Etomidate and propofol inhibit the neurotransmitter release machinery at different sites

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Non-technical summary Alterations in synaptic efficacy are thought to underlie changes in learning and behaviour and are vital to normal neuronal function. Using a variety of different cells and techniques we investigate whether anaesthetics can modify neurotransmitter release as part of their mechanism of action. Our data suggest that inhibition of neurotransmitter release may be a critical mechanism for the actions of anaesthetics like etomidate and propofol. In the future this information may be used to design new generations of clinically useful anaesthetics.

Abstract The mechanism of general anaesthetic action is only partially understood. Facilitation of inhibitory $GABA_A$ receptors plays an important role in the action of most anaesthetics, but is thought to be especially relevant in the case of intravenous anaesthetics, like etomidate and propofol. Recent evidence suggests that anaesthetics also inhibit excitatory synaptic transmission via a presynaptic mechanism(s), but it has been difficult to determine whether these agents act on the neurotransmitter release machinery itself. In the present study we sought to determine whether the intravenous anaesthetics propofol and etomidate inhibit the release machinery. For these studies we used an experimental approach that directly regulated $[Ca^{2+}]_i$ at neurotransmitter release sites, thereby bypassing anaesthetic effects on channels and receptors in order to allow anaesthetic effects on the neurotransmitter release machinery to be examined in isolation. The data show that clinically relevant concentrations of propofol and etomidate inhibited the neurotransmitter release machinery in neurosecretory cells and in cultured hippocampal neurons. md130A is a mutant form of syntaxin with a truncated C-terminus. Overexpressing md130A in PC12 cells completely eliminated the reduction in neurotransmitter release produced by propofol, without affecting release itself. In contrast, overexpressing md130A in PC12 cells had little or no effect on the response to etomidate. These results suggest that both propofol and etomidate inhibit neurotransmitter release by a direct interaction with SNAREs and/or SNARE-associated proteins but they do so at different sites.

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Abbreviations GFP, green fluorescent protein.

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Introduction

Most, but not all, general anaesthetics facilitate $GABA_A$ receptor activity thereby enhancing inhibitory synaptic transmission. Modulation of $GABA_A$ receptors in this manner is known to be an important part of the mechanism of action for many anaesthetics. Currently intravenous general anaesthetics are believed to produce anaesthesia primarily through the facilitation of $GABA_A$ receptors, while volatile anaesthetics, which also facilitate $GABA_A$ receptors activity, have also been shown to inhibit presynaptic glutamate release via one or more presynaptic mechanisms (Perouansky *et al.* 1995; Maclver *et al.* 1996; Westphalen & Hemmings, 2003, 2006).

Although not extensive, some work provides for the possibility that intravenous anaesthetics, like inhalational anaesthetics, may also inhibit presynaptic glutamate release (Kendall & Minchin, 1982; Buggy *et al.* 2000; Westphalen & Hemmings, 2003). For instance, clinically relevant concentrations of the intravenous anaesthetic propofol were found to dose-dependently inhibit 4AP-evoked release of radiolabelled glutamate from rat cerebrocortical synaptosomes (Westphalen & Hemmings, 2003). While these data suggest a presynaptic site of action for propofol, the presynaptic targets of intravenous anaesthetics are largely unknown.

We have previously shown that the commonly used inhalational anaesthetic isoflurane dose-dependently inhibits the mammalian neurotransmitter release machinery (Herring *et al.* 2009). In the present study, we set out to determine if commonly used intravenous general anaesthetics are capable of influencing the mammalian neurotransmitter release machinery as well, in order to determine whether inhibition of the neurotransmitter release machinery represents a common mechanism for general anaesthetics. To accomplish this goal, we observed the effect of two commonly used intravenous anaesthetics, propofol and etomidate, on evoked neurotransmitter release independent of anaesthetic modulation of channels and receptors. To prevent actions of anaesthetics on channels or receptors from altering neurotransmitter release, we used experimental protocols that kept membrane potential constant, but which allowed $[Ca^{2+}]_i$ to be elevated by a known amount. These protocols allowed us to probe interactions between anaesthetics and the release machinery directly. We observed that clinically relevant concentrations of both propofol and etomidate dramatically inhibited the neurotransmitter release machinery of PC12 cells, chromaffin cells and cultured rat hippocampal neurons. md130A is a syntaxin 1A mutant, which was first shown to reduce sensitivity to general anaesthetics in *C. elegans* md130A heterozygotes (van Swinderen *et al.* 1999) and which we subsequently showed blocked isoflurane's ability to inhibit neurotransmitter release (Herring *et al.* 2009). Overexpression of the syntaxin 1A mutant, md130A, in PC12 cells completely blocked propofol's ability to inhibit the neurotransmitter release machinery, but had little or no effect on the response to etomidate. Taken together, these data suggest that the neurotransmitter release machinery is targeted by both etomidate and propofol, but that the anaesthetics interact with different components of the machinery, one which is blocked by the syntaxin 1A mutant (propofol) and the other which is not (etomidate).

Methods

Ethical information

Most of the experiments outlined in this manuscript were carried out in PC12 cells. No approval is required for these cells. The hippocampal neurons were prepared for us by a central core facility run by the University of Chicago. The core facility has been approved by the animal use committee at the University of Chicago (protocol no. 71794). All experiments were carried out according to the guidelines laid down by The University of Chicago's animal welfare committee. Rats were anaesthetized with 4% isoflurane via inhalation and then killed by removal of their hearts. Chromaffin cells were prepared from adrenal glands obtained from a local slaughterhouse. We have read the explanation of journal policy and UK regulations on animal experimentation as given in 'Reporting ethical matters in *The Journal of Physiology*: standards and advice' (Drummond, 2009) and our experiments comply with the policies and regulations.

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^{-1} gentamicin in a humidified 7% CO_2 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 h prior to recording. Chromaffin cells were prepared by digestion of bovine adrenal glands with collagenase and purified by density gradient centrifugation. The cells were plated on collagen-coated glass coverslips (22 mm × 22 mm) at a density of roughly 0.15 × 106 cells cm^{-2}. Fibroblasts were effectively suppressed with cytosine-arabinoside (10 μM), leaving relatively pure chromaffin cell cultures. Hippocampal neuron cultures were prepared from embryonic day 18 Sprague–Dawley rats as previously described (Wang *et al.* 2006).

Amperometric measurement of catecholamine release

Carbon fibre electrodes were fabricated and used as previously described by 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 analysed with a series of macros written in Igor Pro (Wavemetrics Inc.) and kindly supplied to us by Dr Eugene Mosharov.

There can be some variation between experiments. Although there is significant variation week-to-week and even day-to-day, we observed modest cell-to-cell variation in experiments done on the same day using the same cultures. 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. 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 Ca^{2+}-free solution (1), the cell was permeabilized with 20 μM digitonin (Ca^{2+} free) for 25 s (2), and then stimulated for 2–3 min with a solution containing 100 μM Ca^{2+} (3). The cell was allowed to recover for 2 min in Ca^{2+}-free media (4), and the cycle began again at step (2). Cells were stimulated 4–5 times in this way. For cells treated with anaesthetic, propofol (Sigma-Aldrich Co., St Louis, MO, USA) or etomidate (Hospira, Inc., Lake Forest, IL, USA) was introduced into the bath 25 s prior to stimulation and was present throughout the recording. This was done in order to maximize anaesthetic exposure time. The stimulation step (3) producing the greatest amount of release was analysed. The recording solutions had standard compositions previously described in Grabner *et al.* (2005).

Whole-cell patch clamp stimulation protocol

Whole-cell patch electrodes were pulled from micro-haematocrit capillary tubes (Drummond Scientific Co., Broomall, PA, USA), fire-polished and filled with an internal solution that contained 100 μM Ca^{2+}, 145 mM NaCl, 2.0 mM KCl, 10 mM Hepes, 1 mM Na_2ATP and 1.0 mM $MgCl_2$, pH 7.2 NaOH, osmolarity 300 mosmol kg^{-1}. 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, USA). 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 etomidate (8 μM) 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 were collected for 2.5 min. Data analysis started 15 s after breaking the membrane with the patch pipette to allow $[Ca^{2+}]_i$ equilibration. This 15 s 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 + etomidate (8 μM) throughout the duration of the recording. Multiple exposures to anaesthetic or washout of anaesthetic were not possible as exocytotic activity gently declined with time after breaking into the cell.

Optical measurement of evoked RH414 release

RH414 is a fluorescent styryl dye used to monitor neurotransmitter release (Betz *et al.* 1992). 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, USA) for 75 s. 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 60× water objective (0.512 um $pixel^{-1}$). Light of 530–550 nm from a high power 100 W Hg arc lamp was used for excitation, and emitted light was filtered through a 590LP filter. Images were captured using Metamorph software (Molecular Devices). 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 s. Prior to stimulation neurons were washed for 4 min with HBSS + anaesthetic or HBSS alone. Neurons were then exposed to 5 μM ionomycin in HBSS + anaesthetic or 5 μM ionomycin in HBSS alone. Fluorescent synapses were monitored 40 s 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 s 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 propofol or etomidate. A Student's *t* test was used to assess differences between the two conditions. Tau values were determined by fitting fluorescent intensity plots with a second-order exponential decay function, $y = y_0 + A_1 e^{-x/t1} + A_2 e^{-x/t2}$. (The t_1 and t_2 are time constant 1 and 2 of the double exponential function). The fluorescence traces for all nerve terminals in a given condition were then aligned at the initial point of de-staining and averaged.

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 GATCTAGACTCAACCATCTCTCCTTGTAATA TCAAAAATTCCACAAATCTGGCTCTCCACCAG. 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 h post transfection.

Immunoblotting

Levels of syntaxin and actin in PC12 cells were assessed using the following antibodies: syntaxin (no. 573831, Calbiochem), β-actin (JLA20; Developmental Systems Hybridoma Bank, University of Iowa) and horseradish peroxidase-labelled anti-mouse (Jackson Immuno-Research). ECL Advance reagents (Amersham/GE Healthcare) were used for detection of the horseradish peroxidase-labelled secondary antibodies.

Statistical analysis

A Student's *t* test was used to assess differences between populations of cells.

Results

Propofol inhibits the neurotransmitter release machinery of chromaffin cells and PC12 cells

Exocytosis was evoked in digitonin-permeabilized chromaffin cells in the presence and absence of propofol (5 μM). This method of stimulating cells directly regulates $[Ca^{2+}]_i$ at the neurotransmitter release sites, while bypassing any requirement for activation of channels and receptors. Figure 1*A* plots a representative amperometric current observed in a digitonin-perforated cell exposed to Ca^{2+} (100 μM) for 2.5 min (as indicated), in the absence of propofol, while Fig. 1*B* plots a representative amperometric current observed in a cell exposed to propofol (5 μM). Treatment of chromaffin cells with propofol (5 μM) resulted in a 26% reduction in the number of amperometric events observed when compared to control cells (Fig. 1*C*). This difference was significant ($P = 0.02$, $n = 22$).

Propofol inhibited the neurotransmitter release machinery in a dose-dependent fashion over a range of clinically relevant concentrations, reported to be 0.4–10 μM (Krasowski & Harrison, 1999; Sprung *et al.* 2001; Hadipour-Jahromy & Daniels, 2003). Different concentrations of propofol (1, 10, 50 and 100 μM) were applied to permeabilized chromaffin cells; each of these concentrations reduced the number of amperometric events per 2.5 min stimulation by 20, 28, 35 and 33%, respectively. Figure 1*D* plots the number of amperometric events observed as a function of propofol concentration. These data were fitted with a standard dose–response equation. The inset plots the same data on a linear scale to better illustrate the saturation of the response to propofol. The EC_{50} provided by the fitting function (see legend) was 0.79 μM propofol. The propofol concentration required for general anaesthesia in mammals has been reported to be ~0.4 μM (Franks & Lieb, 1994) in water. Dose-dependent effects on quantal amplitude or kinetics were not observed for propofol or etomidate (data not shown). Together, these data indicate that propofol has a statistical and biologically important dose-dependent effect on the release machinery at concentrations spanning this anaesthetic's clinically effective range.

On each day of recording, amperometric measurements were made from a similar number of experimental and control cells. This strategy reduced day-to-day variation.

Chromaffin cells are difficult to transfect. Our subsequent studies used PC12 cells, which allow for straightforward transfection with high efficiency. In addition, this allowed us to test propofol in a different kind of cell. Propofol (at either 0.5 μM or 5 μM) was found to significantly reduce neurotransmitter release in PC12 cells (Fig. 2). Figure 2*A* and *B* plot representative amperometric currents observed in digitonin-perforated PC12 cells exposed to Ca^{2+} (100 μM) for 2.5 min (as indicated) in the presence and absence of propofol (5 μM), respectively. Treatment of PC12 cells with propofol (5 μM) resulted in a 51% reduction in the number of amperometric events observed compared to control cells (Fig. 2*C*). This difference was significant ($P = 0.02$, $n = 16$). A box chart plots the range of these data in Fig. 2*D*. Similarly,

treatment of PC12 cells with 0.5 μM propofol resulted in a significant reduction (by 46%, $P < 0.04$) in the number of amperometric events observed compared to control cells (Fig. 2*E*). A box chart plots the range of these data in Fig. 2*F*. These data suggest that propofol inhibits the neurotransmitter release machinery at clinically relevant concentrations by reducing the number of vesicles released in response to stimulation, in both chromaffin and PC12 cells.

Proprofol's inactive analogue, 2,4-diisopropophenol, does not inhibit the neurotransmitter release machinery of PC12 cells

To determine if inhibition of the neurotransmitter release machinery is specific to agents that produce anaesthesia, we investigated the effects of proprofol's inactive analogue, 2,4-diisopropophenol, on evoked neurotransmitter release from permeablized PC12 cells. 2,4-diisopropophenol is identical to propofol in terms of its molecular composition and hydrophobicity but does not produce anaesthesia. Inhibition of the neurotransmitter release machinery was not observed when cells were exposed to 2,4-diisopropophenol (5 μM; $n = 10$) (Fig. 2*G*). Both the propofol and the 2,4-diisopropophenol used in this study were purchased as pure compounds without carriers or preservatives. The data in Fig. 2*G* serve as a control for DMSO since it was used in the propofol and 2,4-diisopropophenol experiments at identical levels. These data clearly demonstrate the selective nature of the inhibitory actions of propofol on the neurotransmitter release machinery.

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

Exocytosis was also elicited in digitonin-permeabilized PC12 cells in the presence and absence of etomidate (8 μM). Figure 3*A* plots a representative amperometric current observed in a digitonin-perforated cell exposed to Ca^{2+} (100 μM) for 2.5 min (as indicated), in the absence of etomidate while Fig. 3*B* plots a representative amperometric current observed in a cell exposed to etomidate (8 μM). Treatment of PC12 cells with etomidate (8 μM) resulted in a 43% reduction in the number of amperometric events observed when compared to control cells (Fig. 3*C*). This difference was significant ($P = 0.03$, $n = 29$).

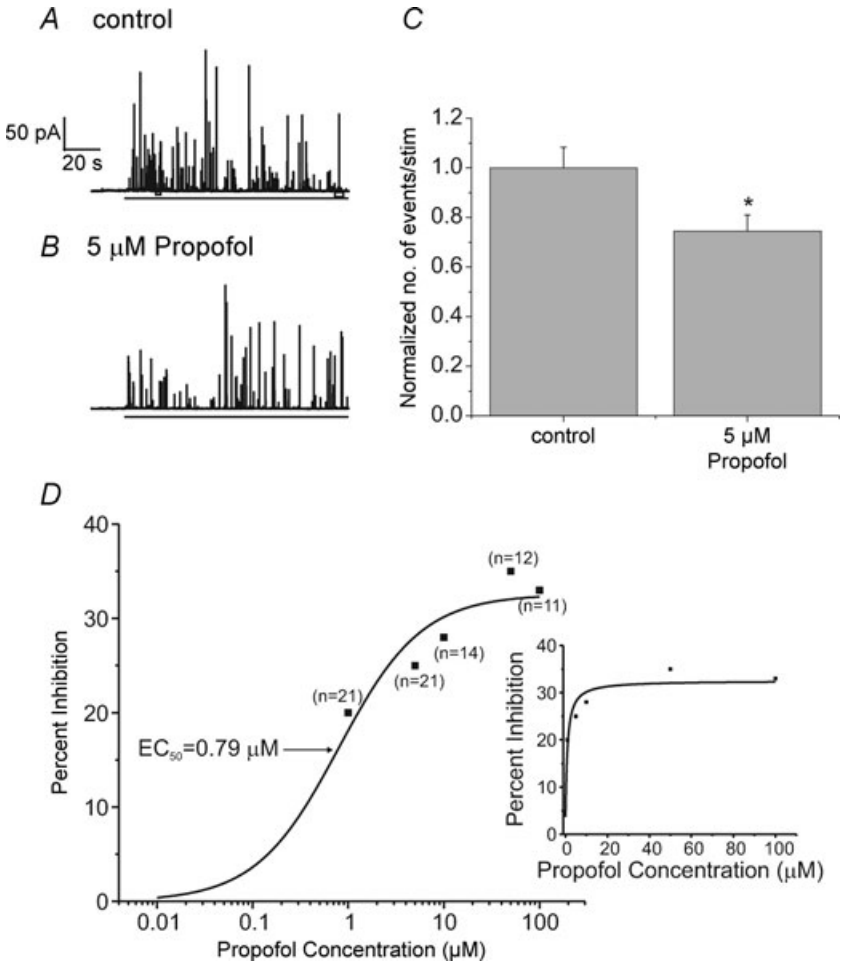

Figure 1. Propofol inhibits neurotransmitter release in permeabilized chromaffin cells
Digitonin-permeabilized cells were exposed to Ca^{2+} (100 μM), indicated by the bars below the current traces, to elicit neurotransmitter release. *A* and *B*, representative amperometric recordings in the absence of propofol ('control') and in the presence of propofol (5 μM). *C*, bar chart plots the normalized average number of events in the absence (control) and presence of propofol (5 μM). Propofol-treated cells produced 26% fewer amperometric events when compared to control cells. $^*P < 0.05$ (Student's *t* test), $n = 22$. Error bars correspond to S.E.M. Each data set was normalized to the mean of the control group. *D*, mean inhibition of neurotransmitter release plotted as a function of propofol concentration ($\log_{10}$). The number of cells studied at each concentration is indicated above or below each data point. Data were fitted with $Y = Y_{max} \times 1/1 + (EC_{50}/X)$. Y is the percentage of release inhibited. X is the propofol concentration. EC_{50} is defined as the concentration that produced 50% of the maximal response. This equation assumes 1:1 binding. Inset re-plots data on a linear scale to show saturation at higher propofol concentrations.

Etomidate was also found to inhibit the neurotransmitter release machinery in a dose-dependent fashion. Three additional concentrations of etomidate (2, 40 and 100 μM) were applied to permeabilized PC12 cells; these concentrations reduced the number of amperometric events per 2.5 min stimulation by 24, 56 and 51%, respectively. Figure 3*D* plots the number of amperometric events observed as a function of the log of the etomidate concentration. The inset plots the same data on a linear scale. The EC_{50} provided by the fitting function (see legend) was 2.62 μM etomidate. Clinically relevant concentrations of etomidate are reported to be $<$8.7 μM (Giese & Stanley, 1983). Together, these data indicate that in addition to propofol, etomidate also has a significant and dose-dependent effect on the release machinery at concentrations spanning this anaesthetic's clinically effective range.

Etomidate was applied in a propylene glycol preservative. Inhibition of the neurotransmitter release machinery was not observed when cells were exposed to propylene glycol at a concentration identical to that of the 40 μM etomidate experiment (Fig. 3*E*, $n = 17$). These

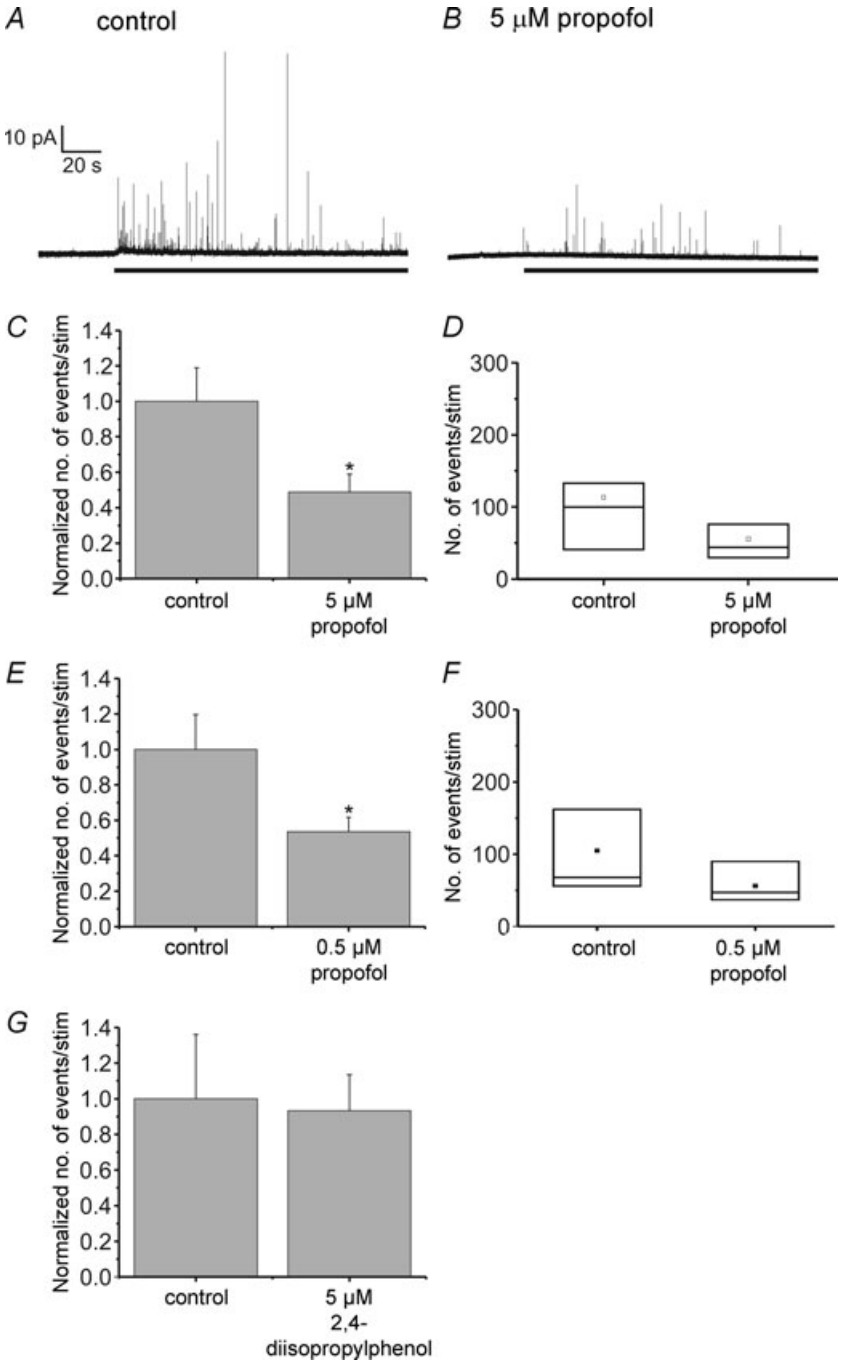

Figure 2. Propofol inhibits neurotransmitter release in permeabilized PC12 cells
Digitonin-permeabilized cells were exposed to Ca^{2+} (100 μM), indicated by the bars below the traces, to elicit neurotransmitter release. *A* and *B*, representative amperometric recordings in the absence of propofol ('control') and in the presence of propofol (5 μM). *C*, bar chart plots the normalized average number of events in the absence (control) and presence of propofol (5 μM). Propofol-treated cells produced 51% fewer amperometric events when compared to control cells. $^*P < 0.05$ (Student's *t* test), $n = 16$. Each data set was normalized to the mean of the control group. *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. *E*, bar chart plots the normalized average number of events in the absence ('control') and presence of propofol (0.5 μM). Propofol-treated cells produced 46% fewer amperometric events when compared to control cells. $^*P < 0.04$ (Student's *t* test); $n = 18$ control cells and 19 propofol-treated cells. Each data set was normalized to the mean of the control group. *F* 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. The symbol represents the mean value. *G*, proprofol's inactive analogue, 2,4-diisopropophenol (5 μM), does not inhibit neurotransmitter release. Bar chart plots the normalized average data from control cells and 2,4-diisopropophenol-treated cells. There was no significant difference between control and 2,4-diisopropophenol-treated cells. $P = 0.87$ (Student's *t* test); $n = 10$.

data clearly demonstrate that the preservative was not the source of the inhibition.

Etomidate inhibits the neurotransmitter release machinery in patch-clamped PC12 cells

To ensure that etomidate inhibited the neurotransmitter release machinery and not digitonin permeabilization a patch pipette was used to dialyse cells with a 100 μM Ca^{2+} solution in order to stimulate catecholamine release. Figure 4*A* and *B* plots amperometric data from PC12 cells dialysed via a whole-cell patch pipette, in the presence and absence of etomidate (8 μM). The elevated $[Ca^{2+}]_i$ introduced by the patch pipette caused the cells to release catecholamines which were then detected by carbon fibre electrodes. Cells were patch clamped in whole-cell configuration and held at −65 mV, which precluded activation of voltage-gated channels. Data were analysed 15 s after establishing whole-cell conditions

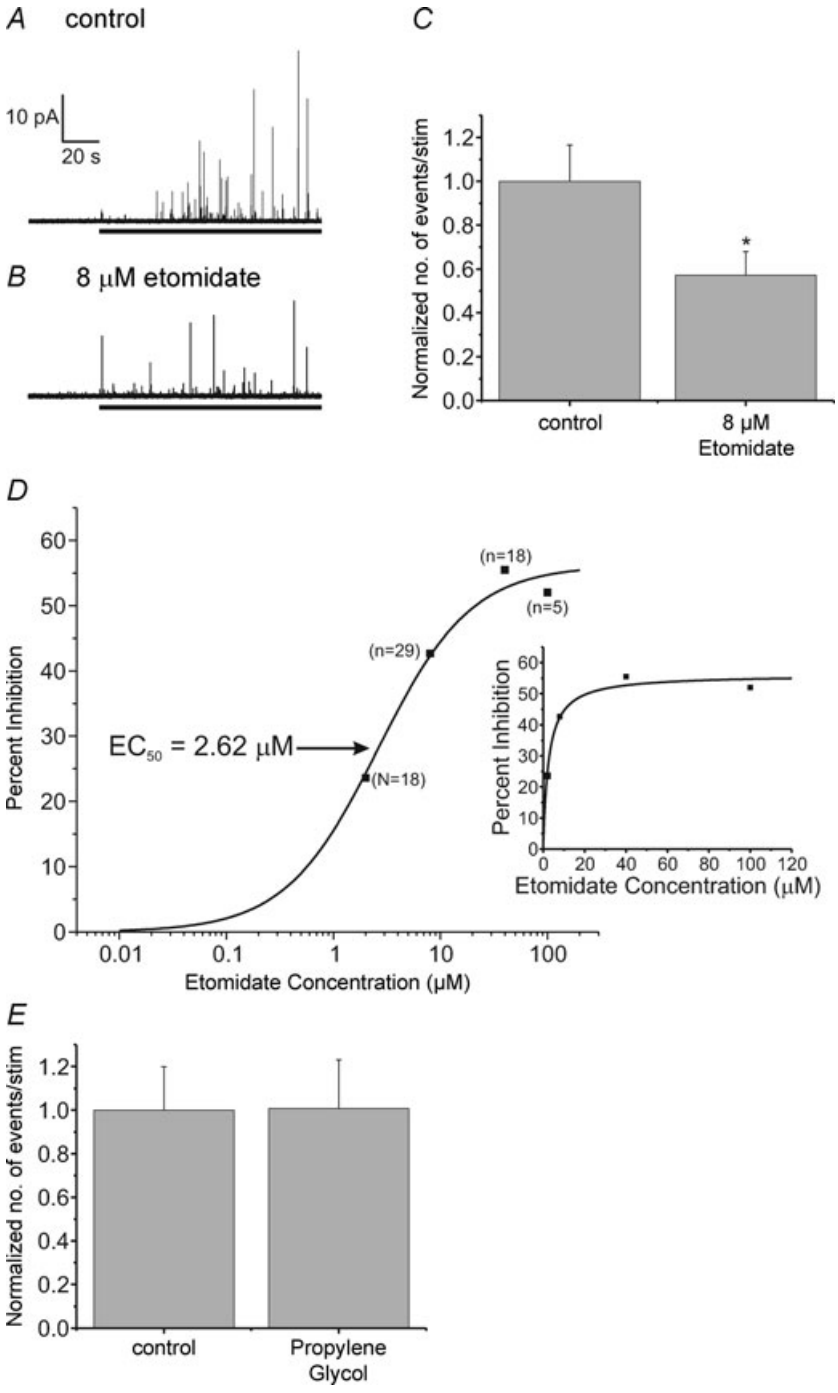

Figure 3. Etomidate inhibits neurotransmitter release in permeabilized PC12 cells
Digitonin-permeabilized cells were exposed to Ca^{2+} (100 μM), indicated by the bars below the traces, to elicit neurotransmitter release. *A* and *B*, representative amperometric recordings in the absence of etomidate ('control') and in the presence of etomidate (8 μM). *C*, bar chart plots the normalized average number of events in the absence and presence of etomidate (8 μM). Etomidate-treated cells produced 43% fewer amperometric events when compared to control cells. $^*P < 0.05$ (Student's *t* test), $n = 29$. *D*, mean inhibition of neurotransmitter release plotted as a function of etomidate concentration ($\log_{10}$). The number of cells studied at each concentration is indicated above or below each data point. Data were fitted with $Y = Y_{max} \times 1/1 + (EC_{50}/X)$. *Y* is the percentage of release inhibited. *X* is the etomidate concentration. This equation assumes 1:1 binding. Inset re-plots data on a linear scale to show saturation at higher etomidate concentrations. Note that each data point represents the average number of events obtained in cells exposed to etomidate divided by the average number of events observed in control cells. *E*, propylene glycol, the preservative for etomidate, did not alter transmitter release. Bar chart plots the normalized average data from control cells and propylene glycol-treated cells. Propylene glycol was used at the same concentration as was present in the 40 μM etomidate experiment. There was no significant difference between control and propylene glycol-treated cells ($P = 0.98$, $n = 17$).

to allow $[Ca^{2+}]_i$ to equilibrate. Etomidate (8 μM) reduced the number of amperometric events during each 2.25 min stimulation period by 56% ($P = 0.047$, $n = 7$; Fig. 4*C*), a value similar to the inhibition observed with digitonin-permeabilized cells (see Fig. 3*C*). These data indicate that etomidate's influence on neurotransmitter release in PC12 cells stems from an inhibition of the neurotransmitter release machinery and confirms the digitonin permeabilization studies.

Propofol and etomidate inhibit the neurotransmitter release machinery of hippocampal neurons

Neurotransmitter release mechanisms are strongly conserved between neurons and secretory cells (Rettig & Neher, 2002). To assess whether propofol and etomidate inhibit neurotransmitter release in central neurons, we studied exocytosis in cultured embryonic hippocampal neurons, after 2 weeks in culture. In order to monitor synaptic vesicle fusion we loaded vesicles with the fluorescent dye RH414 prior to stimulation (Fig. 5*A*). RH414 de-staining following stimulation was monitored over time using time-lapse fluorescence imaging. Exposing neurons to a solution containing the ionophore ionomycin (5 μM), as indicated, evoked exocytosis. Cells treated with propofol (5 μM) showed a significant reduction in exocytosis during ionomycin exposure (Fig. 5*B*). After 2 min of ionomycin exposure the fluorescence of 17 control nerve terminals was reduced an average of $51 \pm 3\%$, while the fluorescence of 37 propofol (5 μM)-treated nerve terminals was reduced an average of $36 \pm 2\%$ ($P < 0.001$; Fig. 5*C*). Therefore propofol (5 μM) was found to inhibit synaptic release, as measured by RH414 de-staining, by 27%. The time course of RH414 release was unaffected by propofol (both curves were well fitted by single exponentials; $\tau_{control} \approx 33$ s and $\tau_{propofol} \approx 29$ s). Cells treated with etomidate (8 μM) also showed a significant reduction in exocytosis during ionomycin exposure (Fig. 5*D*). After 2 min of ionomycin exposure the fluorescence of 38 control nerve terminals was reduced an average of $68 \pm 3\%$, while the fluorescence of 33 etomidate (8 μM)-treated nerve terminals was reduced an average of $54 \pm 3\%$ ($P < 0.001$; Fig. 5*E*). Therefore, etomidate (8 μM) was found to inhibit synaptic release, as measured by RH414 de-staining, by 21%. The time course of RH414 release from control terminals was fitted well with a single exponential, $\tau_{control} \approx 27$ ms. The fluorescence decay curve of etomidate-treated terminals was not well fitted by a single exponential, although visual inspection shows that the de-staining time course of the two curves is similar. These data strongly suggest that in addition to isoflurane, clinically relevant concentrations of both propofol and etomidate inhibit the exocytotic machinery of neurons within the mammalian CNS.

There was some variability in the efficiency of RH414 de-staining, week-to-week. To ensure consistency in the data, recordings from cultured neurons prepared at the same time in the presence and absence of anaesthetic were paired. Also note that there was sometimes de-staining prior to stimulation; this could be observed both in control conditions and in the presence of anaesthetic (see Fig. 5*D*).

md130A overexpression blocks the effect of propofol on the neurotransmitter release machinery

md130A is a point mutation in the syntaxin 1A gene resulting in the loss of part of the H3 and all of the C-terminal transmembrane domain (van Swinderen *et al.* 1999). Previously we demonstrated that the overexpression of the syntaxin mutant, md130A, in wild-type PC12 cells completely blocked the ability of isoflurane to inhibit the neurotrasnsmitter release machinery (Herring *et al.* 2009). To determine if md130A influences the

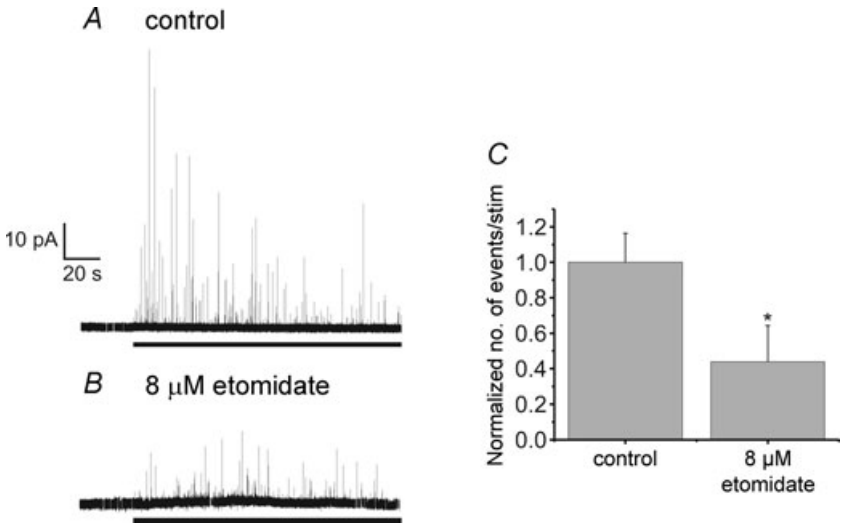

Figure 4. Etomidate inhibits neurotransmitter release in patch-clamped PC12 cells
Cells were dialysed via a patch pipette with Ca^{2+} (100 μM), indicated by the bars below the traces, to elicit neurotransmitter release. *A* and *B*, representative amperometric recordings in the absence ('control') and in the presence of etomidate (8 μM). *C*, bar chart plots the normalized average data from control cells and etomidate-treated cells. During a 2.25 min stimulation etomidate (8 μM)-treated cells produced 56% fewer amperometric events when compared to control cells. *$P < 0.05$ (Student's *t* test), $n = 7$.

sensitivity of the mammalian neurotransmitter release machinery to propofol, PC12 cells were transfected with an md130A expression plasmid (see Methods) and green fluorescent protein (GFP). Western blot analysis was previously used to confirm expression of the mutant (Herring *et al.* 2009). Transfected cells were identified as those expressing GFP. PC12 cells expressing the mutant syntaxin 1A showed no inhibition upon exposure to propofol at either 0.5 μM (Fig. 6*A*) or at 10 μM (Fig. 6*B*), concentrations similar to those shown in Fig. 2. Rather, it appears that propofol might augment release in cells expressing the md130A mutant at the higher propofol concentration, but this difference was not significant ($P = 0.1$, $n = 22$). Further studies in a larger pool of cells will be required to determine whether this augmentation is authentic. The effects of wild-type syntaxin 1A overexpression on the propofol sensitivity of neurotransmitter release were also examined in PC12 cells. Cells transfected with wild-type syntaxin 1A showed a significant reduction in the number of release events per stimulation in the presence of propofol (data not shown), showing that the md130A result was not due to the transfection process itself. Overexpression of md130A or wild-type syntaxin did not affect release rates in PC12 cells. The mean release rate of md130A-transfected PC12 cells was 95% of that observed in wild-type PC12 cells ($P = 0.87$). The mean release rate of syntaxin 1A-transfected cells was 119% of wild-type controls ($P = 0.68$) (Herring *et al.* 2009).

md130A overexpression does not block the effect of etomidate on the neurotransmitter release machinery

Overexpression of md130A was found to have little or no effect on etomidate's ability to inhibit neurotransmitter release from permeablized cells. Etomidate (40 μM) reduced the number of amperometric events observed in md130A-expressing PC12 cells by 54% (Fig. 7*A*). This effect was significant ($P = 0.003$, $n = 20$) and very similar to the 56% reduction observed previously with etomidate (40 μM) in wild-type PC12 cells (Fig. 3*D*).

Etomidate inhibited the neurotransmitter release machinery in a dose-dependent fashion in PC12 cells

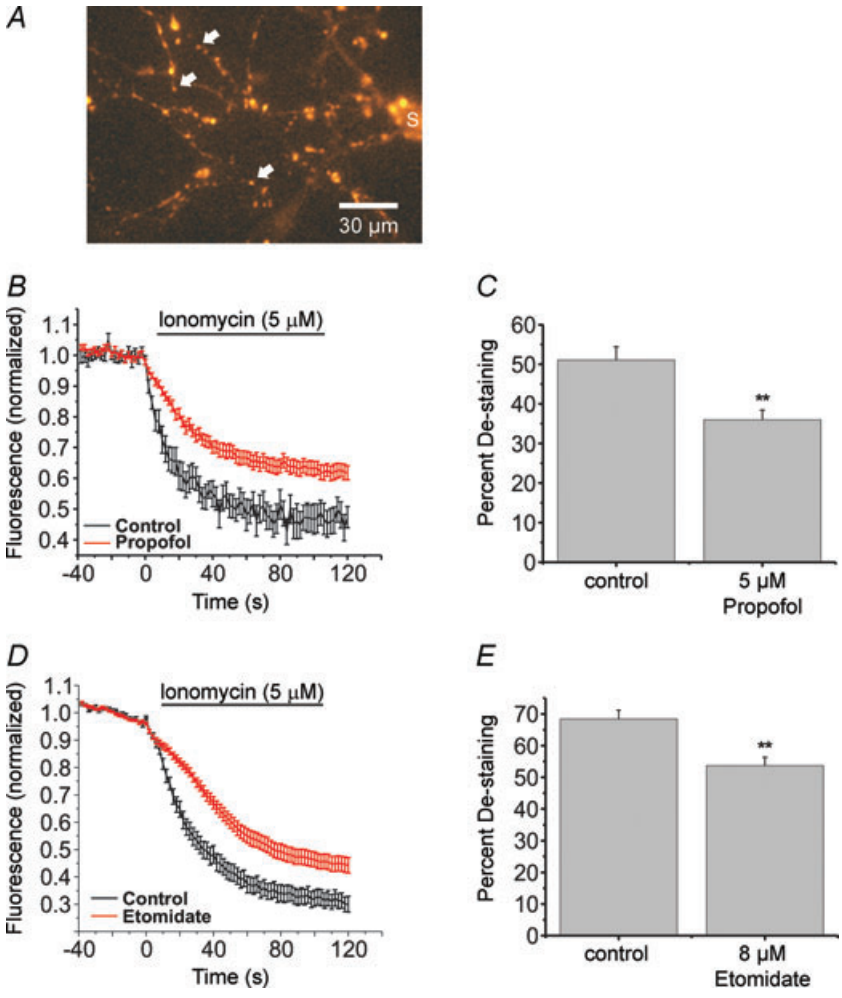

Figure 5. Propofol and etomidate inhibit the neurotransmitter release machinery in rat hippocampal neurons
A, fluorescence micrograph illustrating nerve terminals containing RH414-loaded synaptic vesicles (arrows). S, soma. *B*, fluorescence intensity of terminals in the presence and absence of propofol (5 μM) plotted as a function of time. *C*, average fluorescence intensity of control and propofol-treated terminals 2 min following ionomycin exposure. Stimulation with ionomycin caused a de-staining of 51 ± 3% ($n = 17$) but propofol (5 μM)-treated cells de-stained by 36 ± 2% ($n = 37$), a 27% reduction (**$P < 0.001$, Student's *t* test). *D*, fluorescence intensity of terminals in the presence and absence of etomidate (8 μM) plotted as a function of time. *E*, average fluorescence intensity of control and etomidate-treated terminals 2 min following ionomycin exposure. Stimulation with ionomycin caused a de-staining of 68 ± 3% ($n = 38$) but etomidate (8 μM)-treated cells de-stained by 54 ± 3% ($n = 33$), a 21% reduction (**$P < 0.001$, Student's *t* test).

overexpressing md130A. Four additional concentrations of etomidate (2, 8, 20 and 80 μM) were applied to permeabilized PC12 cells. Figure 7*B* plots the number of amperometric events observed as a function of the log of the etomidate concentration. The EC_{50} provided by the fitting function was 3.9 μM etomidate, a value similar to that observed in wild-type PC12 cells.

Discussion

Previously, we demonstrated that clinically relevant concentrations of the volatile general anaesthetic isoflurane inhibited the neurotransmitter release machinery (Herring *et al.* 2009). Here we show that two intravenous general anaesthetics, propofol and etomidate, also inhibit the neurotransmitter release machinery. Thus, suppression of the release machinery may be a common property of general anaesthetics. Clinically relevant concentrations of both anaesthetics were found to reduce the number of amperometric events observed from digitonin-permeabilized neurosecretory cells. An inactive isomer of propofol, 2,4-diisopropophenol, was found to have no effect on neurotransmitter release from these cells, suggesting neurotransmitter release machinery inhibition is specific to the anaesthesia-producing isomer. Furthermore, clinically relevant concentrations of propofol and etomidate were found to inhibit RH414 release from synaptic vesicles in cultured hippocampal neurons. This finding suggests that intravenous anaesthetics inhibit the neurotransmitter release machinery in neurons found within the mammalian CNS. Overexpression of the syntaxin 1A mutant md130A eliminated the PC12 cell response to propofol but left the response to etomidate unaffected. Previously we demonstrated that the md130A mutation suppressed the response to isoflurane (Herring *et al.* 2009). These data suggest that anaesthetics interact with different components of the release machinery, one that is blocked by the syntaxin 1A mutant (propofol and isoflurane) and the other which is not (etomidate). Since md130A is a syntaxin 1A mutant that is missing the C-terminus including the hydrophobic transmembrane domain, it is possible that isoflurane and propofol interact with the transmembrane segment of syntaxin 1A.

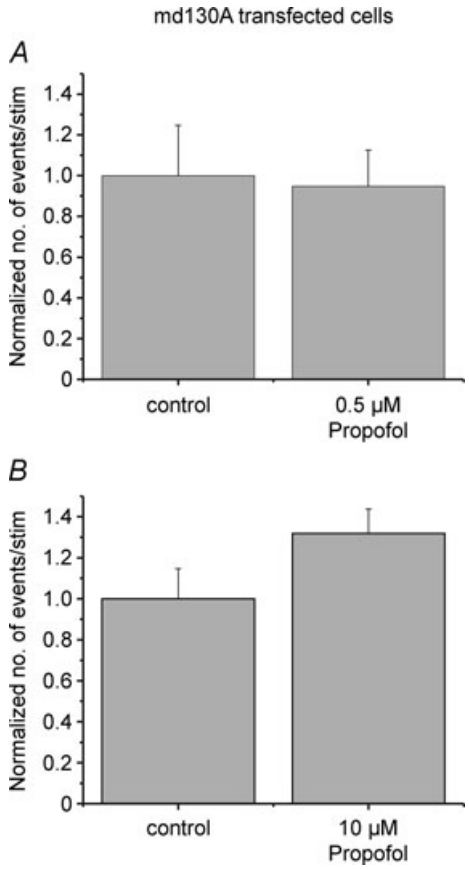

Figure 6. Overexpression of the syntaxin 1A mutant, md130A, eliminates propofol's effect on the neurotransmitter release machinery
A, bar chart plots the normalized average number of amperometric events produced by PC12 cells overexpressing md130A in the absence ('control') and presence of propofol (0.5 μM). There was no significant difference between control and propofol (0.5 μM)-treated cells ($P = 0.865$, $n = 15$). *B*, bar chart plots the normalized average number of amperometric events produced by PC12 cells overexpressing md130A in the absence ('control') and presence of propofol (10 μM). Propofol-treated cells exhibited ~31% more events than control, but this effect did not reach statistical significance ($P > 0.05$, $n = 22$).

It is widely held that intravenous general anaesthetics, such as propofol and etomidate, suppress neuronal activity through $GABA_A$ receptor potentiation. However, a few studies suggest intravenous anaesthetics inhibit neurotransmitter release by acting on one or more presynaptic mechanisms (Ratnakumari & Hemmings, 1997; Buggy *et al.* 2000; Westphalen & Hemmings, 2003). Unfortunately, the literature regarding the ability of intravenous anaesthetics to influence the neurotransmitter release machinery itself is relatively small and unclear. Hemmings and colleagues have reported that high-K^+-induced glutamate release from rat cerebrocortical synaptosomes is largely insensitive to clinical concentrations of propofol (Ratnakumari & Hemmings, 1997; Westphalen & Hemmings, 2003). Therefore, this group concluded that presynaptic Na^+ and K^+ channels, and not the neurotransmitter release machinery, represent the likely presynaptic targets of propofol. In contrast with Hemmings' findings, 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 *et al.* also concluded that inhibition of neurotransmitter release by an anaesthetic, in this case isoflurane, was downstream of pre-synaptic Na^+ channels

and suggested that the anaesthetic might 'disrupt the vesicle release process' (Winegar & MacIver, 2006).

To directly investigate anaesthetic effects on the neurotransmitter release machinery the present study examined evoked release from both neurosecretory cells (PC12 cells and chromaffin cells) and hippocampal neurons, using experimental protocols that elevated $[Ca^{2+}]_i$ at the release sites. These stimulation methods were used in lieu of KCl-evoked release in order to avoid potential confounding effects of anaesthetics on channels and receptors that are known to occur. Our data from individual neurosecretory cells and individual hippocampal neuron synaptic terminals, strongly suggest clinical concentrations of propofol and etomidate inhibit the neurotransmitter release machinery, and is consistent with the study of Buggy *et al.* as well as that of Winegar & MacIver (2006).

The clinical effects of general anaesthetics are dose dependent. If the inhibition of the neurotransmitter release machinery plays a role in the production of anaesthesia, measurable effects on the neurotransmitter release machinery should be observed throughout the clinically effective range of propofol and etomidate (~0.4–10 μM and ~1–8 μM, respectively (Giese & Stanley, 1983; Franks & Lieb, 1994; Sprung *et al.* 2001; Hadipour-Jahromy & Daniels, 2003). Therefore, we tested the effects of six concentrations of propofol (0.5, 1, 5, 10, 50 and 100 μM) and four concentrations of etomidate (2, 8, 40 and 100 μM) on permeabilized neurosecretory cells. The best possible fits of these data suggest that dose-dependent inhibition of the release machinery occurs throughout the clinically relevant ranges of propofol and etomidate. The effects of propofol and etomidate on the neurotransmitter release machinery saturate at concentrations >50 and 40 μM, respectively, which maximally reduce neurotransmitter release by less than 100%. This observation leaves open the possibility that these anaesthetics, along with isoflurane (Herring *et al.* 2009), operate as partial agonists with regards to neurotransmitter inhibition. The calculated EC_{50} for inhibition of the release machinery was 0.79 μM for propofol and 2.62 μM for etomidate. Reassuringly, these concentrations fall well within the clinical ranges of their respective anaesthetics. Such data strongly suggest a biologically important effect of these intravenous anaesthetics on the neurotransmitter release machinery. It is also worth noting that propofol and etomidate exhibited a smaller degree of inhibition in chromaffin cells and hippocampal neurons, as compared to PC12 cells, suggesting that there may be differences in the degree of release machinery block between different kinds of cells. There may also be slight differences in anaesthetic EC_{50} for the different cell types. Even so, in all cases, the amount of block was significant at clinically relevant anaesthetic concentrations.

Expression of a syntaxin 1A truncation mutant, md130A, blocked propofol-mediated inhibition of neurotransmitter release in permeablized PC12 cells, in a manner similar to that previously observed for isoflurane (Herring *et al.* 2009). These data are in agreement with an observed reduction in behavioural sensitivity of *C. elegans* md130A heterozygotes to general anaesthetics (van Swinderen *et al.* 1999). PC12 cells expressing md130A

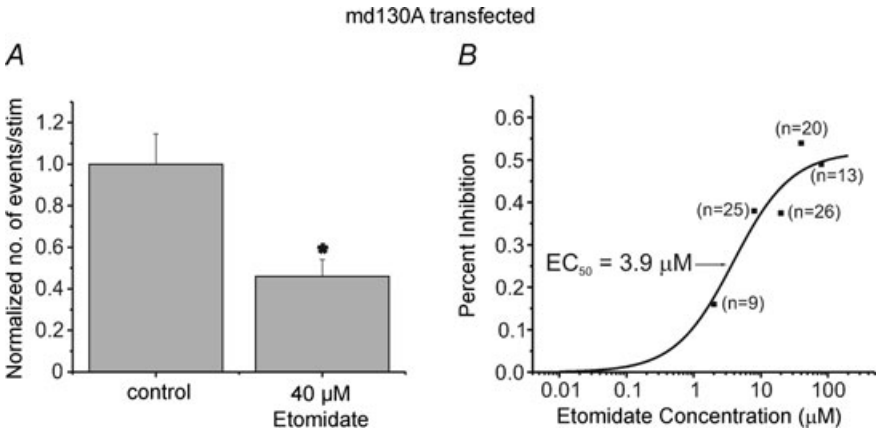

Figure 7. Overexpression of the syntaxin 1A mutant, md130A, had little or no effect on the response to etomidate

A, bar chart plots the normalized average number of amperometric events produced by PC12 cells overexpressing md130A in the absence ('control') and presence of etomidate (40 μM). There was a significant 54% reduction in the number of amperometric events ($P = 0.003$, $n = 20$), produced by etomidate under these conditions. *B*, mean inhibition of neurotransmitter release plotted as a function of etomidate concentration ($\log_{10}$) in md130A-overexpressing cells. Data were fitted with $Y = Y_{max} \times 1/1 + (EC_{50}/X)$. Y is the percentage of release inhibited. X is the etomidate concentration. This equation assumes 1:1 binding. Note that each data point represents the average number of events obtained in cells exposed to etomidate divided by the average number of events observed in control cells.

were no longer inhibited by propofol; in fact, there may have been a small potentiation (see Fig. 6*B*). This effect, which was also seen with isoflurane (Herring *et al.* 2009), did not reach significance in our study and needs further investigation. In blocking the ability of isoflurane and propofol to inhibit neurotransmitter release with md130A, we may have unmasked weaker stimulatory effects of these anaesthetics. In contrast, propofol was effective at inhibiting the neurotransmitter release machinery in cells overexpressing exogenous wild-type syntaxin 1A (Herring *et al.* 2009). It is also interesting that, like isoflurane (Herring *et al.* 2009), overexpression of md130A was found to completely block the effects of propofol on the neurotransmitter release machinery despite the presence of endogenous syntaxin 1A. PC12 cells overexpressing wild-type syntaxin appear to behave in a manner undistinguishable from wild-type cells (Herring *et al.* 2009), suggesting that md130A suppression of the isoflurane and propofol responses was not simply an overexpression artifact.

The details of syntaxin's involvement in the response to isoflurane and propofol remain unclear. It is possible that these anaesthetics suppress neurotransmitter release by binding to syntaxin 1A, a hypothesis supported by NMR binding studies demonstrating the ability of general anaesthetic molecules to bind to syntaxin monomers (Nagele *et al.* 2005). In this scenario the truncation of the C-terminal portion present in the md130A mutant may produce a functional form of syntaxin lacking an anaesthetic binding pocket. It is currently unknown whether the md130A mutant is capable of supporting exocytosis, but the observation that *C. elegans* homozygous for md130A are not viable suggest that neurotransmitter release may be impaired (van Swinderen *et al.* 1999). An alternate hypothesis concerning anaesthetic actions on the release machinery has recently been put forth by Crowder and colleagues whereby anaesthetic molecules may inhibit the recruitment to the plasma membrane of the syntaxin activator UNC-13, thereby reducing syntaxin 1A activation (Metz *et al.* 2007). Crowder and colleagues suggest that the md130A mutant may bind to UNC-13, preventing the association of anaesthetic molecules with the syntaxin activator. Our data are consistent with both models of md130A action. To date our own attempts to completely suppress endogenous syntaxin function using RNAi, in order to address these issues more directly, have not been successful (Cahill, AL, Fox, AP & Xie, Z, unpublished observations).

Unlike isoflurane and propofol, md130A overexpression in permeablized PC12 cells had little or no effect on the inhibition of neurotransmitter release produced by etomidate. Although the EC_{50} of etomidate in md130A-overexpressing cells appears slightly shifted relative to control, the difference is not significant and some concentrations produced almost identical inhibition. For example, 40 μM etomidate elicited an almost identical suppression of release in both wild-type and md130A cells (compare Figs 3 and 7). If isoflurane and propofol suppress neurotransmitter release via an interaction with syntaxin 1A, our data do not necessarily exclude syntaxin 1A as a candidate effector of etomidate. Etomidate molecules are larger and less hydrophobic than isoflurane and propofol molecules. Thus, it might interact with a different site on syntaxin 1A, one that is less hydrophobic.

Regardless of how propofol, etomidate and isoflurane interact 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 seem to suggest biologically relevant inhibition of the release machinery by isoflurane, it is unclear whether this mechanism participates in the production of the anaesthetic state. In the future it will be necessary to generate knockout or transgenic animals in which the effects of anaesthetics on the release machinery are blocked in order to determine the relative contribution, if any, of this mechanism to the production of anaesthesia.

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Author contributions

B.E.H.: conception and design of the experiments, collection, analysis and interpretation of data, drafting the article. K.McM.: collection, analysis and interpretation of data. C.M.P.: collection and analysis of data. J.M.: preparation of reagents and drafting the article. A.P.F.: conception and design of the experiments and drafting the article. Z.X.: conception and design of the experiments, collection, analysis and interpretation of data, drafting the article.

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