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Structure and function of SNARE and SNARE-interacting proteins

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Abstract. This review focuses on the so-called SNARE (soluble *N*-ethyl maleimide sensitive factor attachment protein receptor) proteins that are involved in exocytosis at the pre-synpatic plasma membrane. SNAREs play a role in docking and fusion of synaptic vesicles to the active zone, as well as in the Ca^{2+}-triggering step itself, most likely in combination with the Ca^{2+} sensor synaptotagmin. Different SNARE domains are involved in different processes, such as regulation, docking, and fusion. SNAREs exhibit multiple configurational, conformational, and oliogomeric states. These different states allow SNAREs to interact with their matching SNARE partners, auxiliary proteins, or with other SNARE domains, often in a mutually exclusive fashion. SNARE core domains undergo progressive disorder to order transitions upon interactions with other proteins, culminating with the fully folded post-fusion (*cis*) SNARE complex. Physiological concentrations of neuronal SNAREs can juxtapose membranes, and promote fusion *in vitro* under certain conditions. However, significantly more work will be required to reconstitute an *in vitro* system that faithfully mimics the Ca^{2+}-triggered fusion of a synaptic vesicle at the active zone.

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1. Introduction

The maintenance of distinct organelles within the eukaryotic cytosol is essential for survival. Within each organelle, conditions can be optimized for disparate biochemical processes, but the exchange of material between these organelles requires the merger of two phospholipid membranes (Ferro-Novick & Jahn, 1994; Rothman, 1994). Intracellular membrane or vesicle fusion involves a highly conserved family of proteins termed SNAREs (soluble N-ethyl maleimide sensitive factor attachment protein receptors). One particular example of vesicular fusion is exocytosis, defined as fusion of an intracellular trafficking vesicle with the plasma membrane. Constitutive exocytosis comprises all fusion processes where vesicles are generated, transported, and undergo exocytosis continuously. In contrast, regulated exocytosis is triggered by second messengers such as Ca^{2+} in response to activation or membrane depolarization (Burgess & Kelly, 1987; Jahn, 2004).

Synaptic neurotransmitter release is an example of regulated exocytosis. Synaptic vesicles are recruited to the active zone in the presynaptic membrane, but do not readily fuse. Instead, an average of 10 vesicles are stably docked at the active zone awaiting an action potential (Heuser & Reese, 1977; Schikorski & Stevens, 1997; Harlow *et al.* 2001; Rosenmund *et al.* 2003). Exocytosis is triggered within $\sim 0{\cdot}2$ ms of the Ca^{2+} influx that follows arrival of an action potential (Li *et al.* 1995b; Martin, 2003). There is also a background rate of fusion of about one per minute per synapse in the absence of action potentials. Although extremely rapid, neurotransmitter release is a probabilistic process, with only one fusion event for every 5–10 Ca^{2+} signals (Dobrunz & Stevens, 1997). This low-release probability means that usually at most one synaptic vesicle per synapse undergoes exocytosis upon depolarization (Pabst *et al.* 2000). Thus, regulation of neurotransmission occurs at the level of synaptic vesicle release probability.

SNAREs are linked directly to Ca^{2+} triggering of exocytosis, most likely in conjunction with a Ca^{2+} sensor (Sorensen *et al.* 2002; Sakaba *et al.* 2005). Numerous auxiliary proteins have been found to be essential for Ca^{2+}-dependent neurotransmitter release, such as synaptotagmin, complexin, Munc18, and Munc13. Genetic rescue experiments with mutants of synaptotagmin have left little doubt that synaptotagmin is a Ca^{2+} sensor for neurotransmission (Fernandez-Chacon *et al.* 2001), but the mechanism of action of the synaptotagmin · SNARE · membrane fusion machinery remains a matter of intense research (Arac *et al.* 2003; Shin *et al.* 2003; Weimer & Jorgensen, 2003; Tucker *et al.* 2004; Bai *et al.* 2004b).

SNAREs are the targets of clostridial neurotoxin (CNT) light-chain proteases (Schiavo *et al.* 1992). CNTs are the causative agents of flaccid or spastic paralysis occurring in botulism and tetanus diseases. Proteolysis of SNAREs by CNTs causes disruption of the ability of SNAREs to promote synaptic vesicle docking and fusion, thus disrupting neurotransmission.

In this review I focus on the biochemical, biophysical, and structural properties of SNAREs, their complexes, and their binding partners. I have restricted the discussion mostly to SNAREs involved in plasma membrane fusion, in particular in synaptic vesicle exocytosis, and to their binding partners for which a direct interaction with SNARE proteins has been established by biophysical or biochemical methods. It should be noted that there are many other proteins that play a fundamental role in synaptic neurotransmission and neuronal development upstream of SNARE function, such as the Sec6/8 tethering complexes (TerBush *et al.* 1996; Kee *et al.* 1997; Whyte & Munro, 2002), and Rab proteins and their effectors (Ferro-Novick & Novick, 1993; Simons & Zerial, 1993). However, no direct interactions between these factors and SNAREs have been established at this point. It is also possible that there are proteins acting downstream from neuronal SNARE function (Hiesinger *et al.* 2005) along the lines of what has been observed for vacuolar fusion (Wickner & Haas, 2000; Reese *et al.* 2005). Thus, SNAREs and their binding partners form only one part, albeit a very important part, of the complex system of synaptic neurotransmission.

2. SNAREs

2.1 Individual SNAREs

The importance of SNARE proteins for synaptic neurotransmission was established by studies indicating that SNAREs are the targets of CNT proteases (Blasi *et al.* 1993; Schiavo *et al.* 1992, 1993, 1995; Yamasaki *et al.* 1994a, b). This discovery coincided with the finding that SNAREs are binding proteins of *N*-ethylmaleimide sensitive factor (NSF) in conjunction with the soluble

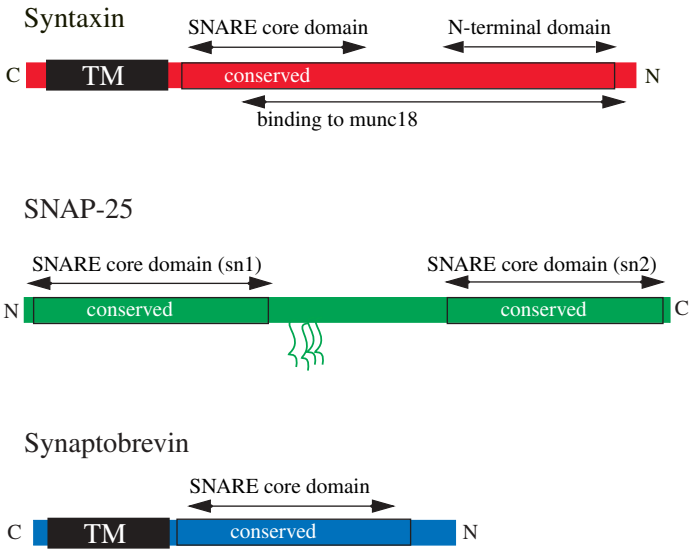

Fig. 1. Primary domain structure of neuronal SNAREs. Primary structure diagram for syntaxin (red), SNAP-25 (green), and synaptobrevin (blue). TM, Transmembrane domain. The SNARE core domains are defined through the 16 layers as found in the crystal structure of the neuronal SNARE complex (Sutton *et al.* 1998). For SNAP-25, the palmitoylation sites are indicated by green lines.

NSF attachment proteins (SNAPs) that had been shown to be involved in vesicle trafficking in the Golgi (Malhotra *et al.* 1988; Söllner *et al.* 1993). More recent experiments have confirmed the crucial role of SNAREs from a genetic perspective as well. For example, in knockout mice lacking the SNARE synaptobrevin (also called VAMP, vesicle-associated membrane protein), the rates of both spontaneous and Ca^{2+}-triggered synaptic vesicle fusion were decreased (Schoch *et al.* 2001). The lack of synaptobrevin was partially compensated for by the presence of cellubrevin, a synaptobrevin homolog (McMahon *et al.* 1993). In the absence of both synaptobrevin and cellubrevin, secretion was entirely abolished in double-knockout mice without affecting biogenesis or docking of chromaffin granules (Borisovska *et al.* 2005). Synaptobrevin is also essential for fast synaptic-vesicle endocytosis: in synaptobrevin-deficient synapses, altered shape and size of synaptic vesicles were observed and stimulus-dependent endocytosis was delayed (Deak *et al.* 2004).

Studies of SNAP-25 (synaptosome-associated protein of 25 kDa) knockout mice showed that Ca^{2+}-triggered release was abolished (Washbourne *et al.* 2002). A systematic analysis of genes required for function or development of *Caenorhabditis elegans* neuromuscular junctions by a high-throughput RNA interference screen also implied that the SNAREs synaptobrevin and SNAP-25 are essential (Sieburth *et al.* 2005).

The domain structure of the neuronal SNAREs syntaxin, synaptobrevin, and SNAP-25 are shown in Fig. 1. In this review I refer to the syntaxin 1A isoform as simply syntaxin, synaptobrevin II as synaptobrevin, and SNAP-25A as SNAP-25 unless specified otherwise. Both syntaxin and synaptobrevin have a C-terminal transmembrane domain and an adjacent domain that is involved in interacting with SNAREs – I will refer to these domains as SNARE core domains. Neuronal syntaxin has a folded N-terminal domain. Neuronal synaptobrevin has a short unstructured N-terminal region while for other synaptobrevins this region is missing or it is

replaced by a folded N-terminal domain, the so-called longin domain (see Section 2.1.1.2). Neuronal SNAP-25 is a soluble protein that consists of two SNARE core domains (also termed sn1 and sn2 respectively), and a linker that contains four palmitoylated cysteine residues. There is considerable varation among other members of the SNAP-25 family: some of them only contain one SNARE core domain, others contain a N-terminal domain as well.

2.1.1 Structure of individual SNAREs

The cytoplasmic domains of synaptobrevin and its yeast homolog Snc1 (both involved in plasma membrane exocytosis) are unfolded in solution as determined by circular dichroism (CD) (Fasshauer *et al.* 1997b) and solution NMR experiments (Fiebig *et al.* 1999; Hazzard *et al.* 1999). Isolated SNAP-25 is also unfolded as assessed by CD experiments (Fasshauer *et al.* 1997a); unfortunately, the tendency of isolated SNAP-25 to form oligomers has prevented solution NMR studies. The cytoplasmic domains of syntaxin and its yeast homolog Sso1 are partially folded as determined by NMR experiments (Dulubova *et al.* 1999; Fiebig *et al.* 1999).

The SNARE core domain fragment (sometimes called the H3 domain) of syntaxin can form a homotetramer consisting of two pairs of parallel α-helices that are anti-parallel to each other (Misura *et al.* 2001b) (Fig. 2*a*). Modeling studies showed that the phenylalanine residue 216 prevents formation of a parallel homotetramer, thus probably favoring physiologically more important interactions with other SNAREs. It should be noted that the possible physiological role of this syntaxin homotetramer is uncertain, but this structure along with all the other structural studies of SNAREs (Section 2.2.11) demonstrate the conformational and oliogomeric variability of SNAREs.

2.1.1.1 *Three-helix bundle N-terminal regulatory domains.* Syntaxin and certain other SNAREs contain an independently folded domain at the N-terminus that is connected to the SNARE core domain by a flexible linker (Margittai *et al.* 2003a). This domain may be involved in regulation of SNARE complex assembly (discussed in Section 2.2). In addition, N-terminal domains can have other functions as well. A striking example is provided by the yeast SNAP-25 homologs Sec9 and Spo20: they both interact with the same SNAREs (Snc2 and Sso1) via their SNARE core domains, but yet they play different roles during yeast development by interactions of their respective N-terminal domains with other factors (Neiman *et al.* 2000). Furthermore, the Lethal giant larvae (Lgl) tumor suppressor which is conserved from yeast to mammals interacts with the N-terminal domain of Sec9, and a model has been proposed where the Lgl family functions in cell polarity by regulating SNARE-mediated membrane delivery events at the cell surface (Gangar *et al.* 2005).

In the syntaxin subfamily, the N-terminal domain consists of an anti-parallel bundle of three α-helices that are structurally conserved despite divergence in primary structure. For example, very similar structures are found for the N-terminal domain of neuronal syntaxin (Fernandez *et al.* 1998; Lerman *et al.* 2000) (Fig. 2*b*), and for the N-terminal domain of yeast Vam3, a distant syntaxin homolog (Dulubova *et al.* 2001). The structure of the N-terminal domain of syntaxin 6, a SNAP-25 homolog, is also similar to that of neuronal syntaxin despite low sequence similarity (Misura *et al.* 2002).

Not all N-terminal domains are essential; for example, the N-terminal domain of the syntaxin homolog Vam3 can be removed without effect on vacuole fusion (Wang *et al.* 2001b). Part of the flexible linker between the SNARE domain and the regulatory N-terminal domain can be

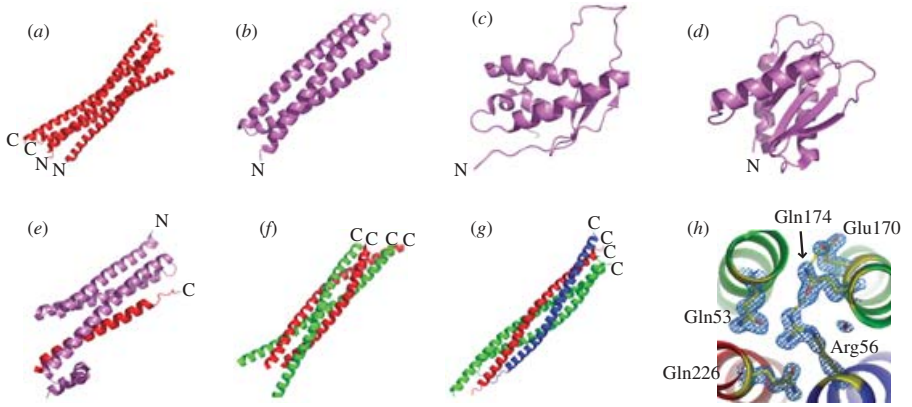

Fig. 2. Structures of SNAREs and SNARE complexes. (*a*) X-ray crystal structure of anti-parallel homotetramer formed by the syntaxin SNARE core domain (PDB ID 1HVV) under certain crystallization conditions (Misura *et al.* 2001b). (*b*) X-ray crystal structure of the N-terminal domain of syntaxin (PDB ID 1EZ3) (Lerman *et al.* 2000). (*c*) Solution NMR structure of the Vam7 (a SNAP-25 homolog) PX N-terminal domain structure (PDB ID 1KMD) (Lu *et al.* 2002). (*d*) X-ray crystal structure of the Sec22 (a synaptobrevin homolog) N-terminal domain (longin domain) ((PDB ID 1IFQ) (Gonzalez *et al.* 2001). (*e*) X-ray crystal structure of the closed conformation of the yeast Sso1, syntaxin homolog (PDB ID 1FIO) (Munson *et al.* 2000) (red: SNARE core domain; purple: N-terminal domain). The N-terminus of the N-terminal domain, and the C-terminus of the SNARE core domain of Sso1 are indicated. (*f*) X-ray crystal structure of the homodimer of the heterodimer consisting of the sn1 N-terminal domain of SNAP-25 (green) and the SNARE core domain of syntaxin (red) (PDB ID 1JTH) (Misura *et al.* 2001a). The C-terminii are indicated. (*g*) X-ray crystal structure of the core of the neuronal *cis* SNARE complex consisting of synapotbrevin (blue), SNAP-25 (green), and syntaxin (red) (PDB ID 1SFC) (Sutton *et al.* 1998). The C-terminii are indicated. (*h*) Close-up view of the ionic layer at the center of the neuronal SNARE complex at 1·4 Å resolution (PDB ID 1N7S) consisting of syntaxin Gln226, synaptobrevin Arg56, SNAP-25 (sn1) Gln53, and SNAP-25 (sn2) Gln174. SNAP-25 Gln174 is stabilized by a hydrogen bond involving SNAP-25 Glu170. An ordered water molecule is also visible that is hydrogen-bonded to Arg56. The electron density map is superimposed on the structure (blue mesh) (Ernst & Brunger, 2003).

involved in interactions with other proteins, such as found in the complex between nsec1 and syntaxin (Misura *et al.* 2000) (see also Section 3.4).

2.1.1.2 *Other N-terminal domains.* SNAREs can contain N-terminal domains other than the three-helix bundle found in neuronal syntaxin. For example, the yeast SNAP-25 homolog Vam7 (involved in vacuolar fusion) has a N-terminal PX domain that binds to PtdIns(3)P; its structure reveals a structurally conserved core that is similar to that of other PX domains (Lu *et al.* 2002) (Fig. 2*c*).

Longins are synaptobrevin homologs that are characterized by a conserved N-terminal domain with a profilin-like fold called a longin domain (Rossi *et al.* 2004; Uemura *et al.* 2005). The structurally conserved longin domains play a role in vacuolar and subcellular targeting. The N-terminal domain of Sec22, a synaptobrevin homolog involved in endoplasmic reticulum (ER)/Golgi membrane trafficking, shows a mixed α-helical/β-sheet fold (Gonzalez *et al.* 2001) (Fig. 2*d*). Its structure is very similar to that of Ykt6 (Tochio *et al.* 2001). Despite similar N-terminal and SNARE core domains, Sec22 and Ykt6 have different functions. Sec22 is anchored via a transmembrane domain and functions in both anterograde and retrograde trafficking between the ER and the Golgi. By contrast, Ykt6 is lipid-anchored and is required for

protein trafficking at the Golgi, endosomes and the yeast vacuole. Even though Ykt6 and Sec22 have very similar longin domain structures, the longin domain of Sec22 is always in an open conformation, i.e. it does not interact with its corresponding SNARE core domain, in contrast to Ykt6 which can adopt a closed conformation.

2.1.1.3 *Interactions between domains within the same SNARE.* The structure of the yeast homolog of syntaxin, Sso1 (Munson *et al.* 2000) exhibits a closed conformation where the N-terminal domain interacts with part of the SNARE domain (Fig. 2*e*). Syntaxin switches between a closed and an open conformation with a relaxation time of 0·8 ms as assessed by single-molecule fluorescence correlation spectroscopy (Margittai *et al.* 2003b). Mutations have been introduced that cause the conformation to remain open (Dulubova *et al.* 1999; Munson & Hughson, 2002). These mutations accelerate assembly of the ternary SNARE complex. Similar to the longin family, not all N-terminal domains of syntaxins form a closed conformation with the SNARE core domain; for example, in vti1b and syntaxin 8, the N-terminal domains do not interact with their adjacent SNARE core domains (Antonin *et al.* 2002a).

2.1.2 Palmitoylation

Synaptobrevin (Veit *et al.* 2000), SNAP-25 (Hess *et al.* 1992), and the yeast SNAREs Snc1, Syn8, and Tlg1 (Valdez-Taubas & Pelham, 2005) are palmitoylated at cysteine residues *in vivo* close to their respective transmembrane domains. Thus, lipid modification of SNARE proteins might contribute to the regulation of the synaptic vesicle cycle. It has also been proposed that palmitoylation protects the transmembrane domains of certain SNAREs from mis-sorting to the vacuole (Valdez-Taubas & Pelham, 2005).

2.1.3 Phosphorylation

SNAP-25 can be substrate for cAMP-dependent protein kinase (PKA)-dependent phosphorylation; over-expression of SNAP-25 mutated in the Thr138 phosphorylation site eliminated the effect of PKA inhibitors on the vesicle-priming process (Hepp *et al.* 2002; Nagy *et al.* 2004). Phosphorylation of SNAP-23 has been found to regulate exocytosis in mast cells (Hepp *et al.* 2005).

2.2 Assembly of SNARE complexes

As mentioned in Section 2.1.1, isolated SNARE core domains are largely unstructured (Fasshauer *et al.* 1997a, b; Fiebig *et al.* 1999; Hazzard *et al.* 1999). Upon complex formation, major structural changes occur for some of the binary combinations of syntaxin, SNAP-25, and synaptobrevin (Fasshauer *et al.* 1997a), as well as for the ternary SNARE complex (Fasshauer *et al.* 1997b). These disorder-to-order transitions are conserved among other members of the SNARE family, as was shown by studies of the yeast SNARE proteins Snc1, Sso1, and Sec9 (Fiebig *et al.* 1999).

2.2.1 Binary syntaxin · SNAP-25 complex

Upon complex formation between Sso1 and Sec9 (yeast homologs of syntaxin and SNAP-25 respectively), significant structure is induced in the C-terminal half (i.e. the membrane proximal

half) of the SNARE core domain compared to the closed conformation of isolated Sso1 (Fiebig *et al.* 1999). This binary complex of yeast SNAREs has a 1:1 stoichiometry in contrast to the neuronal syntaxin · SNAP-25 complex that adopts a mixture of 1:1 and 2:1 complexes (Fasshauer *et al.* 1997a) (see below).

The closed conformation of Sso1 consists of a four-helix bundle formed by part of the SNARE core domain and the N-terminal domain (Munson *et al.* 2000) (Fig. 2*e*), and this bundle is flanked on both sides by large flexible regions (Fiebig *et al.* 1999). Sso1 switches to an open state when its SNARE core domain binds Sec9. Formation of the binary complex induces additional α-helical structure in previously unstructured regions. The interacting core of the binary complex between Sso1 and Sec9 likely consists of a three-helix bundle to which Sec9 contributes two core SNARE domains and Sso1 provides the third SNARE core domain. In this binary complex, the 24 C-terminal residues of Sso1 remain unstructured (Fiebig *et al.* 1999). Conformational switching of the SNARE core domain, via the N-terminal domain, thus provides a key regulatory mechanism in SNARE assembly.

Studies of the syntaxin · SNAP-25 complex by site-directed spin-label electron paramagnetic resonance (EPR) spectroscopy suggested that this particular binary SNARE complex is more structured than the Sso1 · Sec9 complex, including the C-terminal region and the mid-section around the center layer of the helical bundle (Zhang *et al.* 2002). However, it should be noted that these results contradict earlier studies that suggested that this complex has a disordered mid-section (Xiao *et al.* 2001), and that the C-terminal region is disordered (Margittai *et al.* 2001). In any case, the structure of 2:1 form of the syntaxin · SNAP-25 complex consists of a parallel four-helix bundle as assessed by site-directed spin-labeling EPR measurements (Xiao *et al.* 2001).

The 2:1 syntaxin · SNAP-25 complex probably represents a kinetic dead-end intermediate state. Indeed, the relevant state for synapbrevin binding is a transient 1:1 syntaxin · SNAP-25 complex (Fasshauer & Margittai, 2004), perhaps similar to that of the Sso1 · Sec9 binary complex. Consistent with the importance of the 1:1 state of this binary complex, binding of synaptobrevin to the binary complex was inhibited by an excess of syntaxin. Unfortunately, no molecular structure is available for the syntaxin · SNAP-25 1:1 complex due to its transient nature.

In vivo studies in live endocrine cells suggested the presence of a binary complex between syntaxin and SNAP-25 that apparently only requires the amino-terminal (sn1) SNARE core domain of SNAP-25 (An & Almers, 2004). Interestingly, a complex was crystallized between sn1 and the SNARE core domain of syntaxin (Misura *et al.* 2001a) (Fig. 2*f*). However, this complex consisted of a dimer of complexes resulting in a four-helix bundle, so the crystallized structure probably represents a kinetic dead-end. Nevertheless, the interactions between sn1 and the syntaxin core domain may be related to the state of the binary complex observed by (An & Almers, 2004).

2.2.2 Binary syntaxin · synaptobrevin interaction

A small increase in helicity occurs upon mixing of syntaxin and synaptobrevin (Fasshauer *et al.* 1997b), and NMR experiments show a weak interaction between these SNAREs (Hazzard *et al.* 1999). These results are particularly relevant considering that *in vitro* docking and fusion of liposomes to deposited bilayers can be accomplished with just synaptobrevin and syntaxin in different membranes, despite the absence of SNAP-25 (Woodbury & Rognlien, 2000; Bowen *et al.* 2004; Liu *et al.* 2005) (see also Section 4). Studies of SNAP-25 knockout mice revealed a phenotype where vesicle docking and stimulus-independent (spontaneous) fusion persisted

although Ca^{2+}-triggered release was abolished (Washbourne *et al.* 2002). In a follow-up study, it was shown that over-expression of a SNAP-25 homolog rescued Ca^{2+}-dependent fusion (Sorensen *et al.* 2003). Clearly, from a physiological perspective, SNAP-25 is essential for Ca^{2+}-dependent fusion, and it also provides some interactions with the Ca^{2+} sensor synaptotagmin (see Section 3.2). Furthermore, the yeast homolog Sec9 is essential for constitutive fusion (Brennwald *et al.* 1994), again pointing at the importance of this SNARE *in vivo*.

2.2.3 Binary synaptobrevin · SNAP-25 interaction

No significant interaction has been found between the cytoplasmic domains of synaptobrevin and SNAP-25 *in vitro* (Fasshauer *et al.* 1997b). Nevertheless, experiments by Scheller and co-workers using a cracked PC12 cell system indicated that an interaction may exist between synaptobrevin and SNAP-25 *in vivo* (Chen *et al.* 2001). In their proposed model, syntaxin is initially bound by nsec1. During Mg^{2+} ATP and temperature-dependent priming, synaptobrevin and SNAP-25 are brought together and interact reversibly as a partially zippered complex. The SNARE core domain of syntaxin is held back by the Ca^{2+} sensor. When Ca^{2+} arrives, the sensor is removed from the syntaxin core domain, allowing the SNARE complex to zipper up towards the membrane proximal C-terminus of the bundle, resulting in membrane fusion. Clearly, this model is very different from models that involve binary syntaxin · SNAP-25 complexes to which synaptobrevin binds (Fasshauer & Margittai, 2004).

2.2.4 Ternary *cis* (post-fusion) SNARE complex

The ternary SNARE complex consisting of syntaxin, synaptobrevin, and SNAP-25 can be readily isolated from neuronal cell extracts (Otto *et al.* 1997). The membrane anchors are not required for the assembly of the SNARE complex, so many early biophysical and structural studies were carried out with recombinant proteins in the absence of the transmembrane domains and palmitoylated cysteins. These truncated SNAREs readily form ternary complex which can be purified by chromatography (Fasshauer *et al.* 1997a, b; Rice *et al.* 1997). The ternary SNARE complex exhibits remarkable thermal and chemical stability; one of the hallmarks of the neuronal SNARE complex is resistance to disassembly in the presence of sodium dodecyl sulfate (SDS) without boiling although this is not a conserved property of SNAREs (the yeast SNARE complex is not resistant to SDS). Limited proteolysis of the neuronal SNARE complex revealed a core complex consisting of the four SNARE core domains of syntaxin, synatobrevin, and SNAP-25, without the regulatory N-terminal domain of syntaxin. This core complex has similar biophysical properties as the full-length complex (Fasshauer *et al.* 1998a; Poirier *et al.* 1998a).

Ternary SNARE complex formation induces major disorder-to-order transitions in SNARE core domains (Rice *et al.* 1997; Fiebig *et al.* 1999), in addition to those observed in the binary complexes discussed above. These structural transitions were studied by solution NMR for the yeast SNAREs: structural transitions occured in Sso1 and Snc1 upon formation of the ternary complex of Sso1, Sec9 and Snc1 (Fiebig *et al.* 1999); Sec9 was not labeled due to limited expression and hence its structural transition could not be studied by NMR. Upon complex formation, structure is induced in the entire C-terminal region of the cytoplasmic domain of Sso1 and in about two thirds of the cytoplasmic domain of Snc1. Binding of Snc1 to the binary complex only affects the C-terminus of Sso1, suggesting that its N-terminal domain is

conformationally independent from the core SNARE complex. Electron microscopy (EM) images of the entire synaptic fusion complex (Hanson *et al.* 1997b) also support the notion that the N-terminal domain is peripheral to the complex, connected only by a flexible linker. Based on the unstructured nature of Sec9 in isolation (Rice *et al.* 1997) and formation of helical structure in the assembled ternary complex (Sutton *et al.* 1998), one can conclude that the core domains of Sec9 also undergo a major disorder-to-order transition.

The neuronal SNARE core complex in the post-fusion state (*cis* state, see Section 2.2.8) consists of a parallel four-helix bundle (Sutton *et al.* 1998; Ernst & Brunger, 2003) (Fig. 2*g*). The core of the four-helix bundle of the SNARE complex is composed of 16 primarily hydrophobic layers formed by interacting side-chains from each of the four α-helices. The periodicity of these layers is a highly conserved property across the entire SNARE family. At the center of the core complex, a conserved ionic layer is present consisting of an arginine and three glutamine residues contributed from each of the four α-helices (Fig. 2*h*). This ionic layer is sealed off against solvent by adjacent hydrophobic layers, but it contains a buried water molecule.

A high-resolution structure of the neuronal SNARE core complex at 1·4 Å resolution revealed a stabilizing salt bridge, sites of hydration, and conformational variability at the ionic layer (Ernst & Brunger, 2003) (Fig. 2*h*). This particular complex was obtained by slight truncation of the previously characterized core complex (Fasshauer *et al.* 1998a). This truncated SNARE complex is monomeric and monodisperse, and it retains binding to synaptotagmin (Ernst & Brunger, 2003).

A model of the SNARE core complex was obtained independently by site-directed spin-labeling EPR experiments; the overall features of this model were consistent with the crystal structure although it lacked detail. In particular, the ionic layer interactions were not correctly predicted (Poirier *et al.* 1998b).

Fluorescence resonance energy transfer (FRET) efficiency measurements and quick-freeze/deep-etch EM of suitably labeled SNARE complexes had indicated earlier the parallel nature of the syntaxin · synaptobrevin interactions (Hanson *et al.* 1997b; Lin & Scheller, 1997). However, the bulk FRET measurements by Lin & Scheller (1997) were not consistent with the parallel extended conformation of the SNARE complex observed in the crystal structures. A possible reason for this discrepancy is discussed in Section 2.2.7.

The structure of the post-fusion SNARE core complex is evolutionarily conserved. For example, the structure of the endosomal SNARE core complex containing four separate SNARE proteins (syntaxin 7, syntaxin 8, vti1b, and endobrevin/VAMP-8) is very similar to that of the neuronal SNARE complex despite limited sequence similarity (Antonin *et al.* 2002b). However, subtle structural variations are evident that characterize the different SNARE subfamilies.

2.2.5 Classification of Q- and R-SNAREs

Sequence alignments of the most conserved regions in the core domains of the neuronal SNAREs were mapped onto the crystal structure of the SNARE complex (Fasshauer *et al.* 1998b). It was found that the layers of interacting amino acid side-chains in the center of the four-helix bundle are highly conserved, including the ionic layer (Fig. 2*h*). Mutations in these layers are known to reduce complex stability and can cause defects in membrane traffic, even in distantly related SNAREs (Fasshauer *et al.* 1998b). On the basis of the conservation of the ionic layer, SNARE proteins were reclassified into Q-SNAREs (containing a glutamine at the ionic

layer position) and R-SNAREs (containing an arginine at the ionic layer position), and it was proposed that fusion-competent SNARE complexes should consist of four-helix bundles composed of three Q-SNAREs and one R-SNARE. The 3Q-1R requirement for fusion-competent SNARE complexes has been confirmed by mutagenesis studies (Katz & Brennwald, 2000; Ossig *et al.* 2000; Graf *et al.* 2005) (see also Section 2.5).

The two SNARE core domains that are supplied by SNAP-25 in the neuronal case can also be supplied by individual proteins (Fukuda *et al.* 2000). An example is provided by the aforementioned endosomal SNARE complex consisting of syntaxin 7, syntaxin 8, vti1b, and endobrevin/VAMP-8 (Antonin *et al.* 2002b). An extensive statistical analysis revealed 7–12 Qa-SNAREs (syntaxins), 5–9 Qb-SNAREs (SNAP-25 sn1), 4–8 Qc-SNAREs (SNAP-25 sn2), and 5–9 R-SNAREs (synaptobrevins) in the genomic databases of *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* (Bock *et al.* 2001).

2.2.6 Promiscuity of SNARE interactions

The primary sequence conservation of the structural core of the SNARE complex casts doubts on the targeting role of SNAREs for vesicle trafficking, as originally proposed by the SNARE hypothesis (Rothman, 1994). Indeed, very similar biophysical and biochemical properties were obtained for *in vitro* complexes consisting of artificial combinations of SNAREs that are localized to different compartments *in vivo* (Fasshauer *et al.* 1999; Yang *et al.* 1999). Furthermore, some SNAREs can function at several different transport steps *in vivo*. Thus, SNAREs are probably not the sole determinants of vesicle targeting specificity. Rather, the observed SNARE localizations may be important for the interactions with other factors such as Sec1/Munc18 (SM) proteins that interact with non-conserved SNARE residues (Misura *et al.* 2000). However, *in vitro* liposome fusion assays revealed that fusion is limited to combinations resembling the compartmental localization of intracellular trafficking for yeast SNAREs (Fukuda *et al.* 2000; McNew *et al.* 2000a; Parlati *et al.* 2000, 2002) (see Section 4). Perhaps fusion is influenced by more subtle differences than overall complex stability.

2.2.7 Anti-parallel configurations

Single molecule FRET efficiency measurements revealed a surprising characteristic of SNARE complex assembly: a mixture of parallel as well as anti-parallel configurations was found involving the SNARE core domains of syntaxin and synaptobrevin as well as those of syntaxin and SNAP-25 (Weninger *et al.* 2003). The subpopulation with the parallel four-helix bundle configuration could be greatly enriched by an additional purification step in the presence of denaturant, indicating that the parallel configuration is the energetically most favorable state. This explains why only the parallel configuration was found in the crystal structures of SNARE complex and in other biophysical studies that involved highly purified SNARE complex samples. Inter-conversion between the configurations was not observed on the hour time scale.

Consistent with these studies, the aforementioned bulk FRET studies of the SNARE complex (Lin & Scheller, 1997), performed without extensive purification, can now be explained by the presence of a mixture of parallel and anti-parallel configurations in the sample. Likewise, the non-zero FRET efficiency measurements between syntaxin C-terminally fused to blue fluorescent protein (BFP) and synaptobrevin N-terminally fused to green fluorescent protein

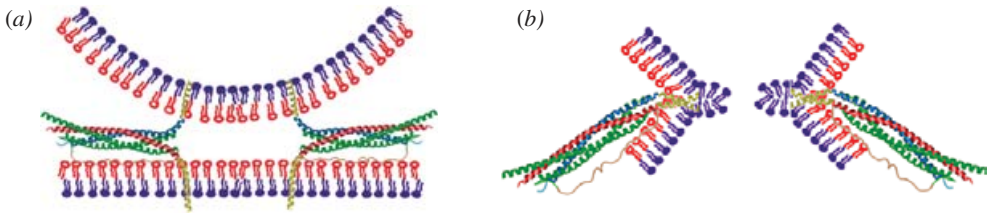

Fig. 3. *Cis* and *trans* states. (*a*) Partially assembled *trans* state of two SNARE complexes that dock a vesicle to a target membrane. This model was obtained by modifying the membrane proximal end of the crystal structure of the neuronal SNARE complex in order to allow the transmembrane domains to enter into the juxtaposed membranes. The transmembrane domains were assumed to be helical. The connecting region between the transmembrane domains and the core complex are likely flexible. Two SNARE complexes are shown; the exact number is uncertain (Section 5). (*b*) Fully assembled *cis* state of the SNARE complexes after fusion and pore formation.

(GFP) in 20S particles (NSF, α-SNAP, and SNARE complex) (Hohl *et al.* 1998) could be due to the partial presence of anti-parallel SNARE complexes. Interestingly, when SNARE complex was obtained from brain extracts, all SNARE complexes appeared to be parallel as observed in electron micrographs (Hohl *et al.* 1998).

To investigate if such mixtures exist in the membrane environment of docked liposomes, single-molecule FRET efficiency studies were carried out to determine the configuration of the SNARE complexes involved in docking liposomes to deposited bilayers (Bowen *et al.* 2004). The anti-parallel population was ∼1/5 the size of the parallel population. Thus, liposome docking to a supported bilayer favors the assembly of SNAREs into the parallel configuration compared to studies in solution where the majority of unpurified complexes can be found in the anti-parallel configuration. An open question is do such anti-parallel configurations exist in the physiological context and if so, are they regulated by chaperones?

2.2.8 *Cis* and *trans* states

Numerous biochemical, structural, and genetic studies have lent support to the zipper model which states that SNARE complex assembly begins in *trans* (i.e. residing on opposite membranes), with separate SNAREs on the donor and acceptor membranes, and ends with formation of a *cis* complex (i.e. residing on the same membrane) (Fig. 3), and that directional folding of SNAREs into a highly stable parallel four-helix bundle drives membrane fusion (Bai & Pagano, 1997; Hanson *et al.* 1997a; Fasshauer *et al.* 1998b; Fiebig *et al.* 1999; Parlati *et al.* 1999). It should be pointed out that, in addition to juxtaposing membranes through SNARE complex formation, the SNARE transmembrane domains may also participate in the later stages of fusion (Langosch *et al.* 2001), for example by promoting inner leaflet mixing and transition through a putative hemifusion intermediate (Xu *et al.* 2005).

In support of the zipper model, SNAREs exist in a partially assembled loose complex prior to the arrival of the Ca^{2+} signal *in vivo* (Xu *et al.* 1998, 1999; Chen *et al.* 2001). Further evidence for such a partially assembled complex was recently obtained by EPR measurements *in vitro* (Zhang *et al.* 2005). In this state, the SNAREs are still susceptible to cleavage by a subset of CNTs (Foran *et al.* 1994; Hua & Charlton, 1999; Blas *et al.* 2005) (see also Section 3.1). Furthermore, *cis* SNARE-complex specific antibodies do not interact with the partially assembled complex.

2.2.9 Time scale of assembly

Assembly of *trans* SNARE complexes *in vitro* exhibits a significant hysteresis (Fasshauer *et al.* 2002) as seen by both chemical and thermal CD denaturation experiments. Similarly, studies of yeast SNAREs exhibited slow binary complex formation between Sso1 and Sec9, caused by interference through the N-terminal domain of Sso1 (Nicholson *et al.* 1998). Studies of the same SNAREs by EPR revealed that the time scale of complex formation shows little difference along the length of the complex with rate constants ranging from 3000 to 5000 s^{-1} M^{-1} (Zhang *et al.* 2004). When full-length proteins reconstituted into liposomes were used the assembly rates were about one order of magnitude slower.

2.2.10 Oligomerization

While the cytoplasmic domains of both synaptobrevin and syntaxin are monomeric, SNAP-25 has a slight tendency to form oliogomers, and both the binary syntaxin · SNAP-25 and the ternary syntaxin · SNAP-25 · synaptobrevin complex tend form higher-order oligomers (Fasshauer *et al.* 1997b). In contrast, the cytoplasmic domains of the yeast SNAREs Snc1, Sso1, and Secp9 are monomeric, while the ternary complex forms higher-order oliogomers (Rice *et al.* 1997).

The transmembrane domains of synaptobrevin also have a weak tendency for oliogomerization although it is unclear if this interaction is significant (Bowen *et al.* 2002) (see Section 2.3). One possible mechanism for SNARE complex oligomerization is domain swapping; indeed using a mixed population of SNAP-25 species with labels placed at different sites, EPR measurements revealed domain swapping at concentrations that promote SNARE complex oliogomerization (Kweon *et al.* 2002a). The linker that connects the two SNAP-25 SNARE core domains is clearly long enough to accommodate such domain swapping with SNARE complexes that are adjacent to each other. Thus, SNARE domain swapping could play a role in formation of the elusive SNARE fusion pore although the existence of such a species has yet to be shown.

2.2.11 Summary of assembly pathways

From the studies reviewed thus far, one can deduce that at a simplistic level, neuronal SNAREs generally have three conformational states: first, the closed and open conformations of uncomplexed syntaxins and the unstructured or flexible conformations of synaptobrevin and SNAP-25; second, the binary complex of syntaxin and SNAP-25; and third, the ternary complex of syntaxin, SNAP-25, and synaptobrevin. As mentioned in Section 2.1.1.3, the closed conformation of uncomplexed syntaxin contains a four-helix bundle made up of the regulatory N-terminal domain and roughly half of the SNARE core domain. A similar conformation of syntaxin has been observed in the crystal structure of syntaxin in the syntaxin · nSec1 complex (Section 3.4).

Syntaxin switches to an open state upon binding to SNAP-25. In this open state, binding to the other SNAREs is mediated by the SNARE core domains. Formation of binary and ternary complexes is associated with increasing induction of α-helical structure in previously unstructured or flexible regions of the SNARE core domains. The available data also suggest that SNARE complex assembly begins distal to the membrane surfaces and proceeds toward them.

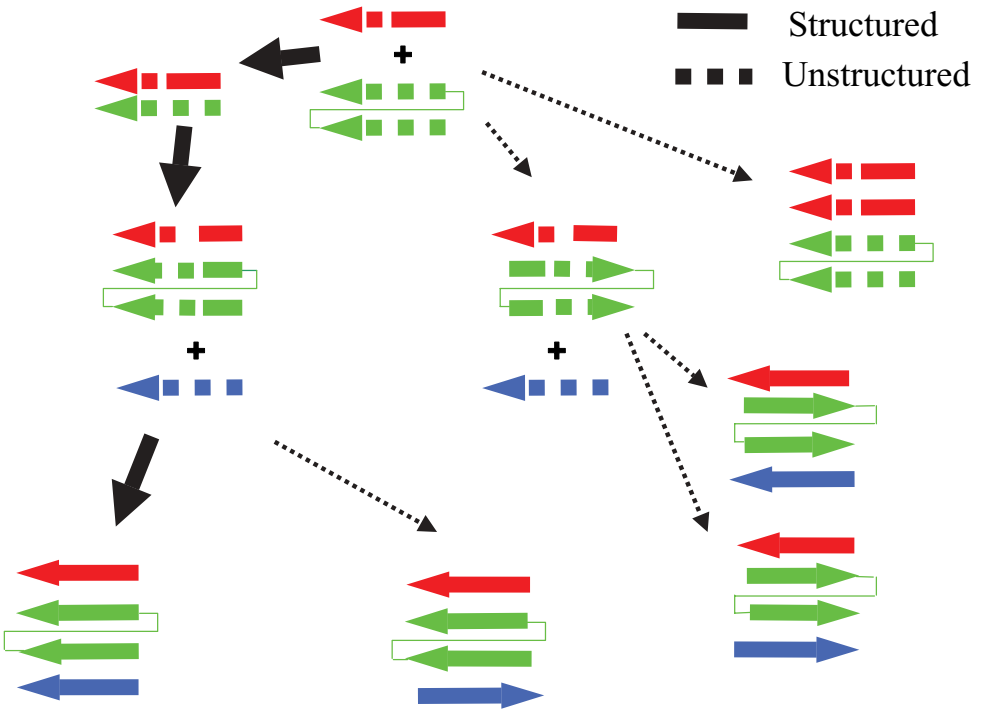

Fig. 4. Assembly pathway of the neuronal SNAREs core domains. The assembly of the core domains of the SNARE complex proceeds via intermediate states. The primary assembly pathway is indicated by solid thick arrows. It starts with binary complex formation between syntaxin (red) and SNAP-25 (green), and ends with the formation of the ternary SNARE complex by addition of synaptobrevin (blue). A putative intermediate state is also shown that consists of the syntaxin core domain and the sn1 domain of SNAP-25 (An & Almers, 2004). Dead-end intermediates, such as anti-parallel complexes (Weninger *et al.* 2003) or the syntaxin · SNAP-25 2:1 complex (Fasshauer & Margittai, 2004) are also shown (branches indicated by dashed arrows).

At a more detailed level, a more complex picture emerges: other binary complex combinations are possible (syntaxin · synaptobrevin, or synaptobrevin · SNAP-25), although these interactions are weak, at least *in vitro*. Futhermore, anti-parallel configurations are possible. A summary of some of these configurations and conformations is shown in Fig. 4.

This complex picture of SNARE complex configurations, conformations, and oligomeric states is further compounded by the observation of additional conformations of SNAREs involved in interactions with other proteins (see Sections 3.1.1 and 3.4).

2.3 Transmembrane domains

The membrane proximal region of the SNARE core domain of isolated syntaxin was studied by spin-labeling EPR with syntaxin reconstituted in large unilamellar vesicles; it was found that this region of syntaxin is unstructured, but inserted into the membrane (Kim *et al.* 2002). Likewise, the membrane proximal region of syntaxin involved in *cis* SNARE complexes was found to be incorporated into the phospholipids headgroup region (Kweon *et al.* 2002b). A similar experiment with yeast plasma membrane SNAREs indicated that the membrane proximal region of the syntaxin homolog Sso1 is partially structured near the membrane; these studies involved

full-length Sso1, the SNARE component of Sec9, and the cytoplasmic domain of synaptobrevin (Zhang *et al.* 2005).

For synaptobrevin it was found that two membrane-proximal tryptophan residues insert into the membrane and that the protein backbone inserts into the membrane at an oblique angle; these spin-labeling EPR experiments used a *cis* SNARE complex consisting of full-length synaptobrevin, the SNARE core domain of syntaxin and SNAP-25 (Kweon *et al.* 2003a). SNARE complex formation was found to be more rapid when the two tryptophan residues were replaced with serines suggesting that the incorporation of the membrane proximal region of isolated synaptobrevin may reduce or regulate SNARE complex formation (Kweon *et al.* 2003b).

Mutations of some residues within the transmembrane segment of syntaxin altered neurotransmitter flux and pore conductance in PC12 cells transfected with syntaxin mutants. The residues that influenced neurotransmitter release lay along one face of the putative transmembrane helix of syntaxin. This prompted the authors to propose a model of a syntaxin pore consisting of 5–8 transmembrane segments (Han *et al.* 2004b).

The importance of the yeast synaptobrevin homolog Snc1 transmembrane domain was studied by an *in vitro* assay by Rothman and colleagues (Weber *et al.* 1998); it was found that this domain plays an essential role since truncation of half of the transmembrane domain inhibited inner leaflet mixing, suggesting an essential role of the Snc1 transmembrane domain in the final stages of fusion (Xu *et al.* 2005).

Reconstitution of syntaxin and synaptobrevin in giant unilamellar vesicles (GUV) revealed that SNAREs have an intrinsic preference for the liquid-disordered phase when phase-separating lipids were used in preparation of the GUVs (Bacia *et al.* 2004). Thus, it is unlikely that SNAREs are localized to so-called rafts (putative liquid-ordered regions of biological membranes). In a related study, syntaxin was found to be concentrated in 200 nm large, cholesterol-enriched regions of the plasmalemma (Lang *et al.* 2001). Cholesterol depletion causes dispersion of these clusters which is associated with a strong reduction in the rate of secretion in PC12 cells. Similarly, cholesterol-dependent clustering of syntaxin and SNAP-23 has been observed in endothelial cells (Predescu *et al.* 2005).

Homodimerization of synaptobrevin can be induced by the transmembrane domain (Laage & Langosch, 1997; Laage *et al.* 2000; Roy *et al.* 2004). However, this interaction is weak questioning a physiological importance, as assessed by SDS–PAGE and a genetic assay to examine the homodimerization of the synaptobrevin transmembrane domain in detergents and the *E. coli* inner membrane, respectively (Bowen *et al.* 2002). Indeed, recombinant synaptobrevin readily forms irreversible dimers and higher-order oligomers in detergents that are highly dependent on solublization conditions.

2.4 *In vivo* conformational studies

To study the role of SNARE conformational changes *in vivo*, Neher and co-workers introduced a conformation-dependent antibody that blocked SNARE assembly but not disassembly (Xu *et al.* 1999). In chromaffin cells, an increase in intracellular Ca^{2+} leads to an exocytotic burst followed by sustained secretion. The burst can be further resolved into two kinetically distinct components, which suggests the presence of two separate pools of vesicles. In the presence of the antibody, the sustained component was largely blocked, the burst was slightly reduced, and one of its kinetic components was eliminated. Thus, a coupling exists between SNARE assembly and the kinetics of exocytosis. The existence of loose *trans* SNARE complexes was also inferred by

the differential effect of the CNTs tetanus and BoNT/B light-chain proteases (Hua & Charlton, 1999).

Microscopy experiments with plasma membrane sheets of PC12 cells showed that the activity of membrane-resident SNAREs is not down-regulated by control proteins but is constitutively active even if not engaged in fusion events (Lang *et al.* 2002). Thus, SNAREs are constitutively active in the native membrane and do not have to be activated by other factors.

2.5 Structure-based mutagenesis studies

Known mutations in the highly conserved layers of the SNARE complex reduce complex stability and cause defects in membrane traffic even in distantly related SNAREs (Fasshauer *et al.* 1998b). Replacing one of the glutamines with arginine in the yeast exocytotic SNARE complex is either lethal or causes a conditional growth defect that is compensated for by replacing the R-SNARE arginine with glutamine (Katz & Brennwald, 2000; Ossig *et al.* 2000 Graf *et al.* 2005). These ionic layer rotation experiments revealed also that a particular SNARE can be involved in more than one cognate SNARE complex, for example Sed5 is involved in a complex with Bos1p, Sec22, and Bet1, as well as in a SNARE complex with Ykt6.

Mutations in two sites on the surface of the SNARE complex formed by acidic and hydrophilic residues of SNAP-25 (D58/E170/Q177) and synaptobrevin (S75/E78/T79), which were found to coordinate divalent cations in the neuronal SNARE complex crystal structure (Sutton *et al.* 1998) interfered with Ca^{2+} triggering of exocytosis when over-expressed in chromaffin cells (Sorensen *et al.* 2002). However, recent results argue against the direct binding of divalent cations to these sites (Chen *et al.* 2005) suggesting that these sites may rather be involved in interactions with Ca^{2+}-binding proteins such as synaptotagmin.

Botulinum neurotoxin serotype E inhibition of norepinephrine release in permeabilized PC12 cells can be rescued by adding a 65 amino acid C-terminal fragment of SNAP-25 (Chen *et al.* 1999). Mutations were carried out along the hydrophobic face of this fragment that resulted in SNARE complexes with different thermostabilities, and these mutants rescued exocytosis to different extents in PC12 cells. The ability of the mutants to rescue exocytosis and the thermostabilities of the mutant complexes were shown to be correlated. Mutations of hydrophobic residues also affected the maximal rate of exocytosis (Chen *et al.* 2001).

3. SNARE-interacting proteins

3.1 Clostridial neurotoxins

One of the most compelling studies that clearly established the importance of SNAREs for neurotransmission was the discovery that they are the targets of CNTs (Schiavo *et al.* 1992). The CNTs block neurotransmitter release by delivering their Zn^{2+}-dependent protease components to the presynaptic side of chemical synapses where they proteolyse SNAREs. The CNT family of structurally and functionally related toxins includes botulinum neurotoxin (BoNT) serotypes A-G and tetanus neurotoxin (TeNT). The target sites for all CNTs show significant variation among the different serotypes, but they were all found to cluster in the SNARE core domains (Blasi *et al.* 1993; Schiavo *et al.* 1993, 1995; Yamasaki *et al.* 1994a, b).

CNTs are the causative agents of the neuroparalytic diseases botulism and tetanus (Dolly *et al.* 1984; Humeau *et al.* 2000) and they are considered potential agents for bioterror attacks

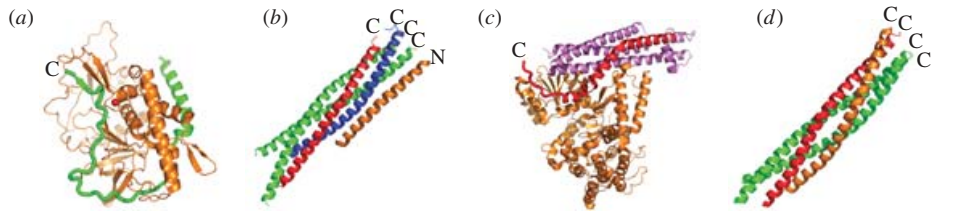

Fig. 5. Complexes between SNAREs and other proteins. (*a*) X-ray crystal structure of the complex between the sn2 domain of SNAP-25 (green) and the light-chain protease of botulinum neurotoxin A (orange) (PDB ID 1XTG) (Breidenbach & Brunger, 2004). The C-terminus of the sn2 domain is indicated. (*b*) X-ray crystal structure of the complex between complexin (orange) and the neuronal SNARE complex (color code as in Fig. 2*g*) (PDB ID 1KIL) (Chen *et al.* 2002). The C-terminii of the SNARE domains and the N-terminus of complexin are indicated. (*c*) X-ray crystal structure of the complex between nsec1 (orange) and syntaxin (purple: N-terminal domain, red: SNARE core domain) (PDB ID 1DN1) (Misura *et al.* 2000). The C-terminus of the syntaxin SNARE core domain is indicated. (*d*) X-ray crystal structure of the complex between tomosyn (orange), and the SNARE core domains of syntaxin (red), and SNAP-25 (green) (PDB ID 1URQ) (Pobbati *et al.* 2004).

(Wein & Liu, 2005). Despite being an extremely lethal poison, CNTs are also widely used for therapeutic medical uses. BoNT serotype A (BoNT/A) is used for alleviating symptoms of disorders including paralytic strabismus, blephoraspasm, cervical dystonia, stroke, and severe hyperhydrosis (Charles, 2004).

3.1.1 Structures

The crystal structures of full-length BoNT/A and BoNT/B revealed a domain organization consisting of the receptor binding domain, the translocation domain, and the light chain (LC) protease domain (Lacy *et al.* 1998; Swaminathan & Eswaramoorthy, 2000). In addition, crystal structures of several apo CNT LC proteases have been determined, including those of BoNT/A (Lacy *et al.* 1998; Segelke *et al.* 2004), BoNT/B (Hanson & Stevens, 2000; Swaminathan & Eswaramoorthy, 2000), BoNT/E (Agarwal *et al.* 2004), BoNT/F (Agarwal *et al.* 2005), and TeNT (Breidenbach & Brunger, 2005; Rao *et al.* 2005). Despite targeting different SNARE sites (with the exception of BoNT/B and TeNT), the CNT LC proteases share the same overall fold with differences primarily limited to surface features.

The striking overall similarity of the CNT LC proteases, especially their active sites, provided little insight into their ability for target discrimination. The first X-ray structure of a CNT LC-substrate complex, that of the BoNT/A · SNAP-25 complex, has revealed that BoNT/A depends on an extensive array of exosites (substrate-binding sites remote from the active site) to provide a substrate-specific boost to catalytic efficiency (Breidenbach & Brunger, 2004) (Fig. 5*a*). SNAP-25 binding to BoNT/A-LC follows a path similar to the toxin's 'belt' region which links the heavy and light chains prior to translocation of the light chain (Lacy *et al.* 1998; Swaminathan & Eswaramoorthy, 2000). It should be noted that a previously reported structure of the BoNT/B · synaptobrevin complex (Hanson & Stevens, 2000) had no electron density for the substrate (Rupp & Segelke, 2001; Breidenbach & Brunger, 2004); thus, no structural information is currently available about the BoNT/B · synaptobrevin complex.

The highly unusual extended enzyme/substrate interface employed by BoNT/A serves to properly orient its conformationally variable SNARE target such that the scissile peptide bond is

placed within close proximity of the enzyme's catalytic motif. Notably, many of the interactions that impart substrate specificity occur on the face of the protease opposite from its active site, and the carboxy-terminus of the substrate induces a conformational change in the active site pocket likely rendering the protease competent for catalysis (Breidenbach & Brunger, 2005). The multi-site binding strategy employed by BoNT/A accounts for the extreme selectivity of this enzyme, both in terms of the particular SNARE that is cleaved as well as the particular cleavage site. Based on the high structural and sequence similarity amongst the clostridial LCs, other serotypes may employ similar mechanisms for substrate recognition (Breidenbach & Brunger, 2005).

Notably, the structure of the BoNT/A · SNAP-25 complex illustrates the extent of substrate that must be available for efficient proteolysis to occur. Despite the fact that BoNT/A cleaves only nine residues from the C-terminus of SNAP-25, numerous exosite-binding residues span nearly 60 residues of the SNAP-25 sn2 core domain. Thus, BoNT/A likely cannot efficiently hydrolyze SNAP-25 if any portion of the sn2 core domain is already incorporated into a ternary SNARE complex or bound to a regulatory factor. Thus, CNT proteolysis of SNAP-25 may not impact initial stages of SNARE complex formation since only the N-terminal core domain appears to be involved in formation of a Ca^{2+}-sensitive binary complex with syntaxin prior to synaptobrevin binding (An & Almers, 2004). The extent of SNARE interactions with other CNT serotypes remains to be determined, but kinetic analyses indicate a similar behavior where other CNTs require stretches of substrates between 30–60 residues regardless of scissile bond location (Foran *et al.* 1994; Yamasaki *et al.* 1994a; Cornille *et al.* 1997).

3.1.2 Interactions

BoNT and TeNT LCs are some of the most selective proteases known (Oost *et al.* 2003). Available crystal structures of CNT proteases suggest they likely employ a catalytic strategy similar to the general Zn^{2+}-metalloprotease thermolysin because their primary catalytic residues adopt a similar geometry (Lacy *et al.* 1998; Swaminathan & Eswaramoorthy, 2000; Agarwal *et al.* 2004; Breidenbach & Brunger, 2005; Rao *et al.* 2005). Oddly, the CNT proteases do not appear to recognize a consensus site, or even have rigorous requirements for particular side-chains flanking the scissile bond (Schmidt & Bostian, 1997). Consistent with the crystal structure of the BoNT/A · SNAP-25 complex (Breidenbach & Brunger, 2004), the CNT proteases generally require long stretches of their target SNAREs for optimal efficiency (Foran *et al.* 1994; Yamasaki *et al.* 1994a; Schmidt & Bostian, 1995; Cornille *et al.* 1997; Schmidt & Bostian, 1997; Vaidyanathan *et al.* 1999). Point mutations in the exosites remote from the scissile bond can dramatically reduce CNT protease efficiency (Rossetto *et al.* 1994; Schmidt & Bostian, 1995; Pellizzari *et al.* 1996; Breidenbach & Brunger, 2004).

3.1.3 Function

The toxins are synthesized as single polypeptide chains of ~150 kDa but must be post-translationally modified by a bacterial or tissue protease for activation (Weller *et al.* 1989, 1991). The active form of the toxin consists of the 50 kDa LC protease and a 100 kDa 'heavy chain' (Helting *et al.* 1979). Though proteolytically nicked, the chains remain covalently and reversibly linked via a disulfide bond until exposed to reducing conditions such as nerve cytosol (Schiavo *et al.* 1990). Each CNT contains three functionally distinct components which participate in a

four-step mechanism of CNT intoxication involving neurospecific binding, receptor-mediated endocytosis, endosomal translocation, and SNARE proteolysis (Humeau *et al.* 2000; Turton *et al.* 2002; Lalli *et al.* 2003). The receptors for CNTs are composed of both proteins and gangliosides. Interestingly, the luminal domain of synaptotagmin (discussed in the following section) is involved in the receptor binding for BoNT/B (Dong *et al.* 2003). The toxin heavy chains largely serve as the delivery systems for their corresponding LC protease components. The Zn^{2+}-binding motif identified in CNT LC primary structures (His-Glu-x-x-His) indicates they might employ a Zn^{2+}-dependent proteolytic activity in their biochemical mechanism of action (Kurazono *et al.* 1992). Indeed, the crystal structure of the BoNT/A · SNAP-25 complex (Breidenbach & Brunger, 2004) confirmed that the directionality of the substrate matches that of an inhibitor bound to thermolysin (Holden *et al.* 1987).

3.2 Synaptotagmin

Synaptic exocytosis is triggered by Ca^{2+} upon the arrival of an action potential (Katz & Miledi, 1967). Strong evidence points to synaptotagmin as the receiver for the Ca^{2+} signal in the neuron. Synaptotagmin has been characterized in several isoforms in neurons and non-neuronal cells (Li *et al.* 1995a); in this review we refer to the synaptotagmin I isoform as simply synaptotagmin unless specified otherwise. All synaptotagmin isoforms are composed of a short intravesicular (luminal) amino-terminal region, a single membrane-spanning domain, a lysine- and arginine-rich region, and two homologous C2 domains, termed C2A and C2B. C2 domains were first characterized in conventional protein kinase C isoforms (cPKC) (Parker *et al.* 1986).

Synaptotagmins I and II are localized on synaptic vesicles (Perin *et al.* 1990). The C2A and C2B domains interact, in a Ca^{2+}-dependent manner with acidic lipids (Brose *et al.* 1992; Davletov & Sudhof, 1993; Chapman & Jahn, 1994; Fernandez *et al.* 2001; Bai *et al.* 2004a), SNAP-25 and syntaxin (both individually and in a binary complex), and the ternary SNARE complex (Bennett *et al.* 1992; Söllner *et al.* 1993; Chapman *et al.* 1995; Kee & Scheller, 1996; Schiavo *et al.* 1997; Ernst & Brunger, 2003; Rickman & Davletov, 2003). In addition, the C2B domain can promote oligomerization in a Ca^{2+}-dependent fashion (Chapman *et al.* 1996a). Genetic studies in mice, *Drosophila*, and *C. elegans* revealed that synaptotagmin I is essential for synaptic transmission (DiAntonio *et al.* 1993; Littleton *et al.* 1993; Nonet *et al.* 1993; Geppert *et al.* 1994). Mice carrying a mutant synaptotagmin I gene died immediately after birth and cultured hippocampal neurons from the mutant mice exhibited a selective defect in Ca^{2+}-triggered neurotransmitter release while Ca^{2+}-independent release was normal (Geppert *et al.* 1994).

Most synaptotagmins bind to phospholipids and syntaxins in a Ca^{2+}-dependent manner, albeit with slightly different properties (Ullrich *et al.* 1994; Li *et al.* 1995a, b). The exceptions are synaptotagmins IV, VIII, XI, and XII (Hilbush & Morgan, 1994; Ullrich *et al.* 1994; Li *et al.* 1995b; Thompson, 1996; von Poser *et al.* 1997) which contain amino-acid substitutions in the C2A domains that apparently abolish Ca^{2+} binding. The close homolog to synaptagmin I, synaptotagmin IX, does not interact with SNAREs and the C2B domain does not interact with phospholipids; nevertheless synaptotagmin IX appears to assist Ca^{2+}-triggered exocytosis when studied in permabilized PC12 cells (Shin *et al.* 2004). Furthermore, although synaptotagmins III and VI are expressed primarily in the brain, they are not enriched on synaptic vesicles (Butz *et al.* 1999). The fact that the various synaptotagmins are co-expressed in synapses but not co-localized on synaptic vesicles suggests that they may have distinct functions.

3.2.1 Structures

Structural information about synaptotagmins is sparse, and no atomic resolution structural information is available at this time about the interactions with phospholipids and other binding partners. The X-ray crystal structure of the C2A domain of synaptotagmin I revealed a β-sandwich fold with a cluster of three Ca^{2+}-binding loops at the apex of the fold (Sutton *et al.* 1995). Upon Ca^{2+} binding, few structural changes occured in the divalent cation-binding pocket of the C2A domain, apart from changes of the side-chain rotamers of the Ca^{2+}-coordinating aspartate residues and a general decrease in flexibility of the domain (Sutton *et al.* 1995; Shao *et al.* 1998).

The bound Ca^{2+} ions quench the negative electrostatic potential within the divalent cation-binding pocket of the C2 domains, and the more positive potential from peripheral basic residues dominate the interaction between synaptotagmin I and target proteins (Shao *et al.* 1997). Analysis by NMR spectroscopy and site-directed mutagenesis showed that the interaction between synaptotagmin I and syntaxin is mediated by the cooperative action of basic residues surrounding the Ca^{2+}-binding sites of the C2A domain and is driven by a change in the electrostatic potential of the C2A domain induced by Ca^{2+} binding. This electrostatic switch mechanism may also regulate the interaction of the C2A domain with phospholipid membranes (Davletov *et al.* 1993).

A solution NMR structure of the C2B domain of synaptotagmin I (Fernandez *et al.* 2001), and a crystal structure of the C2A–C2B fragment of synaptotagmin III (Sutton *et al.* 1999) are also available, although a structure of a full-length synaptotagmin has been elusive. The C2A–C2B fragment of synaptotagmin III shows different numbers of potential divalent cation-binding sites in both binding pockets (three for the C2A domain, and only one potential site for the C2B domain). In contrast, two potential Ca^{2+}-binding sites were identified in the structure of the C2B domain of synaptotagmin I, indicating structural diversity among different synaptotagmin isoforms. Similar to the C2A domain, Ca^{2+} binding to the C2B domain induces an increase in stability, but no major structural changes (Fernandez *et al.* 2001).

3.2.2 Glycosylation and palmitoylation

The luminal N-terminal domain of synaptotagmin is glycosylated and appears to play a role in localization of synaptotagmin isoforms (Han *et al.* 2004a). An intravesicular N-glycosylation site of synaptotagmin I was found that, in combination with the cytosolic C2 domains, directs synaptotagmin I to synaptic vesicles (Han *et al.* 2004a). Localization of synaptotagmins I and VII is normally restricted to synaptic vesicles and the plasma membrane respectively. A swap of the intralumenal domains produced a corresponding swap of the localization of these synaptotagmin isoforms. Synaptotagmin also has a palmitoylation site (Chapman *et al.* 1996b).

3.2.3 Interactions

The tandem C2 domains of synaptotagmin I cooperate to mediate binding to SNAREs: lengthening the linker that connects C2A and C2B selectively disrupted this interaction, and expression of the linker mutants in PC12 cells resulted in reductions of exocytosis (Bai *et al.* 2004b). The interaction between synaptotagmin I and SNAREs is weak, with an estimated K_D in

the micromolar range (Tucker *et al.* 2004). This weak association has precluded atomic resolution structural studies up to this point. Single-molecule fluorescence microscopy studies have revealed first glimpses at the interactions between a C2A–C2B fragment of synaptotagmin I and the SNARE complex (Bowen *et al.* 2005). FRET efficiency interactions were found between the C2B domain of synaptotagmin I and the membrane-proximal portion of the SNARE complex, but only in the presence of Ca^{2+}. Few high FRET efficiency interactions were observed to the C2A domain. Thus, the low number of FRET events observed between C2A and the SNARE-labeling sites suggests that the C2A domain does not closely interact with the SNARE core complex.

Biochemical studies using bead-based pulldown and native gel shift assays suggested Ca^{2+}-independent interactions between synaptotagmin and detergent-solublized or truncated *cis* SNARE complexes (Sutton *et al.* 1998; Rickman & Davletov, 2003), although Ca^{2+}-dependent interactions have also been reported (Gerona *et al.* 2000). In contrast, the single-molecule fluorescence experiments revealed a clear Ca^{2+} dependence of FRET efficiencies between several labeling pairs, confirming the Ca^{2+} dependence of the synaptotagmin · SNARE interaction (Bowen *et al.* 2005). Perhaps the confinement in beads or gels produced the apparent Ca^{2+} independence of the synaptotagmin I · SNARE interaction observed in gel shift and pulldown experiments (Cann, 1989).

The labeling-site combinations with high FRET efficiency in the presence of Ca^{2+} place structural constraints on the synaptotagmin · SNARE interactions (Bowen *et al.* 2005). The labeling sites of the SNARE complex involved in such high FRET efficiency interactions were near the ionic layer and the membrane proximal region, in qualitative agreement with biochemical studies that implied the membrane proximal region of the SNARE complex in synaptotagmin binding (Chapman *et al.* 1995; Kee & Scheller, 1996; Schiavo *et al.* 1997; Davis *et al.* 1999; Gerona *et al.* 2000). Since the fluorescent probes were in a loop distal to the Ca^{2+}-binding sites, the appearance of high FRET efficiency between the SNARE complex and C2B is more consistent with the Ca^{2+}-binding sites oriented away from the SNARE complex rather than in direct contact. This would leave the Ca^{2+}-binding sites accessible for phospholipid binding, allowing concurrent binding of both SNAREs and membrane although the presence of 30% phosphatidyl serine (PS) in the membrane appears to interfere with the SNARE interaction (Arac *et al.* 2003).

A Ca^{2+}-dependent interaction (requiring at least 0·25 mm Ca^{2+}) between the C2A domain of synaptotagmin I and the ATPase p97/VCP was reported by both GST-pulldown assays as well as co-immunopreciptations (Sugita & Sudhof, 2000). Subsequent studies have revealed that synaptotagmin can be a substrate for ER-associated degradation that involves the ATPase p97/VCP (DeLaBarre *et al.*, unpublished observations).

It should be noted that owing to an accidental mutation (Gly374Asp) in the C2B domain of the original synaptotagmin I clone, it was not discovered until later that C2B is also a Ca^{2+}-sensing module (Desai *et al.* 2000; Mackler *et al.* 2002). Thus, some earlier *in vitro* studies of recombinant synaptotagmin I may have been affected by this mutation prior to the correction of the synaptotagmin I clone.

3.2.4 Function

Exocytosis proceeds by two mechanisms. Full fusion occurs when the vesicle and plasma membranes merge. Alternatively, in what is termed ‘kiss-and-run’, vesicles can release

transmitter during transient contacts with the plasma membrane. Synaptotagmin I appears to regulate the choice between full fusion and kiss-and-run, with Ca^{2+} binding to the C2A and C2B domains playing an important role in this choice (Wang *et al.* 2003).

The possibility that synaptotagmin regulates putative fusion pores was investigated with amperometry to monitor exocytosis of single dense-core vesicles; over-expression of synaptotagmin I prolonged the time from fusion pore opening to dilation, whereas synaptotagmin IV shortened this time (Wang *et al.* 2001a). Kinetic measurements of neurotransmitter release revealed that the release of neurotransmitter involving both small synaptic vesicles and large dense-core vesicles is fast with a rise time of less than 60 μs, suggesting rapid opening of the putative fusion pore (Bruns & Jahn, 1995). Over-expression of synaptotagmin I prolonged the time from fusion pore opening to dilation, whereas synaptotagmin IV shortened this time. Thus, synaptotagmin probably plays a role in the opening of the fusion pore, perhaps by associating with the *trans* SNARE complex and/or lipids (Wang *et al.* 2001b).

Synaptotagmin I binds phosphatidylinositol 4,5-bisphosphate (PIP_2), a plasma membrane lipid with an essential role in exocytosis and endocytosis, in both a Ca^{2+}-independent and a Ca^{2+}-dependent mode (for a review of lipid regulation, see Rohrbough & Broadie, 2005). It has been suggested that the Ca^{2+}-independent mode of membrane binding predisposes synaptotagmin I to penetrate PIP_2-harboring target membranes in response to Ca^{2+} with submillisecond kinetics (Bai *et al.* 2004a). The importance of PIP_2 is also underscored by experiments that show that the level of plasmalemma PIP_2 regulates the size of releasable vesicles in chromaffin cells (Milosevic *et al.* 2005).

3.2.5 A controversy

A controversy regarding the Ca^{2+}-sensing role of synaptotagmin erupted a few years ago when experiments in *Drosophila* by Schwarz and colleagues suggested that mutation of an aspartate in the Ca^{2+}-binding site of the C2A domain of synaptotagmin I did not significantly affect the Ca^{2+}-dependent properties of neurotransmission in *Drosophila*, although the mutation disrupted Ca^{2+}-dependent syntaxin binding to the C2A domain (Robinson *et al.* 2002). In contrast, Südhof and colleagues carried out experiments in mice that demonstrated a correlation between Ca^{2+} affinity and Ca^{2+} neurotransmitter release (Fernandez-Chacon *et al.* 2001). These experiments used point mutations of the isolated C2A domain that produced a two-fold reduction of Ca^{2+} affinity with no change in structure as assessed by solution NMR. Furthermore, the Ca^{2+} dependence of the C2A-B fragment to liposome binding was reduced two-fold while little change to syntaxin binding was observed within the accuracy of the experiment. Using knock-in mice, the mutant full-length synaptotagmin produced a marked decrease in evoked excitatory post-synaptic currents (EPSCs) that represent Ca^{2+}-triggered fusion, but had no effect on the Ca^{2+}-independent sucrose response.

It is possible to explain the results by the Schwarz group by arguing that binding experiments involving isolated syntaxin are not relevant for the *in vivo* function of synaptagmin whereas the Ca^{2+}-dependent liposome binding is more critically correlated with the Ca^{2+}-sensing step of neurotransmitter release. This controversy illustrates that experiments involving isolated domains or domain interactions should be used with caution when trying to assess the function of the protein in the context of the entire fusion machinery that involves SNAREs, synaptotagmin, phospholipids, and possibly other factors.

3.3 Complexin (synaphin)

Knockout experiments in mice showed that complexin (also called synaphin) is essential for the Ca^{2+} dependency of synaptic vesicle release with a phenotype related to that of synaptotagmin knockout mice (Marz & Hanson, 2002). However, complexin has no obvious Ca^{2+}-binding site and its precise function is still unknown although it has been implied in promoting oliogomerization of SNARE complexes *in vivo* (Tokumaru *et al.* 2001); thus far, however, it has not been possible to obtain this effect with recombinant proteins *in vitro*.

Complexin is a soluble protein of molecular weight ~15 kDa that shows rapid and high-affinity binding to the SNARE complex without any major conformational changes upon binding (McMahon *et al.* 1995; Pabst *et al.* 2002). Solution NMR studies of complexin and its interactions with SNAREs revealed an α-helical region involved in SNARE binding and an unstructured portion (Pabst *et al.* 2000). Binding to individual SNAREs was not observed, and NSF-mediated disassembly of the SNARE complex was not affected by complexin. The X-ray crystal structures of the mammalian and squid SNARE · complexin complexes (Bracher *et al.* 2002; Chen *et al.* 2002) revealed that complexin binds in an anti-parallel α-helical conformation to the groove between the synaptobrevin and syntaxin α-helices of the SNARE complex (Fig. 5*b*). Based on these structures it was suggested that complexin stabilizes the assembled SNARE complex although relevance of this hypothesis is unclear, especially considering the intrinsic high stability of the SNARE complex (Fasshauer *et al.* 1997b).

Kinetic binding studies between complexin and the *cis* SNARE complex were carried out by stopped-flow fluorescence and isothermal titration calorimetry (ITC) (Pabst *et al.* 2002) and by single-molecule fluorescence microscopy (Bowen *et al.* 2005). The measured K_D values were in the nanomolar range with fast on and off rates although the observed quantities were somewhat different between the bulk- and single-molecule experiments. These differences can be explained by differences in experimental conditions: the stopped-flow and ITC studies used a soluble protease-resistant core of SNARE complex in solution to study binding while the single-molecule experiments used a SNARE complex containing full-length syntaxin and SNAP-25 reconstituted into a lipid bilayer. The single-molecule experiments revealed a transient nature of the complexin · SNARE interaction despite its relatively high affinity. FRET efficiency distributions involving a label attached to the unstructured N-terminal portion of complexin were broader than those seen for the structured SNARE-binding region of complexin (Bowen *et al.* 2005).

3.4 Sec1/Munc18 (SM) proteins

SM proteins are cytosolic proteins that include seven members in vertebrates (munc18-1, munc18-2, munc18-3, Sly1, Vps45, Vps33a, and Vps33b, and four in yeast (Sec1, Sly1, Vps45p, and Vps33p). The crystal structures of munc18 (nsec1) and Sly1 are similar (Bracher *et al.* 2000; Misura *et al.* 2000; Bracher & Weissenhorn, 2001, 2002) and they reveal that SM proteins bind to the partially folded core domain of syntaxin: the N-terminal half of the SNARE core domain is fully folded and α-helical while the C-terminal half shows a mixture of helical and random coil conformations (Fig. 5*c*). More structure is induced in the C-terminal half of syntaxin compared to the closed conformation of the syntaxin homolog Sso1 (cf. Figs 2*e* and 5*c*).

The function of SM proteins in membrane fusion is unclear. Proposed functions include a role in vesicle docking or assisting SNARE complex formation. Furthermore, the interactions with SNARE proteins exhibit striking differences. Munc18 forms a complex with the closed

conformation of syntaxin while it does not interact with the *cis* SNARE complex; the crystal structure of the nsec1 · syntaxin complex shows an extensive array of interactions at the interface (Misura *et al.* 2000). In contrast, yeast Sec1 interacts with assembled SNARE core complexes consisting of yeast Sso1, Snc1, and Sec9 (Carr *et al.* 1999). Finally, the yeast and mammalian syntaxins from the ER (Ufe1 and syntaxin 18), the Golgi (Sed5 and syntaxin 5), and the *trans*-Golgi network and early endosomes (Tlg2 and syntaxin 16) bind to their corresponding SM via short N-terminal sequences that precede the N-terminal syntaxin domain as revealed in the crystal structure of the SM protein Sly1 and a fragment of Sed5 (Bracher & Weissenhorn, 2002). Interactions between the mammalian homologs Sly1 and syntaxin 5 were further analyzed by NMR spectroscopy (Arac *et al.* 2005). Comparison with the crystal structure of the complex between the yeast homologs Sly1 and Sed5 (Bracher & Weissenhorn, 2002), shows that Sed5 binding induces a major conformational change in Sly1.

Mutations that disrupt the closed conformation of syntaxin, i.e. that maintain a permanently open conformation, abolish interaction with munc18 (Dulubova *et al.* 1999). In neuroendocrine cells, over-expression of a munc18 mutant lacking any syntaxin binding actually has a stimulatory effect on secretion (Schutz *et al.* 2005), suggesting that interactions with other factors are also important for the function of munc18. Consistent with these results, syntaxin levels are reduced by 70% in munc18 knockout mice, however, the residual syntaxin is still correctly targeted to synapses and efficiently forms SDS-resistant SNARE complexes, demonstrating that munc18 is not required for syntaxin function as such (Toonen *et al.* 2005).

3.5 Munc13

Munc13 is an ∼200 kDa protein that is essential for priming of synaptic vesicles to the release-ready state (Varoqueaux *et al.* 2002), and is also involved in presynaptic plasticity (Rhee *et al.* 2002; Rosenmund *et al.* 2002; Junge *et al.* 2004). It has been suggested that Munc13 catalyzes the transition from the closed to the open state of syntaxin. Indeed, double-knockout (Munc13 and syntaxin) *C. elegans* mutants are rescued by a constitutively open syntaxin (Richmond *et al.* 2001), and a fragment of Munc13 interacts with syntaxin as assessed by yeast-two hybrid assays and co-sedimentation (Betz *et al.* 1997). However, no biophysical studies of this putative interaction are available yet.

The solution NMR structure of the C1 domain of Munc13 was determined (Shen *et al.* 2005). The structure resembles that of PKC C1 domains with some notable differences in the ligand-binding site partly occluding the binding site. The N-terminal end of Munc13 binds to RIMs which are proteins that are involved in vesicle priming and regulate short- and long-term presynaptic plasticity; this interaction may localize Munc13 to the active zone (Betz *et al.* 2001). A ternary Rab3/RIM/Munc13 interaction may localize synaptic vesicles to the priming machinery (Dulubova *et al.* 2005).

3.6 Tomosyn and amisyn

Tomosyn is a soluble protein that contains a SNARE core domain which forms a stable complex with syntaxin and SNAP-25 (Hatsuzawa *et al.* 2003). The crystal structure of this complex (Pobbati *et al.* 2004) is very similar to that of the neuronal SNARE complex (cf. Figs 2*g* and 5*d*), i.e. tomosyn plays the role of a placeholder for synaptobrevin in this complex. Despite the structural similarity of the tomosyn · syntaxin · SNAP-25 and the synaptobrevin · syntaxin · SNAP-25

complexes, the surface residues are different for both complexes, preventing binding of complexin to the tomosyn · SNAP-25 · syntaxin complex. Tomosyn is, therefore, a soluble factor that directly competes with synaptobrevin in the formation of SNARE complex and may thus regulate exocytosis.

Amisyn is a brain-enriched protein with a tomoysn-like SNARE core domain that binds specifically to syntaxin and syntaxin 4 both *in vitro* and *in vivo*. The amisyn · syntaxin · SNAP-25 complex exhibits greater thermostability than the neuronal SNARE complex (Scales *et al.* 2002). Similar to tomosyn, amisyn can thus act as a placeholder for synaptobrevin by forming non-fusogenic complexes.

3.7 Synaptophysin

The majority of synaptobrevin is bound to the vesicle protein synaptophysin in detergent extracts (Edelmann *et al.* 1995). No syntaxin was found in this complex when synaptophysin-specific antibodies were used for immunoprecipitation. Conversely, no synaptophysin was associated with the synaptobrevin · syntaxin complex when syntaxin-specific antibodies were used for immunoprecipitation. Thus, the synaptobrevin pool bound to synaptophysin is not available for binding to syntaxin and SNAP-25, and vice versa.

Synaptophysin in also a cholesterol-binding protein since the synaptophysin · synaptobrevin interaction critically depends on a high cholesterol content in the membrane of synaptic vesicles. Variations in the availability of cholesterol may promote or impair synaptic vesicle fusion by interfering with this complex (Mitter *et al.* 2003).

3.8 VAP

VAP proteins (VAP stands for VAMP-associated protein) were characterized as potential binding partners of synaptobrevin by yeast-two hybrid and GST pull-down experiments (Skehel *et al.* 1995). However, using cytoplasmic domains for both synaptobrevin and VAP, no interactions could be detected (Kaiser *et al.* 2005). Rather, the N-terminal major sperm protein (MSP) homology domain of VAP interacts with so-called FFAT motifs that are responsible for localizing a number of proteins to the cytosolic surface of the ER and to the nuclear membrane. The intermediate coiled-coil domain of VAP promotes dimerization of VAP (Kaiser *et al.* 2005). Any association between VAP and synaptobrevin would, therefore, have to be mediated by their C-terminal transmembrane domains.

3.9 NSF and α-SNAP

N-ethylmaleimide-sensitive factor (NSF) is an evolutionarily conserved ATPase that is required for intracellular trafficking and fusion events. NSF, as the name implies, was discovered as a protein that is inactivated by *N*-ethylmaleimide (Malhotra *et al.* 1988). Based on *in vitro* assays for vesicular transport between Golgi membranes, NSF was originally thought to be a fusion protein directly involved in membrane fusion (Block *et al.* 1988; Malhotra *et al.* 1988). Later it was determined that NSF is involved in priming membranes for fusion, i.e. acting before membrane docking and fusion occurs, specifically by disassembly of *cis* SNARE complexes (Banerjee *et al.* 1996; Haas & Wickner, 1996; Mayer *et al.* 1996). Before NSF can act, soluble highly conserved co-factors, termed SNAPs (soluble NSF attachment proteins) (Whiteheart *et al.* 1993) must bind

to SNAREs. NSF disassembles SNARE complexes upon ATP hydrolysis, re-setting the SNARE proteins for another round of membrane docking and fusion. NSF only acts on the *cis* state (as opposed to the *trans* state) of the SNARE complex as studies of SNARE complex disassembly during sperm acrosomal exocytosis have shown (Blas *et al.* 2005).

Another potential function of NSF is related to glutamate receptor cycling in and out of the synaptic post-synaptic membrane through endo- and exocytosis (Luscher *et al.* 1999) since NSF also interacts with the C-terminal tail of the GluR2 receptor (Nishimune *et al.* 1998). This interaction requires SNAP proteins as well (Hanley *et al.* 2002), although no SNARE proteins have been implied for this putative function of NSF.

NSF is essential for synaptic exocytosis *in vivo*, as was found early by the comatose mutation in *D. melanogaster*. This mutation (G274E in *Drosophila* NSF) leads to neuroparalysis when the temperature is raised from 25 to 37 °C (Pallanck *et al.* 1995). At the restrictive temperature, *comatose* flies accumulate synaptic vesicles and SNARE complexes, consistent with a role for NSF in disassembly of SNARE complexes (Littleton *et al.* 1998). Studies of the mammalian NSF homolog showed that this mutation causes a conformational change in NSF that disrupts binding to SNARE complexes and ATPase activity (Muller *et al.* 1999).

Further evidence for the importance of NSF *in vivo* was provided by a particular mutant (G89D) of the homolog of NSF in *S. cervisiae*, Sec18, which causes a defect in constitutive fusion by disrupting the ability of Sec18 to bind to its SNAP homolog, Sec17 (Horsnell *et al.* 2002).

3.9.1 Structures

The domain organization of NSF consists of an N domain followed by tandem copies (termed D1 and D2) of a specific ATP-binding domain, known as the AAA (ATPases associated with cellular activities) domain. The AAA domain consists of as two subdomains: the conserved α/β subdomain contains the nucleotide-binding site and the so-called AAA homology region (Neuwald *et al.* 1999; Lupas & Martin, 2002) while the α-helical subdomain shows significant variation in terms of sequence and structure among members of the AAA family (DeLaBarre & Brunger, 2003). The functional oligomeric state of NSF is hexameric, as determined by a variety of biophysical techniques, including analytical ultracentrifugation, transmission EM, and multi-angle laser light scattering (Fleming *et al.* 1998).

The crystal structure of the hexameric NSF D2 domain (Lenzen *et al.* 1998; Yu *et al.* 1998) suggest how ATP binding may stabilize the hexamer by the interaction of three well-ordered and conserved lysines with the nucleotide. The first interaction is from the N-terminal subdomain in a conserved Walker A motif. The second is from the neighboring protomer which interacts with the γ-phosphate, and may interfere with the hydrolytic activity of D2 by destabilizing the transition state of the hydrolysis reaction or hindering nucleophilic water molecules. It may also contribute an ATP-dependent interaction between protomers. The third originates from the C-terminal subdomain and also interacts with the γ-phosphate.

The N-terminal domain of NSF is required for binding to the SNAP · SNARE complex and for the disassembly reaction (Nagiec *et al.* 1995). The structures of the N-terminal domain of NSF (May *et al.* 1999; Yu *et al.* 1999), of the yeast homolog Sec18 (Babor & Fass, 1999), and of the homologous VAT protein of the archaebacterium *Thermoplasma acidophilum* (Coles *et al.* 1999) are nearly identical, illustrating the structural conservation of this part of NSF. The N-terminal domain is composed of two sub-domains joined by a non-conserved short linker region: a double Ψ-Ψ barrel and an α-β roll. The Ψ loops of the double Ψ-Ψ barrel have been implicated

as a substrate recognition motif in other proteins (Castillo *et al.* 1999). The interface between the two sub-domains forms a groove that is a likely site of interaction with the C-terminal portion of α-SNAP (Yu *et al.* 1999).

The only available crystal structure of a SNAP protein is that of the yeast homolog of α-SNAP, Sec17. The structure consists of a twisted sheet of α-helical hairpins and a globular C-terminal domain that is primarily composed of α-helical hairpins (Rice & Brunger, 1999). Sec17 is structurally related to several other α/α proteins known to mediate protein–protein interactions as part of larger assemblies: tetratricopeptide repeats (TPRs; Das *et al.* 1998), 14-3-3 (Yaffe *et al.* 1997), HEAT repeats (Groves *et al.* 1999), and clathrin heavy-chain repeats (Ybe *et al.* 1999). The Sec17 twisted sheet has local similarity to the structure of the TPRs from protein phosphatase 5, but the overall twist of the sheet of α-helical hairpins in the two structures is different suggesting that the twist may play a role in interactions with their respective binding partners.

Full-length NSF has been studied by EM in projection and three-dimensional reconstructions (Hanson *et al.* 1997b; Fleming *et al.* 1998). A cryo-EM density map of NSF was obtained in complex with α-SNAP, the cytoplasmic portion of the neuronal SNARE complex, and a mixture of ADP and ATP by cryo-EM at 11 Å resolution (Furst *et al.* 2003). This assembly is commonly referred to as the 20S complex for its sedimentation behavior. The appearance of this particular 20S complex is roughly similar to lower resolution images of a 20S complex that contains full length SNAREs (Hohl *et al.* 1998). However, the latter 20S complex contains a pronounced rod-like feature that was presumably produced by the transmembrane domains of the SNAREs and a surrounding detergent micelle. The stoichiometry of the 20S complex purified from brain extracts has been determined to consist of three copies of α-SNAP and one copy of NSF using amino-acid analysis of isolated 20S complexes (Wimmer *et al.* 2001).

The cryo-EM density map of the 20S complex (Furst *et al.* 2003) suggests an anti-parallel, head-to-head orientation for the D1 and D2 domains of NSF. This appears to be a major difference to p97/VCP, another tandem AAA protein, which has a parallel orientation of both domains (Brunger & DeLaBarre, 2003). Consistent with this major structural difference between NSF and p97/VCP, a sequence comparison revealed the presence of a long region of random coil near the predicted NSF D1–D2 interface that has very little homology in terms of either primary sequence or tertiary structure with the corresponding region in p97/VCP (Brunger & DeLaBarre, 2003).

Deep etch images of NSF (Hanson *et al.* 1997b) suggested that NSF is capable of large domain changes, in particular upon ADP nucleotide release resulting in a splayed structure. The motion has been primarily attributed to the N domains and it has been suggested that these domains might pull the SNARE complex apart (Hanson *et al.* 1997b). Furthermore, nucleotide depletion by apyrase incubation leads to a dissociation of NSF into monomers, as suggested by electron micrographs (Hanson *et al.* 1997b). Conversely, the presence of ATP shields NSF from limited proteolysis *in vitro* (Hanson *et al.* 1997b). Structural changes have also been observed in VCP/p97 during the hydrolysis cycle as observed by crystallography, cryo-EM, and small-angle X-ray scattering of representative nucleotide states of VCP/p97 (Rouiller *et al.* 2002; Davies *et al.* 2005; DeLaBarre & Brunger, 2005).

3.9.2 Interactions

The interaction of NSF with SNAREs requires the presence of the SNAP proteins. Since available structural data of the 20S complex are only at low resolution (Hohl *et al.* 1998; Furst

et al. 2003), little is currently known about specific interactions between NSF, SNAPs, and SNAREs.

The curvature of the grooves of the four-helix bundle of the SNARE complex is similar to the curvature of the twisted sheet of the SNAP homolog Sec17. EM and mutagenesis studies of SNAP · SNARE complexes suggested that SNAP coats the SNARE complex along most of its length (Hohl *et al.* 1998). Residues that are conserved in a variety of homologous SNAP sequences map predominantly to one face and to one ridge of the structure of Sec17. Four general configurations for a SNAP · SNARE complex were modeled, two longitudinal modes wherein the long axis of SNAP is roughly coincident with the long axis of the SNARE complex, and two transverse modes wherein the long axis of the SNAP is roughly orthogonal to the long axis of the SNARE complex (Rice & Brunger, 1999). SNAP may function as rigid levers, transmitting force generated by conformational changes in NSF to drive disassembly of SNARE complexes (Rice & Brunger, 1999).

Studies by site-directed mutagenesis revealed that mutations in charged residues distributed over a concave surface formed by the N-terminal nine α-helices of α-SNAP affect its ability to bind synaptic SNARE complex and promote its disassembly by NSF. Replacing basic residues on this surface with alanines reduced SNARE complex binding and disassembly, whereas replacing acidic residues with alanines enhanced α-SNAP efficacy (Marz *et al.* 2003). The SNAP-interacting region of the SNARE complex overlaps with their core complex forming regions (Hanson *et al.* 1995; Hayashi *et al.* 1995; Kee *et al.* 1995). However, in a model based on this mutagensis study, the C-terminal globular domain of α-SNAP had to be removed in order to avoid clashes with the SNARE complex (Marz *et al.* 2003).

A handful of positively charged residues in the NSF N domain have been identified as important for SNAP and/or SNARE binding (Matveeva *et al.* 2002). Of these residues, Arg67 seems to be of particular importance, as it is the only residue that is strictly conserved in all known N domain sequences (Zhang *et al.* 2000). This arginine residue is located on a helix that points away from the putative substrate-binding groove mentioned above. Since the entire hexameric NSF protein is required to efficiently interact with adaptor and substrate proteins (Nagiec *et al.* 1995), it must be assumed that the orientation of N with respect to the rest of the protein is important for adaptor and substrate binding. Thus, the interactions disrupted by these mutations (Matveeva *et al.* 2002) do not necessarily reflect direct interactions between the ATPase, adaptors, and substrate, but could be considered more of an overall structural perturbation to the inter-domain communication during nucleotide hydrolysis.

3.9.3 Phosphorylation

Phosphorylation of NSF has been reported (Matveeva *et al.* 2001). The co-factor α-SNAP can also be phosphorylated, resulting in reduced affinity to the SNARE complex (Hirling & Scheller, 1996).

3.9.4 Function

Disassembly of the SNARE complex by the action of the ATPase NSF and the co-factor α-SNAP has been studied in bulk by a variety of biochemical methods, including gel electrophoresis involving a radiolabeled synaptobrevin (Hayashi *et al.* 1995), sensitivity to neurotoxin cleavage (Otto *et al.* 1997), SDS resistance of the SNARE complex combined with SDS–PAGE with and without boiling (Scales *et al.* 2001), and fluorescently labeled SNAREs (Marz *et al.* 2003).

In NSF, the first (D1) domain is known to provide most, if not all, of the ATPase activity while the second (D2) is primarily responsible for hexamerization (Whiteheart *et al.* 1994a; Nagiec *et al.* 1995). The D1 domains have relatively low ATP-binding affinity whereas the D2 domains have a high ATP-binding affinity (Matveeva *et al.* 1997). Indeed, ATP was found in the crystal structure of the D2 domain (Yu *et al.* 1998). It cannot be ruled out, however, that some ATP hydrolysis by the D2 domain occurs during complex disruption in the context of the 20S complex.

NSF does not directly bind to α-SNAP but only in the presence of syntaxin, either individually or in a complex with other SNAREs. The ATPase activity of NSF is stimulated by the complex of α-SNAP · syntaxin or by α-SNAP · syntaxin · SNAP-25, but not by the α-SNAP · syntaxin · SNAP-25 · synaptobrevin complex (Matveeva & Whiteheart, 1998).

The K266A and E329Q mutants in the D1 domain of NSF disrupt nucleotide binding and hydrolysis, respectively, presumably by affecting critical interactions with the nucleotide in the D1 domain (Whiteheart *et al.* 1994b). These mutations also affect disassembly since they inhibit α-SNAP release (Colombo *et al.* 1998). Consistent with the hexamerization role of the D2 domain, the K549A, D604Q, and K631D mutations in the D2 domain have little effect on ATPase activity.

Both the D1 and D2 domains include a distinctive motif termed the second region of homology (SRH) common to all AAA proteins. In hexameric NSF, several SRH residues become *trans* elements of the ATP-binding pocket (Lenzen *et al.* 1998; Yu *et al.* 1998). Mutation of two conserved arginine residues in the NSF-D1 SRH (R385A and R388A) do not effect basal or SNAP-stimulated ATPase activity; however, neither mutant undergoes ATP-dependent release from SNAP · SNARE complexes. Another mutation, D359K, also does not affect nucleotide hydrolysis activity but limits NSF release from SNAP · SNARE complexes. The S491L mutation in the D1–D2 linker region affects stimulation of ATPase activity in NSF, while in Sec18, the corresponding mutation eliminates Sec17 binding (Horsnell *et al.* 2002).

The ionic-layer glutamine of syntaxin is required for efficient α-SNAP and NSF-mediated dissociation of the complex; when this residue is mutated, the SNARE complex still binds to α-SNAP and NSF and is released through ATP hydrolysis by NSF, but the complex no longer dissociates into SNARE monomers (Scales *et al.* 2001).

As mentioned above NSF has been observed to undergo phosphorylation at Ser237 (Matveeva *et al.* 2001). Mutation of Ser237 to Glu, to mimic phosphorylation, results in a hexameric form of NSF that does not bind to SNAP · SNARE complexes, whereas the S237A mutant does form complex. The sensitivity of NSF to *N*-ethylmaleimide is related to numerous cysteine residues, some of which are presumably on the surface of the molecule (Malhotra *et al.* 1988). NSF is also sensitive to hydrogen peroxide which inhibits NSF hydrolysis and SNARE complex disassembly by oxidation of selected cysteine residues (Matsushita *et al.* 2005). Specifically, it was found that Cys264 is crucial for the sensitivity of NSF to hydrogen peroxide; mutation of this residue to threonine eliminates the sensitivity, while not affecting the SNARE complex disassembly reaction.

4. Reconstitution of SNARE-mediated vesicle fusion

Rothman and co-workers developed the first *in vitro* assay to study SNARE-mediated vesicle docking and fusion (Weber *et al.* 1998). Recombinant syntaxin, SNAP-25 and synaptobrevin were reconstituted into separate liposomes (Table 1). In most experiments, only lipid mixing was

Table 1. *Fusion experiments with reconstituted SNAREs*

	Co-expr.	Lipid composition	SNARE complex formation sensor	Lipid mixing sensor	Content mixing sensor	Typical protein* concentration	Temp.	Set-up
Nickel *et al.* (1999), Weber *et al.* (1998)	Yes with or without Habc	82%/15% POPC/DOPS	None	Dequenching Rhodamine DPPE	DNA duplex formation with ^{33}P label	750 Sb per 45 nm vesicle 75 Sx · SNAP-25 per 45 nm vesicle	37 °C	Bulk SUV-SUV
Tucker *et al.* (2004)	Yes without Habc	82%/15% POPC/DOPS	None	FRET NBD-PE Rhodamine-DPPE	None	90 Sb per 50 nm vesicle 80 Sx · SNAP-25 per 50 nm vesicle	37 °C	Bulk SUV-SUV
Schuette *et al.* (2004)	No without Habc	50%/20%/10%/10%/ 10% POPC/POPE/ POPS/PIP_2/CH	FRET sb-Cter-Alexa594 sy-Cter- Alexa488	Dequenching Rhodamine DPPE	None	80 Sb per 30 nm vesicle 80 Sx per 30 mm vesicle	37 °C	SUV-SUV
Bowen *et al.* (2004)	No with Habc	90%/10% egg PC/brain PS or 100% egg PC	FRET sb[Ser28Cys]-Cy5 sy[Ser193Cys]- Cy3	None	Dequenching Calcein	30 Sb per 50 nm vesicle 100 Sx/μm^2 250 nm SNAP-25	20 °C or 37 °C	SMTIRF SUV-bilayer
Fix *et al.* (2004)	Yes without Habc	82%/15% POPC/DOPS	None	Dequenching Rhodamine DPPE	None	750 Sb per 45 nm vesicle 5000 Sx · SNAP-25/ μm^2	37 °C	Bulk SUV-bilayer
Liu *et al.* (2005)	Yes with Habc	85%/15% DOPC/POPS	None	Dequenching TMR-DHPE	None	100 Sb per 50 nm vesicle 100 Sx · SNAP-25/ μm^2	37 °C	Bulk SUV-bilayer
Dennison *et al.* (in press)	No with Habc	32%/25%/15%/20%/8% DOPC/DOPE/SM/CH/ DOPS or 35%/30%/15%/ 20% DOPC/DOPE/SM/CH	FRET Sb[Ser28Cys]-R Sx[Ser193Cys]-F SNAP- 25 [Gln20Cys]- R	FRET BODIPY500-PC and BODIPY530- PE	Dequenching Tb^{3+}/DPA	3·3 Sb/20 nm vesicle 1·4 Sx/20 nm vesicle	23 °C or 37 °C	PEG-induced SUV-SUV

F, Fluorecein maleimide; R, tetramethylrhodamine maleimide; CH, cholesterol; DOPC, 1,2-dioleoyl-3-sn-phosphatidylcholine; DOPS, 1,2-dioleoyl-3-sn-phosphatidylserine; DOPE, 1,2-dioleoyl-3-sn-phosphatidylethanolamine; SM, bovine brain sphingomyelin; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; POPS, 1,2 1-palmitoyl, 2-oleoyl phosphatidylserine; TMR-DHPE, N-(tetramethylrhodamine)-1,2-dipeptadecanol phosphatidylethanolamine; PIP_2, phosphatidylinositol 4,5-bisphosphate; Egg PC, egg phosphatidylcholine; Brain PS, brain phosphatidylserine; Habc, N-terminal domain of syntaxin; PEG, polyethylene glycol; SMTIRF, single molecule total internal reflection fluorescence microscopy, i.e. single dyes were observed; Bulk, fluorescence measurements of large numbers of dyes; Co-expr., co-expression of syntaxin and SNAP-25; Sb, synaptobrevin; Sx, syntaxin.

* Concentrations as published or estimates based on published protein to lipid ratios.

probed except in one case where duplex formation of oliognucleotides was used to assay content mixing (Nickel *et al.* 1999). An increase in the number of rounds of fusion within a time interval resulted from removal of the N-terminal domain of syntaxin (Parlati *et al.* 1999). Fusion was sensitive to particular SNARE pairings (McNew *et al.* 2000a; Parlati *et al.* 2000, 2002) despite promiscuity observed *in vitro* (Fasshauer *et al.* 1999; Yang *et al.* 1999) (see also Section 2.2.6).

The effect of membrane anchors was investigated by replacement of the SNARE transmembrane domains with covalently attached lipids (McNew *et al.* 2000b; Melia *et al.* 2002). Replacing either syntaxin or synaptobrevin transmembrane domains with a phospholipid prevented fusion, but still allowed docking of vesicles. The membrane proximal region of synaptobrevin could be modified by helix-breaking proline residues with little effect on the assay, and insertion of a flexible linker has a moderate effect with increasing influence for longer linkers (McNew *et al.* 1999).

While these experiments for the first time demonstrated that SNAREs are capable of docking and fusion of liposomes in an *in vitro* system under certain artificial conditions, they suffered from three potential deficiencies. First, the protein density in the liposomes was generally too high (750 synaptobrevins per 45 nm vesicles) compared to the physiological density of roughly 70 synaptobrevins per 50 nm synaptic vesicles (R. Jahn, personal communication). Second, the fusion events were infrequent or slow, resulting in rounds of fusion that occurred over a minute time scale, orders of magnitude slower than individual fusion events that occur in synaptic neurotransmission. For comparison, in goldfish bipolar neurons, the population of readily releasable vesicles is $\sim$2000 per neuron and the fusion rate is $\sim$120 ms^{-1}, so the presynaptic response can be submillisecond (von Gersdorff & Matthews, 1994). In calyx of Held nerve terminals the entire population of $\sim$4000 readily releasable vesicles exocytoses with a fusion rate of $\sim$0·6 ms^{-1} at 40 μM Ca^{2+} (Wolfel & Schneggenburger, 2003). Third, kinetic measurements of individual fusion events were not possible.

Attempts have been made to address these concerns with the assay by Weber *et al.* (1998). Docking of liposomes was observed using physiological protein:lipid ratios in the work by Schuette *et al.* (2004) (Table 1). SNARE complex formation was assayed with C-terminal FRET labels and lipid mixing was observed with liposome dye dequenching (Table 1). Both signals were highly correlated and application of BoNT/E or TeNT disrupted both processes. Although no content mixing indicator was used, fusion was inferred indirectly by an increase of liposome size as observed by EM. However, the ensemble time scale of the observed fusion events was still on the minute time scale and no individual fusion events could be discerned.

The absence of a Ca^{2+} sensor in these *in vitro* experiments may account for the slow time scale of fusion. There is overwhelming evidence that synaptotagmin is the required Ca^{2+} sensor (see Section 3.2). However, in one study it has been suggested that the fusion probability between liposomes and deposited bilayers can be greatly enhanced by the addition of divalent cations (Ca^{2+} and Mg^{2+}) but in the absence of synaptotagmin (Fix *et al.* 2004) (Table 1). It should be noted that these experiments were carried out at very high protein concentrations (Table 1). Furthermore, this result is in contrast to two independent studies that do not show any Ca^{2+} dependence of SNARE-mediated fusion in the absence of synaptotagmin (Bowen *et al.* 2004; Liu *et al.* 2005). Furthermore, Rizo and co-workers carried out NMR experiments to study the affinity of putative Ca^{2+}-binding sites on the SNARE complex (Fasshauer *et al.* 1998b; Chen *et al.* 2005), and found that most of these sites are not specific to Ca^{2+} and have low affinity.

In a different study, enhanced lipid mixing upon addition of the cytoplasmic domain of synaptotagmin and Ca^{2+} was reported at physiological SNARE concentrations albeit with high

synaptotagmin concentration (~10 μM) (Tucker *et al.* 2004) (Table 1). An extension of this study showed distinct concentration ranges for three synaptotagmin isoforms (I, VII, and IX) for divalent cations (Ca^{2+}, Ba^{2+}, and Sr^{2+}) (Bhalla *et al.* 2005). Mutations that selectively reduced binding to SNAREs by increasing the linker between the C2A and C2B domains reduced stimulation of lipid mixing. However, two other reports produced negative results: no Ca^{2+} dependence was observed using full-length synaptotagmin (minus the luminal domain) (Mahal *et al.* 2002), and no effect by the synaptotagmin cytoplasmic domain was observed in the presence or absence of Ca^{2+} (Bowen *et al.* 2004). Clearly, these discrepancies call for further study.

Individual docking and fusion events were studied by using single-molecule microscopy (Bowen *et al.* 2004) (Table 1). At a physiological concentration of synaptobrevin in liposomes and syntaxin · SNAP-25 in deposited bilayers, efficient SNARE-dependent docking was observed. Furthermore, FRET efficiency experiments using suitably placed labels demonstrated for the first time directly the formation of *trans* SNARE complexes upon docking. Fusion events were also observed, although they occurred relatively infrequently, and after laser-induced heating. The time scale of these individual fusion reactions was faster than the time resolution of the camera employed in these experiments, indicating that fusion is intrinsically faster than 100 ms (Fig. 6). A surprising result was found when SNAP-25 was left out: docking and fusion still occurred. As discussed in Section 2.2.2, there is a weak interaction between syntaxin and synaptobrevin that appears to be sufficient for docking and fusion, at least *in vitro*. Clearly, for a fully functional Ca^{2+}-sensitive system, one would expect that SNAP-25 is required since it interacts with synatotagmin (see Section 3.2) and manipulations of SNAP-25 (e.g. by the action of CNTs) affect neurotransmission (Sakaba *et al.* 2005).

A similar liposome/bilayer topology was used in an independent study by (Liu *et al.* 2005), although the bilayer preparation and lipid compositions were quite different from that of Bowen *et al.* (2004) and only lipid mixing was monitored. SNARE-dependent docking of liposomes was observed at the start of the experiment. Lipid mixing was monitored by dequenching of liposomes that initially included dye-labeled lipids under quenched conditions (Table 1). Fusion events were thus inferred from dequenching and subsequent decay of the lipid dyes due to diffusion within the deposited bilayer. After initiation of the experiment, 65% of the docked vesicles exhibited lipid mixing within less than 25 ms after docking. When the concentration of syntaxin · SNAP-25 was increased ~100-fold (similar to that of Fix *et al.* 2004), only few events were observed. Atomic force microscopy revealed that a high syntaxin · SNAP concentration produces large aggregates which may hinder lipid mixing and fusion.

The experiments by Bowen *et al.* (2004) and Liu *et al.* (2005) agree on three aspects: first, individual 'events' (content mixing observed by Bowen *et al.* 2004, or lipid mixing observed by Liu *et al.* 2005) are fast (faster than 100 and 25 ms respectively). Second, Ca^{2+} has no effect on this SNARE-only system. Third, SNAP-25 is not required for SNARE-mediated docking and fusion. There are also major differences between the two experiments. Only thermally induced fusion events were observed by Bowen *et al.* (2004), in contrast to the burst of lipid-mixing events at the start of the experiment by Liu *et al.* (2005). It should be noted, however, that the experimental set up by Bowen *et al.* (2004) prevented measurement of events at the early stage since the system was equilibrated to establish single-molecule conditions and to avoid non-specific liposome binding. It is thus possible, that initial 'events' might have also occurred in the experiments by Bowen *et al.* (2004), albeit unobservable. In summary, the studies by Liu *et al.* (2005) and Bowen *et al.* (2004) both produce individual events on the millisecond timescale, while they differ in terms of what is being observed: Liu *et al.* (2005) observed lipid mixing events

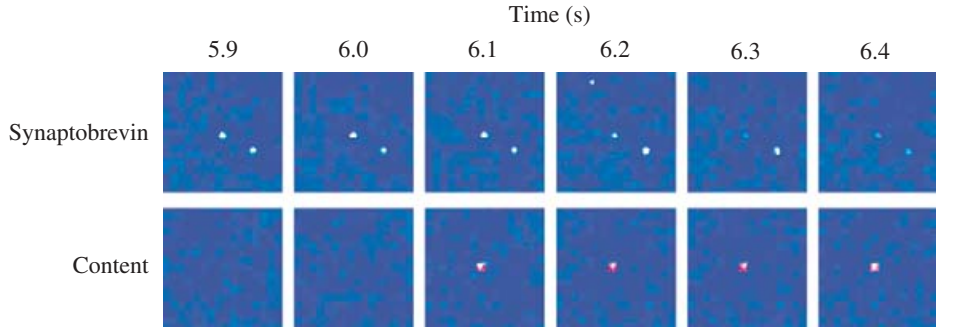

Fig. 6. Individual fusion event observed by single-molecule microscopy. Liposomes containing the content dye calcein were reconstituted with dye-labeled synaptobrevin molecules and then introduced above PC/PS bilayers with reconstituted syntaxin in complex with SNAP-25 (for details see Bowen *et al.* 2004). The images represent a single 11 μm × 11 μm patch of membrane with docked liposomes observed in two different spectral ranges to detect the content dye and synaptobrevin dye fluorescence. Two liposomes are docked to the bilayer in the view field as indicated by the synaptobrevin dyes. A single fusion event occurs at 6 s as indicated by the sudden appearance of a bright content dye signal. The increase of content dye fluorescence is due to dequenching. Fusion proceeds faster than the time resolution of the camera used in this experiment; in other words the fusion reaction is faster than 100 ms.

that occurred spontaneously, while thermally observed fusion events were observed by Bowen *et al.* (2004).

The notion that *trans* SNARE complexes alone are insufficient for fusion but require a trigger such as the Ca^{2+} sensor synaptotagmin or thermal heating (*in vitro*) to promote fusion (Bowen *et al.* 2004) is supported by experiments of SNARE-containing vesicles that are brought into contact by a low concentration of poly(ethylene glycol) (PEG) (Dennison *et al.* in press) (Table 1). In this experimental set-up the neuronal SNARE complex alone did not trigger fusion as determined by a content-mixing indicator. SNAREs did promote PEG-triggered fusion by favoring formation of a putative 'stalk' intermediate. These studies also revealed that high protein-to-lipid ratios for syntaxin, and to a lesser degree for synaptobrevin, cause liposomes to lose intergrity, calling into question studies that were carried out at high protein concentrations.

A putative hemifusion intermediate was observed at physiological lipid:protein ratios using yeast plasma membrane SNAREs (Xu *et al.* 2005) although no content mixing indicator was used. Similarly, for vacuole fusion, a hemifusion intermediate was observed that is arrested by addition of fusion inhibitors (Reese *et al.* 2005). The authors suggested that formation of *trans* SNARE complexes precedes hemifusion although *trans* SNARE complex formation was only measured indirectly rather than by covalently attached fluorescent probes. Further progression to pore opening required additional factors that showed GTP sensitivity, Ca^{2+}, calmodulin and V_o (subunit of the vacuolor V-type ATPase; Bayer *et al.* 2003) dependence. Interestingly, the V_o subunit a1 appears to be involved in a late step in synaptic vesicle exocytosis in *Drosophila* (Hiesinger *et al.* 2005).

Additional insights into SNARE – membrane interactions have been reported by using the Langumir–Blodgett trough to obtain single planar phospholipids bilayers supported on PEG cushions (Zhao & Tamm, 2003). It was found that reconstituted synaptobrevin can readily exchange between the supported bilayer and vesicles in solution (i.e. unbound) (Zhao & Tamm, 2003). However, little transfer was observed in the single-molecule microscopy experiments by Bowen *et al.* (2004), although this difference could be due to differences in the experimental

conditions. Second, it was found that reconstituted syntaxin · SNAP-25 binary complexes partition into a mobile and fixed fraction in deposited bilayers; the mobile fraction was significantly reduced in the presence of negatively charged lipids, such as PS or PIP_2 (Wagner & Tamm, 2001). Similarly, Bowen *et al.* (2004) observed a significant fraction of immobile reconstituted syntaxin molecules in deposited bilayers that were obtained by liposome condensation on the quartz surface; some of the reduced mobility may be related to interactions between syntaxin transmembrane domains involving upside-down syntaxin molecules whose cytoplasmic domain interacts with the surface (Bowen *et al.* 2004).

Inverted or 'flipped' SNAREs were used to fuse cells rather than liposomes (Hu *et al.* 2003). The flipped SNARE promoted complete fusion or hemifusion. Two-thirds of the fusion events were permanent and approximately one third were reversible (Giraudo *et al.* 2005). The SNARE cytoplasmic domain with a lipid anchor was shown to be sufficient for hemifusion, but not for fusion.

5. Number of SNAREs involved in docking and fusion

How many SNARE complexes are sufficient and required for fusion? Is there a fusion pore? These questions are still open, although there are some estimates that generally suggest a low number of SNARE complexes involved in exocytosis. One such study involved a permeabilized PC12 cell system (Hua & Scheller, 2001). Upon injection of the cytosolic domain of synaptobrevin exocytosis is inhibited. The increase in the inhibition of fusion upon an increase of synaptobrevin concentration was best fit to a function involving three SNARE complexes. Atomic force microscopy of reconstituted SNAREs in deposited bilayers shows the presence of ring-like structures (Cho *et al.* 2002, 2005). A model based on interacting syntaxin transmembrane segments suggested 5–8 complexes involved in the formation of a putative fusion pore (Han *et al.* 2004b). Using single-molecule fluorescence microscopy, the number of SNARE complexes required for docking of liposomes to deposited bilayers was estimated to be even lower, as little as one complex per docked liposome (Bowen *et al.* 2004). Further experiments are clearly required to address these important questions.

6. Concluding remarks

The results discussed in this review provide overwhelming evidence that SNAREs are at the very center of the synaptic vesicle fusion machinery. SNAREs are not only involved in aspects of docking and fusion of synaptic vesicles to the active zone, they also play an important role in the Ca^{2+}-triggering step itself (Sakaba *et al.* 2005), most likely in combination with the Ca^{2+} sensor synaptotagmin. Different domains of the SNAREs are involved in different processes: N-terminal domains are involved in regulation of exocytosis, the SNARE core domains are involved in formation of the SNARE complex that sets the stage for fusion, and the transmembrane domains are likely involved in modulating fusion intermediate states. Futhermore, SNAREs have a multitude of binding partners.

The SNARE core domains exhibit a plethora of configurational (i.e. parallel *vs.* anti-parallel), conformational, and oliogomeric states. These different states allow SNAREs to interact with their matching binding partners, auxiliary proteins, or with other SNARE domains, often in a mutually exclusive fashion. SNAREs undergo progressive disorder-to-order transitions upon

interactions with binding partners [cf. Figs 2*e*, 5*c*, and 2*g* for the syntaxin core domain (in red), and Figs 5*a* and 2*g* for the SNAP-25 sn2 domain], culminating with the fully folded *cis* SNARE complex (Figs 2*g* and 3*b*). It is likely that these protein folding events are coupled to aspects of vesicle docking and fusion.

Physiological concentrations of neuronal SNAREs are sufficient to juxtapose membranes and promote lipid mixing *in vitro*. Different studies have come to contradictory conclusions if SNAREs alone are also sufficient to promote content mixing in the absence of other factors. It is, however, encouraging that the kinetics of the observed individual fusion reactions is fast, on the millisecond timescale. Clearly, additional factors will have to be included in these *in vitro* studies, since the neuronal fusion machinery is designed not to fuse until a Ca^{2+} signal arrives *in vivo*. Ultimately, it is hoped that an *in vitro* system could be established that mimics some of the pertinent properties of Ca^{2+}-triggered synaptic vesicle fusion. Such a system would contribute to the understanding of the molecular machinery of vesicle fusion since biophysical experiments could be performed that may not be achievable *in vivo*. Furthermore, such an *in vitro* model system could become a test bed for novel pharmaceutical drug development.

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