The pre-synaptic fusion machinery
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Here, we review recent insights into the neuronal pre-synaptic fusion machinery that releases neurotransmitter molecules into the synaptic cleft upon stimulation. The structure of the pre-fusion state of the SNARE/complexin-1/synaptotagmin-1 synaptic protein complex suggests a new model for the initiation of fast Ca\(^ {2+}\)-triggered membrane fusion. Functional studies have revealed roles of the essential factors Munc18 and Munc13, demonstrating that a part of their function involves the proper assembly of synaptic protein complexes. Near-atomic resolution structures of the NSF/sSNAP/SNARE complex provide first glimpses of the molecular machinery that disassembles the SNARE complex during the synaptic vesicle cycle. These structures show how this machinery captures the SNARE substrate and provide clues as to a possible processing mechanism.

Ca\(^ {2+}\)-triggered synaptic vesicle fusion

The SNARE complex assembly provides the energy for membrane fusion [3,4]. The SNARE proteins synaptobrevin-2/VAMP2, on the synaptic vesicle in conjunction with syntaxin-1A and SNAP-25A on the plasma membrane initiate vesicle fusion by forming a trans-SNARE complex before Ca\(^ {2+}\)-triggering. While SNAREs are essential for neurotransmitter release, a Ca\(^ {2+}\)-sensor to trigger fusion is also required because SNAREs themselves do not exhibit any Ca\(^ {2+}\) sensitivity. In the case of fast synchronous neurotransmitter release, this task is accomplished by synaptotagmin-1 [5,6], a member of the synaptotagmin family of proteins. Synaptotagmins contain a single transmembrane-spanning domain and tandem C-terminal cytoplasmic C2 domains, termed C2A and C2B (or C2AB together) [7,8]. Additionally, the cytoplasmic protein complexin (we focus on complexin-1 in this review) also plays critical roles in regulating neurotransmitter release [9,10]. The characterization of all of these factors begs a number of mechanistic questions: How do SNAREs, synaptotagmins, and complexins achieve fusion in less than a millisecond upon Ca\(^ {2+}\)-triggering; how are they situated between the membranes, and how is the process regulated?

In the past, synaptotagmins have been primarily viewed as factors that activate fusion upon Ca\(^ {2+}\)-binding. However, recent structural and functional studies suggest that fast (submillisecond) Ca\(^ {2+}\)-triggered synaptic vesicle fusion begins with release of inhibition upon Ca\(^ {2+}\) binding to synaptotagmin [11**,12]. Central to this new model of Ca\(^ {2+}\)-triggering are atomic resolution structures of the SNARE/synaptotagmin-1 [13'] and SNARE/complexin-1/synaptotagmin-1 complexes (Figure 1a) [11**], combined with

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Synaptic fusion complex. (a) Crystal structure of the SNARE/complexin-1/ synaptotagmin-1 complex [11**], PDB ID SWSC (red: syntaxin-1A, green: SNAP-25A, blue: synaptobrevin-2, light blue: central helix of complexin-1, orange: C2B domains of synaptotagmin-1, gray: C2A domains of synaptotagmin-1). (b) Superposition of representative members of the C2 domain superfamily 2.60.40.150 generated by CATH [89] (http://www.cathdb.info) with the structure of the SNARE/complexin-1/synaptotagmin-1 complex (red: syntaxin, green: SNAP-25, blue: synaptobrevin, gold: synaptotagmins, purple: Doc2 and Rabphilin, gray: 40 representative structures out of 346 C2 domains with known structure). (c) Release of inhibition model of Ca\(^{2+}\)-triggering. Note that it is unknown as to which membrane the Ca\(^{2+}\)-bound synaptotagmin-1 C2 domains might interact with. For illustration purposes, we placed it at the synaptic vesicle membrane, although the presence of PIP\(_2\) increases the binding affinity between Ca\(^{2+}\)-bound synaptotagmin-1 and the membrane, so it also possible, or even likely, that all synaptotagmin-1 C2 domains localize to the plasma membrane upon Ca\(^{2+}\)-binding.

Functional studies in neuronal cultures and in reconstituted systems. The SNARE/complexin-1/synaptotagmin-1 structure was determined in the absence of Ca\(^{2+}\). We also note that membrane domains, the membrane proximal regions of the SNARE complex, and the Habc domain of syntaxin-1A were excluded in the constructs used for crystallization. In the trans-SNARE complex, the membrane proximal regions of syntaxin-1A and synaptobrevin-2 are probably flexible, sampling many conformations and allowing them to tether the trans-SNARE complex to their respective membrane domains. Thus, the crystal structure likely represents the well-folded core of the pre-fusion state before Ca\(^{2+}\)-triggering.

This SNARE/complexin-1/synaptotagmin-1 structure reveals a tripartite interface between one synaptotagmin-1 C2B domain and both the SNARE complex and complexin-1 [11**] (Figure 1a). This tripartite interface only forms when complexin-1 is bound to the SNARE complex. Simultaneously, a second synaptotagmin-1 C2B domain interacts with the other side of the SNARE complex via a different, pairwise interface that is independent of complexin-1, as also described in structural studies with SNAREs and synaptotagmin-1 [13**]. Structure-guided mutagenesis coupled with isothermal titration calorimetry solution-binding studies and electrophysiological experiments in neuronal cultures showed that both
Ca\textsuperscript{2+}-triggered synchronous neurotransmitter release and suppression of spontaneous release depend on synaptotagmin-1 C2B residues involved in both the SNARE/complexin-1/synaptotagmin-1 tripartite and the SNARE/synaptotagmin-1 primary interfaces\cite{11**,13**}. Moreover, in a reconstituted assay of synaptic vesicle fusion\cite{14–16,17**} the effects of mutations, deletions, or truncations of these interfaces generally track with electrophysiological observations in neurons. Both interfaces map to distinct regions on the surface of the synaptotagmin-1 C2B domain, and they are separate from the polybasic region implicated in membrane interactions\cite{18,19}. Interestingly, the Ca\textsuperscript{2+} +-binding loops of the synaptotagmin C2B domains are not involved in either interface.

Among the most striking structural features of the tripartite interface is the continuation of the complexin-1 central helix into the HA α-helix of synaptotagmin-1 (Figure 1a). The HA α-helix is structurally conserved in C2B domains of all synaptotagmins, as well as the homologous C2B domains of the synaptic proteins Doc2b and Rabphilin, both of which have tandem C2 domains similar to synaptotagmins. In contrast, there is no HA α-helix in all other C2 domains of known structure (Figure 1b). Note, that there is no HA α-helix in the so-called C2B domain of Munc13, but the three C2 domains of Munc13 are separated in sequence (i.e. there are no tandem C2 in Munc13)\cite{20}.

The primary SNARE/synaptotagmin-1 interface occurs in very different crystal forms\cite{11**,13**}, and regardless of the presence of complexin bound to the other side of the SNARE complex. The primary interface is identical for apo-bound, Ca\textsuperscript{2+}-bound, or Mg\textsuperscript{2+}-bound synaptotagmin-1. Additionally, the importance of some of the residues involved in the primary interface was subsequently confirmed in neurons by two other groups\cite{21,22}. In addition to the primary SNARE/synaptotagmin-1 interface\cite{13**}, other bipartite interactions between the SNARE complex and the synaptotagmin-1 C2B domain may exist, as suggested by single molecule FRET experiments in both the absence or presence of Ca\textsuperscript{2+}\cite{23} and by solution NMR studies in the presence of Ca\textsuperscript{2+}\cite{24**}. However, the data from both studies were insufficient to determine unique high-resolution structures of these alternative interfaces. Moreover, the placement of the lanthanide labels on SNAP-25 (D166C and D414C) and mutations of synaptotagmin-1 (R398Q and R399Q) in the NMR study\cite{24**} likely disrupt the primary interface\cite{13**}. Nevertheless, both solution studies\cite{24**} suggest possible alternative conformations of the SNARE/synaptotagmin complex, especially in the presence of Ca\textsuperscript{2+}.

At least two SNARE complexes are probably involved in Ca\textsuperscript{2+}-triggered fusion\cite{26,27}, but it has not yet been possible to visualize these complexes. For example, the resolution of cryo-electron tomography images (cryo-ET) of synapses\cite{28,29} was insufficient to localize synaptic protein complexes. However, cryo-ET studies of proteoliposomes with reconstituted synaptic proteins revealed proteinaceous contacts with a variety of morphologies between the vesicle membranes\cite{30}. The observed contacts span inter-membrane distances of 40–60 Å. At that separation, lipids alone would be unable to bridge the membranes, as the critical distance at which lipid stalks form is <9 Å\cite{31}. Thus, one can conclude from this study that the pre-fusion SNARE/complex-1/synaptotagmin-1 complex keeps the membranes in close juxtaposition, but short of actually promoting fusion. Two or more synaptic complexes involved in Ca\textsuperscript{2+}-triggered fusion could potentially interact with each other via Syt1 C2B domains\cite{11**,12}, but the precise quaternary arrangement remains to be elucidated.

On the basis of the available structures and functional data, a new model of action-potential evoked, Ca\textsuperscript{2+}-triggered synaptic vesicle fusion has emerged\cite{12} (Figure 1c). In the pre-fusion state, the SNARE/complexin-1/synaptotagmin-1 complex is formed. Upon Ca\textsuperscript{2+} binding, the synaptotagmin-1 molecule involved in the tripartite interface is dislodged, for example by binding and inserting into a nearby membrane or by binding to a different region on the SNARE complex. We note that it is unknown as to which membrane the Ca\textsuperscript{2+}-bound C2 domains of synaptotagmin-1 would bind to, although the presence of PIP\textsubscript{2} in the plasma membrane increases the binding affinity\cite{18,19}. In turn, the primary SNARE/synaptotagmin-1 interface may assist in inducing membrane curvature upon Ca\textsuperscript{2+} binding\cite{13**,32,33}. With the disruption of the tripartite interface, the trans-SNARE complex then fully zippers, and the membranes move close enough to fuse. This release-of-inhibition model of Ca\textsuperscript{2+}-triggered fusion may explain the rapid (submillisecond) speed of the process because all the factors to promote fusion are already in place once the fusion block is released. We note that this release-of-inhibition model is independent of the actual number of SNARE complexes involved at the docking site of a primed synaptic vesicle, assuming that the presence of one or more inhibited complexes would prevent fusion.

This model of the inhibited pre-fusion SNARE/complexin-1/synaptotagmin-1 complex provides a mechanistic explanation as to why certain mutations of the Ca\textsuperscript{2+} +-binding region of the C2B domain of synaptotagmin-1 have dominant negative effects on both evoked and spontaneous neurotransmitter release\cite{11**,34,35}. In other words, expression of mutant synaptotagmin-1 reduces evoked release and upregulates spontaneous release in the presence of endogenous wildtype synaptotagmin-1. Also, complexin knockdown abrogates these dominant negative effects\cite{11**}. When some of the tripartite complexes with mutant synaptotagmin-1 molecules participate in a docked synaptic vesicle, these
mutant complexes would prevent the membranes from moving close enough for triggered fusion because Ca\textsuperscript{2+} cannot bind to these mutant synaptotagmin-1 molecules.

We note that the molecular mechanisms of spontaneous release are probably, or even likely, different from those of evoked release and involve a Ca\textsuperscript{2+} sensor other than synaptotagmin-1 [36]. The release-of-inhibition model is meant to explain action-potential evoked synchronous release. However, we note that disruption of either the tripartite or the primary interface in the SNARE/complexin-1/synaptotagmin-1 complexes increases spontaneous release rate [11**, so the presence of such complexes may also modulate spontaneous release.

Regulated assembly of the synaptic pre-fusion complex
As discussed, the pre-fusion SNARE/complexin-1/synaptotagmin-1 complex (Figure 1a) is probably important for establishing the primed state of synaptic vesicles (i.e. for the association of synaptic vesicles with presynaptic proteins that enables them to undergo fast Ca\textsuperscript{2+}-triggered fusion). Regulation of the formation of the primed state is critical as well. Several factors are involved in priming, including Munc18 and Munc13, but the molecular basis of the priming function of these molecules had been unclear until recently. Munc18 and its homologues (also referred to as SM proteins) are required components for all membrane trafficking pathways, as exemplified by the complete block of synaptic vesicle fusion upon Munc18-1 knockout in mice [37]. At the molecular level, Munc18 captures free syntaxin-1A, locking it into a heterodimeric complex that kinetically prevents formation of the ternary SNARE complex [38,39] (Figure 2a).

To enable ternary neuronal SNARE complex formation, another factor is required. One such factor is Munc13, a primarily brain-specific, cytoplasmic protein in the

![Figure 2](currentopinioninstructuralbiology.com)}
presynaptic terminal implicated in synaptic vesicle priming and short-term synaptic plasticity [1,40]. At the molecular level, Munc13 accomplishes two tasks: (1) catalyzing the transit of syntaxin from the syntaxin/Munc18 complex into the ternary trans-SNARE complex [41–43] and (2) promoting proper assembly of the SNARE complex in conjunction with Munc18 (i.e. ensuring the parallel configuration of all components of the SNARE complex) [17**]. In vivo, both Munc13 and Munc18 are required to promote proper SNARE complex assembly because genetic deletion of Munc13 cannot be fully rescued with a mutant of syntaxin that bypasses the Munc13 requirement in vitro [17**,44].

Why is it necessary to assist the proper assembly of the SNARE complex? When soluble fragments of SNARE proteins are simply mixed in solution, improper (e.g. anti-parallel) configurations may occur [45,46**]. It turns out that Munc13-1 promotes the proper parallel subconfiguration of syntaxin-1A and synaptobrevin-2 when assembling the ternary SNARE complex [17**]. Additionally, Munc13 also cooperates with Munc18 to promote the proper syntaxin-1A/SNAP-25A subconfiguration within the assembled ternary SNARE complex [17**]. Together, Munc13 and Munc18 can be viewed as assembly factors for establishing the functional subconfiguration of the ternary SNARE complex assembly.

The cooperation of Munc18 and Munc13 in promoting the proper SNARE complex assembly explains the severe effect of deletion of Munc18 and Munc13 in neurons [37,47]. Moreover, the localization of Munc13 to the plasma membrane and to synaptic vesicle docking sites may be regulated by Ca^{2+} binding to the C2 domains of Munc13 [48], as well as by interactions with other factors, including calmodulin [49] and Doc2 [50]. Such a membrane localization may in turn modulate the functions of Munc13 in priming, pre-synaptic plasticity, and augmentation. Finally, and consistent with their molecular functions, Munc18 and Munc13 substantially increased the efficiency of Ca^{2+}-triggered vesicle fusion in a reconstituted fusion assay [17**].

The molecular mechanism by which Munc13 and Munc18 promote proper SNARE complex assembly is still unclear, although there is some information about their molecular interactions [17**,**43,51–54,55**]. To begin, syntaxin-1A forms a tight complex with Munc18 [38,39] (Figure 2a), although this complex appears specific to neuronal exocytosis. For example, the situation is quite different for a yeast homologue of Munc18, the yeast homotypic fusion and vacuole protein-sorting (HOPS) protein Vps33. Crystal structures of the yeast SNARE proteins Vam3 and Nvy1 bound to Vps33 show fairly different interactions and suggest that Vps33 alone may promote proper assembly of ternary yeast SNARE complexes [56**] (Figure 2b). Similar interactions may also exist for the neuronal SNAREs and Munc18 as suggested by NMR [52] and single molecule pulling experiments [57**]. However, Munc18 alone appears to be insufficient to promote the proper configurations of all components of the neuronal SNARE complex because a constitutively ‘open’ mutant of syntaxin-1A does not rescue the essential role of Munc13 in neurons [17**].

Although there is no homolog for the MUN of Munc13 in yeast, the molecular architecture of entire HOPS complex [58] includes a relatively long, flat central region that bears some superficial resemblance to the shape of the MUN domain. Interestingly, the fold of the MUN domain is similar to that of several other proteins, including the Sec3, Sec15, Exo70 components of the exocyst tethering complex [59].

How does Munc13 then cooperate with Munc18 to promote proper assembly of the neuronal SNARE complex? The most complete structure of Munc13 to date is the structure of its C1C2BMUN fragment [60**] (Figure 2c). In this structure, the C2B domain interacts with the MUN domain of Munc13. Both syntaxin-1A and synaptobrevin-2 weakly interact with the MUN domain [17**,51,53,54,55**]. The membrane proximal region of synaptobrevin-2 binds to the MUN domain; this interaction is essential for Munc13 function [55**].

Deletion of either the C1 or C2B domain of Munc13, or Ca^{2+} binding to the C2B domain, increases evoked release in Caenorhabditis elegans, suggesting that the molecule exists in two states—an autoinhibited state and an activated state [61**]. The crystal structure of the C1C2BMUN domain of Munc13 was determined in the presence of Ca^{2+}, so it presumably corresponds to the active state. Of course, it possible that the activation relates to the interactions between Munc13 and the membrane as opposed to a conformational change in Munc13 [62].

**Post-fusion SNARE complex disassembly**
Following vesicle fusion, the ternary SNARE complex must be disassembled so that the individual SNARE proteins can be recycled. In concert with the adaptor protein SNAP, the ATPase NSF disassembles the ternary SNARE complex upon ATP hydrolysis [63–65]. NSF is a member of the AAA+ family which consists of two ATPase rings (D1 and D2 domains, known as Type II AAA+) and an N-terminal domain. Most eukaryotic organisms have only one NSF gene. By contrast, there are three homologues of SNAP, with αSNAP being the most widely studied. NSF, SNAPs, and SNAREs form the so-called 20S complex, the starting state for the disassembly process. The molecular mechanism of NSF-mediated SNARE complex disassembly is still unknown.

Years ago, crystal structures of the N and D2 domains of NSF were determined [66–69]. Subsequently, crystal structures
of the distantly related ATPase p97 [70–72] were determined. Early EM reconstructions of NSF and the NSF/ SNAP/SNARE complex followed [73,74]. However, these earlier EM studies suffered from relatively low resolution and the imposition of symmetry on the reconstructions that obscured the SNARE complex and its interactions with SNAPs and NSF (see Extended Data Figure 5 and Supplemental Discussion in Ref. [75**]), and despite many attempts, crystallization of full-length NSF and the NSF/ αSNAP/SNARE complex was unsuccessful. In 2015, a major breakthrough was achieved by determining cryo-EM structures of NSF at 4.2 Å and of the NSF/αSNAP/SNARE complex at 7.6 Å resolution without imposing symmetry [75**]. Two key factors led to these higher resolution EM reconstructions: (1) much improved sample homogeneity and (2) advances in cryo-EM technology, especially the availability of direct electron detectors. The higher sample homogeneity was achieved by ensuring a specific nucleotide-bound state [75**] and by building on previous biochemical and biophysical studies of the NSF-mediated SNARE complex disassembly [76,77].

Recently, an even higher-resolution reconstruction of the NSF/αSNAP/SNARE complex was obtained at 4.5 Å resolution [78**]. This reconstruction showed the interaction between the SNAREs with both NSF and αSNAPs in unprecedented detail (Figure 3a). Two αSNAP molecules bind to the ternary SNARE complex primarily along a large, negatively charged region at the center of the SNARE complex. Although the αSNAP stoichiometry is different for the two reconstructions of the NSF/αSNAP/SNARE complex (related to the absence of the SNAP-25A linker in the previous reconstruction [75**]), the interactions of the common two αSNAP molecules are nearly identical for all structures determined to date. The interacting regions on the SNARE complex and the two αSNAP molecules have complementary electrostatic features that may be a key contributor to this preferred binding mode. Taken together, these structures suggest that the 2:1 αSNAP: SNARE configuration is likely conserved in all NSF/ αSNAP/SNARE complexes.

Perhaps most importantly, a series of direct interactions between the SNARE complex and NSF were observed. A large portion of the SNAP-25A N-terminus was found within the pore of the D1 ATPase ring of NSF, consistent with a ratchet-like threading mechanism before disassembly (Figure 3b). This configuration is reminiscent of other recent structures of Type II AAA+ proteins in complex with peptides, suggesting a common, conserved mechanism for substrate unfolding. Some recent examples of AAA proteins with model substrates include: casein for Hsp104 [79] and Vps4 binding a peptide from the substrate Vps2 [80]; inhibited-substrate complexes with the 26S proteasome [81,82], and a substrate complex with inhibited PAN-proteasome [83]. More broadly, this suggests that different parts of AAA proteins and their adapters are responsible for substrate recognition and initiation of the unfolding or disassembly process.

A single molecule FRET disassembly assay enabled characterization of the disassembly process under a variety of conditions [84]. These studies revealed that NSF/αSNAP is capable of also disassembling anti-parallel SNARE complexes, suggesting a role in quality control of SNARE complex formation in conjunction with Munc18 and Munc13. Moreover, complexin-1 reduces the rate of NSF/αSNAP -mediated SNARE complex disassembly [84,85], probably by interfering with binding of one of the two conserved SNAP molecules found in structures of the NSF/αSNAP/SNARE complex. The interference by complexin on disassembly is more pronounced for a trans-SNARE complex situated between a synaptic vesicle and the plasma membranes [86***].

**Summary and outlook**

In summary, recent structures of the pre-fusion SNARE/ complexin-1/synaptotagmin-1 complex, the C1C2BMU1 fragment of Munc13, and the NSF/ αSNAP/SNARE complex are important new pieces in the puzzle of neuronal exocytosis (Figure 4). Yet, the molecular mechanisms are still unclear.
The key roles of Munc13 in priming and short-term plasticity may be related to the role of the MUN domain in proper assembly of the SNARE complex [17**]. This function of the MUN domain may in turn be subject to regulation by the other domains of Munc13. For example, Ca$^{2+}$-binding to the C2B domain [87], the synergy between C1, C2B, and C2C domains [48], and the Ca$^{2+}$-dependent interaction between calmodulin and Munc13 [49] may localize Munc13 to sites of docked synaptic vesicles and increase its ability to assist in proper SNARE complex assembly. Clearly, the underlying molecular mechanisms of Munc13 and Munc18 remain to be uncovered.

The conformation of the \textit{trans}-SNARE/complexin-1/synaptotagmin-1 complex within its native environment (that is, between the synaptic and plasma membranes) is unknown. In accordance with the abovementioned cryo-ET studies [30], the SNARE/complexin-1/synaptotagmin-1 complexes form a protein stalk that juxtaposes the membranes but keeps them far enough away to reduce the chances of membrane fusion. Yet, the resolution of the cryo-ET study was insufficient to draw any conclusions about the quaternary arrangements. Finally, the molecular steps after Ca$^{2+}$-binding to the synaptotagmin C2 domains should be visualized. In other words, what happens to the synaptotagmin C2 domains, the SNARE complex, complexin, and the membranes around the emerging fusion pore?

The recent structures of the NSF/αSNAP/SNARE complex provide evidence for loading of the full-length neuronal SNARE complex via the N-terminal residues of one of the SNARE proteins (SNAP-25A). How does complete disassembly then proceed? Is the N-terminus of SNAP-25A further translocated or threaded through the pore of the D1 ATPase ring? Such a translocation would exert a pulling force on the remaining membrane-anchored SNAP-25A, likely destabilizing interactions with syntaxin-1A and synaptobrevin-2—a model appealing in its simplicity. In any case, regardless of the mechanism, single molecule disassembly studies suggest that
the maximum number of ATPs required for complete disassembly cannot exceed 12 [88], ruling out processive mechanisms requiring many rounds of ATP hydrolysis. Future studies of conformational intermediates will be essential in testing these models and for uncovering the molecular mechanism of NSF/αSNARE-mediated disassembly of the SNARE complex.

Conflict of interest statement
Nothing declared.

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References


Single molecule FRET studies and fusion experiments with reconstituted SNAREs, synaptotagmin-1, and complexin-1 show that Munc18 and Munc13 cooperate to properly assemble the ternary SNARE complex. Either Munc18 or Munc13 alone is insufficient for proper assembly.


NMR studies of complexes between Ca2+-bound synaptotagmin-1 and the SNARE complex suggest that there are multiple binding modes between these molecules in solution.


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57. Structures of complexes between the Munc18 homolog Vps33 and the vacuolar yeast SNARE Vam3 and between Vps33 and Nvy1 suggest that Vps33 provides a template for SNARE complex assembly.


59. Single molecule force measurements suggest that Munc18 helps to promote proper assembly of the SNARE complex.


63. In this work, the first structure of the C12BMUN fragment of Munc13 is presented.


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The EM structure of the NSF/αSNAP/SNARE complex at ~3.9 Å resolution reveals a direct interaction between SNAP-25A and the D1 ring pore of NSF.


This study shows that the properly assembled trans-SNARE complex is protected against NSF/αSNAP disassembly when Munc18 and Munc13, complex-1, and synaptotagmin-1 are present.

