

SNARES AND MUNC18 IN SYNAPTIC VESICLE FUSION

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The release of neurotransmitters by Ca^{2+} -triggered synaptic vesicle exocytosis is an exquisitely regulated process that is fundamental for interneuronal communication. This process involves several steps and is controlled by a protein machinery that must prevent release before Ca^{2+} entry into presynaptic terminals, and yet must rapidly induce release on Ca^{2+} influx. Extensive studies of the components of this machinery have indicated that SNAREs and Munc18-1 are central proteins for membrane fusion during exocytosis. An increasing amount of information derived from a convergence of structural, physiological and genetic studies is providing important insights into the mechanism of neurotransmitter release.

Neurotransmitter release is restricted to specialized, electron-dense sites on the presynaptic plasma membrane that are known as active zones. Synaptic vesicles dock at active zones and then undergo a priming reaction that prepares them for exocytosis when Ca^{2+} channels open in response to an action potential. Release occurs very quickly, less than 0.5 ms after Ca^{2+} influx¹. The speed of synaptic transmission is also facilitated by the close apposition of the active zones to electron-dense sites on the postsynaptic plasma membrane — so-called ‘postsynaptic densities’ — that are enriched in neurotransmitter receptors.

Extensive research has led to the identification of numerous proteins that are involved in neurotransmitter release, and to the idea that release is mediated by cascades of protein–protein interactions². Homologues of some of these proteins have been found to govern membrane traffic in different cellular compartments of organisms ranging from yeast to humans, resulting in the belief that most types of intracellular membrane fusion share a common mechanism^{3,4}. These proteins include **Sec18**/*N*-ethylmaleimide-sensitive fusion protein (**NSF**) homologues, **Sec17**/soluble NSF attachment proteins (**SNAPs**), SNAP receptors (SNAREs), **Sec1**/Munc18 homologues (SM proteins) and small GTPases of the Rab family. In addition, proteins such as **synaptotagmin 1**, **Munc13-1** and the complexins are crucial for Ca^{2+} -triggered exocytosis,

but not for other membrane-traffic events, and are specialized for the tight spatial and temporal regulation of neurotransmitter release.

The SNAREs and the SM protein **Munc18-1** have emerged as probably the most central components of the exocytotic apparatus, and attractive models of their functions have been developed. The SNAREs have been proposed to account for the specificity of membrane fusion⁵ and to directly execute fusion by forming a tight complex (the SNARE or core complex) that brings the synaptic vesicle and plasma membranes together^{6–8}. Munc18-1 has been suggested to assist in the formation of this complex, and models of the roles of other presynaptic proteins generally revolve around their interactions with SNAREs and/or Munc18-1. Although these concepts are widely accepted, abundant data argue for substantial promiscuity in SNARE–SNARE interactions, and the relationship between SNARE-complex formation and membrane fusion is still intensely debated^{8–14}. In addition, recent results have revealed different modes of SNARE–SM-protein coupling in various membrane-trafficking systems, indicating that the steps that lead to membrane fusion might be less conserved than was previously thought^{15–17}. Hence, research on the mechanism of neurotransmitter release is at a fascinating stage, with much available data and exciting emerging principles; at the same time, there is a sense that we are only beginning to understand its complexity. In this review, we

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ELECTRON PARAMAGNETIC RESONANCE (EPR). A spectroscopic technique that detects chemical species that have unpaired electrons. EPR spectroscopy can provide molecular structural details that are inaccessible by any other analytical tool.

COILED COIL
A polypeptide structural motif that is formed by two or more intertwined α -helices. Coiled-coil domains often participate in protein–protein interactions, but can also be involved in intramolecular interactions that stabilize protein structures.

attempt to distinguish between established concepts, notions that are probably oversimplifications, and ideas that are plausible but remain to be demonstrated. Particular attention is devoted to the abundant three-dimensional structural information that has been obtained by electron microscope (EM), ELECTRON PARAMAGNETIC RESONANCE (EPR), X-ray and nuclear magnetic resonance (NMR) studies, which have yielded many new insights and have provided a framework within which to understand the mechanism of neurotransmitter release.

SNAREs

The core complex. The SNAREs constitute a large family of proteins that are characterized by ~60-residue sequences known as SNARE motifs, which have a high propensity to form COILED COILS and often precede carboxy-terminal transmembrane regions^{9,18–20}. All steps of the secretory and endocytic pathways that have been studied in yeast and higher eukaryotes require SNARE proteins^{9,18,20}. Much of what we know about SNARE function has come from the analysis of synaptic SNAREs, which include the synaptic vesicle protein **synaptobrevin/VAMP** (for vesicle-associated membrane protein) and the plasma-membrane proteins **syntaxin 1** and **SNAP25** (for synaptosomal-associated protein of 25 kDa; no relation to SNAPs).

The functional importance of the synaptic SNAREs was first shown by the observation that they constitute the specific targets for clostridial neurotoxins, which inhibit neurotransmitter release^{21–24}, and later by genetic

experiments in *Drosophila*^{25,26}. In addition, synaptobrevin, syntaxin 1 and SNAP25 were found to form a large complex (the 20S complex) with NSF and SNAPs, which had been found to be essential for Golgi transport²⁷. Together with the known involvement of homologues of these proteins in membrane traffic in yeast, these findings led to a model of intracellular membrane traffic in which recognition between SNAREs in transport vesicles (v-SNAREs) and those in target membranes (t-SNAREs) mediates vesicle docking and targeting specificity (the SNARE hypothesis)^{27,28}. The SNAREs were also proposed to serve as membrane binding sites for SNAPs and NSF, which were believed to be components of the fusion apparatus, leading to their designation as SNAP receptors. The three synaptic SNAREs were later shown to form a complex — the SNARE or core complex — that binds to NSF through SNAPs and is disassembled by the ATPase activity of NSF²⁹. In fact, only the core complex (and not the individual SNAREs) binds with high affinity to SNAPs³⁰. The core complex was found to be resistant to cleavage by clostridial neurotoxins and to denaturation by SDS, highlighting its extremely high stability³¹, and to require the SNARE motifs of synaptobrevin, syntaxin 1 and SNAP25 (REFS 31,32).

The results obtained with clostridial neurotoxins^{21–24} and studies of yeast vacuolar fusion³³ provided evidence that v-SNAREs and t-SNAREs are required on opposing membranes for biological membrane fusion. However, even before the SNARE hypothesis was proposed, it was noted that clostridial neurotoxins interferes not with the recognition of synaptic vesicles and active zones, but

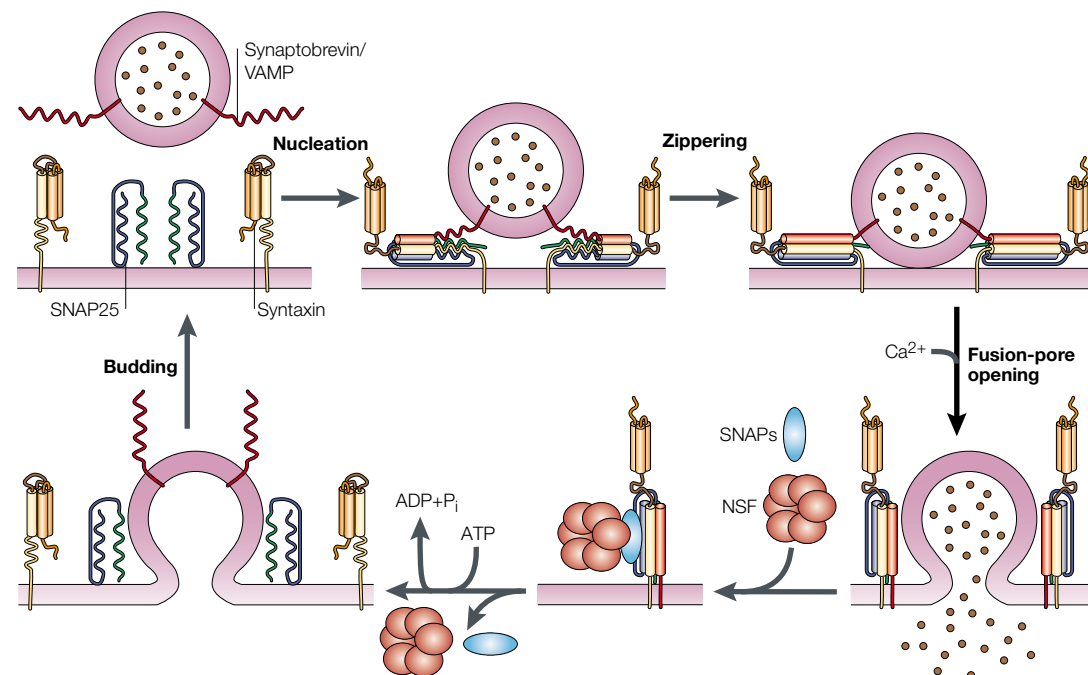


Figure 1 | Cycle of assembly and disassembly of the SNARE complex in synaptic vesicle exocytosis. Syntaxin exists in a closed conformation that needs to open to initiate core-complex assembly (nucleation). ‘Zippering’ of the four-helix bundle towards the carboxyl terminus brings the synaptic vesicle and plasma membranes towards each other, which might lead to membrane fusion. After fusion, *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs) disassemble the *cis*-core complexes that remain on the same membrane to recycle them for another round of fusion. SNAP25, synaptosomal-associated protein of 25 kDa; SNARE, SNAP receptor.

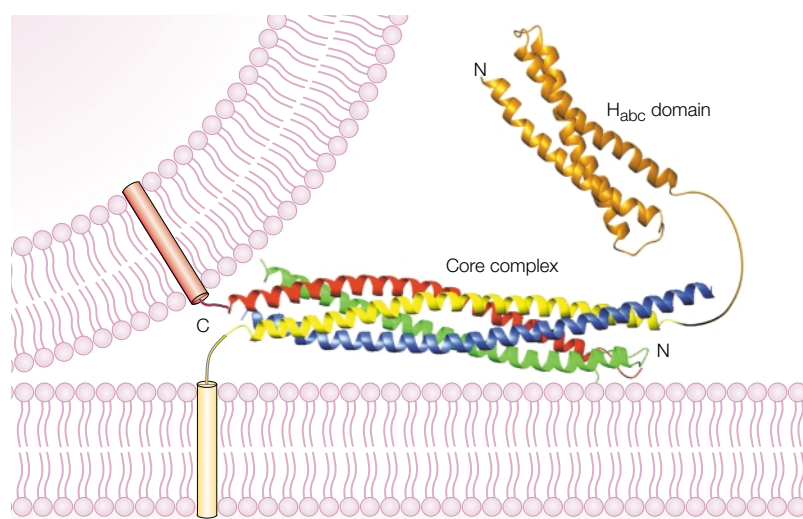


Figure 2 | Model of the neuronal SNAREs assembled into the core complex. The ribbon diagrams represent the crystal structure of the core complex⁴¹ and the NMR structure of the amino-terminal H_{abc} domain of syntaxin 1 (REF. 101). The H_{abc} domain is coloured in orange and the SNARE (SNAP receptor) motifs are colour coded as follows: synaptobrevin, red; syntaxin 1, yellow; synaptosomal-associated protein of 25 kDa (SNAP25) amino terminus, blue; SNAP25 carboxyl terminus, green. The cylinders represent the transmembrane regions of synaptobrevin and syntaxin 1, which are inserted into the synaptic vesicle and plasma membranes, respectively. The curved lines represent short sequences that connect the SNARE motifs and the transmembrane regions, as well as the linker region between the H_{abc} domain and the SNARE motif of syntaxin 1. All ribbon diagrams were prepared with the program MOLMOL¹⁵⁷.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). A spectroscopic technique that is based on the transfer of energy from the excited state of a donor moiety to an acceptor. The transfer efficiency depends on the distance between the donor and the acceptor. FRET is often used to estimate distances between macromolecular sites in the 20–100-Å range, or to study interactions between macromolecules *in vivo*.

KINETIC TRAP
A metastable state that cannot evolve or evolves slowly to a thermodynamically more stable state owing to a high energy barrier.

AAA SUPERFAMILY
A family of proteins that share a homologous ATPase module. Proteins from this family participate in diverse cellular processes, including membrane traffic, proteolysis and DNA replication.

rather with fusion itself (reviewed in REF. 34). Experiments with *Drosophila* mutants confirmed that SNAREs are not required for docking²⁶. In addition, NSF was found to function in a prefusion step in exocytosis and in yeast vacuolar fusion, but not in fusion itself^{35,36}. EM⁶ and FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)⁷ studies later showed that the SNARE motifs of synaptobrevin and syntaxin 1 bind in a parallel fashion. As these SNARE motifs are adjacent to the carboxy-terminal transmembrane regions of synaptobrevin and syntaxin 1, which are anchored at the synaptic vesicle and plasma membrane, respectively, these crucial observations led to the proposal that SNARE-complex formation brings the two membranes together and might cause membrane fusion. In this model, SNAPs and NSF are required to disassemble the core complex, recycling the SNAREs for another round of fusion (FIG. 1).

The synaptic core complex is formed by four SNARE motifs (two from SNAP25 and one each from synaptobrevin and syntaxin 1)^{37,38} that are unstructured in isolation^{38,39}, but form a parallel four-helix bundle on assembly^{40,41}. The crystal structure of the core complex (FIG. 2) revealed that the helix bundle is highly twisted and contains several salt bridges on the surface, as well as layers of interior hydrophobic residues⁴¹. However, a polar layer in the centre of the complex is formed by three glutamines (two from SNAP25 and one from syntaxin 1) and one arginine (from synaptobrevin). The interior residues are more conserved than the surface residues, and the polar layer is present throughout the SNARE family. These observations led to the reclassification of SNAREs as Q-SNAREs (contributing glutamine) and R-SNAREs

(contributing arginine), and to the proposal that all core complexes consist of four-helix bundles that are formed by three Q-SNAREs and one R-SNARE, the SNARE motifs that are homologous to those of SNAP25 usually being provided by two separate SNAREs¹⁹. This proposal has been supported by biophysical and reconstitution studies^{5,42–46}, and the crystal structure of a mammalian endosomal SNARE complex is remarkably similar to that of the neuronal complex, despite the low level of sequence conservation⁴⁶. Both classifications of SNAREs (v/t and Q/R) are useful in different contexts and are widely used.

Core-complex disassembly. After fusion, core complexes that remain on the same membrane need to be disassembled to recycle the SNAREs for another round of fusion, thereby completing the ‘SNARE cycle’ (FIG. 1). Core complexes on the same membrane are referred to as *cis*-core complexes to distinguish them from the *trans*-core complexes that are formed by SNAREs on opposing membranes before fusion. The need to disassemble *cis*-core complexes is even more clear for homotypic membrane fusion, in which both membranes have a full complement of SNAREs that can form core complexes. Disassembly is widely believed to be achieved by the ATPase activity of NSF, which binds to the core complex through SNAPs²⁹. Reconstitution experiments have suggested that NSF/SNAPs disassemble only *cis*-core complexes⁴⁷, but studies of yeast vacuolar fusion indicate that the yeast NSF/SNAP homologues, Sec18/Sec17, can disassemble *trans*-SNARE complexes¹⁰. In addition, NSF/SNAPs can disassemble four-helix bundles formed by syntaxin and SNAP25 that constitute KINETIC TRAPS and could hinder core-complex assembly⁴⁸.

EM and X-ray studies have provided insights into the mechanism of core-complex disassembly by NSF/SNAPs. NSF forms a hexameric ring structure⁶. Each NSF subunit contains three domains — an amino-terminal domain that is responsible for substrate binding⁴⁹, and two ATP-binding domains from the AAA SUPERFAMILY, D1 and D2. The ATPase activity of the D1 domain is crucial for core-complex disassembly, whereas the D2 domain has little ATPase activity^{49,50} and is responsible for hexamerization^{51,52}. The D2 hexamer has a flat pinwheel shape with a small hole in the centre, and each D2 protomer contains a nucleotide-binding subdomain and a carboxy-terminal helical subdomain^{51,52} (FIG. 3a). The amino-terminal domains of NSF^{53,54} and Sec18 (REF. 55) also contain two subdomains, one with a double-ψ β-barrel fold and another that forms an α/β-roll (FIG. 3b). The interface between these subdomains, which is rich in basic residues, was proposed to be the binding site for SNAPs⁵⁴. The yeast SNAP homologue Sec17 contains a twisted sheet of α-helical hairpins and a globular α-helical domain at the carboxyl terminus⁵⁶ (FIG. 3c). The curvature of the twisted sheet is similar to that of the grooves in the core complex, suggesting that SNAPs might wrap around the core complex⁵⁶. EM images indicate that SNAPs coat the surface of the core complex, that the SNAP/SNARE complex binds to one end of a stacked

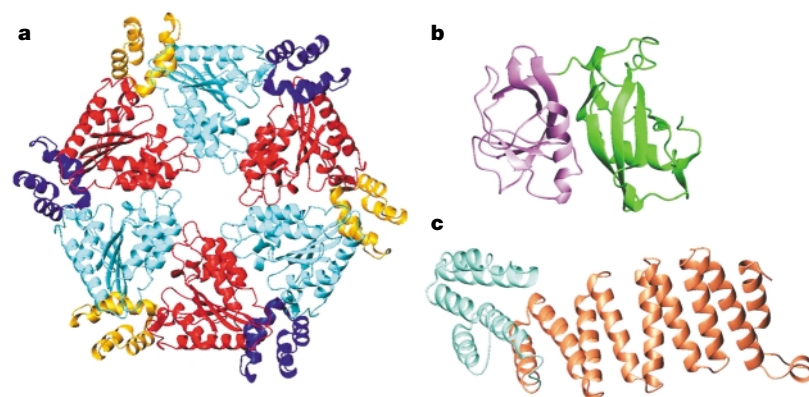


Figure 3 | Crystal structures of NSF and Sec17. **a** | The *N*-ethylmaleimide-sensitive fusion protein (NSF) D2 domain^{51,52}. **b** | The NSF amino-terminal^{53,54} domain. **c** | The yeast soluble NSF-attachment protein (SNAP) homologue Sec17 (REF. 56). In **a**, the protomers that form the NSF D2-domain hexamer have been coloured alternately in cyan/blue and red/orange; the nucleotide-binding subdomains are coloured in cyan and red, and the carboxy-terminal helical subdomains in blue and orange. In **b**, the double- ψ β -barrel of the NSF amino-terminal domain is coloured in violet, and the α/β -roll is coloured in green. In **c**, the sheet of helical hairpins from Sec17 is coloured in coral, with the carboxy-terminal globular domain in aquamarine.

double-ring structure that is formed by NSF⁵⁷, and that NSF undergoes large conformational changes following ATP hydrolysis⁶. These results have led to several models of how NSF/SNAPs disassemble the core complex, including one in which conformational changes in NSF that are concomitant with ATP hydrolysis untwist the SNARE four-helix bundle using SNAPs as a lever^{6,56,57}.

NSF was originally proposed to act as a fusion protein²⁹. Consistent with this idea, microinjection experiments with NSF peptides pointed to a role for NSF in regulating the kinetics of neurotransmitter release⁵⁸. However, analysis of temperature-sensitive paralytic mutations in *Drosophila* NSF (*comatose*) showed the importance of NSF for core-complex disassembly *in vivo*. A mutation that disrupts the ATPase activity of NSF leads to the accumulation of SNARE complexes on synaptic vesicles, and to a slow block in synaptic transmission⁵⁹ that is reflected in a progressive decrease in neurotransmitter release on repetitive stimulation⁶⁰. This is consistent with the proposed role of NSF in SNARE recycling. Further analysis of *comatose* mutants confirmed the role of NSF in disassembling core complexes *in vivo*⁶¹, and indicated that this activity follows synaptic vesicle fusion⁶². However, mammalian NSF that carries the mutation that disrupts the ATPase activity of *Drosophila* NSF is still active in a Golgi *in vitro* fusion assay, even though core-complex disassembly is impaired⁶³. This result indicates that NSF might have a function that is distinct from core-complex disassembly, but it is also possible that residual ATPase activity in the mutant is sufficient to support fusion in this assay.

SNAREs and membrane fusion. The proposal that SNARE-complex assembly causes membrane fusion is attractive because the energy that is released on the formation of this highly stable helix bundle could be used to overcome the energy barrier for fusion — a strikingly similar mechanism to that proposed for viral membrane fusion^{6,7,64}. In addition, it is generally thought that

multiple core complexes could cooperate in membrane fusion by forming ring structures around the site of bilayer merger. However, the high kinetic stability of the core complex has hindered the measurement of the energy released on assembly and the estimation of how many core complexes might be needed to cause membrane fusion. A marked hysteresis was observed when core-complex denaturation/renaturation was studied as a function of the temperature or the concentration of CHAOTROPIC AGENTS, and thermodynamic equilibrium could not be reached even with incubations that lasted for several days⁶⁵.

Reconstitution experiments with synaptobrevin and syntaxin/SNAP25 incorporated into separate liposomes led to the conclusion that the SNAREs do indeed constitute the minimal machinery necessary for intracellular membrane fusion⁸. However, these experiments revealed only lipid mixing; they did not show a smooth fusion process with the characteristics that would be expected for physiological membrane fusion (bilayer merger and FUSION PORE formation without the disordered rupture of membranes⁶⁶). The study provided no evidence for four hallmarks of standard *in vitro* fusion assays: EM data showing increased vesicle size on fusion, as well as vesicle integrity before and after fusion; inhibition of fusion by INVERTED CONE LIPIDS that destabilize STALKS; content mixing; and an absence of leakiness. Content mixing and leakiness were studied later⁶⁷, but with a method that was based on duplex formation between oligonucleotides that might be too large for an assessment of leakiness. A small percentage of content mixing occurred, but there was an even larger percentage of initial leakiness, despite the size of the probes. Perhaps most worrisome is the fact that the v-SNARE vesicles were loaded with a very high protein:lipid ratio⁸, which can render the liposomes unstable and prone to fusion. On the other hand, the slow rate of lipid mixing that was observed in these experiments (over a period of minutes to hours⁸) has often been invoked to question their physiological relevance; however, it seems plausible that more optimal spatial organization and the help of other proteins could accelerate fusion *in vivo*. Two additional observations further emphasize the importance of establishing whether or not the lipid mixing that was induced by the SNAREs represents a physiological fusion reaction rather than an irrelevant activity that can be observed for many polybasic or AMPHIPATHIC POLYPEPTIDES. First, NSF can promote vesicle fusion depending on the lipid composition^{68,69}, but it is unlikely that NSF functions directly in fusion (see above). Second, the transmembrane regions of the SNAREs alone can cause the fusion of synthetic liposomes⁷⁰.

In experiments using liposomes with a lipid composition more closely related to that of synaptic membranes, reconstituted synaptobrevin was unable to bind to syntaxin/SNAP25, either in soluble form or when incorporated into separate liposomes, and no fusion occurred⁷¹. In addition, no SNARE complexes were observed when native synaptic vesicles were mixed with synthetic liposomes that contained syntaxin and SNAP25 in the absence of Ca^{2+} , but the addition of Ca^{2+}

CHAOTROPIC AGENT

An agent that disorganizes the structure of water and can denature proteins by disrupting hydrophobic interactions.

FUSION PORE

A pore that is formed after the two leaflets of two lipid bilayers merge to form a single continuous bilayer and the 'bottle neck' at the site of fusion expands outwards radially. In synaptic vesicle exocytosis, opening of the fusion pore connects the luminal side of the vesicles with the extracellular space, allowing neurotransmitter release.

INVERTED CONE LIPIDS

Lipids that have large head groups relative to the diameter of the acyl chain.

STALKS

Metastable states that are widely believed to be intermediates in membrane fusion and involve merger of the proximal leaflets of the two bilayers at small contact sites.

AMPHIPATHIC POLYPEPTIDE

A polypeptide with both hydrophobic and hydrophilic surfaces.

induced membrane fusion and robust core-complex formation. These results indicate that Ca^{2+} helps to promote the availability of synaptobrevin for core-complex assembly, and that assembly coincides with fusion, although the rate of fusion was also slow (lasting for minutes) in this assay. Studies of the ability of SNAP25 peptides to rescue exocytosis in PC12 CELLS that were treated with BOTULINUM NEUROTOXIN E also indicated that core-complex formation is concomitant with membrane fusion⁷². However, although this assay provided a powerful tool to examine structure–function relationships for SNAP25 (REFS 72,73), the slow rate of exocytosis in this system (minutes) hinders the resolution of the final molecular events that lead to membrane fusion.

There is some evidence that core-complex assembly precedes fusion-pore opening in synaptic vesicle exocytosis. Studies with superfused synaptosomes showed that inactivation of NSF increases the number of core complexes without triggering neurotransmitter release, consistent with the idea that core-complex assembly and disassembly might be a dynamic process under ‘resting’ conditions¹². In these synaptosomes, inhibition of NSF enhanced the amount of release that was observed on stimulation, indicating that core-complex formation primes vesicles for exocytosis. *In vivo* FRET experiments have also indicated that SNARE complexes assemble before synaptic vesicle exocytosis⁷⁴. These results are consistent with a model in which SNARE-complex formation executes membrane fusion, whereas other factors control subsequent fusion-pore opening. Such a two-stage process would offer an attractive explanation for the speed and regulation of neurotransmitter release, but alternative explanations of these data are equally possible.

Data from other systems have also indicated that core-complex assembly does not coincide with fusion-pore opening, and have even questioned the role of SNAREs in membrane fusion. Experiments with sea urchin egg secretory vesicles indicated that core complexes are, in fact, disrupted by Ca^{2+} in a step before membrane fusion¹¹. In studies of fusion between two populations of vacuoles that contained either v-SNAREs or t-SNAREs, *trans*-core complexes were formed and could be disassembled by Sec18/Sec17 without inhibiting the subsequent mixing of vacuolar contents¹⁰. Further analyses of yeast vacuolar fusion indicated that Ca^{2+} released from the vacuole, calmodulin and protein phosphatase 1 function downstream of SNARE complexes^{75,76}. In addition, it was proposed that the V0 subunit of the vacuolar proton pump, which is present in most intracellular and vesicular membranes, directly mediates membrane fusion by a proteinaceous-pore mechanism¹⁴. Although this proposal is intriguing, it seems inconsistent with the finding that V0 is less crucial than the SNAREs for viability¹⁴, and with the observation that V0 subunits do not accumulate at the vertices of contacting vacuoles, where fusion occurs⁷⁷.

Although genetic experiments have supported the importance of SNAREs for membrane fusion, they have also indicated that SNAREs might not be essential for fusion *per se*, but that they act as catalysts or cofactors. In

yeast, the lethal phenotype that is caused by the deletion of *Snc1* and *Snc2* can be rescued by mutations in genes that control lipid metabolism⁷⁸. On the other hand, the deletion of syntaxin or synaptobrevin abolishes evoked neurotransmitter release in *Drosophila*, but spontaneous miniature release events (minis) still occur in the absence of synaptobrevin^{26,79,80}. Similarly, the deletion of syntaxin results in complete paralysis in *Caenorhabditis elegans*⁸¹, but some movement persists in synaptobrevin nulls⁸². In synaptobrevin-knockout mice, Ca^{2+} -triggered release is decreased more than 100-fold, but ~10% of the minis and of the release induced by hypertonic sucrose remains¹³. Deletion of SNAP25 in mice also abolishes Ca^{2+} -evoked release, but increases the frequency of minis at the neuromuscular junction⁸³. The remaining fusion that is observed in these experiments might arise from compensation by other SNAREs. However, as no closely related ‘cognate’ SNAREs are present, such compensation would imply a lack of specificity of SNARE function in fusion. It is possible that SNAREs act as catalysts or cofactors that facilitate the action of the fusion machinery, and that this role is more crucial under the tight temporal constraints of Ca^{2+} -triggered release¹³. Studies of exocytosis in CHROMAFFIN CELLS have, in fact, indicated that the core complex might act as part of the Ca^{2+} -sensing apparatus⁸⁴. In summary, there is little doubt that a primary function of SNAREs is to bring the two membranes together, but the key question is whether another factor(s) is more crucial and actually executes the fusion reaction after core-complex formation. Understanding the functions of other proteins that are crucial for fusion, particularly SM proteins, will be necessary to clarify these issues.

Specificity of SNARE–SNARE interactions. The main postulate of the SNARE hypothesis is that recognition between v-SNAREs and t-SNARE dictates vesicle targeting specificity^{27,28}. However, several yeast SNAREs have been implicated in multiple SNARE complexes, and v-SNAREs cannot be the sole determinants of the destination of a vesicle, as they are recycled after fusion through vesicles that are destined for a different compartment²⁰. In addition, synaptic vesicles release neurotransmitters only at active zones, despite the presence of syntaxin 1 and SNAP25 throughout the plasma membrane⁸⁵. The initial contact between membranes is generally mediated by large fibrous proteins or multimeric protein complexes — a recognition event that is known as ‘tethering’ and generally involves Rab proteins (reviewed in REF 86). The fact that Rab proteins are the most diverse membrane-traffic proteins in the genomes completed so far bodes well for a role in transport specificity, although they might also participate in other functions (reviewed in REF 87). It is worth noting that the debate on the specificity of SNARE interactions has been muddled by problems of terminology. There is little doubt that SNAREs have biologically specific functions, but their specificity can arise from sequences outside the SNARE motif, or from surface residues of the SNARE motif that do not participate in core-complex formation and might interact with other proteins. These

PC12 CELLS

Neuron-like cells that are derived from a malignant neural crest tumour (a pheochromocytoma).

BOTULINUM NEUROTOXIN E

A lethal neurotoxin that is produced by clostridium bacteria.

CHROMAFFIN CELLS

Cells of the adrenal gland that store and secrete catecholamines. They are termed ‘chromaffin’ because of the ability of chromium salts to stain them.

aspects should be distinguished from the question that has attracted much attention — to what extent the interactions between SNARE motifs that form the core complex provide a further level of specificity that ensures that vesicles fuse with only the appropriate membranes.

Interactions between coiled coils involve some specificity, but also show promiscuity, because they are promoted by multiple contacts between strings of hydrophobic residues. This promiscuity has been illustrated for the SNARE motif of syntaxin 1: as well as forming the core complex, it can oligomerize by itself into a four-helix bundle⁸⁸, and can form two other four-helix bundles — one containing the two SNARE motifs of SNAP25 and two copies of the syntaxin 1 SNARE motif⁸⁹, and another that includes two copies each of the syntaxin 1 SNARE motif and the SNAP25 amino-terminal SNARE motif⁴⁸. Promiscuity in SNARE-complex formation is favoured by the fact that interior residues of SNARE motifs are more highly conserved than surface residues¹⁹. Not surprisingly, some non-cognate combinations of SNARE motifs form core complexes with stabilities that are comparable to those of cognate SNARE complexes^{90,91}. Some stable non-cognate SNARE complexes that were observed biochemically did not support exocytosis in PC12 cells⁹². However, this is an example in which specificity might arise from interactions between surface residues of the core complex and other proteins, rather than from SNARE–SNARE interactions. The complexity of analysing SNARE specificity is further illustrated by the observation that overexpression of the yeast late endosomal syntaxin **Pep12** can partially substitute for the absence of the vacuolar syntaxin **Vam3** *in vivo*, but that replacement of the SNARE motif of Vam3 with that of Pep12 abolishes vacuolar fusion⁹³.

The reconstitution assays that were used to suggest SNARE-induced membrane fusion⁸ have also been applied to the study of the specificity of SNARE-complex formation using different combinations of yeast SNAREs^{5,44,45}. The results led McNew *et al.*⁵ to conclude that membrane-traffic specificity is encoded largely by the SNAREs, and that fusion assays that are used to test for specificity should include transmembrane regions, rather than being restricted to the analysis of complex formation by isolated SNARE motifs. However, the latter conclusion was limited by the fact that McNew *et al.* did not describe whether the non-cognate core complexes that were identified biochemically^{90,91} could cause membrane fusion. The first conclusion — that SNAREs largely encode targeting specificity — also needs to be qualified, because the reconstitution results would predict a substantial percentage of non-desirable fusion events if specificity depended only on SNARE pairing. Thus, vesicles containing Snc1 or Snc2 v-SNAREs would be able to fuse with the plasma membrane, the trans-Golgi network (TGN) and early endosomes, and also partially with vacuoles, according to the fusion assays^{5,44}. It is also unclear to what extent the reconstitution results faithfully reflect *in vivo* SNARE–SNARE interactions.

A comprehensive search for **Sed5**-containing SNARE complexes that can mediate lipid mixing did not yield any complex involving **Vti1** (REF. 45), whereas strong evidence exists for a functional interaction between Vti1 and Sed5 in Golgi transport *in vivo*^{94,95}. Moreover, a strict requirement for the presence of three t-SNAREs on one membrane and a v-SNARE on the other membrane was indicated by the reconstitution experiments^{44,45}. However, the assignment of **Bos1** as a Golgi t-SNARE on the basis of these data is uncertain, as Bos1 seems to be required on vesicles rather than on Golgi membranes *in vivo*⁹⁶. Finally, a potential limitation of the reconstitution experiments is the formation of kinetic traps by t-SNAREs⁴⁸, which might underlie the observation that some t-SNAREs require activation by external factors to mediate lipid mixing with the corresponding v-SNARE⁴⁴.

The arginine and glutamine residues that form the polar layer of the SNARE complex seem to contribute significantly to the specificity observed in v-SNARE–t-SNARE interactions, although they are not the only determinants. All combinations of v- and t-SNAREs that induced lipid mixing contained only one arginine in the polar layer, and the most promiscuous t-SNAREs — the plasma-membrane **Sso1/Sec9** — mediate lipid mixing with all yeast R-SNAREs that precede a trans-membrane region, but not with any Q-SNARE^{5,44,45}. On the other hand, studies of the functional consequences of mutations in the polar layer have yielded diverse results, ranging from no discernible functional defects to severe impairment of fusion by Q-to-R substitutions that leave two arginines in the polar layer^{72,93,97,98}. Independent of its contribution to specificity, the polar layer seems to be involved in core-complex disassembly by NSF/SNAPs⁹⁹.

SNARE amino-terminal regions. In addition to a SNARE motif and a carboxy-terminal transmembrane region, syntaxin 1 contains an amino-terminal region that spans two-thirds of its sequence and is crucial for forming a tight complex with the neuronal SM protein Munc18-1 (REF. 100). This region includes an autonomously folded three-helix bundle called the H_{abc} domain (FIG. 2), which is preceded by a short amino-terminal sequence and is followed carboxy-terminally by a linker region that separates it from the SNARE motif¹⁰¹. In isolated syntaxin 1, the H_{abc} domain folds back onto the SNARE motif, forming a ‘closed conformation’ that is required for Munc18-1 binding, but differs from the ‘open conformation’ of syntaxin 1 in the core complex³⁹. Thus, syntaxin 1 must experience a large conformational change to switch between its complex with Munc18-1 and the core complex. The crystal structure of syntaxin 1 bound to Munc18-1 showed how the amino-terminal half of the SNARE motif packs against the H_{abc} domain, whereas the carboxy-terminal half, which is unstructured in isolated syntaxin 1, does not contact the H_{abc} domain and forms an irregular, partially helical structure that is induced by Munc18-1 binding¹⁰² (FIG. 4a,b). The linker region also forms a partially helical structure at the opposite end of the molecule.

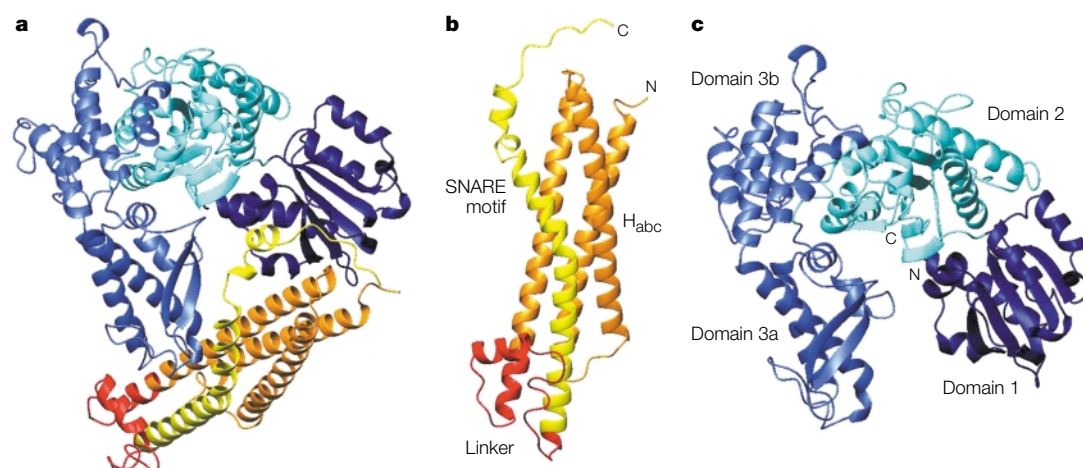


Figure 4 | **Structure of the syntaxin-1/Munc18-1 complex**¹⁰². **a** | Structure of the complex showing how Munc18-1 wraps around the closed conformation of syntaxin 1. **b** | Closed conformation of syntaxin 1 bound to Munc18-1. The orientation has been chosen to illustrate how the SNARE (SNAP receptor) motif (yellow) forms an α -helix that binds to a groove of the amino-terminal H_{abc} domain (orange) and then departs at the carboxyl terminus, forming an irregular, partially helical structure. The linker region (red) forms a small globular subdomain at the bottom of the structure that includes helical and irregular structure, with one of the helices representing an extension of helix C from the H_{abc} domain. **c** | Structure of Munc18-1 bound to syntaxin 1; its different domains are coloured in different shades of blue.

Syntaxin homologues that function in the same cellular compartment in organisms from yeast to mammals show sequence homology in their amino-terminal regions^{16,101}, but there is generally no clear homology in this region between syntaxins from different compartments. However, the domain structure of syntaxin 1 is shared by all syntaxins studied so far, including most of the seven yeast syntaxins^{16,17,103,104}, although the different parts of the amino-terminal region can vary in length. The yeast plasma-membrane syntaxin Sso1 also forms a closed conformation that probably regulates core-complex assembly^{42,43,103}, but does not bind to the corresponding SM protein¹⁵. Yeast viability requires the H_{abc} domain of Sso1 (REF. 103) but not the closed conformation¹⁰⁵, indicating that this domain has a crucial function that is distinct from the regulation of core-complex formation. On the other hand, the H_{abc} domain of Vam3 is not essential for yeast vacuolar fusion⁹³. Vam3 and two further yeast syntaxins, Tlg2 and Pep12, adopt a default open conformation^{17,104}, showing that the closed conformation is not a general feature of syntaxins. Importantly, three yeast syntaxins (Tlg2, Sed5 and Ufe1) and their mammalian homologues (syntaxin 16, syntaxin 5 and syntaxin 18) contain a conserved amino-terminal peptide motif that precedes the H_{abc} domain and mediates binding to the corresponding SM proteins^{16,17} (see below). These results indicate that the amino-terminal regions of syntaxins have some common features, but they also show differences that might underlie distinct but evolutionarily conserved functions in different compartments. Many SNAREs outside the syntaxin family also contain amino-terminal regions with folded domains that have diverse functions (for example, REFS 106,107). Thus, although much attention has been focused on the SNARE motifs, sequences outside the SNARE motif also have crucial roles that have been largely overlooked but are now beginning to be elucidated.

SM proteins

Essential for membrane fusion. SM proteins are hydrophilic 60–70-kDa polypeptides that share homology evenly throughout their sequence, indicating that no particular domain is associated with their primary function. The first SM protein to be identified, **UNC-18**, was discovered in genetic screens for uncoordinated phenotypes in *C. elegans*¹⁰⁸; Sec1 was later identified in screens for genes involved in the yeast secretory pathway¹⁰⁹. The mammalian homologue of UNC-18, Munc18-1 (also called nSec1 or RbSec1), was isolated by virtue of its ability to bind to syntaxin 1 (REF. 100). Further studies have indicated that all types of intracellular membrane traffic require an SM protein, similar to the requirement for SNAREs (reviewed in REF. 9).

Mutations in SM proteins completely block membrane fusion^{110–114}. The sensitivity of electrophysiological analyses of mammalian synapses showed the absolute requirement for Munc18-1 for neurotransmitter release. Ca^{2+} -triggered release, minis or exocytosis evoked by hypertonic sucrose or by α -LATROTOXIN were not observed in Munc18-1-knockout mice¹¹⁴. These observations indicate that SM proteins are essential for membrane fusion, but their functions remain undefined. Analysis of chromaffin cells from Munc18-1-knockout mice indicated a role for Munc-18 in the docking of secretory vesicles to the plasma membrane¹¹⁵, but docking was unaltered in brain synapses¹¹⁴, showing that the most crucial function of Munc18-1 is downstream of docking. A late role for SM proteins in membrane fusion is also indicated by the observation that the yeast vacuolar SM protein **Vps33** is enriched in the vertices of the vacuole–vacuole contact area in which membrane fusion occurs⁷⁷, and that Sec1 accumulates at sites of secretion in the yeast plasma membrane¹⁵. In addition, Sec1 binds to assembled SNARE complexes and its localization to sites of secretion depends on core-complex assembly,

α -LATROTOXIN
An agent that normally causes massive neurotransmitter release.

which seems to be normal in *sec1-1* mutants at non-permissive temperatures, despite the fact that more than 95% of secretion is blocked¹¹⁶. These results indicate that Sec1 might function after SNARE-complex formation, but the precise point of action of SM proteins in fusion remains unclear.

Syntaxin-1–Munc18-1 interaction. The observation that Munc18-1 binds tightly to syntaxin 1 (REFS 100,117,118), and that the yeast SM proteins **Sly1** and **Vps45** also bind to syntaxin homologues^{119–122}, indicated that SM proteins generally function by interacting with syntaxins. However, the finding that Munc18-1 binds to the closed conformation of syntaxin 1 (REF. 39) and competes with core-complex formation¹²³ indicates that this interaction is inhibitory. This idea is supported by the observation that a mutation that decreases the affinity of syntaxin for **Rop**, the *Drosophila* Munc18-1 homologue, increases neurotransmitter release¹²⁴. Moreover, overexpression of Rop or syntaxin inhibits release, whereas simultaneous overexpression of both suppresses this defect¹²⁵. However, overexpression of Munc18-1 increases exocytosis in chromaffin cells¹¹⁵. The physiological relevance of the syntaxin-1–Munc18-1 interaction was further shown by the observation that the levels of syntaxin 1 are reduced by 70% in Munc18-1-knockout mice¹¹⁴. Although the role of the syntaxin-1–Munc18-1 interaction is unclear from these results, they provided evidence for its importance *in vivo* and indicated that the relative levels of syntaxin 1 and Munc18-1 could be crucial for the efficiency of neurotransmitter release.

The finding that a mutation in the linker region between the H_{abc} domain and the SNARE motif of syntaxin 1 (L165A, E166A) disrupts the syntaxin 1 closed conformation and Munc18-1 binding showed that the closed conformation is crucial for this interaction³⁹. The crystal structure of the Munc18-1–syntaxin-1 complex (FIG. 4a) confirmed these conclusions, showing that Munc18-1 wraps around the syntaxin 1 closed conformation¹⁰². The structure revealed that Munc18-1 contains three domains (FIG. 4c). Domain 1 has an α/β architecture and spans the 134 amino-terminal residues. Domain 2 also has an α/β fold and includes residues 135–245 and 480–592. Domain 3 (residues 246–479) constitutes a large insertion between two strands of domain 2 and can be divided into two subdomains — one that also contains a mixture of α - and β -structure (subdomain 3a), and another that is composed of a cluster of α -helices (subdomain 3b). The three domains are arranged in an arch shape with a central cavity of ~ 15 Å that is lined primarily by domains 1 and 3a. Syntaxin 1 binds to this cavity, making extensive contacts with both domains of Munc18-1 through the H_{abc} domain and the SNARE motif.

The structural work led to the proposal that an unidentified factor helps to dissociate the syntaxin-1–Munc18-1 complex or causes conformational changes in Munc18-1, after which Munc18-1 provides a platform for core-complex assembly in a concerted fashion¹⁰². Differences between the crystal structure of isolated Sso1 and the structure of syntaxin 1 bound to Munc18-1 also

indicated that Munc18-1 binding might facilitate core-complex formation by changing the closed conformation of isolated syntaxin 1 (REF. 103). However, the crystal structure of isolated Munc18-1 from squid¹²⁶ did not reveal large differences from the structure observed in the complex with syntaxin 1, and NMR studies indicate that the closed conformation of isolated syntaxin 1 is stabilized, rather than altered, by Munc18-1 binding (unpublished results). In addition, these models seem to be inconsistent with the fact that core complexes can be easily assembled *in vitro* in the absence of Munc18-1, and Munc18-1 inhibits this assembly¹²³. It is plausible that a concerted mechanism of Munc18-1 release and SNAP25/syntaxin binding to syntaxin 1 results in more efficient formation of core complexes *in vivo*, but it is difficult to explain in this case why deletion of Munc18-1 blocks exocytosis completely, and why such a function is not structurally conserved in other SM-protein–SNARE interactions (see below).

Releasing syntaxin 1 from Munc18-1. Two large proteins that are localized at the active zone — **UNC-13**/Munc13 and **RIM** (Rab3-interacting molecule) — are attractive candidates for mediating the transition between the syntaxin-1–Munc18-1 complex and the core complex. *C. elegans* UNC-13 (REF. 127) and its mammalian homologues, the Munc13s (REF. 128), have been implicated in priming synaptic vesicles to a fusion-competent state¹²⁹. UNC-13 mutations in *C. elegans*¹³⁰ and *Drosophila*¹³¹, and double knockouts of Munc13-1 and Munc13-2 in mice¹³², completely block neurotransmitter release. RIM is an effector of **Rab3**, the predominant Rab protein associated with synaptic vesicles¹³³. RIM mutants in *C. elegans* show a severe decrease in neurotransmitter release that might result from a defect in synaptic vesicle priming¹³⁴, and deletion of one RIM isoform (RIM1 α) in mice decreases the release probability¹³⁵.

A direct role for Munc13 in releasing the syntaxin-1–Munc18-1 complex was indicated by the finding that a carboxy-terminal region of Munc13-1 binds to the amino-terminal region of syntaxin 1 (REF. 136), and that an amino-terminal region of *C. elegans* UNC-13 displaces syntaxin from UNC-18 (REF. 137). On the other hand, RIM and Munc13 interact functionally, pointing to a role for RIM in regulating the priming activity of Munc13 (REF. 138). Further support for these ideas was provided by rescue experiments using a mutant form of syntaxin that exists in a constitutively open conformation and disrupts Munc18-1 binding³⁹. Whereas wild-type syntaxin could not rescue the absence of neurotransmitter release caused by an UNC-13 mutation in *C. elegans*, the open form of syntaxin restored release almost completely¹³⁹. Similarly, neurotransmitter release was restored to normal levels in a *C. elegans* RIM mutant by the open form of syntaxin, but not by wild-type syntaxin¹³⁴. These results raise the possibility that the syntaxin-1–Munc18-1 complex represents an ‘off state’ in which the two proteins inhibit each other, and release of this clamp by the concerted action of Munc13 and RIM is restricted spatially by their localization to the active zone. This model implies a link between Rab and SNARE function, and emphasizes the

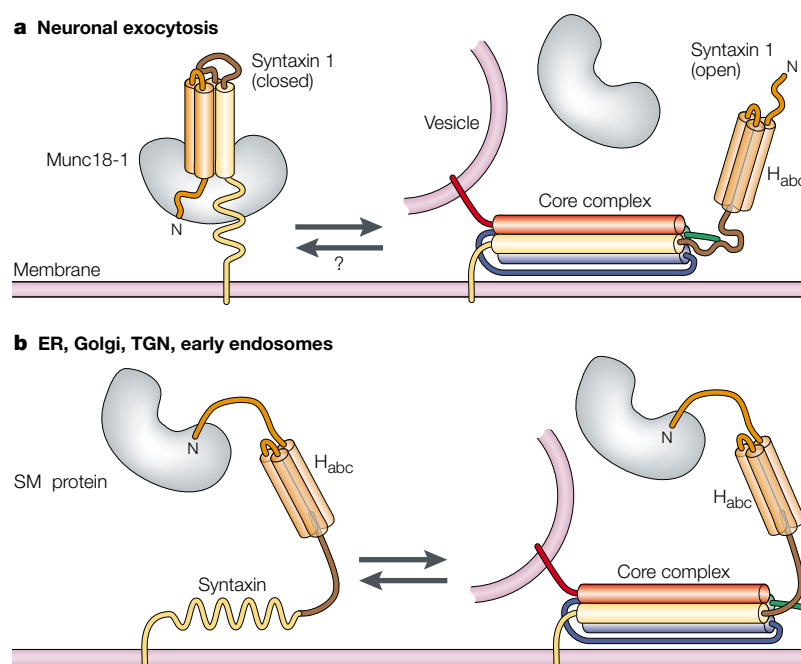


Figure 5 | Different modes of coupling between syntaxins and SM proteins. a | In neuronal exocytosis, the interaction between Munc18-1 (grey) and the closed conformation of syntaxin 1 (yellow and orange)^{39,102} is incompatible with formation of the core complex by the SNARE (SNAP receptor) motifs of syntaxin, synaptobrevin and synaptosomal-associated protein of 25 kDa (SNAP25)¹²³ (same colour scheme as that in FIG. 2). **b** | The interaction of syntaxin amino-terminal peptide motifs with Sec1/Munc18 homologues (SM proteins) that occurs in the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN) and the early endosomes is fully compatible with core-complex formation^{16,17}.

importance of ensuring that exocytosis happens only at the right time and the right place for an exquisitely regulated process such as neurotransmitter release. However, the efficient rescue of the UNC-13 and RIM phenotypes by the open syntaxin form indicates that further factors should restrict exocytosis to the active zone. Alternative mechanisms to regulate the formation and disassembly of the syntaxin-1–Munc18-1 complex have been proposed. Tomosyn, a large neuron-specific SNARE protein, binds tightly to syntaxin 1 and displaces Munc18-1 (REF. 140), although it is unclear how tomosyn would be released from syntaxin 1 if this interaction were relevant. In addition, phosphorylation of Munc18-1 by protein kinase C (REF. 141) and cyclin-dependent kinase 5 (REF. 142) hinders binding to syntaxin 1. Munc18-1 also binds to other proteins, such as mints¹⁴³ and Doc2 (REF. 144), although the roles of these interactions remain unclear. It will therefore be crucial to test whether Munc13 and RIM can release the block of core-complex formation that is caused by the binding of Munc18-1 to syntaxin 1, or whether other proteins are involved.

Different modes of SM-protein–SNARE coupling. Originally, all SM proteins were assumed to function by binding to closed conformations of syntaxins. However, recent studies indicate that the mode of interaction between syntaxin 1 and Munc18-1 represents a specialization rather than a paradigm. For example, Sec1 binds to core complexes that contain the plasma-membrane

syntaxin Sso1, rather than to isolated Sso1, although the nature of this interaction is unclear¹⁵. Moreover, the yeast and mammalian syntaxins that function at the TGN and in early endosomes (Tlg2/syntaxin 16), and in the endoplasmic reticulum (Ufe1/syntaxin 18) and the Golgi (Sed5/syntaxin 5), bind tightly to the corresponding SM proteins (Vps45 and Sly1) through a short amino-terminal peptide motif^{16,17}. This binding mode is strikingly different from the syntaxin-1–Munc18-1 interaction (FIG. 5), is evolutionarily conserved and seems to represent the most general mode of syntaxin–SM-protein coupling. Importantly, this binding mode is compatible with core-complex formation and might recruit SM proteins to the sites of fusion, allowing SM proteins and SNAREs to carry out active functions simultaneously. It should be noted that other mechanisms of SNARE–SM-protein coupling also occur in yeast, and the same SM protein can be coupled to diverse SNAREs by distinct mechanisms in different cellular compartments¹⁷.

These observations and the diversity of the proteins that interact with SM proteins in different systems raise the possibility that there is no common primary function of all SM proteins. However, because of the homology of SM proteins and their essential nature for all types of intracellular membrane traffic, it seems more likely that they do have a universal, primary function that is subject to different mechanisms of regulation. Such a universal function might be related to the regulation of core-complex assembly, as Vps45 is required for co-immunoprecipitation of the TGN/endosomal Tlg SNAREs¹⁴⁵, and Sly1 seems to prevent the formation of non-cognate core complexes involving Sed5 (REF. 146). But it is unclear why, in this case, SM proteins are so essential for membrane fusion — perhaps even more essential than the SNAREs themselves.

Complexins

Neurotransmitter release occurs under tight spatial and temporal constraints that require specialized proteins, in addition to proteins that are generally involved in intracellular membrane fusion. Several of these specialized proteins have been found to interact with SNAREs^{2,9,18}, although this might arise, in part, from their 'sticky' nature. Particularly important for the last steps of neurotransmitter release are synaptotagmin 1 and the complexins (also known as synaphins). Synaptotagmin 1 acts as a Ca^{2+} sensor during release¹⁴⁷ and binds to SNAREs *in vitro*, but these interactions have not been characterized structurally and it seems that the most crucial activity of this protein is Ca^{2+} -dependent phospholipid binding¹⁴⁷. The literature on synaptotagmin 1 and other members of the synaptotagmin family has recently been reviewed¹⁴⁸. Below, we focus on complexins, as exciting results about their interactions with SNAREs have recently been described.

Complexin 1 and **complexin 2** are evolutionarily conserved, closely related proteins of 15–16 kDa that bind to neuronal SNARE complexes and are highly enriched in the brain, where they co-localize with the neuronal SNAREs^{149–151}. Knockout of complexins in

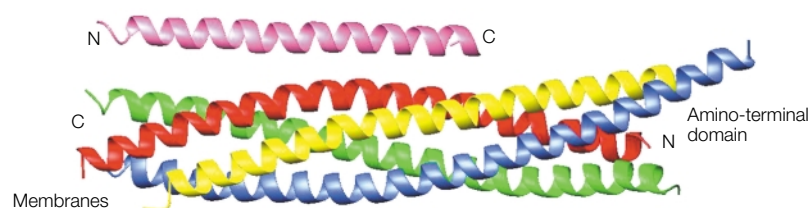


Figure 6 | **Three-dimensional structure of the complexin-SNARE complex**¹⁵⁶. Complexin is coloured in pink and the SNARE (SNAP receptor) motifs are coloured as in FIG. 2. The approximate locations of the membranes and the amino-terminal domain of syntaxin 1 are indicated.

mice showed that these proteins are essential for normal Ca^{2+} -triggered neurotransmitter release, but not for release triggered by hypertonic sucrose¹⁵², indicating that complexins function in the Ca^{2+} -dependent step of exocytosis. A late role in exocytosis was also indicated by microinjection experiments in squid giant synapses with a peptide that interferes with the complexin-SNARE complex¹⁵³. These observations were surprising, because the binding of complexin to the core complex is not dependent on Ca^{2+} (REFS 149,154).

NMR and biochemical studies showed that complexins lack a tertiary structure but include a central α -helical region that coincides with the most highly conserved region of their sequence and is responsible for stoichiometric 1:1 binding to the core complex¹⁵⁵. Complexins bind to the core complex very rapidly (practically within the diffusion limit) and with high

affinity (dissociation constant ~ 10 nM)¹⁵⁴. The crystal structure of the complexin-1-SNARE complex (FIG. 6) showed that complexins bind in an antiparallel α -helical conformation to the central region of the interface between synaptobrevin and syntaxin, without substantially altering the structure of the core complex¹⁵⁶. Binding involves a network of salt bridges, hydrogen bonds and hydrophobic interactions between complexin and the SNARE motifs of synaptobrevin and syntaxin, indicating that complexin might act as a 'tape' that seals and stabilizes the interface between the synaptobrevin and syntaxin SNARE motifs. Deuterium-exchange experiments confirmed this proposal and showed that the stabilizing effect of complexin binding propagates beyond the contact region, towards the carboxy-terminal, membrane-proximal region of the core complex¹⁵⁶. As the interface between synaptobrevin and syntaxin bears the strong repulsive forces between the synaptic vesicle and the plasma membranes, these observations led to a model in which the primary function of complexin is to 'freeze' a primed state that involves a fully assembled SNARE complex that is crucial for fast, Ca^{2+} -triggered neurotransmitter release¹⁵⁶ (FIG. 7).

The model shown in FIG. 7 proposes that priming occurs in more than one step and assumes that the first priming step involves partial formation of the SNARE complex, perhaps after release of the syntaxin-Munc18-1 complex. This partially assembled complex might be in equilibrium with a second primed state that involves full assembly of the SNARE complex and is stabilized by complexin binding. Hypertonic sucrose might release both primed states, but only the second state might lead to fast neurotransmitter release on Ca^{2+} influx. A key aspect of this model is that the need for a protein such as complexin arises from the stringent time requirements of this process (<0.5 ms after Ca^{2+} influx), although a back-and-forth equilibrium between half- and fully assembled states might suffice for efficient, constitutive membrane fusion on longer timescales¹⁵⁶. Note that complexins have also been proposed to function by promoting core-complex assembly and oligomerization¹⁵³, although these results could not be reproduced in another study¹⁵² and the oligomerization model is inconsistent with other data (see REFS 154,156). However, alternative models of complexin function are possible. For instance, part of the complexin central helix does not contact the core complex and could be involved in recruiting other proteins of the release machinery¹⁵⁶. Further electrophysiological and biochemical studies will be required to test this and other possibilities.

Concluding remarks

Great progress in the characterization of proteins involved in neurotransmitter release has been made in recent years, but many pressing questions need to be answered to gain a basic understanding of the mechanisms involved. The million-dollar question is 'what is the function of Munc18-1?' But it will also be crucial to establish rigorously whether SNAREs execute membrane fusion, or whether other factors act after core-complex formation. Further aspects of neurotransmitter

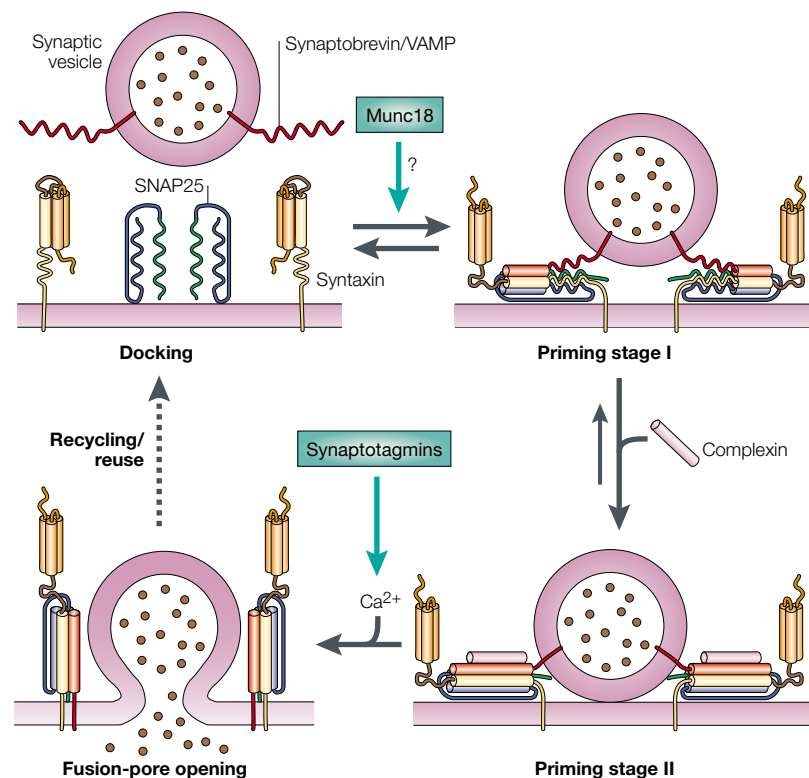


Figure 7 | **Model of complexin function.** The crucial aspect of this model is that priming might occur in two steps that involve partial and full assembly of the core complex. Only the fully assembled state, which is stabilized by complexin, can support fast, Ca^{2+} -triggered neurotransmitter release. SNAP25, synaptosomal-associated protein of 25 kDa.

release that have not been discussed here include the mechanism of action of the Ca^{2+} sensor synaptotagmin 1, the function of Rab3, and the structural and regulatory roles of lipids in membrane fusion. Characterizing the structure and interactions of Munc13 and RIM, as well as those of other large proteins that are located at the active zone, such as aczonin/piccolo and bassoon, will help to elucidate their functions and to build a picture of the structural organization of the active zone. However, a *tour de force* that combines biochemistry with genetics and several biophysical techniques will be required to achieve this goal. Although much attention

has been paid to understanding how membrane fusion is 'turned on', an emerging theme is the importance of the off state(s) for a signalling process that is as tightly regulated, both spatially and temporally, as neurotransmitter release. Thus, it will not be surprising if some of the central components of the release machinery turn out to have both inhibitory and active roles. As the basic mechanisms that explain how fusion is turned off and on are elucidated, the next big challenge will be to understand how the efficiency of release is regulated in processes of presynaptic plasticity that are believed to underlie information processing in the brain.

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References 90 and 91 show that non-cognate combinations of SNAREs can form highly stable complexes, indicating that SNAREs are more promiscuous than was previously thought.

This paper provides some of the most convincing evidence that a SNARE can function in more than one membrane-traffic pathway.

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