Anesthesiology 2005; 103:768–78

Volatile Anesthetics Bind Rat Synaptic Snare Proteins

Peter Nagele, M.D., J. Brett Mendel, B.A.,† William J. Placzek, B.A.,† Barbara A. Scott, B.S.,‡ D. André d'Avignon, Ph.D.,§ C. Michael Crowder, M.D., Ph.D.‖*

Background: **Volatile general anesthetics (VAs) have a number of synaptic actions, one of which is to inhibit excitatory neurotransmitter release; however, no presynaptic VA binding proteins have been identified. Genetic data in *Caenorhabditis elegans* have led to the hypothesis that a protein that interacts with the presynaptic protein syntaxin 1A is a VA target. Motivated by this hypothesis, the authors measured the ability of syntaxin 1A and proteins that interact with syntaxin to bind to halothane and isoflurane.**

Methods: **Recombinant rat syntaxin 1A, SNAP-25B, VAMP2, and the ternary SNARE complex that they form were tested. Binding of VAs to these proteins was detected by ^{19}F-nuclear magnetic resonance relaxation measurements. Structural alterations in the proteins were examined by circular dichroism and ability to form complexes.**

Results: **Volatile anesthetics did not bind to VAMP2. At concentrations in the clinical range, VAs did bind to SNAP-25B; however, binding was detected only in preparations containing SNAP-25B homomultimers. VAs also bound at clinical concentrations to both syntaxin and the SNARE complex. Addition of an N-terminal His_6 tag to syntaxin abolished its ability to bind VAs despite normal secondary structure and ability to form SNARE complexes; thrombin cleavage of the tag restored VA binding. Thus, the VA binding site(s) has structural requirements and is not simply any α-helical bundle. VAs at supraclinical concentrations produced an increase in helicity of the SNARE complex; otherwise, VA binding produced no gross alteration in the stability or secondary structure of the SNARE complex.**

Conclusion: **SNARE proteins are potential synaptic targets of volatile anesthetics.**

VOLATILE anesthetics (VAs) such as diethyl ether and its halogenated analogs isoflurane and halothane are widely believed to produce anesthesia by action on synaptic transmission. In the vertebrate nervous system, VAs enhance inhibitory neurotransmission and reduce excitatory neurotransmission.[1] At inhibitory synapses, the most prominent effect of VAs is postsynaptic, where VAs potentiate γ-aminobutyric acid type A receptors and glycine receptors.[2–4] At excitatory synapses, VAs have their greatest effect presynaptically, where they inhibit transmitter release.[5–11] The presynaptic VA target(s) has not been identified.

In the nematode *Caenorhabditis elegans*, a mutation in the *unc-64* gene, which encodes the ortholog of the mammalian presynaptic t-SNARE syntaxin 1A, strongly antagonizes the behavioral effects of VAs acting at concentrations within the range of that used in human anesthesia.[12] This mutation alters the consensus sequence for the splice donor site of the sixth intron, resulting in the synthesis of a truncated syntaxin along with a reduced amount of full-length product. The truncated mutant products lack the C-terminal transmembrane domain and latter half of the H3 helical domain and act semidominantly to antagonize VAs. The semidominance of its phenotype indicates that the mutation does not produce VA resistance due to the loss of a syntaxin structural component, *e.g.*, loss of the VA binding site. Rather, the genetic data argue that the truncated syntaxin interacts with another molecule and thereby blocks VA binding to or action on the target. Further, overexpression of wild-type syntaxin can suppress the VA resistance produced by the truncated form, arguing that wild-type syntaxin and the truncated syntaxin compete for this target. These genetic data have led to the hypothesis that a protein that normally interacts with syntaxin is the primary VA target in *C. elegans*.

Syntaxin interacts with multiple proteins, two of which are the SNARE proteins SNAP-25 and VAMP (also known as synaptobrevin). These three proteins form a ternary complex (the SNARE complex) that is thought to mediate synaptic vesicle fusion with the presynaptic membrane.[13,14] Both the SNARE complex and syntaxin are particularly good candidates to bind VAs because both are thought to form 4-α-helical bundles with large hydrophobic cavities within each bundle.[15–17] Structurally similar synthetic 4-α-helical bundles have been shown to bind VAs with affinities in the clinical range,[18–20] and a putative 4-α-helical structure in the γ-aminobutyric acid type A receptor formed by transmembrane domains has been implicated as a potential binding site for VAs.[21,22]

As ligands in binding assays, VAs pose some unique challenges. VAs are highly hydrophobic and are presumed to have relatively low affinities for their relevant binding sites because their EC_{50} values are hundreds of micromolars. Standard binding assays on membrane fractions are difficult to interpret because the membranes are essentially an unsaturable reservoir. VAs are also, of course, volatile, making standard binding assays with

* Adjunct Instructor, Department of Anesthesiology, Washington University School of Medicine. Attending Anesthesiologist, Department of Anesthesiology and General Intensive Care, Medical University of Vienna. † Undergraduate Research Assistant, ‡ Senior Research Technician, Department of Anesthesiology, ‖ Associate Professor, Department of Anesthesiology and Department of Molecular Biology/Pharmacology, Division of Biology and Biomedical Sciences, Washington University School of Medicine. § Director High Resolution NMR Facility, Department of Chemistry, Washington University.

Received from the Departments of Anesthesiology and Molecular Biology/Pharmacology, Washington University School of Medicine, and the Department of Chemistry, Washington University, St. Louis, Missouri; and the Department of Anesthesiology and General Intensive Care, Medical University of Vienna, Vienna, Austria. Submitted for publication February 5, 2005. Accepted for publication May 18, 2005. Supported by the National Institutes of Health/National Institute of General Medical Sciences, Bethesda, Maryland, and the Austrian Science Fund, Vienna, Austria.

Address reprint requests to Dr. Crowder: Department of Anesthesiology, Washington University School of Medicine, Box 8054, 660 South Euclid Avenue, St. Louis, Missouri 63110. Address electronic mail to: crowderm@morpheus.wustl.edu.

radiolabeled VAs hazardous. Therefore, alternative methods have been used, including photoaffinity labeling and spectroscopy-based techniques.[23–27] These methods have been used to demonstrate binding of VAs at near-clinical concentrations to model proteins. However, binding of VAs to plausible integral membrane protein targets has not been reported. Direct measurement of VA binding to any protein that might be involved in mechanisms of anesthesia has not been reported. Using nuclear magnetic resonance (NMR)–based methods, we report here evidence for VA binding at clinical concentrations to the presynaptic SNARE complex and to its component t-SNAREs.

Materials and Methods

Protein Expression Constructs

The basic SNARE protein expression constructs used have been described in detail.[28] Rat syntaxin 1A was expressed from a modified pET-11 vector (pHO2c) to produce syntaxin$_{1\text{-}265}$ with a C-terminal His$_6$ tag and no transmembrane domain.[28] Full-length rat SNAP-25B (1-206) with a C-terminal His$_6$ tag was expressed in a modified pET-11 vector (pHO2d).[28] The cytoplasmatic domain of rat VAMP 2 (synaptobrevin 2) (196) was subcloned into pET-15b (Novagen, Madison, WI) to produce a protein with an N-terminal His$_6$ tag.[28] To make a syntaxin construct with both an N-terminal and C-terminal His$_6$ tag (H6:STX:H6), the 799-bp *Nde*I–*Eco*RI fragment from pHO2c was ligated into similarly cut pET-28a. The entire coding sequence and insertion sites were confirmed by dye-terminator sequencing.

Protein Expression, Purification, and Characterization

All recombinant His$_6$-tagged fusion protein constructs were expressed in BL21(DE3)pLysS or BL21Star®(DE3)pLysS bacteria (Stratagene, La Jolla, CA). Cells were grown in TB (terrific broth) at 37°C to an A_{595} of approximately 0.6 OD and induced with 0.5 mM IPTG and harvested after 4 h. Except for SNAP-25B multimer preparations, proteins were purified under nondenaturing conditions by Ni^{2+}-NTA affinity chromatography (Qiagen, Valencia, CA) as described in Fasshauer *et al.*,[28] except that a modified lysis buffer was used to decrease nonspecific binding (500 mM NaCl, 20 mM imidazole, 50 mM NaH_2PO_4, 20 mM β-mercaptoethanol, pH 8.0). Imidazole was increased stepwise (50 mM, 200 mM, 400 mM) to elute the proteins. Proteins were dialyzed against fast protein liquid chromatography buffer (FPLC) (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT); syntaxin and SNAP-25 were further purified by anion exchange chromatography (Mono-Q-column) on an ÄKTA FPLC (Amersham Bioscience, Piscataway, NJ) using a linearly increasing NaCl gradient in FPLC buffer (100–1,000 mM). Peak fractions were pooled and dialyzed against phosphate-buffered saline (PBS) (1.47 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and, if necessary, concentrated by ultrafiltration to a final concentration of 2–4 mg/ml. Protein concentration was measured by absorption at 595 nm by Bradford assay. For SNAP-25B preparations containing mostly multimer, the purification protocol was identical except for the following: β-Mercaptoethanol was not used, and the lysis buffer contained 300 mM NaCl and 10 mM imidazole. The N-terminal His$_6$ tag on H6:STX:H6 was cleaved for some experiments with thrombin according to manufacturer's protocol (Invitrogen, Carlsbad, CA).

Purified recombinant proteins were characterized by gel electrophoresis, circular dichroism (CD) spectroscopy, and Western blots to ensure adequate purity and correct size and helicity. Gel electrophoresis was performed as described by Fasshauer *et al.* [28] Sodium dodecyl sulfate (SDS)-PAGE was performed with a 5% stacking gel (Tris, pH 6.8) and a 10% resolving gel (Tris, pH 8.8). Except where noted, samples were incubated for 5 min either at room temperature (unboiled) or at greater than 95°C (boiled) before being loaded onto the gel. Nondenaturing PAGE was performed in a similar fashion, except that SDS was not used.

Formation of Ternary SNARE Complex

Monomeric SNAP-25B, syntaxin 1A, and VAMP2 were mixed together in an equimolar stoichiometric ratio (1:1:1) in PBS buffer and incubated overnight with rocking at 22°C. This sample was further purified by anion exchange chromatography on a Mono-Q-column. The correct formation of the SNARE complex was confirmed by formation of an SDS- and heat-resistant complex that migrated at the correct size on SDS-PAGE and, upon boiling, separated into the three monomers.

CD Spectroscopy

Circular dichroism spectroscopy experiments were performed on a Jasco J-600 spectrometer (Jasco, Inc., Easton, MD) in quartz cuvettes with 0.1 cm path length at 22°C. Samples were diluted in PBS buffer to a final concentration of 5 μM. Spectra (200–250 nm) were an average of five scans.

Western Blots

To confirm the identity of the purified proteins, Western blots were performed. Mouse anti-human monoclonal antibodies against VAMP2 (Chemicon MAB333; Chemicon, Inc., Temecula, CA) and against human syntaxin 1A (Sigma S0664; Sigma-Aldrich, Inc., St. Louis, MO) and goat anti-human polyclonal antisera against SNAP-25B (Santa Cruz SC-7538; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used and visualized with goat anti-mouse (Sigma A9309 [Sigma-Aldrich, Inc.] for syntaxin; Chemicon AP130P [Chemicon, Inc.] for

VAMP2) or rabbit anti-goat HRP-conjugated secondary antibodies according to manufacturers protocols. Specificity was confirmed by lack of immunoreactivity with the other SNARE proteins.

NMR Measurements of Anesthetic Binding

For measurement of binding of VAs to SNARE proteins, the difference between the ^{19}F-NMR transverse relaxation times (T2) of free and protein-bound VA was used.[23,24,27] Two hundred thirty-two microliters protein sample in PBS buffer diluted with 88 μl D_2O to a final concentration of 20 μM was injected into gastight vials containing VAs at various concentrations. The protein was incubated at 22°C with the anesthetic for 30 min, with vortexing approximately every 5 min. Pilot experiments with binding to bovine serum albumin showed this time was sufficient for full partitioning of anesthetic into the aqueous phase by extraction into heptane and quantification by GC[29] and maximal binding to protein by NMR. Anesthetic concentrations are expressed as the aqueous phase concentration. After equilibration, the protein sample was transferred *via* a gastight syringe into a gastight NMR-tube (Shigemi, Allison Park, PA). The ^{19}F-NMR relaxation measurements were performed at 500 MHz on a Varian Inova-500 (Varian, Inc., Palo Alto, CA) instrument equipped with Nalorac Bio-Quad probe (Nalorac, Corp., Martinez, CA). The observe channel of this probe was readily tuned to ^{19}F, where a 90° pulse length of 12.5 μs is routinely achieved. All spin–spin relaxation information was measured at 22°C by the method of Carr, Purcell, Meiboom, and Gill[30] on the single CF_3 fluorine resonance present in both halothane and isoflurane. The collection parameters for all relaxation measurements were an interpulse delay time of 200 μs, preparation time of 10 s, and four scans collected at each of 16 echo evolution times ranging from 4 to 4,000 ms, except where noted. Spin–spin relaxation data were analyzed using Bayesian probability theory for curve fitting to estimate decay constants from the spin echoes. All data were well fit to a single exponential decay; in all cases, fitting to a biexponential curve significantly worsened the fit ($P < 0.01$). Therefore, the anesthetic seems to be in fast exchange between bound and free states. This software (Bayes Analyze) is incorporated into Varian VNMR software (Varian Inc.) and has been developed specifically to extract and statistically analyze NMR parameters (frequencies, amplitudes, decay constants, etc.) from time domain free induction decay data. Curves were compared for statistical differences in decay constants by simultaneous curve fitting and F test using Graphpad Prism software (GraphPad Software, Inc., San Diego, CA). $P < 0.01$ was the threshold for statistical significance.

Results

Recombinant rat syntaxin 1A (hereafter referred to as syntaxin), SNAP-25B (hereafter referred to as SNAP-25), and VAMP2 (hereafter referred to as VAMP) were made with either an N-terminal or C-terminal His_6 tag (fig. 1A), using pET-based expression constructs.[28] The proteins were sufficiently pure as judged by PAGE (fig. 1B) and were the correct proteins as judged by their appropriate migration and immunoreactivity on Western blots (figs. 1B and C). By circular dichroism, syntaxin was found to be highly helical and induced helicity in SNAP-25 and VAMP by forming the SNARE complex (fig. 1D), similar to previous reports.[28] In addition, the SNARE complex was both SDS and heat resistant (see figure 6). Syntaxin and SNAP-25 antibodies labeled at least three SDS-resistant complexes migrating at approximately 55, 120, and 200 kd. Lower and higher order species of these sizes have been typical for SNARE complexes composed of wild-type syntaxin, SNAP-25, and VAMP and are thought to be otherwise identical noncovalently interacting monomeric and multimeric SNARE complexes, respectively.[31–33] We conclude that our recombinant SNARE proteins and complex are pure and structurally similar to previous reports.

Halothane and Isoflurane Do Not Bind to Recombinant Rat VAMP

We measured binding of VAs to the SNARE proteins and the SNARE complex by ^{19}F-NMR relaxation measurements of the CF_3 moiety of isoflurane and halothane. The spin–spin transverse relaxation time (T2) of smaller molecules such as VAs decreases when bound to larger molecules such as proteins. Using the effects on T2, binding of VAs to model proteins such as bovine serum albumin has been demonstrated.[23,24] In PBS buffer, the halothane and isoflurane relaxation times were highly reproducible (figs. 2A and B); note that all of the data points from five independent experiments for isoflurane and seven for halothane are shown and generally overlie one another. Recombinant VAMP did not significantly reduce the T2 of either halothane or isoflurane (figs. 2A and B). In addition, a crude host bacterial lysate without the expression vector that was passed over an Ni-NTA column did not reduce T2 (data not shown). Therefore, under these conditions, halothane and isoflurane do not bind with an affinity detectable by NMR to VAMP or bacterial lysate.

Halothane and Isoflurane Bind to SNAP-25

Unlike VAMP2, SNAP-25 significantly reduced the T2 of both halothane and isoflurane, indicating binding of a significant fraction of VA to SNAP-25 (figs. 3A and B). We performed a large number of T2 measurements with isoflurane and found a great deal of variability depending on the protein preparation (fig. 3B). With some prepa-

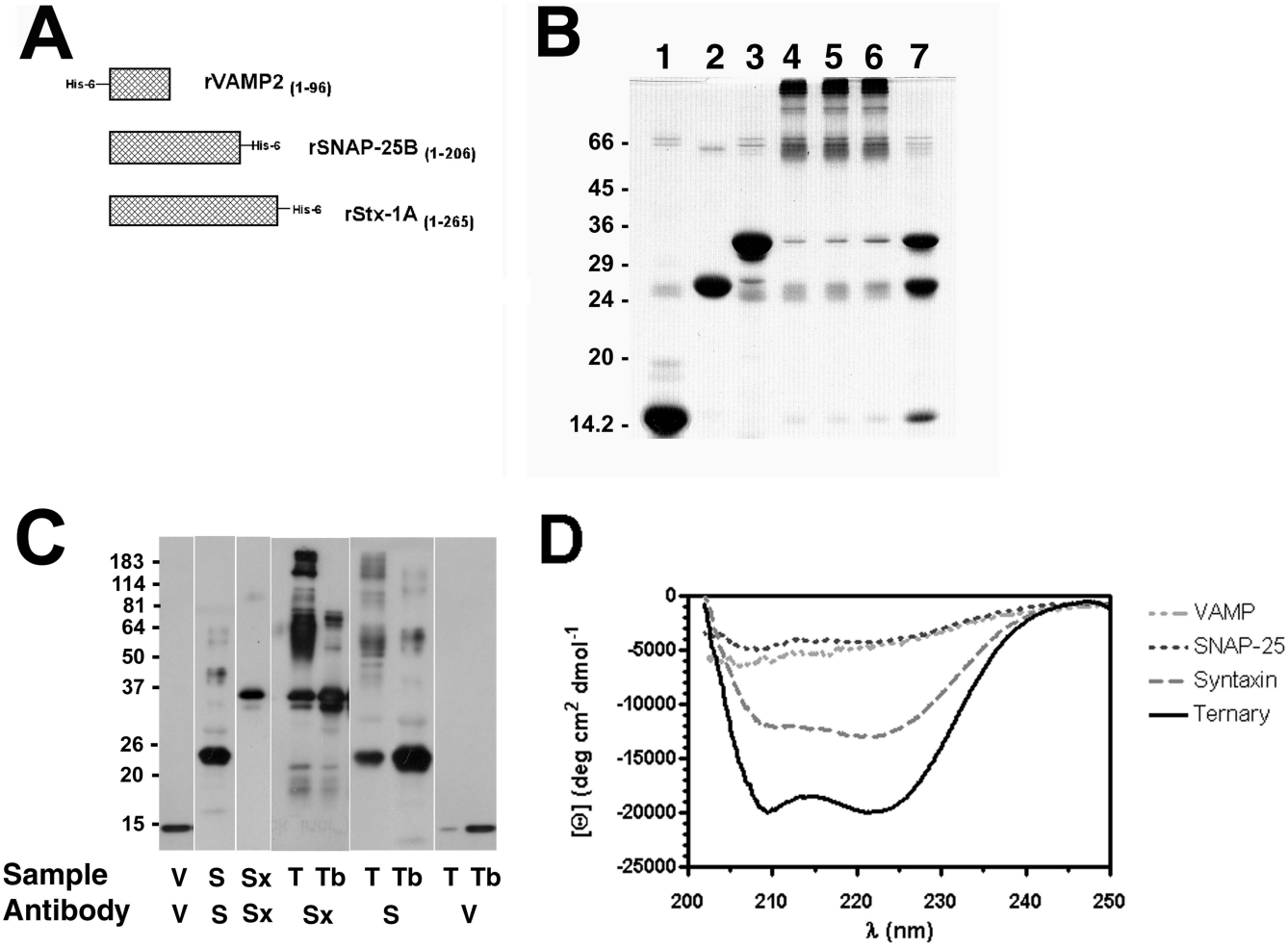

Fig. 1. Characterization of recombinant SNARE proteins used in study. (*A*) Schematics of expression constructs used for synthesis of recombinant rat SNARE proteins. All constructs coded for full-length proteins minus the C-terminal transmembrane domains in rVAMP2 and rSTX-1A (syntaxin 1A). Location of the His_6 tags are denoted. (*B*) Coomassie-stained sodium dodecyl sulfate (SDS)-PAGE of fast protein liquid chromatography buffer–purified recombinant proteins. *Lanes*: 1: rVAMP2; 2: rSNAP-25B; 3: rSTX-1A; 4: ternary complex incubated in 0% SDS at 23°C; 5: ternary complex (0.1% SDS); 6: ternary complex (0.5% SDS); 7: ternary complex (0.5% SDS—boiled); migration of size markers in kilodaltons is denoted. (*C*) Western blots of the recombinant SNARE proteins as monomers and in the ternary complex. S = SNAP-25B; Sx = syntaxin-1A; T = ternary complex; Tb = ternary complex boiled; V = VAMP2. (*D*) Circular dichroism spectra of recombinant SNARE proteins and the SNARE complex.

rations, T2s were consistently low, indicative of binding; other preparations gave consistently high T2s similar to that measured in buffer. The affinity of SNAP-25 for VAs did not correlate with its ability to form ternary complex or its purity. Upon examination by nondenaturing PAGE, we discovered that SNAP-25 preparations varied in the amount of higher-molecular-weight (MW) species; these higher-MW species migrated as a single band on denaturing PAGE at approximately 26 kd and immunoreacted with SNAP-25 antibody (figs. 1C and 3C). Subsequent ^{19}F-NMR experiments showed that isoflurane bound to preparations containing SNAP-25 multimers (*e.g.*, preparation 2) but did not significantly bind to preparations with almost exclusively monomer (*e.g.*, preparation 8) (figs. 3D and E). The T2 varied with isoflurane concentration from the lowest concentration of isoflurane measured (0.13 mM) to approximately 1 mM and then remained constant up to 5.1 mM (figs. 3D and E). These data are consistent with binding sites on SNAP-25 for isoflurane that saturate around 1 mM. Although the affinity of isoflurane for SNAP-25 cannot be estimated precisely without other methods, a four-parameter logistic fit of the variation in T2 gives an apparent Kd of 330 ± 122 μM, which is similar to the isoflurane EC_{50} for anesthesia in humans (human EC_{50} = 310 μM).[34] However, SNAP-25 multimers have not been well documented *in vivo*; therefore, the physiologic importance of VA binding to SNAP-25 is unclear.

Halothane and Isoflurane Bind to Syntaxin

The T2 values of both halothane and isoflurane were significantly decreased by syntaxin, indicative of binding (figs. 4A and B). However, the binding of either anesthetic was not apparently concentration dependent, at least in a concentration range that starts at the EC_{50} for anesthesia for these VAs and extends 15- to 25-fold

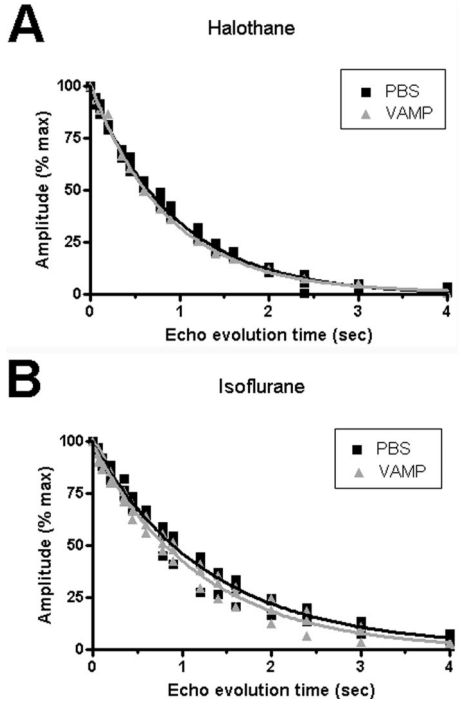

Fig. 2. Lack of binding of volatile anesthetics to VAMP by ^{19}F-nuclear magnetic resonance. Fluorine spin–spin relaxation curves of the CF_3 moiety of either halothane or isoflurane were obtained by a Carr, Purcell, Meiboom, and Gill pulse sequence.[30] (*A*) The amplitude of the halothane (0.96 mM) CF_3 spin echo signal, normalized to the initial maximal amplitude at 4 ms, is plotted against echo evolution time in the presence of 20 μM VAMP or phosphate-buffered saline (PBS) buffer. T2s (number of independent measurements): VAMP, 0.883 ± 0.027 s (n = 4); PBS, 0.959 ± 0.014 s (n = 7). VAMP T2 not significantly different from PBS. (*B*) Isoflurane (0.87 mM) spin–spin relaxation curves for VAMP and PBS. T2s: VAMP, 1.20 ± 0.065 s (n = 4); PBS, 1.297 ± 0.065 s (n = 7). VAMP T2 not significantly different from PBS.

higher (figs. 4C and D). In addition, halothane and isoflurane did not seem to compete for binding to syntaxin (fig. 4E). The lack of concentration dependence of the T2 can be explained if higher affinity sites with longer bound lifetimes and multiple lower affinity sites with shorter bound lifetimes exist on syntaxin.[23] For example, using identical NMR techniques coupled with partitioning measurements by gas chromatography, Dubois and Evers[23] showed bovine serum albumin had both low- and high-affinity binding sites that resulted in the T2 never approaching the T2 of buffer. This property of apparent lack of saturability of the binding sites was explained by the relatively low T2 of bound VA molecules *versus* free. For bovine serum albumin, the low- and high-affinity sites were calculated to produce T2s for bound halothane of 6 and 9 ms, respectively; even for the low-affinity sites, this is 140-fold lower than the T2 in buffer. Therefore, even if a small fraction of VA is bound, it will produce a significant reduction in the observed T2 and an apparent lack of saturability. Subsequently, x-ray crystallographic studies have confirmed the presence of multiple halothane binding sites on bovine serum albumin with varying affinities.[35] Resolution of low- and high-affinity binding sites by observance of concentration dependence of T2 would require NMR measurements at lower VA concentrations. However, the signal/noise ratio was insufficient to measure accurately T2s at [VA] below 100 μM (*e.g.*, see fig. 3E).

Alternatively, syntaxin could somehow nonspecifically alter the T2s of VAs in a way that does not represent binding, perhaps by changing the physical properties of the solution or by weak interaction of VAs all along the surface of the protein. To test this hypothesis, we set out to make truncated syntaxins that might retain their physical properties but lose VA binding affinity. As a precursor to making these mutant syntaxins, we built an expression construct that produced an otherwise identical recombinant syntaxin but with the addition of a thrombin-cleavable N-terminal His_6 tag along with the existing C-terminal His_6 tag (H6:STX:H6). Surprisingly, we found that this doubly tagged syntaxin did not bind isoflurane (fig. 4F). H6:STX:H6 had a CD spectra and ability to form SNARE complex similar to STX:H6 (fig. 4G and data not shown). Thrombin cleavage of the N-terminal His_6 tag restored ability of the syntaxin to bind VAs, demonstrating that the protein retained the capacity to bind VAs (fig. 4F). Therefore, despite the apparent lack of saturable or competitive binding sites, VA binding to syntaxin can be blocked without denaturation or changes in secondary structure. This result argues for specific binding sites on the protein, access to which is blocked by the N-terminal tag. Alternatively, more subtle structural changes that do not alter complex formation and are not detectable by CD could obliterate VA binding sites.

Halothane and Isoflurane Bind to the SNARE Complex

The SNARE complex forms a 4-α-helical bundle with a hydrophobic interior that, like syntaxin, is a good candidate to bind VAs. The SNARE complex did significantly reduce the T2 of both halothane and isoflurane, indicative of binding (figs. 5A and B). As for syntaxin, the T2 of isoflurane and halothane in the presence of the SNARE complex did not vary over the concentration ranges tested (figs. 5C and D). Similarly, we could not detect competition between isoflurane and halothane for binding sites on the SNARE complex (fig. 5E). Again, the apparent lack of saturable binding sites can be explained by multiple binding sites with varying affinities. The halothane and isoflurane T2 values were similar in the presence of syntaxin and the SNARE complex. If an equal fraction of VA were bound to the SNARE complex and syntaxin, the observed T2 should be reduced more by the larger molecular species, the SNARE complex. Given that the T2s values are nearly identical, the fraction of VA bound to the SNARE complex must be smaller than that bound to syntaxin. Alternatively, we considered the possibility that VAs bound to contaminating

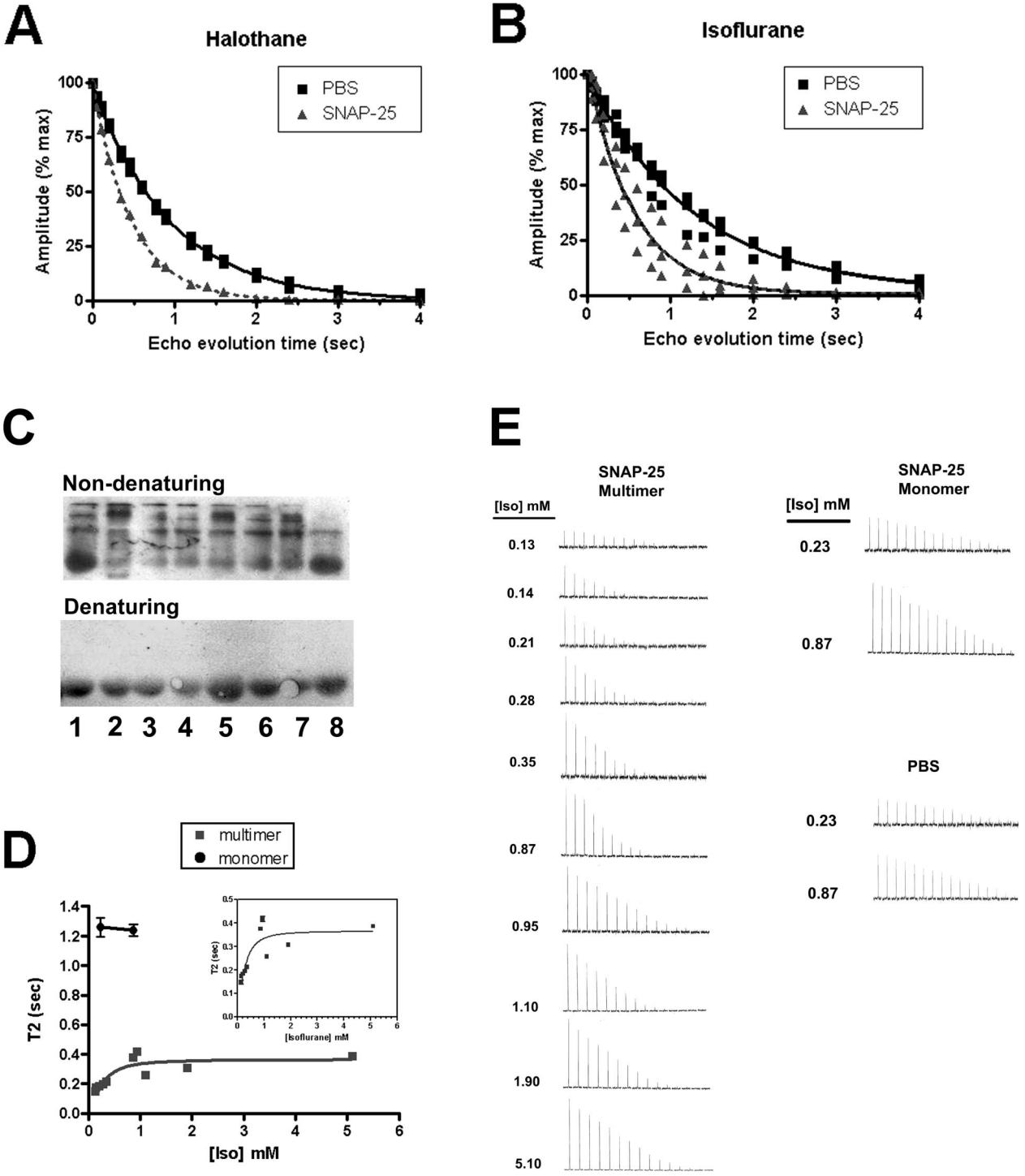

Fig. 3. Volatile anesthetics bind to SNAP-25 homomers. (*A*) Spin–spin relaxation curves for halothane (0.96 mM) in the presence of 20 μM SNAP-25 or phosphate-buffered saline (PBS) buffer. T2s: SNAP-25, 0.47 ± 0.01 s (n = 1); PBS, 0.936 ± 0.012 s (n = 5); $P < 0.001$ by nonlinear regression, $F_{1,90} = 585.4$. (*B*) Spin–spin relaxation curves for isoflurane (0.87 mM) in the presence of 20 μM SNAP-25 or PBS buffer. T2s: SNAP-25, 0.869 ± 0.071 s (n = 8); PBS, 1.30 ± 0.057 s (n = 5); $P < 0.01$ by nonlinear regression, $F_{1,202} = 10.98$. (*C*) Western blot with rat SNAP-25 antibody of nondenaturing and denaturing PAGE of eight different SNAP-25 preparations. Aliquots from the same preparation were loaded onto each gel in the same order. (*D*) T2 values plotted against isoflurane concentration for SNAP-25 preparations containing mostly multimer (preparation 2) and mostly monomer (preparation 8). (*Inset*) The multimer curve is shown on a different scale and was fit by a four-parameter logistic equation: Kd apparent = 330 ± 120 μM; slope = 1.86 ± 1.06; Ymax = 0.365 ± 0.045 s. The curve was constrained to a Y minimum of 0.1 s. Iso = isoflurane. (*E*) Spin–spin relaxation data used to calculate the T2s in *D*. Note that the array sampling times for 0.13- and 0.87-mM multimers, all monomer, and PBS data are different from all other arrays, which are 0.004, 0.056, 0.1, 0.148, 0.2, 0.276, 0.348, 0.448, 0.6, 0.78, 0.9, 1.2, 1.4, 1.6, 2.0 s. The array for 0.13 mM is 0.004, 0.008, 0.016, 0.032, 0.064, 0.08, 0.1, 0.2, 0.16, 0.2, 0.256, 0.3, 0.448, 0.516, 0.6 s. The arrays for 0.87 multimer, monomer, and PBS are 0.004, 0.056, 0.1, 0.2, 0.348, 0.448, 0.6, 0.78, 0.9, 1.2, 1.4, 1.6, 2, 2.4, 3, 4 s.

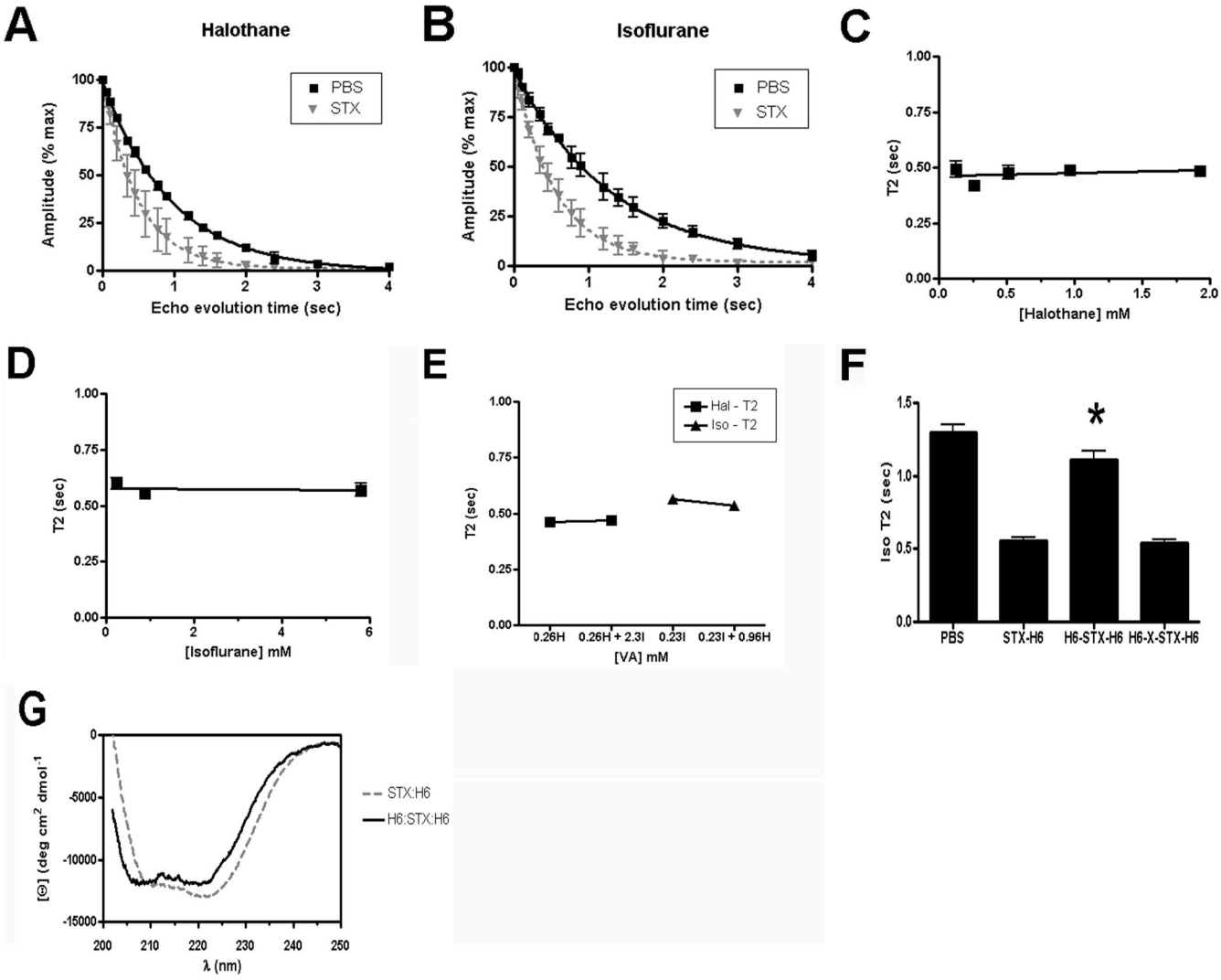

Fig. 4. Volatile anesthetics bind to syntaxin 1A. (*A*) Halothane binds to syntaxin 1A. Spin–spin relaxation curves for halothane (0.96 mM) in the presence of 20 μM syntaxin 1A or phosphate-buffered saline (PBS) buffer. Values are mean ± SD (syntaxin: n = 8; PBS: n = 7). T2s: Syntaxin, 0.490 ± 0.023 s; PBS, 0.959 ± 0.014 s; $P < 0.001$ by nonlinear regression, $F_{1,234} = 143.6.4$. (*B*) Isoflurane binds to syntaxin 1A. Spin–spin relaxation curves for isoflurane (0.87 mM) in the presence of 20 μM syntaxin or PBS buffer. T2s: syntaxin 1A, 0.554 ± 0.021 s (n = 6); PBS, 1.30 ± 0.057 s (n = 5); $P < 0.001$ by nonlinear regression, $F_{1,170} = 204.5$. (*C*) Halothane concentration–response relation for syntaxin 1A T2s. The T2 values are fit by a line with a slope not significantly different from 0. (*D*) Isoflurane concentration–response relation for syntaxin 1A T2s. The values are fit by a line with a slope not significantly different from 0. (*E*) Lack of competition between halothane and isoflurane for binding to syntaxin 1A. Halothane and isoflurane were added simultaneously for competition experiments and allowed to incubate for 30 min before nuclear magnetic resonance data collection. (*F*) Addition of an N-terminal His_6 tag antagonizes binding of isoflurane to syntaxin. The syntaxin $1A_{1-265}$ coding sequence was subcloned into PET-28A to allow for the expression of an N-terminal– and C-terminal–tagged $syntaxin_{1-265}$ (H6-STX-H6) and for thrombin-cleavage of the N-terminal His_6 tag (H6-X-STX-H6). All proteins were fast protein liquid chromatography buffer purified and tested at a concentration of 20 μm. T2 values are from a pooled fit of measurements of at least three independent protein preparations. * $P < 0.01$ *versus* STX-H6 and H6-X-STX-H6 by simultaneous nonlinear regression. (*G*) Comparison of the circular dichroism spectra of single- and double-tagged syntaxin.

syntaxin rather than ternary complex itself. To examine this hypothesis, we diluted our syntaxin preparation so that the concentration of syntaxin, as assessed by band intensity on a Coomassie-stained gel, was similar to the residual syntaxin monomer in the ternary complex preparations. By serial dilution, we estimated that the residual syntaxin in the ternary complex preparation was at most 2 μM. The isoflurane (0.87 mM) T2 in 2 μM syntaxin was 1.31 ± 0.095 s, which is significantly different than the isoflurane T2 of ternary complex ($P < 0.0001$, $F_{1,60} =$ 20). In addition, we formed ternary complex with the doubly His_6-tagged syntaxin (H6:STX:H6). The ternary complex formed with H6:STX:H6 bound isoflurane, producing a T2 similar to that of complex containing STX:H6 (fig. 5F). Given that H6:STX:H6 and the other components forming the ternary complex do not bind VAs (monomeric SNAP-25 preparations were used for all ternary complex preparations), the VA binding species must be the ternary complex and not contaminating SNARE component proteins.

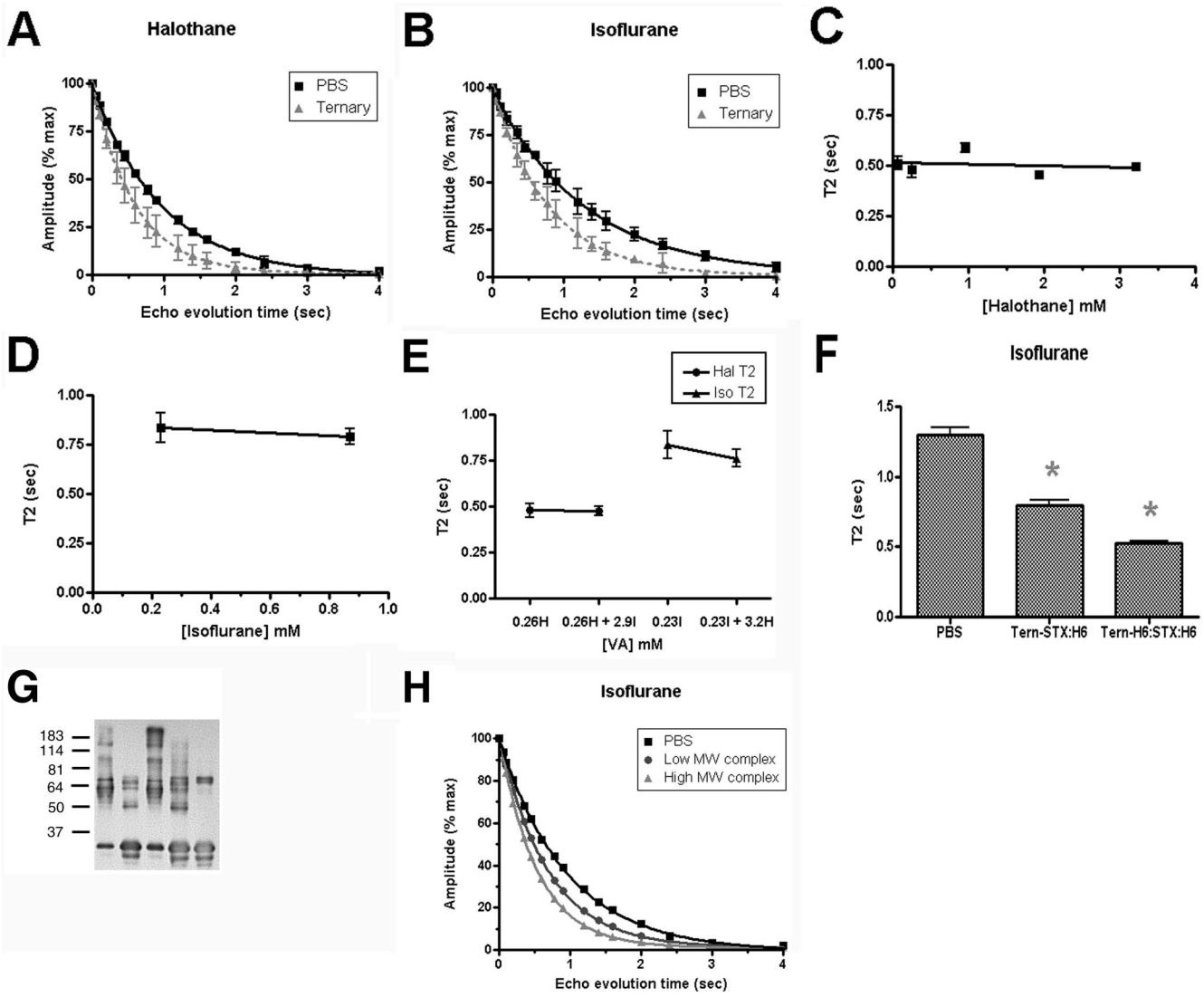

Fig. 5. Volatile anesthetics bind the SNARE complex. (*A*) Halothane binds to the SNARE complex. Spin–spin relaxation curves for halothane (0.96 mM) in the presence of 20 μM SNARE complex or phosphate-buffered saline (PBS) buffer. Values are mean ± SD. T2s (number of independent measurements): ternary complex, 0.588 ± 0.021 s (n = 9); PBS, 0.959 ± 0.014 s (n = 8); $P < 0.001$ by nonlinear regression, $F_{1,250} = 121.6$. (*B*) Isoflurane binds to the SNARE complex. Spin–spin relaxation curves for isoflurane (0.87 mM) in the presence of 20 μM SNARE complex or PBS buffer. T2s (number of independent measurements): ternary complex, 0.793 ± 0.0397 s (n = 3); PBS, 1.297 ± 0.0574 s (n = 5); $P < 0.001$ by nonlinear regression, $F_{1,222} = 55.02$. (*C*) Halothane concentration *versus* T2 in the presence of STX:H6 SNARE complex. The slope of the line is not significantly different from 0. (*D*) Isoflurane concentration *versus* T2 in the presence of STX:H6 SNARE complex. The T2 at 0.23 mM not significantly different from T2 at 0.87 mM. (*E*) Lack of competition between halothane (Hal) and isoflurane (Iso) for binding to ternary complex. (*F*) Comparison of isoflurane T2s in the presence of SNARE complex containing double-His_6-tagged syntaxin *versus* single-tagged syntaxin. * $P < 0.0001$ *versus* PBS T2. (*G*) Western blot with syntaxin antibody of fast protein liquid chromatography buffer purified SNARE complex to isolate low- and high-MW species. Peak fractions eluted from a shallow ionic strength gradient were analyzed by PAGE for SNARE complex size. Fractions containing primarily low-MW or high-MW SNARE complex were pooled, dialyzed against PBS, and separated on PAGE before staining with a syntaxin antibody. Migration of proteins of known molecular weight are shown to the *left*. *Lanes* from *left* to *right*: 1: unboiled low-MW SNARE complex pooled fractions; 2: boiled; 3: unboiled high-MW SNARE complex; 4: boiled; 5: syntaxin monomer used for SNARE complex formation. (*H*) Spin–spin relaxation curves for the low- and high-MW SNARE complexes in *G*. $T2_{Low\ MW} = 0.682 \pm 0.022$; $T2_{High\ MW} = 0.532 \pm 0.021$; [Isoflurane] = 0.87 mM; [Low-MW SNARE complex] = 11.9 μM; [High-MW SNARE complex] = 10.7 μM. $T2_{Low\ MW} > T2_{High\ MW}$, $P < 0.001$ by nonlinear regression, $F_{1,28} = 1{,}010$; $T2_{Low\ MW}$ and $T2_{High\ MW} < T2_{PBS}$, $P < 0.001$, $F_{1,28}$ (Low MW *vs.* PBS) = 445.2, $F_{1,28}$ (High MW *vs.* PBS) = 1,566.

As shown in figure 1, syntaxin, SNAP-25, and VAMP form lower- and higher-MW SNARE complexes, which are thought to represent noncovalent monomeric and multimeric SNARE complexes.[31–33] We asked whether the monomeric and/or multimeric SNARE complex species bound isoflurane. After FPLC purification of primarily monomeric or multimeric SNARE complexes (fig. 5G), isoflurane T2 measurements were performed with the two complexes (fig. 5H). Both the primarily low-MW complex and the higher-MW complex significantly reduced the isoflurane T2 compared with buffer, and the higher-MW complex had a significantly lower T2 than

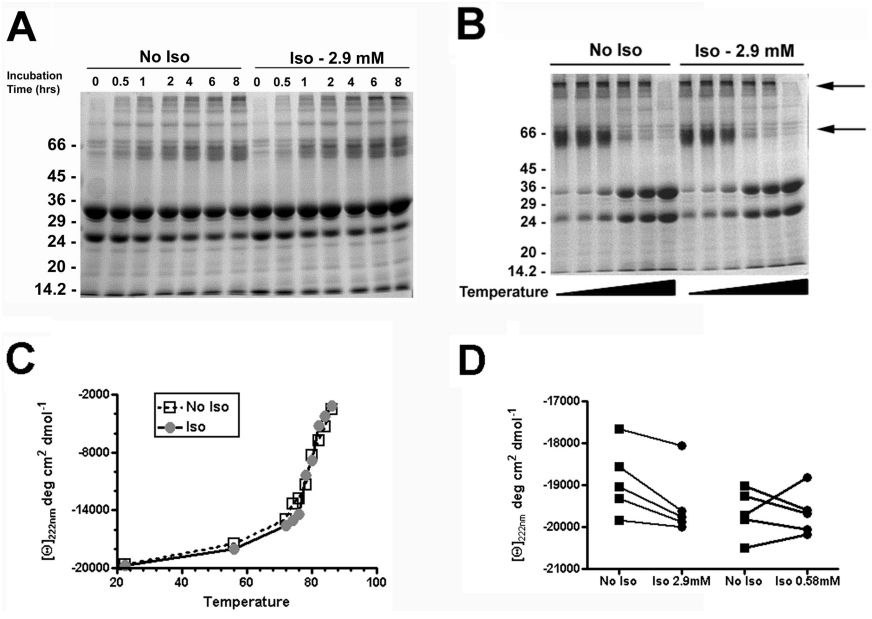

Fig. 6. Volatile anesthetic binding alters the secondary structure but not stability of the SNARE complex. (*A*) SNARE monomers were mixed at time 0 and allowed to form ternary complex at 22°C in the presence or absence of 2.9 mM isoflurane (Iso) for the indicated incubation times before separation by sodium dodecyl sulfate (SDS)-PAGE. (*B*) Thermal denaturation of the complex in the presence and absence of isoflurane. SNARE complex was incubated for 2 h in gastight vials in the presence or absence of 2.9 mM isoflurane then incubated for 25 min at various temperatures in the 0.5% SDS loading buffer; the samples were then separated on a 10% polyacrylamide gel with 0.1% SDS and visualized by Coomassie stain. The incubation temperatures were identical for the two conditions (*left* to *right*: 22°, 30°, 40°, 50°, 60°, 70°C). (*C*) Thermal stability of SNARE complex as measured by helical content in the presence and absence of 2.9 mM isoflurane. SNARE complex was incubated for 15 min at the indicated temperatures in gastight vials in an atmosphere of 5.0 vol% (= 2.9 mM at 22°C) isoflurane or in air. The incubation was followed immediately by transfer to a gastight cuvette and measurement of the circular dichroism spectra. (*D*) Mean residue ellipticity (Θ) at 222 nm of the SNARE complex in the presence and absence of isoflurane. Five independent preparations of fast protein liquid chromatography buffer–purified ternary complex were divided into two aliquots and allowed to sit at room temperature for 2 h in 2.9 or 0.58 mM isoflurane or in air. The circular dichroism spectra were then measured at 20°C. *Lines* connect paired aliquots from the same preparation incubated in the absence or presence of isoflurane. The absolute value of Θ was increased relative to air by 2.9 mM but not 0.58 mM isoflurane ($P < 0.01$, paired *t* test).

the lower-MW complex, as is expected because of the presumed slower diffusion of the heavier species. Therefore, we conclude that the SNARE complex whether as a monomer or a multimer binds isoflurane.

What are the consequences of VA binding to the ternary complex? One attractive possibility that could explain VA inhibition of transmitter release is that VA binding destabilizes the complex. Given that denaturation of the complex *in vitro* requires boiling, disassembly of the complex by VAs is unlikely at room temperature; rather, instability of the complex might manifest as an apparent slowing of complex formation, a reduction in the melting temperature of the complex, and/or a reduction in helicity. However, the rate and extent of complex formation was not grossly altered by isoflurane (fig. 6A). The thermal stability of the complex was also not detectably reduced by isoflurane (fig. 6B). Similarly, circular dichroism measurements detected no additional reduction in helicity by isoflurane with increasing temperature. Rather, at intermediate temperatures, isoflurane seemed to increase helicity (fig. 6C). CD measurements at more physiologic temperatures confirmed that 2.9 mM but not 0.58 mM isoflurane slightly but significantly increased the helicity of the SNARE complex (fig. 6D).

Discussion

Firefly luciferase was the first protein shown to bind VAs.[36] Although clearly not a clinically relevant VA target, the unequivocal demonstration that VAs can bind to a protein was an important discovery accelerating a paradigm shift in theories of anesthesia away from membrane targets to protein. In the ensuing 20 yr, multiple additional proteins have been shown to bind VAs.[23,37,38] Only a handful of these might reasonably mediate the clinical effects of VAs, and no proteins localized to the synapse are among them.[38] In particular, a good presynaptic VA candidate target has been elusive. With the demonstration of shortening of isoflurane and halothane T2 times, indicative of VA immobilization, by a subset of SNARE proteins and the SNARE complex, this work reports the first direct evidence for presynaptic volatile anesthetic binding proteins.

In considering whether SNARE proteins might be relevant VA binding proteins, a few criteria are fundamental. First, relevant targets should bind VAs at concentrations in the range that produces general anesthesia. The aqueous EC_{50}s for anesthesia in rat are 290 μM for halothane and 350 μM for isoflurane. SNAP-25, syntaxin, and the SNARE complex detectably bound halothane and isoflurane at the lowest concentrations measured, each below 230 μM. Therefore, the SNARE VA-binding proteins bind halothane and isoflurane at relevant concentrations. Second, a relevant VA target should function in the nervous system, specifically in synaptic transmission. Certainly, syntaxin and the SNARE complex meet this criterion. SNARE proteins and the SNARE complex are mediators of evoked neurotransmitter release at most, if not all, chemical synapses. However, the physiologic relevance of SNAP-25 multimers is unclear.

A third criterion for the relevance of a VA target is that the binding should alter the function of the target in a way that might produce anesthesia. The CD spectrum of the SNARE complex was altered by supraclinical concentrations of isoflurane, indicative of a gross secondary structural alteration by VAs. Clinical concentrations might also produce structural changes not detectable by CD, which measures only the overall helical content of the protein. Alternatively, clinical concentrations of VAs may bind to existing pockets in the SNARE complex and produce little structural change, as has been shown for binding to bovine serum albumine.[35] If binding of VAs to the SNARE complex has a physiologic effect *in vivo*, a reasonable mechanism might be that VA binding to the SNARE complex alters calcium responsiveness of SNARE complex–mediated fusion or somehow alters the ability of the complex to catalyze opening of a membrane fusion pore.

A conundrum posed by our results is the reconciliation of synapse selective VA action with a putative VA target present in most, if not all, chemical synaptic terminals. VAs have been shown to inhibit neurotransmitter release from a variety of excitatory synapses across multiple phyla.[5–12,39] However, release from γ-aminobutyric acid–mediated (GABAergic) and peptidergic terminals is relatively resistant to VAs.[40,41] Further, potassium-evoked transmitter release, which is a more direct means of producing SNARE complex–mediated fusion, is relatively resistant to VAs.[42] Therefore, if VA binding to SNARE proteins is indeed relevant to inhibition of transmitter release, the effect of the VA binding or the binding itself must depend on other synapse-specific factors.

Two particularly intriguing synapse-specific proteins are mUNC-13 and SNAP-25. mUNC-13 is a highly conserved syntaxin-binding protein that promotes neurotransmitter release. Different isoforms of mUNC-13 are present at GABAergic and glutamatergic synapses in vertebrates.[43–45] Our genetic data in *C. elegans* show that the N-terminus of syntaxin, which has known interactions with only a few synaptic proteins, one of which is UNC-13, can dominantly antagonize VA action on transmitter release.[12] The findings reported herein coupled with the genetic results suggest a model where binding and/or the effect of binding to SNARE proteins/complex depend on mUNC-13. This model predicts that in *C. elegans*, the truncated syntaxin acts by binding to UNC-13 and by an unknown mechanism antagonizes VA binding to t-SNAREs and/or the SNARE complex. Another difference between GABAergic and glutamatergic transmission, at least in rat hippocampal neurons, seems to be the presence of SNAP-25.[46] Verderio *et al.* [46] were unable to detect either known SNAP-25 isoform in rat GABAergic hippocampal neurons. Further, they showed that the normal differential calcium responses of glutamatergic and GABAergic neurons were dependent on the presence or absence of SNAP-25. These surprising results remain to be confirmed in other species and brain regions; nevertheless, these data offer the possibility that neuronal subtype specific expression of SNAP-25 might explain how VAs can selectively inhibit neurotransmitter release depending on the levels or role of SNAP-25 in those neurons.

A final criterion for the relevance of a VA binding protein is that ablation of the target, either pharmacologically or genetically, results in alterations in sensitivity to VAs. No vertebrate organisms have been reported to be highly resistant to clinical concentrations of VAs. To our knowledge, the only organism reported to be highly VA resistant is the *C. elegans* mutant *unc-64(md130)*. As described in the introduction, this syntaxin mutation produces a truncated syntaxin that dominantly antagonizes VA action. These genetic data were in fact the motivation for examining binding of VAs to SNARE proteins. However, it is not clear how a truncated syntaxin could antagonize VA binding or the effect of binding if the VA target is syntaxin, SNAP-25, or the SNARE complex. At least in the case of syntaxin, binding is clearly sensitive to relatively small structural changes as shown by loss of binding with the doubly His_6-tagged protein. If SNARE proteins are relevant VA targets, we can explain our genetic results in *C. elegans* with a model where truncated syntaxin alters posttranslational modification of or interaction with an accessory protein in a way that reduces the affinity of the SNARE protein/complex for VAs. A direct test of the requirement for SNARE proteins in VA action by mutation is complicated by their essential role in transmitter release and normal behavior. Future work will be aimed at identifying SNARE proteins that normally mediate neurotransmitter release but do not bind VAs to genetically test the relevance of SNARE proteins as VA targets.

Besides the relevance of the protein itself, the relevance of the structural motifs, to which VAs are binding in the SNARE proteins, warrants consideration. Of course, at this time, the VA binding sites on the SNARE proteins have not been defined. However, for syntaxin and the SNARE complex, the interior of the 4-α-helical bundles that they form is a good candidate. Synthetic 4-α-helical bundles have been shown to bind VAs with affinities in the clinical range. Based on quenching of the fluorescence of an interiorly located tryptophan, the site of binding was predicted to be within the interior of the bundle.[18–20] X-ray crystallographic and spectroscopic data indicate that the interior of the 4-α-helical bundle of syntaxin and the SNARE complex is a hydrophobic environment that might bind hydrophobic VAs. The ability of an N-terminal His_6 tag to block binding of VAs to syntaxin argues that the presence of a 4-α-helical bundle is not itself sufficient for VA binding; rather, relatively subtle changes near but not within the α-helical bundles are capable of blocking binding. This argues that the VA binding site is near the N-terminus or that VAs gain

access to their binding site from the N-terminus. The increase in helicity by higher concentrations of isoflurane can be explained by binding within the helical bundle and further stabilizing the hydrophobic interactions that form the bundle. Future studies will be aimed at defining the binding sites on the SNARE proteins and using genetic approaches in *C. elegans* to determine their relevance to anesthetic behavioral effects.

The authors thank Alex Evers, M.D. (Henry E. Mallinckrodt Professor of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri), and Jason Berilgen (Technician, Washington University, St. Louis, Missouri) for encouragement and initiation of this project. The authors also thank Phyllis Hanson, Ph.D. (Associate Professor of Cell Biology, Washington University School of Medicine), and Mike Nonet, Ph.D. (Associate Professor of Anatomy and Neurobiology, Washington University School of Medicine), for expression constructs and for their advice and assistance throughout the project with protein expression and purification.

References

1. Pocock G, Richards CD: Excitatory and inhibitory synaptic mechanisms in anaesthesia. B J Anaesth 1993; 71:134–47

2. Jones MV, Brooks PA, Harrison NL: Enhancement of gamma-aminobutyric acid-activated Cl- currents in cultured rat hippocampal neurones by three volatile anaesthetics. J Physiol 1992; 449:279–93

3. Lin LH, Chen LL, Zirrolli JA, Harris RA: General anesthetics potentiate gamma-aminobutyric acid actions on gamma-aminobutyric $acid_A$ receptors expressed by Xenopus oocytes: Lack of involvement of intracellular calcium. J Pharmacol Exp Ther 1992; 263:569–78

4. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994; 367:607–14

5. Zorychta E, Capek R: Depression of spinal monosynaptic transmission by diethyl ether: Quantal analysis of unitary synaptic potentials. J Pharmacol Exp Ther 1978; 207:825–36

6. Takenoshita M, Takahashi T: Mechanisms of halothane action on synaptic transmission in motoneurons of the newborn rat spinal cord in vitro. Brain Res 1987; 402:303–10

7. Kullmann DM, Martin RL, Redman SJ: Reduction by general anaesthetics of group Ia excitatory postsynaptic potentials and currents in the cat spinal cord. J Physiol 1989; 412:277–96

8. Schlame M, Hemmings Jr HC: Inhibition by volatile anesthetics of endogenous glutamate release from synaptosomes by a presynaptic mechanism. ANESTHESIOLOGY 1995; 82:1406– 16

9. Perouansky M, Baranov D, Salman M, Yaari Y: Effects of halothane on glutamate receptor–mediated excitatory postsynaptic currents. ANESTHESIOLOGY 1995; 83:109–19

10. Miao N, Frazer MJ, Lynch IIIC: Volatile anesthetics depress Ca^{2+} transients and glutamate release in isolated cerebral synaptosomes. ANESTHESIOLOGY 1995; 83:593– 603

11. MacIver MB, Mikulec AA, Amagasu SM, Monroe FA: Volatile anesthetics depress glutamate transmission *via* presynaptic actions. ANESTHESIOLOGY 1996; 85:823–34

12. van Swinderen B, Saifee O, Shebester L, Roberson R, Nonet ML, Crowder CM: A neomorphic syntaxin mutation blocks volatile-anesthetic action in Caenorhabditis elegans. Proc Natl Acad Sci U S A 1999; 96:2479–84

13. Jahn R, Grubmuller H: Membrane fusion. Curr Opin Cell Biol 2002; 14:488–95

14. Rizo J, Sudhof TC: Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci 2002; 3:641–53

15. Sutton RB, Fasshauer D, Jahn R, Brunger AT: Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 1998; 395:347–53

16. Poirier MA, Xiao W, Macosko JC, Chan C, Shin YK, Bennett MK: The synaptic SNARE complex is a parallel four-stranded helical bundle. Nature Structural Biol 1998; 5:765–9

17. Lerman JC, Robblee J, Fairman R, Hughson FM: Structural analysis of the neuronal SNARE protein syntaxin-1A. Biochemistry 2000; 39:8470–9

18. Johansson J, Gibney B, Rabanal F, Reddy K, Dutton P: A designed cavity in the hydrophobic core of a four-α-helix bundle improves volatile anesthetic binding affinity. Biochemistry 1998; 37:1421–9

19. Johansson JS, Scharf D, Davies LA, Reddy KS, Eckenhoff RG: A designed four-alpha-helix bundle that binds the volatile general anesthetic halothane with high affinity. Biophys J 2000; 78:982–93

20. Manderson GA, Johansson JS: Role of aromatic side chains in the binding of volatile general anesthetics to a four-alpha-helix bundle. Biochemistry 2002; 41:4080–7

21. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL: Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature 1997; 389:385–9

22. Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, Rampil IJ, Trudell J,Vissel B, Eger IIEI: Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. Anesth Analg 2003; 97:718– 40

23. Dubois BW, Evers AS: 19F-NMR spin-spin relaxation (T2) method for characterizing volatile anesthetic binding to proteins: Analysis of isoflurane binding to serum albumin. Biochemistry 1992; 31:7069–76

24. Dubois BW, Cherian SF, Evers AS: Volatile anesthetics compete for common binding sites on bovine serum albumin: A 19F-NMR study. Proc Natl Acad Sci U S A 1993; 90:6478–82

25. Eckenhoff RG, Johansson JS: Molecular interactions between inhaled anesthetics and proteins. Pharmacol Rev 1997; 49:343–68

26. Miller KW: The nature of sites of general anaesthetic action. Br J Anaesth 2002; 89:17–31

27. Xu Y, Seto T, Tang P, Firestone L: NMR study of volatile anesthetic binding to nicotinic acetylcholine receptors. Biophys J 2000; 78:746–51

28. Fasshauer D, Otto H, Eliason WK, Jahn R, Brunger AT: Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. J Biol Chem 1997; 272:28036–41

29. Stern RC, Towler SC, White PF, Evers AS: Elimination kinetics of sevoflurane and halothane from blood, brain, and adipose tissue in the rat. Anesth Analg 1990; 71:658–64

30. Meiboom S, Gill D: Modified spin-echo method for measuring nuclear relaxation times. Rev Sci Instrum 1958; 29:688–91

31. Hao JC, Salem N, Peng XR, Kelly RB, Bennett MK: Effect of mutations in vesicle-associated membrane protein (VAMP) on the assembly of multimeric protein complexes. J Neurosci 1997; 17:1596–603

32. Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Sudhof TC, Niemann H: Synaptic vesicle membrane fusion complex: Action of clostridial neurotoxins on assembly. EMBO J 1994; 13:5051–61

33. Poirier MA, Hao JC, Malkus PN, Chan C, Moore MF, King DS, Bennett MK: Protease resistance of syntaxin.SNAP-25.VAMP complexes: Implications for assembly and structure. J Biol Chem 1998; 273:11370–7

34. Franks NP, Lieb WR: Selective actions of volatile general anaesthetics at molecular and cellular levels [published erratum appears in Br J Anaesth 1993; 71:616]. Br J Anaesth 1993; 71:65–76

35. Bhattacharya AA, Curry S, Franks NP: Binding of the general anesthetics propofol and halothane to human serum albumin: High resolution crystal structures. J Biol Chem 2000; 275:38731–8

36. Franks NP, Lieb WR: Do general anaesthetics act by competitive binding to specific receptors? Nature 1984; 310:599–601

37. Eckenhoff RG: Promiscuous ligands and attractive cavities: How do the inhaled anesthetics work? Mol Interv 2001; 1:258–68

38. Eckenhoff MF, Chan K, Eckenhoff RG: Multiple specific binding targets for inhaled anesthetics in the mammalian brain. J Pharmacol Exp Ther 2002; 300: 172–9

39. Nishikawa K, Kidokoro Y: Halothane presynaptically depresses synaptic transmission in wild-type *Drosophila* larvae but not in halothane-resistant (har) mutants. ANESTHESIOLOGY 1999; 90:1691–7

40. Pashkov VN, Westphalen RI, Hemmings Jr HC: General anesthetics do not affect release of the neuropeptide cholecystokinin from isolated rat cortical nerve terminals. ANESTHESIOLOGY 2002; 97:1500–6

41. Westphalen RI, Hemmings Jr HC: Selective depression by general anesthetics of glutamate versus GABA release from isolated cortical nerve terminals. J Pharmacol Exp Ther 2003; 304:1188–96

42. Lingamaneni R, Birch ML, Hemmings Jr HC: Widespread inhibition of sodium channel–dependent glutamate release from isolated nerve terminals by isoflurane and propofol. ANESTHESIOLOGY 2001; 95:1460–6

43. Augustin I, Rosenmund C, Sudhof TC, Brose N: Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 1999; 400:457–61

44. Junge HJ, Rhee JS, Jahn O, Varoqueaux F, Spiess J, Waxham MN, Rosenmund C, Brose N: Calmodulin and Munc13 form a Ca2+ sensor/effector complex that controls short-term synaptic plasticity. Cell 2004; 118:389–401

45. Rosenmund C, Sigler A, Augustin I, Reim K, Brose N, Rhee JS: Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. Neuron 2002; 33:411–24

46. Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M: SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. Neuron 2004; 41:599–610