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Synaptic vesicle fusion

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Abstract

The core of the neurotransmitter release machinery is formed by SNARE complexes, which bring the vesicle and plasma membranes together and are key for fusion, and by Munc18-1, which controls SNARE-complex formation and may also have a direct role in fusion. In addition, SNARE complex assembly is likely orchestrated by Munc13s and RIMs, active-zone proteins that function in vesicle priming and diverse forms of presynaptic plasticity. Synaptotagmin-1 mediates triggering of release by Ca²⁺, probably through interactions with SNAREs and both membranes, as well as through a tight interplay with complexins. Elucidation of the release mechanism will require a full understanding of the network of interactions among all these proteins and the membranes.

The immense variety and complexity of the functions performed by the nervous system rely on the ability of neurons to communicate with each other in defined and precisely timed patterns. Such precise timing is enabled in part by the fast speed of synaptic transmission. It takes as little as 100 μs from the arrival of an action potential to the release of neurotransmitters by Ca²⁺-evoked synaptic vesicle exocytosis¹. These high speeds arise because, after synaptic vesicles dock onto specialized sites of the plasma membrane called active zones, a priming reaction(s) leaves the vesicles in a metastable state that is ready for fast Ca²⁺-triggered fusion with the plasma membrane. Neurotransmitter release does not just constitute a means to send signals between neurons; acute, dynamic as well as long-term changes in the efficiency of release during presynaptic plasticity processes shape the properties of neural networks and underlie some forms of information processing in the brain¹. Synaptic exocytosis is thus an exquisitely regulated form of intracellular membrane fusion, and understanding the complexity of this process requires characterization of not only the mechanism of membrane fusion but also how fusion is controlled to occur at the right time and with the correct probability.

Intense research that started more than 20 years ago has led to the identification of many proteins involved in neurotransmitter release. Among the components of this machinery are

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proteins that have homologs in most types of intracellular membrane fusion and include the Sec1/Munc18-1 (SM) protein Munc18-1; *N*-ethylmaleimide sensitive factor (NSF); soluble NSF attachment proteins (SNAPs); the SNAP receptors (SNAREs) synaptobrevin (also known as VAMP, for vesicle-associated membrane protein), syntaxin-1 and SNAP-25 (no relation to SNAPs); and small GTPases from the Rab3 family. These proteins have conserved properties that underlie general mechanisms of docking, fusion and recycling, but some of them also show unique features that are likely to have evolved to meet the tight regulatory requirements of synaptic exocytosis. In addition, release also depends on diverse specialized proteins that control different steps leading to exocytosis.

The focus of the field changed gradually from identification and initially characterization of components to detailed studies of how they function. This research is increasingly illustrating the complexity of the network of intermolecular interactions that controls synaptic exocytosis, showing that great advances have been made but much remains to be learned in order to reach a true understanding of the mechanism of release. In this review, we primarily discuss mechanistic aspects, with an emphasis on recent data but also describing earlier key results. We focus on a few proteins that have emerged as crucial players in this system, including the SNAREs and Munc18-1 as the core components of the general fusion machinery, and specialized proteins with critical roles in vesicle priming and presynaptic plasticity (Munc13s and RIMs) or in the Ca^{2+} -triggered step of release (synaptotagmin-1 and complexins). Many reviews have recently covered these and other facets of the vast literature that is accumulating in this field^{1–8}, and other reviews in this issue of *Nature Structural & Molecular Biology* discuss diverse aspects of intracellular membrane fusion.

SNAREs

SNARE proteins govern most types of intracellular membrane traffic and are characterized by sequences called SNARE motifs that have a high propensity to form coiled coils. The key roles in synaptic exocytosis of the synaptic vesicle SNARE synaptobrevin and the plasma membrane SNAREs syntaxin-1 and SNAP-25 (see domain diagrams in Fig. 1a) were demonstrated by the finding that they are the targets of clostridial neurotoxins and by genetic experiments (reviewed in refs. 1,5,8). The three proteins form a highly stable ‘SNARE complex’ that is disassembled upon binding to SNAPs and NSF through the ATPase activity of NSF^{9,10}. The SNARE complex consists of a parallel four-helix bundle formed by the SNARE motifs of the three neuronal SNAREs^{11,12} (Fig. 1b). Because the SNARE motifs of syntaxin-1 and synaptobrevin are adjacent to their transmembrane regions, the parallel arrangement of the helices indicated that the SNARE complex should bring the vesicle and plasma membranes into close proximity and suggested that complex assembly could provide the energy required for fusion¹³ (Fig. 1c, left panel). Indeed, reconstitution experiments with synaptobrevin and syntaxin-1–SNAP-25 incorporated into separate populations of liposomes indicated that the SNAREs alone can induce membrane fusion¹⁴. Although the relevance of this finding has been debated for diverse reasons (see below and ref. 4.), the reconstitution approach has provided an invaluable tool to study the components of the release machinery in a membrane environment and to correlate their ability to govern membrane merger *in vitro* with functional studies *in vivo*. Another strategy

that provides complementary information involves monitoring fusion between cells bearing flipped SNAREs on their plasma membranes¹⁵.

The notion that formation of *trans*-SNARE complexes between two membranes is key for fusion is now widely accepted, and the activity of SNAPs–NSF is thus believed to be required for disassembly of *cis*-SNARE complexes to recycle the SNAREs. Much of the recent research on the mechanism of release has thus focused on studying how other critical factors govern SNARE complex assembly and/or cooperate with the SNAREs in membrane fusion (see below), as well as how assembly, fusion and coupling with these factors depend on distinct SNARE sequences. Particularly important for control of SNARE complex assembly is the long N-terminal region of syntaxin-1 containing an autonomously folded three-helix bundle called the H_{abc} domain¹⁶ (Fig. 1a,b). In isolated syntaxin-1, this domain folds back onto the SNARE motif, resulting in a so-called ‘closed conformation’ that hinders SNARE complex assembly¹⁷ (Fig. 1d, left panel). In addition to mediating coupling with Munc18-1 (see below), the syntaxin-1 closed conformation is likely to be important for preventing reassembly of the SNARE complex after disassembly by SNAPs–NSF. Moreover, removing the syntaxin-1 N-terminal region in coexpressed syntaxin-1–SNAP-25 heterodimers enhances the rate of SNARE-induced liposome fusion¹⁸, suggesting that this region may have a further role in controlling SNARE complex formation downstream of heterodimer formation¹⁹.

SNARE complex assembly *in vitro* is also hindered by the tendency of syntaxin-1 and SNAP-25 to form 2:1 heterodimers²⁰ wherein synaptobrevin is replaced by a second copy of syntaxin-1 in the four-helix bundle²¹. Indeed, syntaxin-1 and SNAP-25 have a natural tendency to associate into diverse four-helix bundles *in vitro* and are highly promiscuous⁵. However, coexpression of syntaxin-1 and SNAP-25 does yield 1:1 heterodimers^{19,22} and, in the original reconstitution assays¹⁴, was required to observe liposome fusion, suggesting that coexpression leads to a metastable state that is kinetically unreachable by mixing separately expressed proteins. Conversely, recent data have shown that syntaxin-1 reconstituted into lipid bilayers at very low protein-to-lipid (P/L) ratios can form stable 1:1 heterodimers with SNAP-25 that interconvert between two- and three-helix bundle configurations²³. Although the 1:1 heterodimers formed under these conditions or by coexpression may have a different nature, both types of heterodimer bind to Munc18-1 and Munc13-1, among other factors^{22,23}, and these interactions stabilize the three-helix bundle²³. Thus, binding of these factors to either type of 1:1 heterodimer is likely to lead to the same three-helix bundle configuration of the syntaxin-1–SNAP-25 SNARE motifs that is expected to serve as the acceptor complex for synaptobrevin. These findings suggest that a crucial task of the release apparatus is to help form this acceptor complex and to prevent unwanted interactions of the promiscuous SNARE motifs of syntaxin-1 and SNAP-25.

Binding of synaptobrevin to pre-formed syntaxin-1–SNAP-25 heterodimers is believed to start at the N terminus of its SNARE motif and then ‘zipper’ toward the C terminus (Fig. 1c). This notion is supported by several lines of evidence, including the differential inhibition of release caused by clostridial neurotoxins⁸, antibodies²⁴ or mutations at the N- and C-terminal regions of the SNARE complex²⁵. Furthermore, biophysical data showed that the N-terminal half of the SNARE complex is extremely stable²⁶ and can fold

independently of the C terminus^{20,25} and that synaptobrevin and syntaxin-1–SNAP-25 anchored at apposed lipid bilayers form an intermediate state in which about 70% of the SNARE complex is assembled²⁷. This intermediate has a very high stabilization energy ($35 k_B T$), and assembly of the C terminus of the SNARE complex is expected to provide further energy. Because several SNARE complexes (estimated between 3 and 17; ref. 8.) are believed to form a ring around the site of fusion, the bulk energy of formation of these complexes easily exceeds the energy required for membrane fusion²⁸. However, it is still unclear whether all of this energy or only that provided by C-terminal assembly can be transduced to cause fusion, whether more energy is provided by other factors, and how the available energy is transduced into fusion. Note also that the transmembrane regions of synaptobrevin and syntaxin-1 are critical in SNARE-mediated liposome fusion²⁹ and probably lower the energy barrier to fusion by destabilizing the lipid bilayers³⁰, but it is unknown to what extent.

Liposome fusion assays showed that the SNAREs alone could force the merger of two membranes¹⁴, but the efficiency of fusion depends highly on the P/L ratios and the homogeneity of the liposomes^{31,32}. The P/L ratios required to observe fusion lead to liposome leakiness³³, probably because high densities of the SNARE transmembrane regions compromise membrane integrity. Note, however, that the low fusion efficiency arises in part from inhibition by the syntaxin-1 H_{abc} domain or by formation of 2:1 syntaxin-1–SNAP-25 heterodimers, and peptides spanning the C terminus of the synaptobrevin SNARE motif can release both types of inhibition, facilitating the N- to C-terminal zippering and allowing highly efficient fusion in a few minutes^{19,20}. Still, single-molecule spectroscopy studies showed that multiple SNARE complexes (~ 12) can assemble between vesicles containing low synaptobrevin/lipid ratios and supported bilayers containing syntaxin-1–SNAP-25 without inducing membrane fusion³⁴. A plausible rationalization for all these results is that a limited number of assembling SNARE complexes can diffuse to the center of the intermembrane space and remain assembled without inducing fusion (Fig. 1c, right panel); accumulation of many SNARE complexes may cause sufficient strain on the membranes to force them to merge³², but such accumulation may compromise membrane integrity. More efficient fusion mechanisms can be envisaged by cooperation with other factors⁴ (see below).

The observation of SNARE complex formation without fusion suggests that there is some degree of flexibility in the linker between the SNARE motifs and transmembrane regions of synaptobrevin and syntaxin-1 (ref. 4), in contrast to the assumption in early models of SNARE-mediated fusion that these sequences form continuous helices. It is noteworthy in this context that inclusion of helix-breaking residues in the synaptobrevin linker does not alter Ca^{2+} -evoked exocytosis³⁵ or the rate of liposome fusion³⁶, but the linker sequences contain basic residues that bind to membranes³⁷ and could help in coupling SNARE-complex assembly to mechanical action on the membranes. Such coupling was suggested by the findings that inserting flexible sequences in the synaptobrevin linker strongly inhibits Ca^{2+} -triggered exocytosis³⁸ and that that inhibition depends steeply on the length of the linker³⁵. Interestingly, inhibition of spontaneous release in synapses³⁸ and the slow phase of exocytosis in chromaffin cells³⁵ depend less steeply on the linker length, which

correlates with the effects of similar linkers on SNARE-mediated liposome fusion³⁶. These findings suggest that the primed state that is ready for fast Ca^{2+} -evoked exocytosis may involve hemifusion of the vesicle and plasma membranes³⁵. Overall, the results summarized above show that elucidating how SNARE complex assembly is coupled to membrane fusion remains as a key challenge in this field and emphasize the importance of understanding how other factors govern assembly and may cooperate with the SNAREs in fusion.

Munc18-1

Like the SNAREs, SM proteins are crucial for most types of intracellular membrane traffic^{1,6}, as emphatically shown by the total abrogation of neurotransmitter release observed in Munc18-1 knockout mice³⁹. However, the function(s) of SM proteins has been enigmatic. The finding that Munc18-1 binds tightly to syntaxin-1 suggested that Munc18-1 is functionally coupled to the SNAREs and forms part of the fusion machinery⁴⁰, but this interaction was found to require the closed conformation of syntaxin-1 (ref. 17) and to hinder SNARE complex formation⁴¹. Moreover, X-ray crystallography showed that Munc18-1 forms an arch-shaped structure with a cavity in the middle where syntaxin-1 binds (Fig. 1a,b) and that the closed conformation is clearly incompatible with the SNARE complex⁴². Although it was proposed that Munc18-1 may somehow assist in SNARE-complex assembly to explain its critical function in release^{17,42}, the structural and biochemical data seemed to contradict this notion.

Key insights clarifying SM protein function came from studies of SNAREs and SM proteins from diverse membrane compartments. The yeast SM protein involved in exocytosis, Sec1p, was shown to bind to cognate SNARE complexes rather than to isolated Sso1p, the yeast plasma membrane syntaxin^{43,44}. Although Sso1p forms a closed conformation⁴⁵ and all syntaxins contain an H_{abc} domain^{46–48}, it became clear that the closed conformation is not a general feature of syntaxins^{46,48}, and the syntaxins from the ER, Golgi, TGN and early endosomes were found to bind tightly to their cognate SM proteins through a short N-terminal sequence (NTS; Fig. 1a), in an interaction that is compatible with the SNARE complex^{47–49}. The apparently confusing picture emerging from these findings has been recently reconciled by several studies showing that many SM proteins, including Munc18-1, bind to SNARE complexes and that the syntaxin NTS most often contributes to binding (refs. 50,51 and references cited therein; note that the recent observation of Munc18-1 binding to the SNARE complex contrasts with results of an earlier study⁴¹).

These results suggest that SM-protein binding to SNARE complexes underlies the general mode of coupling between these two critical protein families and most often involves the syntaxin NTS, whereas the binary complex of Munc18-1 with closed syntaxin-1 represents a specialization of regulated exocytosis. Both types of interaction of Munc18-1 with the neuronal SNAREs involve the syntaxin-1 NTS (Fig. 1b,d), which plays a crucial role in exocytosis and was proposed to provide a pivot point for the transition between the two complexes⁵². Conversely, recent data suggested that the NTS needs to be released from Munc18-1 for SNARE complex assembly to occur⁵³, but it is unclear whether such release is required in the presence of other factors. Indeed, although Munc18-1 binding stabilizes syntaxin-1 *in vivo*³⁹ and seems to be key for transport of syntaxin-1 to the plasma

membrane⁵⁴, this binary interaction poses an energetic barrier to SNARE complex assembly, and other factors such as Munc13s must be required to promote the transition to the SNARE complex. Despite its apparently inhibitory interaction with closed syntaxin-1, Munc18-1 is likely to play an additional, active role in SNARE complex assembly, as suggested by the Munc18-1–SNARE complex interaction. Such a role may involve providing a template to form the syntaxin-1–SNAP-25 acceptor complex for synaptobrevin, as Munc18-1 also binds to syntaxin-1–SNAP-25 heterodimers^{22,23,55}. This proposal is supported by the finding that Munc18-1 facilitates assembly of SNARE complexes between synaptobrevin and coexpressed syntaxin-1–SNAP-25 heterodimers⁵¹. Note also that recent evidence⁵⁶ suggests multiple roles for Munc18-1 throughout the steps leading to exocytosis, including a function in vesicle priming that could arise from its action in SNARE complex assembly.

As SNAREs alone can form SNARE complexes *in vitro*, a key question is whether a general function for Munc18-1 and SM proteins in promoting SNARE complex assembly can explain the strong phenotypes observed upon genetic deletion of these proteins; or, perhaps they play a direct, key role in membrane fusion. Genetic experiments indeed suggested a function for Sec1p downstream of SNARE complex formation⁵⁷, and a model for how Munc18-1 might participate in fusion was proposed considering that diffusion of assembling SNARE complexes to the center of the intermembrane space may hinder application of mechanical force to the membranes (Fig. 1c; see above)⁴. This model predicts that binding of a bulky protein(s) such as Munc18-1 to the assembling SNARE complexes may prevent such diffusion and provide an asymmetry in the resulting Munc18-1–SNARE complex assemblies to allow energy transduction to the membranes (Fig. 1d)⁴. Although the findings that Munc18-1 binds to SNARE complexes^{50,51} and strongly enhances SNARE-mediated liposome fusion⁵¹ support this model, it is still unclear whether Munc18-1 stimulates fusion indirectly (that is, by promoting SNARE complex assembly) or directly. Recent evidence favors the later possibility⁵⁸, but further research will be necessary to demonstrate whether and how Munc18-1 directly contributes to fusion.

Munc13s and RIMs

Presynaptic active zones are closely apposed to postsynaptic densities and are formed by large proteins that facilitate rapid mobilization, fusion and recycling of synaptic vesicles, enabling the precise timing and localization of synaptic transmission². Among these proteins, Munc13s and RIMs (unc13 and unc10, respectively, in *Caenorhabditis elegans*) play prominent roles in vesicle priming and diverse forms of presynaptic plasticity. Figure 2a shows the multidomain structures of Munc13-1 and RIM1 α , two of the major isoforms of these protein families in brain; other isoforms contain the same or only a subset of these domains. Note the abundance of C₂ domains in both Munc13s and RIMs. These widespread protein modules adopt characteristic β -sandwich structures and commonly function as Ca²⁺ binding modules⁵⁹, but the Munc13 C₂A domain and both RIM C₂ domains are Ca²⁺-independent^{60–62}, and only the Munc13 C₂B domain is predicted to bind Ca²⁺.

The key role of Munc13s for priming is illustrated by the total abrogation of spontaneous and evoked release observed upon deletion of Munc13-1 and the closely related Munc13-2

in mice, or of the homologs in *C. elegans* and *Drosophila melanogaster* (reviewed in ref. 2). Crucial for this function is the MUN domain, which is sufficient to rescue release in Munc13-1/2 double-knockout mice⁶³. The observation that a constitutively open syntaxin mutant¹⁷ partially rescues release in *unc13* nulls in *C. elegans* suggested that *unc13* and Munc13s play a role in the conformational transition of syntaxin-1 (ref. 64). This role was initially attributed to an interaction with the syntaxin-1 N-terminal region, but the Munc13-1 MUN domain does not bind to isolated syntaxin-1 (refs. 22,63). However, the MUN domain does bind to membrane-anchored SNARE complexes and to syntaxin-1–SNAP-25 heterodimers^{22,23}. These results support the idea that Munc13s function in opening syntaxin-1, but perhaps by a mechanism different from that originally envisioned, acting in concert with Munc18-1 to form the syntaxin-1–SNAP-25 heterodimer and promote vesicle priming. It is also worth noting that the protein CAPS, which also contains a MUN domain⁶³, has also been implicated in vesicle priming, suggesting that priming may depend generally on MUN domains. These bulky domains could also play a direct role in fusion through their interaction with the SNARE complex, as proposed above for Munc18-1 (Fig. 1d), but this notion has not been tested.

A function for Munc13s in presynaptic plasticity in addition to priming was shown by the observation of distinct changes in synaptic amplitudes upon high-frequency stimulation in the presence of either Munc13-1 or Munc13-2, as well as by the functional effects of mutations in the C₁ domain and a calmodulin-binding (CaMb) sequence^{65–68} (see Fig. 2a). Coupling of the Munc13 C₂A domain to RIMs and Rab3 may provide a link to further forms of plasticity (see below). These observations led to the hypothesis that various factors involved in distinct presynaptic plasticity processes may regulate release efficiency by altering intramolecular interactions of their receptor domains with the Munc13 MUN domain, thus controlling its priming activity and, as a consequence, the vesicular release probability⁶³.

RIM1 α was originally identified as a Rab3 effector, but RIMs have multiple functions beyond this role⁶⁹, as only a subset of RIMs contain the N-terminal Rab-binding region, and a much stronger impairment of neurotransmitter release is observed upon deletion of RIM1 α and the closely related RIM2 α in mice or of *unc10* in *C. elegans* than is observed in the absence of Rab3s (reviewed in ref. 69; note that the function of Rab3s is still unclear and that the mild inhibition of release observed in their absence contrasts with the strong blocks in membrane traffic caused by mutations in other Rab proteins¹). The phenotype of *unc10* nulls in *C. elegans* and the finding that this phenotype can be partially rescued by the open syntaxin mutant⁷⁰ suggested that *unc10* and RIMs play a role in vesicle priming that is functionally linked to *unc13* and Munc13s. Such a link has been demonstrated by the reduction in Munc13-1 levels observed in RIM1 α knockout mice⁷¹, by the observation of a direct interaction between the N-terminal regions of RIM1 α and Munc13-1 (ref. 72), and by the impairment of vesicle priming caused by interference with this interaction^{72,73}. RIM1 α shows additional interactions with other active zone proteins that suggest a role in organizing the active zone^{69,71}. Moreover, mossy fiber long-term potentiation is abolished in RIM1 α knockout mice⁷⁴, as observed in Rab3A knockout mice⁷⁵, and short-term

plasticity is altered^{71,76}. The severe deficits in memory and learning observed in RIM1 α knockout mice⁷⁷ emphasize the importance of these varied functions.

The mechanisms of RIM function are poorly understood. The Munc13-1–RIM interaction has been well characterized and shown to involve the Munc13-1 C₂A domain and a sequence at its C terminus, which wrap around the RIM ZF domain⁶⁰ (Fig. 2b). The Munc13-1 C₂A domain also forms a tight homodimer (Fig. 2c) that is incompatible with Munc13–RIM heterodimerization, although there is only a slight overlap between the surfaces involved in homo and heterodimerization⁶⁰ (Fig. 2d). Conversely, the interactions of Munc13 and Rab3 with RIM can occur simultaneously and thus lead to a tripartite complex⁷³. RIM binds to Rab3A through two α -helices adjacent to the ZF finger⁷³, as expected from the crystal structure of a complex between Rab3A and rabphilin, another Rab3 effector⁷⁸ (Fig. 2e), and from the homology between the rabphilin and RIM N-terminal sequences. Superposition of the Munc13-1–RIM heterodimer with a homology model of the RIM–Rab3A complex suggests that some steric clashes between Munc13-1 and Rab3A occur in the tripartite Munc13-1–RIM–Rab3A complex (Fig. 2f), but they can be relieved by a slight structural rearrangement⁶⁰ (Fig. 2g). Overall, these results suggest that a switch from a Munc13-1 homodimer to a Munc13-1–RIM heterodimer, perhaps influenced by Rab3s, may regulate the activity of the MUN domain in priming and provide a link between priming and diverse forms of presynaptic plasticity that depend on RIMs and Rab3s (refs. 60,73). However, this model remains to be tested, and it has also been proposed that the Munc13-1–RIM–Rab3A complex may help to recruit Munc13-1 and synaptic vesicles at release sites in the active zone⁷³. Distinguishing these possibilities and characterizing in detail the relevant interactions of the highly conserved C₂ domains will be crucial to understanding RIM function.

Synaptotagmin-1 and complexins

Key for the Ca²⁺-triggering step of release are synaptotagmin-1 and complexins^{1,3,4,7} (Figs. 3 and 4). Synaptotagmin-1 is a synaptic vesicle protein with two C₂ domains, the C₂A and C₂B domains, that adopt similar β -sandwich structures and bind three and two Ca²⁺ ions, respectively, through loops at the top of the sandwich^{79–82} (Fig. 3a,b). These top loops also mediate Ca²⁺-dependent phospholipid binding to both C₂ domains^{82–84}. Mutations that decrease or increase the apparent Ca²⁺ affinity of synaptotagmin-1 lead to parallel changes in the Ca²⁺ sensitivity of release^{85,86}, showing that synaptotagmin-1 acts as a Ca²⁺ sensor in release and that Ca²⁺-dependent phospholipid binding to both C₂ domains is key for this function. However, mutations in the Ca²⁺-binding ligands of the C₂B domain impair release much more severely than analogous mutations in the C₂A domain^{87,88}, indicating that Ca²⁺ binding to the C₂B domain is more critical for release. Among several potential explanations for these findings^{4,7}, particularly attractive is the observation that the C₂B domain mediates simultaneous binding to two membranes through its Ca²⁺-binding loops and the abundant basic residues around its surface, thus showing that the C₂B domain can bring two membranes together as the SNAREs do, but through a Ca²⁺-dependent mechanism⁸⁹ (Fig. 3d).

In addition to membrane binding, diverse interactions between SNAREs and synaptotagmin-1 have been described (reviewed in refs. 1,3,4,7), and those involving SNARE complexes are the most likely to couple their functions in triggering release. Although the relevance of Ca^{2+} -dependent interactions of synaptotagmin-1 with soluble SNARE complexes was questioned because phospholipids compete with these interactions⁹⁰, synaptotagmin-1 actually binds simultaneously to membranes and SNARE complexes if they are anchored on the membranes⁹¹, resulting in a quaternary SNARE–synaptotagmin-1– Ca^{2+} –phospholipid (SSCAP) complex⁹². These findings can be rationalized by considering the highly charged nature of these proteins (Fig. 3d) together with a model that was built from a mutagenesis analysis of SSCAP complex formation⁹² (Fig. 3b) and is compatible with single-molecule spectroscopy data⁹³. The model envisions the relevant synaptotagmin-1/SNARE complex interaction as being mediated by a polybasic region on the side of the C₂B domain β -sandwich (Fig. 3b) (but note that this region has also been implicated in diverse interactions including phospholipid binding^{4,7}). An attractive feature of this model of the SSCAP complex is that the top Ca^{2+} -binding loops and the bottom side of the C₂B domain can still interact with the apposed membranes to bring them together in cooperation with the SNAREs; moreover, the highly positive electrostatic potential generated by the C₂B domain and the C terminus of the SNARE complex could help induce negative curvature on the membranes to bend them and induce fusion^{89,92} (Fig. 3d).

This overall model has been supported by theoretical calculations⁹⁴ and is consistent with multiple functional studies, including those showing a predominant role of the C₂B domain in release⁹². Note, however, that basic residues in the C₂A domain that are functionally important have also been implicated in SNAP-25 binding⁹⁵ and that alternative models have also been proposed. Thus, the vesicle and plasma membranes might be hemifused before Ca^{2+} influx, and the positive electrostatic potential of the C₂B domain may help to open the fusion pore^{92,94}. Another model predicts that synaptotagmin-1 causes fusion by inducing positive curvature in the plasma membrane⁹⁶ (Fig. 3e); this model is also supported by theoretical calculations⁹⁶ and is compatible with the induction of negative curvature proposed in Figure 3d (membrane bending involves both types of curvature; Fig. 3f). However, the significance of the liposome tubulation experiments supporting the induction of positive membrane curvature by synaptotagmin-1 is unclear because they used high concentrations of negative stain⁹⁶ and only a small degree of tubulation is observed in aqueous solution⁸⁹. Regardless of these models, a role for synaptotagmin-1 in fusion has been supported by the observation that Ca^{2+} -bound synaptotagmin-1 can enhance SNARE-mediated liposome fusion^{97,98}.

The function of complexins is intimately coupled to that of the SNAREs and synaptotagmin-1. Complexins are largely unstructured in solution⁹⁹ and bind tightly to the SNARE complex¹⁰⁰, forming an α -helix that interacts in an antiparallel fashion with the synaptobrevin and syntaxin-1 SNARE motifs²⁶ (Fig. 4b). The observations that complexin inhibits SNARE-induced cell-cell or liposome fusion and that Ca^{2+} -bound synaptotagmin-1 releases this inhibition led to the proposal that complexins act as fusion clamps^{101,102}. This model is consistent with biophysical data suggesting that synaptotagmin-1 displaces complexin-I from membrane-anchored SNARE complexes and with the observation that

increasing the local concentration of complexin-I by fusing it to the N terminus of synaptobrevin strongly impairs synchronous release¹⁰³. Furthermore, a marked increase in spontaneous release is observed in *Drosophila* complexin null mutants¹⁰⁴. However, a merely inhibitory role for complexin cannot explain the severe impairment in release observed in complexin-I/II double-knockout mice¹⁰⁵ and the finding that the other two mammalian complexin isoforms (III and IV) rescue this phenotype¹⁰⁶. Taking into account these findings and the observation that complexin binding stabilizes the C terminus of the SNARE complex²⁶, it was proposed that complexins have dual roles and that complexin binding to the SNARE complex generates a metastable state that serves as a substrate for synaptotagmin-1 to trigger fast release¹⁰³. Studies of sperm acrosomal fusion indicated a similar synaptotagmin-complexin interplay¹⁰⁷, suggesting that this interplay is not limited to neuronal exocytosis.

Studies of the ability of complexin-I mutants to rescue release in complexin-I/II double knockout mice have illustrated the complexity of complexin function and shown that SNARE complex binding is necessary but not sufficient for this function¹⁰⁸. Mutations that disrupt binding to the SNARE complex abolish rescue, but rescue is also abrogated by deleting the 26 N-terminal residues of complexin-I, which preserves the SNARE interaction; intriguingly, partial rescue was observed upon deletion of the 46 N-terminal residues. These results led to the proposal that residues 27–46, which form the ‘accessory helix’ preceding the SNARE complex-binding region defined by crystallography (residues 48–70), play an inhibitory role, perhaps by replacing the C terminus of the synaptobrevin SNARE motif in the SNARE complex¹⁰⁸ (Fig. 4d; left panel). Recent data showing that complexin-I binds to syntaxin-1–SNAP-25 heterodimers^{22,23} have provided some support for this proposal. The rescue data also revealed that the N terminus is essential for complexin function and must be involved in an as-yet-unidentified interaction that is critical for release¹⁰⁸. This notion suggests that SNARE-complex binding provides an attachment point for complexin so that it may perform this role, but it seems unlikely that complexin is then fully displaced by synaptotagmin-1 from the SNARE complex upon Ca^{2+} influx. Note that the model of the SSCAP complex built by monitoring complexin-I displacement from SNARE complexes by various synaptotagmin-1 mutants (Fig. 3b) predicts that the C₂B domain collides with the accessory helix but not with the SNARE-binding region of complexin-I (ref. 92) (Fig. 4c). Hence, the complexin-I displacement assays may reflect a local competition and weakening of the complexin–SNARE complex interaction due to steric hindrance, rather than a full competition between complexin-I and synaptotagmin-1 for SNARE complex binding (Fig. 4d). Moreover, co-flotation assays have indicated that complexin and synaptotagmin-1 can indeed bind simultaneously to SNARE complexes¹⁰².

These observations suggest that both complexin-I and synaptotagmin-1 are bound to the SNAREs after Ca^{2+} influx and that Ca^{2+} induces a rearrangement of interactions that leads to fast neurotransmitter release through the concerted action of all these proteins, although the exact nature of this rearrangement is unclear. Note that Ca^{2+} -free synaptotagmin-1 binds to membrane-anchored syntaxin-1–SNAP-25 heterodimers²³, but it is unknown whether this interaction is compatible with complexin binding. In addition, X-ray crystallography recently revealed an intramolecular interaction between the C₂ domains in Ca^{2+} -free

synaptotagmin-1 (ref. 109) (Fig. 3c), which, although not observed in solution⁸⁹, could influence how Ca^{2+} rearranges the release machinery. Hence, elucidating the conformational and binding status of synaptotagmin-1 before Ca^{2+} influx will be as crucial to understand the mechanism of release as testing the emerging ideas about its Ca^{2+} -triggered action.

Perspective

Studies of the neurotransmitter release machinery have led to some well-established conclusions about the functions of its components and to several models for their mechanism of action, some of which are depicted in Figures 1–4. However, many of the ideas discussed above remain as likely but undemonstrated, or they are purely speculative, and critical questions need to be answered before all these ideas can be integrated into a unified model of the mechanism of neurotransmitter release. It seems clear that the SNARE complex is central in membrane fusion and that many factors interact with this complex (Fig. 5) to control its assembly and/or membrane fusion, but most of these interactions are not well-understood, and it is unclear whether they occur simultaneously or sequentially. Perhaps the most crucial question for understanding not only synaptic exocytosis but also intracellular membrane fusion in general is whether SM proteins are directly involved in fusion, in addition to having a role in SNARE-complex assembly. Munc13s and RIMs appear to control the conformational transition of syntaxin-1 and various forms of presynaptic plasticity, but the mechanisms of these functions remain to be defined, and it is plausible that Munc13s also have a direct role in fusion. Synaptotagmin-1 and complexins are clearly involved in the Ca^{2+} -triggered step of release, and both may play a role in fusion, but the exact mechanism of their interplay with the SNAREs is still enigmatic. Also unclear are the functional differences between synaptotagmin-1 and other synaptotagmin isoforms that are likely to play related but slightly different roles in different types of exocytosis¹. But perhaps most unclear of all is the mechanism of vesicle docking, since removal of any of these proteins does not impair docking in vertebrate central nervous system synapses, but increasing evidence suggests that Munc18-1, Munc13s and syntaxin-1 are actually key for docking in chromaffin cells and *C. elegans* synapses^{110–113}. Hence, it appears that the basic mechanism of docking that operates in these other systems may be redundant with vesicle tethering mechanisms in the active zones of vertebrate central nervous system synapses.

Consideration of three emerging themes will be critical for unraveling the mechanism of release. First, while there is a natural tendency to assign either active or inhibitory roles to the components of the release machinery, it is becoming clear that several components may play dual roles through diverse interactions with other proteins or the membrane. Second, interactions of two components with a common target often involve largely different but slightly overlapping binding sites (Figs. 2d,f and 4c), which may facilitate rapid rearrangements of interactions during docking, priming and fusion⁶⁰. Third, the influence of membranes on interactions of the SNARE complex with synaptotagmin-1 (refs. 92,103) and the Munc13-1 MUN domain²² underlines the importance of working with reconstituted systems to study the interactions within the release machinery, which is not surprising given the very nature of the biological process controlled by this machinery. Thus, further development of methods for structural studies of protein complexes on a membrane or,

ideally, between two membranes will be crucial for reaching a detailed understanding of the complex mechanism of Ca²⁺-triggered neurotransmitter release.

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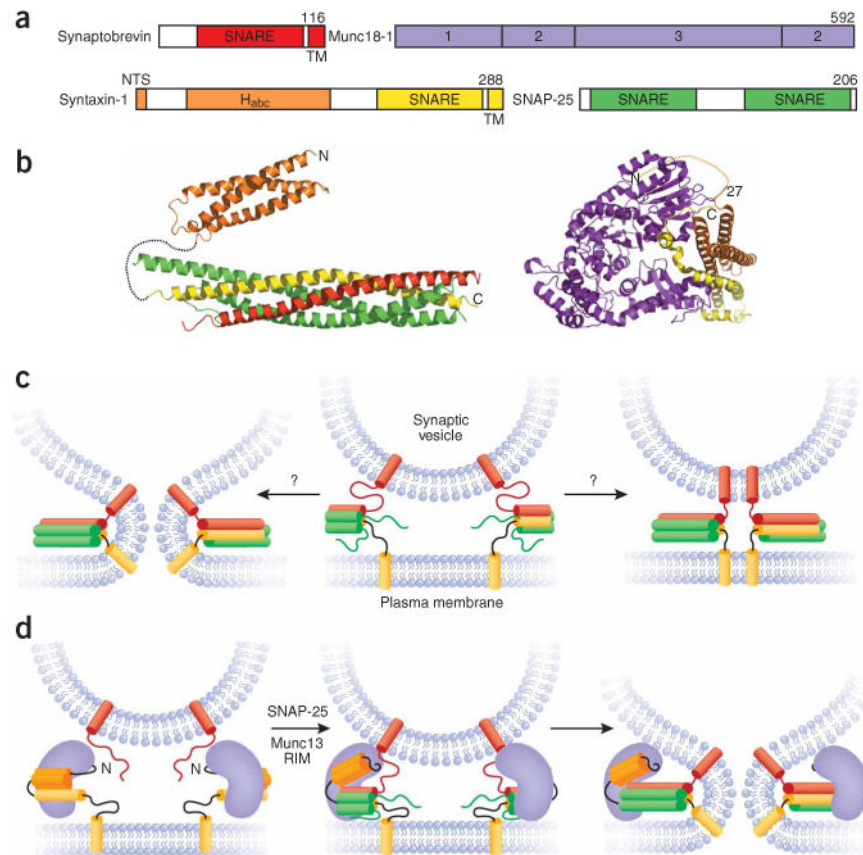


Figure 1. SNAREs and Munc18-1. **(a)** Domain diagrams of the neuronal SNAREs and Munc18-1. The number of residues of each protein is indicated above each diagram on the right. The same color coding for these proteins is used in all other figures. TM, transmembrane. **(b)** Ribbon diagrams of the structures of the SNARE complex¹¹ and the syntaxin-1 H_{abc} domain¹⁶ (shown connected by a dashed curve that represents the syntaxin-1 sequence linking them) and of the binary complex between Munc18-1 and the closed conformation of syntaxin-1⁴². The orange dashed curve indicates that the syntaxin-1 NTS also participates in Munc18-1 binding^{52,53}, even though it was not observed in the structure of the complex. The N and C termini of syntaxin-1 are indicated in both diagrams. **(c)** Partially assembled SNARE complexes (center) and how they could induce membrane fusion as they fully assemble (left) or could remain fully assembled between the two membranes (right) if the linkers between the SNARE motifs and transmembrane regions are flexible. **(d)** The closed conformation of syntaxin-1 bound to Munc18-1 (left) and Munc18-1 bound to partially assembled SNARE complexes (center) that could induce fusion as the SNARE complexes fully assemble (right). Munc13s and RIMs are likely to mediate the transition between the two complexes.

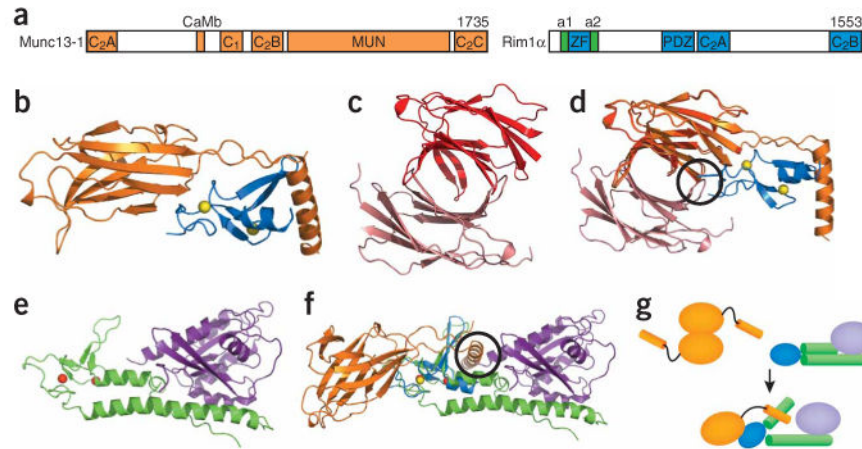


Figure 2.

Munc13s and RIMs. **(a)** Domain diagram of Munc13-1 and RIM1 α . The number of residues of each protein is indicated above each diagram on the right. The helices adjacent to the RIM1 α ZF domain, which are involved in Rab3 binding, are labeled a1 and a2. The same color-coding is used in **b,d-g**. **(b)** Structure of the complex between the RIM2 α ZF domain and the N-terminal region of Munc13-1, including the C₂A domain60. Yellow spheres, zinc ions. **(c,d)** Structure of the Munc13-1 C₂A domain homodimer60 **(c)** and superposition with the Munc13-1/RIM2 α heterodimer **(d)**. The two protomers of the Munc13-1 C₂A domain homodimer are shown in red and salmon to distinguish them from the Munc13-1 C₂A domain in the heterodimer. **(e)** Structure of Rab3A (purple) bound to the rabphilin N-terminal region containing its ZF domain and the two adjacent helices involved in Rab3 binding (green)78. Red spheres, zinc ions. **(f)** Superposition of the Munc13-1–RIM2 α and Rab3A–rabphilin structures shown in **b** and **e** using the two ZF domains for the superposition. Black circles, regions of overlap between the RIM2 α ZF domain and one Munc13-1 C₂A domain protomer of the homodimer in **d**, and between Rab3A and the Munc13-1 α -helix at the C terminus of the C₂A domain in **f**. **(g)** Model summarizing the structural rearrangements and changes in protein-protein interfaces proposed to occur during the switch from the Munc13-1 homodimer to the Munc13-1–RIM–Rab3 tripartite complex.

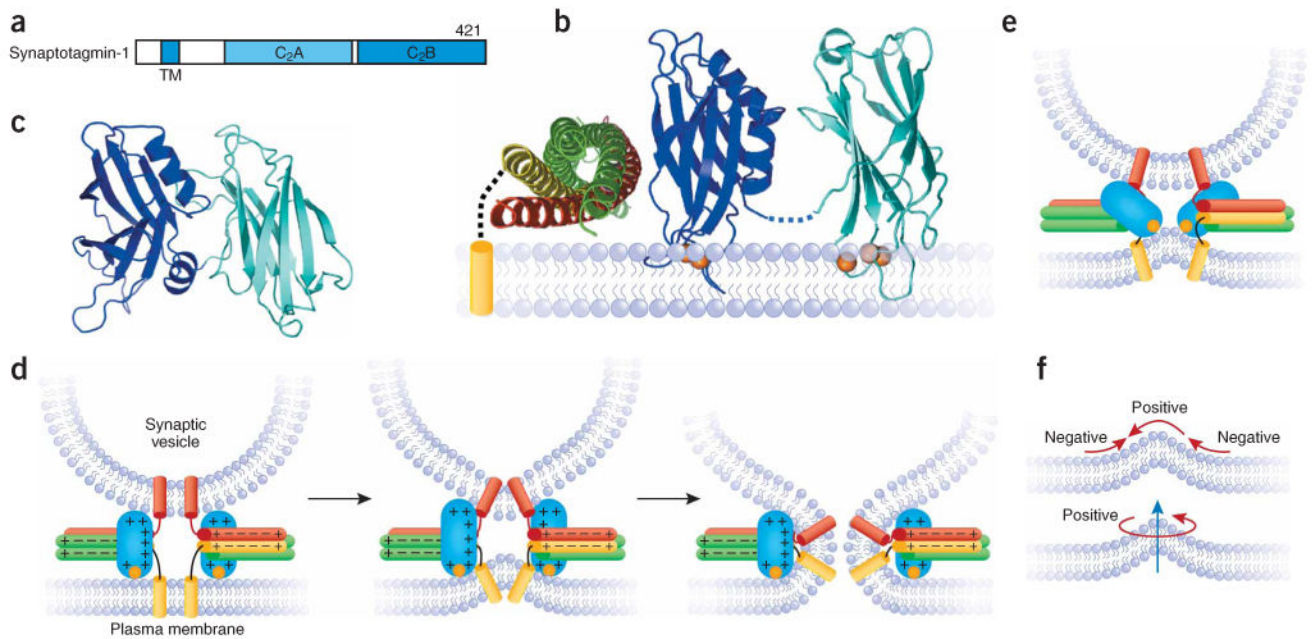


Figure 3.

Syntaptotagmin-1 and its coupling to SNAREs and membranes. **(a)** Domain structure of syntaptotagmin-1, with the number of residues indicated in the top right corner. TM, transmembrane. **(b)** Model of the SSCAP complex built from the structures of the SNARE complex¹¹ and the Ca²⁺-bound syntaptotagmin-1 C₂ domains^{81,82} and a mutagenesis analysis of SSCAP complex formation⁹². Orange spheres, Ca²⁺ ions; dashed black curve, the linker between the syntaxin-1 SNARE motif and transmembrane region. Note that it is uncertain whether the syntaptotagmin-1 Ca²⁺-binding loops bind to the plasma membrane or the synaptic vesicle membrane. **(c)** Crystal structure of the tandem syntaptotagmin-1 C₂ domains in the absence of Ca²⁺; the structure involves an antiparallel interaction between the two domains that needs to be disrupted to allow Ca²⁺ binding to the C₂A domain¹⁰⁹. **(d)** Model of how the syntaptotagmin-1 C₂B domain could cooperate with the SNAREs in triggering membrane fusion upon Ca²⁺ influx by binding to both membranes and the C terminus of the SNARE complex. Each orange circle represents the two Ca²⁺ ions bound to the C₂B domain. The + and – signs illustrate the electrostatic charge distribution of the C₂B domain and the SNARE complex. The model assumes that syntaptotagmin-1 interacts with the SNARE complex through the polybasic region on the side of the C₂B domain, as in **b**. This interaction is weak in solution but is likely to be strengthened by colocalization of syntaptotagmin-1 and the SNARE complex on one membrane, which at the same time may increase binding specificity by disfavoring irrelevant interactions existing in solution between these highly charged molecules⁹². The C₂A domain is not shown in this model for simplicity, but could play a related role. **(e)** Model of how the Ca²⁺ binding loops of the syntaptotagmin-1 C₂ domains (only the C₂B domain is shown for simplicity) could help to cause membrane fusion by inducing positive curvature on the plasma membrane⁹⁶. **(f)** Two diagrams showing the types of curvature involved in membrane bending and illustrating that such bending requires both positive and negative curvature.

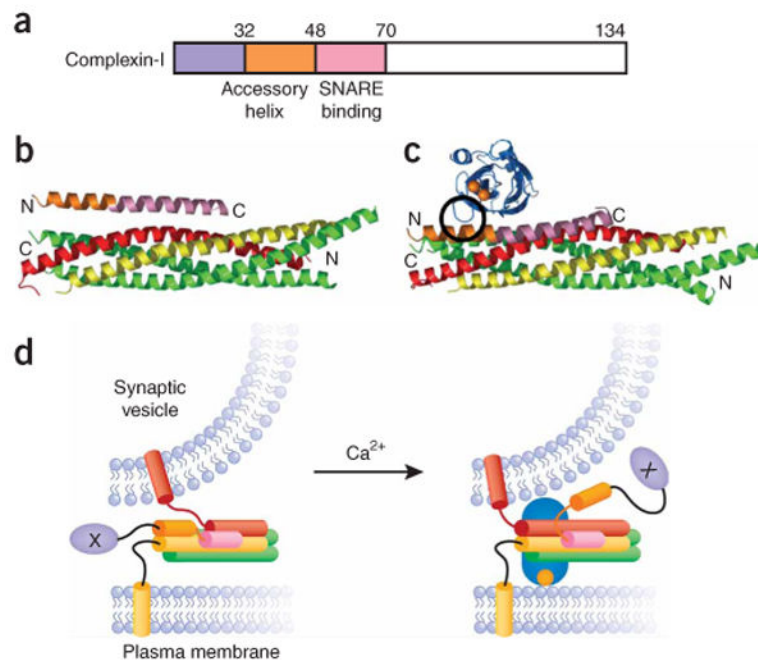


Figure 4.

Complexins and their coupling to SNAREs and synaptotagmin-1. **(a)** Domain diagram of complexin-I. Residue numbers are indicated above the diagram. The same color-coding is used in the remaining panels. **(b)** Structure of a complexin-I fragment bound to the SNARE complex²⁶. The N and C termini of the complexin-I fragment and the SNARE complex are indicated. **(c)** Superposition of the crystal structure shown in **b** with the model of the SCCAP complex shown in Figure 3b (omitting the membrane and the C₂A domain). Black circle, the region of overlap between the C₂B domain and the accessory helix of complexin-I. **(d)** Models of how binding of the accessory helix to the C terminus of the SNARE complex could hinder full SNARE complex assembly and thus inhibit fusion (left), and how this inhibition could be released by partial competition between the synaptotagmin-1 C₂B domain and complexin for binding to the C terminus of the SNARE complex (right). The N terminus of complexin is shown as a purple ellipse with an X in the center to indicate an as-yet-unidentified interaction of this region that is critical for complexin function. The orientation of this N-terminal region in both diagrams is arbitrary.

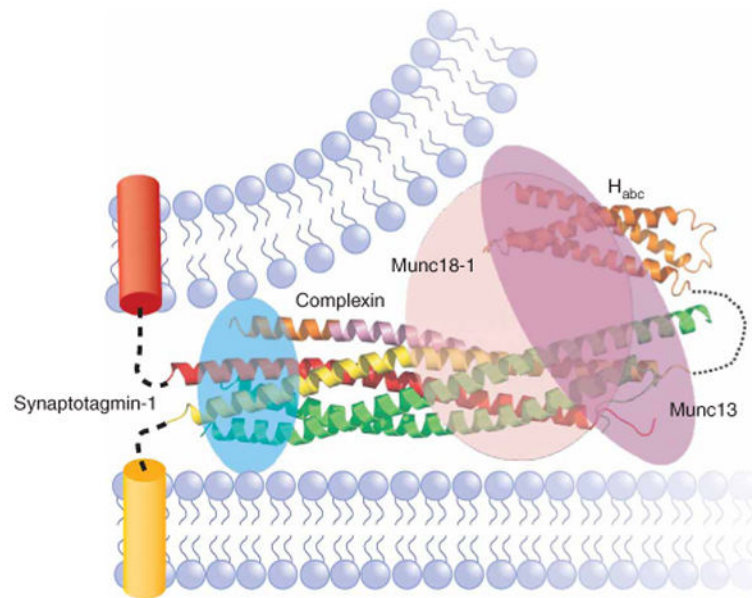


Figure 5.

Diagram illustrating the notion that Munc18-1, Munc13, complexin and synaptotagmin-1 bind to the SNARE complex. The ribbon diagram shows the structure of the complexin-I–SNARE complex²⁶, which illustrates the only one among these interactions that has been revealed at atomic resolution. The binding site of synaptotagmin-1 on the four-helix bundle is inferred from mutagenesis⁹², whereas those of Munc18-1 and Munc13 are unknown. The figure intends to illustrate that all these SNARE complex interactions may be compatible, mutually exclusive, or partially competitive, and that resolving this issue as well as how these interactions occur during the steps that lead to exocytosis will be critical to understand the mechanism of release.