 nM—10 μM) inhibition of 4AP-evoked release (with basal effect subtracted) were fitted to concentration-effect curves by least-squares analysis to estimate I_{max} and IC_{50} with standard errors (Prism v.4.0; GraphPad Software, San Diego CA). Each curve fit was concurrently tested for the bottom of the curve (I_{max}) differing significantly from 0. If the null hypothesis ($I_{\text{max}} = 0$) was not rejected, curve fit

analyses were performed with I_{\max} constrained to 0. Sum ΔFR values for all single concentration experimental groups followed Gaussian distributions, with some significantly differing in variance, as determined by a modification of the Kolmogorov and Smirnov test and Bartlett's test, respectively. Significant differences between mean sum ΔFR values and between concentration-effect curve parameters were determined by unpaired t -test with Welch correction for variances that were not assumed to be equal (Prism v. 4.0; GraphPad Software, San Diego, CA). Sum ΔFR values obtained for 4AP-evoked release in the presence of continuously applied anesthetics were compared to the corresponding values read from inhibition curves with the basal effect subtracted by paired t -test.

Results

4-Aminopyridine-Evoked Release

Stimulation of rat cerebrocortical synaptosomes with 1 mM 4AP in the presence of 1.9 mM free external Ca^{2+} evoked both L-[^3H]glutamate and [^{14}C]GABA release (Figs. 1-4). Total release (sum ΔFR) was greater for [^{14}C]GABA release compared to L-[^3H]glutamate, as previously reported (Westphalen and Hemmings, 2003b).

Isoflurane

Isoflurane inhibited glutamate and GABA release in a concentration-dependent manner (Fig. 1). Concentration-effect curves showed that isoflurane inhibited 4AP-evoked release of L-[^3H]glutamate (Fig. 1A) and [^{14}C]GABA (Fig. 1B), both in the absence and presence of Ca^{2+} . At concentrations up to 1 mM isoflurane, maximal inhibition of L-[^3H]glutamate release was greater than maximal inhibition of [^{14}C]GABA release. However, at concentrations > 1 mM isoflurane, sum ΔFR values increased from maximal inhibition. This augmentation of release paralleled the effects on basal release previously observed at high isoflurane concentrations (Westphalen and Hemmings, 2005), as evident in Figure 1A & B. Subtraction of the effects of isoflurane on basal release from 4AP-evoked release yielded data that fitted sigmoidal concentration-effect curves with 100% inhibition for both L-[^3H]glutamate release ($I_{\text{max}} = 0$, $P = 0.84$; Fig. 1C) and [^{14}C]GABA release ($I_{\text{max}} = 0$, $P = 0.37$; Fig. 1D), and were comparable to release evoked during continuously perfused anesthetic (see figure legend Fig. 1). Isoflurane inhibited evoked L-[^3H]glutamate release with greater potency than [^{14}C]GABA release in the presence of Ca^{2+} ($P = 0.0001$; Table 1). This selective inhibition by isoflurane is not due to the lower fractional release of glutamate vs. GABA evoked by 1 mM 4AP as shown previously by matching fractional release by lowering 4AP concentration (Westphalen and Hemmings, 2003b).

In the absence of Ca^{2+} , isoflurane inhibited 4AP-evoked release of L-[^3H]glutamate with lower potency than [^{14}C]GABA release ($P < 0.0001$; Table 1), with efficacies of 100% for both ($I_{\text{max}} = 0$, $P = 0.77$ and $P = 0.78$, respectively). Isoflurane inhibited Ca^{2+} -dependent and Ca^{2+} -independent 4AP-evoked L-[^3H]glutamate release at clinically relevant concentrations ($\text{IC}_{50} = 0.58$ mM; 1.7 times MAC) with the same potency ($P = 0.98$), and both with greater potency ($P < 0.0001$) than Ca^{2+} -dependent 4AP-evoked [^{14}C]GABA release ($\text{IC}_{50} = 1.06$ mM; 3.0 times MAC; Table 1). Isoflurane inhibited Ca^{2+} -independent 4AP-evoked [^{14}C]GABA release with greater potency than Ca^{2+} -dependent release ($P < 0.0001$; Table 1).

Enflurane

Enflurane inhibited 4AP-evoked release of L-[^3H]glutamate and [^{14}C]GABA in a concentration-dependent manner (Fig. 2A&B). Maximum inhibition of L-[^3H]glutamate release was indeterminable from these data, which trended negative at concentrations > 0.8 mM, apparently as a consequence of the inhibitory effect of enflurane on basal glutamate release (Westphalen and Hemmings, 2005). With basal release subtracted, enflurane inhibited both L-[^3H]glutamate and [^{14}C]GABA release by 100% ($I_{\text{max}} = 0$, $P = 0.98$ and $P = 0.25$, respectively; Fig. 2C&D). The potency of enflurane for inhibition of 4AP-evoked L-[^3H]glutamate release was greater than that for [^{14}C]GABA release ($P < 0.0001$; Table 1). In the absence of Ca^{2+} , enflurane completely inhibited 4AP-evoked release of L-[^3H]glutamate and [^{14}C]GABA ($I_{\text{max}} = 0$, $P = 0.57$ and $P = 0.12$, respectively; Table 1; Figs. 2C&D). Enflurane inhibited Ca^{2+} -independent [^{14}C]GABA release with greater potency than Ca^{2+} -dependent release ($P < 0.0001$; Table 1). Enflurane inhibited Ca^{2+} -dependent and Ca^{2+} -independent 4AP-evoked L-[^3H]glutamate release at clinically relevant concentrations ($\text{IC}_{50} = 0.72$ mM; 1.0 times MAC) with the same potency ($P = 0.66$), but with greater potency ($P = 0.0002$) than Ca^{2+} -dependent 4AP-evoked [^{14}C]GABA release ($\text{IC}_{50} = 2.34$ mM; 3.1 times MAC; Table 1). Enflurane inhibited Ca^{2+} -independent 4AP-

evoked [^{14}C]GABA release with greater potency than Ca^{2+} -dependent release ($P < 0.0001$; Table 1).

Halothane

Halothane inhibited 4AP-evoked release of L- ^3H]glutamate and [^{14}C]GABA in a concentration-dependent manner (Fig. 3A&B). With basal release subtracted, halothane inhibited both L- ^3H]glutamate and [^{14}C]GABA release by 100% ($I_{\text{max}} = 0$, $P = 0.84$ and $P = 0.78$, respectively; Figs. 3C&D). Halothane inhibited 4AP-evoked L- ^3H]glutamate release with greater potency than [^{14}C]GABA release ($P < 0.001$; Table 1). In the absence of Ca^{2+} , halothane inhibited 4AP-evoked release of L- ^3H]glutamate and [^{14}C]GABA with similar potencies ($P = 0.93$) and 100% efficacy ($I_{\text{max}} = 0$, $P = 0.88$ and $P = 0.08$, respectively; Table 1; Figs. 3C&D). Halothane inhibited Ca^{2+} -dependent L- ^3H]glutamate release at clinically relevant concentrations ($\text{IC}_{50} = 0.56$ mM; 1.6 times MAC) with the same potency as Ca^{2+} -independent 4AP-evoked L- ^3H]glutamate release ($P = 0.085$; Table 1). Halothane inhibited Ca^{2+} -dependent [^{14}C]GABA release ($\text{IC}_{50} = 0.76$ mM; 2.2 times MAC) with less potency than Ca^{2+} -independent release ($P = 0.008$; Table 1).

Tetrodotoxin

Tetrodotoxin inhibited 4AP-evoked release of L- ^3H]glutamate and [^{14}C]GABA in a concentration-dependent manner (Fig. 4A&B). With basal effects subtracted, tetrodotoxin showed incomplete efficacy for inhibition of both 4AP-evoked L- ^3H]glutamate release (66% inhibition; $I_{\text{max}} = 0$, $P = 0.0024$) and [^{14}C]GABA release (46% inhibition; $I_{\text{max}} = 0$, $P = 0.0022$) in the presence of Ca^{2+} (Figs. 4C&D). Tetrodotoxin inhibited 4AP-evoked L- ^3H]glutamate and [^{14}C]GABA release with the same potency ($P = 0.78$; Table 1). The effects of tetrodotoxin on Ca^{2+} -independent 4AP-evoked L- ^3H]glutamate and [^{14}C]GABA release were not statistically

significant with basal effects subtracted (Figs. 4C&D). Thus, curve parameters for Ca^{2+} -dependent inhibition of evoked release of glutamate and GABA were the same (Table 1). However, inhibition of Ca^{2+} -dependent glutamate release was complete ($I_{\text{max}} = 0$, $P = 0.86$), but was incomplete for inhibition of Ca^{2+} -dependent GABA release ($I_{\text{max}} = 0$, $P = 0.026$). This difference in maximal inhibition of Ca^{2+} -dependent release by tetrodotoxin between glutamate and GABA was also statistically significant by a comparative curve fit ($P = 0.044$).

Tetrodotoxin-Insensitive 4-Aminopyridine-Evoked Release

In the presence of Ca^{2+} and 1 μM tetrodotoxin (a concentration that maximally inhibited 4AP-evoked L-[^3H]glutamate and [^{14}C]GABA release; Figs 4C&D), the tetrodotoxin-insensitive 4AP-evoked [^{14}C]GABA release was significantly inhibited by 0.7 mM isoflurane ($P < 0.001$), 1.8 mM enflurane ($P < 0.001$) or 0.7 mM halothane ($P < 0.001$) (Fig. 5). Inhibition of the residual tetrodotoxin-insensitive 4AP-evoked L-[^3H]glutamate release was not significant for any anesthetic.

Discussion

Isoflurane, enflurane and halothane all selectively inhibited Ca^{2+} -dependent 4AP-evoked glutamate release compared to GABA release. Taking into consideration anesthetic-specific effects on basal release (Westphalen and Hemmings, 2005), the volatile anesthetics inhibited glutamate release with greater potencies within a clinically relevant concentration range and with full efficacies. This refined analysis clarifies the finding of different efficacies when basal effects are not considered (Westphalen and Hemmings, 2003b). Since depolarization-evoked vesicular transmitter release is Ca^{2+} -dependent, these results support preferential inhibition of evoked vesicular glutamate release over GABA release by a variety of clinically used volatile anesthetics. The effects of volatile anesthetics on both basal and evoked release of the major excitatory and inhibitory transmitters may combine to depress overall CNS neuronal activity. The significant actions of volatile anesthetics on basal release highlight the need to consider these effects when analyzing drug effects on evoked release data.

Different volatile anesthetic potencies for inhibition of glutamate vs. GABA release by volatile anesthetics suggest underlying physiological difference(s) between glutamatergic and GABAergic nerve terminals resulting in distinct transmitter-selective pharmacologies. Voltage-gated Na^+ channels are important in the generation of neuronal action potentials that trigger exocytosis of synaptic vesicles (Tibbs et al., 1989; Südhof, 2004). Mammalian Na^+ channels show selective densities within and between neurons (Goldin, 2001; Novakovic et al., 2001), can modulate transmitter release (Engelman and MacDermott, 2004), and are inhibited by volatile anesthetics (see below). Thus, inhibition by volatile anesthetics of evoked neurotransmitter release might occur via Na^+ channel block. This hypothesis is supported by the present demonstration that 4AP-evoked release is also inhibited by the selective Na^+ channel blocker, tetrodotoxin. Moreover, volatile anesthetics inhibit 4AP-evoked (action potential-evoked) transmitter release with greater potency than release evoked by elevated K^+ (Schlame and

Hemmings, 1995; Ratnakumari et al., 2000; Westphalen and Hemmings, 2003b; Hemmings et al., 2005b), which implicates an anesthetic target upstream of Ca^{2+} entry. Additional support for volatile anesthetic inhibition of mammalian neuronal Na^+ channels includes anesthetic inhibition of synaptosomal influx of $^{22}\text{Na}^+$ and [^3H]batrachotoxinin-A binding to nerve terminal Na^+ channels (Ratnakumari and Hemmings, 1998), inhibition of heterogeneously expressed brain Na^+ channels (Rehberg et al., 1996; Shiraishi and Harris, 2004), and inhibition of Na^+ currents in isolated rat dorsal root ganglion neurons (Ratnakumari et al., 2000) and isolated neurohypophysial nerve terminals (Ouyang et al., 2003; Ouyang and Hemmings, 2005).

Isoflurane, enflurane and halothane completely inhibited Ca^{2+} -dependent 4AP-evoked glutamate and GABA release with potencies comparable to inhibition of mammalian voltage-gated Na^+ channels (Rehberg et al., 1996; Ouyang et al., 2003). In contrast, the selective Na^+ channel blocker tetrodotoxin completely inhibited Ca^{2+} -dependent 4AP-evoked glutamate release, but only partially inhibited GABA release. This is consistent with a previous study that showed tetrodotoxin-resistant Ca^{2+} -dependent 4AP-evoked inhibitory postsynaptic currents (IPSCs) in rat hippocampus (Akaike, 2002). Preferential inhibition of autaptic excitatory postsynaptic currents (EPSCs) vs. IPSCs by Na^+ channel blockers in hippocampal neuron microcultures (Prakriya and Mennerick, 2000) suggests fundamental differences in the distribution and/or function of Na^+ channels between glutamate and GABA nerve terminals. The potency of tetrodotoxin in inhibiting both glutamate and GABA release corresponds to the tetrodotoxin-sensitive Na^+ channel subtypes $\text{Na}_v1.1$, 1.2, 1.3, 1.6 and/or 1.7 (Novakovic et al., 2001; Lai et al., 2004). Perhaps the tetrodotoxin-insensitive fraction of 4AP-evoked GABA release involves expression of tetrodotoxin-resistant Na^+ channel subtypes (e.g. $\text{Na}_v1.8$ and/or 1.9; Novakovic et al., 2001; Lai et al., 2004) in a subset of GABAergic terminals. Variability in depolarization threshold and/or release probability of vesicular transmitter exocytosis between nerve terminals containing the same (Rosenmund et al., 1993; Murthy et al., 1997) and/or

different transmitters (this study, Prakriya and Mennerick, 2000; Akaike, 2002) may provide the basis for preferential inhibition of evoked glutamate vs. GABA release by volatile anesthetics. Since inhibition of 4AP-evoked glutamate and GABA release by tetrodotoxin was neither totally effective nor transmitter selective in potency, volatile anesthetic effects on transmitter release, which were fully efficacious and transmitter selective, can only partially be attributable to Na⁺ channel inhibition or require the distinct state-dependent Na⁺ channel blocking effects of volatile anesthetics (Ouyang et al., 2003). The ability of clinical concentrations of volatile anesthetics to inhibit the tetrodotoxin-insensitive component of 4AP-evoked GABA release further supports the involvement of additional mechanism(s). GABAergic synaptic diversity in release probability, number of release sites, presynaptic GABA_A receptor subtypes, modulation by endogenous and exogenous factors (Cherubini and Conti, 2001), and tetrodotoxin-insensitive nicotinic mediated increases in GABA release (Zhu and Chiappinelli, 2002) suggest possible mechanisms for transmitter-selective anesthetic effects.

Other potential presynaptic targets of volatile anesthetics include K⁺ and Ca²⁺ channels (Topf et al., 2003; Franks and Honoré, 2004), ligand-gated ion channels (Franks and Lieb, 1994), cell signaling machinery such as protein kinase C (Hemmings and Adamo, 1998) and G-protein coupled receptors (Yamakura et al., 2001), and synaptic vesicle fusion machinery (Hawasli et al., 2004), all of which can modulate transmitter release (Miller, 1998; Engelman and MacDermott, 2004). Genetic inactivation of components of the presynaptic release machinery differentially affect basal and evoked release (SNAP-25; Washbourne et al., 2001) or evoked glutamate and GABA release (Munc 13-1; Augustin et al., 1999), suggesting these proteins as potential targets for the pathway and transmitter-selective effects of volatile anesthetics. Like Na⁺ channels, these putative anesthetic targets may have distinct patterns of expression within and between neurons that may contribute to transmitter-selective effects.

Preferential inhibition of Ca²⁺-independent 4AP-evoked GABA vs. glutamate release by

isoflurane and enflurane, but not halothane, was in contrast to preferential inhibition of Ca^{2+} -dependent glutamate vs. GABA release by all three anesthetics tested. This suggests more than one presynaptic site of action for these effects. A potential target for anesthetic effects on Ca^{2+} -independent transmitter release is inhibition of reverse transmitter transporter function (Attwell et al., 1993). Inhibition by isoflurane of glutamate uptake into rat cerebrocortical synaptosomes occurs with similar potency ($\text{IC}_{50} = 0.7 \text{ mM}$) to inhibition of Ca^{2+} -independent release, but inhibition of GABA uptake ($\text{IC}_{50} = 0.8 \text{ mM}$) is less potent than inhibition of Ca^{2+} -independent release (Westphalen and Hemmings, 2003a). The maximal efficacy of isoflurane inhibition of glutamate and GABA uptake also varies (Westphalen and Hemmings, 2003a), consistent with a greater role of transporter inhibition to glutamate release than GABA release. In view of the insensitivity of GABA uptake to volatile anesthetics, the greater sensitivity of Ca^{2+} -independent 4AP-evoked GABA release to isoflurane further suggests that nerve terminal GABA transporters are not sensitive to volatile anesthetics.

In summary, three volatile anesthetics consistently inhibited Ca^{2+} -dependent 4AP-evoked glutamate release with greater potency than GABA release, which supports distinct presynaptic targets between glutamatergic and GABAergic nerve terminals. Although glutamate is released from synaptic vesicles at saturating concentrations for its receptors, even moderate reductions in release probability could affect the kinetics of synaptic glutamate concentration (Cavelier et al., 2005) and the release probability of low probability nerve terminals (Prakriya and Mennerick, 2000) to depress excitatory transmission. The less potent inhibition of GABA release by volatile anesthetics is balanced by postsynaptic potentiation of GABA_A receptors such that net inhibitory GABAergic transmission is enhanced (Stucke et al., 2002). The correlation between anesthetic potency *in vivo* (MAC) and potency of evoked glutamate release inhibition support this action as a relevant volatile anesthetic target. The differing potencies for inhibition of Ca^{2+} -dependent and Ca^{2+} -independent 4AP-evoked GABA and glutamate release provide evidence for multiple

transmitter and pathway-specific presynaptic targets. The biological difference(s) between glutamatergic and GABAergic nerve terminals that underlie this selectivity in volatile anesthetic action remains to be determined. Comparable transmitter-selective inhibition was not produced by tetrodotoxin, indicating that a mechanism(s) in addition to Na⁺ channel block must be involved. Selective depression of evoked glutamate vs. GABA release, depressed basal glutamate relative to GABA release, and potentiation of postsynaptic GABA_A receptors provide synergistic mechanisms by which volatile anesthetics may depress excitatory and enhance net inhibitory CNS transmission.

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Footnotes

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Figure Legends

Table 1 Values were derived by fitting data to sigmoidal concentration effect-curves (see Methods). Statistical significance between glutamate and GABA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) and between $-Ca^{2+}$ and Ca^{2+} -dependent release ($\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$) were calculated using $\log IC_{50}$ values by unpaired t -test.

Figure 1 Isoflurane effects on evoked release. Isoflurane effects on **A**, L-[3H]glutamate and **B**, [^{14}C]GABA release evoked by 4AP from rat cortical synaptosomes were fitted to sigmoidal concentration-effect curves. Control data in the presence of Ca^{2+} (glutamate: $n = 12$; GABA: $n = 13$) or absence of Ca^{2+} (glutamate: $n = 9$; GABA: $n = 11$) are presented as mean \pm SD. Basal effects (taken from Westphalen and Hemmings, 2005) were subtracted from evoked release data and fitted to sigmoidal concentration-effect curves (**C&D**). Shaded area highlights the clinical concentration range (0.5 - 2 times MAC). Symbols in gray present sum ΔFR data (mean \pm SD) for 2 min pulses of 1 mM 4AP in the presence of Ca^{2+} , plus ~ 0.5 times MAC (0.19 ± 0.003 mM, $n = 3$), ~ 1 times MAC (0.34 ± 0.03 mM, $n = 4$) or ~ 2 times MAC (0.76 ± 0.04 mM, $n = 7$) concentrations of isoflurane applied continuously, which do not differ from data obtained using the standard pulse application with basal effect subtracted (glutamate: $P = 0.35$, GABA: $P = 0.17$). Release in the absence of anesthetic presents mean of all assay controls.

Figure 2 Enflurane effects on evoked release. Enflurane effects on **A**, L-[3H]glutamate and **B**, [^{14}C]GABA evoked by 4AP from rat cortical synaptosomes were fitted to sigmoidal concentration-effect curves. Control data in the presence of Ca^{2+} (glutamate, $n = 20$; GABA, $n = 18$) or absence (glutamate, $n = 8$; GABA, $n = 8$) of Ca^{2+} are presented as mean \pm SD. Basal effects (taken from Westphalen and Hemmings, 2005) were subtracted from evoked release data and the difference fitted to sigmoidal concentration-effect curves (**C&D**). Shaded area highlights

the clinical concentration range (0.5 - 2 times MAC). Symbols in gray present sum Δ FR data (mean \pm SD) for 2 min pulses of 1 mM 4AP in the presence of Ca^{2+} , plus ~ 0.5 times MAC (0.39 mM \pm 0.007, $n = 3$), ~ 1 MAC (0.74 \pm 0.03 mM, $n = 5$) or ~ 2 times MAC (1.58 \pm 0.02 mM, $n = 5$) concentrations of enflurane applied continuously, which do not differ from data obtained using the standard pulse application with basal effect subtracted (glutamate: $P = 0.09$, GABA: $P = 0.26$). Release in the absence of anesthetic presents mean of all assay controls.

Figure 3 Halothane effects on evoked release. Halothane effects on **A**, L-[^3H]glutamate and **B**, [^{14}C]GABA evoked by 4AP from rat cortical synaptosomes were fitted to sigmoidal concentration-effect curves. Control data in the presence of Ca^{2+} (glutamate, $n = 16$; GABA, $n = 16$) or absence of Ca^{2+} (glutamate, $n = 6$; GABA, $n = 6$) are presented as mean \pm SD. Basal effects (taken from Westphalen and Hemmings, 2005) were subtracted from evoked release data and the difference fitted to sigmoidal concentration-effect curves (**C&D**). Shaded area highlights the clinical concentration range (0.5 - 2 times MAC). Symbols in gray present sum Δ FR data (mean \pm SD) for 2 min pulses of 1 mM 4AP in the presence of Ca^{2+} , plus ~ 0.5 times MAC (0.14 \pm 0.005 mM, $n = 3$), ~ 1 MAC (0.33 \pm 0.02 mM, $n = 5$) or ~ 2 times MAC (0.70 \pm 0.01 mM, $n = 4$) concentrations of halothane applied continuously, which do not differ from data obtained using the standard pulse application with basal effect subtracted (glutamate: $P = 0.06$, GABA: $P = 0.27$). Release in the absence of anesthetic presents mean of all assay controls.

Figure 4 Tetrodotoxin effects on evoked release. Tetrodotoxin effects on **A**, L-[^3H]glutamate and **B**, [^{14}C]GABA evoked by 4AP from rat cortical synaptosomes were fitted to sigmoidal concentration-effect curves. Control data in the presence of Ca^{2+} (glutamate, $n = 7$; GABA, $n = 7$) or absence of Ca^{2+} (glutamate, $n = 6$; GABA, $n = 6$) are presented as mean \pm SD. Basal effects (taken from Westphalen and Hemmings, 2005) were subtracted from evoked release data and the difference fitted to sigmoidal concentration-effect curves (**C&D**). Tetrodotoxin did not completely inhibit L-[^3H]glutamate or GABA release in the presence of

Ca²⁺, or significantly inhibit Ca²⁺-independent L-[³H]glutamate or GABA release. Symbols in gray present sum Δ FR data (mean \pm SD) for 2 min pulses of 1 mM 4AP in the presence of Ca²⁺, and 10 nM ($n = 4$), 100 nM ($n = 4$) or 1 μ M ($n = 6$) tetrodotoxin applied continuously, which do not differ from data obtained using the standard pulse application with basal effect subtracted (glutamate: $P = 0.16$, GABA: $P = 0.10$). Release in the absence of anesthetic presents mean of all assay controls.

Figure 5 Additivity experiments for the effects of volatile anesthetics plus tetrodotoxin on release. Effects of ~ 2 times MAC isoflurane (0.73 ± 0.02 mM, $n = 4$), enflurane (1.79 ± 0.05 mM, $n = 4$) or halothane (0.69 ± 0.12 mM, $n = 4$) on tetrodotoxin (TTX)-insensitive 4AP-evoked release of L-[³H]glutamate and [¹⁴C]GABA in the presence of Ca²⁺ are shown as mean \pm SEM. TTX (1 μ M, $n = 8$) significantly inhibited 4AP-evoked L-[³H]glutamate and [¹⁴C]GABA release vs. control ($\dagger\dagger\dagger P < 0.001$, $n = 8$). In the presence of 1 μ M TTX, anesthetics significantly inhibited [¹⁴C]GABA release ($**P < 0.01$), with no significant additive effect on L-[³H]glutamate release.

Table 1

Inhibition potencies of 4-aminopyridine-evoked L-[³H]glutamate and [¹⁴C]GABA release.

Drug	Transmitter	Potency (IC ₅₀)		
		- Ca ²⁺	+ Ca ²⁺	Ca ²⁺ -dependent
Isoflurane	Glutamate	0.58 ± 0.10 mM *	0.57 ± 0.04 mM ***	0.58 ± 0.06 mM ***
	GABA	0.26 ± 0.06 mM	0.78 ± 0.03 mM	1.06 ± 0.04 mM ^{ttt}
Enflurane	Glutamate	0.64 ± 0.12 mM **	0.67 ± 0.06 mM ***	0.72 ± 0.11 mM ***
	GABA	0.31 ± 0.05 mM	1.48 ± 0.14 mM	2.34 ± 0.29 mM ^{ttt}
Halothane	Glutamate	0.33 ± 0.04 mM	0.41 ± 0.04 mM ***	0.47 ± 0.06 mM *
	GABA	0.34 ± 0.05 mM	0.59 ± 0.04 mM	0.76 ± 0.08 mM ^{tt}
Tetrodotoxin	Glutamate	20 ± 58 nM	18 ± 5.5 nM	18 ± 6.9 nM
	GABA	29 ± 28 nM	20 ± 7.3 nM	15 ± 8.3 nM

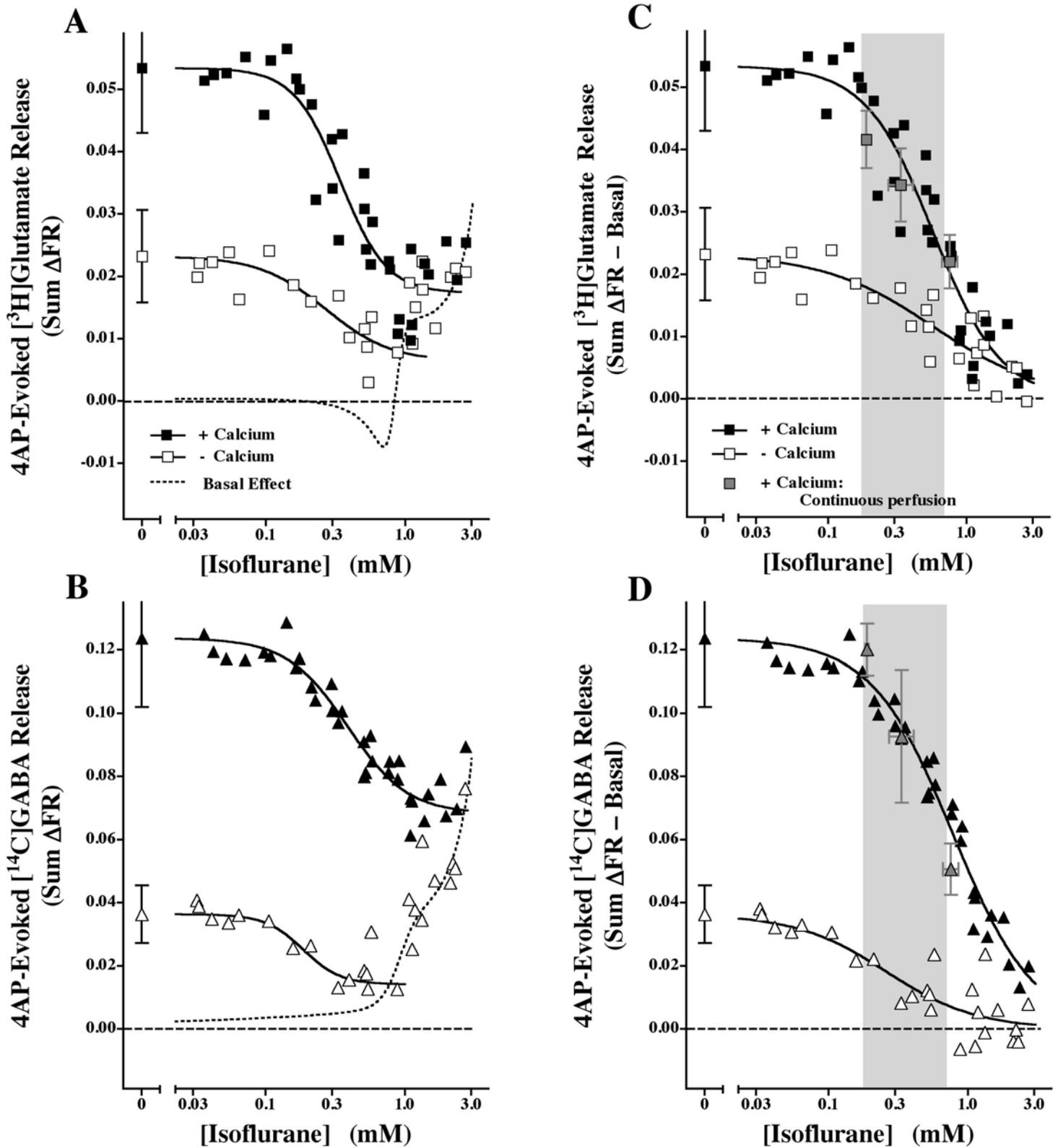


Figure 1

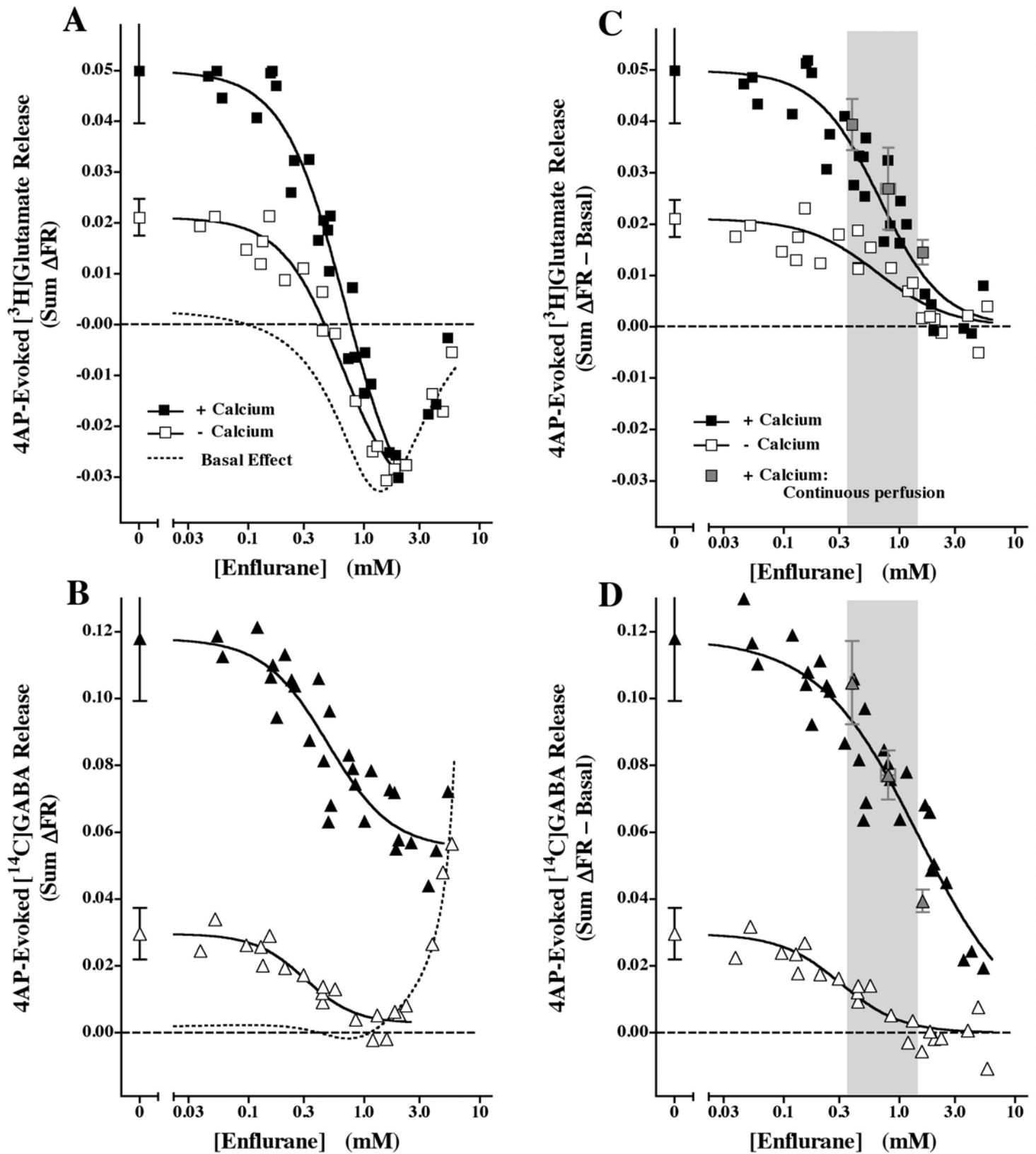


Figure 2

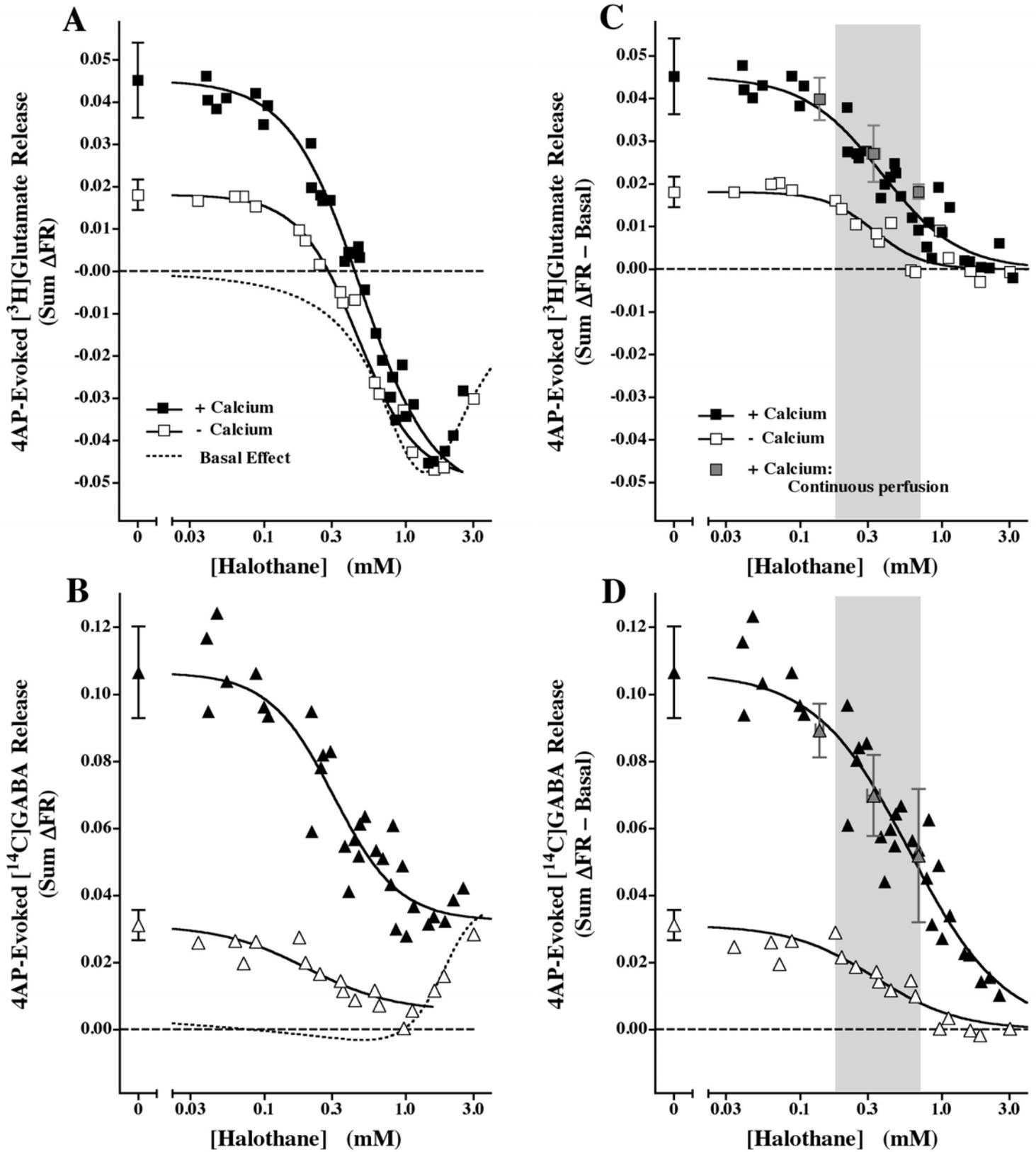


Figure 3

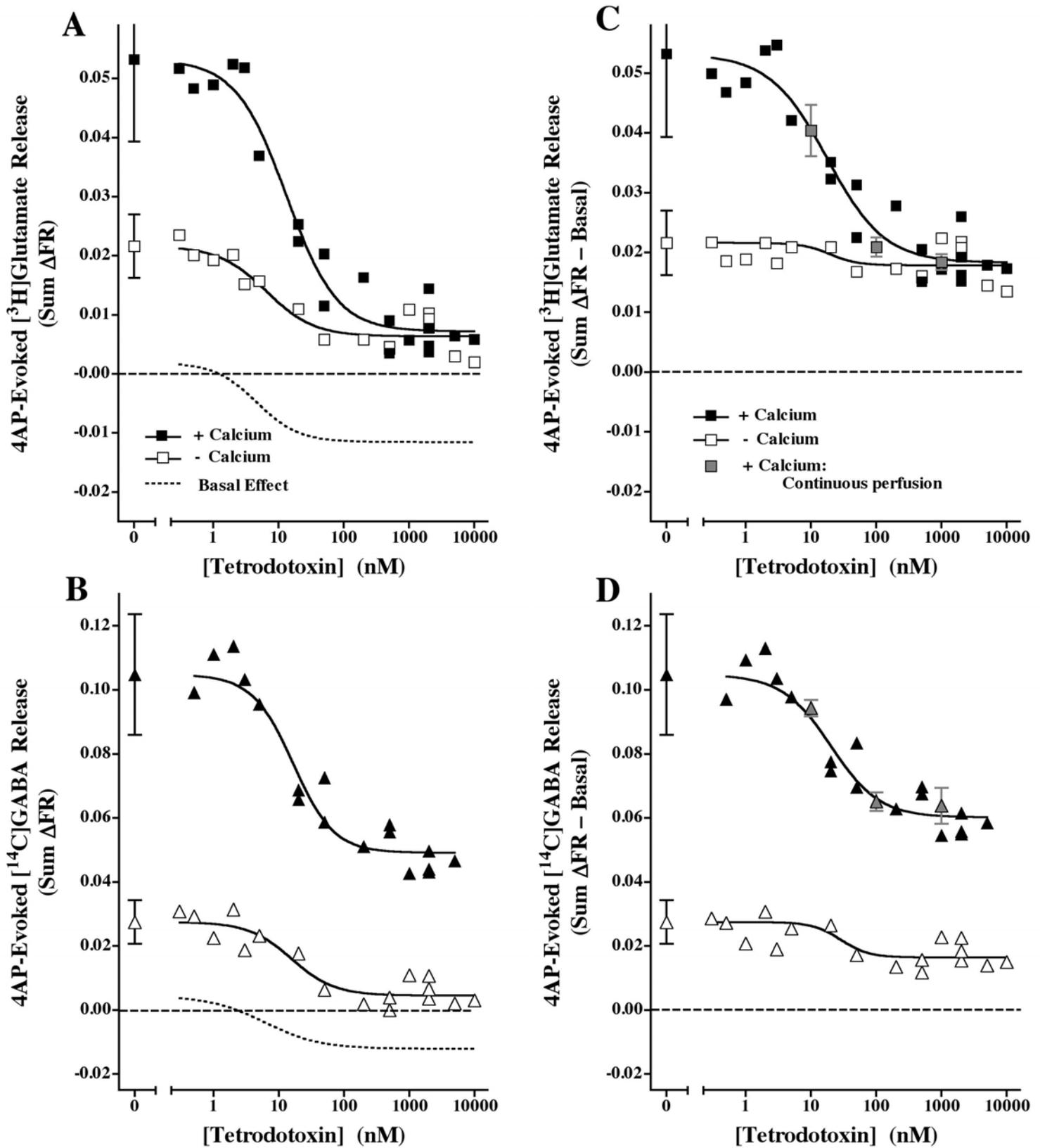


Figure 4

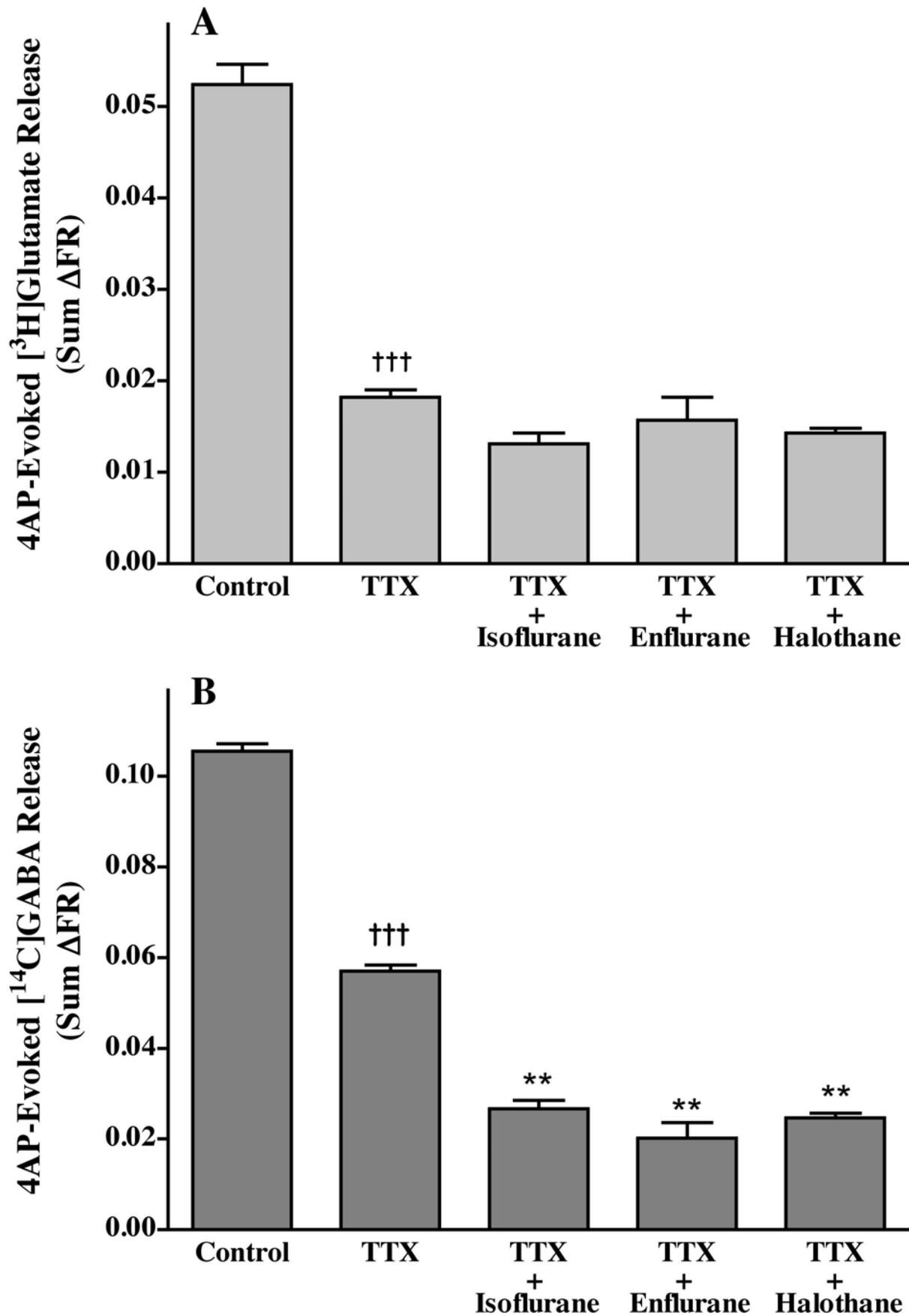


Figure 5