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Molecular mechanisms of fast neurotransmitter release

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Abstract

This review summarizes recent insights in the cooperation of synaptic proteins that are central to synaptic vesicle fusion in presynaptic active zones, including SNAREs, synaptotagmin, complexin, Munc18, and Munc13. Structural and functional studies of the synaptic fusion machinery suggest new molecular models of synaptic vesicle priming and Ca^{2+}-triggered fusion. These studies will be a stepping-stone towards answering the question of how the synaptic vesicle fusion machinery achieves such high speed and sensitivity.

Keywords

synaptic vesicle fusion; fusion protein; action potential; synaptic vesicle priming; Ca^{2+}-triggering

1. Introduction

During synaptic transmission, Ca^{2+} influx into the presynaptic terminal triggers neurotransmitter release. This process involves sensing Ca^{2+}, and subsequently fusing neurotransmitter-filled synaptic vesicles with the presynaptic membrane in less than a millisecond (122, 139). In the neuron, neurotransmitters are released “synchronously” upon action potential arrival in the synaptic terminal, “asynchronously” with a certain delay after an action potential, and “spontaneously” without an action potential; each of these processes likely involves specific sets of synaptic proteins (69). Many, if not most, of the key factors of the core synaptic fusion machinery have been identified, including SNAREs (Soluble *N*-ethylmaleimide sensitive factor Attachment protein REceptor), NSF (N-ethylmaleimide-sensitive factor), SNAP (soluble NSF adaptor protein), synaptotagmin (abbreviated as Syt), complexin (abbreviated as Cpx), Munc18 (mammalian uncoordinated-18) and Munc13 (mammalian uncoordinated-13). Yet, important questions remain: how is the process tightly regulated by Ca^{2+}, and how is it triggered within less than a millisecond, much faster than any other biological membrane fusion process? We will first summarize the current knowledge about each *individual* factor in an encyclopedic fashion (Sections 1–7), and then

discuss recent insights into the cooperation and interplay between these factors for neurotransmitter release (Sections 8–10). The reader may wish to skip to these sections first and consult the sections on the individual factors when more information is needed.

2. SNAREs

In this review, we focus on the neuronal SNAREs and their most extensively studied isoforms. Prior to membrane fusion, synaptobrevin-2 (also called VAMP2 – Vesicle Associated Membrane Protein 2) on the synaptic vesicle, and syntaxin-1A and SNAP-25A on the plasma membrane initially form a *trans* SNARE complex with the transmembrane domains of synaptobrevin-2 and syntaxin-1A in their respective membranes (Fig. 1). Moreover, individual syntaxin-1A molecules form clusters in the plasma membrane that are segregated from phosphatidylinositol-4,5-bisphosphate (PIP2) domains (149). During fusion, the SNARE complex completely zippers into the fully assembled *cis* SNARE complex where the soluble core consists of a parallel four α-helix bundle (144). The synaptobrevin-2 and syntaxin-1A helices of the *cis* SNARE complex extend into the merged membrane (136). The topology of the core of the SNARE complex is a left-handed four-helix coiled coil where the core consists of layers of primarily hydrophobic residues, except for an ionic layer at the center of the complex consisting of three glutamine amino acid residue and one arginine residue (referred to as ionic or zero layer) (39, 144). In addition to this parallel four α-helix bundle configuration, SNAREs can form a variety of non-canonical stoichiometries and configurations *in vitro* (reviewed in ref. (17)), and we will discuss potential physiological roles of these non-canonical assemblies in Section 10.

The assembly of the SNARE complex is thought to provide the energy necessary for membrane fusion (144, 158). Single-molecule optical and magnetic trap pulling experiments suggest that the free energy that is released by the zippering of one SNARE complex is approximately 36 k_BT (42, 103), and that a half-zippered intermediate exists under “loaded” conditions (*i.e.*, when the juxtamembrane regions of the SNARE complex are steadily pulled by a force apparatus), suggesting that the *trans* SNARE complex may consist of a partially zippered complex. However, we note that direct imaging of the *trans* SNARE complex has not yet been achieved. Nevertheless, the estimated free energy that is released by formation of one SNARE complex is somewhat less than the energy that is required to overcome the hydration-force barrier for the formation of a lipid stalk (*i.e.*, the dehydration energy of ~40–90 k_BT that is required to induce stalk formation) (2). Additional energy may be required for pore formation and expansion (28). Consistent with this notion, SNAREs mediate membrane exchange when reconstituted into proteoliposomes (158). As little as one SNARE complex (148) is sufficient for ensemble lipid mixing of proteoliposomes, but at least two synaptobrevin-2 molecules, and presumably two SNARE complexes, are required for fast Ca^{2+}-triggered exocytosis (131). Although these lipid mixing experiments demonstrate that SNAREs mediate membrane exchange, content mixing is the proper correlation for neurotransmitter release since lipid mixing can occur in the absence of full fusion due to metastable hemifusion diaphragms (35, 54, 78). Moreover, dequenching of the fluorescent dyes used in lipid mixing assays may occur due to other phenomena (186). The first genuine content mixing experiment for precisely timed Ca^{2+}-triggered fusion with synaptic proteins was reported by (78, 79) (Fig. 2) (for a review of other assays and more details see (18)).

Over the years, this single-vesicle fusion assay has progressed to include more molecular components with the goal of a more complete physiological reconstitution (35, 80–82) (Section 10).

3. Synaptotagmin

In addition to SNAREs, synaptotagmins (Syts) constitute an evolutionary conserved family of proteins (105) that are composed of an N-terminal single transmembrane-spanning domain, a variable juxtamembrane linker and two C-terminal cytoplasmic C2 domains, termed C2A and C2B, respectively (112). In this review we focus on synaptotagmin-1 (Syt1), which resides on the synaptic vesicle and is vital for synchronous Ca^{2+}-triggered synaptic vesicle fusion (41, 43).

Syt2 and Syt9 are also involved in Ca^{2+}-triggered synchronous neurotransmitter release for different subsets of neurons (167), while Syt7 mediates asynchronous release (5, 160). In addition, Syt1 and Syt7 together act as redundant Ca^{2+} sensors for neuroendocrine exocytosis (125, 141), and they also participate in postsynaptic AMPA receptor exocytosis during long term potentiation (LTP) (164).

Syt1 interacts with anionic membranes and SNARE complexes in both Ca^{2+}-dependent and Ca^{2+}-independent manners (7, 15, 16, 22, 26, 31, 41, 73, 77, 111, 152, 156, 184). Syt1 function and membrane binding is specific to Ca^{2+}, *i.e.*, other divalent cations, such as Sr^{2+}, only weakly trigger synaptic vesicle fusion (38). Under certain experimental *in vitro* conditions, (*i.e.*, in the presence of Ca^{2+}), the Syt1 C2B domain or the C2A-C2B fragment binds to curved membranes or favors deformation of the membrane (60, 93), along with membrane clustering (3). Computer simulations support the notion that both C2 domains cooperate to induce membrane bending (165). Moreover, the linker between the C2 domains is important for Syt1 function in neurons (6, 87), and both C2 domains can insert into the same membrane at the same time upon Ca^{2+}-binding *in vitro* (59). Additionally, the juxtamembrane linker domain—the domain between the transmembrane domain and the C2 domains—is important for Syt1 function in fusion assays (83) and in neurons (85). Thus the relative locations of the C2 domains are critical for proper function, but the precise location of Syt C2 domains before and after Ca^{2+}-triggering remains to be directly visualized.

While mutations in the Ca^{2+}-binding sites of the Syt1 C2A domain can partially rescue Syt1-deficient mammalian neurons, mutations in the Ca^{2+}-binding sites of the Syt1 C2B domain cannot (107, 130, 168). The mutations in the Syt1 C2B domain not only block Ca^{2+}-evoked synchronous release in an intact synapse, but also dominantly inhibit the ability of endogenous wild-type Syt1 to trigger fusion, consequently increasing spontaneous miniature release when co-expressed (84, 164, 185).

SNAREs and Syt1 alone are sufficient to promote Ca^{2+}-triggered proteoliposome lipid mixing (147). However, as mentioned above, lipid mixing does not necessarily correlate with neurotransmitter release since lipid mixing can occur without content mixing. While SNAREs and Syt1 can indeed promote full fusion (*i.e.*, content mixing) upon Ca^{2+}-triggering (35, 78) with a ratio of Ca^{2+}-dependent fusion to Ca^{2+}-independent fusion of ~ 10

(82), Ca^{2+}-triggered fusion with this minimal system is relatively inefficient. As discussed in Section 10, a more complete reconstitution greatly increases the efficiency and synchrony of Ca^{2+}-triggered fusion.

The first crystal structure of a Syt C2 domain (143) revealed that there are no large conformational changes upon Ca^{2+} binding. Rather, the C2 domain acts as an electrostatic switch where Ca^{2+}-binding neutralizes the negative charges in that region as suggested by NMR experiments (127). Structures of complexes between Syts and the SNARE complex have been extremely difficult to obtain due to the multiple binding modes of the components of the complex (15, 26), and it had been questioned if SNARE-Syt interactions are functionally important (110). It is only recently that crystal structures of SNARE/Syt1 and SNARE/Cpx1/Syt1 complexes have been obtained and functionally validated (184, 185) (Section 9).

4. Complexin

The cytoplasmic protein complexin (Cpx) also plays critical roles in neurotransmitter release. Specifically, synchronous evoked neurotransmitter release depends on Cpx (99), and this activating role of Cpx is conserved across all species and different types of Ca^{2+}-induced exocytosis studied to date (21, 55, 61, 67, 94, 97, 119, 173, 175, 177). Cpx also regulates spontaneous release, although this effect is less conserved among species and experimental conditions: for example, in *Drosophila*, spontaneous release increases with knockout of Cpx compared to wildtype neurons (65, 171). Likewise, knockdown in cultured cortical mouse neurons increases spontaneous release compared to wild-type neurons, although knockout of Cpx only affects spontaneous release depending on the particular neuronal cell type (67, 97, 146, 175).

We focus here on the homolog complexin-1 (Cpx1) of the mammalian Cpx family whose primary sequence is highly conserved (96%) in mouse, rat, and human. Cpx1 consists of four domains: the N-terminal domain is important for activation of synchronous Ca^{2+}-triggered release in murine neurons (97, 170, 172) and in isolated chromaffin cells (33); the accessory domain regulates spontaneous release (97); the central domain is required for all functions of Cpx1 and it binds with high affinity (~10 nM) to the neuronal ternary SNARE complex (24, 99, 109); and the C-terminal domain is involved in vesicle priming and binds to anionic membranes in a curvature sensitive fashion (24, 47, 67, 97, 133).

Structurally, in isolation, both the N- and C-terminal domains of Cpx1 are largely unstructured, while the accessory and central domains have α-helical propensity (108). The central domain of Cpx1 binds to the groove formed by the synaptobrevin-2 and syntaxin-1A α-helices in the SNARE complex (14, 24). This interaction is anti-parallel, *i.e.*, the direction of the α-helix of the accessory domain of Cpx1 is anti-parallel to the direction of the α-helices in the ternary SNARE complex.

The accessory domain of Cpx1 is not required for activation of Ca^{2+}-triggered single-vesicle fusion with reconstituted neuronal SNAREs and Syt1 (81), and mutations of this domain have no effect on evoked release in neurons (176). However, mutations of the Cpx1

accessory domain affect spontaneous release (25, 176), and elimination of the accessory domain increases Ca^{2+}-independent single-vesicle fusion (content mixing) (81) compared to wild-type control. In another study, introduction of an α-helix breaking mutation into the accessory domain of Cpx1 increased spontaneous release in *C. elegans* (117).

Functional studies, along with molecular modeling suggested that Cpx1 may inhibit full ternary SNARE complex formation by preventing the C-terminal part of synaptobrevin-2 from binding to the syntaxin-1A/SNAP-25A subcomplex of the ternary SNARE complex (45, 46). However, a subsequent crystal structure of a mutant of Cpx1 (D27L, E34F, R37A) in complex with a partially truncated SNARE complex (containing a truncated synaptobrevin-2 fragment), along with isothermal titration calorimetry (ITC) and light scattering experiments instead found that Cpx1 bridges two partially truncated SNARE complexes: one partial SNARE complex binds to the central domain of Cpx1 while another partial SNARE complex binds weakly to the accessory domain of the same Cpx1 molecule (PDB IDs 3RK3 and 3RL0) (75, 76). Although this weak interaction of the accessory domain is observable by ITC (75, 116), it was not observed by NMR (146), perhaps due to differences in constructs (75, 116). Finally, single-molecule experiments demonstrated that the accessory domain also weakly interacts with the syntaxin-1A/SNAP-25A (binary) complex (27). Moreover, these single-molecule studies showed that both binding modes of the Cpx1 accessory helix are possible, reconciling the previous results. However, the biological context of these weak interactions involving the accessory domain remains to be elucidated.

The C-terminal domain of Cpx1 binds to phospholipids (67, 126) and it is important for vesicle priming in neurons (33, 34, 67). Moreover, Cpx1 without the C-terminal domain does not reduce spontaneous release, but it still activates Ca^{2+}-triggered release in neuronal cultures (67) and in a reconstituted system (82). The C-terminal domain is sensitive to membrane curvature, and it may thus localize Cpx1 to the synaptic membrane (47, 133, 163). Interestingly, the curvature sensing value of the C-terminal domain is comparable to that of α-synuclein (47, 63), *i.e.*, a value that corresponds to the relatively high curvature of synaptic vesicles with a typical diameter of 40 nm. In support of this notion, replacement of the C-terminal domain with plasma membrane-localizing elements, results in increased spontaneous neurotransmission and a prolonged synchronous decay time-constant of NMDA-type glutamate receptor evoked postsynaptic currents in cultured neurons (47).

In conjunction with neuronal SNAREs and Syt1, Cpx1 increases the Ca^{2+}-triggered amplitude and synchrony of proteoliposome fusion, and it also suppresses Ca^{2+}-independent single vesicle fusion (content mixing) (82), resulting in a 50–100-fold ratio of Ca^{2+}-triggered fusion to Ca^{2+}-independent fusion. Moreover, the functions of the four domains of Cpx1 have been reproduced *in vitro* (81, 82). By varying the Cpx1 concentration, these single-vesicle studies suggest that the regulatory effect on Ca^{2+}-independent fusion and the facilitating role on Ca^{2+}-triggered fusion are governed by distinct molecular mechanisms involving different subsets of the four domains of Cpx1. Similarly, genetic experiments in *Drosophila* also suggest distinct mechanisms for activation of fast synchronous release and regulation of spontaneous release (25).

5. Munc18

Sec1/Munc18 (SM) proteins are also required components of all membrane trafficking pathways as exemplified by the block of synaptic vesicle fusion upon Munc18–1 knockout in mice (151). Following disassembly of SNARE complexes with NSF and SNAP, Munc18–1 captures syntaxin-1A, locking it into a “closed” conformation and preventing reassembly of the ternary SNARE complex (91). Munc18–1 binds tightly (K_d ~1–4 nM) to syntaxin-1A in which the N-terminal three helix bundle of syntaxin-1A (H_{abc} domain) forms a *cis* conformation with the syntaxin-1A SNARE motif (H3 domain) (19, 23, 104), in turn hindering SNARE complex formation (114, 174).

Moreover, Munc18 co-localizes with syntaxin and SNAP-25 in fixed cultured neurons and forms nano-clusters in the plasma membrane (113). Interestingly, in neuroendocrine cells, transfection of syntaxin alone results in its mislocalization, whereas co-transfection of both Munc18 and syntaxin results in proper trafficking to the plasma membrane (100, 123). Thus, one function of Munc18 may be to stabilize or “chaperone” syntaxin, which also agrees with the aforementioned knockout studies of Munc18 in mice that showed a reduced level of syntaxin-1 in the plasma membrane (13). The crystal structure of the Munc18–1/syntaxin-1A complex revealed extensive interactions of syntaxin-1A in the closed conformation with the concave surface formed by domains 1 and 3 of Munc18–1 (104), explaining the inability of the syntaxin-1A H3 domain in this closed conformation to interact with SNAP-25A and synaptobrevin-2.

Binding of Munc18–1 to the closed conformation of syntaxin-1A is not always required, as the syntaxin-1A H_{abc} domain is not essential for evoked synaptic vesicle fusion in cultured syntaxin-1A-deficient neurons, although it is required for spontaneous synaptic vesicle fusion (183). In marked contrast, the N-terminal residues of syntaxin-1A are required for binding of the ternary SNARE complex to Munc18–1, and may thereby serve as a recruiting factor for Munc18–1 (118). Munc18–1 binds to the assembled ternary SNARE complex via the syntaxin-1A N-terminal residues, with syntaxin-1A in an “open” conformation (128) (32, 58, 169). In this open conformation, the H3 domain of syntaxin-1A interacts with its partner SNARE motifs to form the SNARE complex (144), while the syntaxin-1A H_{abc} domain is presumably flexibly linked to the four-helix core of the SNARE complex (51, 106).

6. Munc13

Munc13–1 and its homologues are primarily brain-specific, cytoplasmic proteins in the presynaptic terminal that are involved in synaptic vesicle priming and short-term synaptic plasticity (138, 139). Munc13–1 is expressed in all neurons of the rat central nervous system, while Munc13–2 and Munc13–3 are mostly restricted to different brain regions. The ubiquitously expressed splice variant ubMunc13–2 and the Munc13–4 isoform are predominantly expressed in non-neuronal cells (72). Munc13–4 only includes the C2B, MUN, and C2C domains (Domain functions will be detailed below). The UNC-13 and Dunc-13 homologs of Munc13–1 lack the N-terminal C2A domain, as do a brain-specific

splice-variant of bMunc13–2 and the Munc13–3 isoform. Here we will focus on the Munc13–1 isoform.

Early studies found that neurons in mice lacking Munc13–1 exhibit severely limited activity-dependent as well as hypertonic sucrose-induced neurotransmitter release (4). Moreover, deletion of Munc13–1 results in substantially larger distances between synaptic vesicles and active zones as observed by electron microscopy (50, 62, 159). The priming phenotype of Munc13–1 knockout is much stronger than that of other priming factors, including Cpx1 (175), *i.e.*, the size of the readily releasable pool of synaptic vesicles is much smaller in Munc13–1 knockout mice compared to Cpx1 knockout mice. Moreover, Munc13–1, together with Munc18–1, prevents NSF-dependent de-priming of synaptic vesicles (53). Finally, Munc13–1 and −2 double-knockout mice have essentially no release-competent synaptic vesicles (150) and exhibit a severe docking defect (62).

Below, we briefly summarize structural and functional studies for each domain of Munc13–1. Munc13–1 consists of a C1 domain (C1), a calmodulin-binding domain, two C2 domains (C2A, C2B), and a MUN domain, and another C2 domain (C2C). The crystal structure of the C1C2BMUN fragment of Munc13–1 revealed inter-domain interactions between the C1, C2B, and MUN domains and a folded linker region between the C1 and C2B domains (166). An electron cryo-tomography (cryoET) study of vesicles with bound C1C2BMUN revealed filamentous features that suggest hinge bending of this fragment, with the straight conformation being consistent with the crystal structure (44).

Functionally, the C2A domain of Munc13–1 interacts with the Rab3 effector RIM, forming a tripartite complex (36) which is important for the priming function of Munc13–1 and for the presynaptic localization in mice (68). Likewise, the C2A domain of UNC-13 in *C. elegans* is important for regulating both evoked and spontaneous release, as well as its precise localization to the active zone (182).

Munc13–1 and the ubiquitously expressed isoform Munc13–2 contain a conserved calmodulin-binding domain. When calmodulin binding is disrupted by mutation, evoked release upon a single action potential is unchanged, but short-term plasticity and Ca^{2+}-dependent modulation of the readily-releasable pool is reduced (66).

The diacyglycerol (DAG)-phorbol C1 binding domain regulates Munc13–1 functions in short-term synaptic plasticity and priming of synaptic vesicles, although mutation of the site does not affect isolated evoked neurotransmitter release (120). The C2B domain binds to phospholipids in a Ca^{2+}-dependent fashion, with particular specificity for phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP2). Additionally, an interaction between the C2B domain and presynaptic voltage-gated calcium channels modulates use-dependence of the channels (20). Interestingly, deletion of the C2B domain enhances Ca^{2+}-triggered exocytosis in *C. elegans* neurons, suggesting an auto-inhibitory function of this domain (102).

The MUN domain of Munc13–1 has two major roles in vesicle fusion: catalyzing the transit of syntaxin-1A from the syntaxin-1A/Munc18–1 complex into the ternary SNARE complex (10, 91, 178) and promoting the proper assembly of the SNARE complex, in particular the

parallel configuration of syntaxin-1A and synaptobrevin-2 (80). Supporting this latter role, the so-called LE mutant of syntaxin-1A (syntaxin-1A^{LE}) (37) bypasses the requirement of Munc13–1 for the transit of syntaxin-1A into the ternary SNARE complex in presence of SNAP-25A and synaptobrevin-2 *in vitro* (90). It accomplishes this by preferentially adopting an open conformation of syntaxin-1A (155). However, this open syntaxin-1A mutant can only partially restore neurotransmitter release in *unc-13* mutants of *C. elegans* (50), because it does not promote the proper assembly of the SNARE complex, *i.e.*, SNARE complexes with the syntaxin-1A LE mutant are scrambled (80); this observation explains the poor rescue of Munc13 deletion by this mutant. Earlier studies in *C. elegans* suggest a similar function of full-length *unc-13* (121), although the role of the MUN domain has not been directly studied in that system.

Finally, the C2C domain of Munc13–1 also plays a functional role in evoked release in chromaffin granule secretion and neurotransmission in *C. elegans* (92, 137), and in evoked release in priming in mouse neurons (88). It also enhances ensemble lipid and single-vesicle content mixing experiments (80, 88).

Similar to Munc13, CAPS proteins are members of an extended Munc13/CAPS family of proteins that are also important for priming of synaptic vesicles (64). They contain one Munc13 homology domain (MHD) that comprises part of the MUN domain, and a pleckstrin homology domain that interacts with PIP2. It is likely that the MHD domain in CAPS proteins is partially responsible for their function in synaptic priming (30). Both Munc13s and CAPS proteins are probably jointly important for synaptic vesicle priming. In support of a partially redundant function of both molecules, the C1C2BMUN fragment of Munc13 can substitute for deletion of CAPS in Ca^{2+}-triggered dense core vesicle fusion with a planar supported bilayer (74).

7. NSF and SNAPs

Historically, the ATPase NSF was first discovered as a key factor for inter-compartmental transport in eukaryotic cells and, subsequently, SNAREs were found to be substrates for NSF (9, 134). Independently, SNAREs were discovered to be important for neurotransmitter release (11, 12, 140). In concert with the adaptor protein, SNAP, the ATPase NSF disassembles the SNARE complex into individual proteins upon ATP hydrolysis (51, 98, 135). NSF is a member of the AAA+ family consisting of two ATPase rings (known as Type II AAA+), and an N-terminal domain. Most eukaryotic organisms encode only one NSF gene. By contrast, there are three homologues of SNAP, with αSNAP being the most widely studied. Although earlier work suggested that disruption of the SNARE complex by ATP hydrolysis drives fusion (135), subsequent work clarified that it is actually the SNARE complex formation that drives fusion (51, 98). For more structural details of NSF and SNAPs we refer to a recent review (180).

8. Cooperation between Syt1, Cpx1 and SNAREs for evoked release

SNARE/Cpx1/Syt1 synaptic fusion complex

As mentioned in sections 1–3, Syt1, Cpx1 and SNAREs are all important for evoked release upon action potential arrival in the synaptic terminal (97, 168). The structures of the neuronal SNARE/Cpx1 subcomplex (24) and of the SNARE/Syt1 subcomplex (184), along with functional studies, suggested that each of these binary interactions are essential for neurotransmitter release. For example, the 4M mutant of Cpx1 that disrupts binding to the ternary SNARE complex neither rescued deletion of wildtype Cpx1 in neuronal cultures (97), nor increased the Ca^{2+}-triggered amplitude in single-vesicle fusion experiments with reconstituted SNAREs and Syt1 (82). Similarly, disruption of the primary SNARE/Syt1 complex interface by mutations abolished fast synchronous release in cultured neurons and greatly reduced the Ca^{2+}-triggered amplitude in single-vesicle fusion experiments (184).

The primary SNARE/Syt1 interface is structurally conserved in very different crystal packing environments and conditions (Fig. 5) (184, 185), demonstrating that it is a genuine and specific interface. In the presence of anionic phospholipid membranes, the polybasic region of Syt1 C2B primarily interacts with the membrane (111), and this membrane interaction likely stabilizes the SNARE/Syt1 primary interface (184), lowering the dissociation constant by about an order of magnitude (156). Based on primary sequence alignment, the SNARE/Syt1 primary interface is specific for fast Ca^{2+} sensors (Syt1, Syt2, Syt9) (167, 184).

The binary SNARE/Syt1 and SNARE/Cpx1 interactions alone do not explain certain experimental results. For example, mutation of the Ca^{2+}-binding region of the C2B domain of Syt1 has dominant negative effects on both evoked and spontaneous neurotransmitter release (84, 164) while deletion of Cpx1 abrogates these dominant negative phenotypes (185), suggesting a tripartite cooperation between SNAREs, Cpx1, and Syt1. The structure of the *tripartite* SNARE/Cpx1/Syt1 complex (Fig. 6) (185) explained these experimental results for the first time since it revealed a new interface between one Syt1 C2B domain and both the SNARE complex and Cpx1 (Fig. 6) (185). Simultaneously, a second Syt1 C2B domain interacts with the other side of the SNARE complex via the abovementioned SNARE/Syt1 primary interface (Fig. 6). Structure-guided mutagenesis, solution-binding studies by ITC, and electrophysiological recordings demonstrated that both Ca^{2+}-triggered synaptic release and suppression of spontaneous release depend on *both* the SNARE/Cpx1/Syt1 tripartite and the SNARE/Syt1 primary interfaces (185).

For the tripartite interface, the Syt1 C2B domain binds to the SNARE/Cpx1 subcomplex via interactions with both the SNARE and Cpx1 components (Fig. 6). Among the most striking structural features of this tripartite interface is the continuation of the Cpx1 central helix into the α-helix HA of Syt1. Since the α-helix HA is structurally conserved in C2B domains of all synaptotagmins, Doc2b, and Rabphilin, but not present in the Munc13–1 C2B domain and synaptotagmin C2A domains, the SNARE/Cpx1/Syt1 tripartite interface may be more general and involve other Syts. The different types of Syt-regulated exocytosis are mediated by similar Cpx1-dependent fusion mechanisms (Syt1, Syt2, Syt7, Syt9, and Syt10) (21, 49,

125, 167), so it is conceivable that these other Syts could participate in a tripartite interface while another Syt1, Syt2, or Syt9 molecule could be involved in the primary interface.

The tripartite interface involves the central α-helix, but not the accessory helix of Cpx1 or any other part of Cpx1. Indeed, the accessory domain can be entirely eliminated while maintaining the activating function of Cpx1 in single-vesicle fusion experiments with reconstituted SNAREs, Syt1, and Cpx1 (81). The structure of the SNARE/Cpx1/Syt1 complex now explains the functional requirement of the central α-helix of Cpx1 since it is an integral part of the tripartite interface (Fig. 6). On the other hand, the N-terminal domain of Cpx1 is important for activation of synchronous Ca^{2+}-triggered release (33, 97, 170, 172), and it increases the Ca^{2+}-triggered amplitude in single-vesicle fusion (content mixing) experiments when added as an independent fragment in addition to the Cpx1 central domain (81). One possibility is that the Cpx1 N-terminal domain may independently interact with the splayed open *trans* SNARE complex (27). Note that the membrane proximal parts of the *trans* SNARE complex were not included in the crystal structure of the SNARE/Cpx1/Syt1 complex, so it is not surprising that the Cpx1 N-terminal domain is not visible in that crystal structure.

Why did it take so many years since publication of the first crystal structure of the SNARE complex (144) to obtain structures of the SNARE/Syt1 and SNARE/Cpx1/Syt1 complexes? In retrospect, we now know that there are many weak or dynamic interactions between Syt1 and the SNARE complex (15, 26) which obscure the specific interface(s) in solution (185). In order to obtain the SNARE/Syt1 complex, covalently linked chimeras of the components of the complex were designed in order to stabilize the complex (184). Crystallization of the tripartite SNARE/Cpx1/Syt1 complex was achieved by truncation of the 23 C-terminal residues of the cytoplasmic domain of synaptobrevin-2 which prevented formation of the six membrane proximal layers of the SNARE complex, but left the binding interface to the central α-helix of Cpx1 intact (185). This truncated complex mimics a pre-fusion state of the *trans* SNARE complex where the membrane proximal part of the complex is not fully zippered. For crystallization, this feature of the truncated complex was essential since it prevented formation of inter-SNARE complex interactions that would otherwise interfere with formation of the SNARE/Cpx1/Syt1 complex. Moreover, this strategy alleviated the need for covalent linkers between the components.

Supramolecular arrangements of synaptic fusion complexes

The structure of the SNARE/Cpx1/Syt1 complex likely represents the pre-fusion primed state of the system (185), one of likely two or more synaptic complexes involved in Ca^{2+}-triggered fusion (131). These two or more synaptic complexes likely interact with each other. For example, one Syt1 C2B domain could bridge two SNARE complexes via the primary and tripartite interfaces and it could be sandwiched between the two membranes (185). CryoET images of the same proteoliposomes as used for the single-vesicle fusion experiments indeed revealed contacts with a variety of morphologies between the vesicle membranes with a preference for relatively compact point contacts (Fig. 7A) (44), although it remains to be established that it is these point contacts that undergo fast Ca^{2+}-triggered fusion. These contacts are likely proteinaceous since the observed densities of these contacts

are relatively high (Fig. 7B) (44) and the observed separation in the cryoET images between the membranes is 40 to 60 Å, substantially longer than the critical distance at which lipid stalks can form (< 9 Å) (2). Volumetric analysis suggested that the compact point contacts can accommodate two synaptic complexes while the lone contacts can accommodate more complexes (44). Thus, the juxtaposition of protein complexes between membranes prevents fusion until Ca^{2+}-triggering, in remote analogy to the action of viral fusion proteins where fusion only happens when a sufficient number of neighboring viral fusion proteins switch to their fusogenic form (52).

How does Cpx fit into this picture? Cpx1 has at least two conformations when bound to the ternary SNARE complex (27). In one conformation, the accessory domain cooperates with the N-terminal domain of Cpx1 to induce a conformational change at the membrane-proximal C-terminal end of the bound SNARE complex, likely making it similar to the splayed open *trans* SNARE complex. Conversely, if the SNARE complex is in the *trans* conformation, the interaction of the N-terminal domain of Cpx1 can occur, regardless of the accessory domain, consistent with the expendable nature of the accessory domain for evoked release. In the other Cpx1 conformation, Cpx1 can bridge two SNARE complexes, such as, a fully-assembled ternary SNARE complex and a binary SNARE complex, consisting of syntaxin-1A and SNAP-25A, requiring the presence of both the central and accessory domains (27). It has been proposed that Cpx1 could bridge many SNARE complexes in a "zigzag" arrangement (76). However, such an arrangement would be incompatible when Syt1 would bridge SNARE complexes via the primary and tripartite interfaces (185), although the abovementioned bridging of a ternary and a binary SNARE complex would be possible.

In addition to forming a complex with SNAREs and Cpx1, Syts are capable of forming ring-like homo-oligomeric assemblies on membranes containing acidic lipids (153) and in solution (154), although the functional importance of these assemblies has yet to be established. These rings are disrupted by Ca^{2+} when bound to acid phospholipid membranes (153, 179). Finally, synaptobrevin is capable of forming ring-like structures in complex with synaptophysin *in vitro* (1). In contrast to these studies, no rings are observed by electron cryo-tomography of slices of unstained, vitrified frozen-hydrated mouse synapses (40), or electron cryo-tomography of thin sections of high-pressure frozen hippocampal neuronal cultures (29). Moreover, cryoET studies of proteolipsomes with neuronal SNAREs, Syt1, Cpx1, and Munc13 revealed short and long contacts, but no ring-like features, *i.e.*, regular point-group symmetry was not discernible in these cryoET studies (44). Clearly, the long contacts may represent higher order oligomeric, but asymmetric, assemblies. Perhaps multiple membrane contact morphologies co-exist in the neuron and are relevant in different contexts (*e.g.*, fast synchronous release, asynchronous release, and spontaneous release).

Model of unlocking and Ca^{2+}-triggering

Regardless of the supramolecular arrangement of the synaptic protein complexes in the observed contacts between membranes, they appear stably locked into a conformation from which fusion is unlikely considering the observed membrane separation of 40–60 Å (Fig. 7). Consequently, the pre-fusion SNARE/Cpx1/Syt1 complexes would be only partially

zippered (Fig. 8). The readily releasable pool of synaptic vesicles requires both interfaces (185), suggesting that the crystal structure of the SNARE/Cpx1/Syt1 complex represents the primed state. Upon Ca^{2+}-binding to the C2 domains of the primed complex, the SNARE/Cpx1/Syt1 tripartite interface must be unlocked or un-inhibited, allowing membranes to move closer and Ca^{2+}-triggered fusion to commence. It is likely that upon Ca^{2+}-binding, the Syt1 molecule that is involved in the SNARE/Cpx1/Syt1 tripartite interface is dislodged by interacting with the synaptic vesicle membrane (185). This Syt1 dissociation may also induce a conformational change of Cpx1 (27). However, full dislodging of Cpx1 (145) is unlikely considering the tight binding ($K_d \approx$ 10–100 nM) and the relatively slow off-rate of 0.1 s^{-1} between Cpx1 and the *cis* ternary SNARE complex (27). Upon unlocking the primed complex, the SNARE then may fully zipper (144, 158), and trigger fusion, likely in conjunction with the membrane-bending action of the Syt1 molecule (60, 93) that is involved in the primary interface (184).

A model of locked and primed SNARE/Cpx1/Syt1 complexes that juxtapose synaptic and plasma membranes (Fig. 8) explains the dominant negative effect of certain mutations of the Ca^{2+}-binding region of the Syt1 C2B domain (84, 164, 185): the presence of permanently locked complexes would prevent membranes from approaching the critical distance to form lipid stalks (less than 9 Å) (2). As mentioned in Section 3, neuronal SNAREs and Syt1 alone are a minimal system for Ca^{2+}-triggered membrane fusion (35, 78, 82, 147). Thus, the triggering mechanism in this even more minimal system may be similar to that of the unlocked SNARE/Cpx1/Syt1 system after dislodging of the Syt1 molecule that is involved in the tripartite interface. Clearly, high resolution images of the SNARE/Cpx1/Syt1 complexes between membranes, and monitoring the rapid conformational transitions of the complexes upon Ca^{2+}-triggering will be important next steps.

9. Implications for spontaneous release

Syt1, Cpx1, and SNAREs are also important for spontaneous release (33, 65). More specifically, both the primary SNARE/Syt1 and tripartite SNARE/Cpx1/Syt1 interfaces are required for the suppression of spontaneous release in neurons, *i.e.*, elimination of either interface resulted in an increase in mini frequency (185). Why is there an increase in spontaneous fusion frequency when either interface is disrupted? At a structural level, it could simply mean that membranes can get closer when one or more components of the SNARE/Cpx1/Syt1 complex are missing, consistent with the closer membrane separation distance observed by cryoET of proteoliposomes when Cpx1 is absent (44). However, there is an observation that counters this simple membrane separation distance argument: the probability of Ca^{2+}-independent fusion did not increase when Syt1 is absent in single-vesicle fusion experiments with SNAREs and Cpx1 only (82). Despite this, these experiments should be repeated with full reconstitution (80), since in the absence of full reconstitution, improperly formed SNARE complexes may obscure the more subtle effect of suppression of Ca^{2+}-independent fusion.

We would like to emphasize that Ca^{2+}-independent fusion of single-vesicle experiments cannot strictly be compared with spontaneous release in neurons. First, Ca^{2+}-independent fusion probabilities are normalized to the number of associated vesicles. Second, the

frequency of spontaneous release measured in electrophysiological experiments depends on both the number of functional synapses as well as the number of synaptic vesicles that are capable of undergoing spontaneous fusion. Third, there is likely another Ca^{2+}-sensor that competes with Syt1 in binding to the tripartite interface (142). Elimination of the primary interface or presence of the certain Syt1 C2B mutants would affect the binding equilibrium, possibly explaining the increase of spontaneous release in neurons (185).

10. Cooperation between Munc18 and Munc13 for proper SNARE assembly

SNARE assembly is fuzzy

As mentioned above, the two interfaces in the SNARE/Cpx1/Syt1 complex are important for the readily releasable pool of synaptic vesicles (185). The readily releasable pool of synaptic vesicles in turn is established by a process that is referred to as priming, *i.e.,* the association of synaptic vesicles with presynaptic proteins that enables them to undergo fast evoked fusion (70). There are several factors involved in priming, including Munc18 and Munc13, but the molecular basis of the priming function of these molecules had been unknown until recently. There is now evidence that at least one function of priming factors is to ensure the proper assembly of SNARE complexes (80). Why is it necessary to assist the proper assembly of the SNARE complex? When soluble fragments of SNARE proteins are simply mixed in solution, improper configurations may occur (27, 89, 124, 162). In particular, the existence of antiparallel SNARE complex configurations were *directly* probed by using FRET dye pairs (27, 162). As described (162), antiparallel configurations can be suppressed by extensive purification of the ternary SNARE complex, including a urea wash step. Another biochemical method to obtain more fusogenic complexes consists of incubating with C-terminal fragments of synaptobrevin-2 (101, 115) prior to assembling the SNARE complex. Single-molecule FRET experiments now revealed that these C-terminal synaptobrevin fragments promote proper SNARE complex formation (27). Clearly, urea treatments or incubation with synaptobrevin-2 fragments are not physiological mechanisms, but they illustrate the importance of proper SNARE complex assembly in establishing maximum fusogenicity.

Munc13 and Munc18 promote proper SNARE complex assembly

In search for a physiological mechanism that ensures proper SNARE complex formation, Lai *et al.* found that the MUN domain of Munc13–1 promotes the proper parallel subconfiguration between syntaxin-1A and synaptobrevin-2 when assembling the ternary SNARE complex when starting from the syntaxin-1A/SNAP-25A complex, *i.e.*, the MUN domain prevented improper subconfigurations, and it increased the Ca^{2+}-triggered amplitude in single-vesicle fusion (content mixing) experiments (80). Inclusion of the longer C1C2BMUN or C1C2BMUNC2C fragments of Munc13–1 in the fusion assay enhanced the Ca^{2+}-triggered amplitude and fusion ratio in a manner similar to the MUN domain. However, these longer fragments were able to achieve these effects at twenty- and hundred-fold lower concentrations than the MUN domain, respectively.

In addition to its autonomous function of promoting the proper syntaxin-1A/synaptobrevin-2 subconfiguration, Munc13 also cooperates with Munc18–1 to promote the proper syntaxin-1A/SNAP-25A subconfiguration within the assembled ternary SNARE complex (80). Thus, while Munc13 alone is sufficient to promote the proper syntaxin-1A/synaptobrevin-2 subconfiguration starting from the syntaxin-1A/SNAP-25A complex and consequently increasing the Ca^{2+}-triggered fusion amplitude, the proper syntaxin-1A/SNAP-25A subconfiguration is only achieved when starting from the syntaxin-1A/Munc18–1 complex. Moreover, near physiological Ca^{2+}-sensitivity of the assay was achieved when both Munc13–1 and Munc18–1 were included (80). Taken together, Munc13–1 and Munc18–1 are factors for establishing the proper subconfigurations during *trans* ternary SNARE complex assembly (with parallel arrangements of the SNARE components and 1:1:1 stoichiometry) by suppressing improper subconfigurations, thus promoting proper ternary SNARE complex assembly. The cooperation of Munc18–1 and Munc13–1 in promoting the proper SNARE complex assembly suggests that Munc18–1 has an indirect but essential role in enabling high fusion efficiency, perhaps explaining the severe phenotype of deletion of Munc18–1 in neurons (151).

When some of the SNARE complexes around the contact site between the synaptic and plasma membranes are improperly assembled in the absence of Munc13, they have a large effect on morphologies of the contact sites between SV and PM vesicles as observed by cryoET of these vesicles (44). Upon inclusion of Munc13, the contacts are largely restricted to point contacts only, *i.e.,* no long contacts are present and very few “dead-end” hemifusion diaphragms were observed prior to Ca^{2+}-triggering. The ternary SNARE complex interacts with both Syt1 and Cpx1, forming the primed complex (185). These interactions in the SNARE/Cpx1/Syt1 complex may not occur if there are improper subconfigurations within the ternary SNARE complex. In particular, an anti-parallel subconfiguration between syntaxin-1A and synaptobrevin-2 would prevent formation of the SNARE/Cpx1/Syt1 tripartite interface (Fig. 6) (185). Moreover, an anti-parallel subconfiguration between SNAP-25A and syntaxin-1A would prevent formation of the primary SNARE/Syt1 interface since it involves the SNAP-25A and syntaxin-1A components of the SNARE complex (Fig. 5) (184).

Towards more complete reconstitution of synaptic vesicle fusion

Consistent with their molecular functions, Munc18 and Munc13 increased the efficiency of ensemble proteoliposome lipid and content mixing in the presence of SNAREs, Syt1, NSF and αSNAP (91). Moreover, when Munc13 and Munc18 were included, the Ca^{2+}-triggered amplitude of single vesicle content mixing was four-fold higher for a reconstitution with SNAREs, Syt1, Cpx1, NSF, αSNAP (80). Remarkably, the Ca^{2+}-sensitivity of this single-vesicle fusion assay was about 23 μM, which is close to the physiological range (Fig. 3). These improvements in amplitude and sensitivity are achieved by proper assembly of all components of the SNARE complex by Munc18 and Munc13 (Fig. 4) (80).

How do Munc13 and Munc18 function?

The molecular mechanism by which Munc13 and Munc18 promote proper SNARE complex assembly is still a mystery, although there is some information about the molecular

interactions. The MUN domain initially interacts with the Munc18–1/syntaxin-1A complex via a conserved hydrophobic pocket located at the midpoint of the MUN domain (90, 178). Moreover, two conserved residues R151 and I155 in the syntaxin-1 linker region are also critical for the function of Munc13–1, and they likely interact with the MUN domain (155). Single-molecule FRET experiments revealed that the MUN domain does not dissociate the closed Munc18–1/syntaxin-1A complex, but that it rather induces a conformational change in the syntaxin-1A linker region, thus enabling syntaxin-1A to transit into the ternary SNARE complex once synaptobrevin-2 and SNAP-25A are added. In addition to these interactions, the MUN domain weakly interacts with synaptobrevin-2 (80, 132), syntaxin-1A (80, 90), the syntaxin-1A/SNAP-25A complex (48, 161), and the ternary SNARE complex (48).

In some ways, assistance of proper ternary SNARE complex formation by Munc13–1 is reminiscent of certain ATP-independent chaperones such as Trigger Factor that assist protein folding of the nascent polypeptide chain upon synthesis by the ribosome (57). It has been proposed that Trigger Factor functions by shielding exposed hydrophobic residues during folding (71), inhibiting premature folding (96), unfolding preexisting structures and intermediate structures (56), or assisting protein assembly (95). While the MUN domain of Munc13–1 is structurally quite different than Trigger Factor, some of the properties of Trigger Factor also apply to the MUN domain in that both act through a series of weak and transient interactions to ensure a given conformation.

The existence of factors for proper assembly of ternary SNARE complex may be a general phenomenon. In the context of a different SNARE-mediated fusion process, the so-called homotypic fusion and protein sorting (HOPS) complex is essential in promoting maximum efficiency of homotypic vacuolar fusion (186), where the Munc18–1 homolog in the HOPS complex (Vps33) affects vacuolar SNARE-mediated membrane fusion (8). The crystal structures of a complex with the Munc18 homolog Vps33 and the synaptobrevin homolog Nyv1 as well as the complex of Vps33 and the syntaxin homolog Vam3 suggest that Vps33 regulates the proper assembly of Nyv1 and Vam3. Moreover, NMR experiments showed that synaptobrevin-2 interacts with Munc18–1 (132) in a similar fashion as suggested by the Vps33/Nyv1 complex. It is of course possible that the Vps33/Nyv1 interaction is promiscuous and may allow interactions with other SNAREs as well. Such a promiscuous interaction would be consistent with the cooperation of Munc18 and Munc13 in promoting the proper assembly of all components of the neuronal SNARE complex. Finally, there is no Munc13 homolog in homotypic vacuolar fusion and all Munc13 homologues are thought to be involved in Ca^{2+}-triggered exocytosis (72, 86). Thus, the evolution of the Ca^{2+}-triggered fusion machineries deviated from that of constitutive vesicle fusion.

Hypothesis: presynaptic plasticity may be a manifestation of regulation of proper SNARE complex assembly

The key roles of Munc13–1 in priming and short-term plasticity may be related to the role of the MUN domain in proper assembly of the SNARE complex (80). This function of the MUN domain which may in turn be subject to regulation by the other domains of Munc13–1. For example, Ca^{2+}-binding to the C2B domain, the synergy between C1, C2B, and C2C

domains (88), and the Ca^{2+}-dependent interaction between calmodulin and Munc13 (66) may localize Munc13 to sites of docked synaptic vesicles and increase its ability to assist in proper SNARE complex assembly, as corroborated by the concentration dependence of the various Munc13 fragments in single-vesicle fusion experiments (80). Indeed, mutations of the Munc13–2 C2B domain affect neurotransmitter release upon a single action potential as well as short-term synaptic plasticity (129) and mutations in the interface regions between the C1, C2B, and MUN domains have differential effects on evoked release and the readily releasable pool (166), suggesting that the two molecular functions of Munc13 may be differentially regulated in the neuron. Taken together, regulation of presynaptic plasticity via Munc13 may be in part accomplished by its profound molecular function on ensuring proper assembly of the ternary SNARE complex. The starting point for the action by Munc18 and Munc13 is the disassembled SNARE complex mediated by NSF and αSNAP (Fig. 9). Thus, NSF, αSNAP, Munc18, and Munc13 constitute a quality control system that produces maximal fusogenic synaptic complexes.

11. Perspectives and Outlook

There are other important factors that were not reviewed here. In particular, factors that are involved in tethering, regulation through Rab G-proteins, and voltage gated Ca^{2+} channels (68, 157). Clearly, complexes with these factors also need to be imaged at near atomic resolution, and functionally reconstituted. Moreover, there are many proteins in synaptic vesicles with little known function that may deserve closer examination. Ultimately, studying the effects of disease and aging on the synaptic neurotransmitter release machinery and its regulations may provide clues for entirely new therapeutic developments.

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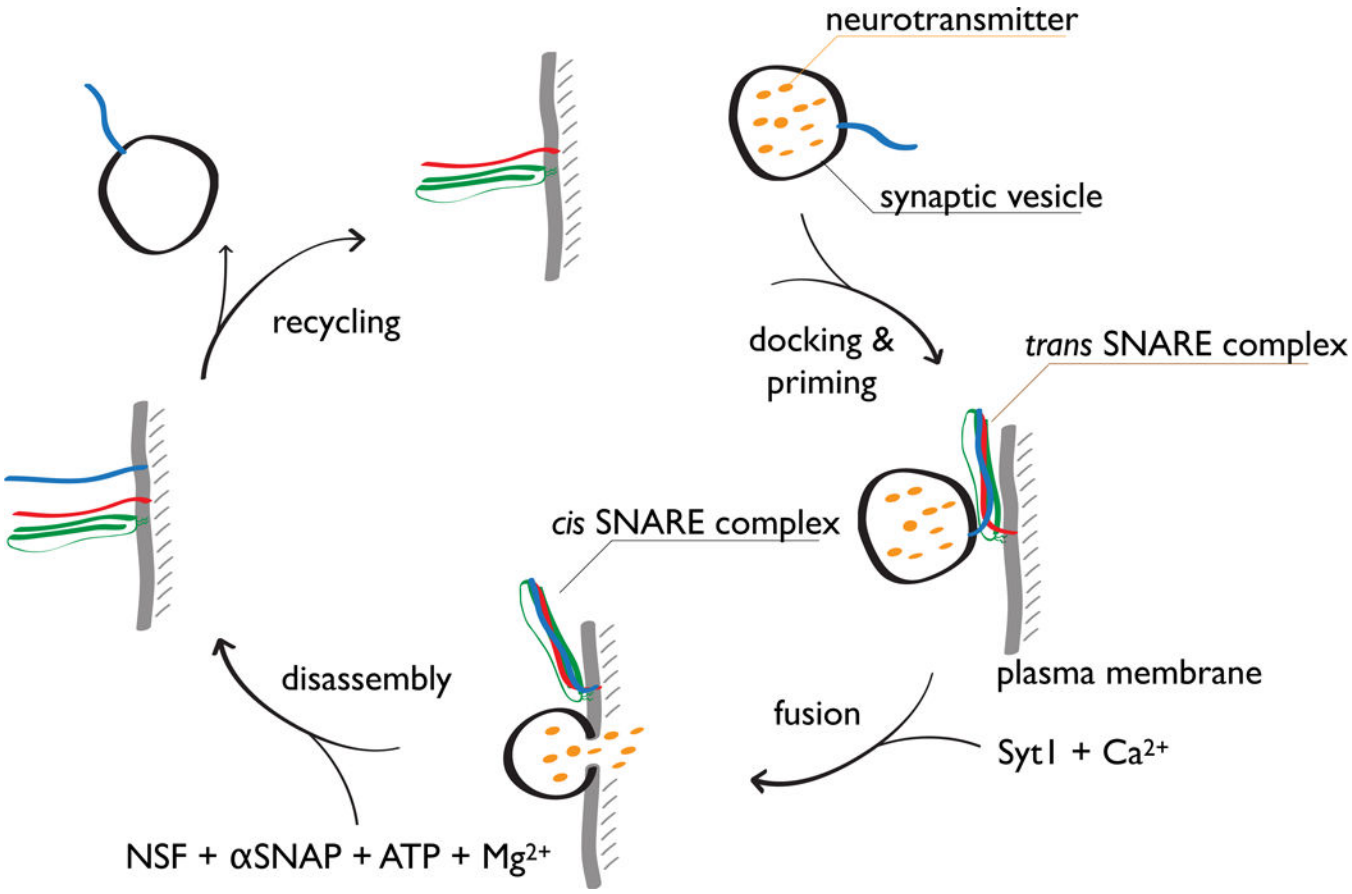

Figure 1. The roles of SNAREs in the synaptic vesicle cycle.

SNAREs form a *trans* complex that juxtaposes membranes after synaptic vesicle docking and priming. In combination with a Ca^{2+}-sensor, evoked fusion occurs upon an action potential. During fusion, SNARE complexes are fully formed (*cis* complex). *Cis* SNARE complexes are disassembled by the ATPase NSF in conjunction with SNAPs and made available for another round of synaptic vesicle formation.

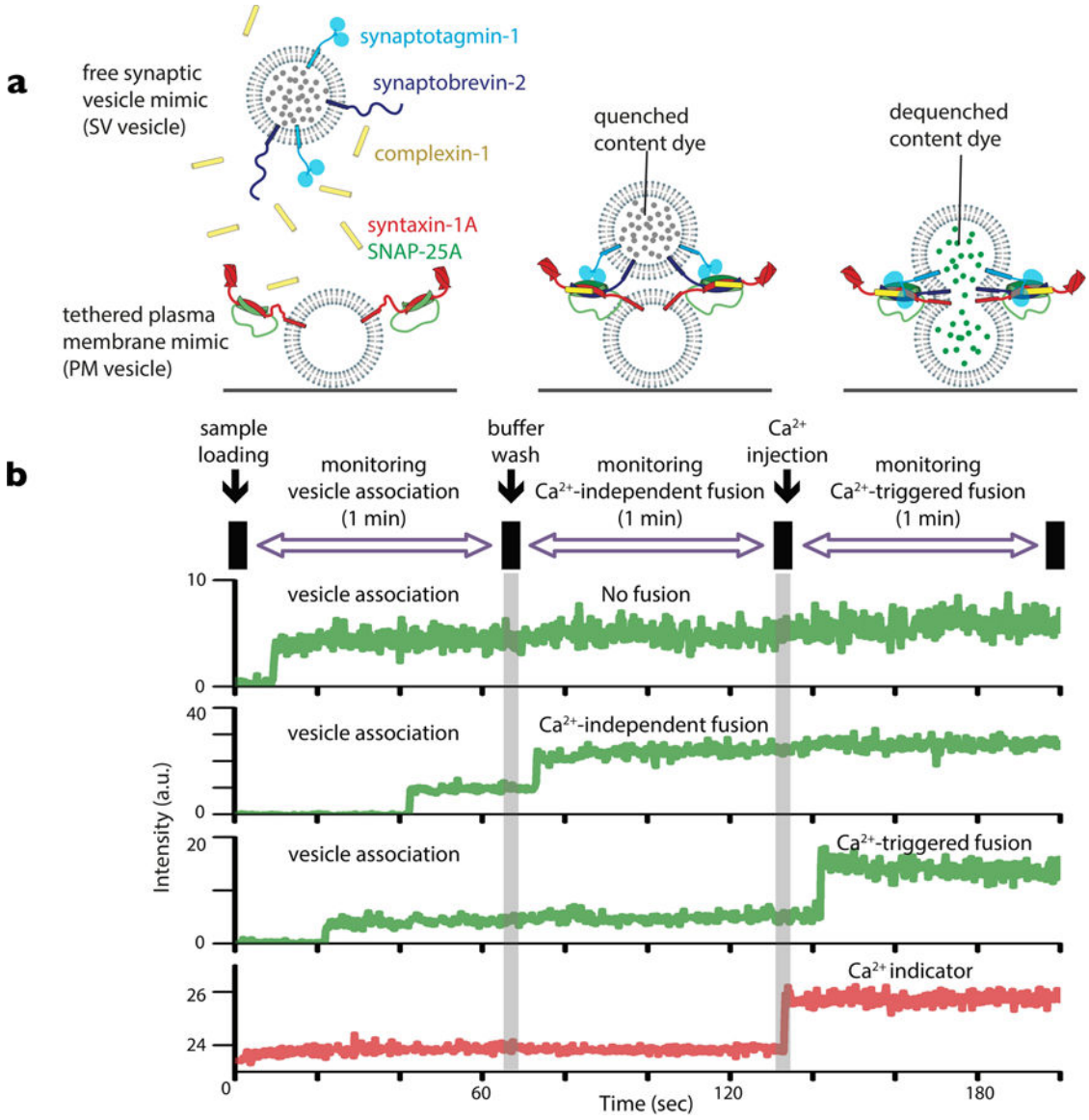

Figure 2. Single-vesicle fusion assay.

(a) The schema shows the initial setup with proteoliposomes with reconstituted synaptobrevin-2 and Syt1 that mimic synaptic vesicles (SV vesicles) and surface-tethered proteoliposomes with reconstituted syntaxin-1A and SNAP-25A that mimic the plasma membrane (PM vesicles). Cpx1 is included in solution. A soluble dye is encapsulated into the SV vesicles, and in some experiments, a lipid dye is incorporated into the SV vesicle membrane. These dyes are at high enough concentration to result in self-quenching of the fluorescence intensity. An increase in volume or surface area, respectively, will produce an increase in the respective fluorescence intensity. **(b)** Ca^{2+}-independent fusion is observed for SV vesicles that are associated with tethered PM vesicles. **(c)** Ca^{2+}-triggered fusion events are observed. For more details see references (35, 78–80, 82).

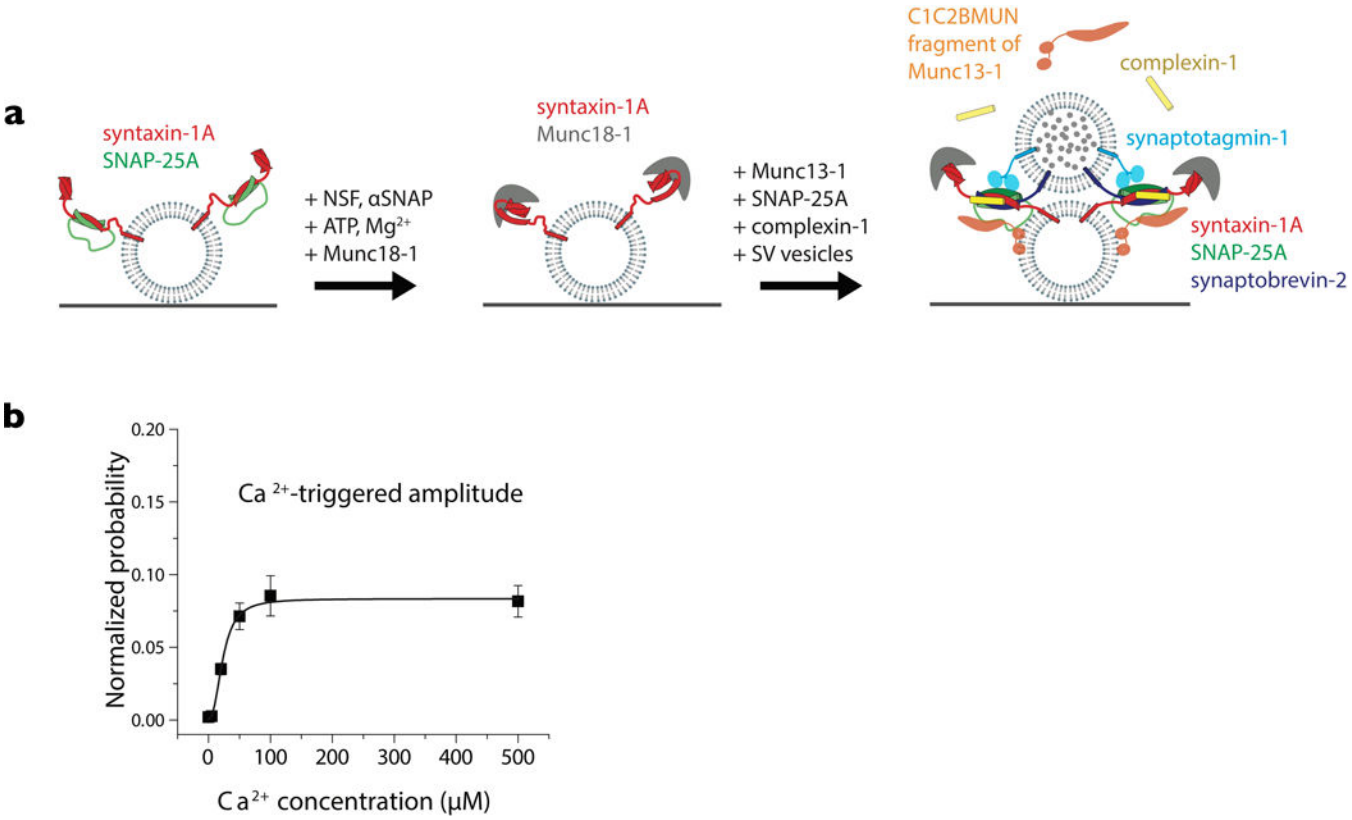

Figure 3. Reconstitution with neuronal SNAREs, Cpx1, Syt1, NSF, αSNAP, Munc18, Munc13.

(a) Prior to the schema shown in Fig. 2a, a step is added that dissociates the syntaxin-1A/SNAP-25A complex by NSF, αSNAP, ATP, Mg^{2+} and concomitant formation of the syntaxin-1A/Munc18 complex. Incubation of the resulting syntaxin-1A/Munc18 vesicles is performed in the presence of Munc13–1, Cpx1, and SNAP-25A. **(b)** Ca^{2+}-sensitivity of the fusion assay using the schema shown in panel a. For more details see (80).

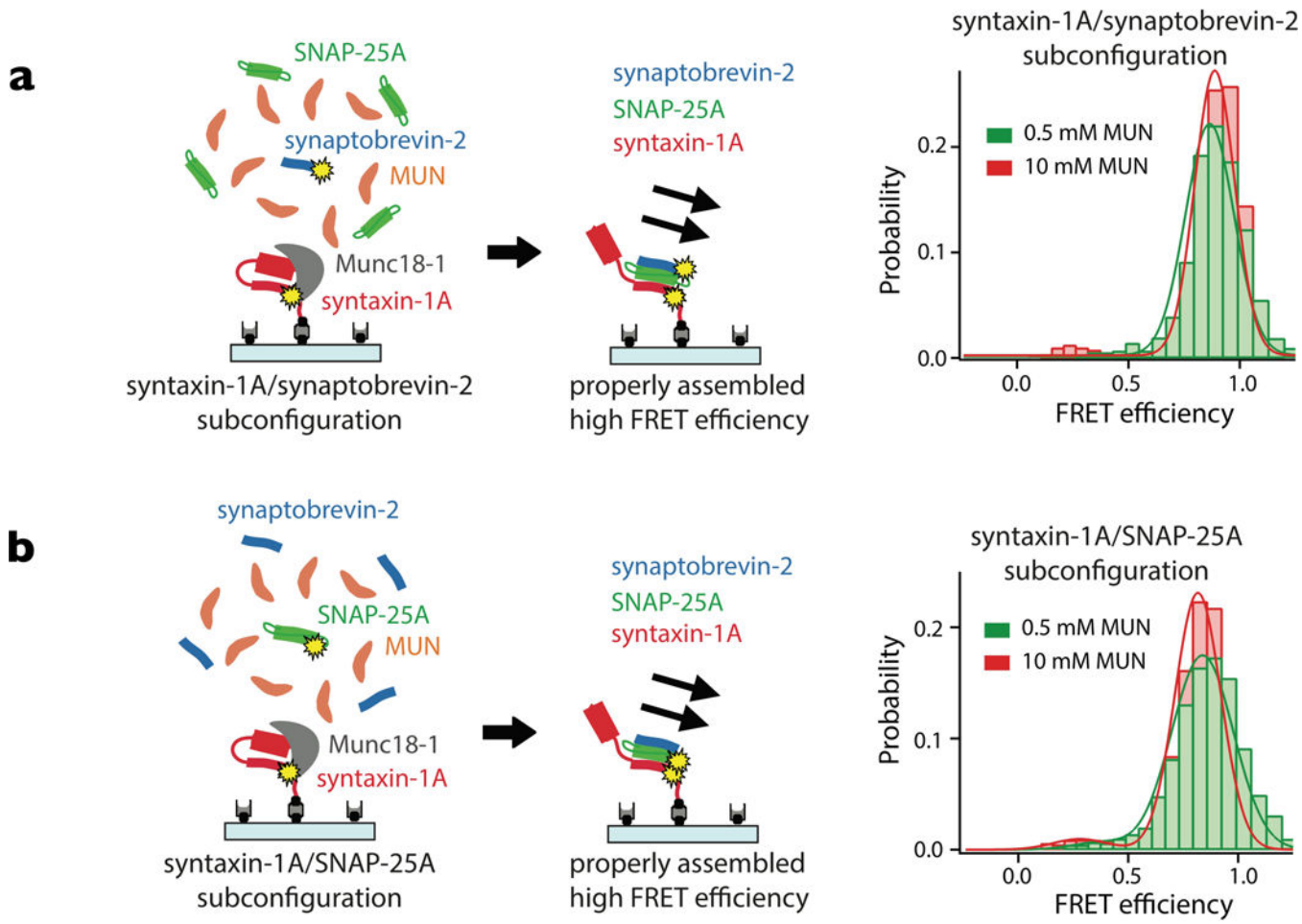

Figure 4. Munc13 and Munc18 assist in proper SNARE complex formation.

Starting from the syntaxin-1A/Munc18 complex, ternary SNARE complex is formed upon addition of the MUN domain of Munc13, SNAP-25A, and synaptobrevin-2. Single-molecule FRET measurements involving pairs of fluorescent dyes indicate that the components of the SNARE complex are parallel with respect to each other. For more details see (80).

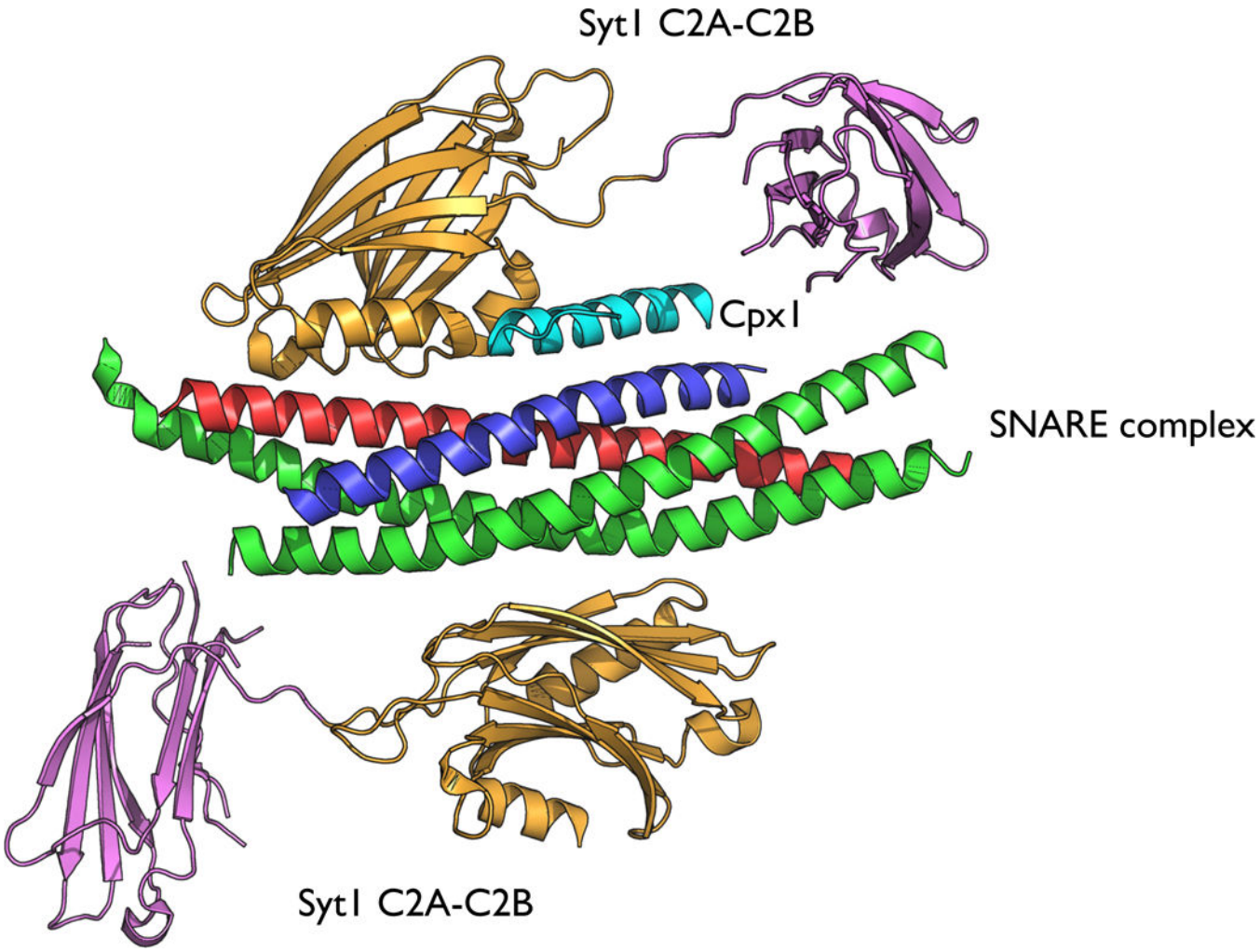

Figure 5. The primary SNARE/Syt1 interface is structurally conserved.

Superposition of the primary interface observed in crystal structures of the Ca^{2+} (3.5 Å resolution, PDB code 5CCG, color) and Mg^{2+} (4.1 Å resolution, PDB code 5CCI, gray/white) bound structures of the SNARE/Syt1 (184), and of the SNARE/Cpx1/Syt1 complex (1.8 Å resolution, PDB code 5W5C, black) (185). Note, that the crystal packing of the SNARE/Cpx1/Syt1 complex is very different from that of the SNARE/Syt1 complexes (185).

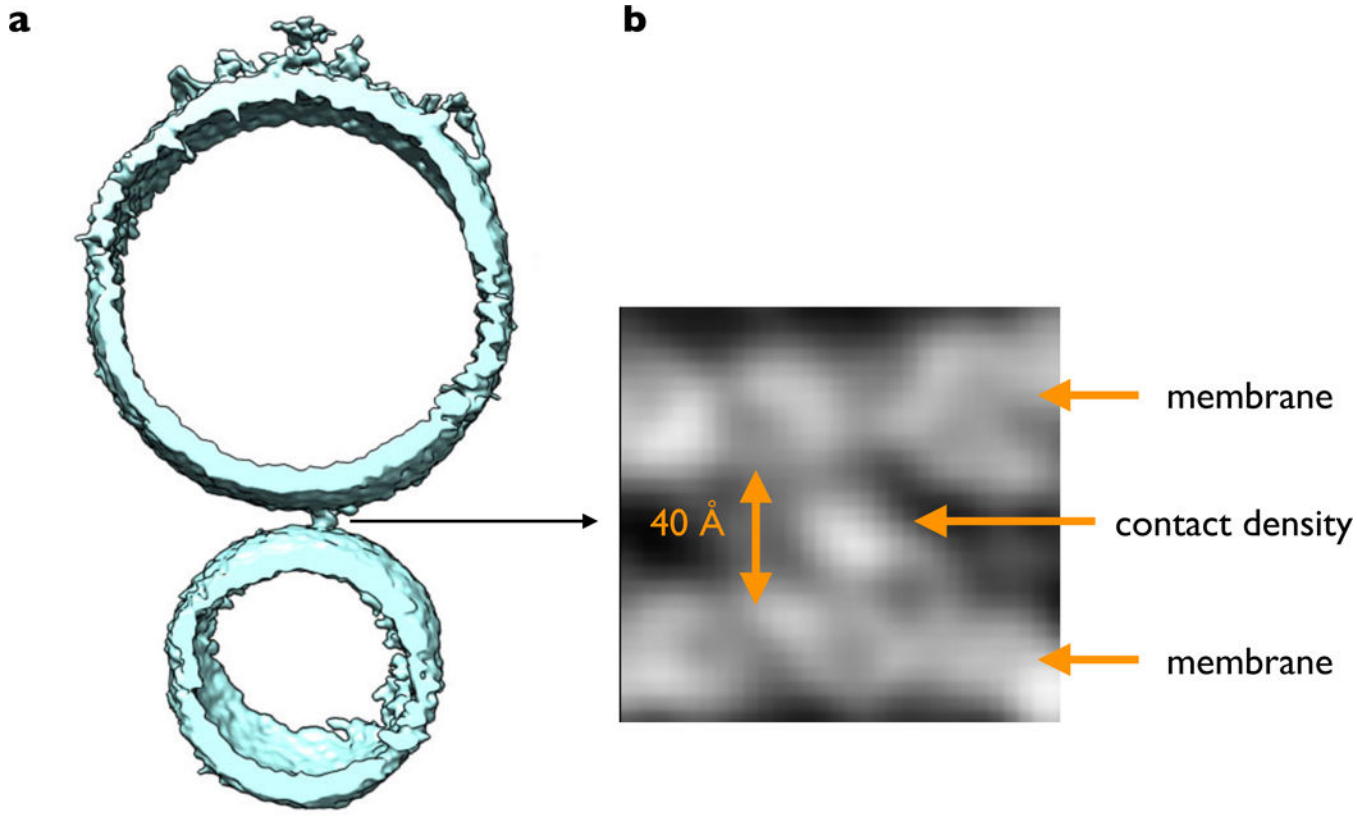

Figure 6. Structure of the SNARE/Cpx1/Syt1 complex.
Shown are both the primary and the tripartite interface. For details see (185).

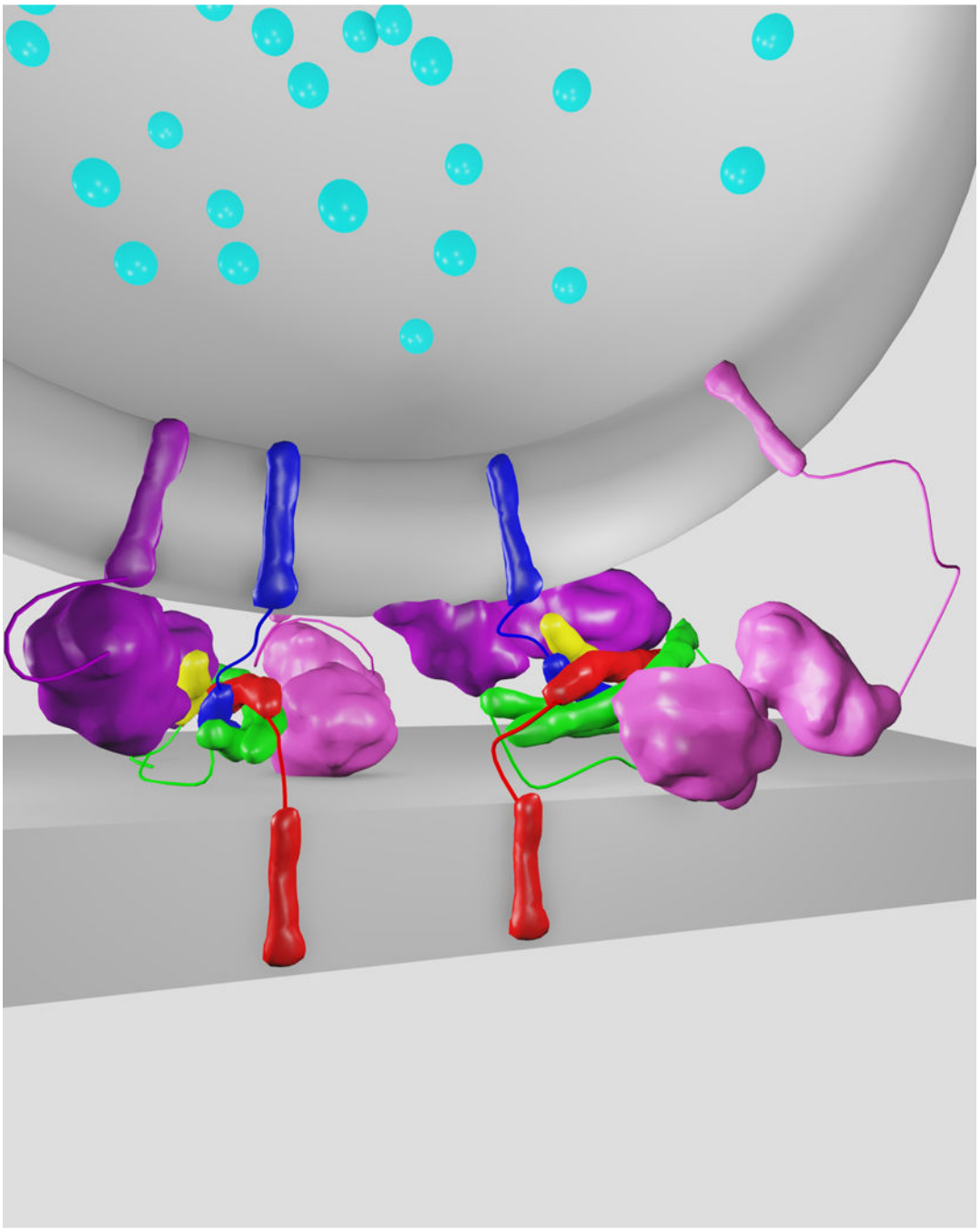

Figure 7. Point contact between proteoliposomes with neuronal SNAREs, Cpx1, Syt1, and Munc13.

(a) Shown is a slice of an isosurface representation of a SV vesicle (bottom) and a PM vesicle (top) in the presence of the C1C2BMUN fragment of Munc13. **(b)** Close-up view of grey scale tomographic 2D-slices of the contact site between the two membranes. For more details see (44).

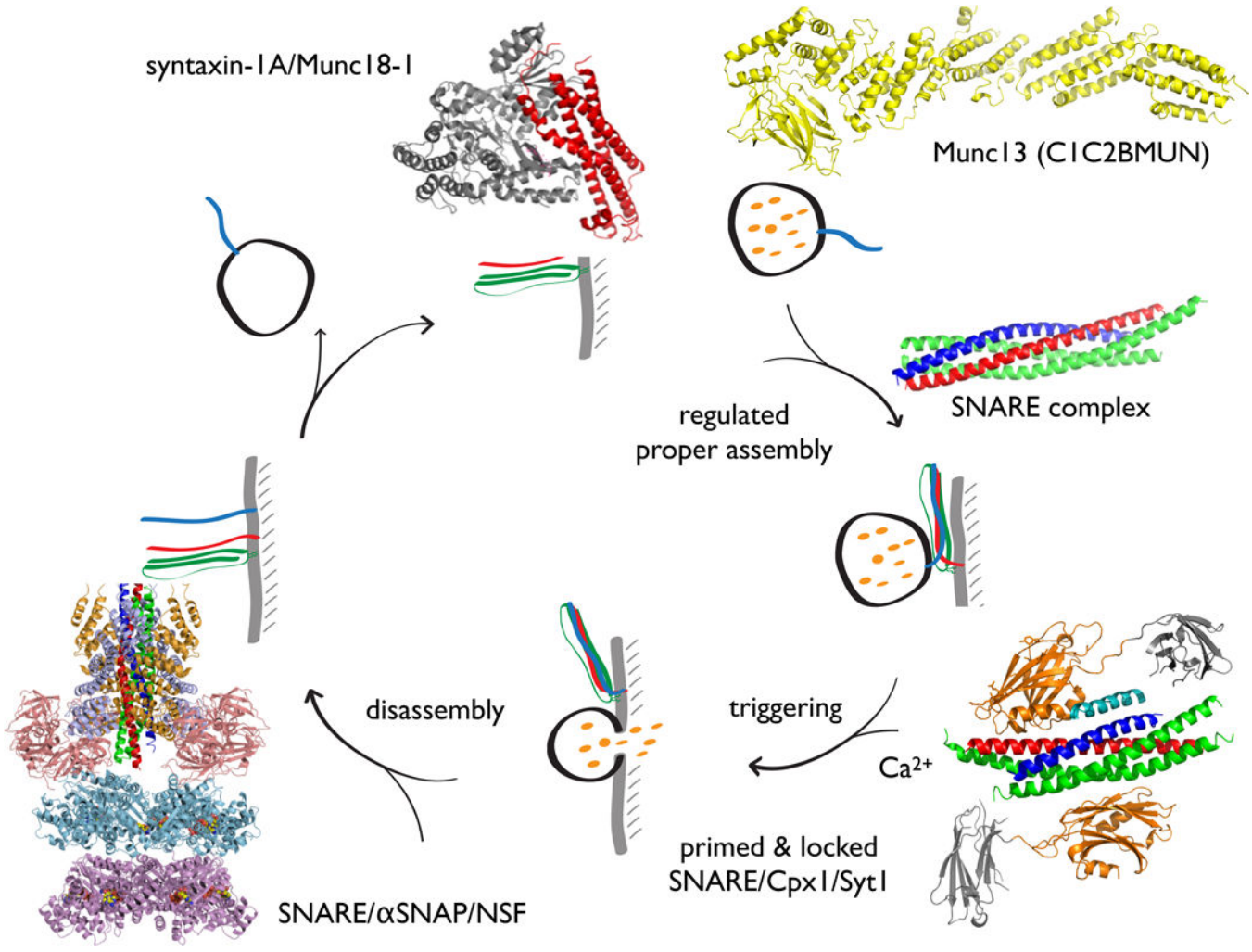

Figure 8. Model of primed pre-fusion SNARE/Cpx1/Syt1 complexes.

In this model, two SNARE/Cpx1/Syt1 complexes (185) form a contact between a synaptic vesicle (top) and the plasma membrane (bottom). Blue: synaptobrevin-2, green: SNAP-25A, red: syntaxin-1A, yellow: Cpx1, light/dark magenta: Syt1s. The molecules and membranes are drawn to scale, with a ~ 40 Å separation between membranes as observed by cryoET (44). Note that the relative arrangement of the two SNARE/Cpx1/Syt1 complexes is unknown. Also, there can be more than two such complexes forming long membrane contacts (44).