Title:

Isoflurane inhibits the neurotransmitter release machinery

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Isoflurane interacts with syntaxin 1A

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Abstract

Despite their importance, the mechanism of action of general anesthetics is still poorly understood. Facilitation of inhibitory GABA\textsubscript{A} receptors plays an important role in anesthesia but other targets have also been linked to anesthetic actions. Anesthetics are known to suppress excitatory synaptic transmission, but it has been difficult to determine whether they act on the neurotransmitter release machinery itself. By directly elevating $[\text{Ca}^{2+}]_i$ at neurotransmitter release sites without altering plasma membrane channels or receptors, we show that the commonly used inhalational general anesthetic, isoflurane, inhibits neurotransmitter release at clinically relevant concentrations, in a dose-dependent fashion in PC12 cells and hippocampal neurons. We hypothesized that a SNARE and/or SNARE-associated protein represents an important target(s) for isoflurane. Overexpression of a syntaxin 1A mutant, previously shown in C. elegans to block the behavioral effects of isoflurane, completely eliminated the reduction in neurotransmitter release produced by isoflurane, without affecting release itself, thereby establishing the possibility that syntaxin 1A is an intermediary in isoflurane’s ability to inhibit neurotransmitter release.

Key words: syntaxin 1A, anesthesia, SNARE, synaptic vesicle release, amperometry.
**Introduction**

Most, but not all, anesthetics are known to facilitate GABA<sub>A</sub> receptor activity thereby enhancing inhibitory synaptic transmission. Modulation of GABA<sub>A</sub> receptors in this manner is known to be an important part of the mechanism of action for many anesthetics. More recent studies, however, have shown that general anesthetics can influence a number of voltage and ligand gated ion channels (Campagna et al. 2003; Chen et al. 2007; Hemmings et al. 2005; Krasowski and Harrison 1999; Petrenko et al. 2007).

Although not extensive, some work has investigated the effects of inhalational anesthetics on neurotransmitter release. For instance, Richards and colleagues showed that low concentrations of anesthetic affected chemical transmission but neither impulse conduction nor cellular electrical properties were affected, raising the possibility of direct modulation of the release machinery (Pocock and Richards 1988; Richards 1972; Richards and White 1975). But the significant number of proteins targeted by general anesthetics has made it difficult to isolate the release machinery in many of these experiments.

In the present study we set out to determine if the clinically used volatile anesthetic, isoflurane, directly influences the mammalian neurotransmitter release machinery. To accomplish this, it is necessary to observe the effect of isoflurane on evoked neurotransmitter release independent of anesthetic modulation of channels and receptors. To prevent actions of anesthetics on channels or receptors from altering neurotransmitter release, we used experimental paradigms that kept membrane potential constant, but which allowed [Ca<sup>2+</sup>]<sub>i</sub> to be elevated by a known amount. These paradigms allowed us to probe interactions between anesthetics and the release machinery directly. We observed that
clinically relevant concentrations of isoflurane dramatically inhibited the neurotransmitter release machinery of PC12 cells and cultured rat hippocampal neurons. The robust nature of the suppression suggests that inhibition of release machinery may represent an important component of the anesthetic state. The hydrophobic nature of isoflurane suggests that it might interact with proteins within the plane of lipid membranes. Thus, we sought to examine release machinery proteins with alterations in their transmembrane domains. The C. elegans md130 syntaxin 1A point mutation results in a truncated form of syntaxin 1A. This mutant syntaxin is missing part of the H3 domain and the entire transmembrane domain; the mutant syntaxin also includes 10 novel amino acids on the carboxy-terminus. The md130A mutation blocked the behavioral effects of isoflurane in C. elegans (van Swinderen et al. 1999). Overexpression of md130A in PC12 cells completely blocked isoflurane’s ability to inhibit the neurotransmitter release machinery. This data suggests a possible role for syntaxin 1A as an intermediary in isoflurane’s ability to inhibit neurotransmitter release.
Materials and Methods

PC12 and neuronal cell culture

PC12 cells were grown on collagen-coated 10 cm Petri dishes in culture medium that consisted of RPMI-1640, 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine, and 10 µg/ml gentamicin in a humidified 7% CO₂ incubator at 37ºC. Culture medium was replaced every other day and cells were passaged once per week. Cells were replated on poly-lysine coated glass coverslips 24 hours prior to recording. Hippocampal neuron cultures were prepared from E18 Sprague-Dawley rats as previously described (Wang et al. 2006).

Amperometric measurement of catecholamine release

Carbon fiber electrodes were fabricated and used as previously described by Grabner et al. 2005 (Grabner et al. 2005). The detection threshold for amperometric events was set at 5 times the baseline root mean squared noise, and the spikes were automatically detected. Amperometric spike features, quantal size, and kinetic parameters were analyzed with a series of macros written in Igor Pro (Wavemetrics Inc.) and kindly supplied to us by Dr. Eugene Mosharov. On each day of recording amperometric measurements were made from a similar number of experimental and control cells. This strategy reduces cell-to-cell variation. A student’s t-test was used to assess differences between populations of cells.
**PC12 cell permeabilization and stimulation**

An amperometric electrode was placed gently against a cell. Following 2 min in a Ca²⁺-free solution (1), the cell was permeabilized with 20 µM digitonin (Ca²⁺-free) for 25 sec (2), and then stimulated for 2-3 min with a solution containing 100 µM Ca²⁺ (3). The cell was allowed to recover for 2 min in Ca²⁺-free media (4), and the cycle began again at step (2). Cells were stimulated 4-5 times in this way. In drug treated cells isoflurane or the non-immobilizer, F6, was introduced into the bath 25 sec prior to stimulation and was present throughout the recording. This was done in order to maximize drug exposure time. The stimulation step (3) producing the greatest amount of release was analyzed. The recording solutions had standard compositions previously described in (Grabner et al. 2005).

**Optical measurement of evoked RH414 release**

Coverslips containing live rat hippocampal neurons were briefly rinsed in HBSS before being placed in a 60 mM KCl loading solution containing 10 µM RH414 (Molecular probes, Eugene, OR) for 75 sec. The coverslips were then put back into HBSS for 1-5 min. RH414 loaded synapses were observed using an Olympus IX81 inverted microscope through a U Plan APO 60x water objective (0.512 um/pixel). 530-550 nm light from a high power 100W Hg arc lamp was used for excitation, and emitted light was filtered through a 590LP filter. Images were captured using MetaMorph. Time-lapse sequences of synaptic fluorescence prior to and following evoked synaptic vesicle exocytosis were made with an acquisition rate of one image every 2 sec. Prior to stimulation neurons were washed for 4
min with HBSS+0.5mM isoflurane or HBSS alone. Neurons were then exposed to 5 µM ionomycin in HBSS+0.5 mM isoflurane or 5 µM ionomycin in HBSS alone. Fluorescent synapses were monitored 40 sec before and ~2 min after ionomycin treatment.

Quantitative analysis of evoked RH414 release

Fluorescent nerve terminals found to undergo de-staining following ionomycin treatment were marked as regions of interest in ImageJ (http://rsb.info.nih.gov/ij/). Circular regions of interest were selected to include the largest portion of the fluorescent spot and include as little background as possible. ImageJ was then used to determine the pixel intensities of each region, which were then averaged together to produce values of local fluorescence intensity in nerve terminals over time. Background fluorescence was subsequently subtracted. The fluorescence intensity of each nerve terminal was then normalized to its average fluorescence value 40 sec prior to ionomycin exposure. The percentage of de-staining following 2 min of ionomycin exposure as well as the time constant of fluorescent decay were determined for each nerve terminal in the control condition and compared to that of nerve terminals exposed to isoflurane. A student’s t-test was again used to assess differences between the two conditions. Tau values were determined by fitting fluorescent intensity plots with a second-order exponential decay function, $y = y_0 + A_1 e^{-x/t_1} + A_2 e^{-x/t_2}$. The fluorescence traces for all nerve terminals in a given condition were then aligned at the initial point of de-staining and averaged.
[Ca\textsuperscript{2+}]\textsubscript{i} measurements using Fura-2

Hippocampal neurons were loaded with Fura-2 and imaged as previously described in chromaffin cells (Xie et al. 2006). For these experiments, peak [Ca\textsuperscript{2+}]\textsubscript{i} was measured.

Measurement of drug concentrations

Isoflurane solutions were prepared and measured as previously described (Jones et al. 1992; Jones and Harrison 1993). Isoflurane was prepared in sealed plastic I-V bags. We have previously found that isoflurane concentrations in the bags and in the bath are remarkably constant for up to 1.5 hours when measured in representative experiments using gas chromatography (GC) (Xie et al. 2006). All isoflurane concentrations in this manuscript are provided in mM. The MAC (minimum alveolar concentration required for immobility in response to a noxious stimulus in 50% of trials (Eger et al. 1965)) equivalents of isoflurane have been reported to be in the range of ~0.3 mM (Franks and Lieb 1996) to ~0.5 mM (Franks and Lieb 1996; Jones and Harrison 1993) at 25°C. The equivalent of 2 MAC was employed for the nonimobilizer, F6. This concentration was estimated to be ~36 µM (Mihic et al. 1994).

Whole-cell patch clamp stimulation protocol

Whole-cell patch electrodes were pulled from microhematocrit capillary tubes (Drummond Scientific Co., Broomall, PA), fire-polished, and filled with an internal solution that contained 100 µM Ca\textsuperscript{2+}, 145 mM NaCl, 2.0 mM KCl, 10 mM HEPES, 1 mM
Na$_2$ATP and 1.0 mM MgCl$_2$, pH 7.2 NaOH, osmolarity 300 mOsm/kg. A single PC12 cell was then selected and a gigaseal was obtained with a patch pipette connected to an Axopatch-1C amplifier (Axon Instruments, Foster City, CA). An amperometric electrode was then gently placed against the opposite side of the cell. This preparation was then washed with either HBSS (Hank’s Balanced Saline Solution) or HBSS containing isoflurane (1 mM) for 4 min. Using suction the cell was then patch clamped in the whole-cell configuration and held at -65 mV throughout the duration of the recording. Amperometric data was collected for 2.5 min. Data analysis started 15 sec after breaking the membrane with the patch pipette to allow [Ca$^{2+}$]$_i$ equilibration. This 15 sec delay ensured a uniform concentration of the 100 µM Ca$^{2+}$ pipette solution inside the cell prior to data acquisition. Cells were continuously washed with HBSS or HBSS+isoflurane (1 mM) throughout the duration of the recording.

**md130A cloning and expression**

A pGMHE vector containing rat syntaxin 1A cDNA was provided by Dr. Richard Tsien. PCR cloning was used to obtain DNA encoding md130A and wild type syntaxin 1A from this vector. The primers used to produce md130A were

GAGAATTCCATGAAGGACCGAACCCA

and

GATCTAGACTCAACCATCTCTCTTGTAATATCAAATTCCACAAATCTGGCTCTCCACCAG.

The primers used to produce wild type syntaxin were

GAGAATTCCATGAAGGACCGAACCCA

and

GATCTAGACTATCCAAAGATGCCCC. The resulting PCR products were cloned into
pcDNA3.1/Neo (Invitrogen), sequenced and purified. Cells were co-transfected with either the md130A or wild type syntaxin plasmid and pEGFP-N1 (BD biosciences) using Lipofectamine 2000. A syntaxin plasmid:pEGFP ratio of 7:1 was used to ensure green cells expressed the desired form of syntaxin. Recordings were made from these cells 48 to 72 hours post transfection.

**Immunoblotting**

Levels of syntaxin and actin in PC12 cells were assessed using the following antibodies: syntaxin (#573831, Calbiochem), β-actin (JLA20; Developmental Systems Hybridoma Bank, University of Iowa) and horseradish peroxidase-labeled anti-mouse (Jackson ImmunoResearch). ECL Advance reagents (Amersham/GE Healthcare) were used for detection of the horseradish peroxidase-labeled secondary antibodies.
Results

 Isoflurane dose-dependently inhibits the neurotransmitter release machinery of PC12 cells

Exocytosis was elicited in digitonin-permeabilized cells in the presence and absence of isoflurane (0.5 mM). Basal (Ca^{2+}-independent) neurotransmitter release is virtually non-existent in permeabilized PC12 cells in Ca^{2+}-free conditions, but robust release is observed upon exposing cells to Ca^{2+}-containing solutions (Graham et al. 2002; Jankowski et al. 1992). Fig. 1A plots a representative amperometric current observed in a PC12 cell upon stimulation. Physiologically, release is evoked by the activation of voltage-gated Ca^{2+} channels. The proximity of Ca^{2+} channels to synaptic release sites suggests that [Ca^{2+}]_i may rise to levels above 100 μM at the vesicle (Llinas et al. 1992). To mimic these levels in our experiments, evoked neurotransmitter release was elicited by exposing digitonin-permeabilized cells to 100 μM Ca^{2+}, for 2 min (as indicated), in the absence of isoflurane (Fig. 1A) or in the presence of isoflurane (0.5 mM; Fig. 1B). The amperometric trace in the presence of isoflurane contained many fewer amperometric events. Control cells had an average of 94 ± 13 (n = 25) amperometric events during a 2 min stimulation while cells exposed to isoflurane (0.5 mM) had 58 ± 11 (n = 24) events (mean ± SEM; fig. 1C). This 38% reduction in the number of amperometric events was significant (P = 0.049). These data suggest that isoflurane inhibits the neurotransmitter release machinery at a clinically relevant concentration by reducing the number of vesicles released.

[On each day of recording, amperometric measurements were made from a similar number of experimental and control cells. This strategy reduces cell-to-cell variation].
Isoflurane was also found to inhibit the neurotransmitter release machinery in a dose-dependent fashion over a range of concentrations (0.3, 0.5, 1, 2, and 3 mM) that include the clinically relevant range (0.3 – 1 mM). Isoflurane at 0.3, 1, 2, and 3 mM reduced the number of amperometric events per 2 min stimulation by 27, 52, 64 and 54%, respectively. Fig. 1D plots the number of amperometric events observed as a function of isoflurane concentration. These data were fit with a standard dose-response equation (see legend). The best possible fit of the data suggests the effects of isoflurane on the neurotransmitter release machinery saturate at concentrations > 1 mM, which maximally reduce neurotransmitter release by ~70%. The inset plots the same data on a linear scale to better illustrate the saturation of the response to isoflurane. 1 mM, 2 mM and 3 mM isoflurane inhibited neurotransmitter release to the same extent. The EC$_{50}$ provided by the fitting function was 0.38 mM isoflurane. Here, the EC$_{50}$ refers to the concentration at which isoflurane reached 50% of its maximal inhibition (~70%). The MAC (minimum alveolar concentration required for immobility in response to a noxious stimulus in 50% of trials (Eger et al. 1965)) equivalent of the isoflurane used in this study has been reported to be ~0.3 mM (Franks and Lieb 1996) at 25°C. Dose-dependent effects on quantal amplitude or kinetics were not observed (data not shown). Together, these data indicate that isoflurane has a statistical and biologically important dose-dependent effect on the release machinery at concentrations spanning this anesthetic’s clinically effective range.

To ensure that isoflurane inhibited the neurotransmitter release machinery and not digitonin-permeabilization a patch pipette was used to dialyze cells with a 100 µM Ca$^{2+}$ solution in order to stimulate catecholamine release. Cells were patch clamped in whole-
cell configuration and held at -65 mV, which precluded activation of voltage-gated channels. Fig. 2A plots a representative amperometric current observed in a patch-clamped cell perfused with a 100 µM Ca^{2+} solution for 2.5 min (as indicated by the bar in the figure), in the absence of isoflurane while Fig. 2B plots a representative amperometric current observed from a cell exposed to isoflurane (1 mM). Isoflurane (1 mM) reduced the number of amperometric events during each 2.5 min stimulation period by 59% (P = 0.014, fig. 2C), a value similar to the inhibition observed with digitonin-permeabilized cells (see fig. 1D). A box chart plots the range of these data in fig. 2D. Effects on quantal amplitude or kinetics were not observed (data not shown). These data indicate that isoflurane’s influence on neurotransmitter release stems from an inhibition of the neurotransmitter release machinery.

To further eliminate the possibility of non-specific effects, experiments were carried out with a non-immobilizing anesthetic analog, 1, 2-dichlorohexaflurocyclobutane (F6). F6, although similar to volatile anesthetics in terms of its composition and hydrophobicity, does not produce anesthesia. This experiment was conducted to determine whether inhibition of the neurotransmitter release machinery is specific to agents capable of producing anesthesia. Inhibition of neurotransmitter release was not observed when cells were exposed to F6 (36 µM; predicted ~2X MAC, see methods). If anything, F6 increased the number of amperometric events observed in permeabilized cells by ~25% (fig. 3), but this difference was not significant (P = 0.29). Further studies in a larger pool of cells will be required to determine whether this small augmentation is significant. Taken together, these data demonstrate the selective inhibitory actions of isoflurane on the neurotransmitter release machinery.
Isoflurane inhibits the neurotransmitter release machinery of hippocampal neurons

Neurotransmitter release mechanisms are strongly conserved between neurons and secretory cells (Rettig and Neher 2002). To assess whether isoflurane inhibited neurotransmitter release in central neurons, we studied exocytosis in cultured embryonic hippocampal neurons. In order to monitor neurotransmitter release synaptic vesicles were loaded with the fluorescent dye RH414 prior to stimulation (fig. 4A). RH414 de-staining was monitored over time using time-lapse florescence imaging. Exposing neurons to a solution containing the ionophore ionomycin (5 µM), as indicated, evoked exocytosis. Synapses treated with isoflurane (0.5 mM) showed a significant reduction in exocytosis during ionomycin exposure (fig. 4B). After 2 min of ionomycin exposure the fluorescence of 58 control nerve terminals was reduced an average of 51 ± 3%, while the fluorescence of 98 isoflurane (0.5 mM)-treated nerve terminals was reduced an average of 37 ± 2% (P < 0.0001, fig. 4C). These results suggest that isoflurane (0.5 mM) inhibits synaptic release, as measured by RH414 de-staining, by 27.5%. The time course of neurotransmitter release was unaffected by isoflurane (both curves were well fit by single exponentials; τ_{control} ~36 ms and τ_{isoﬂurane} ~39 ms). Ionomycin was found to produce identical increases in [Ca^{2+}] in the presence and absence of isoflurane in fura-2 loaded neurons (fig. 4D), a result which suggests that the inhibition of neurotransmitter release results from interaction with the release machinery. Taken together, these data strongly suggest that clinically relevant concentrations of isoflurane inhibit the exocytotic machinery of neurons within the mammalian CNS.
Isoflurane interacts with syntaxin 1A

It is reasonable to assume that isoflurane, a strongly lipophilic molecule, might interact with release machinery proteins within the plane of the plasma membrane. We examined the md130A syntaxin 1A mutant which is missing the entire transmembrane domain (van Swinderen et al. 1999). Behavioral sensitivity to isoflurane is significantly reduced in C. elegans heterozygous for the md130 mutation (van Swinderen et al. 1999). In addition, isoflurane has been shown to bind to syntaxin 1A (Nagele et al. 2005). To determine if md130A influences the isoflurane sensitivity of the mammalian neurotransmitter release machinery, PC12 cells were transfected with an md130A expression plasmid. Western blot analysis was used to confirm expression of the mutant (fig. 5A). Permeablized PC12 cells expressing the mutant syntaxin 1A in addition to endogenous syntaxin 1A showed no inhibition upon exposure to isoflurane (1 mM; fig. 5B). Rather, it appears that isoflurane might augment release in cells expressing the md130A mutant, but this difference was not significant. Further studies in a larger pool of cells will be required to determine whether this augmentation is significant. Cells transfected with wild-type syntaxin 1A showed a ~60% reduction in the number of release events per stimulation \( (P = 0.032; \) fig. 5C) in the presence of isoflurane. Overexpression of md130A and wild-type syntaxin 1A alone did not affect release rates in permeablized PC12 cells. Release from md130A-transfected PC12 cells was 95% of that in wild-type PC12 cells \( (P = 0.87) \). The mean rate of release from cells transfected with unmodified syntaxin 1A was 119% of wild-type controls \( (P = 0.68) \) (data not shown). Taken together, these data
demonstrate the direct involvement of syntaxin 1A in isoflurane’s ability to influence neurotransmitter release.

[Please note that a high concentration of isoflurane (1 mM) was tested to ensure that the md130A mutation completely suppressed the response to isoflurane.]
Discussion

In this study, three different experimental protocols were used to stimulate neurotransmitter release while bypassing confounding anesthetic effects on channels and receptors. In PC12 cells, isoflurane strongly suppressed neurotransmitter release in digitonin-permeablized cells perfused with elevated Ca$^{2+}$ as well as in cells dialyzed with elevated levels of Ca$^{2+}$, using a patch pipette. The amount of inhibition observed using the two different protocols was very similar. Isoflurane also suppressed neurotransmitter release in cultured hippocampal neurons. The fact that clinically relevant concentrations of isoflurane inhibited exocytosis in all three of these paradigms indicates that isoflurane inhibits the neurotransmitter release machinery in different kinds of cells including those found within the mammalian CNS. Furthermore, the failure of F6 to inhibit neurotransmitter release from permeablized PC12 cells suggests that inhibition of neurotransmitter release machinery is specific to agents that produce anesthesia. Finally, overexpression of the syntaxin 1A mutant, md130A, completely eliminated the PC12 cell response to isoflurane, suggesting that this t-SNARE may be involved in isoflurane’s ability to inhibit the mammalian neurotransmitter release machinery. This final result, though, does not preclude the involvement of other release machinery proteins in the response to isoflurane.

It is widely held that general anesthetics influence glutamate neurotransmission via presynaptic sites of action (Maclver et al. 1996; Perouansky et al. 1995; Schlame and Hemmings 1995; Wu et al. 2004). However, the literature is unclear regarding the ability of anesthetics to influence the neurotransmitter release machinery itself. Richards and
colleagues found that low concentrations of anesthetics affected chemical transmission but not impulse conduction or cellular electrical properties in hippocampal neurons (Pocock and Richards 1988; Richards 1972; Richards and White 1975). These observations led them to raise the possibility of direct modulation of the release machinery by general anesthetics. This group later dismissed the involvement of the neurotransmitter release machinery in anesthetic action in favor of presynaptic Na\(^+\) channels after clinical concentrations of isoflurane were found to have little effect on neurotransmitter release from KCl-treated chromaffin cells (Pocock and Richards 1988). Similarly, subsequent studies conducted by Hemmings and colleagues also suggested an insensitivity of KCl-evoked neurotransmitter release to clinical concentrations of isoflurane in cerebrocortical synaptosomes (Lingamaneni et al. 2001; Westphalen and Hemmings 2003). Using a different assay of vesicular fusion in individual neuronal synaptic terminals this group reported a dramatic inhibition of neurotransmitter release (~56%) by 1 mM isoflurane; they attributed most of the effect to an inhibition of pre-synaptic Na\(^+\) channels but ~one third of the response could have been due to suppression of the release machinery (Hemmings et al. 2005). These results are similar to those of Wu et al. who found that clinically relevant concentrations of isoflurane dose-dependently reduce action potential amplitude in the presynaptic terminal of glutamatergic calyx-type synapses in rat brain stem (Wu et al. 2004). Simulations suggested that the effects of isoflurane on the electrical properties of neurons can account for between 62-78% of the inhibitory effects of isoflurane on excitatory post synaptic currents (Wu et al. 2004). In contrast with the studies from Hemmings, Richards and Wu, other groups have reported more robust dose-dependent inhibition of KCl-evoked neurotransmitter release by clinically relevant concentrations of isoflurane (Larsen et al.
and 2 MAC isoflurane inhibited KCl-evoked glutamate release from rat hippocampal slices by 31, 42 and 51%, respectively (Larsen et al. 1994). Furthermore, Miao et al. reported that volatile anesthetics, including isoflurane, dose-dependently inhibited KCl-evoked neurotransmitter release from guinea pig cerebrocortical synaptosomes by ~20-25% per MAC (Miao et al. 1995). Surprisingly, a recent study reported that isoflurane facilitated KCl-evoked norepinephrine release from mouse spinal cord slices at ~0.5 MAC (Rowley and Flood 2008).

In an attempt to directly investigate anesthetic effects on the neurotransmitter release machinery the present study examined evoked release from both neurosecretory cells and hippocampal neurons, using experimental paradigms that elevated \([\text{Ca}^{2+}]\), at the release sites. These methods of stimulation were used in lieu of KCl-evoked release in order to avoid potential confounding effects of anesthetics on channels and receptors. Our data from individual PC12 cells and individual hippocampal neuron synaptic terminals, strongly suggests clinical concentrations of isoflurane inhibit the neurotransmitter release machinery, and is most consistent with reports of dose-dependent inhibition of KCl-evoked release with clinical concentrations of isoflurane reviewed above. At this time it is difficult to draw firm conclusions from the literature, as the effects of anesthetics remain somewhat unclear. This may be due to the fact that anesthetics target a variety of different proteins. For instance, anesthetics appear to target both pre-synaptic \(\text{Na}^+\) channels as well as pre-synaptic TREK channels (Hemmings et al. 2005; Westphalen et al. 2007). Activation of pre-synaptic \(\text{K}^+\) channels along with a concomitant decrease in \(\text{Na}^+\) channel activity may produce significant suppression of neurotransmitter release, when neurons are induced to...
fire action potentials. But cells may exhibit a different response when exposed to high-K⁺ solutions. Neurons typically do not respond to high-K⁺ solutions in a Nernst-like manner, due to the activation of a variety of non-K⁺ conductances (Augustine et al. 2008). In this case, activation of background K⁺ channels by anesthetics may cause cells to depolarize to different potentials in response to elevated K⁺, making comparisons in the presence and absence of anesthetics somewhat difficult. Therefore, we chose to try to elevate Ca²⁺ directly at the release sites in an effort to bypass these effects. But the suppression of neurotransmitter release in intact neurons probably corresponds to a combination of all these effects, as well as inhibitory effects due to the facilitation of GABA_A receptors. Please note that the use of amperometry in these studies precludes the determination of whether isoflurane induces changes in endocytosis.

The clinical effects of general anesthetics are dose-dependent. If the inhibition of the neurotransmitter release machinery plays a role in the production of anesthesia, measurable effects on the neurotransmitter release machinery should be observed throughout isoflurane’s clinically effective range (~0.3-1 mM). Therefore, we tested the effects of five concentrations of isoflurane (0.3, 0.5, 1, 2 and 3 mM) on permeabilized PC12 cells. Dose-dependent inhibition of the release machinery was indeed observed throughout isoflurane’s clinically relevant range and beyond. The best possible fit of the data suggests the effects of isoflurane on the neurotransmitter release machinery saturate at concentrations > 1 mM, which maximally reduce neurotransmitter release by ~70%. The calculated EC₅₀ for inhibition of the release machinery was 0.38 mM. Reassuringly, this concentration is very similar to the MAC value of isoflurane. However, caution must be exercised when drawing quantitative conclusions based on dose-response curves fit to
relatively few data points. Nevertheless, robust inhibitory effects at 0.3 mM and 0.5 mM isoflurane strongly suggest a biologically important effect on the neurotransmitter release machinery.

Interestingly, isoflurane inhibition of total neurotransmitter release peaked at <100%. This observation leaves open the possibility that anesthetics operate as partial agonists with regards to neurotransmitter inhibition. This, coupled with the observation that higher concentrations of isoflurane are able to inhibit 100% of neurotransmitter release in rat synaptosomes and in cultured hippocampal neurons (Hemmings et al. 2005; Westphalen and Hemmings 2003), suggests that multiple mechanisms may come together to produce the complete inhibition observed. This idea is consistent with more recent studies that have sought to determine the relative contributions of multiple presynaptic targets of anesthetic action (Hemmings et al. 2005; Westphalen et al. 2007; Wu et al. 2004).

In this study we have also demonstrated the importance of the SNARE protein, syntaxin 1A, in isoflurane’s ability to inhibit the neurotransmitter release machinery. The expression of a syntaxin 1A truncation mutant, md130A, completely blocked isoflurane-mediated inhibition of neurotransmitter release in permeabilized PC12 cells. This data is in agreement with an observed reduction in behavioral sensitivity of C. elegans md130A heterozygotes to isoflurane (van Swinderen et al. 1999), and suggests that isoflurane-mediated inhibition of the release machinery in mammalian cells is dependent on an interaction between isoflurane and syntaxin 1A. Surprisingly, cells expressing md130A produced more amperometric events when they were stimulated in the presence of isoflurane than in the absence of isoflurane (see Fig. 5B). An increase in amperometric event number was also observed in permeabilized PC12 cells exposed to the non-
immobilizer, F6 (see fig. 3). While in both cases increases in neurotransmitter release failed to reach significance, increasing the number of recordings in these experiments may ultimately lead to significant results. Thus, in blocking isoflurane’s ability to inhibit neurotransmitter release with md130A, we may have unmasked a weaker stimulatory effect. It is also interesting that overexpression of md130A was found to completely block the effects of isoflurane on the neurotransmitter release machinery despite the presence of endogenous syntaxin 1A. One possible explanation is md130A operates in a dominant fashion with regards to endogenous syntaxin 1A. Additional study will be necessary to test these hypotheses.

The details of syntaxin’s involvement in the response to isoflurane remain unclear. While anesthetic interactions with other SNARE and/or SNARE-related proteins are possible, our data raises the possibility that syntaxin 1A is involved in the response to isoflurane. This hypothesis is supported by NMR binding studies demonstrating the ability of isoflurane to bind to syntaxin monomers (Nagele et al. 2005). It is currently unknown whether the md130A mutant is capable of supporting exocytosis, although replacing wild-type syntaxin with the md130A mutant produced C. elegans that were not viable; animals that had both md130A and wild-type syntaxin were viable and were resistant to isoflurane (van Swinderen et al. 1999). Our own studies found that overexpressing the md130A mutant in cells containing endogenous syntaxin resulted in anesthetic insensitive PC12 cells. It is possible that the truncation of the C-terminal portion present in the md130A mutant may produce a functional form of syntaxin lacking the isoflurane binding pocket. It is also possible that md130A, lacking its transmembrane domain, may be more mobile than endogenous syntaxin allowing for preferential access to conjugate SNARE proteins. This
may explain the potential dominant characteristics of md130A. An alternate hypothesis concerning isoflurane’s actions on the release machinery has recently been put forth by Crowder and colleagues whereby isoflurane inhibits the recruitment of the syntaxin activator, UNC-13, to the plasma membrane, thereby reducing syntaxin 1A activation (Metz et al. 2007). This group goes on to speculate that the md130A mutant may bind to UNC-13, preventing the association of isoflurane with the syntaxin activator. Our data is consistent with this model as well. In this scenario the potential of md130A to act dominantly may also be explained as a consequence of increased motility. A soluble md130A might preferentially bind to soluble (unactivated) UNC-13 and prevent the binding of anesthetic molecules to this syntaxin 1A activator. An md130A/UNC-13 association prior to the recruitment of UNC-13 to the plasma membrane (activation) may “protect” this syntaxin 1A activator from anesthetic molecules until md130A can be replaced with endogenous syntaxin. Additional study will undoubtedly be necessary to test these hypotheses or to determine whether other as yet unidentified release machinery proteins play a role in the response to isoflurane.

Regardless of how isoflurane interacts with the release machinery, this mechanism is likely to operate in humans due to the highly conserved nature of the neurotransmitter release machinery among a variety of species that span invertebrates to mammals. While our data seems to suggest biologically relevant inhibition of the release machinery by isoflurane, it is unclear at the present time as to whether this mechanism participates in the production of the anesthetic state. In the future it will be necessary to generate knockout or transgenic animals in which the effects of anesthetics on the release machinery are blocked.
in order to determine the relative contribution of this mechanism to the production of anesthesia.

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References


**Figure Legends**

Fig. 1. Isoflurane inhibits neurotransmitter release in permeabilized PC12 cells. Digitonin-permeabilized cells were exposed to Ca^{2+} (100 µM), indicated by the bars below the traces, to elicit neurotransmitter release. (*A-B*) Representative amperometric recording in the absence of isoflurane and in the presence of isoflurane (0.5 mM). (*C*) Bar chart plots the averaged number of events in the absence (“Control”) and presence of isoflurane (0.5 mM). Control cells produced an average of 94 ± 13 events per stimulation (mean ± SEM, n = 25). Isoflurane-treated cells produced an average of 58 ± 11 events (n = 24), a 38% reduction. *P < 0.05, student’s t-test. (*D*) Mean inhibition of neurotransmitter release plotted as a function of isoflurane concentration (log_{10}). The number of cells studied at each concentration is indicated above or below each data point. Data was fit with Y = Y_{max} × 1 / 1 + (EC_{50} / X). Y is the percentage of release inhibited. X is the isoflurane concentration. This equation assumes 1:1 binding. Inset re-plots data on a linear scale to show saturation at higher isoflurane concentrations. The open data point denotes data obtained from patch dialysis stimulation.

Fig. 2. Isoflurane inhibits neurotransmitter release in patch-clamped PC12 cells. Cells were dialyzed via a patch pipette with Ca^{2+} (100 µM), indicated by the bars below the traces, to elicit neurotransmitter release. (*A-B*) Representative amperometric recording in the absence (“Control”) and in the presence of isoflurane (1 mM). (*C*) Bar chart plots averaged data from control cells and isoflurane-treated cells. During a 2.5-minute stimulation control cells produced an average of 126 ± 22 events (n = 7). Isoflurane (1 mM)-treated cells produced
an average of 51 ± 10 events (n = 6), a 59% reduction. *P < 0.05, student’s t-test. (D) Re-plots
the same data as box plots which span 25% - 75% of each data range. The line in each
box represents the median data point.

Fig. 3. The non-immobilizing agent, 1, 2-dichlorohexafluorocyclobutane (F6), does not
inhibit neurotransmitter release in permeabilized PC12 cells. Digitonin-permeabilized cells
were exposed to Ca$^{2+}$ (100 µM), indicated by the bars below the traces, to elicit
neurotransmitter release. (A-B) Representative amperometric recording in the absence of
F6 (“Control”) and in the presence of F6 (36 µM). (C) Bar chart plots averaged data from
control cells and F6-treated cells. During a 2.5-minute stimulation control cells produced an
average of 103 ± 22 events (n = 14). F6-treated cells produced an average of 137 ± 24
events (n = 15). This difference was not significant, P = 0.29 (student’s t-test). (D) Re-plots
the same data as box plots which span 25% - 75% of each data range. The line in each box
represents the median data point.

Fig. 4. Isoflurane inhibits the neurotransmitter release machinery in rat hippocampal
neurons. (A) Fluorescence micrograph illustrating nerve terminals containing RH414-
loaded synaptic vesicles (arrows). “S”=soma. (B) Fluorescence intensity of terminals in the
presence and absence of isoflurane (0.5 mM) plotted as a function of time. (C) Average
fluorescence intensity of control and isoflurane-treated terminals 2 min following
ionomycin exposure. Stimulation with ionomycin caused a de-staining of 51 ± 3% (mean ±
SEM, n = 58) but isoflurane (0.5 mM) treated cells de-stained by only 37 ± 2% (n = 98), a
27.5% reduction. **P < 0.0001, student’s t-test. (D) Plots normalized average increases in peak $[Ca^{2+}]_i$, in the presence and absence of isoflurane, in response to 5 µM ionomycin.

Fig. 5. Overexpression of the syntaxin 1A mutant, md130A, eliminates isoflurane’s effect on the neurotransmitter release machinery. (A) Cells were transfected with the md130A expression plasmid. This immunoblot illustrates the levels of md130A and endogenous syntaxin expressed in transfected and untransfected PC12 cells. The relative faintness of the 26 kDa md130A band relative to the 33 kDa endogenous syntaxin band is due to the fact that only ~13% of cells were transfected in these experiments. (B) Normalized average number of amperometric events produced by PC12 cells overexpressing md130A in the absence (“Control”) and presence of isoflurane (1 mM). Isoflurane treated cells exhibited ~40% more events than control (n = 15, P > 0.05), student’s t-test. (C) Normalized average number of amperometric events produced by PC12 cells overexpressing wild-type (wt) syntaxin 1A in the absence and presence of isoflurane (1 mM). Isoflurane treatment resulted in ~57% reduction in the number of events (n = 16). *P < 0.05, student’s t-test.
A. Control

B. 0.5 mM isoflurane

C. Graph showing the number of events/stim for control and 0.5 mM isoflurane. The y-axis represents the number of events/stim, and the x-axis represents the concentration.

D. Graph showing the relationship between percent inhibition and isoflurane concentration. The EC50 is 0.38 mM.

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EC50 = 0.38 mM

(n=10)

(n=24)

(n=13)

(n=6)

(n=6)

(n=10)

0.01 0.1 1 10

Isoflurane Concentration

Percent Inhibition
A. Control

B. 1 mM Isoflurane

C. 

D. 

# of events/stim

control 1 mM Isoflurane

20 pA
A. Control

B. F6

C. D.C.

D. Control F6
**Figure A.** Image showing fluorescent staining.

**Figure B.** Graph showing fluorescence (normalized) over time (seconds) with ionomycin (5 μM) treatment.

**Figure C.** Bar graph comparing percent de-staining between control and 0.5 mM isoflurane.

**Figure D.** Bar graph comparing normalized Ca²⁺ increase between control and 0.5 mM isoflurane.
A. md130A transfected cells

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B. md130A transfected cells

C. wt syntaxin 1A transfected cells