Architecture of the synaptotagmin–SNARE machinery for neuronal exocytosis

Qiangjun Zhou1,2, Ying Lai1,2*, Taulant Baca1,2*, Minglei Zhao1,2, Artem Y. Lyubimov1,2, Monarin Uervirojngkorn1,2, Oliver B. Zeldin1,2, Aaron S. Brewster1, Nicholas K. Sauter1, Aina E. Cohen1,2, S. Michael Solits4, Roberto Alonso–Mori5, Matthieu Chollet1, Henrik T. Lemke1, Richard A. Pfuetzner1,2, Ucheor B. Choi1,2, William I. Weis1, Jiajie Diao1,2, Oliver B. Zeldin1,2, Aaron S. Brewster3, Nicholas K. Sauter3, Aina E. Cohen4, S. Michael Soltis4, Roberto Alonso-Mori4, Thomas C. Südhof1 & Axel T. Brunger1,2

Synaptotagmin-1 and neuronal SNARE proteins have central roles in evoked synchronous neurotransmitter release; however, it is unknown how they cooperate to trigger synaptic vesicle fusion. Here we report atomic-resolution crystal structures of Ca2+- and Mg2+-bound complexes between synaptotagmin-1 and the neuronal SNARE complex, one of which was determined with diffraction data from an X-ray free–electron laser, leading to an atomic–resolution structure with accurate rotamer assignments for many side chains. The structures reveal several interfaces, including a large, specific, Ca2+-independent and conserved interface. Tests of this interface by mutagenesis suggest that it is essential for Ca2+-triggered neurotransmitter release in mouse hippocampal neuronal synapses and for Ca2+-triggered vesicle fusion in a reconstituted system. We propose that this interface forms before Ca2+ triggering, moves en bloc as Ca2+ influx promotes the interactions between synaptotagmin-1 and the plasma membrane, and consequently remolds the membrane to promote fusion, possibly in conjunction with other interfaces.

Membrane fusion is essential for many physiological processes in eukaryotic cells, including protein and membrane trafficking, hormone secretion and neurotransmitter release. Evolutionarily conserved SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins have a key role in these processes. Specific combinations of SNARE proteins are located on opposite membranes. Upon zippering into a highly stable four-helix bundle—the SNARE complex—they provide the energy for membrane fusion. However, other factors are essential for regulation of membrane fusion. In particular, several proteins are required for neurotransmitter release in addition to neuronal SNAREs, but it is unknown, at the atomic level, how these factors cooperate with SNAREs to promote synaptic transmission. One key factor is the Ca2+ sensor synaptotagmin, which consists of a short N-terminal luminal segment, a single transmembrane α-helix, an unstructured linker, and two Ca2+-binding C2 domains, termed C2A and C2B, respectively (or C2AB together). There are 16 isoforms of mammalian synaptotagmins that are localized to synaptic and secretory vesicles or the plasma membrane. Among these isoforms, synaptotagmin-1 (Syt1) is a Ca2+ sensor for evoked synchronous neurotransmitter release. Synaptotagmin-2 and synaptotagmin-9 are also involved in evoked synchronous neurotransmitter release for different subsets of neurons. In contrast, synaptotagmin-7 plays a part in ‘slower’ asynchronous release; moreover, these and other synaptotagmins act in other types of exocytosis. In addition to its role in evoked synchronous release, Syt1 also clamps the frequency of miniature spontaneous events.

Syt1 binds in a Ca2+-dependent manner to anionic membranes; during binding, anionic phospholipids and synaptotagmin C2 domains together coordinate calcium ions. The membrane–synaptotagmin interaction has functional significance since the Ca2+-affinity of Syt1 for binding to anionic membranes and the Ca2+-sensitivity of neurotransmitter release are tightly correlated. The Syt1 C2AB fragment can induce vesicle clustering and preferentially binds to curved membranes. Moreover, C2 domains may penetrate the membrane upon Ca2+ binding.

Syt1 also interacts with the neuronal SNARE complex based on immunoprecipitation and pull-down experiments, single molecule fluorescence resonance energy transfer (smFRET), and nuclear magnetic resonance experiments. A gain-of-function mutation in the C2A–binding region of the C2A domain suggested that the Syt1–SNARE interaction may be functionally important, but the molecular basis and the significance of the interaction between Syt1 and the SNARE complex remain unknown.

Several crystal structures of Syt1 C2A and C2B domains, and C2AB fragments, are available, as well as the structure of the neuronal SNARE complex; however, the atomic-resolution structure of the complex between Syt1 and the neuronal SNARE complex (referred to as Syt1–SNARE complex) has been elusive. Single molecule methods allowed the study of the Syt1–SNARE complex under dilute conditions with spatially isolated neuronal SNARE complexes reconstituted in a supported bilayer. The observed smFRET histograms suggested several possible interfaces between Syt1 and the SNARE complex. Other dynamic or approximate models of the C2AB–SNARE complex were obtained by nuclear magnetic resonance (NMR), but cannot be readily compared with the previous smFRET studies or the results presented here because of differences.
in conditions, particular covalent attachment of lanthanide labels, and lack of atomic resolution.

Here we report atomic-resolution crystal structures of a Syt1–SNARE complex in two different crystal forms and in the presence of either Ca\(^{2+}\) or Mg\(^{2+}\). We found several interfaces, including a large structurally and evolutionarily conserved interface that is Ca\(^{2+}\)-independent. Structure-based mutations of this interface disrupt evoked neurotransmitter release in primary neurons and Ca\(^{2+}\)-triggered fusion in a reconstituted system.

**Structure of the Syt1–SNARE complex**

We designed and tested several chimeric constructs involving the Syt1 C2AB fragment (amino acids 141–421) and the neuronal SNARE complex (Extended Data Fig. 1a, b and Methods). We crystallized the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound Syt1–SNARE complexes (Extended Data Fig. 1d, e), and determined their structures (Fig. 1 and Extended Data Table 1). The crystallization conditions were at near-physiological pH and ionic strength (Methods and Extended Data Fig. 1e). We observed two crystal forms for the Ca\(^{2+}\)-bound Syt1–SNARE complex, referred to as 'short unit cell' and 'long unit cell' crystal forms hereafter (Extended Data Figs 2 and 3).

The X-ray free-electron laser (XFEL) of the Linac Coherent Light Source (LCLS) at SLAC National Accelerator Laboratory yielded substantially higher-quality diffraction data than the Advanced Photon Source (APS) NE-CAT microfocus synchrotron beamline at Argonne National Laboratory from similar crystals of the long unit cell crystal form (Extended Data Fig. 2a, b). The electron density maps obtained from the XFEL diffraction data were notably superior to those of the synchrotron data sets (Methods). In particular, the electron density maps calculated from the XFEL data set were of sufficient quality to obtain accurate rotamers for most side chains, including those at the interfaces between molecules (Extended Data Fig. 2d–f). This is one of the first new crystal structures determined using XFEL diffraction data. Moreover, in contrast to the tens of thousands to millions of crystals typically used in XFEL-based crystallography experiments, we obtained a reasonably complete data set from a few hundred images captured from 72 of the 148 crystals exposed to the LCLS XFEL beam (Methods).

**Three distinct Syt1–SNARE interfaces**

The crystal structures of the Syt1–SNARE complex reveal three interfaces between the SNARE complex and the Syt1 C2A and C2B domains (referred to as 'primary', 'secondary' and 'tertiary'), as well as an interface between Syt1 C2 domains (referred to as C2A–C2B interface; Fig. 1a, b and Supplementary Videos 1 and 2). There are two essentially identical instances of the primary interface formed between Syt1 C2B domains and the SNARE complex in the long unit cell crystal form (Fig. 1c, d). The secondary interface involves another Syt1 C2B domain and the SNARE complex, while the tertiary interface involves a Syt1 C2A domain and the SNARE complex (Fig. 1b). These two C2 domains also form the C2A–C2B interface. All three interfaces between Syt1 and the SNARE complex fall within the range of smFRET efficiency histograms obtained previously (Extended Data Fig. 4). These interfaces may suggest how multiple Syt1 and SNARE complexes simultaneously interact in the neuron (see below).

The largest, primary interface between the Syt1 C2B domain and the SNARE complex is very similar in both complexes (Fig. 1b) and in both crystal forms (Extended Data Fig. 5a), suggesting that it is not affected by crystal packing. The primary interface is also very similar in the Ca\(^{2+}\)-bound as well as in the Mg\(^{2+}\)-bound crystal structures (Extended Data Fig. 5d), implying a Ca\(^{2+}\)-independent interface. The residues involved in the primary interface have relatively low temperature factors, among the lowest in these structures (Extended Data Fig. 5e), and the electron densities of the side chains that form this interface are well defined (Fig. 2c, d), suggesting a specific interaction. In a recent study, lanthanide labels were covalently attached to SNAP-25 residues 41 and 166 for pseudo-contact chemical shift NMR measurements between Syt1 and the SNARE complex in the presence of 125 mM thiocyanate; these covalent labels would probably disrupt the primary interface (Fig. 2c), so it is not possible to compare our primary interfaces that occur in the long unit cell crystal form (root-mean-square difference (r.m.s.d.) = 0.34 Å, including Ca atoms of the SNARE complex and the Syt1 C2B domain forming the interface). The middle panel shows the secondary interface. The right panel shows both the tertiary interface and the C2A–C2B interface. e. Rotated view of panel a, but showing the entire asymmetric unit and the symmetry-related Syt1 C2B fragment. Three Syt1 C2AB fragments (designated as I, I′ and II) bind to two SNARE complexes in the asymmetric unit. SNARE (II) only interacts with the C2B domain of one Syt1 C2AB fragment, Syt1 (II), via the same primary interface as observed in complex I. d. A schema corresponding to the structure shown in panel c.
The primary interface is critical

We designed mutations of the critical interacting residues of the primary interface based on the crystal structure and verified that all mutants result in properly folded Syt1 and SNARE complex (Methods and Extended Data Fig. 7). To test the interactions in neurons, we performed co-immunoprecipitation of syntaxin-1A in cultured Syt1 conditional knockout neurons infected with viruses expressing wild-type Syt1 or Syt1 containing the designed C2B mutants (Fig. 3a and Methods). The region II Syt1 mutant (R398Q/R399Q) has been reported previously, and is used here for comparison. Syntaxin-1A was immunoprecipitated from lysates of these neurons, and the presence of co-immunoprecipitated proteins was assayed with monoclonal antibodies against Syt1 and synaptobrevin-2. Mutation of either regions I and II of the primary interface reduced Syt1 binding to the SNARE complex by ~50%, and simultaneous mutation of both regions (referred to as ‘Syt1 quintuple’) reduced binding to ~33% as compared to a Syt1 wild-type construct introduced in the same manner (Fig. 3b, c). These results suggest that both regions are required for efficient Syt1–SNARE binding.

For further investigation of the function of the primary interface between the neuronal SNARE complex and Syt1, we used a single-vesicle content-mixing assay and tested the effect of both Syt1 and SNAP-25 mutants on association, spontaneous fusion and Ca²⁺-triggered fusion of single vesicles with reconstituted full-length neuronal SNAREs, Syt1 and complex-1 (Fig. 3d–g, Extended Data Fig. 8 and Extended Data Table 2). The Syt1 mutants disrupting the interface between the SNARE complex and Syt1 as seen by co-immunoprecipitation (Fig. 3b, c) also reduced vesicle association to similar degrees for the four different Syt1 mutants (left group of mutants in Fig. 3d). Our reconstituted assay also allowed testing of the interacting SNAP-25 residues (middle group of mutants in Fig. 3d), showing significant reduction of vesicle association as well. Thus, as expected, mutations on both the Syt1 and SNARE sides of the primary interface reduce the interaction between them. Likewise, both groups of mutants reduce the amplitude and decrease the synchronization of Ca²⁺-triggered fusion, but they do not affect spontaneous fusion (Fig. 3e–g). In particular, the Syt1 quintuple mutant significantly reduced Ca²⁺-triggered synchronization to the control level without Syt1.

To characterize the interaction between Syt1 and the SNARE complex further, we investigated the effect of ATP, which has an ionic shielding effect on certain cellular processes. We observed no effect on both spontaneous and Ca²⁺-triggered fusion, and only a mild effect on vesicle association (Fig. 3d–g, right control group), suggesting that the functionally important interactions between Syt1 and the SNARE complex are not affected by ionic shielding.

Evoked release requires primary interface

We assayed release electrophysiologically in Syt1 conditional knockout neurons in which endogenous Syt1 was replaced with mutant Syt1 (Fig. 3a and Methods). As expected, removal of Syt1 abolished synchronous release, as monitored by recording evoked inhibitory postsynaptic currents (eIPSCs), a phenotype that can be rescued by re-introduction of wild-type but not mutant Syt1 cDNA (Fig. 4a, b). Notably, the region I mutants retained some rescue ability, in contrast to region II mutants that were nearly non-functional (Fig. 4a, b), including the triple mutant (R281A/R398A/R399A) and the Syt1 quintuple mutant that combines mutations in both regions showed even more severe phenotypes. In contrast, a mutant that affects the polybasic region of Syt1 (R322E/K325E) exhibited a milder phenotype, as reported...
Figure 3 | Mutations of the primary interface affect binding and Ca\(^{2+}\)-triggered single vesicle–vesicle fusion. a, Syt1 quintuple refers to the Syt1 mutant (R281A/E295A/Y338W/R398Q/R399Q). b, SNAP-25 quintuple refers to the SNAP-25 mutant (K40A/D51A/E52A/E55A/D166A). The colour code is specified in the figure. a, Schematic diagram of Syt1 conditional knockout (cKO) mice. The Syt1 exon 2 which contains the transmembrane domain is floxed. cKO recombinase removes exon 2, ablating all cytoplasmic Syt1 sequences. b, Co-immunoprecipitation (Co-IP) of either Syt1 (top row) or synaptobrevin-2 (bottom row) with a syntaxin-1A antibody in Syt1 conditional knockout cultured neurons rescued with the indicated Syt1 mutant constructs. c, Quantification of co-immunoprecipitation of Syt1 normalized to synaptobrevin-2. Results are scaled to Syt1 wild-type levels. All data are means ± s.e.m.; statistical significance was analysed by the Student’s t-test comparing the mutants with wild-type Syt1; **P < 0.01, n = 4 for Syt1 E295A/Y338W and Syt1 quintuple; NS, no significant difference, n = 3 for Syt1 R398Q/R399Q; *P < 0.05, n = 4 for Syt1 R281A/R398A/R399A. d–g, Bar graphs showing the effects of Syt1 and SNAP-25 mutants in fusion of single vesicles with reconstituted neuronal SNAREs, Syt1 and complexin-1 (see Methods and ref. 38). d, Number of associated SV vesicles (see Methods) after incubation of SV vesicles with surface-immobilized PM vesicles (see Methods) for a 1-min period. e, Number of spontaneous fusion events over the subsequent 1-min observation period normalized by the number of associated SV vesicles. f, Synchronization, that is, decay rates (1/τ), of the histograms of fusion events upon 500 µM Ca\(^{2+}\) injection. Error bars are error estimates computed from the covariance matrix upon fitting the corresponding cumulative histograms with a single exponential decay function using a Levenberg–Marquardt technique. g, Amplitude of the first 1-s time bin upon Ca\(^{2+}\) injection. Each value in this panel was normalized by the respective number of fusion events after Ca\(^{2+}\) injection. d–g, All data are means ± s.e.m.; the number of independent repeat experiments are depicted above the bars and in Extended Data Table 2; statistical significance was assessed by the Student’s t-test comparing all other conditions with wild type (**P < 0.005; *P < 0.05). The cumulative fusion histograms are shown in Extended Data Fig. 8. Controls in panels d–g are in the presence of 3 mM ATP and in the absence of SNAP-25 and Syt1. As expected, Ca\(^{2+}\)-triggered fusion required the presence of both SNAP-25 and Syt1; fusion is not affected by the presence of ATP.

Figure 4 | Mutations of the primary interface impair Syt1 function in Ca\(^{2+}\)-triggered release. a, b, Sample traces of evoked IPSCs from single action potentials (a) and quantification of peak amplitudes (b). Tick marks indicate stimulus delivery. All data are means ± s.e.m.; number of cells/independent cultures analysed are depicted above the bars; statistical significance was assessed by one-way analysis of variance comparing all other conditions with wild-type rescue group (**P < 0.001). c–e, Syt1 mutants display facilitation during spontaneous release, instead of depression, during high-frequency stimulation. f, Sample traces of 10 Hz trains; g, quantification of absolute (d) and normalized (e) IPSC amplitudes during the train; numbers of cells/independent cultures analysed are depicted in parentheses in the labels for each of the traces. AP, action potential. h, Syt1 mutants are unable to clamp the frequency of spontaneous IPSCs (sIPSCs). f, Sample spontaneous IPSC traces; g, quantification of event frequency (left) and amplitude (right). All data are means ± s.e.m.; the number of cells/independent cultures analysed are depicted above the bars. Statistical significance was assessed by one-way analysis of variance comparing all other conditions with the wild-type rescue group (**P < 0.001; NS, no significant difference). Previously\(^3\). During high-frequency stimulation, wild-type cultured hippocampal neurons displayed depression while Syt1 conditional knockout neurons showed asynchronous release with robust facilitation (Fig. 4c–e). All Syt1 mutants underwent facilitation, and the severity of the phenotype for each mutant correlated well with the results for single evoked release. Another known consequence of Syt1 removal is the unclamping of spontaneous release. Interestingly, the E295A/Y338W mutant could rescue this phenotype while the region II mutants (or combinations thereof) could not (Fig. 4f, g). There were no differences in spontaneous miniature IPSC amplitudes between wild type and mutant rescues (Fig. 4g). Together, these observations support the notion that the primary Syt1–SNARE interface is critical for the role of Syt1 as a Ca\(^{2+}\) sensor.
Before Ca\(^{2+}\) influx, we assume a membrane juxtaposed, hemifusion-free state (Fig. 5c), the likely starting state for fast Ca\(^{2+}\)-triggered fusion\(^2\), and a partially folded trans-SNARE complex\(^4\). We assume that the Syt1 C2B–SNARE unit can assemble in this state since the primary interface involves residues within the folded region of the trans-SNARE complex. The palmitoylated cysteine residues of SNAP-25 would be able to interact with the plasma membrane, and the membrane-proximal side of syntaxin is closely juxtaposed with the membrane, as inferred by the requirement of tight membrane coupling of syntaxin for evoked release\(^7\). Upon Ca\(^{2+}\) binding to the Syt1 C2B domain, the Ca\(^{2+}\) binding loops partially insert into the membrane\(^23\) with a preference for membrane curvature\(^20,21\), possibly supported by ionic interactions via the polybasic region and with continuing maintenance of the Syt1–SNARE interface. We propose that the Syt1 C2B–SNARE unit moves en bloc as an entity upon Ca\(^{2+}\) triggering. The simultaneous membrane interactions of the Ca\(^{2+}\) binding loops, of the polybasic region, and of the membrane-proximal region of the SNARE complex would therefore require a deformation of the plasma membrane. This morphological change of the plasma membrane juxtaposes the membranes closer than the critical distance (0.9 nm) to promote stalk formation\(^4\) (Fig. 5d), and subsequently leads to fusion pore opening (Fig. 5e).

It is likely that Syt1 engages in multiple functionally relevant interactions with neuronal SNARE complexes, including the other interfaces found in our crystal structures. All of these interactions may be anchored by the primary interface, which is the most stable and extensive contact site. We speculate that multiple SNARE complexes could interact via Syt1 interactions employing some or all of the interfaces (primary, secondary, tertiary, C2A–C2B) that are observed in our crystal structures (Fig. 5f). In addition to the primary and secondary interfaces formed by C2B domains (Fig. 5f, gold), the tertiary interface involves a C2A domain (Fig. 5f, grey). This interface could be involved in displacing complexin from its interaction with the core of the SNARE complex, since complexin binds in the groove between syntaxin-1A and synaptobrevin-2 (ref. 49); partial displacement of complexin may be important for neurotransmitter release\(^40\). Interestingly, the gain-of-function mutation D232N in the Ca\(^{2+}\)-binding region of the C2A domain\(^26\) is close to the tertiary interface and it is also part of the C2A–C2B interface (Fig. 1b). In the proposed network of interactions there are C2A domains (Fig. 5f, purple) that have no interactions with SNARE complexes; we envision that they could interact with a membrane. This entire Syt1–SNARE assembly would thus be poised to confer cooperativity upon Ca\(^{2+}\) triggering on a sub-millisecond timescale.

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METHODS
No statistical methods were used to predetermine sample size.

Strategy for crystallization of the Syt1–SNARE complex. Successful crystallization of the Syt1–SNARE complex involved extensive testing of multiple designs of covalently linked chimaeras between a Syt1 C2AB fragment and different SNARE domain fragments of the neuronal SNARE complex (both SNARE domains of SNAP-25, denoted SNAP-25_N and SNAP-25_C, as well as the SNARE domain of syntaxin–1A). Three different linker lengths for the chimaeras were tested (16, 23 and 37 amino acids) derived from the linker sequence of the human Oct-1 transcription factor13. This particular linker sequence had been used previously to crystallize the Arf-ArfGAP complex. We also tested different truncations of the neuronal SNARE complex13,14. The resulting constructs were screened for protein expression and homogeneity by ion exchange and size-exclusion chromatography. The best constructs resulted in a Syt1–SNARE complex that eluted in a mono-disperse peak in the final size-exclusion chromatography step, and that remained stable in SDS–PAGE without boiling, a hallmark for neuronal SNARE complex formation (Extended Data Fig. 1b). Best results were obtained when the SNARE fragments were truncated as previously described14. The best candidates were used for crystallization trials (see below).

Cloning, expression and purification of the Syt1–SNARE complex. The SNAP-25 isoform used throughout this study is commonly referred to as isoform 2 or isoform 23 and 37 amino acids) derived from the linker sequence of the human Oct-1 transcription factor51 (Extended Data Fig. 1a) (referred to as C2AB-421) was fused to the amino terminus of the C-terminal SNARE domain of rat SNAP-25 (amino acid range 7–83), the rat syntaxin-1A fragment (amino acid range 191–250) and the neuronal SNARE complex54 (Extended Data Fig. 1a). These four protein components and the aggregation propensity at higher concentrations, especially in the presence of Ca2+. To overcome these problems, crystallization trials were performed with chimaeras between Syt1 C2AB and SNAP-25_C, as described above. The best three candidates consisted of three different linker lengths. Initially, thin crystalline plates that diffracted to about 30 Å were obtained from a construct connected by a 23-amino-acid linker. Screening of volatile crystallization additives (such as, methanol, ethanol, 1,2-butanediol) led to improved diffraction to about 10 Å resolution, with sharp Bragg peaks. The complex with a longer 37-amino-acid linker (Syt1–SNARE37aa-linker) was more soluble in SEC buffer compared to the one with the shorter 23-amino-acid linker. Clusters of needle-shaped crystals were obtained for this construct at first. Using a reverse vapour-diffusion method led to thicker crystal plates (Extended Data Fig. 1e), and eventually produced Bragg reflections past 4 Å resolution. There were consistently two crystal forms in the same drop with identical morphologies, referred to as ‘short unit cell’ crystal form and ‘long unit cell’ crystal form, respectively. The difference between the short and long unit cell crystal forms of the Ca2+-bound Syt1–SNARE complex can be approximated by a doubling of the number of complexes, except that one of the interacting Syt1 C2AB fragments is absent (Fig. 1c, Extended Data Fig. 2g and Extended Data Fig. 3d).

Before setting up crystal trays, purified Syt1–SNARE37aa-linker complex (at a concentration of ~20 mg ml−1) was diluted to a final concentration of ~8 mg ml−1 supplemented with CaCl2 and MgCl2. The final buffer contained 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 100 mM MgCl2, 1 mM CaCl2 and 0.5 mM TCEP. The reservoir contained 100 mM HEPES-Na (pH 7.5) and 1% PEG 8000. Equal amounts of protein and reservoir (2 μl) were mixed and incubated at 20 °C. The crystals appeared after 1 to 4 months, and were flash-frozen in a cryo-protecting solution containing the same constituents as the crystallization condition supplemented with 35% (v/v) sucrose. Although Syt1 was initially covalently linked to the C-terminal half of SNAP-25 (SNAP-25_C), the linker was slowly cleaved at ambient temperature (Extended Data Fig. 1c). Moreover, crystal growth required 1 to 4 months and contained entirely cleaved complex (Extended Data Fig. 1b), enabling formation of 2.1 and 3.2 stoichiometries in the asymmetric unit of the short and long unit cell crystal forms, respectively. As expected, the crystal structures are probably not affected by the initial presence of the linker. We simply refer to the resulting crystal structure as that of the Syt1–SNARE complex.

Cryotransfer of the Mg2+-bound Syt1–SNARE complex. Crystals of Mg2+-bound Syt1–SNARE complex were grown using the reverse hanging-drop vapour-diffusion method. Purified Syt1–SNARE37aa-linker complex (at a concentration of ~20 mg ml−1) that was prepared without Ca2+ and in the presence of EDTA (as described above) was diluted to a final concentration of ~4 mg ml−1 and supplemented with 100 mM MgCl2. The final buffer contained 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 100 mM MgCl2 and 0.5 mM TCEP. The reservoir contained 100 mM HEPES-Na (pH 7.5) and 2.5% PEG3350. Similar to Ca2+-bound complex, the linker was also cleaved during crystallization. Mg2+-bound Syt1–SNARE complex crystals appeared after 2 months and were flash-frozen in a cryo-protecting solution containing the same constituents as the crystallization condition supplemented with 20% (v/v) glycerol.

Cryotransfer of the magnesium-unbound form of the Syt1 C2B domain. Crystals of the magnesium-unbound form of the Syt1 C2B domain that were grown by the hanging-drop vapour diffusion method at 20 °C by mixing 2 μl protein solution (at a concentration of ~10 mg ml−1) in a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5 mM TCEP with equal volumes of reservoir solution containing 100 mM Tris-HCl (pH 8.5), 1.5M ammonium sulfate.

XFEL data collection and processing. The diffraction data of crystals of the long unit cell crystal form of the Ca2+-bound Syt1–SNARE complex (Extended Data Table I) were collected at the X-ray Pump Probe (XPP) endstation using the LCSL.
XFEL at the SLAC National Accelerator Laboratory at Stanford University equipped with a Rayonix MX325 X-ray imaging detector. The XFEL beam was focused to 30 μm at a nominal energy of 9 keV and a pulse duration of 40 fs in self-amplified stimulated emission (SASE) mode64-67. Each 40-fs XFEL pulse at the XPP endstation at LCLS delivers 10^12 photons, exceeding a dose of 30 MGy. The dose is so large that the diffraction volume is vaporized after exposure to a single XFEL pulse (Extended Data Fig. 2b). Nonetheless, the 40-fs laser pulse enables the so-called ‘diffraction before destruction’ data collection68-69, whereby the diffraction pattern is recorded before the sample is destroyed.

The diffraction images were obtained from 148 frozen and cryo-protected crystals mounted in conventional cryo-loops at 100 K using a goniometer-based fixed-target sample delivery station70. XFEL diffraction tests of hydrated crystals at room temperature did not show any marked improvement in comparison to cryo-preserved crystals, at least in terms of visible limiting resolution of the observed diffraction pattern. Furthermore, the entire crystal was damaged when exposed at room temperature, requiring many more crystals and much more time to collect a complete diffraction data set. As beam time and sample volume were limited, we decided to collect diffraction data at cryogenic temperature, since it was possible to obtain multiple diffraction images from the same frozen crystal.

In most cases, 2-20 diffraction images (depending on crystal size and quality) were obtained from a single crystal. Since the diffracting volume was destroyed by the XFEL beam, a different volume had to be exposed for every shot (Extended Data Fig. 2b). The crystal was translated at least 100 μm between exposures. During data collection we observed that improved diffraction quality could be achieved by placing consecutive shots far apart (that is, across the length of the crystal from one another), then later ‘filling’ between the first and second shots in the same ‘shuttling’ manner. Finally, care was taken to collect exposures at a variety of spindle (θ) angles to maximize the completeness of the final data set.

Of the 148 crystals used in the experiment, 113 crystals produced 578 images that could be processed; the other crystals did not diffract or showed multiple lattices. The long unit cell form occurred much more frequently (about 80%). Images indexed in the short unit cell crystal form were identified using a hierarchical clustering method71 and were omitted from further processing since it was not possible to obtain a complete data set in this crystal form. Other images were rejected during data processing as described below. The 309 diffraction images (from 72 crystals) in the final selection for the long unit cell crystal form were indexed and integrated with the cctbx.xfel suite of data-processing software72-75, with the diffraction data processing parameters optimized by a grid search procedure (Lyubimov et al., manuscript in preparation). The integrated diffraction data were subsequently scaled, merged and post-refined with the PRIME software76.

We optimized the data integration process using a combination of mosaic quality analysis77, highest number of bright reflections yielded by integration, and overlay of observed reflections on the actual integrated areas to determine whether nearly all visible reflections were integrated, and no unobserved reflections were predicted and integrated. We inspected overlays of diffraction images and predicted reflection positions to fine-tune the computational approaches and optimize the processing of the XFEL data. The iterative scaling and post-refinement approach used by PRIME allowed the construction of a complete diffraction data set from the relatively small number of diffraction images.

Synchrotron beamline data collection. All other diffraction data sets (Extended Data Table 1) were collected using beamline 24-ID-C of the Advanced Photon Source (APS) at Argonne National Laboratory (Argonne, IL). Diffraction data of the best crystals of both the Ca^2+ -bound short unit cell crystal form and the Mg^2+ -bound short unit cell crystal form of the Syt1-SNARE complex were indexed and integrated using the XDS software78, and scaled and merged using the SCALA program in CCP4 package79. Diffraction data of the quintuple mutant of the Syt1 C2B domain were indexed, integrated, scaled and merged using the XDS software80.

Structure determination. The phases for all crystal structures of Syt1–SNARE complex were determined by molecular replacement with Phaser90 using the rat Syt1 C2B domain (PDB code 1UOW) as the search model. The structure was iteratively built and refined using the program Coot92, and phenix.refine93 (Extended Data Table 1). The final model consists of four Syt1 C2B molecules in the asymmetric unit. Ramachandran analysis with MolProbity94 indicated that 98% of the residues are in the favoured regions and none is in disallowed regions for both the Ca^{2+} and Mg^{2+} -bound Syt1-SNARE complexes in the short unit cell crystal form, and that 97% of the residues are in the favoured regions and none is in disallowed regions for the Ca^{2+} -bound Syt1–SNARE complex in the long unit cell crystal form. The quality indicators for the crystal structures of the Syt1–SNARE complex are well within acceptable ranges indicated by the ‘polygon’ plot75 produced by phenix.refine93 and by the validation report of the deposited structures and diffraction data.

The phases for crystal structure of the quintuple mutant of the Syt1 C2B domain were determined by molecular replacement with Phaser90 using the rat Syt1 C2B domain (PDB code 1UOW) as the search model. We suspect that the standard diffraction data statistics (such as the merging R values) of rotation data are better due to the ability to directly measure full reflections (at least by summation of partials) without modelling partiality, which is still a relatively crude process even with the latest post-refinement approaches.

At present, it is difficult to assess the relative quality of XFEL diffraction data studied here with conventional rotation diffraction data measured at a synchrotron. However, we utilized the XFEL data to improve the quality of the resulting electron density maps for diffraction data of crystals collected at the XFEL (Fig. 2c, d and Extended Data Fig. 2c–f). These maps, superior to those of the short unit cell data crystal structure collected at the APS NE-CAT microfocus synchrotron beamline at comparable limiting resolution (Extended Data Fig. 3b, c), enabled us to better investigate the binding interfaces between Syt1 and the SNARE complex, and was thus essential for the structural portion of our study.

Validation and structure analysis. MolProbity95 was used for evaluating the geometry and quality of the models (Extended Data Table 1). The electrostatic...
potential maps were calculated and displayed using the UCSF Chimera package. (Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311)). All other structure figures were prepared with PyMol (DeLano, 2002, The PyMOL Molecular Graphics System, http://www.pymol.org, Schrödinger, LLC). Interface areas were calculated by PISA; note that the commonly used ‘buried surface area’ is twice the ‘interface area’.

**Design of mutations to disrupt the primary interface.** We mutated combinations of residues from a syntaxin-1A and SNAP-25 homologue. Wild-type rat complexin-1 was expressed and purified as previously described. All mutants of Syt1 and SNAP-25 were generated using the Quick Change Site-Directed Mutagenesis kit (Agilent) and expressed and purified using the same protocol.

**Protein reconstitution for single vesicle–vesicle fusion experiments.** Neuronal SNAREs and Syt1 represent a minimal system for Ca\(^{2+}\)-triggered membrane fusion. Recent evidence for this notion came from a reconstituted single vesicle–vesicle assay that discriminates among vesicle association, lipid mixing and content mixing (Abou-Donia et al., 2009). Moreover, addition of complexin-1 greatly enhanced Ca\(^{2+}\)-triggered amplitude and synchronization, and suppressed spontaneous release in this system (Abou-Donia et al., 2009). A variety of complexin-1 truncations and mutations qualitatively reproduced effects observed in neuronal cultures for both spontaneous and Ca\(^{2+}\)-triggered release (Abou-Donia et al., 2009), lending credence to this reconstituted system to investigate mechanistic questions.

We used the same membrane compositions and protein densities as in our previous studies (Abou-Donia et al., 2009). Likewise, the reconstitution protocol was similar to previous studies (Abou-Donia et al., 2009) with several changes as described in ref. 38. Briefly, one class of vesicles was reconstituted with both Syt1, or its mutants, and synaptotagmin-2 to mimic synaptic vesicles (referred to as SV vesicles), while another class of vesicles was reconstituted with syntaxin-1A and SNAP-25 or its mutants to mimic plasma membrane (referred to as PM vesicles), using the previously described lipid compositions. The protein-to-lipid ratios used were 1:200 for synaptotagmin-2 and syntaxin-1A, and 1:1,000 for Syt1 and its mutants. A three- to fivefold excess of SNAP-25 and its mutants (with respect to syntaxin-1A) and 3.5 mol% PIP\(_2\) were added to the protein–lipid mixture for PM vesicles only. Dried lipid films were dissolved in 110 mM \(\beta\)-octyl glucoside (\(\beta\)-OG) buffer containing purified proteins. Detergent-free buffer (20 mM HEPES-Na, pH 7.4, 90 mM NaCl, 0.1% 2-mercaptoethanol) was then added to the protein-lipid mixture until the \(\beta\)-OG concentration reached the critical micelle concentration 24.4 mM. The vesicles were then subjected to size-exclusion chromatography using a Sepharose CL-4B column, packed under near-constant pressure by gravity with a peristaltic pump (GE Healthcare) in a 5 ml column with a 2 ml bed volume, that was equilibrated with buffer V (20 mM HEPES-Na, pH 7.4, 90 mM NaCl, 20 mM EGTA, 0.1% 2-mercaptoethanol) followed by dialysis into 1 l of detergent-free buffer V supplemented with 50 mg of Bio-beads SM2 and 0.8 g l\(^{-1}\) Chelex 100 resin (Bio-Rad, Life Science Research). After 4 h, the buffer was changed with 2 l of fresh buffer V containing Bio-beads and Chelex, and dialysis continued for 12 h. During the preparation of SV vesicles, 50 mM sulforhodamine B (Invitrogen) was present in all solutions before the size-exclusion chromatography step. As described previously (Abou-Donia et al., 2009), the presence and purity of reconstituted proteins was confirmed by SDS–PAGE of the vesicle preparations, and the directionality of the membrane proteins (facing outward) was assessed by chymotrypsin digestion followed by SDS–PAGE. The size distributions of the SV and PM vesicles were analysed by cryo-EM, as described previously (Abou-Donia et al., 2009).

**Single vesicle–vesicle content-mixing assay.** We used the single vesicle–vesicle assay described in ref. 38. Briefly, SV vesicles were labelled with a soluble fluoroscent content dye (sulforhodamine B) at a moderately self-quenching concentration; for simplicity in this work we did not include a lipid dye since we were exclusively interested in the exchange of content, the correlation for neurotransmitter release. The PM vesicles were immobilized on a surface that was passivated with polyethylene glycol (PEG) and functionalized via streptavidin–biotin linkages. SV vesicles were then added in the presence of 2 \(\mu\)M complexin-1. We directly started monitoring the arrival of SV vesicles to surface-immobilized PM vesicles during the first minute acquisition period. A stepwise increase in fluororescence intensity was recorded. A emission of a spot in the field of view was used to monitor the formation of a SV–PM vesicle pair during the fusion event.

Unbound SV vesicles were then removed through extensive washing with vesicle-free buffer, while continuing real-time observation of the fluorescence intensity; consequently we did not observe any additional SV–PM vesicle associations after the washing step. While continuing the observation for another 1 min period, a second step-wise increase of fluorescence intensity appeared for some fraction of the associated vesicles, which indicated Ca\(^{2+}\)-independent, that is, spontaneous fusion events (referred to as the spontaneous fusion period). Next, we injected 500 \(\mu\)M Ca\(^{2+}\) solution, and continued monitoring for another minute, referred to as the Ca\(^{2+}\)-triggered fusion period. For associated SV vesicles that did not undergo spontaneous fusion during the second period, a step-wise increase in fluorescence intensity during the third period indicated a Ca\(^{2+}\)-triggered fusion event. To determine the temporal arrival of Ca\(^{2+}\) in the evanescent field of our TIR microscope setup, soluble Cy5 dye was added with the Ca\(^{2+}\) buffer to monitor the emergence of fluorescence intensity. Thus, our improved single vesicle–vesicle assay enables one to monitor the association of SV vesicles,
spontaneous, and Ca\textsuperscript{2+} triggered fusion events during the same data acquisition. Further details can be found in ref. 38.

Previous reconstitutions often employed lipid mixing, rather than content mixing, between vesicles with reconstituted neuronal SNAREs\textsuperscript{51}. For example, increased ensemble lipid mixing was observed upon addition of the soluble Syt1 C2AB fragment and Ca\textsuperscript{2+} (ref. 83). However, in retrospect, this result was probably caused by an effect on vesicle association since multivalent binding of the soluble Syt1 C2AB fragment can induce vesicle clustering\textsuperscript{84}. Moreover, subsequent work revealed major differences between using the soluble C2AB fragment and reconstituted, full-length Syt1 (refs 84, 85). More importantly, assays based on lipid mixing alone can produce misleading results since lipid mixing can occur without content mixing\textsuperscript{86}. Our single vesicle-vesicle assay thus uses full-length Syt1 and it monitors content mixing, a correlate for neurotransmitter release\textsuperscript{72,85,86}.

**Syt1 conditional knockout mice.** Syt1 conditional knockout mice were generated by the European Conditional Mouse Mutagenesis Program (EUComm) and are available from the European Mouse Mutant Archive (EMMA) (EM:06829). Exon 2 of Syt1 containing the transmembrane domain is floxed and its removal results in a frameshift that produces a truncated protein. The Syt1 targeted mice were first crossed to FlPe mice to remove a gene trap cassette surrounded by Sfl sites. This cross yielded the conditional knockout mice as schematically shown in Fig. 3a. Exposure to Cre recombinase results in the total absence of synchronous release which is typical for Syt1 knockout neurons (Fig. 4a, b).

**Neuronal cultures.** Neuronal cultures were produced from wild-type and Syt1 conditional knockout mice as previously described\textsuperscript{87}. Hippocampal were dissected from P0 pups, dissociated by papain digestion, and plated on Matrigel-coated glass coverslips. Neurons were cultured in vitro in MEM supplemented with B27 (Gibco), glucose, transferrin, fetal bovine serum and Ara-C (Sigma), and were analysed after 14–16 days.

**Lentivirus production.** For rescue experiments, we used a lentiviral construct carrying a synapsin promoter, an optional rat Syt1 cDNA, internal ribosome entry site (IRES), and a GFP–Cre recombinase fusion sequence. The control plasmid (TB592) contained no rescuing cDNA, with rescuing plasmids carrying additional knockout neurons infected with viruses expressing the desired mutants.

**Electrophysiological recordings in cultured neurons.** Recordings were performed essentially as previously described\textsuperscript{88}. The whole-cell pipette solution contained 40 mM CsCl, 90 mM K-gluconate, 1.8 mM NaCl, 1.7 mM MgCl\textsubscript{2}, 5 mM Mg\textsuperscript{2+}-ATP, 0.4 mM Mg\textsuperscript{2+}-EGTA, 10 mM HEPES, 2 mM Na\textsuperscript{2+}-ATP, 0.4 mM Na\textsuperscript{2+}-EGTA, 10 mM phosphocreatine, and 10 mM QX-314 (pH 7.4, adjusted with NaOH). The bath solution contained 40 mM NaCl, 5 mM Mg\textsuperscript{2+}, 2 mM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 10 mM HEPES, 10 mM glucose (pH 7.4, adjusted with NaOH). Evoked synaptic responses were triggered by a bipolar electrode. GABA-R-mediated IPSCs were pharmacologically isolated with CNQX (20 \textmu M) in the bath solution and recorded at a 407–234 (2015).

**Results**

**Syt1 and its role in synaptic vesicle release.** Syt1 is essential for vesicle docking and priming during nerve terminal stimulation\textsuperscript{108}. Consequently, Syt1 mutants exhibit defects in vesicle release from nerve terminals\textsuperscript{9}. The first indication that Syt1 might play a role in synaptic vesicle release was the observation that Syt1+/– mice exhibit impaired facilitation and potentiation\textsuperscript{109}. These effects were shown to be due to an altered vesicle release mechanism in the Syt1+/– mice\textsuperscript{108}.

**Syt1 and synaptic vesicle release.** Syt1 is a calcium sensor that triggers vesicle release in response to an increase in intracellular calcium concentration. This function is mediated by the C2A domain of Syt1, which binds to the calmodulin-like domain (CaM-LD) of synaptotagmin\textsuperscript{110}. The CaM-LD binds to the C2A domain of Syt1, resulting in the activation of the C2B domain, which then binds to the synaptobrevin-2 (SNAP-25) and synaptophysin-2 (Syn-2) proteins to form a synapse-dependent fusion complex\textsuperscript{111}. This complex is then spontaneously activated by the influx of calcium ions through voltage-gated calcium channels, resulting in vesicle release\textsuperscript{111,112}.
Extended Data Figure 1 | Purification and crystallization of the Syt1–SNARE complex. a, Diagram of the Duet co-expression vectors (Novagen) that express the fragments of the neuronal SNARE complex and the C2AB-linker-SNAP-25_C chimera used for purification and crystallization of the Syt1–SNARE37aa-linker complex. The rat syntaxin-1A and His-tagged rat synaptobrevin-2 fragments were cloned into the vector pACYCDuet-1; the C2AB-linker-SNAP-25_C chimera and the SNAP-25_N fragment were cloned into the vector pETDuet-1 with amino acid ranges labelled. Dashed lines represent the 37-amino-acid linker (see Methods). b, The purified Syt1–SNARE37aa-linker complex eluted as a single peak during size-exclusion chromatography (profile on the left). Left gel: Coomassie-blue-stained SDS–PAGE gel of the purified Syt1–SNARE37aa-linker complex (unboiled and boiled). Right gel: Coomassie-blue-stained SDS–PAGE gel of dissolved crystals of the Syt1–SNARE complex that were grown over a period of 2 months starting from purified Syt1–SNARE37aa-linker (unboiled and boiled). Although Syt1 was initially covalently linked to SNAP-25_C, the linker was cleaved during crystallization. The comparison between boiled and unboiled lanes is a hallmark showing that neuronal SNARE complex is fully formed. c, Boiled Coomassie-blue-stained SDS–PAGE gel of the purified Syt1–SNARE37aa-linker complex in solution at ambient temperature at the specified time after purification. Cleavage is apparent on day one and progresses slowly over several days. d, Schema showing the commonly used vapour-diffusion technique: the drop contains a lower concentration of the precipitant than the reservoir. e, Schema showing a reverse vapour-diffusion method that was used for crystallization of the Ca\(^{2+}\)-bound Syt1–SNARE complex: the drop contains a higher concentration of the precipitant than the reservoir.
Extended Data Figure 2 | Diffraction images, electron density maps and crystal packing of the Syt1–SNARE complex in the long unit cell crystal form. a, Only one out of 85 screened crystals in the long unit cell crystal form diffracted to 4.1 Å resolution at the APS NE-CAT microfocus synchrotron beamline (a total of 105 crystals were screened with 20 that indexed in the short unit cell crystal form). b, A total of 61 out of ~72 crystals in the long unit cell crystal form diffracted to at least 3.5 Å resolution at the LCLS XFEL (a total of 148 crystals were diffracted, out of those 113 crystals produced 578 images that could be processed; 35 crystals did not diffract or showed multiple lattices). These exposures were taken along the crystal c axis. The left upper pictures in a and b show images of loop-mounted crystals after X-ray exposure. c, \( mF_o - DF_c \) annealed omit map (Methods) of the Ca\(^{2+}\)-bound Syt1–SNARE complex in the long unit cell crystal form using diffraction data collected at the LCLS XFEL; omitted residues within region I of the primary interface (residues 335–340 in Syt1 and 159–166 in SNAP-25) are coloured cyan. The contour level is 2.3σ. d–f, Representative \( 2mF_o - DF_c \) electron density maps of the Ca\(^{2+}\)-bound Syt1–SNARE complex in the long unit cell crystal form using diffraction data collected at the LCLS XFEL. The contour level is 1.5σ. g, Views of the crystal lattice perpendicular to the bc (left) and to the ac (right) planes of the Ca\(^{2+}\)-bound Syt1–SNARE complex in the long unit cell crystal form. The particular layer shown on the right corresponds to the red arrowhead in the left panel (only a slice corresponding to the layer is shown, creating the appearance of two disconnected groups of molecules—these groups are actually connected via interactions with the neighbouring layers). The red dashed oval indicates the ‘missing’ Syt1 C2AB fragment compared to the short unit cell crystal form (Extended Data Fig. 3d).
Extended Data Figure 3 | Asymmetric unit, electron density maps and crystal packing of the Syt1–SNARE complex in the short unit cell crystal form. a, Asymmetric unit of the Ca$^{2+}$-bound Syt1–SNARE complex in the short unit cell crystal form at 3.6 Å resolution using diffraction data collected at the APS NE-CAT microfocus synchrotron beamline (Extended Data Table 1). The colour code is the same as in Fig. 1c. Two Syt1 C2AB fragments (distinguished by the designators I and I') bind to the same SNARE complex in the asymmetric unit (see schema). b, m$F_o$ - $DF_c$ annealed omit map (Methods) of the Ca$^{2+}$-bound Syt1–SNARE complex in the short unit cell crystal form collected at the APS NE-CAT microfocus synchrotron beamline; omitted residues within region I of the primary interface (residues 335–340 in Syt1 and 159–166 in SNAP-25) are coloured cyan. The contour level is 2.3σ. Left side, without B-factor sharpening; right side, with B-factor sharpening. c, Representative $2mF_o$ - $DF_c$ electron density map of the Ca$^{2+}$-bound Syt1–SNARE complex for the short unit cell crystal form using diffraction data collected at the APS NE-CAT microfocus synchrotron beamline. The contour level is 1.5σ. Left side, without B-factor sharpening; right side, with B-factor sharpening. The sharpening B-factor ($-55\text{ Å}^2$) was set to make the lowest atomic B-factor of the short unit cell crystal form comparable to that of the long unit cell crystal form. Even with B-factor sharpening, the electron density map of the long unit cell crystal form collected at the LCLS XFEL is superior to that of the short unit cell crystal form. d, Views of the crystal lattice perpendicular to the bc (left) and to the ac (right) planes of the Ca$^{2+}$-bound Syt1–SNARE complex in the short unit cell crystal form. The particular layer shown on the right corresponds to the red arrowhead in the left panel. The unit cell is outlined by a black box.
Extended Data Figure 4 | Single-molecule FRET efficiency distributions of the Syt1–SNARE complex versus FRET efficiency values calculated from the Syt1–SNARE interfaces observed in the crystal structure. Shown are histograms of intermolecular single molecule FRET (smFRET) efficiency values that were measured between pairs of covalently attached organic labels on the Syt1 C2AB fragment and the SNARE complex (also shown as large spheres superimposed on the interfaces observed in the crystal structure). Arrowheads indicate FRET efficiencies calculated from the crystal structure of the Ca$^{2+}$-bound Syt1–SNARE complex in the long unit cell crystal form (complex I) for the primary, secondary and tertiary interfaces, using the methods and approximations described in ref. 28 to simulate the positions of dye centres in order to calculate the FRET-efficiency values. Only the dye pair combinations between the nearest C2 domain (including the C2A–C2B linker) and the SNARE complex were calculated for the three interfaces. Note that owing to the presence of transitions between different states the histograms reflect a combined effect of interaction interfaces. The label at position A61 would have disrupted the tertiary interfaces between the C2A domain and the SNARE complex, explaining the discrepancy for these labels (indicated by open triangles). In retrospect, the top smFRET-derived model and the primary interface observed in the crystal structure primarily differed in the orientation of the C2B domain. Moreover, the top smFRET-derived model predicted the approximate location of the primary interface on the neuronal SNARE (see Fig. 4c in ref. 28).
a. Primary interface
long unit cell crystal form vs short unit cell crystal form

b. Secondary interface
long unit cell crystal form vs short unit cell crystal form

c. Other interfaces
long unit cell crystal form vs short unit cell crystal form

d. Ca²⁺-bound vs Mg²⁺-bound Syt1-SNARE complex
(Short unit cell crystal form)

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Extended Data Figure 5 | Comparison of the two crystal forms and the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound crystal structures of the Syt1–SNARE complex.

a, Superposition of the primary interfaces of the Ca\(^{2+}\)-bound Syt1–SNARE complex structure in the long unit cell crystal form (gold and bright orange) and in the short unit cell crystal form (white). The primary interface is very similar in both crystal forms: the r.m.s.d. for the primary interface between both crystal forms is 0.38 Å (bright orange) and 0.42 Å (white) for complex I and complex II, respectively (including Cα atoms of the SNARE complex and the Syt1 C2B (I) domain forming the interface).

b, Superposition of complex I in the long unit cell crystal form with the asymmetric unit of the short unit cell crystal form, but only showing the secondary interface (light-blue shaded disk) between Syt1 C2B (I’9) and the SNARE complex (I). The bottom panels show close-up views of the secondary interface: left, interacting residues (sticks and balls); right, a 90° rotated view of the view shown in the left panel. The Syt1 C2B (I’9) domain is rotated by 16° between the two crystal forms and, as a consequence, the interactions between residues R281, K288 and R398 of the Syt1 C2B (I’9) domain and residues E224 and E228 of syntaxin-1A are slightly changed by this rotation. Notably, residues Syt1 R281, K288 and R398 are involved in both the primary (Fig. 2) and secondary interfaces.

c, Superposition of complex I in the long unit cell crystal form with the asymmetric unit of the short unit cell crystal form, showing all interfaces.

d, Superposition of the Ca\(^{2+}\)-bound (white) and Mg\(^{2+}\)-bound (black) crystal structures of the Syt1–SNARE complex, both in the short unit cell crystal form. The lower left panel shows a close-up view of the primary interface, indicating that it is very similar in both the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound crystal structures. The Syt1 C2B domain that forms the secondary interface (light-blue shaded disk) is rotated by 19° between the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound complexes. The lower-right panel is a rotated view of the complex, also showing the tertiary interface (light-green shaded disk), and the C2A–C2B interface that involves asymmetry-related Syt1 C2A domain (I’9) (grey shaded disk).

e, B-factor coloured cartoon representations of the asymmetric units of the Ca\(^{2+}\)-bound long unit cell crystal form (top), the Ca\(^{2+}\)-bound short unit cell crystal form (bottom left), and the Mg\(^{2+}\)-bound short unit cell crystal form (bottom right) of the Syt1–SNARE complex. Note that the primary interfaces have relatively low B-factors, similar to the majority of the structure, while parts of the C2A and C2B domains involved in the secondary and tertiary interfaces have higher B-factors, possibly indicating increased flexibility.
Extended Data Figure 6 | Sequence alignments of Syt1, SNAP-25 and syntaxin-1A from different homologues. 

**a**, Sequence alignment of Syt1 homologues, showing the sequences around the primary interface of the Syt1–SNARE complex. Note that rat Syt5 refers to UniProt ID Q925C0, zebrafish Syt9 refers to GeneBank accession number AA152175, rat Syt9 refers to UniProt ID P47861, and human Syt9 refers to UniProt ID O00445.

**b**, Electrostatic potential surfaces of the known crystal structures of synaptotagmin-1, synaptotagmin-3, synaptotagmin-4 and synaptotagmin-7; the dashed rectangles indicate the regions that correspond to the primary interface regions I and II of the Syt1–SNARE complex.

**c**, Sequence alignment of different SNAP-25 homologues, showing the sequences around the primary interface of the Syt1–SNARE complex.

**d**, Sequence alignment of different syntaxin homologues, showing a sequence range around the primary interface of the Syt1–SNARE complex. In all panels, the interacting residues of the primary interface are indicated by solid circles and coloured boxes for region I (cyan) and region II (red/orange).
Extended Data Figure 7 | Syt1 mutants and SNARE complexes with SNAP-25 mutants are well folded. a, Top panels: CD spectra of wild-type and mutant Syt1 C2B domains in the absence of Ca^{2+}. Bottom panels: thermal denaturation was monitored by molar ellipticity at a wavelength of 216 nm in the absence of Ca^{2+} (black) and in the presence of 5 mM Ca^{2+} (red). The specified melting temperatures were estimated as the mid-point of the melting curves (Methods). b, Superposition of the Syt1 C2B domains from the Ca^{2+}-bound Syt1–SNARE complex in the short unit cell crystal form (gold), the crystal structure of the quintuple mutant (R281A/E295A/Y338W/R398A/R399A) of the Syt1 C2B domain (green), and the crystal structure of the isolated Syt1 C2B domain (white, PDB code 2YOA). c, d, Representative m2Fo – DFo electron density maps of the crystal structure of the quintuple mutant of the Syt1 C2B domain (Extended Data Table 1) contoured at 2.0σ. The labels refer to the mutated residues. e, Overlay of SEC profiles of full-length Syt1 mutant proteins used in the single vesicle–vesicle fusion assay (Fig. 3d–g). f, Coomassie-blue-stained SDS–PAGE with and without boiling of neuronal SNARE complexes formed by full-length SNAP-25 and its mutants, syntaxin-1A and synaptobrevin-2, using the proteins that were used in the single vesicle–vesicle fusion assay (Methods).
Ca\textsuperscript{2+}-triggered fusion

Spontaneous fusion

Ca\textsuperscript{2+}-triggered fusion

Spontaneous fusion

Ca\textsuperscript{2+}-triggered fusion

Spontaneous fusion

Mock injection
Extended Data Figure 8 | Probability of fusion versus time upon 500 μM Ca$^{2+}$ injection and spontaneous fusion for Syt1 and SNAP-25 mutants. Shown are the data that were used to generate Fig. 3d–g. The number of independent experiments and analysed events are provided in Extended Data Table 2. a–d, Cumulative histograms of probability of fusion versus time for Syt1 mutants upon 500 μM Ca$^{2+}$ injection (a) and spontaneous fusion (b), and SNAP-25 mutants upon 500 μM Ca$^{2+}$ injection (c) and spontaneous fusion (d). e–g, Control experiments: e, Ca$^{2+}$-triggered fusion; f, spontaneous fusion with 3 mM ATP, without SNAP-25 or Syt1; and g, mock injection without Ca$^{2+}$. 
## Extended Data Table 1 | Crystallographic data and refinement statistics

| Data collection | Ca\textsuperscript{2+}-bound Syt1–SNARE complex (long unit cell crystal form) | Ca\textsuperscript{2+}-bound Syt1–SNARE complex (short unit cell crystal form) | Mg\textsuperscript{2+}-bound Syt1–SNARE complex (short unit cell crystal form) | Quintuple mutant of Syt1 C2B |
|----------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------------|
| Beamline       | SLAC-LCLS                                                                        | APS-NECAT                                                                        | APS-NECAT                                                                        | APS-NECAT                   |
| Space group    | P2\textsubscript{1};2/\textsubscript{1}                                           | P2;2/2                                                                           | P2;2/2                                                                           | P2;2/2                      |
| Cell dimensions|                                                                                  |                                                                                  |                                                                                  |                              |
| a, b, c (Å)    | 69.6, 171.1, 291.9                                                              | 69.1, 171.6, 146.9                                                              | 69.1, 171.8, 146.6                                                              | 68.9, 107.6, 110.8          |
| α, β, γ (°)    | 90.0, 90.0, 90.0                                                                 | 90.0, 90.0, 90.0                                                                 | 90.0, 90.0, 90.0                                                              | 90.0, 90.0, 90.0            |
| Resolution (Å) | 20.0-3.5  (3.62-3.50)\textsuperscript{*}                                        | 50.0-3.60 (3.73-3.60)                                                           | 85.9-4.10 (4.32-4.10)                                                          | 50.0-1.65 (1.69-1.65)       |
| R\text{merge} (%)(rotation)\textsuperscript{1} | 39.7 (32.2)                                                                        | 92.7 (35.5)                                                                       | 99.7 (64.6)                                                                     | 99.7 (78.3)                 |
| R\text{merge} (%)(still)\textsuperscript{1} | 6.7 (74.1)                                                                         | --                                                                               | 10.4 (64.6)                                                                    | 6.40 (91.7)                 |
| CC1/2          |                                                                                   | 12.3 (1.9)                                                                        | 8.8 (1.9)                                                                       | 18 (2.3)                    |
| Completeness (%)| 87.6 (65.6)                                                                        | 99.7 (98.7)                                                                       | 95.9 (97.0)                                                                     | 99.0 (98.5)                 |
| Multiplicity (rotation)\textsuperscript{1} | 5.0 (1.8)                                                                          | 14.5 (12.4)                                                                       | 3.4 (3.5)                                                                      | 6.7 (6.8)                   |
| Multiplicity (still)\textsuperscript{1} | --                                                                                | --                                                                               | --                                                                              | --                          |

### Refinement

| Resolution (Å) | 20.0-3.50 (3.62-3.50) | 50.0-3.60 (3.73-3.60) | 85.9-4.10 (4.32-4.10) | 50.0-1.65 (1.71-1.65) |
|----------------|-----------------------|-----------------------|-----------------------|-----------------------|
| No. reflections| 39174 (2884)          | 20846 (2004)          | 13519 (1320)          | 98575 (9676)          |
| R\text{merge} | 0.322 / 0.353         | 0.249 / 0.289         | 0.276 / 0.323         | 0.150 / 0.178         |
| No. atoms     | Protein: 10890         | 6506                  | 6510                  | 5033                  |
|                | Ca\textsuperscript{2+} | 19                    | 7                     | 0                     |
|                | Mg\textsuperscript{2+} | 0                     | 0                     | 0                     |
| B-factors     | Protein: 49            | 158                   | 194                   | 23.1                  |
|                | Ca\textsuperscript{2+} | 32                    | 187                   | --                    |
|                | Mg\textsuperscript{2+} | --                    | --                    | 202                   |
| R.m.s. deviations | Bond lengths (Å) | 0.003                  | 0.004                  | 0.003                  | 0.019                  |
|                | Bond angles (°) | 0.758                  | 0.862                  | 0.714                  | 1.763                  |

\textsuperscript{*}Values in parentheses are for the highest resolution shell.
\textsuperscript{1}“(rotation)” refers to rotation diffraction data collected at the APS synchrotron and “(still)” refers to still diffraction data collected at the LCLS XFEL.
## Extended Data Table 2 | Data summary table for the single vesicle–vesicle fusion experiments with Syt1 and SNAP-25 mutants

|                  | No. of spontaneous fusion events | No. of Ca\(^{2+}\) triggered fusion events | Total no. of analyzed events (no. of associated vesicle pairs) | No. of independent experiments (N) |
|------------------|----------------------------------|-------------------------------------------|---------------------------------------------------------------|-----------------------------------|
| Syt1 WT          | 90                               | 167                                       | 3764                                                         | 5                                 |
|                  | 100                              | 262                                       | 4632                                                         | 6                                 |
|                  | 49                               | 152                                       | 3035                                                         | 5                                 |
|                  | 64                               | 173                                       | 3564                                                         | 4                                 |
|                  | 159                              | 256                                       | 6503                                                         | 9                                 |
| **Syt1 mutants** |                                  |                                           |                                                              |                                   |
| E295A/Y338W      | 113                              | 246                                       | 5872                                                         | 12                                |
| R398Q/R399Q      | 166                              | 217                                       | 6724                                                         | 7                                 |
| R281A/R398A/R399A| 133                              | 271                                       | 5134                                                         | 7                                 |
| Syt1 quintuple   | 49                               | 143                                       | 5241                                                         | 7                                 |
| **SNAP-25 mutants** |                                |                                           |                                                              |                                   |
| K40A/D166A       | 61                               | 139                                       | 2549                                                         | 7                                 |
| D51A/E52A/E55A   | 81                               | 150                                       | 2717                                                         | 7                                 |
| SNAP-25 quintuple| 105                              | 261                                       | 6741                                                         | 9                                 |
| **Control**      |                                  |                                           |                                                              |                                   |
| +ATP             | 59                               | 129                                       | 2830                                                         | 5                                 |
| −SNAP-25         | 24                               | 36                                        | 3040                                                         | 5                                 |
| −Syt1            | 57                               | 58                                        | 2379                                                         | 5                                 |
| Mock             | --                               | 25                                        | 1947                                                         | 5                                 |