Dynamic binding mode of a Synaptotagmin-1–SNARE complex in solution

Kyle D Brewer1–3, Taulant Bacaj1,5, Andrea Cavalli6,7, Carlo Camilloni7, James D Swarbrick8, Jin Liu1–3,9, Amy Zhou1–3, Peng Zhou1,5, Nicholas Barlow8, Junjie Xu1–3, Alpay B Seven1–3, Eric A Prinslow1–3, Rashmi Voleti1–3, Daniel Häussinger10, Alexandre M J Bonvin11, Diana R Tomchick1,2, Michele Vendruscolo7, Bim Graham8, Thomas C Südhof4,5 & Josep Rizo1–3

Neurotransmitter release is governed by a sophisticated protein machinery1,2. Central components of this machinery are the SNAREs synaptobrevin, syntaxin-1 and SNAP-25, which form a tight four-helix bundle3,4 that brings the synaptic vesicle and plasma membranes together and is key for membrane fusion5 (schematic in Supplementary Fig. 1a). Ca2+ triggering of fast neurotransmitter release is executed by Syt1 (ref. 6) via its two C2 domains. The C2A and C2B domains bind multiple Ca2+ ions through loops at the top of β-sandwich structures6–9, and Ca2+-dependent membrane binding through these loops is key for Syt1 function6. Ca2+ binding to the C2B domain appears to have a predominant role in release10, which may arise from the ability of C2B to bind simultaneously to two membranes11,12. The function of Syt1 in release also depends on interactions with the SNAREs13 and is tightly coupled to complexes14–16, small soluble proteins with active and inhibitory roles in release17–19. Complexins bind to the SNARE complex through a central α-helix, and they also contain an accessory α-helix20 (Supplementary Fig. 1a) that inhibits release19,21, probably because of repulsion with the membranes22.

These and other advances have led to the reconstitution of synaptic-vesicle fusion with eight central components of the release machinery23, but fundamental questions remain about the mechanism of release. This uncertainty arises in part from the lack of high-resolution structures of Syt1–SNARE complexes. Thus, it is unclear which of the diverse Syt1–SNARE interactions reported24 are physiologically relevant. Syt1 interacts with isolated syntaxin-1 and SNAP-25 (refs. 25–28), although it is unknown whether SNARE-complex binding involves these interactions, and distinct regions of SNAP-25 have been implicated in such binding29,30. Some studies have reported that SNAP-complex binding involves a polybasic region on the side of C2B30–32 (Fig. 1a), but other studies have implicated the bottom of C2B33 or other weak binding sites of Syt1 that contribute to aggregation with the SNARE complex34. It is also puzzling that Syt1 and a complexin-I fragment spanning the central and accessory α-helices (CpxI(26–83)) bind simultaneously to the SNARE complex in solution and yet compete for binding to SNARE complexes on membranes35.

The study described here results from 15 years of attempts to elucidate the structure of Syt1–SNARE complexes and used sensitive NMR methods36 to measure lanthanide-induced pseudocontact shifts (PCSs)37 induced on Syt1 fragments by lanthanide probes attached to the SNARE complex. Our data delineate a dynamic structure in which binding is mediated by adjacent acidic regions from syntaxin-1 and SNAP-25, and by the basic concave side of the Syt1 C2B-domain β-sandwich, including residues from the polybasic region. The physiological relevance of this dynamic structure is supported by the parallel effects caused by mutations in basic residues of the C2B domain on SNARE-complex binding in vitro and on Syt1 function in neurons. Moreover, the observed Syt1–SNARE complex binding

1Department of Biophysics, University of Texas Southwestern Medical Center, Dallas, Texas, USA. 2Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, USA. 3Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas, USA. 4Department of Molecular and Cellular Physiology, Stanford University Medical School, Stanford, California, USA. 5Howard Hughes Medical Institute, Stanford University Medical School, Stanford, California, USA. 6Institute for Research in Biomedicine, Bellinzona, Switzerland. 7Department of Chemistry, University of Cambridge, Cambridge, Cambridge, UK. 8Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia. 9Department of Chemistry, Southern Methodist University, Dallas, Texas, USA. 10Department of Chemistry, University of Basel, Basel, Switzerland. 11Bijvoet Center for Biomolecular Research, Faculty of Science, Utrecht University, Utrecht, the Netherlands. Correspondence should be addressed to J.R. (jose@arnie.swmed.edu).

Received 19 January; accepted 27 April; published online 1 June 2015; doi:10.1038/nsmb.3035
mode potentially explains why Syt1 competes with CpxI(26–83) for binding to the SNARE complex on membranes but not in solution. Although our results need to be interpreted with caution (as discussed below), they are consistent with a model in which binding of Syt1 to the SNARE complex places the Syt1 C2B domain in an ideal position to release the inhibition caused by the CpxI accessory α-helix and to bridge the two membranes, thus allowing Syt1 to cooperate with the SNAREs in membrane fusion.

RESULTS

The SNARE complex binds to the C2B-domain polybasic region

To overcome the tendency of Syt1–SNARE complexes to aggregate in the presence of Ca2+, we used a Syt1 fragment containing both C2 domains (C2AB) and exhibiting improved behavior35, together with a buffer containing 125 mM thiocyanate, a chaotropic anion that disrupts ionic interactions between flexible side chains (discussed below). Indeed, reverse experiments with 2H-15N–labeled SNARE complexes did not reveal substantial shifts (Supplementary Fig. 1). The limited solubility of the SNARE complex hindered the assignment of its side chain resonances20 and hence the analysis of perturbations in side chain chemical shifts to map the Syt1-binding site.

Figure 1 A polybasic region of the Syt1 C2B domain binds to the SNARE complex. (a) Ribbon diagram of the Syt1 C2B domain showing the side chains that form the polybasic region, other basic residues that were mutated in this study, and Val283, Arg398 and Arg399 at the bottom of the domain. Basic residues are colored in blue and Val283 in green. Ca2+ ions are represented by yellow spheres. N and C represent the N and C termini, respectively. (b) 1H-15N TROSY-HSQC spectra of 2H-15N–C2AB (50 μM) in the absence (black contours) and presence of 10, 20 or 40 μM SNARE complex (green, red and blue contours, respectively). The values obtained for the axial and rhombic components of the tensor, Δχax and Δχ2, (10–32 m²) are 35.1 and 2.9, respectively, for the SC166 tensor (b) and 15.9 and 6.7, respectively, for the SC41 tensor (e). (c,f) Ribbon diagrams of the SNARE complex (yellow, syntaxin; red, synaptobrevin; blue and green, SNAP-25 N-terminal and C-terminal SNARE motifs, respectively) with isosurfaces representing regions with positive (blue) and negative (red) PCSs, contoured at ±0.8 p.p.m. with the SC166 (c) and SC41 (f) tensors. The tensor centers are indicated with black spheres. The same color coding for the SNAREs is used in all figures.
Pseudocontact shifts from the SNARE complex to Syt1 C2B

The existence of sparsely populated states hindered structural analyses of C2AB–SNARE complexes with paramagnetic relaxation effects but is less of an obstacle for studies with lanthanide-induced PCs37 (Supplementary Note 1). After attempts for several years with different lanthanide-chelating tags placed at diverse positions (Supplementary Note 1), we used SNARE complexes labeled with a 1,4,7,10 tetraazacyclododecane-tetraacetic acid (DOTA)-based tag called C2 (no relation to the term C2 domain)30,41 loaded with Dy3+. SNARE complexes labeled with Dy3+-C2 on residues 41 or 166 of SNAP-25 (below referred to as SC41Dy or SC166Dy, respectively) exhibited strong PCs that could be fit to unique anisotropic magnetic susceptibility tensors (Δχ tensors; Fig. 2).

To analyze the Syt1–SNARE-complex binding mode, we used 15N-2H–ILV-13CH3–C2B labeled Syt1 fragments specifically 1H,13C-labeled at isoleucine, leucine and valine methyl groups (15N-2H–ILV-13CH3–labeling) for optimal relaxation properties36. Most of the PCs induced by SC41Dy or SC166Dy on Syt1 C2AB were observed for cross-peaks from C2B, and very similar PCs were observed when, instead of C2AB, we used the isolated C2B domain or the C2B domain with an R398Q R399Q mutation38 that hinders aggregation with the SNARE complex (Fig. 3a,b, Supplementary Fig. 2a,b and Online Methods). Because the latter yielded the best-quality data, and Arg398 and Arg399 might mediate binding modes that promote aggregation, we performed structural analyses with PC data obtained from 1H,15N TROSY-TROSY HSQC and 1H-13C heteronuclear multiple quantum coherence (HMBC) spectra of 15N-2H–ILV-13CH3–C2B R398Q R399Q mutant in the absence of 30 μM SC166Dy (a) or SC41Dy (b) before (red contours) or after (black contours) tag removal. (c,d) Ribbon diagrams of Syt1 C2B showing PCs induced by SC166Dy (c) or SC41Dy (d). Amide hydrogens and methyl carbons are shown as spheres color coded according to the measured PCs (dark blue, >−0.06 p.p.m.; blue, 0.04 to 0.06 p.p.m.; cyan, 0.02 to 0.04 p.p.m.; pale cyan, 0.008 to 0.02 p.p.m.; red, −0.04 to −0.06 p.p.m.; salmon, −0.02 to −0.04 p.p.m.; light pink, −0.008 to −0.02 p.p.m.); Yellow spheres represent Ca2+ ions. (e,f) Models of C2B bound to the SNARE complex built manually to match the C2B PCs with the SC166 (e) and SC41 (f) tensors represented by isosurfaces (as in Fig. 2c,f). (g–j) Correlations between experimental PCs induced on C2B by SC166Dy (g,h) or SC41Dy (i,j) and PCs calculated with the 166- and 41-manual models with the optimized SC166 (g) and SC41 (i) tensors (illustrated in Fig. 2c,f, respectively) or slightly modified tensors (h,j) (Supplementary Note 7). Correlation coefficients (r) and slopes (m) are indicated.

Figure 3 PCs induced by SC166Dy and SC41Dy on the Syt1 C2B domain. (a,b) 1H-13C HMBC spectra of 30 μM 15N-2H–ILV-13CH3–C2B R398Q R399Q mutant in the presence of 30 μM SC166Dy (a) or SC41Dy (b) before (red contours) or after (black contours) tag removal. (c,d) Ribbon diagrams of Syt1 C2B showing PCs induced by SC166Dy (c) or SC41Dy (d). Amide hydrogens and methyl carbons are shown as spheres color coded according to the measured PCs (dark blue, >−0.06 p.p.m.; blue, 0.04 to 0.06 p.p.m.; cyan, 0.02 to 0.04 p.p.m.; pale cyan, 0.008 to 0.02 p.p.m.; red, −0.04 to −0.06 p.p.m.; salmon, −0.02 to −0.04 p.p.m.; light pink, −0.008 to −0.02 p.p.m.); Yellow spheres represent Ca2+ ions. (e,f) Models of C2B bound to the SNARE complex built manually to match the C2B PCs with the SC166 (e) and SC41 (f) tensors represented by isosurfaces (as in Fig. 2c,f). (g–j) Correlations between experimental PCs induced on C2B by SC166Dy (g,h) or SC41Dy (i,j) and PCs calculated with the 166- and 41-manual models with the optimized SC166 (g) and SC41 (i) tensors (illustrated in Fig. 2c,f, respectively) or slightly modified tensors (h,j) (Supplementary Note 7). Correlation coefficients (r) and slopes (m) are indicated.

Slight alterations of PCs within SC41Dy (5–20%; Supplementary Fig. 2f), thus showing that C2B does not contact residue 166 but is close to residue 41. Nevertheless, we analyzed the SC41Dy data to examine the consistency with the SC166Dy data. We also note that addition of CpxI caused either no changes or very small changes in the PCs induced by SC166Dy (Supplementary Fig. 2c), thus indicating that the binding sites for Syt1 and CpxI on the SNARE complex in solution are distinct but proximal.

Dynamic nature of the Syt1–SNARE complex in solution

We could not apply standard methods used for stable protein complexes37 to derive a structure of the C2B–SNARE complex from the PCs induced on C2B by SC166Dy (Online Methods and Supplementary Fig. 3) or SC41Dy. Because these PCs exhibit defined patterns of positive-negative values when mapped onto the structure of C2B (Fig. 3c,d), we manually matched these patterns with the positive-negative lobes of the Δχ tensors obtained from the PCs measured within SC166Dy and SC41Dy (referred to as C2AB and SC41Dy, respectively; Fig. 2a) while keeping contact between C2B and the SNAREs. The C2B PCs calculated with the resulting models (referred to as the 166- and 41-manual models, respectively; Fig. 3e,f) and the SC166 or SC41 tensors have reasonable correlations with the measured PCs, albeit with large slopes (Fig. 3g,i).
The abundance of basic residues from C2B and acidic residues from the SNAREs cooperate in triggering release. We anticipate that the development of restrained MD simulations incorporating replica-averaged PCS restraints, as described for residual dipolar couplings,, will allow a more extensive exploration of the ensemble of C2B–SNARE-complex orientations to fit the PCS data better. Importantly, despite the relative uncertainty remaining about this ensemble, our results yield a clear picture of the nature of the Syt1–SNARE-complex interactions that predominate under our conditions, including models that can be used to probe the functional importance of these interactions and to elucidate how Syt1 and the SNAREs cooperate in triggering release.

The PCS data establish that the polybasic region of the C2B domain binds to a polyacidic region formed by residues from syntaxin-1 and SNAP-25 (examples in Fig. 5a–d). a result consistent with the high sensitivity of Syt1–SNARE-complex interactions to ionic strength. The abundance of basic residues from C2B and acidic residues from the SNAREs in the binding interface provide a natural explanation for the dynamic nature of Syt1–SNARE-complex interactions. Thus, any single structure would represent one of many binding modes existing in solution. Even with the caveat just mentioned, common features observed in the manual and MD models lead to defined predictions about which residues are most important for binding. A central feature of C2B PCSs for representative MD structures and optimization of population weights (Online Methods) yielded some degree of correlation between the population-averaged and measured PCSs, with slopes much closer to 1 than those obtained with individual models (Fig. 4e,f). The correlations are rather modest, indicating that additional structures are needed to fully account for the observed PCSs, but these results illustrate how conformational averaging decreases the calculated PCSs to values closer to those observed experimentally.

Overall, our analysis shows that the Syt1 C2B–SNARE-complex binding mode is highly dynamic under our conditions. The 166-MD (or 166-manual) model and the 41-manual model can be considered to be representative structures located approximately at the center of the ensemble of binding modes. Differences between the two models could arise from slight perturbation of the binding ensemble caused by the tag in SC41Dy (described above), but, given the uncertainty of this analysis, the two models are quite similar (Fig. 4c) and verify the consistency of the two PCS data sets.

The Syt1–SNARE-complex binding mode in solution

To explore the range of structures that form the ensemble of C2B–SNARE-complex binding modes, we performed extensive MD simulations with chemical-shift restraints. Importantly, the 166-MD model and 41-manual model are located in the middle of the ensemble of structures visited during the simulations (Fig. 4d), thus showing a striking consistency with the PCS data. Calculation of C2B PCSs for representative MD structures and optimization of population weights (Online Methods) yielded some degree of correlation with the consistency of the two PCS data sets.
is that the concave surface of the C2B β-sandwich is oriented toward the SNARE complex (Fig. 5a,b). Thus, although the polybasic β-strand of C2B (residues 321–327) was previously viewed as a functional unit, the observed orientation predicts key distinctions between the functional importance of basic side chains in this region. R322, K325 and K327 from this β-strand and K313 from the adjacent β-strand are oriented toward the SNARE complex and hence are likely to have critical roles in binding; in contrast, K324 and K326 are not directly oriented toward the SNARE complex and are predicted to have less prominent roles.

There is more variability in the residues of the SNARE complex that bind to C2B in the different models. In the 166-MD model, the C2B concave side exhibits multiple ionic interactions with an acidic region of syntaxin-1 (E224, E228, D231 and E234) but is also near an acidic region of SNAP-25 (D51, E52 and E55) (Fig. 5c). In the 41-manual model, C2B interacts primarily with this SNAP-25 acidic region (Fig. 5b). A detailed analysis is not useful in this case because we built the model manually, but multiple structures from the chemical shift–restrained MD simulations placed C2B in locations close to that observed in the 41-manual model (example in Supplementary Fig. 5; referred to as the 41-MD model) and illustrate how K313, R322, K325 and K327 of C2B can establish multiple salt bridges with the SNAP-25 acidic region (Fig. 5d).

This analysis shows that the large number of charges in the C2B polybasic concave side and the polyacidic region formed by syntaxin-1 and SNAP-25 allow formation of multiple salt bridges in many different orientations, explaining the dynamic nature of the binding mode. Importantly, our results also provide a clear explanation for the finding that Syt1 C2AB and CpxI(26–83) bind simultaneously to soluble SNARE complex but compete for binding to membrane-anchored SNARE complex35. Thus, the binding sites of the SNARE complex for CpxI(26–83) and C2B are distinct, allowing simultaneous binding in solution; however, Ca2+-induced binding of C2B to a membrane would cause strong steric and electrostatic repulsion between the membrane and the CpxI accessory helix, both of which are negatively charged (Fig. 5e).

**Mutagenesis analysis of Syt1–SNARE-complex binding**

To test the Syt1–SNARE-complex binding mode derived from our PCS data, we used an assay46 that monitors the decrease in intensity of the strongest methyl resonance (SMR) in 1D 13C-edited 1H NMR spectra of 13C-labeled C2AB upon binding to unlabeled SNARE complex (Fig. 6a).

In initial experiments performed in the absence of KSCN, to extend a recent study34, we found that single-residue substitutions in the C2B polybasic region did not alter binding strongly (Supplementary Fig. 6a,b). Given the large number of charges at the binding surface of C2B, we prepared 13C-labeled C2AB mutants in which two basic residues were replaced with glutamates and then analyzed binding in 125 mM KSCN to minimize interactions that contribute to aggregation, as we used for the PCS measurements. Importantly, three double mutations that replaced basic residues in the concave side of C2B (K313E K325E, R322E K325E and K325E K327E) strongly impaired binding to the SNARE complex (Fig. 6b), whereas binding was not
affected by double mutations in basic residues (K297E R388E and K354E R388E; Fig. 1a). Moreover, double mutation of the two residues located in the polybasic region but not in the concave side of C2B (K324E K326E) impaired binding but to a much lesser extent than the mutations in the concave side (Fig. 6b). The apparent Kd values derived from these data (Supplementary Fig. 6c) give a semiquantitative idea of the effects of the mutations and clearly confirm the conclusion that the concave side of C2B is primarily responsible for binding to the SNARE complex under these conditions. Furthermore, we performed titrations of wild-type (WT) 13C-C2AB with SNARE complexes containing double mutations (SNAP-25 E52K E55K and syntaxin-1 E228K D231K) in residues of the polyacidic regions of syntaxin-1 and SNAP-25 that form the primary binding sites for C2B, according to the PCS data. These mutations markedly impaired binding, whereas a control mutation in another acidic region (SNAP-25 E24K E27K) had no effect (Fig. 6c and Supplementary Fig. 6d). These results provide strong support for the binding mode derived from the PCS data.

Because Ca2+-dependent phospholipid binding is crucial for Syt1 function5, we analyzed the effects of selected double mutations in C2B on this activity with a fluorescence resonance energy transfer (FRET) assay (Fig. 6d). The relative effects of the mutations on the apparent Kd values observed in these titrations were distinctly different from those caused upon SNARE-complex binding: R322E K325E had the most marked effect on Ca2+-dependent phospholipid binding; K313E K325E and K297E R388E had similar moderate effects; and the control K354E R388E mutation had the smallest effect (Fig. 6e). We also analyzed the effects of these mutations on Ca2+-independent binding of C2B to phosphatidylinositol 4,5-bisphosphate (PIP2), which has been proposed to direct Syt1 to the plasma membrane42. Because binding of C2B to PIP2-containing liposomes is weak and not easily detectable (for example, by FRET assays48), we again used 1D 13C-edited 1H NMR spectra of 13C-C2AB, which are expected to exhibit marked decreases in SMR intensity even for small percentages of binding. Liposomes containing 1% PIP2 (1 mM total lipid) caused a 40% decrease in the SMR intensities of 15C-labeled wild-type C2AB (Fig. 6f), thus confirming that a fraction of C2AB binds to the liposomes, but this binding is weak. The double mutations in the polybasic region, but not the control K354E R388E mutation, abolished binding regardless of whether the side chains were in the concave side of C2B (Fig. 6f). These data confirm that the C2B polybasic region mediates Ca2+-independent PIP2 binding to Syt1 (ref. 47) and suggest that mutations in this region disrupt PIP2 binding more indiscriminately than they disrupt SNARE-complex binding.

A question that arises is whether PIP2 could prevent binding of Syt1 to the SNARE complex before Ca2+ influx. To address this question, we first examined the effects of the C2B double mutations on Ca2+-independent binding of C2B to the SNARE complex. We used 1D 13C-edited 1H NMR spectra and KSCN-free buffer because C2B–SNARE complexes aggregate less in the absence of Ca2+, and these conditions facilitate the observation of Ca2+-independent binding, which is weaker than Ca2+-dependent binding44. The relative effects of the three double mutations in the C2B polybasic region (Fig. 6g)
Correlation of Syt1–SNARE-complex binding with Syt1 function

To investigate the functional relevance of the Syt1–SNARE-complex binding mode described above, we performed electrophysiological rescue experiments. As previously described\(^6\), lentiviral expression of WT Syt1 rescued evoked release in Syt1-knockout (KO) neurons (Fig. 7a–c). Three single Syt1 mutants with substitutions in residues from the polybasic region (K313E, R322E and K326E) rescued evoked release almost as efficiently as WT Syt1 (Fig. 7a–c), in correlation with the finding that such mutations do not markedly impair C\(_2\)AB–SNARE-complex binding (Supplementary Fig. 6a,b). Importantly however, two double mutations in the concave side of C\(_2\)B (K313E K325E and R322E K325E) strongly impaired rescue of evoked release in Syt1-KO neurons, whereas we observed much milder effects for the control double mutant (K354E R388E) and the mutant with substitutions in residues of the polybasic region that are not in the concave side (K313E K324E) (Fig. 7d–f). These differences in rescue activities do not arise from inefficient protein overexpression because all double mutants were overexpressed at similar levels (Supplementary Fig. 7a–d). These results establish a striking correlation between the disruption of Syt1 function in neurons and the impairment of C\(_2\)AB–SNARE-complex binding (Fig. 6b) caused by the double mutations.

We also analyzed spontaneous release in Syt1-KO neurons rescued with the R322E K325E and K324E K326E double mutants.  

paralleled those observed in the presence of Ca\(^{2+}\) (Fig. 6b), thus supporting the notion that the primary Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent C\(_2\)AB–SNARE-complex binding modes are similar under these conditions. To test whether PIP\(_2\) binding to Syt1 precludes binding to the SNARE complex, we used a competition assay monitoring 1D \(^{13}\)C-edited \(^1\)H NMR spectra of SNARE complex containing \(^{15}\)N-\(^2\)H–ILV-\(^{13}\)CH\(_3\)-labeled syntaxin-1. Liposomes containing 1% PIP\(_2\) (3 mM total lipid) had no effect on the SMR intensity of 15 \(\mu\)M SNARE complex (Fig. 6h), thus showing that the SNARE complex does not bind to the liposomes, whereas 15 \(\mu\)M WT C\(_2\)AB caused a marked decrease in the SMR intensity, thus reflecting partial release of C\(_2\)AB from the SNARE complex (Fig. 6h); however, only 38% of the signal was recovered even with a large excess of PIP\(_2\) (6 mM total lipids; Fig. 6i). Hence, PIP\(_2\) hinders but does not abrogate Ca\(^{2+}\)-independent binding of C\(_2\)AB to the SNARE complex.
Figure 8 Disruption of Syt1–SNARE-complex binding correlates with impairment of the Ca\(^{2+}\)-triggered step of release. (a) Sample traces of evoked IPSCs observed in Syt1-KO cultured neurons rescued with wild-type (WT) or double-mutant Syt1 cDNAs as a function of extracellular Ca\(^{2+}\) concentration. The same neuron was recorded at the indicated Ca\(^{2+}\) concentrations. (b,c,f,g) Peak eIPSC amplitude (b,c) or total charge transfer in a single action potential (f,g), as a function of extracellular Ca\(^{2+}\) concentration in the rescue experiments with WT or double-mutant Syt1. Data were fit with Hill equations to determine EC\(_{50}\)s and Hill coefficients. The data are presented in absolute values (b,f) or are normalized to the limiting values at infinite extracellular Ca\(^{2+}\) derived from the fits (c,g). In the top of b, numbers indicate number of neurons/independent cultures analyzed. (d,e,h,i) Summary graphs of the EC\(_{50}\)s and Hill coefficients calculated from the data in b and f. (j) Coimmunoprecipitation with syntaxin-1 (Synt IP), performed in Syt1-KO neurons rescued with WT or R322E K325E mutant Syt1 by incubation with a polyclonal antibody to syntaxin-1 and western blot analysis with monoclonal antibodies to Syt1 or Syb2. rSyt1, rat Syt1; pre, preimmune sera. (k) Quantitative analysis of coimmunoprecipitation of WT and R322E K325E Syt1s with antibody to syntaxin-1. The amount of signal for each condition is quantified as a percentage of the input level in that condition. All data are means ± s.e.m. Statistical significance (d,e,h,i) was assessed by one-way ANOVA (*P < 0.05).

As described earlier\(^{29}\), overexpression of WT Syt1 suppressed the large increase in spontaneous release observed in excitatory and inhibitory Syt1-KO neurons without altering the amplitudes of individual miniature excitatory and inhibitory postsynaptic currents (Fig. 7g–l). Overexpression of all double mutants led to similar suppression of the increased miniature currents, thus suggesting that Syt1–SNARE-complex interactions are not critical for clamping the secondary Ca\(^{2+}\) sensor that mediates the increase in spontaneous release in Syt1-KO neurons.

We furthermore measured the dependence of evoked release on extracellular Ca\(^{2+}\) in Syt1-KO neurons rescued with WT Syt1 or the two double mutants (Fig. 8). Analysis of the Ca\(^{2+}\) dependence of both amplitude and charge transfer of inhibitory postsynaptic currents (IPSCs) further illustrated the strong impairment of evoked release caused by the R322E K325E mutation and showed that the half-maximal effective concentration (EC\(_{50}\)) for extracellular Ca\(^{2+}\) is significantly increased for rescue with this mutant compared to rescue with WT Syt1 (Fig. 8d,h). In contrast, rescue with the K324E K326E mutant did not cause a significant shift in the EC\(_{50}\), as compared to the WT rescue, but did result in a slight decrease in release at higher extracellular Ca\(^{2+}\) concentrations. These results suggest that Syt1–SNARE-complex interactions may play a key part in Ca\(^{2+}\) triggering of release. To examine whether the R322E K325E mutation impairs Syt1-SNARE interactions in neurons, we performed coimmunoprecipitations with an antibody to syntaxin-1. The R322E K325E mutation caused a 62% decrease in the amount of Syt1 that coimmunoprecipitated with syntaxin-1 without significantly affecting the coimmunoprecipitation of synaptobrevin used as a control (Fig. 8j,k). This result further supports the notion that the partial impairment of neurotransmitter release caused by the R322E K325E mutation arises because of partial disruption of Syt1-SNARE interactions.

DISCUSSION

Syt1–SNARE-complex interactions are probably key for coupling Ca\(^{2+}\) sensing to membrane fusion during neurotransmitter release. This work illustrates the difficulties involved in studying such interactions and shows how a PCS-based approach allowed us to define a dynamic structure that represents the preferred Syt1–SNARE-complex binding mode under the specific conditions of our experiments. Our results need to be interpreted with caution because of the potential existence of other binding modes that may be occluded under our conditions. However, the relevance of our PCS-derived structure is supported by biochemical and functional data, and its dynamic nature may be an intrinsic feature that enables the fast speed of neurotransmitter release, concordantly with the increasing realization that dynamics can be key for biological function\(^{51}\). The dynamic Syt1–SNARE-complex structure suggests a possible mechanism for how Syt1 relieves the inhibition caused by CpxI and supports a speculative model whereby Syt1 brings the synaptic vesicle and plasma membrane together upon Ca\(^{2+}\) influx, cooperating with the SNAREs in membrane fusion (Supplementary Fig. 8).

Our analysis shows that no single structure of the C\(_{2}\)B–SNARE complex can fit all the PCS data, which hinders application of standard computational tools to interpret these data. The manual procedure used to match the C\(_{2}\)B PCS patterns with the lobes of the tensors determined with the SNARE-complex PCSs does yield an approximate but unambiguous definition of the preferred location and orientation of C\(_{2}\)B with respect to the SNARE complex under our conditions, thus leading to a clear picture of the binding mode that readily explains its dynamic nature. Although the data used for our structural analysis were acquired in 125 mM KSCN with R398Q R399Q mutant C\(_{2}\)B, the measured PCSs parallel those observed with WT C\(_{2}\)B and C\(_{2}\)AB (Supplementary Fig. 2a,b,d,e), and the validity of the derived binding mode is supported by in vitro binding assays (Fig. 6b,c), physiological data (Figs. 7 and 8) and coimmunoprecipitation...
The present dynamic structure of the Syt1–SNARE complex is consistent with an attractive model for how Syt1 relieves the inhibition of neurotransmitter release caused by CpxI (refs. 14–16) and cooperates with the SNAREs in membrane fusion (Supplementary Fig. 8). The model postulates that, before Ca$^{2+}$ influx, CpxI binds to partially assembled SNARE complexes (Supplementary Fig. 8a) via similar interactions to those defined here; this is supported by our Ca$^{2+}$-independent CpxI–SNARE-complex binding assays (Fig. 6g) and by studies suggesting that Ca$^{2+}$-independent binding of Syt1 to syntaxin-1–SNAP-25 heterodimers involves the same surfaces$^{32,53}$. Notably, although the interaction is weaker in the absence of Ca$^{2+}$ (Supplementary Note 2), it should be strongly enhanced by colocalization and could cooperate with binding of Arg398 and Arg399 to the plasma membrane. In this putative primed state, CpxI could be simultaneously bound to the SNARE complex because the CpxI- and Syt1-binding sites are distinct, and the negatively charged accessory helix of CpxI would inhibit release because of repulsion with the vesicle membrane$^{22}$ (Fig. 5e and Supplementary Fig. 8a). According to our structure, in this primed state, the CpxI domain would be ideally positioned to quickly bind simultaneously to the vesicle membrane via its Ca$^{2+}$-binding loops and to the plasma membrane through the bottom side upon Ca$^{2+}$ influx (Supplementary Fig. 8b). Our model proposes that this action forces melting of the CpxI accessory helix (Supplementary Fig. 8b and Supplementary Note 3) and occurs concomitantly with full SNARE-complex zipper formation, leading to membrane fusion and neurotransmitter release. This model is based in part on the observation that simultaneous binding of Syt1 to two membranes brings them within 4 nm of each other (refs. 11, 55), and it accounts for the critical role of Arg398 and Arg399 in neurotransmitter release$^{12}$. The proposed action of Syt1 would probably require some reorientation of CpxI with respect to the SNARE complex for optimal efficiency. The dynamic nature of the CpxI–SNARE-complex binding mode may be a key feature facilitating such rearrangement.

Although the model in Supplementary Figure 8 potentially explains a large amount of experimental evidence, further research will be required to test its relevance and to address several unresolved issues. First, the change in Ca$^{2+}$-dependent phospholipid binding to the R322E K325E mutant (Fig. 6d,e) correlates with the shift in the Ca$^{2+}$ dependence of release caused by this mutant (Fig. 8). This shift can also be explained by the disruption of SNARE-complex binding caused by the R322E K325E mutation, given the synergy between Ca$^{2+}$ and SNARE-complex binding to Syt1 (Supplementary Note 2), and the correlation between disruption of Ca$^{2+}$-dependent phospholipid binding and impairment of Syt1 function is only partial (Figs. 6d,e and 7). These observations argue against the notion that the functional effects caused by the double mutants arise from alteration of phospholipid binding, but the finding that the mutations can affect binding both to the SNARE complex and to phospholipids underlines the limitations of studying these interactions separately because they are likely to influence each other (Supplementary Note 4).

Second, there are additional binding modes between Syt1 and the SNAREs$^{29,31–34}$ that, although less populated under our specific conditions than the mode defined here, could be critical for function (Supplementary Note 5). Third, although the effects of the CpxI double mutations on SNARE-complex binding, as compared to their effects on PIP$_2$ binding, correlate better with disruption of Syt1 function (Figs. 6b,f and 7), and our competition assays (Fig. 6i) suggest that the presence of PIP$_2$ in the plasma membrane should not abrogate binding of Syt1 to the SNARE complex, the interplay between interactions of Syt1 with PIP$_2$ and the SNARE complex needs to be further investigated (Supplementary Note 6). Fourth, although our model is consistent with the importance of Ca$^{2+}$-binding to the Syt1 C3B domain$^{10}$, normal release also depends on the C2A domain$^{56,57}$. In our C2B–SNARE-complex structure, C2A emerges at the N terminus of C2B, on the opposite side of the SNARE-binding region (Fig. 5e). This positioning would allow C2A to bind to the vesicle and/or plasma membranes and to cooperate with the action of C2B in triggering release. However, there is some evidence that C2A–SNARE interactions are important for release$^{13,58}$, and there are weak C2A–SNARE-complex interactions that contribute to aggregation of C2AB–SNARE complexes in solution$^{34}$ but could be functionally important.

Challenging structural studies of Syt1–SNARE-complex interactions on membranes, or ideally between two membranes, will probably be required to resolve these and other issues. Despite all these concerns, the dynamic structure of the Syt1–SNARE complex described here provides a framework for rationalization of the available data and will serve as a guide for future research in this field.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates of five energy-minimized structures that illustrate the dynamic Syt1 C2B domain–SNARE-complex binding mode have been deposited in the Protein Data Bank under accession code 2N1T. The ensemble includes the 166-MD model and four of the structures from the chemical shift–restrained MD simulations that contribute to the optimized population-weighted PCS data of Figure 4e,f. The five conformers deposited must be considered to be just a few of the many conformers that form the dynamic ensemble existing under our conditions, but they illustrate the types of interactions between the Syt1 C2B domain and the SNARE complex that mediate this dynamic binding mode.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank L. Kay for fruitful discussions and F. Peters (Max Planck Institute for Biophysical Chemistry) for generously providing a sample for Cys-Pha-TAHA-labeling. The DD2 console of one of the Agilent 600-MHz NMR spectrometers used for the research presented here was purchased with shared instrumentation grant S1BR026461 from the US National Institutes of Health (to M.K. Rosen; University of Texas Southwestern Medical Center). The authors acknowledge the Texas Advanced Computing Center at the University of Texas at Austin for providing high-performance computer resources that have contributed to the research results reported within this paper. This work was supported by Welch Foundation grant I-1304 (to J.R.), Australian Research Council (ARC) Discovery Grant DP150100383 (to B.G.), ARC Future Fellowship FT130100838 (to B.G.), Swiss National Science Foundation grant 200021_130263 (to D.H.) and US National Institutes of Health grants K99NS087086 (to T.B.) and NS040944 (to J.R.).
1. Brunger, A.T., Weninger, K., Bowen, M. & Chu, S. Single-molecule studies of the neuronal SNARE fusion machinery. *Ann. Rev. Biochem.* 78, 903–928 (2009).

2. Rizo, J. & Sudhof, T.C. The membrane fusion enigma: SNARES, Sec1/Munc18 proteins, and their accomplices—guilty as charged? *Annu. Rev. Cell Dev. Biol.* 28, 279–308 (2012).

3. Poirier, M.A. et al. The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Biol.* 5, 765–769 (1998).

4. Sutton, R.B., Fasshauer, D., Jahn, R. & Brunger, A.T. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 395, 347–353 (1998).

5. Hanson, P.I., Roth, R., Morisaki, H., Jahn, R. & Heuser, J.E. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/dry-etch electron microscopy. *Cell* 90, 523–535 (1997).

6. Fernández-Chacón, R., Rodríguez, M., Gómez-Carrion, M., Sudhof, T.C. & Rizo, J. Ca2+-independent SNARE complex is a phospholipid binding machine. *Neuron* 32, 1057–1069 (2001).

7. Xue, M., Wang, I.C., Shao, X., Sudhof, T.C. & Rizo, J. Ca2+-dependent binding of synaptotagmin to SNAP-25 regulates the interaction between two major substates of RNase A using NMR chemical shifts. *J. Am. Chem. Soc.* 133, 3968–3971 (2011).

8. Schmitz, C. & Bonvin, A.M. Protein-protein HADDocking using exclusively nonspecific interactions for label-free detection of low-abundance plasma proteins. *Bioinformatics* 22, 2118–2125 (2006).

9. Chapman, E.R., Han, S. & Jahn, R. Ca2+-dependent SNARE complex is a phospholipid complex in neurotransmitter release. *J. Biol. Chem.* 275, 255–259 (1992).

10. Li, et al. Ca2+-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* 375, 594–599 (1995).

11. Peng, Y., Lane, E.C., Kowalczyk, J.A. & Martin, T.F. The C terminus of SNAP25 is essential for Ca2+-dependent binding of synaptotagmin to SNARE complexes. *J. Biol. Chem.* 275, 6328–6336 (2000).
ONLINE METHODS

Protein expression and purification. The expression and purification of fragments spanning the SNARE motifs of rat synaptobrevin 2 (residues 29–93), rat syntaxin-1A (residues 191–253), and human SNAP-25 (residues 11–82 and 141–203) from a pGEX-KT vector, and rat Syt1 C2B (residues 271–421), C2B R398Q R399Q mutant and C2AB (residues 131–421 and 140–421) from a pGEX-KG vector were previously described1,12,25. Constructs to express single-cysteine SNARE mutants were obtained by site-directed mutagenesis with PCR and custom-designed primers. Unlabeled proteins were expressed in Esherichia coli BL21(DE3) cells in LB medium. For uniformly $^{13}$C-$^{15}$N-labeled proteins, we used M9 minimal expression medium with $^{13}$C-glucose as the sole carbon source (3 g per L of culture) and $^{15}$NH$_4$Cl as the sole nitrogen source (1 g/L). Perdeuterated proteins were produced with M9 expression medium in 99.9% D$_2$O with $^{13}$C-glucose as the sole carbon source (3 g/L) and $^{15}$NH$_4$Cl as the sole nitrogen source (1 g/L). ILVM methyl-labeling was achieved by addition of [3,3-$^{2}$H$_2$]-cysteine (80 mg/L), [3,$^{13}$C]-methionine (250 mg/L) (Cambridge Isotope Laboratories) to the cell cultures 30 min before isopropyl $\beta$-D-thiogalactopyranoside (IPTG) induction. SNARE-complex assembly was achieved as previously described26 by mixture of the SNARE domains in equimolar ratio, except that the assembly reaction was incubated at room temperature while the mixture was rotated.

Paramagnetic labeling. The SNARE complex does not contain native cysteines. To label with paramagnetic tags, we obtained diverse single-cysteine mutants of the SNAREs (described below). For labeling of SNARE cysteine mutants with paramagnetic tags, the protein was treated with 10 mM DTT, which was subsequently removed by gel-filtration chromatography on a Superdex 75 column in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl for MTSL-labeling, the protein was concentrated to 40–60 µM and incubated overnight at 4°C with a ten-fold molar excess of MTSL from a 40 mM stock in dimethyl sulfoxide. The excess MTSL was removed by concentration-dilution in the gel-filtration buffer with a 3-kDa molecular-weight-cutoff filter before being assembled into the SNARE complex. The SNARE complex was then buffer-exchanged into 25 mM Tris-DCI, pH 7.4, 125 mM KSCN, 1 mM CaCl$_2$ and 10% D$_2$O with a 10-kDa molecular-weight-cutoff filter. KSCN salt was used in these and other experiments to limit nonspecific interactions and to prevent precipitation of the Syt1–SNARE complex in the presence of Ca$^{2+}$. The MTSL nitroxide radical was reduced when needed by addition of 1 mM ascorbic acid and 1 mM sodium dithionite from 100 mM stocks adjusted to pH 7.4, prepared immediately before use. For lanthanide-labeling with Dy$^{3+}$-DOTA-M8 or Dy$^{3+}$-C2, the protein after gel filtration was concentrated to 30–100 µM and incubated 10 min at room temperature with a three-fold molar excess of the tag. After tag-incubation, the other SNAREs were added for assembly and incubated at room temperature with overnight rotation. A final concentration of 1 M NaCl was also added before mixture of the SNAREs to prevent precipitation. To minimize the amount of free isotopically labeled SNARE left after SNARE-complex assembly, nonisotopically labeled SNAREs were included in 40% excess. The SNARE complex was buffer-exchanged at 25 °C into 25 mM Tris-HCl, pH 7.4, 500 mM NaCl with a 10-kDa molecular-weight-cutoff filter to remove the excess tag and SNAREs and then exchanged to 25 mM Tris-DCI, pH 7.4, 125 mM KSCN, 1 mM CaCl$_2$, 10% D$_2$O. The samples were reduced when needed by the addition of 0.3 mM TCEP from a 30 mM stock.

NMR spectroscopy. All NMR spectra were acquired at 25°C on Varian INOVA spectrometers operating at 600 or 800 MHz, equipped with cold probes. $^{1}$H-$^{13}$C HMQC and $^{1}$H-$^{15}$N HSQC-TROSY spectra were acquired under the conditions indicated in the figure legends with 10% D$_2$O as the solvent. Total acquisition times were 4–56 h. NMR data were processed with NMRPipe26 and analyzed with NMRView26. All data for PCS measurements were acquired in 25 mM Tris-HCl, pH 7.4, 125 mM KSCN, 1 mM CaCl$_2$ and 10% D$_2$O.

Measurement of PCSs. Analysis of NMR data was based on the assignments that are available for most of the resonances of the Syt1 C2B domains and for the SNARE-complex backbone28,20,61. To measure PCSs within the SNARE complex, we acquired $^{1}$H-$^{15}$N TROSY-HSQC spectra of 60–80 µM samples of SNARE complex $^{1}$H-$^{15}$N-labeled at the syntaxin-1 or SNN SNARE motifs (with the Dy$^{3+}$-C2 tag on residue 166 of SNAP-25) or at the synaptobrevin and syntaxin-1 SNARE motifs (with the Dy$^{3+}$-C2 tag on residue 41 of SNAP-25). PCSs were calculated from the differences in the chemical shifts observed before and after removal of the Dy$^{3+}$–C2 tag by reduction with 1 mM TCEP. Because of the relatively low sensitivity of the spectra at the concentrations used, cross-peaks from NH groups within less than ~18 Å from the lanthanide were broadened beyond detection, and the chemical shifts of all the NH groups for which we could measure PCSs were not affected by introduction of a diamagnetic tag. Hence, the procedure used to measure PCSs is equivalent to using control spectra with SNARE complex tagged with a nonparamagnetic lanthanide. Errors in the measurements were estimated from the reproducibility of the chemical shifts obtained manually from contour plots of repeated spectra. The PCSs measured within the SNARE complex with Dy$^{3+}$–C2 tag on residue 166 or 41 of SNAP-25 were used to derive the corresponding $\Delta$χ tensors (referred to as SC166 and SC41 tensors, respectively; Fig. 2) in Numbat25. To analyze how sensitive the tensor parameters are to small variations in the coordinates of the center, we recalculated the tensor while constraining the center to be at different random locations within 4 Å of the optimal tensor center derived without constraints. Selected tensors among those obtained were used to reanalyze the fits between PCSs measured on C$_2$B and the calculated values (for example, Fig. 3h,i).

Small PCSs on many cross-peaks of the $^{1}$H-$^{13}$C HMQC and $^{1}$H-$^{15}$N TROSY-HSQC spectra of 30 µM C$_2$B were induced by 20 µM SCH41Dy or SC166Dy (example in Supplementary Fig. 2a,d), but higher concentrations to saturate binding more fully, and hence increase the PCSs, led to aggregation. Because the C$_2$A domain contributes to aggregation44, and all the stronger PCSs observed in C$_2$AB correspond to cross-peaks from the C$_2$B domain, we used a Syt1 fragment containing only the C$_2$B domain (residues 270–421) and were able to obtain good-quality data upon addition of 30 µM SC41Dy or SC166Dy (Supplementary Fig. 2b,e). The PCSs observed for C$_2$B were parallel to those observed for C$_2$AB (Supplementary Fig. 2a,b,d,e), thus showing that removal of the C$_2$A domain did not alter the major binding mode. Very similar PCSs were also observed for C$_2$B with the R398Q R399Q mutation (Fig. 3a,b). We used the PCSs measured for this mutant (referred to as C$_2$B for simplicity) for further analysis because it yielded the best-quality data, and the PCSs are less likely to be influenced by weak binding modes that lead to aggregation. From $^{1}$H-$^{15}$N HSQC titrations (such as those shown in Fig. 1b), we estimated that binding was about 66% saturated under the conditions of the experiments. To account for the incomplete binding, all PCSs measured on C$_2$B were multiplied by a factor of 1.5. Because of the small size of the PCSs and because the digital resolution was much higher in the $^1$H dimension, we measured PCSs on only $^1$H nuclei.

Structural analysis with PCSs. We built the 166 and 41 manual models (Fig. 3e,f) in PyMOL (http://www.pymol.org) by manual translation and rotation of the C$_2$B domain with respect to the SNARE complex, trying to optimally match the pattern of negative-negative PCSs with the positive-negative lobes of the SC166 and SC41 tensors, respectively, while also having the C$_2$B domain within van der Waals contact with the SNARE complex. To try to obtain structures of the C$_2$B–SNARE complex compatible with the SC166Dy PCS data computationally, we used Numbat to calculate a $\Delta$χ tensor from the PCSs induced on C$_2$B (referred to as C$_2$B166 tensor; Supplementary Fig. 3a) and observed a good correlation between measured PCSs and those calculated with the tensor (Supplementary Fig. 3c). However, this tensor has a considerably different shape from the tensor derived with the SNARE-complex PCSs (the SC166 tensor; comparison of Supplementary Fig. 3a,b,c), and C$_2$B does not contact the SNARE complex in the model obtained by superimposing the two tensors with PyMOL (Supplementary Fig. 3b). Moreover, the C$_2$B PCSs calculated with this model and the SC166 tensor have only a modest correlation with the measured PCSs (Supplementary Fig. 3d). Because of the axial symmetry of the SC166 tensor, we used PyMOL to manually rotate the C$_2$B domain with respect to the SNARE complex, trying to optimally match the pattern of negative-negative PCSs with the positive-negative lobes of the SC166 and SC41 tensors, respectively, while also having the C$_2$B domain within van der Waals contact with the SNARE complex. The resulting model yields a similar correlation between calculated and measured C$_2$B PCSs (Supplementary Fig. 3e). However, in this model C$_2$B would clash with CpxI if Syt1 and CpxI were bound around the vertical axis (in the orientation of Supplementary Fig. 3b), trying to maintain a similar orientation and distance of C$_2$B with respect to the long axis of the SC166 tensor, as observed after the initial superposition of the C$_2$B166 and SC166 tensors, but placing C$_2$B within van der Waals contact of the SNARE complex (Supplementary Fig. 3f).
is larger than the distance from C$_2$B to the center of the SC166 tensor in the 166-manual model (Supplementary Fig. 3g). Because fast motions of a lanthanide with respect to a molecule attenuate the observed PCs and yield a distorted tensor with a center that is further away from the molecule than the real position of the lanthanide$^{45}$, this analysis indicates that the PCs induced by SC166Dy on C$_2$B reflect motional averaging and that the manual model constitutes a better representation of the center of the ensemble of binding modes than models derived from superposition of the SC166 and C$_2$B166 tensors.

We also attempted to obtain structures of the C$_2$B–SNARE complex that fit the SC166Dy PCS data with HADDOCK-PCS$^{42}$, but we did not obtain any structures exhibiting good correlations between observed and calculated PCs. Similar results were obtained with the SC41Dy PCS data. To try to account for the decreases in PCs expected to be caused by motional averaging and/or for the possibility that we overestimated the population of the major binding mode, we attempted to find structures that fit the measured PCSs multiplied by $x$ factors from 2 to 7 with HADDOCK-PCS. We did not obtain structures with a good fit for the SC41Dy data. With the SC166Dy data, we did obtain structures that had consistent orientations of C$_2$B and had good correlations between measured and calculated PCs, as illustrated in Supplementary Figure 4a for a representative structure obtained with an x factor of 5 (referred to as the 166 HADDOCK model). However, these structures exhibited a limited number of salt bridges between C$_2$B and the SNAREs, and the pattern of positive-negative PCs did not match well with the positive-negative lobes of the SC166 tensor (Supplementary Fig. 4b). This overall analysis emphasizes the danger of attempting to fit dynamically averaged PCS data to single structures and shows that the large slopes in Figure 3g,h and Supplementary Figure 4c arise to a large extent from motions of C$_2$B with respect to the SNARE complex.

To aid in the analysis of PCs, we performed unrestrained MD simulations starting from the 166 HADDOCK model (Supplementary Note 8) and generated a chemical shift–based ensemble with replica-averaged metadynamics (RAM) simulations$^{44}$ (Supplementary Note 9). To explore whether the PCS measurements on C$_2$B could be fit with ensemble-averaged values, we selected one representative structure from each of the 73 clusters of the chemical shift–based ensemble. For each structure, we calculated an SC166 tensor and an SC41 tensor with the PCs measured within the SNARE complex, in Numbat. These tensors were used to calculate the C$_2$B PCs for each structure. We then used MATLAB to find population weights for the 73 structures that minimize the r.m.s. deviation between the population-averaged PCs and the experimental values. The calculations were performed separately for PCs induced by SC166Dy and SC41Dy, thus leading to the correlations presented in Figure 4e,f.

Syt1–SNARE-complex binding assays. 1D 13C-edited $^1$H NMR spectra for SNARE-complex binding assays were obtained by acquiring the first trace of C$_2$B and generating a chemical shift–based ensemble with replica-averaged metadynamics (RAM) simulations$^{44}$ (Supplementary Note 9). To explore whether the PCS measurements on C$_2$B could be fit with ensemble-averaged values, we selected one representative structure from each of the 73 clusters of the chemical shift–based ensemble. For each structure, we calculated an SC166 tensor and an SC41 tensor with the PCs measured within the SNARE complex, in Numbat. These tensors were used to calculate the C$_2$B PCs for each structure. We then used MATLAB to find population weights for the 73 structures that minimize the r.m.s. deviation between the population-averaged PCs and the experimental values. The calculations were performed separately for PCs induced by SC166Dy and SC41Dy, thus leading to the correlations presented in Figure 4e,f.

Syt1–KO rescue experiments. Neuronal cultures were produced from WT (C57BL/6J) and Syt1-KO (also in a C57BL/6J background) mice as previously described$^{85}$. Hippocampi were dissected from P0 pups, either male or female, dissociated by papain digestion and plated on Matrigel-coated glass coverslips. Neurons were cultured for 14–16 d in vitro in MEM (Gibco) supplemented with B27 (Gibco), glucose, transferrin, FBS and Ara-C (Sigma). For rescue experiments, a rat Syt1 cDNA (carrying mutations when desired) was introduced into a lentiviral construct that has been previously described$^{66}$. To make viruses, human embryonic kidney 293T cells (ATCC) were cotransfected with the lentiviral vector and three packaging plasmids. Supernatant containing the viruses was collected 48 h after transfection and was used to infect hippocampal neuronal cultures at DIV4. Cultures were used for biochemical or physiological analyses at DIV14–16. All animal studies were approved by the Stanford University Institutional Review Board and performed in accordance with Stanford University Animal Care and Use guidelines. Although no statistical method was used to predetermine sample size, randomized experiments were performed by an experimenter blind to the status of the treatment groups.

Electrophysiological recordings in cultured neurons. Recordings were performed essentially as previously described$^{87}$. The whole-cell pipette solution contained 135 mM CsCl, 10 mM HEPES, 1 mM EGTA, 1 mM Na-GTP, 4 mM Mg-ATP and 10 mM QX-314, pH 7.4 (adjusted with CsOH). The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 10 mM HEPES, 10 mM glucose, pH 7.4 (adjusted with NaOH) and 2 mM MgCl$_2$, unless otherwise indicated. Synaptic currents were monitored with a Multiclamp 700B amplifier (Molecular Devices). Extracellular stimuli were controlled with a Model 2100 Isolated Pulse Stimulator (A-M Systems) synchronized with the Clampex 9 or 10 data-acquisition software (Molecular Devices). Evoked synaptic responses were triggered by a bipolar electrode. GABA–R–mediated IPSCs were pharmacologically isolated with CNQX (20 μM) and AP-5 (50 μM) in the bath solution and recorded at a −70 mV holding potential. Because the intracellular solution contains high internal Cl$^-$ levels, IPSCs evoke large inward currents. mIPSCs were monitored in the presence of tetrodotoxin (1 μM) in addition to the compounds listed above. Miniature events were analyzed in Clampfit 9.02 (Molecular Devices) with the template matching search and a minimal threshold of 5 pA, and each event was visually inspected for inclusion or rejection. For Ca$^{2+}$-titrations, eIPSCs were measured for each cell at multiple Ca$^{2+}$ concentrations starting at 2 μM Ca$^{2+}$; this was followed by measurement of the higher, then lower, Ca$^{2+}$ concentrations. For all electrophysiological experiments, the experimenter was blind to the condition genotype of the cultures analyzed.

Immunoprecipitation and quantitative immunoblotting. Cultured Syt1-KO neurons were solubilized in PBS (with 1 mM CaCl$_2$ and 0.2% Triton X-100, pH 7.4) supplemented with protease inhibitors (Roche) for 1 h. The lysate was cleared by centrifugation at 16,000g for 10 min at 4 °C, and immunoprecipitation...
was performed by incubation with polyclonal antibodies to syntaxin-1 (438B) that have been used previously in multiple studies (for example, refs. 68,69) or with preimmune sera for 1 h at 4 °C; this was followed by incubation with 15 µl of a 50% slurry of protein A–Sepharose beads (GE Healthcare) for 2 h at 4 °C. Beads were washed 4× with 1 ml extraction buffer, and bound proteins were eluted with 2× SDS sample buffer containing 100 mm DTT and were boiled for 20 min at 100 °C.

Coprecipitated proteins were separated by SDS-PAGE; this was followed by detection with monoclonal antibodies against rat Syt1 (604.4, Synaptic Systems) and synaptobrevin-2 (clone 69.1, Synaptic Systems) (validation provided by the manufacturer). To allow for quantitative detection, dye-conjugated secondary antibodies were used (IRDye 800CW donkey anti-mouse IgG, Li-cor), membranes were scanned in an Odyssey scanner (Li-cor), and quantification was performed with Image Studio software (Li-cor).

59. Delaglio, F. et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293 (1995).
60. Johnson, B.A. & Blevins, R.A. NMR View: a computer-program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 (1994).
61. Shao, X., Fernandez, I., Sudhof, T.C. & Rizo, J. Solution structures of the Ca2+-free and Ca2+-bound C2A domain of synaptotagmin I: does Ca2+ induce a conformational change? Biochemistry 37, 16106–16115 (1998).
62. Chen, X., Tang, J., Sudhof, T.C. & Rizo, J. Are neuronal SNARE proteins Ca2+ sensors? J. Mol. Biol. 347, 145–158 (2005).
63. Schmitz, C., Stanton-Cook, M.J., Su, X.C., Otting, G. & Huber, T. Numbat: an interactive software tool for fitting Deltachi-tensors to molecular coordinates using pseudocontact shifts. J. Biomol. NMR 41, 179–189 (2008).
64. Camilloni, C. & Vendruscolo, M. Statistical mechanics of the denatured state of a protein using replica-averaged metadynamics. J. Am. Chem. Soc. 136, 8982–8991 (2014).
65. Maximov, A., Pang, Z.P., Tervo, D.G. & Sudhof, T.C. Monitoring synaptic transmission in primary neuronal cultures using local extracellular stimulation. J. Neurosci. Methods 161, 75–87 (2007).
66. Pang, Z.P., Cao, P., Xu, W. & Sudhof, T.C. Calmodulin controls synaptic strength via presynaptic activation of calmodulin kinase II. J. Neurosci. 30, 4132–4142 (2010).
67. Bacaj, T. et al. Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. Neuron 80, 947–959 (2013).
68. Zhou, P. et al. Syntaxin-1 N-peptide and Habc-domain perform distinct essential functions in synaptic vesicle fusion. EMBO J. 31, 829–841 (2012).