Interaction of anesthetics with neurotransmitter release machinery proteins

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We have previously shown that the commonly used inhalational anesthetic isoflurane dose-dependently inhibits the mammalian neurotransmitter release machinery in neurons and secretory cells (Herring et al. 2009). We have also shown that the intravenous anesthetics propofol and etomidate inhibit the neurotransmitter release machinery (Herring et al. 2011). In contrast, drugs with similar composition and hydrophobicity, but which do not produce anesthesia, did not inhibit neurotransmitter release (Herring et al. 2009, 2011). These previous studies suggested that the t-SNARE syntaxin 1A may be an intermediary in the inhibitory effects of general anesthetics on neurosecretion. Other evidence that syntaxin 1A may be a target of isoflurane includes the demonstration that isoflurane binds to syntaxin 1A (Nagele et al. 2005) and that the Caenorhabditis elegans syntaxin 1A mutation (md130A) has diminished behavioral sensitivity to isoflurane (van Swinderen et al. 1999). In our previous studies, we found that overexpression of the syntaxin 1A mutant md130A in PC12 cells completely blocked the inhibitory responses to both isoflurane and propofol but had no effect on the response to etomidate (Herring et al. 2009, 2011). These results suggested that general anesthetics may target SNARE or SNARE-associated proteins, but that different anesthetics may target different proteins. The goal of the present study was to examine the possible role of other SNARE and SNARE-associated proteins in anesthesia more closely.

To prevent actions of anesthetics on channels or receptors from altering neurotransmitter release, we use an experimental paradigm that keeps membrane potential constant but allows the intracellular Ca2+ concentration ([Ca2+]i) to be elevated by a known amount. This paradigm allows the interaction between anesthetics and the release machinery to be directly probed. Using an RNA interference (RNAi) strategy, we derived three knockout PC12 cell lines that have stably reduced levels of selected release machinery proteins. The first line had virtually undetectable levels of SNAP-25 (Cahill et al. 2006), and the second cell line had no detectable expression of synaptotagmin I (Cahill et al. 2006; Moore et al. 2006). In the third cell line, SNAP-25 was completely ablated and there was a partial knockdown of SNAP-23. Neurotransmitter release was observed in all three PC12 cell lines after stimulation. The effects of isoflurane, propofol, and etomidate were tested in these three cell lines. The anesthetics were all less effective at suppressing neurotransmitter release, thereby bypassing anesthetic effects on channels and receptors, allowing anesthetic effects on the neurotransmitter release machinery to be examined in isolation. Three different PC12 cell lines, which had the expression of different release machinery proteins stably suppressed by RNA interference, were used in these studies. Interestingly, there was still significant neurotransmitter release when these knockdown PC12 cells were stimulated. We have previously shown that etomidate, isoflurane, and propofol all inhibited the neurotransmitter release machinery in wild-type PC12 cells. In the present study, we show that knocking down synaptotagmin I completely prevented etomidate from inhibiting neurotransmitter release. Synaptotagmin I knockdown also diminished the inhibition produced by propofol and isoflurane, but the magnitude of the effect was not as large. Knockdown of SNAP-25 and SNAP-23 expression also changed the ability of these three anesthetics to inhibit neurotransmitter release. Our results suggest that general anesthetics inhibit the neurotransmitter release machinery by interacting with multiple SNARE and SNARE-associated proteins.

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It is generally accepted that most general anesthetics facilitate GABA_A receptor activity, thereby enhancing inhibitory synaptic transmission, and that this alteration in GABA_A receptor activity is an important component of anesthetic action. This is especially true for intravenous general anesthetics, where anesthetic potency is thought to be produced primarily through the facilitation of GABA_A receptors. Volatile anesthetics, on the other hand, while they facilitate GABA_A receptor activity, also have a variety of other targets. General anesthetics have also been shown to inhibit presynaptic glutamate release via one or more presynaptic mechanisms (MacIver et al. 1996; Perouansky et al. 1995; Westphalen and Hemmings 2003, 2006).
MATERIALS AND METHODS

Ethical information. All of the experiments outlined in this report were carried out in PC12 cells. No approval is required for these cells.

PC12 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 37°C. Culture medium was replaced every other day, and cells were passaged once per week. Cells were replated on poly-lysine-coated glass coverslips 24–48 h prior to recording. After ~8 wk in culture cells were discarded, and previously frozen stocks were thawed and then kept in culture.

Amperometric measurement of catecholamine release. Carbon fiber electrodes were fabricated and used as previously described by Grabner et al. (2005). Each carbon fiber electrode was positioned such that it gently pressed against the PC12 cell in order to record amperometric events, which correspond to the currents produced by the carbon fiber’s oxidation of the neurotransmitter content of a single vesicle. 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) and kindly supplied to us by Dr. Eugene Mosharov (Department of Neurology, Columbia University, New York, NY).

Cell-to-cell variation can be a problem between experiments. The most important source of variability appears to be change in cellular properties with time. PC12 cells are kept as frozen stocks. After an aliquot of cells was defrosted, it typically took 2–3 wk for the cultures to recover fully from freeze. After cultures are kept in the incubator for several months, the cells stop secreting even with strong stimuli. There could be significant cell-to-cell variation in experiments performed on different days. Cell-to-cell variation in experiments done on the same day with the same cultures was modest. Thus, for each recording for an experimental group, a control cell was added on the same day at about the same time. Without a matching control the experiment was not used. Cells that exhibited no neurotransmitter release after stimulation, a relatively rare occurrence, were removed from the data set. A Student’s t-test was used to assess differences between populations of cells.

PC12 cell permeabilization and stimulation. An amperometric electrode was positioned against a cell. After 2 min in a Ca2+-free solution (step 1), the cell was permeabilized with 20 μM digitonin (Ca2+-free) for 25 s (step 2) and then stimulated for 2–3 min with a solution containing 100 μM Ca2+ (step 3). The cell was allowed to recover for 2 min in Ca2+-free medium (step 4), and the cycle began again at step 2. Cells were stimulated three or four times in this way, in order to identify the largest response. For cells treated with anesthetic, isoflurane, propofol (Sigma-Aldrich, St. Louis, MO), or etomidate (Hospira, Lake Forest, IL) was introduced into the bath 25 s prior to stimulation and was present throughout the recording. This was done in order to maximize anesthetic exposure time. The stimulation step (step 3) producing the greatest amount of release was analyzed. The recording solutions had standard compositions previously described by Grabner et al. (2005). [Isoflurane solution was prepared and measured as previously described (Jones et al. 1992; Jones and Harrison 1993; Xie et al. 2006). Briefly, isoflurane solutions were prepared by injection of a pure liquid form of isoflurane with gastight syringes into evacuated saline bags containing defined volumes of extracellular solution. Isoflurane was then applied to the chromaffin cell preparation via the extracellular solution. Because isoflurane can slowly leak from the bags, they were only used for a short period before they were discarded and replaced with fresh bags. The concentration of isoflurane in the bath was accurate (Xie et al. 2006)].

Encapsulation. The plasmid used for generating short hairpin (sh)RNAs (pshRNA/Neo) was constructed in our lab as described previously (Cahill et al. 2006). The following regions were selected as targets: bp 663–681 of synaptotagmin cDNA (NM_00103368: AGACATTGGGAAACCGTCTG), bp 355–373 of SNAP-25 cDNA (AF245227: GTTGGATGGGAAGGCAGCA), and bp 727–745 of SNAP-23 cDNA (NM_022689: CAAGAATCGCATGAGGATT) for knockdown of rat synaptotagmin, SNAP25, and SNAP-23, respectively. pshRNA/Neo-synaptotagmin and pshRNA/Neo-SNAP25 were transfected into PC12 cells with Lipofectamine 2000 to generate the synaptotagmin and SNAP-25 knockdown cells, respectively. The SNAP-25 knockdown cell line was further transfected with pshRNA/HisD-SNAP-23 to generate SNAP-23 and SNAP-25 double-knockdown cells. The cells were replated one day after transfection at a lower density and selected with 100 μg/ml G418 (or 2 mM histidinol) for 3–4 wk until discrete colonies were formed. Individual colonies were isolated, grown up, and tested by PCR for the incorporation of the plasmid into genomic DNA. The cell lines with the largest reduction of synaptotagmin, SNAP-25, and SNAP-23 were identified by immunoblotting with the following antibodies: SNAP-25 (ANR-001; Alomone), SNAP-23 (DS-19; Sigma), synaptotagmin I (mAb48; Developmental Systems Hybridoma Bank, University of Iowa), β-actin (JLA20; Developmental Systems Hybridoma Bank), and horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch).

For each knockdown, the stable cell lines that were constructed transmitted the knockdown indefinitely from weekly passage to weekly passage (Cahill et al. 2006). To prevent phenotypic drift (which can be a problem with PC12 cells), the cultures were used for only ~12–14 wk before reverting to frozen stocks from an early passage. At no time was any reversal of knockdown observed. The protein suppression shown in Figs. 1 and 2 is representative of the cell lines.

Immunofluorescence. PC12 cell lines (original or with gene knockdown) were cultured on Lab-Tek Chamber Slides (Nalge Nunc International) and were washed with PBS, fixed with 4% paraformaldehyde in PBS for 25 min at room temperature, quenched 10 min with 75 mM NH4Cl and 20 mM glycine, permeabilized, and blocked with 0.2% Triton X-100 in PBS complemented with 10% FBS for 30 min at 37°C and then incubated 2 h at 37°C with the primary antibodies against SNAP25 antibody (Santa Cruz), anti-SNAP23 antibody (Sigma), and anti-synaptotagmin antibody (Developmental Studies Hybridoma Bank). Next, the cells were incubated for 1 h with either Alexa Fluor 594-labeled goat anti-rabbit secondary antibody or Alexa Fluor 488-labeled goat anti-mouse secondary antibody (Invitrogen). After washing, the slides were mounted with VectaShield (Vector Laboratories, Burlingame, CA) and analyzed with an Eclipse E600 microscope (Nikon) and a ×40 objective.

Statistical analysis. A Student’s t-test was used to assess differences between populations of cells.

RESULTS

Details of the derivation of two of the three knockdown PC12 cell lines used in this study have been previously published (Cahill et al. 2006; Roden et al. 2007). Figures 1 and 2 show the extent of knockdown of SNAP-25, SNAP-23, and synaptotagmin I in each of the three lines. In the case of synaptotagmin I and SNAP-25, barely detectable levels of these proteins are present after the RNAi knockdown (Figs. 1 and 2). In the case of the SNAP-25/SNAP-23 double knockdown, levels of SNAP-23 were reduced by >60% (Figs. 1 and 2). Figure 2 shows that synaptotagmin I, SNAP-25, and SNAP-23 were distributed uniformly in wild-type PC12 cells and that the knockdown was also uniform throughout the cells. This latter result was not surprising, as each cell line was derived from a single cell and thus represents a clonal line. These cell lines are used throughout the rest of this report to
determine the effects of release machinery proteins on anesthetic actions.

Exocytosis was elicited by permeabilizing cells with digoxin in the presence of 100 μM extracellular Ca\(^{2+}\), in the presence and absence of isoflurane. This method of stimulating cells directly regulates [Ca\(^{2+}\)\_]e at the neurotransmitter release sites, while bypassing any requirement for activation of channels and receptors. On each day of recording, amperometric measurements of catecholamine secretion were made from a similar number of experimental and control cells. This strategy reduced day-to-day variation. The proximity of Ca\(^{2+}\) channels to synaptic release sites suggests that [Ca\(^{2+}\)\_]e may rise to levels above 100 μM at the vesicle (Llinas et al. 1992).

Although previous studies with these cell lines found significant differences in amperometric event properties when comparing wild-type and synaptotagmin I knockdown cell lines (Moore et al. 2006), we observed no such differences (see Discussion for explanation). Amperometric traces were similar (Fig. 3A), as were half-times (Fig. 3B) and the number of molecules released per event (Fig. 3C). The only difference observed in our study was that the number of events per stimulus was significantly reduced in the SNAP-25/SNAP-23 double knockdown cells [wild type: 94.8 ± 9.4 (n = 25) events per stimulus; synaptotagmin I KD: 98.8 ± 8.9 (n = 25); SNAP-25 KD: 84.04 ± 9.9 (n = 25); SNAP-25/SNAP-23 double KD: 51.88 ± 8.1 (n = 25)]. The fact that the amperometric properties were similar between the different cell lines facilitated comparisons and meant that any changes in release rate elicited by anesthetics were easily quantifiable.

Effects of isoflurane, propofol, and etomidate on catecholamine secretion in synaptotagmin I knockdown PC12 cells. We have previously shown that isoflurane significantly inhibits neurotransmitter release in PC12 cells and hippocampal neurons at clinically relevant concentrations (Herring et al. 2009). In this earlier study 0.5 mM isoflurane significantly reduced the number of amperometric events by ~38% (P < 0.05, n = 24), but it had no effect on amperometric event amplitude, charge, or kinetics. This experiment has been repeated. Figure 4A and B, plot representative amperometric currents obtained in the absence (Fig. 4A) and the presence of isoflurane (0.5 mM, Fig. 4B; 0.5 mM isoflurane is a concentration of ~1.6 MAC; a MAC is the minimum alveolar concentration of an anesthetic required for immobility in response to a noxious stimulus in 50% of trials) in wild-type PC12 cells. The amperometric trace in the presence of isoflurane contained many fewer amperometric events. On average isoflurane significantly inhibited the number of amperometric events by ~39% (Fig. 4C; P < 0.01, n = 19). The data in Fig. 4C have been normalized to the number of events observed in the absence of isoflurane.

A representative amperometric current observed in a digoxin-perforated synaptotagmin I knockdown PC12 cell exposed to Ca\(^{2+}\) (100 μM) in the absence of isoflurane is shown in Fig. 5A. A representative amperometric current observed in a cell exposed to isoflurane (0.5 mM) is shown in Fig. 5B. Treatment of synaptotagmin I knockdown cells with isoflurane resulted in an ~18% reduction in the number of amperometric events observed compared with control cells (Fig. 5C). This difference was not significant (P = 0.17, n = 16). Compared with the results shown in Fig. 4, the results in Fig. 5 suggest that knocking down synaptotagmin I lessened the response to isoflurane.

Ioflurane at 1 mM represents a dose rarely used clinically (>3 MAC) but one that is especially convenient for testing in order to determine whether the synaptotagmin I knockdown suppressed the response to isoflurane. This concentration of isoflurane significantly reduced neurotransmitter release by >60% in wild-type PC12 cells (Herring et al. 2009). Figure 5D plots the response of synaptotagmin I knockdown PC12 cells to 1 mM isoflurane and shows that the anesthetic reduced the amount of neurotransmitter released by ~34%, a difference that was not significant (P = 0.12, n = 15). The net effect of knocking down synaptotagmin I was to lessen the effect of isoflurane by about half compared with that observed in wild-type cells, at both 0.5 and 1 mM.

We have previously shown that propofol and etomidate significantly inhibit neurotransmitter release in PC12 cells, chromaffin cells, and hippocampal neurons at clinically relevant concentrations (Herring et al. 2011). In this earlier study 5 μM propofol significantly reduced the number of amperometric events by ~51% (P < 0.05, n = 16), but it had no effect on amperometric event amplitude, charge, or kinetics. This experiment has been repeated in wild-type PC12 cells. The bar chart in Fig. 6A plots the normalized average number of events in the absence (“control”) and presence of propofol (5 μM). Propofol-treated cells exhibited 49.1% fewer amperometric events compared with control cells (P < 0.02, n = 12), a reduction very similar to that observed in our earlier study (Herring et al. 2011). Figure 6B plots the normalized representative amperometric currents obtained in the absence and the presence of etomidate (10 μM). Etomidate-treated cells exhibited 47.3% fewer amperometric events, a value very similar to that of our earlier study (Herring et al. 2011).

Suppressing synaptotagmin I expression had an even larger effect on propofol’s ability to suppress neurotransmitter release. Previously we had shown that propofol inhibited the neurotransmitter release machinery in a dose-dependent fashion over a range of clinically relevant concentrations (Herring et al. 2009), reported to be 0.4–10 μM (Hadipour-Jahromy and Daniels 2003; Krasowski and Harrison 1999; Sprung et al. 2001). In wild-type PC12 cells, 5 μM propofol produced a
significant ~51% reduction in neurotransmitter release (Herring et al. 2011). In contrast, 5 μM propofol had almost no effect on neurotransmitter release in synaptotagmin I knockdown PC12 cells (Fig. 7A; \( P = 0.84, n = 15 \)).

Suppression of synaptotagmin I had the largest effect on etomidate’s ability to inhibit neurotransmitter release. We had previously shown that etomidate inhibited neurotransmitter release in a dose-dependent manner, with 40 μM etomidate inhibiting neurotransmitter release in wild-type PC12 cells by ~56% (Herring et al. 2011). In contrast, 40 μM etomidate potentiated neurotransmitter release in synaptotagmin I knockdown PC12 cells (Fig. 7B). In this case, etomidate augmented the number of amperometric events by ~71%. This difference was not significant (\( P = 0.065, n = 12 \)). Synaptotagmin I appears to play a key role in the inhibitory response to etomidate. [Large concentrations of propofol and etomidate were tested to determine whether the synaptotagmin I knockdown completely suppressed the response to anesthetic.]

**Effects of isoflurane, propofol, and etomidate on catecholamine secretion in SNAP-25 knockdown PC12 cells.** In SNAP-25 knockdown PC12 cells 1 mM isoflurane reduced the average number of amperometric events by ~54% (\( P = 0.03, n = 21 \)), a response that was not different from that observed in wild-type PC12 cells (Fig. 8A). In contrast, the responses to propofol and to etomidate were diminished by the knockdown of SNAP-25. Figure 8B shows that propofol (5 μM) reduced the amount of neurotransmitter release by ~27%. This response, about half that observed in wild-type PC12 cells, was not significant (\( P = 0.09, n = 31 \)). Etomidate (40 μM) reduced neurotransmitter release by ~8% (Fig. 8C; \( P = 0.73, n = 25 \)), a response only about one-seventh that observed in wild-type PC12 cells. Thus knockdown of SNAP-25 expression reduced the inhibitory effect of propofol and almost completely eliminated the response to etomidate but did not change the inhibition produced by isoflurane.
Effects of isoflurane, propofol, and etomidate on catecholamine secretion in SNAP-25/SNAP-23 knockdown PC12 cells. Previous studies have shown that knocking down SNAP-25 leaves significant neurosecretory activity in both chromaffin and PC12 cells (Cahill et al. 2006; Sorensen et al. 2003), a result that was attributed to a partial redundancy of SNAP-25 with SNAP-23. Therefore we tried to produce a PC12 cell line in which both SNAP-25 and SNAP-23 were knocked down. Our efforts were met with mixed success. At best, SNAP-25 was reduced below the limits of detection while SNAP-23 was reduced by 60%. We were never able to achieve complete knockdown of both proteins; some SNAP-23 was still present in all surviving cell lines. In our best SNAP-25/SNAP-23 knockdown PC12 cells 1 mM isoflurane reduced the number of events by 18% ($P = 0.35$, $n = 30$), a smaller response than that observed in wild-type PC12 cells (Fig. 9A). In contrast, the response to propofol was almost exactly the same as that observed in wild-type PC12 cells; 5 μM propofol reduced the number of amperometric events by ~51% (Fig. 9B; $P = 0.01$, $n = 30$). This result was surprising given that SNAP-25 suppression alone inhibited much of the response to propofol (see Fig. 8B). The added partial suppression of SNAP-23 restored the complete response to propofol. The response to etomidate (10 μM) in the SNAP-25/SNAP-23 PC12 cells was somewhat similar to that observed in the SNAP-25 knockdown cells. There was no inhibition of neurotransmitter release by etomidate in the SNAP-25/SNAP-23 PC12 cells. In fact, it appeared that etomidate potentiated neurotransmitter release, but this increase was not significant (Fig. 9C; $P = 0.39$, $n = 18$). Thus suppressing both SNAP-25 and SNAP-23 expression

Fig. 3. Amperometric events recorded from knockdown cell lines were similar to those from wild-type cells under our stimulation conditions. A: representative traces showing 2.5 min of data from wild-type PC12 cells and the 3 knockdown cell lines used in these studies, as indicated. B: averaged half-times of amperometric events showing no clear difference between the different cell lines (W-T, wild-type PC12 cells; Syt-1, synaptotagmin I knockdown; SNAP-25, SNAP-25 knockdown; SNAP-25/23, SNAP-25/SNAP-23 double knockdown). Data from 25 different cells were used for each group shown. C: average number of molecules of neurotransmitter released per amperometric event for the 4 different cell lines. No clear difference was observed. $n = 25$ for each of the 4 groups.

Fig. 4. Isoflurane inhibits neurotransmitter release in permeabilized wild-type PC12 cells. Digitonin-permeabilized cells were exposed to Ca\textsuperscript{2+} (100 μM), as indicated by the bar underneath the traces, to stimulate release. A and B: representative amperometric traces in the absence (A) and presence (B) of isoflurane (0.5 mM). C: averaged number of events in the absence (“Control”) and the presence of isoflurane (0.5 mM). Isoflurane inhibited release by ~39% ($n = 19$), a significant reduction ($^*P < 0.01$, Student’s $t$-test).
Fig. 5. Effect of isoflurane on neurotransmitter release in permeabilized synaptotagmin I knockdown PC12 cells. Digitonin-permeabilized cells were exposed to Ca²⁺ (100 μM), indicated by the bars below the traces, to elicit neurotransmitter release. A and B: representative amperometric recording in the absence (A) and presence (B) of isoflurane (0.5 mM). C: normalized average number of amperometric events produced by synaptotagmin I knockdown PC12 cells in the absence (“Control”) and presence of isoflurane (0.5 mM). There was an ~18% reduction in the number of events. The difference was not significant (P = 0.17, n = 16). D: averaged number of events in the absence (“Control”) and presence of isoflurane (1 mM). Isoflurane reduced the amount of neurotransmitter release by ~34%. The difference was not significant (P = 0.12, n = 15). Data were normalized to the mean of the control group throughout the study.

Fig. 6. Propofol and etomidate inhibit neurotransmitter release in permeabilized PC12 cells. Digitonin-permeabilized cells were exposed to Ca²⁺ (100 μM) to elicit neurotransmitter release. A: normalized average number of events in the absence (“Control”) and presence of propofol (5 μM). Propofol-treated cells produced 49.1% fewer amperometric events compared with control cells. *P < 0.02 (Student’s t-test); n = 12. B: normalized average number of events in the absence (“Control”) and presence of etomidate (10 μM). Etomidate-treated cells produced 47.3% fewer amperometric events compared with control cells. *P < 0.002 (Student’s t-test); n = 14.

had a large effect on the response to isoflurane and no effect on propofol and completely eliminated the response to etomidate.

DISCUSSION

Previous studies from our lab and others have suggested that, in addition to facilitating GABA_A receptor activity, certain general anesthetics may interact with the SNARE and SNARE-associated proteins that make up the neurotransmitter release machinery (Herring et al. 2009, 2011). In this study we have attempted to identify more specifically which proteins of the neurotransmitter release machinery are necessary for the inhibitory action of isoflurane, propofol, and etomidate on catecholamine secretion from PC12 cells. Using RNAi methods, we have derived knockdown PC12 cell lines that express nearly undetectable levels of synaptotagmin I or SNAP-25. A third cell line expressed almost no detectable SNAP-25 and had significantly reduced levels of SNAP-23. We studied catecholamine secretion in these cell lines in the presence and absence of the anesthetics with a permeabilization technique that allowed direct control of intracellular Ca²⁺ levels sufficient to elicit secretion. This approach was taken in order to avoid potential confounding effects of anesthetics on channels and receptors. In wild-type PC12 cells isoflurane, propofol, and etomidate have all been shown to inhibit catecholamine secretion from permeabilized cells (Herring et al. 2009, 2011). This effect is specific to the anesthetics, as isoflurane and propofol were applied without carriers or preservatives while propylene glycol, the preservative for etomidate, has no effect on neurotransmitter release (Herring et al. 2009). In the present study we show that knockdown of synaptotagmin I or SNAP-25 or dual knockdown of SNAP-25 and SNAP-23 prevents or at least reduces the inhibitory effect of all three anesthetics. This suggests that these SNARE and SNARE-associated proteins may mediate at least some of the effects of general anesthetics. Interestingly, neurotransmitter release is maintained after knockdown of the different release machinery proteins. This raises the possibility that there is functional redundancy with other proteins, some of which may be anesthetic insensitive or at least less sensitive.
sensors (Lynch and Martin 2007). Significant evidence exists that synaptotagmin I, II, VII, and IX operate as Ca\textsuperscript{2+} sensors in neuronal and neuroendocrine secretion (Cao et al. 2011). There have been conflicting claims about the importance of various synaptotagmin isoforms in neurosecretory PC12 and chromaffin cells; roles for isoforms I, III, V, VII, and IX have all been proposed (Fukuda 2004; Fukuda et al. 2002, 2004; Lynch and Martin 2007; Schonn et al. 2008; Sugita et al. 2002). Some studies have shown that neurotransmitter release persisted in PC12 cells, even after synaptotagmin I was suppressed by RNAi, albeit at a reduced level (Moore et al. 2006).

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**Fig. 8.** Isoflurane, but not propofol or etomidate, maintains its inhibitory effect on neurotransmitter release on SNAP-25 knockdown PC-12 cells. A: averaged number of events in the absence (“Control”) and presence of isoflurane (1 mM). Isoflurane produced a significant reduction of \( \sim 54\% \) in the number of amperometric events (\( *P = 0.03, n = 21 \)). B: averaged number of events in the absence (“Control”) and presence of propofol (5 \( \mu \)M). Propofol reduced the number of amperometric events by \( \sim 27\% \), which was not significant (\( P = 0.09, n = 31 \)). C: averaged number of events in the absence (“Control”) and presence of etomidate (40 \( \mu \)M). Etomidate produced an insignificant reduction of \( \sim 8\% \) on the number of amperometric events (\( P = 0.73, n = 25 \)). Data were normalized to the mean of the control group. Note that the inhibitory effect of isoflurane on neurotransmitter release was largely intact while the inhibitory effect of etomidate was significantly reduced on SNAP-25 knockdown PC12 cells. The effects of propofol on neurotransmitter release were moderately reduced on SNAP-25 knockdown PC12 cells.

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**Fig. 9.** The inhibitory effect of isoflurane and etomidate, but not propofol, on neurotransmitter release is reduced on SNAP-25/SNAP-23 knockdown PC12 cells. A: averaged number of events in the absence (“Control”) and presence of isoflurane (1 mM). Isoflurane produced a significant reduction of \( 54\% \) in the number of amperometric events (\( *P = 0.03, n = 21 \)). B: averaged number of events in the absence (“Control”) and presence of propofol (5 \( \mu \)M). Propofol reduced the number of amperometric events by \( \sim 27\% \), which was not significant (\( P = 0.09, n = 31 \)). C: averaged number of events in the absence (“Control”) and presence of etomidate (40 \( \mu \)M). Etomidate did not show any reduction of \( 8\% \) on the number of amperometric events (\( P = 0.73, n = 25 \)). Data were normalized to the mean of the control group. Note that the inhibitory effects of etomidate and isoflurane, but not propofol, on neurotransmitter release were significantly reduced on SNAP-25/SNAP-23 knockdown PC12 cells.
studies have suggested that deletion of synaptotagmin I from PC12 had no effect on release (Fukuda et al. 2002) or, alternatively, that release levels were similar but exhibited altered kinetics (Lynch and Martin 2007). It has been suggested that there is functional redundancy between synaptotagmin I and synaptotagmin IX in PC12 cells (Lynch and Martin 2007). Additionally, permeabilization of cells followed by exposure to 100 μM Ca²⁺ corresponds to a very strong stimulus, which may maximize secretion even under nonoptimal conditions.

Thus our results showing relatively robust Ca²⁺-dependent neurotransmitter release from PC12 cells deficient in synaptotagmin I should come as no surprise. Even so, there were clear differences in the efficacy of isoflurane, propofol, and etomidate in their ability to inhibit neurotransmitter release in synaptotagmin I-deficient PC12 cells compared with wild-type cells. Perhaps most interesting is the observation that the suppression of synaptotagmin I did not alter anesthetic actions in a uniform manner and that the differences were dramatic. Suppression of synaptotagmin I had the largest effect on etomidate and the smallest on isoflurane, with propofol being intermediate. This result suggests that the anesthetics bind to different functional sites, a conclusion reinforced by previous work from our lab that showed that overexpressing a syntaxin 1A mutant, md130A, in PC12 cells had large effects on neurotransmitter release but did not alter the efficacy of etomidate at all (Herring et al. 2011).

The data presented in this report are different from those of Moore et al. (2006), who reported significant differences in number of amperometric events per stimulation and amperometric properties like half-times and number of molecules of neurotransmitter per event in synaptotagmin I knockdown PC12 cells compared with wild-type PC12 cells. We observed no such differences in our study. This discrepancy is likely due to the method of stimulation employed, in that previous studies stimulated cells with high-K⁺ solutions while we used digitonin permeabilization followed by exposure to Ca²⁺. Undoubtedly, the [Ca²⁺]i levels achieved in our studies were much higher and more maintained throughout the stimulus than those elicited by high K⁺. We have previously validated the stimulation protocol by directly dialyzing cells with known Ca²⁺ concentrations (Herring et al. 2009, 2011). In an elegant study, Aratlejo and colleagues showed that quantal content in chromaffin cells depended on Ca²⁺ levels (Elhamdani et al. 2001). Higher [Ca²⁺], completely emptied vesicles, while lower levels did not. We interpret the difference between our study and this earlier study to be that once synaptotagmin I was knocked down high-K⁺ stimulation did not provide sufficient Ca²⁺ to empty vesicles completely because of altered Ca²⁺ sensitivity. Digitonin permeabilization followed by exposure to 100 μM Ca²⁺, the conditions used in the present report, elevated [Ca²⁺], to a level at which vesicular release rates were maintained and the vesicles emptied completely compared with wild-type cells. The large stimulus used in our study maximized release and allowed for direct comparisons between wild-type and knockdown PC12 cells.

Roden et al. (2007) found that VMAT1 expression was reduced in synaptotagmin I knockdown PC12 cells. Wouldn’t that give rise to altered vesicle neurotransmitter content? Possibly not. It is generally thought that the copy number of VMAT per vesicle is small. Higher expression of VMAT per vesicle would load the vesicles more quickly, but the final neurotransmitter concentration would depend on proton levels, due to VATPase activity, and not VMAT number, as long as there was sufficient time to load vesicles completely. Catecholamine content would only be altered in cells that are heavily stimulated, as they would not have time to refill their vesicles. In our study we stimulated naive cells, most likely with completely filled vesicles, even with small numbers of VMAT per vesicle.

In a similar manner, suppression of SNAP-25 in PC12 cells reduced but did not eliminate neurotransmitter release when cells were stimulated with high K⁺ (Cahill et al. 2006; Ray et al. 1997). Using a cell line deficient in SNAP-25, we observed differential effects on anesthetic actions. There appeared to be little or no effect on the efficacy of isoflurane to inhibit neurotransmitter release, while propofol’s actions were clearly diminished. The largest effects were observed in the response to etomidate, where reduction of neurotransmitter release was completely eliminated. As before, these results suggest that anesthetics interact with different components of the release machinery.

It has previously been suggested that there is functional redundancy between SNAP-25 and SNAP-23 (Delgado-Martinez et al. 2007; Sorensen et al. 2003). We attempted to suppress both SNAP-25 and SNAP-23 in the same PC12 cell; unfortunately, only a partial suppression (~60%) of SNAP-23 (see Fig. 1) was achieved. In this cell line we also observed differential effects on anesthetic actions. There appeared to be little or no effect on the efficacy of propofol to inhibit neurotransmitter release, while isoflurane’s actions were clearly diminished. The largest effects were observed in the response to etomidate, where reduction of neurotransmitter release was completely eliminated and potentiation was observed. These results are consistent with the hypothesis that anesthetics interact with different components of the release machinery.

It was previously shown that a syntaxin 1A mutant could suppress the actions of anesthetics in C. elegans (van Swinderen et al. 1999), and we showed that overexpressing this same protein suppressed isoflurane and propofol’s actions in PC12 cells (Herring et al. 2009, 2011). NMR binding studies have demonstrated the ability of general anesthetic molecules to bind to syntaxin monomers (Nagele et al. 2005). This result opens the possibility that syntaxin 1A might be an important site for anesthetic action (but see Metz et al. 2007 for an alternate hypothesis recently put forth by Crowder and colleagues whereby anesthetic molecules may inhibit the recruitment to the plasma membrane of the syntaxin activator UNC-13, thereby reducing syntaxin 1A activation). Thus we tried to construct a PC12 cell line deficient in syntaxin 1A. This effort was not successful.

Our results suggest interactions between anesthetics and release machinery proteins. There is a considerable literature supporting the idea 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), but the literature regarding the ability of anesthetics to influence the neurotransmitter release machinery itself remains sparse and unclear. Some studies are consistent with the possibility of direct modulation of the release machinery by general anesthetics. For instance, Richards and colleagues found that low concentrations of anesthetics affected
chemical transmission but not impulse conduction or cellular electrical properties in hippocampal neurons (Richards 1972; Richards and White 1975). 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). Hemmings and colleagues also suggested an insensitivity of KCl-evoked neurotransmitter release to clinical concentrations of anesthetic in cerebrocortical synaptosomes (Lingamaneni et al. 2001; Ratnakumari and Hemmings 1997; Westphalen and Hemmings 2003). They concluded that presynaptic Na⁺ channels or K⁺ channels were responsible for most of the effect (Hemmings et al. 2005). Wu et al. 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). Needless to say, alterations in Na⁺ channel function can easily give rise to altered presynaptic release. In any case, some of the studies described above are difficult to interpret, as anesthetics target K⁺ channels directly (Patel et al. 1999). Anesthetics like isoflurane activate TREK/TASK-type K⁺ channels (Franks and Honore 2004; Westphalen et al. 2007). These channels are widely distributed, and they help set the resting potential (Yost 2003). One predicts that as anesthetics increase the K⁺ conductance high-K⁺ stimulation would depolarize neurons or synaptosomes to a greater extent, thereby masking effects on the release machinery.

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. 1994; Laijenko et al. 1999; Miao et al. 1995). Buggy and colleagues found that high-K⁺-evoked release from rat cerebrocortical slices was markedly inhibited by a clinically relevant concentration of propofol (Buggy et al. 2000). In addition, Winegar and MacIver also concluded that inhibition of neurotransmitter release by an anesthetic, in this case isoflurane, was downstream of presynaptic Na⁺ channels and suggested that the anesthetic might “disrupt the vesicle release process” (Winegar and MacIver 2006). Our own results showing clear suppression of the neurotransmitter release machinery are most consistent with these latter reports.

Our results show that isoflurane, propofol, and etomidate appear to interact with multiple release machinery proteins. In previous work we found that overexpression of a syntaxin 1A mutant, m130A, suppressed the inhibitory actions of isoflurane and propofol, suggesting that syntaxin 1A represents a necessary component for the inhibitory effects of these anesthetics. Here knockdown of synaptotagmin I attenuated the inhibitory effects of isoflurane on neurotransmitter release, which may indicate that synaptotagmin I participates in the formation of an isoflurane binding pocket with syntaxin 1A. Knockdown of SNAP-25 did not prevent inhibition of release by isoflurane, while co-knockdown of SNAP-25 and SNAP-23 attenuated the effects of isoflurane. These data may indicate that SNAP-23 can substitute for SNAP-25 in permitting isoflurane action or, alternatively, show a preferential interaction of isoflurane with SNAP-23-containing SNARE complexes. Propofol inhibition of release was completely blocked by the knockdown of synaptotagmin I and attenuated by knockdown of SNAP-25. Co-knockdown of both SNAP-25 and SNAP-23 restored propofol-mediated inhibition. These results exhibit two particularly noteworthy features. First, robust neurotransmitter release was observed after every release machinery alteration, and second, every alteration produced some change in anesthetic efficacy. These results suggest that viable but unique release machinery complexes formed even after the alterations were made and that each one appeared to interact with anesthetic in an independent manner. These data are consistent with a model in which propofol preferentially interacts with a binding pocket created by synaptotagmin I, SNAP-25, and perhaps other release machinery proteins. This binding pocket might provide a more hydrophobic environment to stabilize the anesthetics. Inhibition of neurotransmitter release with etomidate was also prevented by knockdown of synaptotagmin I, in that a reduction in the expression level of synaptotagmin I appeared to convert etomidate from an inhibitor of release to a potentiator. Synaptotagmin I may therefore represent an essential component for etomidate-mediated release of inhibition, and a reduction in synaptotagmin I expression may unmask an ability of etomidate to promote neurotransmitter release. Knocking down SNAP-25 also prevented etomidate from inhibiting neurotransmitter release, consistent with a role for synaptotagmin I and SNAP-25 in the creation of a binding pocket necessary for etomidate-mediated inhibition. While the present data suggest that multiple SNARE proteins contribute to the inhibitory effects of general anesthetics on neurotransmitter release, additional work will be necessary to determine whether unique binding sites created by SNARE complexes are responsible for the distinct effects of isoflurane, propofol, and etomidate in PC12 cells deficient in the expression of specific SNARE proteins.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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