

Chapter 4

Fusion Machinery: SNARE Protein Complex

Ira Milosevic and Jakob B. Sørensen

Abstract SNARE proteins constitute the minimal machinery needed for membrane fusion. SNAREs operate by forming a complex, which pulls the lipid bilayers into close contact and provides the mechanical force needed for lipid bilayer fusion. At the chemical synapse, SNARE-complex formation between the vesicular SNARE VAMP2/synaptobrevin-2 and the target (plasma membrane) SNAREs SNAP25 and syntaxin-1 results in fusion and release of neurotransmitter, synchronized to the electrical activity of the cell by calcium influx and binding to synaptotagmin. Formation of the SNARE complex is tightly regulated and appears to start with syntaxin-1 bound to an SM (Sec1/Munc18-like) protein. Proteins of the Munc13-family are responsible for opening up syntaxin and allowing sequential binding of SNAP-25 and VAMP2/synaptobrevin-2. N- to C-terminal “zippering” of the SNARE domains leads to membrane fusion. An intermediate, half-zipped, state represents the “primed” vesicle, which is ready for release when C-terminal SNARE assembly is triggered by synaptotagmin. Following fusion, the SNAREs are recycled by the action of the AAA-ATPase NSF (N-ethylmaleimide-sensitive factor). In recent years, the lipid requirements for the SNARE mechanism have been scrutinized, and roles for the “noncanonical” SNAREs in the synapse are emerging, yet much remains to be learned about the spatial and temporal regulation of fusion.

Keywords Exocytosis • Membrane fusion • SNARE protein • Synapse • Neuron • Synaptic vesicle

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4.1 SNARE Complex and Membrane Fusion: Introduction

Many fundamental cellular functions rely on the transport processes through membrane fusion because cells are subdivided into subcellular compartments by lipid bilayer membranes. Membrane fusion is a cooperative and synchronized process characterized by three central steps, although each of these steps represents a complex sequence of events on its own (Risselada and Grubmuller 2012). In the first step, two bilayers come into very close contact, sometimes as close as 1 nm. During the second step, an initial lipid structure develops that connects two bilayers. In the third step, this structure transforms into a funnel-like structure, the fusion pore, which connects the two membranes and allows flux of interior content (e.g., neurotransmitters). The exact mechanisms underlying this complex sequence of events are not fully understood, in spite of the fact that some aforementioned intermediates have been directly observed (Risselada and Grubmuller 2012).

One of the most important, as well as best-studied, examples of membrane fusion is neurotransmitter release at the neuronal synapse, a process which requires fusion of synaptic vesicles with the presynaptic plasma membrane. The current prevailing view is that regulated neurotransmitter release at the neuronal synapse relies on the same basic proteinaceous machinery as other membrane trafficking events (Jahn and Fasshauer 2012; Sudhof 2013). Soluble NSF attachment protein (SNAP) receptors (SNAREs) are presently considered the core constituents of the protein machinery responsible for membrane fusion. Most members of the SNARE protein family were identified by their possession of a conserved homologous stretch of 60–70 amino acids, referred to as the SNARE motif (Klopper et al. 2007; Terrian and White 1997; Weimbs et al. 1998). Four SNARE motifs assemble spontaneously into a thermostable, sodium dodecyl sulfate- and protease-resistant coiled-coil bundle, called the SNARE core complex (Antonin et al. 2000a; Fasshauer et al. 1998a; Sollner et al. 1993b; Sutton et al. 1998). Heptad repeats in components of the core complex form 16 conserved layers of interacting amino acid side chains, which are arranged perpendicularly to the axis of the complex. All layers except one contain hydrophobic amino acids. The unique central layer, termed the “0-layer,” is hydrophilic and consists of three glutamines (Q_s) and one arginine (R) stabilized by ionic interactions (Sutton et al. 1998). Based on this characteristic, SNARE proteins are classified into four subfamilies: Q_a -, Q_b -, Q_c -SNAREs (Q -SNAREs contribute a glutamine residue to the 0-layer), and R-SNAREs (R-SNAREs contribute an arginine residue to the 0-layer) (Fig. 4.1a, b) (Bock et al. 2001; Fasshauer et al. 1998b). All analyzed SNARE complexes to date have a $Q_aQ_bQ_cR$ composition and consist of four SNARE proteins, with the exception of the neuronal synaptic SNARE complex, which is comprised of three SNARE proteins: synaptosome-associated protein of 25 kDa (SNAP-25; (Oyler et al. 1989)), syntaxin-1 (Bennett et al. 1992), and synaptobrevin-2/vesicle-associated membrane protein (VAMP) 2 (Baumert et al. 1989; Trimble et al. 1988).

The fusogenicity of the SNAREs relies on their differential localization: synaptobrevin/VAMP is a vesicular protein, whereas syntaxin-1 and SNAP-25

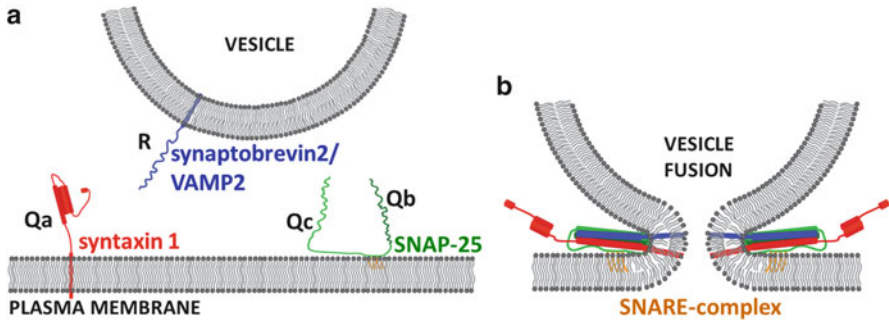


Fig. 4.1 Schematic of the three neuronal SNARE proteins that mediate synaptic vesicle fusion by forming a SNARE complex: syntaxin-1 (red), SNAP-25 (green) and synaptobrevin-2/VAMP2 (blue). The color coding for these proteins is used in all other figures. (a) Syntaxin-1 (Qa) and SNAP-25 (Qbc) are present in the plasma membrane, while synaptobrevin-2/VAMP2 (R) is in the synaptic vesicle. (b) Formation of SNARE complex composed of syntaxin-1, SNAP-25, and synaptobrevin-2/VAMP2 drives fusion of the vesicle with the plasma membrane

are attached to the plasma membrane. Assembly of a SNARE complex between the membranes (in *trans*) leads to fusion, driven by the energy liberated during SNARE-complex formation. During fusion, the membranes merge and the SNARE proteins find themselves in the same membrane, forming what is referred to as a *cis*-SNARE complex. SNARE-complex disassembly by the ATPase NSF is needed to recycle SNAREs for another round of fusion (see below). The central role of the SNARE complex in exocytosis at the neuronal synapse is well documented. The first demonstration came from studies with neurotoxins that selectively cleave SNAREs and potently inhibit exocytosis (Montecucco and Schiavo 1995; Niemann et al. 1994). Tetanus toxin cleaves synaptobrevin/VAMP, while botulinum neurotoxins (BoNT) A, B, and C cleave SNAP-25, synaptobrevin/VAMP, and syntaxin, respectively. Reconstitution studies of purified proteins in liposomes indicated that SNAP-25, syntaxin-1, and synaptobrevin-2/VAMP2 are sufficient to fuse membranes and can be viewed as representing a “minimal fusion machinery” (Weber et al. 1998). Another study expressed the same SNARE proteins on the cellular surface and detected spontaneous cell-to-cell fusion, showing that SNAREs are sufficient to fuse biological membranes (Hu et al. 2003).

SNAP-25 (Table 4.1) is a Q_{bc} -SNARE and contributes two of the four α -helices to the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). This arrangement is unique to the SNAP-25 family, which is involved in fusion of vesicles with the plasma membrane, whereas in intracellular fusion reactions, the Q_b - and Q_c -SNARE motifs are supplied by separate proteins (Fukuda et al. 2000). The SNARE motifs are present at the N- and C-termini of SNAP-25 and are separated by a central cysteine-rich membrane targeting domain (also called a linker domain; (Sutton et al. 1998)). SNAP-25 is highly conserved among species, with little variation in length. The two alternatively spliced variants, SNAP-25a and SNAP-25b, have high homology, differing only by nine amino acids (Bark and Wilson 1994). They are efficiently targeted to the plasma membrane due to the

palmitoylation of the four cysteine residues in the linker domain (Gonzalo et al. 1999; Hess et al. 1992). SNAP-25 is most abundant in brain, where an interesting developmental shift in the expression of the isoforms has been described: SNAP-25a is more abundant in embryonic brain, whereas the expression of SNAP-25b increases robustly after birth to become the predominant isoform in most, but not all, adult brain areas (Bark et al. 1995; Boschert et al. 1996). Impairment of this switch towards the SNAP-25b isoform in mice leads to premature mortality and a change in short-term plasticity in hippocampal synapses (Bark et al. 2004). Ablation of the SNAP-25 gene in mice results in embryonic lethality (Washbourne et al. 2002), and Ca^{2+} -triggered secretion from neuroendocrine cells without SNAP-25 is nearly abolished (Sorensen et al. 2003). Both SNAP-25 isoforms can rescue secretion when expressed in SNAP-25 knock-out cells, but the SNAP-25b isoform is more efficient in driving vesicle priming than the SNAP-25a isoform (Sorensen et al. 2003), which has been attributed to a more efficient binding of synaptotagmin-1, the calcium sensor for exocytosis, by SNAP-25b (Mohrman et al. 2013).

Syntaxin-1 (Table 4.1) is a prototypic Q_a -SNARE and it contributes one of the four α -helices forming the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). This 35 kDa plasma membrane protein consists of a transmembrane domain, a SNARE motif, and an N-terminal H_{abc} -domain (Bennett

Table 4.1 Major proteins involved in synaptic vesicle exocytosis at the neuronal synapse

Exocytic protein	Function
Syntaxin	Plasma membrane transmembrane protein and central component of SNARE complex; involved in docking, priming, and fusion
SNAP25	Plasma membrane-anchored protein and central component of SNARE complex; involved in docking, priming, and fusion
VAMP/ synaptobrevin	Synaptic vesicle transmembrane protein and central component of SNARE complex; involved in priming and fusion
Synaptotagmin	Ca^{2+} sensor, binds $\text{PI}(4,5)\text{P}_2$, lipids, and the SNARE complex and regulates fusion through docking, priming, and fusion triggering
Complexin	Binds and regulates SNARE complex
Munc13	Involved in synaptic vesicle priming; opens syntaxin within Munc18 and interacts with RIM
Munc18	Involved in synaptic vesicle docking and priming; binds syntaxin in the closed configuration
NSF	Disassembles SNARE complex
α - and β -SNAP	Cofactor to NSF
Rab3	Regulates synaptic vesicle cycle; interacts with RIM and rabphilin
RIM1	Involved in synaptic vesicle priming; interacts with synaptotagmin, Munc13, and RimBPs (Rim-binding proteins)
N-, P-, Q-type Ca^{2+} channels	Regulate calcium influx to the presynaptic terminus and subsequently trigger exocytosis
PIPK1 γ	Key $\text{PI}(4,5)\text{P}_2$ -synthesizing enzyme at the presynaptic plasma membrane

et al. 1992). Two paralogs, syntaxin-1A and syntaxin-1B, share high homology and localization to the plasma membrane (Bennett et al. 1992). While their expression pattern largely overlaps, with only subtle changes in distribution, it is essentially restricted to neuronal and neuroendocrine cells (Ruiz-Montasell et al. 1996). The N-terminal H_{abc}-domain of syntaxin-1 is implicated in the regulation of protein accessibility. This region reversibly binds to the SNARE motif and can maintain two distinct syntaxin-1 conformations. In the “open” conformation (H_{abc}-domain not bound to the SNARE motif), the protein is able to form a functional SNARE complex, whereas in the “closed” conformation (H_{abc}-domain bound to the SNARE motif) it is not (Dulubova et al. 1999). In *Drosophila*, deletion of the syntaxin-1A homologue completely blocks neurotransmitter release (Schulze et al. 1995). In mouse, genetic ablation of syntaxin-1A does not lead to dramatic phenotypes, indicating that syntaxin-1B is sufficient to maintain vital functions (Fujiwara et al. 2006; Gerber et al. 2008), but long-term potentiation was affected due to a defect in the catecholamine systems (Fujiwara et al. 2006; Mishima et al. 2012).

Synaptobrevin/VAMP (Table 4.1) is a prototypic R-SNARE and contributes one of the four α -helices that compose the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). It is an abundant 13 kDa synaptic vesicle protein with a central SNARE motif, a C-terminal transmembrane region, and a proline-rich N-terminus. Two of the best-studied isoforms, synaptobrevin-1/VAMP1 and synaptobrevin-2/VAMP2, differ mainly in the hydrophobic C-terminus and in the poorly conserved N-terminus (Elferink et al. 1989; Trimble et al. 1988). Although a partial overlap in their expression pattern is apparent, synaptobrevin-1 and synaptobrevin-2 are differentially distributed in the brain, suggesting specialized functions for each isoform. Synaptobrevin-2/VAMP2 is, in general, more evenly distributed, while synaptobrevin-1/VAMP1 expression is located predominantly in neurons with somatomotor function (Trimble et al. 1990). Ablation of the synaptobrevin-2/VAMP2 gene is postnatally lethal (Schoch et al. 2001). Evoked synaptic exocytosis from hippocampal neurons without synaptobrevin-2/VAMP2 is severely decreased, but fusion is not completely abolished (Schoch et al. 2001). The vesicles that fuse in the absence of synaptobrevin-2/VAMP2 are unable to endocytose and recycle quickly, implying that synaptobrevin-2/VAMP2 is also necessary for rapid synaptic vesicle endocytosis (Deak et al. 2004). Synaptobrevin-2 is by far the most abundant synaptic vesicle protein, with 60–70 copies per vesicle (Takamori et al. 2006).

Fusion mediated by SNAREs only is relatively slow, due to uncoordinated fusion events. Given that neurons require high levels of spatially and temporally coordinated activity, SNARE proteins in neurons are tightly regulated at different stages of their generation and action: transcriptional regulation of gene expression, targeting to the correct compartment membranes, functionality in targeted membranes, posttranslational modification (e.g., phosphorylation), assembly and disassembly of the SNARE complex, and fusion triggering by calcium. Consequently, many accessory factors that modulate multiple SNARE complexes are needed to ensure the sophisticated control that characterizes membrane fusion in neurons (Sudhof 2013).

4.2 SNARE-Assembly Mechanism and Fusion

4.2.1 SNARE-Complex Assembly I: Role of Closed Syntaxin and Munc18-1

Syntaxin-1 is found in dense clusters with diameters of 50–70 nm on the plasma membrane (Barg et al. 2010; Lang et al. 2001; Rickman et al. 2010; Sieber et al. 2007). The lipids phosphatidylinositol-4,5-bisphosphate (PIP₂) and cholesterol both participate in clustering syntaxin-1 (Honigsmann et al. 2013; Lang et al. 2001; van den Bogaart et al. 2011a) – but see Murray and Tamm (2009). The syntaxin-1 clusters are so dense that they are thought to preclude the formation of the SNAP-25/syntaxin acceptor complex for synaptobrevin/VAMP and possibly even the closed conformation of syntaxin-1 (Sieber et al. 2007) (Fig. 4.2a). SNAP-25 also forms clusters, which are distinct or partly overlapping with the syntaxin clusters (Knowles et al. 2010; Lang et al. 2001). The first event leading to SNARE-complex assembly and fusion is therefore arguably the recruitment of syntaxin-1 and SNAP-25 from these clusters. This might happen spontaneously by lateral diffusion of SNAREs into the membrane from the rim of clusters, resulting in a certain abundance of reactive syntaxin-1 in the membrane, in equilibrium with clustered and thus unavailable syntaxin-1 or SNAP-25 (Bar-On et al. 2009, 2012; Lang et al. 2002), or conversely the vesicles might themselves induce recruit SNAREs to the underlying membrane (Barg et al. 2010; Knowles et al. 2010).

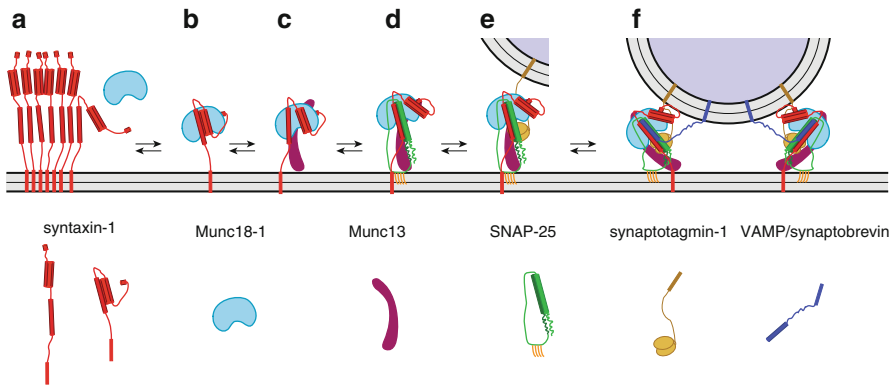


Fig. 4.2 Overview of events leading up to formation of a fusion complex between vesicle and plasma membrane. (a) Syntaxin-1 (and SNAP-25) exists in the plasma membrane as clusters of ~70 nm diameter. (b) Munc18-1 binds to syntaxin-1 in its closed configuration, where the H_{abc}-domain is folded back on the SNARE domain. (c) Munc13 proteins bind to and open syntaxin-1 within syntaxin/Munc18 dimers. (d) Open syntaxin can now bind to SNAP-25. (e) Vesicle docking in adrenal chromaffin cells involves binding of synaptotagmin to syntaxin/SNAP-25 dimer. (f) Synaptobrevin-2/VAMP2 binds to the acceptor complex, creating the fusogenic complex. Note that it is unknown whether Munc13 and Munc18 are still bound to the complex at this time. Also note that complexin, which probably binds to the assembling SNARE-complex, has been left out

Once outside the clusters, syntaxin-1 can associate with SNAP-25 in various configurations (An and Almers 2004; Freedman et al. 2003; Halemani et al. 2010; Laage et al. 2000; Margittai et al. 2001; Misura et al. 2001a; Rickman et al. 2010).

Munc18-1/nsec-1 (Table 4.1) is a soluble kidney-shaped protein, which stabilizes closed syntaxin by binding the H_{abc}-H3 helical bundle through its central cavity (Misura et al. 2000) (Fig. 4.2a, b). This naturally leads to the expectation that Munc18-1 is a negative regulator of secretion, but knockout experiments revealed that Munc18-1 and its homologues are required for fusion in the cell (Harrison et al. 1994; Hosono et al. 1992; Verhage et al. 2000). Further experiments showed that membrane-anchored syntaxin can form SNARE complexes even when bound to Munc18-1 (Zilly et al. 2006). The issue of the essential positive function of Munc18-1 in exocytosis has still not been resolved and remains one of the most interesting open questions in the exocytosis field (for a recent review on SNAREs and Munc18, see (Rizo and Sudhof 2012)). One important function for Munc18 is its binding to syntaxin-1 in the closed configuration during trafficking of both proteins to the cell membrane (Medine et al. 2007; Rowe et al. 1999). In this configuration, Munc18-1 protects the cell from the formation of ectopic SNARE complexes when syntaxin-1 traffics through the Golgi-TGN area (Rowe et al. 1999). Accordingly, in the Munc18-1 knockout mouse, the level of syntaxin-1 is reduced (by about 70 %), although part of the syntaxin-1 population of syntaxin-1 is still properly localized at synapses (Toonen et al. 2005). Thus, the function of Munc18-1 as a chaperone for syntaxin is important, but not sufficient to explain the complete arrest of secretion in Munc18-1 knockout mice (Verhage et al. 2000). It appears likely that initial recruitment of syntaxin from the dense clusters on the plasma membrane might be regulated by Munc18-1 binding to syntaxin-1 adopting the closed configuration at the edge of syntaxin clusters (Fig. 4.2a).

Munc18-1 exerts several actions along the exocytotic pathway. In the absence of Munc18 (or Unc18), vesicle docking to the plasma membrane is absent (Voets et al. 2001; Weimer et al. 2003). This might be expected from the chaperone function of Munc18, because docking also depends on syntaxin-1 expression (de Wit et al. 2006; Hammarlund et al. 2007). However, expression of a Munc18-1 mutant in chromaffin cells, which rescued syntaxin abundance, was unable to rescue docking or secretion, and, conversely, expression of Munc18-2 rescued syntaxin abundance and docking, but not secretion (Gulyas-Kovacs et al. 2007). Expression of a mutant defective in syntaxin binding stimulated release in PC12-cells (Schutz et al. 2005). Therefore, Munc18-1 must play a positive role downstream from docking and distinct from binding to closed syntaxin. Further experiments showed that overexpression of SNAP-25 in Munc18-1 null cells, which presumably increases the amount of syntaxin/SNAP-25 dimers, fully rescued vesicle docking, but secretion remained fully depressed (de Wit et al. 2009). The vesicular docking factor in chromaffin cells was identified as synaptotagmin-1 (de Wit et al. 2009), whereas there is conflicting evidence as to whether VAMP-2/synaptobrevin-2 is involved in this step (Borisovska et al. 2005; Wu et al. 2012). Synaptotagmin-1 seems to be necessary for docking in chromaffin cells by binding

to SNAP-25, presumably within SNAP-25/syntaxin dimers (Mohrmann et al. 2013), but Munc18-1 carries out an essential role in fusion downstream of this step.

The different functions of Munc18-1 along the exocytotic pathway might correlate with different binding configurations with the SNAREs. One configuration is the aforementioned binding to closed syntaxin; another is the binding to the assembled (or assembling) SNARE complex (Carr et al. 1999; Dulubova et al. 2007; Khvotchev et al. 2007; Shen et al. 2007). Both configurations include an interaction with an N-terminal peptide of syntaxin-1 (Burkhardt et al. 2008; Dulubova et al. 2007; Shen et al. 2007). Interfering with the N-terminal peptide reduces Munc18 stimulation of lipid mixing in vitro (Schollmeier et al. 2011; Shen et al. 2007) and exocytosis in vivo (Deak et al. 2009; Khvotchev et al. 2007; Zhou et al. 2013a). Mutations in Munc18 (and its homologue in *Caenorhabditis elegans*), which abolish binding to the N-terminal syntaxin, also strongly reduce synaptic transmission in some (Deak et al. 2009; Johnson et al. 2009), but not all (Meijer et al. 2012) investigations. In in vitro fusion assays, it was surprisingly found that only the N-terminal part of syntaxin, and not the Habc-domain, is essential for Munc18-1 dependent stimulation of vesicle fusion (Rathore et al. 2010; Shen et al. 2010), and furthermore, in *C. elegans*, the N-terminal domain of syntaxin could be transferred onto SNAP-25 without loss of synaptic transmission (Rathore et al. 2010). This indicates that the N-terminal domain of syntaxin acts to recruit Munc18-1 to the assembled (or assembling) SNARE complex to perform its essential stimulating function. However, it is still unclear what this function is.

One possibility is that Munc18-1 assists in nucleating or finalizing SNARE-complex assembly (Shen et al. 2007). This is supported by the observation that the stimulatory role of Munc18-1 in in vitro fusion assays depends on the exact nature of the R-SNARE and is abolished upon mutation of synaptobrevin-2 or its replacement by other isoforms (Schollmeier et al. 2011; Shen et al. 2007). Biochemical experiments have shown that the four-helix SNARE bundle binds to the same cavity in Munc18, which interacts with the H_{abc}-domain of closed syntaxin (Xu et al. 2010), but binding to the SNARE complex has a lower affinity than binding to closed syntaxin (Burkhardt et al. 2008; Xu et al. 2010). Binding was also found to occur directly between the 3a-domain of Munc18-1 and the C-terminal end of the synaptobrevin/VAMP SNARE domain (Xu et al. 2010), which would localize this part of Munc18-1 right at the fusion pore. Notably, the 3a-domain has been shown by random and targeted mutagenesis to be essential for fusion (Boyd et al. 2008; Martin et al. 2013). The basic conceptual problem with the hypothesis that Munc18-1 stimulates SNARE-complex assembly is that SNAREs assemble readily to drive membrane fusion in vitro without the need for Munc18-1 (Schuette et al. 2004; Weber et al. 1998). Consequently, in in vitro fusion assays, Munc18-1 only has a relatively mild effect on fusion and it only becomes stimulatory after preincubation of liposomes under conditions that probably result in initial SNARE-complex formation. Thus, it is currently hard to understand from these findings why Munc18-1 should be absolutely required for SNARE-complex formation in the cell.

It has been suggested that Munc18 might directly catalyze lipid mixing itself, once it is brought into contact with the fusion site by binding to the SNAREs (Rizo and Sudhof 2012). In this model, assembly of the SNAREs would bring the membranes together by exerting force on them, leading to tight apposition and dehydration of the two membranes. Munc18 might then act to stimulate lipid splaying, which might be the rate-limiting step for fusion (see below), and would result in the formation of a lipid stalk. Possibly, Munc18 could provide a surface over which the lipids might bend, catalyzing fusion. While there is no direct evidence to support this model – and it does not account for the difference between in vitro and in vivo findings – it is in agreement with the notion that all SNARE-driven fusion events in the cell require a SM-protein.

4.2.2 SNARE-Complex Assembly II: Opening Syntaxin and Roles of Munc13/CAPS

Since the closed conformation of syntaxin blocks SNARE-complex assembly, opening syntaxin is a key step towards membrane fusion. This opening step must be correlated with a change in interaction mode of Munc18-1. Replacement of wild-type syntaxin with a mutated version – the so-called LE-mutant (Dulubova et al. 1999), which tends to adopt the open conformation more readily (Dulubova et al. 1999) – promoted spontaneous release and increased vesicular release probability (Gerber et al. 2008), presumably because of facilitated formation of SNARE complexes between vesicles and plasma membrane.

In the cell, the opening of syntaxin must be followed or accompanied by binding to SNAP-25, which yields the acceptor complex for synaptobrevin-2/VAMP2 (Fig. 4.2c, d). A role for Munc18-1 in catalyzing this step by keeping syntaxin in a half-open configuration has been proposed based on biochemical studies (Burkhardt et al. 2008). Structural studies have led to the suggestion that a conformational change in Munc18-1 might drive the formation of the acceptor complex while syntaxin remains bound to Munc18-1 (Christie et al. 2012; Colbert et al. 2013; Hu et al. 2011), although these studies did not agree on whether the N-terminal part of syntaxin would engage Munc18-1 to open or close syntaxin.

Proteins of the Munc13/CAPS family (Table 4.1) act by mediating or stimulating the opening of syntaxin, probably within Munc18/syntaxin dimers (Fig. 4.2c). These proteins contain a catalytic so-called Mun domain, which mediates vesicle priming (Basu et al. 2005; Stevens et al. 2005). In the absence of Munc13 proteins, neurosecretion is completely abolished in neurons (Augustin et al. 1999; Richmond et al. 1999; Varoqueaux et al. 2002), whereas overexpression strongly stimulates priming in adrenal chromaffin cells (Ashery et al. 2000). Munc13 interacts directly with syntaxin, with the SNARE complex and with Munc18 (Ma et al. 2011). These rather weak interactions might play key roles in opening syntaxin within syntaxin/Munc18 dimers and allowing SNARE-complex assembly (Ma et al. 2011). The

important role of Munc13 proteins in opening syntaxin was demonstrated in *Drosophila*, where expression of the LE-mutated open syntaxin partly overcomes the secretion defect in Munc13-deficient neurons (McEwen et al. 2006; Richmond et al. 2001). However, in mouse open syntaxin-1B did not overcome the lethal phenotype of the Munc13-1 knockout (Gerber et al. 2008).

CAPS-1 and CAPS-2 also contain a Mun domain, in addition to a PIP₂-binding pleckstrin homology domain, which are both essential for their function (Grishanin et al. 2002; Khodthong et al. 2011). Deletion of CAPS-1 and CAPS-2 leads to a subtle phenotype in neurons, where vesicles can be primed only transiently, by increases in the basal calcium concentration, but soon fall back to the non-primed state (Jockusch et al. 2007). Therefore, there might be two different priming pathways, one governed by Munc13 proteins and one by CAPS proteins. In fusion of dense-core vesicles, CAPS acts at the priming step (Elhamdani et al. 1999; Liu et al. 2008; Speidel et al. 2008), and it has been further shown that CAPS binds to the individual SNAREs and orchestrates the formation of the SNARE complex (Daily et al. 2010; James et al. 2009). Thus, CAPS proteins might play similar roles as Munc13 proteins, but the presence of a PH domain in CAPS and C1- and C2-domains in Munc13 might confer the proteins with different regulatory properties. In fusion of dense-core vesicles, the absence of CAPS proteins can be partly overcome by expression of open syntaxin (Hammarlund et al. 2008; Liu et al. 2010), indicating the similarity in function between Munc13 and CAPS in opening syntaxin. However, Munc13 overexpression could not compensate for CAPS deficiency, or vice versa, (Jockusch et al. 2007; Liu et al. 2010); therefore, CAPS and Munc13 proteins must perform other, distinct, functions in the exocytotic cascade.

Deletion of the H_{abc}-domain of syntaxin – while maintaining the N-terminal peptide intact – supported fast evoked release in syntaxin-1-deficient neurons, but the RRP and spontaneous release were depressed (Zhou et al. 2013b). This finding, together with the only partial rescue of Munc13 or CAPS deficiencies by LE-mutated open syntaxin, indicates that although the closed conformation of syntaxin-1 is inhibitory for SNARE-complex formation per se, the ability to open syntaxin up – presumably catalyzed by Munc13 or CAPS proteins – plays a specific positive role in vesicle priming, which cannot be overcome by constitutively open syntaxin. This seems to play the largest role during sustained calcium elevations, where priming has to be fast to keep up with fusion. This correlates well with the fact that Munc13 proteins are stimulated by calcium and diacylglycerol (Lipstein et al. 2013; Rhee et al. 2002; Shin et al. 2010). Thus, the opening of syntaxin by Munc13 might be a pivotal regulation point of the synaptic cycle, and the regulatory domains of Munc13 (and CAPS) might have developed to link the activity of G-protein-regulated receptors and the calcium concentration to the synaptic priming speed.

The above establish the involvement of the major players in (calcium-independent) exocytosis and neurotransmitter release: the SNARE proteins, Munc18, and Munc13. In a striking experiment, it was shown that the essential functions of SNAREs, Munc18, and Munc13 can be reconstituted in vitro in a

fusion assay (Ma et al. 2013). In these experiments, Munc18 displaced SNAP-25 from syntaxin, and fusion then became Munc13 dependent. Thus, it was concluded that syntaxin being bound to Munc18 in the closed conformation is the starting point for fusion (Fig. 4.2a–c), not “free” syntaxin/SNAP-25 dimers, which would be susceptible to spontaneous disassembly by the activity of α -SNAP/NSF (see below). These syntaxin/Munc18 dimers then bind SNAP-25 and synaptobrevin-2/VAMP2 through the action of Munc13 (Fig. 4.2d), in a pathway which is resistant to α -SNAP/NSF action (Ma et al. 2013). It should be noted that this conclusion does not contradict the observation that the LE-mutated “open syntaxin” can partly bypass the need for Munc13 *in vivo*, because recent studies have shown that the LE-mutated syntaxin in fact binds to Munc18 in a closed configuration with only slightly lower affinity than wild-type syntaxin (Burkhardt et al. 2008; Colbert et al. 2013). But when bound to Munc18, LE-mutated syntaxin allows formation of the SNARE complex without further cofactors (Burkhardt et al. 2008). Therefore, LE-mutated syntaxin might be called “open” in the sense that it allows spontaneous formation of the SNARE complex within syntaxin/Munc18 dimers.

4.2.3 SNARE-Complex Assembly III: Zippering the SNARE Bundle

Productive formation of the syntaxin/SNAP-25 dimer probably takes place while syntaxin remains bound to Munc18-1 (see above). Biochemical experiments and experiments in adrenal chromaffin cells are consistent with the idea that this dimer binds to synaptotagmin, which docks vesicles to the SNARE acceptor complex (de Wit et al. 2009; Mohrmann et al. 2013; Rickman et al. 2004, 2006) (Fig. 4.2e). A specific step corresponding to synaptotagmin binding to SNAP-25/syntaxin has not been detected in neurons, where morphological docking as detected by the electron microscope is driven by another complex consisting of RIM (Rab3-interacting molecule), RIM-BP (RIM-binding protein), and Rab3 or Rab27 (Sudhof 2013). Nevertheless, it is likely that the same sequence of events takes place in neurons, even though it does not correspond to a visible phenotype in EM micrographs. The next event on the path to vesicle fusion is most likely synaptobrevin/VAMP binding to the acceptor complex to form the ternary SNARE complex, which drives membrane fusion itself (Fig. 4.2f). This is probably a heavily regulated step towards fusion. The protein tomosyn, which carries a SNARE motif and is classified as an R-SNARE, binds to the SNAP-25/syntaxin dimer to block or limit vesicle priming, which then requires synaptobrevin-2/VAMP2 to replace tomosyn in a poorly characterized fashion (for a review, see (Ashery et al. 2009)).

The main framework for our thinking about the SNARE-assembly step is the “zipper hypothesis” (Hanson et al. 1997a; Lin and Scheller 1997), which was suggested following the realization that SNARE-complex formation aligns the SNARE domains in parallel (Hanson et al. 1997b; Sutton et al. 1998). This placed

the C-terminal membrane anchors of synaptobrevin-2/VAMP2 and syntaxin in the same end of the complex, suggestive of a mechanism where N- to C-terminal assembly (“zippering”) brings the membranes closer and closer together, until fusion results. In the simplest possible version of this model, the complex would be linked to the membrane via stiff linker between the SNARE domains and transmembrane anchors, linking complex formation directly to deformation of the membranes (see below).

Experiments relying on deletion studies, peptide interference in cells, and *in vitro* fusion assays uniformly support the idea that ternary SNARE-complex formation starts in the N-terminal end and progresses towards the C-terminus (Chen et al. 2001; Fasshauer and Margittai 2004; Matos et al. 2003; Melia et al. 2002; Pobbati et al. 2006; Xu et al. 1999b). It is the formation of the SNARE complex, which leads to structuring of the SNARE domains of SNAP-25 and synaptobrevin-2/VAMP2, which are unstructured when free in solution (Fasshauer et al. 1997). However, the α -helical SNARE motifs are fairly promiscuous, which *in vitro* can lead to formation of several other products, such as a 2:1 complex, where the binding site for synaptobrevin-2/VAMP2 is occupied by a second syntaxin (Fasshauer and Margittai 2004; Xiao et al. 2001). This structure – as well as others (Misura et al. 2001a, b) – represents off-pathway products (kinetic traps), which are unproductive for fusion and therefore exacerbate the very slow kinetics of SNARE-driven fusion *in vitro*. Blocking formation of these alternative complexes results in markedly sped up *in vitro* fusion (Pobbati et al. 2006). In the cell, it is likely that the formation of the SNAP-25/syntaxin acceptor complex while bound to Munc18 protects against the formation of off-pathway complexes, which might be yet another function for Munc18 (Rizo and Sudhof 2012).

Experiments performed in cells support the idea of N- to C-terminal assembly of the SNARE complex. Infusion of an antibody, which blocked SNARE-complex assembly, into chromaffin cells led to the suggestion that the SNARE complex might coexist in two different states, a “loose” and a “tight” state, before exocytosis (Xu et al. 1999b). Differences in the activity dependence of neurotransmission block caused by tetanus toxin (TeNT) and botulinum neurotoxin D (BoNT/D), which bind to the N- or the C-terminal end of the synaptobrevin-2/VAMP2 SNARE domain, respectively, led to the suggestion that the N-terminal, but not the C-terminal, half of the SNARE domain is shielded before fusion (Hua and Charlton 1999).

Mutagenesis studies have shown that mutating the hydrophobic layers in the middle of the SNARE bundle to cause a local destabilization has very different consequences depending on where along the bundle the mutation is placed. Mutations in the middle of the bundle – or towards the N-terminal end – caused a decrease in forward vesicle priming rate, whereas mutations in the most C-terminal layers – layers +7 and +8 – cause a depression in fusion speed (Sorensen et al. 2006; Walter et al. 2010; Weber et al. 2010; Wei et al. 2000). The depression in speed was graded when several destabilizing mutations were compared, and – importantly – temperature unfolding experiments showed that C-terminal mutations caused the C-terminal end of the complex to disassemble at lower temperatures, while the

N-terminal end of the complex remained unaffected, indicating that the two ends of the SNARE complex fold independently (Sorensen et al. 2006). Conversely, a mutation in syntaxin layer +7, which tightens the C-terminal end, caused increased spontaneous and evoked release in *Drosophila* (Lagow et al. 2007). Thus, fusion rate or, equivalently, fusion probability might correlate directly with the stability of the very C-terminal end of the SNARE bundle.

Since N-terminal assembly correlates with vesicle priming, whereas C-terminal assembly causes vesicle fusion, some mechanism must arrest further SNARE complex zippering after initial N-terminal assembly until arrival of the calcium signal (but see below for a different view). This mechanism could in principle be (1) intrinsic to the SNARE complex, such that two sub-domains fold independently, separated by an energy barrier; (2) the repulsion between the membranes, which will put up an energy barrier for C-terminal assembly; or (3) an accessory protein, which arrests the SNARE complex in the half-zipped state. In fact, it is likely that all of these mechanisms contribute to the partial assembly of SNARE complexes. First, elegant experiments using forced unzipping and re-zipping of single SNARE complexes (without accessory proteins) by optical or magnetic tweezers have revealed that the N-terminal and C-terminal halves of the SNARE bundle fold/unfold in discrete steps (Gao et al. 2012; Min et al. 2013). This is followed by zippering of the linker and transmembrane domains to yield the fully zippered complex (Stein et al. 2009). Interestingly, the N- and C-terminal halves seem to assemble as binary switches, so that further intermediate assembly steps are not discernible. The assembly of the N-terminal half of the SNARE complex arranges the C-terminal domains of the acceptor complex into the same structure as the *cis*-complex (Li et al. 2014), indicating that N-terminal assembly sets the stage for later C-terminal assembly. Second, the same experiments showed that, with a repulsion force on the C-terminal end of the SNAREs between 12pN and 20pN (Gao et al. 2012), or between 11 pN and 34 pN (Min et al. 2013), the SNARE complex would rest in a state where only the N-terminal end was assembled. Above this range, the N-terminal end would disassemble. Thus, it is likely that repulsion between membranes would keep the SNARE complex in a partially assembled state. Third, recent *in vitro* and *in vivo* experiments have found that the accessory protein complexin binds to the SNARE complex and inserts its so-called accessory helix into the C-terminal end of the partially assembled complex, where it blocks further assembly until calcium binds to synaptotagmin and relieves the complexin block [e.g., (Giraudo et al. 2006; Kummel et al. 2011; Malsam et al. 2012; Tang et al. 2006; Xue et al. 2007)] (See chapter on complexins; Chap. 6). Because the binding site for complexin is created during formation of the SNARE complex (Chen et al. 2002; Pabst et al. 2000), the two former mechanisms might be required to arrest the SNARE-complex formation long enough for complexin to bind.

Not all data support the view that assembly of the C-terminal half of the SNARE complex leads to membrane fusion. Fully assembled SNARE complexes – or SNARE complexes only slightly frayed at the very C-terminal end – have been identified between non-fused – or hemifused – membranes (Hernandez et al. 2012; Shin et al. 2014). Thus, full assembly of the SNARE domains into a complex might

not be sufficient to fuse the membranes. According to this view, it might be the zippering of the linker domains and transmembrane anchors that triggers fusion, assisted by calcium binding to synaptotagmin. This scenario is consistent with mutagenesis conducted in cells, because the only mutations that were found to selectively compromise secretion rate were located in layers +7 and +8, and indeed it was suggested that the “partially assembled” complex might only need to assemble layer +8 (Walter et al. 2010). Thus, whether SNARE-complex assembly is arrested at the zero layer in the cell is unclear.

The physical movements in the SNAREs associated with membrane fusion in living cells have been detected using inter- and intramolecular FRET (fluorescence resonance energy transfer) (An and Almers 2004; Degtyar et al. 2013; Wang et al. 2008; Zhao et al. 2013). These studies have detected changes in FRET reflecting SNARE-complex formation, or other conformational changes, before and after fusion. However, because of the long exposures required to detect the small FRET signals, it has been hard to establish unequivocally which signals are derived from changes before or after membrane fusion. Recently, this problem was overcome using a four-electrode electrochemical detector array on a glass coverslip to detect and localize fusing vesicles in chromaffin cells in TIRF microscopy independently of the FRET signal (Zhao et al. 2013). Averaging the FRET signal around many fusing vesicles led to the detection of a FRET signal, which preceded release by ~90 ms. The FRET probe used [SCORE for SNARE complex reporter (An and Almers 2004)] was an intramolecular SNAP-25 probe constructed to display FRET upon SNARE-complex formation. Since 90 ms is probably too fast to be caused by vesicle priming in chromaffin cells, this experiment detected a structural change in SNAP-25 linked to membrane fusion itself. This might be interpreted as evidence that the second SNARE motif of SNAP-25 only joins the SNARE complex at the time of fusion (An and Almers 2004). Alternatively, the FRET signal might reflect a more subtle movement, for instance, a rotation, associated with final SNARE-complex zippering.

Recently, it was suggested that synaptotagmin might act as a distance regulator, which would prevent SNARE-complex assembly entirely until calcium influx (Jahn and Fasshauer 2012; van den Bogaart et al. 2011b). In this model, the SNARE complex acts as a “one-shot” device, which assembles uninterrupted to fuse the membranes as soon as the N-terminal ends engage. Thus, in this model there is no function for a partially assembled SNARE complex. While this model is attractive in that it can explain the FRET signal detected immediately before fusion (Zhao et al. 2013), it is hard to reconcile with the known effects of complexin on vesicle priming and fusion, since complexin cannot bind until the SNARE complex has formed. It further appears inconsistent with the finding that mutations in the N-terminal end of the SNARE complex selectively affect vesicle priming, a reaction that takes part upstream of calcium influx.

4.2.4 SNARE-Complex Assembly IV: Fusing the Membranes

Whereas a consistent picture is emerging regarding the steps leading up to membrane fusion, membrane merger itself remains incompletely understood. This is due, at least in part, to the fact that the membrane merger process cannot be studied directly in living cells, whereas *in vitro* systems might not reproduce essential features of the cellular fusion process. Nevertheless, recent years have seen substantial progress using indirect methods and mathematical simulations that allow us to paint a preliminary picture, which will undoubtedly become more complete as cellular methods for the measurement of lipid mixture with high temporal resolution are developed.

It is commonly assumed that SNARE-dependent membrane fusion follows the same basal steps as protein-less membrane fusion (Chernomordik and Kozlov 2003) (Fig. 4.3). Initially, the proximal leaflets approach each other (Fig. 4.3a), which results in dehydration of the lipid head groups, splaying of lipids (Fig. 4.3b) and lipid stalk formation, where the proximal leaflets are fused. This lipid stalk can now either expand, yielding a hemifused state and then a fusion pore, or the lipid stalk can directly yield a fusion pore. There is extensive evidence that SNARE-mediated fusion can pass through the hemifused state (Abdulreda et al. 2008; Giraud et al. 2005; Lu et al. 2005; Reese et al. 2005; Wong et al. 2007) (Fig. 4.3c). The next step is fusion pore generation by breaking the hemifused membrane to yield the fully fused state (Fig. 4.3d). In synaptic transmission, neurotransmitter release starts as soon as the fusion pore is generated, and for small synaptic vesicles, it is expected that even the formation of a transient fusion pore might be enough to empty the vesicle for neurotransmitter (Bruns and Jahn 1995). In contrast, for dense-core vesicles, transmitter might be released both

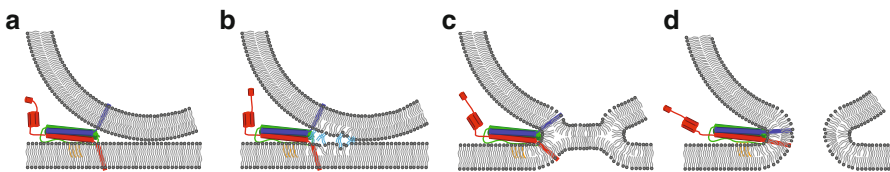


Fig. 4.3 Final SNARE-mediated fusion of membranes. Note that only the SNARE complex has been drawn, even though complexin and synaptotagmin also are expected to participate at this stage (a) Formation of the SNARE complex leads to close apposition between the membranes, leading to dehydration of the leaflets and approach of lipid head groups from the two membranes. (b) The next step is probably lipid splaying, i.e., some lipids will flip out of the leaflet and form bridges with lipids from the opposing leaflet. Splayed lipids have been drawn in cyan. (c) After formation of a lipid stalk (not shown), the fusion pathway might transit through the hemifused state, where distal leaflets meet to form a bilayer. (d) The C-terminal ends of syntaxin and synaptobrevin-2/VAMP2 cause destabilization of the hemifused state and lead to full membrane fusion, which relaxes bending stress in the SNAREs and leads to a fully zippered complex. See Risselada and Grubmüller (2012) for a more detailed account of the last events leading to membrane fusion

during fusion pore formation and as the fusion pore expands (Albillos et al. 1997; Zhou et al. 1996).

How is SNARE-complex assembly linked to the fusion of the lipid membranes? One view sees the conclusion of the SNARE-catalyzed membrane fusion process as a highly structured and reproducible superstructure, often consisting of a large number (5–15) of SNARE complexes arranged in a radial and symmetrical fashion around the nascent fusion pore (Megighian et al. 2013; Montecucco et al. 2005). Whereas most investigators assume that the fusion pore would be lined by lipids, in some models this lipid pore would be preceded by a transient proteinaceous pore lined by the transmembrane domains of syntaxin and synaptobrevin-2/VAMP2 (Han et al. 2004). Recent findings make such a fixed high-order structure unlikely. *In vitro* experiments have shown that a single SNARE complex suffices to fuse membranes (Shi et al. 2012; van den Bogaart et al. 2010), whereas three complexes are necessary for keeping the fusion pore open (Shi et al. 2012). This fits very well with titration experiments of all three SNAREs in living cells, which is consistent with two to three SNARE complexes being enough to cause fast fusion (Arancillo et al. 2013; Mohrmann et al. 2010; Sinha et al. 2011). Similar results had been obtained previously in cracked-open PC12 cells (Hua and Scheller 2001). Even though more experiments are needed to understand whether the number of engaging SNARE complexes is fixed or variable, and whether it is stochastic or under regulatory control, these experiments are most easily reconciled with a model wherein SNAREs fuse membranes essentially as independent devices resulting in variable overall stoichiometry, which might be reflected in the multiple kinetic components of exocytosis. This is further in agreement with the fact that no mechanism that would organize ring-formed higher-order SNARE structures in the cell has so far been established. One such possible mechanism would be domain sharing by SNAP-25, i.e., the two SNARE domains of SNAP-25 might join different but neighboring complexes, which might organize multiple SNARE complexes in a ring-formed structure (Tokumaru et al. 2001). However, this arrangement was not supported by experiments in chromaffin cells (Mohrmann et al. 2010).

The assumption that the energy of formation of the SNARE complex drives membrane fusion has prompted considerations about whether the energy of a single SNARE complex would suffice to overcome the energy barrier for fusion. Theoretical calculations using continuum models have placed the energy barrier for membrane fusion at $\sim 40 k_B T$ (k_B is the Boltzmann constant; T is the absolute temperature) (Cohen and Melikyan 2004; Kuzmin et al. 2001). This estimate has been compared to the energy released by forming a single SNARE complex as measured in a surface force apparatus ($\sim 35 k_B T$) (Li et al. 2007) or as measured using optical tweezer manipulation of single complexes ($\sim 65 k_B T$) (Gao et al. 2012). However, isothermal calorimetry resulted in markedly lower estimates of the energy release by a SNARE complex, around $\sim 19 k_B T$ (Wiederhold and Fasshauer 2009). The large discrepancy between those three studies (note that $\sim 2.3 k_B T$ corresponds to a factor 10 difference in equilibrium constant at physiological temperatures) remains unexplained but is likely related to the difficulty in

obtaining reversible measurements within an experimentally accessible time scale, combined with the fact that assembly and disassembly displays hysteresis (Fasshauer et al. 2002). In any case, it is unlikely that all of the energy liberated by the formation of a SNARE complex can be harnessed for membrane fusion. Assembly of the C-terminal half of the SNARE complex liberates less energy than assembly of the N-terminal end (Wiederhold and Fasshauer 2009), even though, in the optical tweezers experiment, the C-terminal end still released an impressive $\sim 28 k_B T$ (Gao et al. 2012). It is formation of the C-terminal end that couples to membrane fusion, whereas assembly of the N-terminal end drives vesicle priming (see above). Therefore, it is important to understand whether assembly of the N-terminal end results in a tense structure that stores energy eventually used for C-terminal assembly. However, destabilizing mutations in the N-terminal end did not compromise fusion speeds in chromaffin cells (Sorensen et al. 2006; Walter et al. 2010; Wiederhold et al. 2010), whereas in neurons such mutations actually slightly increased release probability (Weber et al. 2010). This is most easily reconciled with the assembly of the N-terminal end of the SNARE complex resulting in a stable structure (i.e., a minimum in the energy landscape), which might result in an increase in the effective size of the energy barrier for fusion. Thus, partial SNARE-complex assembly might actually increase the energy barrier for fusion, in order to set the state for fast calcium-triggered fusion. This is exactly what is expected in the “complexin as a clamp” model (see above), where tight assembly of the N-terminal end of the SNARE complex leads to complexin binding and clamping of C-terminal SNARE-complex assembly. Another possibility is that a similar feature is encoded in the SNARE complex itself and then exacerbated by interaction with complexin/synaptotagmin.

The energy barrier for membrane fusion is lowered by $\sim 10 k_B T$ during the arrival of an action potential (Rhee et al. 2005). Thus, a low number of SNARE complexes (down to a single one) might release enough energy to fuse the membranes with a rate consistent with neurotransmission. Simulations using coarse-grain models have resulted in lower estimates for the overall fusion barrier [reviewed in (Risselada and Grubmuller 2012)]. Notably, several energy barriers could be distinguished. The first consists of close approach and dehydration of the membranes. The next is the occurrence of splayed lipids, i.e., lipids with exposed hydrophobic tails reaching over towards the opposite membrane. This state might constitute the main energy barrier, while the stalk is at a local energy minimum (Markvoort and Marrink 2011; Risselada and Grubmuller 2012). Expansion of the fusion pore might constitute another downstream energy barrier (Katsov et al. 2004). SNARE assembly will likely be able to overcome the first energy barrier and cause close membrane apposition. It has been suggested that Munc18 might directly act to induce lipid mixing (Rizo and Sudhof 2012), which would help overcome the second energy barrier. Similarly, it has been found that synaptotagmins help expand the fusion pore (Bai et al. 2004b; Wang et al. 2001, 2006). Thus, it is likely that we will come to see the SNAREs as part of a larger fusion machine, which has to overcome several distinct energy barriers.

Whatever the exact nature of the energy barrier(s), if the energy of formation of the SNARE complex is harvested to fuse the membranes, then force must be transduced to the membranes via the linkers between the SNARE domains and the transmembrane domains (TMD) of syntaxin-1 and synaptobrevin-2/VAMP2. Force transduction might take place by the formation of continuous α -helices throughout the linker and TMD, as found in a crystal structure that included those regions (Stein et al. 2009). Because α -helices are stiff structures, progressive formation of a continuous α -helix from the N- to the C-terminal would link the conformation of synaptobrevin-2/VAMP2 to membrane deformation. However, mutagenesis experiments have shown that α -helical continuity per se is not absolutely required for membrane fusion. In *in vitro* fusion assays, inserting helix breakers (five amino acids, including two prolines) in synaptobrevin-2/VAMP2 did not inhibit lipid mixing, whereas insertion in syntaxin reduced fusion by a factor of two (McNew et al. 1999). Insertion of flexible domains into the linkers progressively reduced lipid mixing, and again syntaxin was more susceptible than synaptobrevin-2/VAMP2, indicating that the SNARE complex acts asymmetrically on the two membranes. Simulations have shown that the syntaxin linker has considerable stiffness, making the molecule adopt an upright posture in the membrane (Knecht and Grubmüller 2003), while the synaptobrevin-2/VAMP2 linker is more flexible and might be partly inserted into the membrane (Ellena et al. 2009; Kweon et al. 2003).

Experiments in chromaffin cells and cultured neurons have shown that insertion of additional flexible sequences of 2–8 amino acids in the synaptobrevin-2/VAMP2 linker compromises vesicle priming, induces a longer delay before secretion starts, and slows down release from individual vesicles (Bretou et al. 2008; Guzman et al. 2010; Kesavan et al. 2007). Surprisingly, in chromaffin cells expressing a synaptobrevin-2/VAMP2 with a 6-amino acid addition in linker, secretion – when it got under way after a longer delay – was as fast as in the wild-type situation (Kesavan et al. 2007). With a linker encompassing 22 additional amino acids, secretion was indistinguishable from synaptobrevin-2/VAMP2 knockout cells (Kesavan et al. 2007). In neurons, insertion of three or seven amino acids in the linker of syntaxin-1 eliminated the ability to support evoked release (Zhou et al. 2013a). Further studies have identified two tryptophans in the synaptobrevin-2/VAMP2 linker as essential in imposing a fusion clamp, which favors evoked rather than spontaneous fusion (Fang et al. 2013; Maximov et al. 2009). Overall, there is little doubt that the linkers of syntaxin and synaptobrevin-2/VAMP2 are important for transducing force to the membranes as part of the evoked release mechanism (see discussion of spontaneous release below).

In a very recent and fascinating study, it was demonstrated that replacing the TMDs of both syntaxin and synaptobrevin-2/VAMP2 with lipid anchors, which inserted into only one membrane leaflet, still allowed a substantial amount of fast evoked release from central neurons (Zhou et al. 2013a). This was unexpected, because a previous study performed *in vitro* was unable to reconstitute fusion upon anchoring the SNARE domains to lipids (McNew et al. 2000). One important

difference between the studies – apart from the obvious difference between *in vitro* studies and studies carried out in cells – is that in the 2000 study, SNARE domains were linked to single lipids, whereas in the 2013 study, the SNAREs were linked to longer palmitoylation stretches, which presumably conferred a much more solid lipid anchor, consisting of several lipid moieties, onto the SNAREs. These considerations are thus in line with the idea that the main function of the TMD regions of the SNAREs is to transfer force to the membranes, whereas the details of how this is achieved appear to be of secondary importance.

Likewise, the finding that fast release was still present, although depressed in magnitude, after insertion of 4–5 amino acids in the linker of synaptobrevin-2/VAMP2 (Kesavan et al. 2007), indicates that the exact properties of the synaptobrevin-2/VAMP2 linker – while obviously important to optimize release of neurotransmitter – are not crucial to obtain membrane fusion *per se*. It is interesting to compare linker mutations with mutations in the C-terminal layers of the SNARE complex, where even single-point mutations, or deletions of single amino acids, lead to severe phenotypes *in vivo* (Criado et al. 1999; Gil et al. 2002; Sorensen et al. 2006; Walter et al. 2010) and *in vitro* (Hernandez et al. 2012; Siddiqui et al. 2007). Thus, it appears that although the stability and detailed topology of the SNARE bundle are very important, linker domains and transmembrane domains are less restricted. Nevertheless, analysis of single-vesicle fusion events has shown that the SNAREs add force to drive membrane fusion not only during fusion pore formation but also during the subsequent expansion of the fusion pore (Bretou et al. 2008; Guzman et al. 2010; Kesavan et al. 2007).

Thus, within the wild-type proteins, the SNARE TMDs are likely to play an important role in fusion pore formation and expansion. Deletion of the C-terminal half of the synaptobrevin-2/VAMP2 TMD was found to suppress secretion in PC12 cells (Fdez et al. 2010), possibly due to arrest in the hemifused state. Dimerization of the TMD of synaptobrevin-2/VAMP2 has also been demonstrated (Laage and Langosch 1997; Laage et al. 2000), but the mutation that eliminated dimerization did not inhibit fusion (Fdez et al. 2010). Addition of one or two amino acid residues to the very C-terminal (i.e., the intravascular) end of synaptobrevin-2/VAMP2 inhibited fusion in chromaffin cells, according to the transfer energy of the residues from water to the membrane (Ngatchou et al. 2010). This led to the suggestion that the C-terminal end of the TMD is pulled into the membrane – driven by formation of the SNARE complex – leading to disruption of the hemifused stalk or membrane, and fusion pore formation.

Simulations have provided important insights into the final events leading up to membrane fusion [reviewed in (Markvoort and Marrink 2011; Risselada and Grubmuller 2012)]. Molecular dynamics simulations confirmed experimental findings that a single or a few SNARE complexes can drive fusion and identified important roles for the SNARE linkers and TMRs in inducing lipid disordering that eventually leads to stalk formation (Risselada et al. 2011). Following formation of the hemifused state, the C-terminal ends of the TMRs help formation of the fusion pore, which releases bending stress in the SNAREs by placing both charged C-terminal ends into the hydrophilic pore (Lindau et al. 2012; Risselada

et al. 2011), consistent with the crystal structure (Stein et al. 2009). One simulation also identified important roles for dimerization (Risselada et al. 2011). These findings all contrast to a certain degree with the experimental evidence mentioned above that dimerization is not necessary and that the TMD and linkers can be manipulated without losing fusogenicity. However, it is important to remember that until molecular dynamics simulations have been carried out on these mutants, it is unclear to what extent they might have furnished the system with alternative pathways of fusion that might substitute for specific endogenous properties.

In conclusion, there is compelling – even overwhelming – evidence to conclude that SNARE-complex formation liberates energy, which is transferred to the membrane through the linkers and TMD as a corresponding force to drive membrane fusion. This force participates in closely aligning the membranes, leading to dehydration of the lipid head groups, at which point the fusion process becomes governed by poorly characterized lipid-protein and lipid-lipid interactions. It appears most likely that the subsequent events are lipid splaying, stalk formation, and the formation of a hemifusion intermediate, which gives way to the formation of a fusion pore and release of the vesicular content [for a model incorporating a transient proteinaceous pore, see (Jackson 2010)]. The SNAREs continue to drive fusion throughout these stages, but it seems likely that the last part of the process can proceed in several different ways.

4.3 Disassembly and Recycling of the SNARE Proteins

After synaptic vesicle fusion, the tight *cis*-SNARE complexes need to be disassembled, thereby recycling the SNAREs for subsequent fusion reactions (Fig. 4.4). The SNARE-complex disassembly process is achieved by the action of a complex consisting of the ATPase NSF and its cofactors α - β - γ -SNAP (Sollner et al. 1993a) (Table 4.1). α -SNAP is known to bind to the *cis*-SNARE complexes and, in turn, to recruit and activate NSF, which liberates individual SNAREs from the complex. α -SNAP and its two homologues, β - and γ -SNAP, were originally discovered as factors required for recruiting NSF to membranes in cell-free transport assays (Clary and Rothman 1990; Whiteheart et al. 2001). Both NSF and α -SNAP have been shown to actively participate in all intracellular processes involving membrane fusion.

In spite of the fact that SNARE proteins are in vast excess, they still may become limiting during the vesicle cycle if they are not rapidly recycled. The best example is given by the analysis of a temperature-sensitive mutation in the *Drosophila* homologue of NSF, *comatose* or dNSF-1, which revealed pronounced synaptic depression during repetitive stimulation (Littleton et al. 1998, 2001). These and other data (Banerjee et al. 1996; Xu et al. 1999a) led to the realization that NSF is not necessary for fusion per se but required to recycle the SNAREs. Likewise, it was recently reported that mammalian neurons without α -SNAP and with a hypomorphic β -SNAP level displayed additional rundown during phases of high

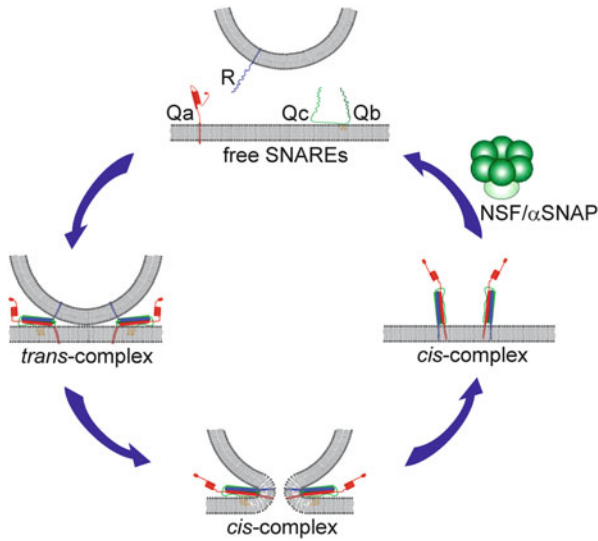


Fig. 4.4 Schematic of the canonical SNARE protein cycle. SNARE components: syntaphin-1 (*red*), SNAP-25 (*green*), and synaptobrevin-2/VAMP2 (*blue*). Membrane fusion through formation of a *trans*-SNARE complex proceeds from a loose state (in which only the N-terminal portion of the SNARE motifs is “zipped up”) to a tight state (in which the zipping process is mostly completed), and this is followed by the opening of the fusion pore. During fusion, the *trans*-complex relaxes into a *cis*-configuration. Dissociation of the *cis*-complex and repriming requires the energy input of ATP hydrolysis and is achieved through the binding of the ATPase protein NSF (N-ethylmaleimide-sensitive factor) together with SNAPs (soluble NSF attachment proteins) that function as cofactors

synaptic activity (Burgalossi et al. 2010), demonstrating the importance of NSF’s cofactors in priming new vesicles for release.

While *cis*-SNARE-complexes should act as substrates for α - β - γ -SNAP and NSF in order to be disassembled for another round of fusion, *trans*-SNARE complexes should be resistant, lest their disassembly would block fusion. Different mechanisms have been suggested to protect *trans*-SNARE complexes from disassembly, including steric hindrance by the membrane or resistance of partially assembled complexes to SNAP binding (Weber et al. 2000). In a recent study, it was found that formation of the *trans*-SNARE complex within the confined environment of Munc18 and Munc13 protects *trans*-SNARE complexes from NSF-mediated disassembly (Ma et al. 2013).

Generally, it is believed that the activity of NSF is so high that it acts soon after fusion to disassemble the resultant *cis*-SNARE complexes and to liberate the SNAREs that drove the merger of membranes. Consequently, in the resting state most SNAREs in the membrane of neurosecretory cells are uncomplexed (Lang et al. 2002), and when blocking the action of NSF, secretion runs down only slowly, whereas blocking the function of syntaxin leads to a much faster block of secretion

(Littleton et al. 1998). However, there is some evidence that NSF and α - β -SNAP could have an acute function immediately prior to vesicle fusion (Burgalossi et al. 2010; Kuner et al. 2008). This was taken to indicate that under some conditions, α -SNAP/NSF-dependent priming occurs immediately prior to the fusion step and is needed to free the SNAREs from the inactive *cis*-complex state (Kuner et al. 2008). However, it is also possible that NSF and α - β -SNAP interact with the *trans*-SNARE complex in a way which has yet to be determined.

4.4 SNAREs, Lipids, and Membrane Fusion

Apart from the SNAREs and associated proteins, other cellular factors also regulate the kinetics, the extent of fusion and the preparation of vesicle for release. Among those factors, membrane lipids are especially noteworthy [for review see (Chasserot-Golaz et al. 2010; Darios et al. 2007)]. Since lipids are the main constituents of the fusing membranes, modifying lipids can directly change the intrinsic fusogenic properties of membranes. In addition, lipids act to recruit and/or activate a large number of different proteins to create a local environment in which exocytosis takes place.

Although it has been known since the 1950s that stimulation of pancreatic cell secretion leads to increased phosphorylation of phosphatidylinositides (PIs) (Hokin and Hokin 1953), it is still an ongoing work to characterize all the PI molecules that are needed for the exocytic process. This is partially due to the PI versatility given that the inositol head group of PI can be reversibly phosphorylated at various positions, resulting in seven naturally occurring PIs. All PIs show distributions restricted to well-characterized membrane territories and can be rapidly interconverted by specialized lipid kinases and phosphatases, which add or remove specific phosphate groups; some forms are also broken down by phospholipases (Cremona and De Camilli 2001; Di Paolo and De Camilli 2006).

PI(4,5)P₂ is a key PI player in regulated exocytosis as well as endocytosis in neurons and neuroendocrine cells [reviewed by (Cremona and De Camilli 2001; Martin 2001; Saheki and De Camilli 2012)]. The canonical pathway of PI metabolism places PI(4)P as the precursor of PI(4,5)P₂. In this pathway, PI4-kinases (PI4Ks) phosphorylate PI to produce PI(4)P, which then serves as a substrate for PIP5-kinases (PI5Ks). Two types of PIP5Ks are responsible for the PI(4,5)P₂ synthesis and each exists as several isoforms. For example, phosphatidylinositol 4-phosphate 5-kinase I γ (PI4P5K-I γ) is the major isoform that produces PI(4,5)P₂ at the neuronal active zone (Wenk et al. 2001). To account for the complex demands for PI(4,5)P₂ at the active zone, PI4P5K-I γ is tightly regulated by Ca²⁺, Arf6, phosphorylation, and phosphatidic acid, the product of phospholipase D activity [see also below; (Aikawa and Martin 2003; Fruman et al. 1998)].

The first clue suggesting a direct role of PI(4,5)P₂ in regulated exocytosis came from studies on permeabilized chromaffin and PC12 cells which showed that PI(4,5)P₂ was required for an ATP-dependent priming step preceding Ca²⁺-triggered

fusion (Eberhard et al. 1990; Hay et al. 1995). A search for the cytosolic factors required for this energy requiring priming step in permeabilized neuroendocrine cells led to the identification of two enzymes involved in the PI metabolism: a phosphatidylinositol transfer protein (PITP; (Hay and Martin 1993)) and a PI4P5K (Hay et al. 1995). A model was suggested in which PI was delivered to the vesicle membrane via PITP, phosphorylated to PI(4)P by vesicular protein PI4K-II, and finally converted to PI(4,5)P₂ by PI4P5K recruited from the cytoplasm (Hay et al. 1995). In subsequent work it was observed that the PI(4,5)P₂-binding PH domain from PLCδ₁ became localized to the plasma membrane and inhibited Ca²⁺-dependent exocytosis in chromaffin cells (Holz et al. 2000). Further, overexpression of the PI4P5K-Iγ caused an increase in the plasmalemmal PI(4,5)P₂ level and the primed vesicle pool, whereas overexpression of a membrane-tagged PI(4,5)P₂ phosphatase eliminated plasmalemmal PI(4,5)P₂ and inhibited secretion, showing that the balance between the generation and degradation rates of the plasmalemmal PI(4,5)P₂ directly regulates vesicle priming (Milosevic et al. 2005). Dual roles of PI(4,5)P₂ in both exocytosis and endocytosis suggest that this lipid may control the plasma membrane trafficking and a model in which a PI cycle is nested within the secretory vesicle cycle was proposed (Cremona and De Camilli 2001).

Many proteins involved in regulated exocytosis have been shown to interact with PI(4,5)P₂ in vitro, and based on these interactions, it can be postulated that PI(4,5)P₂ has a function in vesicle docking, in priming, and in particular the fusion reaction. Most notably, PI(4,5)P₂ binds to synaptotagmin family members and CAPS proteins (Bai et al. 2004a; Loyet et al. 1998; Schiavo et al. 1996; van den Bogaart et al. 2012). Other relevant interactions include binding of PI(4,5)P₂ to Mints, which bind Munc18s and are implicated in docking (Okamoto and Sudhof 1997), and rabphilin 3, an effector of Rab3 proteins, which might control SNARE-complex formation (Chung et al. 1998). Molecular details of how PI(4,5)P₂ forms a platform for vesicle recruitment have recently been proposed (Honigsmann et al. 2013). Specifically, synaptotagmin-1 was shown to interact independently of calcium with the polybasic linker region of syntaxin-1 already associated with PI(4,5)P₂ at the plasma membrane. This interaction might cause vesicle docking at least in vitro (Kim et al. 2012).

Besides PI(4,5)P₂, PI hydrolysis products or other PIs may act as recruitment or signaling factors to prime secretory vesicles for fusion. Diacylglycerol (DAG) production through hydrolysis of PI(4,5)P₂ by phospholipase C is now considered to be needed for the priming process, owing to the activation of protein kinase C and Munc13, which then modulate the function of syntaxin-1 (Bauer et al. 2007). DAG is further hydrolyzed by DAG lipases to liberate fatty acids and monoacylglycerols. PI(3)P is located on a subpopulation of neurosecretory vesicles and positively regulates secretion (Meunier et al. 2005). In addition, PIKfyve kinase that can produce PI(3,5)P₂ from PI(3)P on secretory vesicles has been proposed to negatively affect exocytosis (Osborne et al. 2008), yielding an opposite effect and revealing how fine-tuning of membrane fusion by PIs can potentially control the number of vesicles undergoing priming. Finally, synaptic PI(3,4,5)P₃ has recently been shown to contribute to syntaxin clustering and exocytosis (Khuong et al. 2013).

Phosphatidylserine (PS) and cholesterol are involved in the spatial definition of exocytotic sites [recently reviewed by (Ammar et al. 2013)]. In the plasma membrane, PS is mainly present in the inner membrane leaflet and it contributes substantially to its negative charge. PS is necessary for synaptotagmin binding and thus for fusion triggering (Zhang et al. 2009; Zhang and Jackson 2010). It was recently found that exocytosis is associated with outward translocation of PS, which in turn is required for compensatory endocytosis (Ory et al. 2013). Cholesterol depletion provided a clue for a role of cholesterol in neurosecretory cell exocytosis (Chamberlain et al. 2001), which was supported by additional biochemical and imaging experiments implying that SNARE proteins concentrate in cholesterol-dependent clusters (Lang et al. 2001).

Growing evidence also supports a role for phosphatidic acid (PA) during exocytosis: the local formation of PA by phospholipase D1 underneath the vesicle regulates the fusion competency of secretory vesicles docked at the plasma membrane of neurosecretory cells, suggesting a direct role in membrane fusion (Vitale et al. 2001; Zeniou-Meyer et al. 2007). Because PA is a cone-shaped lipid, it will promote the formation of bend lipid structures displaying negative curvature, which is required during formation of the hemifusion state. Several constituents and regulators of the fusion machinery have also been shown to bind to PA, including NSF, small GTPases, and syntaxin-1 (Jang et al. 2012). As mentioned above, PA is an essential cofactor of PI4P5K-I γ , which produces PI(4,5)P₂, which in turn recruits and activates phospholipase D, suggesting a positive feedback loop in the synthesis of PI(4,5)P₂ and PA (Jang et al. 2012).

Finally, fatty acids have been proposed to play an important function in membrane fusion. Arachidonic acid, omega-3, and omega-6 unsaturated fatty acids were found to directly promote SNAP-25/syntaxin-3 assembly and the formation of the ternary SNARE complex leading to dendrite expansion (Darios and Davletov 2006). In chromaffin cells, arachidonic acid promoted vesicle docking and increased quantal size (Garcia-Martinez et al. 2013). It remains to be seen whether endogenous levels of free fatty acids suffice to stimulate the SNARE mechanism.

In summary, lipids play multiple roles in membrane fusion, acting either individually, sequentially, or simultaneously with other lipids. The rapid enzymatic production and degradation of lipids has the potential to modify the physiological function at the synapse within seconds or minutes without the need for protein synthesis/degradation. Further studies will be needed to understand the interplay between lipids and SNAREs in regulation membrane fusion.

4.5 Noncanonical SNAREs in Synaptic Transmission

Spontaneous miniature release is the release of single neurotransmitter quanta in the absence of an action potential. Even though most spontaneous release events are triggered by calcium – similar to evoked release – there is much evidence to show that spontaneous events are subject to separate regulation and that at least some of

the vesicle fusion events follow a different mechanistic route [for a recent review, see (Ramirez and Kavalali 2011)]. Although spontaneous release cannot transfer time-locked information from one neuron to another, it can nevertheless be important for controlling firing in the postsynaptic cell (Carter and Regehr 2002). Whether the fusion machinery and the vesicles themselves are different from those that support evoked release is, however, controversial.

Knockout of SNAP-25 or synaptobrevin-2/VAMP2 almost eliminates evoked release, whereas spontaneous release is affected much less (Bronk et al. 2007; Deitcher et al. 1998; Delgado-Martinez et al. 2007; Schoch et al. 2001; Schulze et al. 1995; Washbourne et al. 2002). Indeed, normalizing spontaneous release in SNAP-25 and synaptobrevin-2/VAMP2 knockouts to the primed vesicle pool would lead to frequencies at least as high as in wild-type neurons (Bronk et al. 2007; Delgado-Martinez et al. 2007; Schoch et al. 2001). The most likely explanation for this is substitution by non-cognate SNAREs, which form complexes with syntaxin-1 and support spontaneous release. For instance, exogenously expressed SNAP-23 can fully restore spontaneous release in SNAP-25 KO neurons, whereas evoked release is strongly asynchronous (Delgado-Martinez et al. 2007). As another example, endogenous or exogenous cellubrevin effectively substitutes for synaptobrevin-2/VAMP2 in its absence, although it seems to play no role in the presence of synaptobrevin-2/VAMP2 (Borisovska et al. 2005; Deak et al. 2006). These findings imply that SNAREs are partly interchangeable in the cell as long as the 3Q:R-rule is observed (Fasshauer et al. 1998b), but only a few of them – the neuronal SNAREs – are able to effectively link to complexin and synaptotagmin to support evoked release. In contrast, knockout or reduction of the syntaxin-1 level appears to reduce both spontaneous and evoked release in parallel (Arancillo et al. 2013; Stewart et al. 2000; Zhou et al. 2013a). Thus, syntaxin-1 might be special and not amenable to substitution. The most likely explanation is the structure of this SNARE, combined with the interaction with Munc18-1 and Munc13 proteins, which are necessary to open up syntaxin-1 as an obligatory part of the vesicular priming machinery.

Likewise, mutation of SNAP-25 and synaptobrevin-2/VAMP2 often leads to milder phenotypes for spontaneous release than for evoked release. This is true for insertions in the synaptobrevin-2/VAMP2 linker domain (Deak et al. 2006; Guzman et al. 2010) and for deletion of two phenylalanines within the linker (Fang et al. 2013; Maximov et al. 2009). Furthermore, mutations inside the SNARE bundle of SNAP-25 around the middle of the complex increased spontaneous release rates (Weber et al. 2010). These and other findings indicate that the structural requirements for spontaneous release are easier to fulfill than those for evoked release. In contrast, upon destabilizing mutation in the C-terminal end of the SNAP-25 SNARE motif, spontaneous release suffered even more than evoked release (Weber et al. 2010). Conversely, a mutation in syntaxin that tightened the C-terminal end of the SNARE complex led to an increase of both evoked and spontaneous release (Lagow et al. 2007). These findings indicate that an obligatory prerequisite for spontaneous release is the firm assembly of the C-terminal end of the SNARE bundle, just as for evoked release. Since this is the process that initiates

lipid splaying and stalk formation (see above), the membrane fusion pathway itself might be conserved between spontaneous and evoked release.

What is then the difference between evoked and spontaneous release in terms of SNARE-complex assembly? As explained above, the prerequisite for evoked release is the presence of a fusion clamp, which is most likely engaged in the partly zippered SNARE complex, preventing C-terminal assembly. This clamp might include complexin and synaptotagmin, or the repulsion between the membranes, but it is likely that it also depends on the details of the SNARE-complex assembly pathway. Firm N-terminal assembly might stabilize the vesicle in a trough in the energy landscape, which sets up an additional energy barrier for fusion compared to the unprimed vesicle. Conversely, a looser assembly of the N-terminal end might allow the complex to “skip over” the clamped state, progressing directly to C-terminal assembly and release (Weber et al. 2010). This might explain why mutations will often disinherit spontaneous release, whereas evoked release is much more susceptible to mutation and substitution by other SNAREs.

Synaptic vesicles contain several other SNAREs in addition to synaptobrevin-2/VAMP2, including vti1a and VAMP4 (Antonin et al. 2000b; Raingo et al. 2012; Ramirez et al. 2012; Takamori et al. 2006). In recent years, it has been found that these SNAREs might participate in synaptic vesicle fusion, which results in either spontaneous or asynchronous release. VAMP-4 expression is able to restore evoked release in synaptobrevin-2/VAMP2 knockout neurons, but the resulting release is asynchronous release, which is more susceptible to the calcium buffer EGTA (Raingo et al. 2012). Conversely, knockdown of VAMP-4 under some circumstances attenuated asynchronous release, and using pHluorin assays, it was shown that VAMP-4 traffics independently of synaptobrevin-2/VAMP2 (Raingo et al. 2012). Finally, it was shown that VAMP-4 – as a R-SNARE – is able to substitute for synaptobrevin-2/VAMP2 and form a SNARE complex with syntaxin-1 and SNAP-25; however, this SNARE complex did not bind complexin or synaptotagmin-1, which accounts for the asynchronicity of release.

Vti1a is a Q_b -SNARE and is therefore expected to take up the same position in a SNARE complex as the N-terminal domain of SNAP-25. It has been mainly associated with intracellular fusion events, such as endosome fusion and endosome-to-Golgi fusion (Brandhorst et al. 2006; Mallard et al. 2002). Using pHluorin fusion proteins, one group concluded that vti1a traffics on spontaneously fusing synaptic vesicles, and knockdown of vti1a reduced the frequency of spontaneous release (Ramirez et al. 2012). However, vti1a-pHluorin-carrying vesicles fused during prolonged stimulation trains, although this was less prominent than for synaptobrevin-pHluorin (Hoopmann et al. 2010; Ramirez et al. 2012), showing that vti1a-carrying vesicles are not entirely resistant to calcium. It was concluded that vti1a is resident on vesicles that do not contain synaptobrevin-2/VAMP2 (Ramirez et al. 2012). It remains to be understood what the vti1a SNARE partners for this fusion pathway would be, since synaptobrevin-2/VAMP2 as an R-SNARE is not expected to be replaceable by a Q_b -SNARE. VAMP7/TI-VAMP is an R-SNARE belonging to the longin family of R-SNAREs with extended N-terminal domains (Filippini et al. 2001). VAMP7 was identified as another

marker for the resting vesicle pool, vesicles of which cycle only spontaneously or reluctantly (Hua et al. 2011). However, differences in the cycling between VAMP-7 and vti1a make it unlikely that they reside in the same vesicle population or participate in the same SNARE complex (Ramirez and Kavalali 2012). Indeed, it was recently shown that Reelin, a secreted glycoprotein, stimulates spontaneous release by specifically recruiting VAMP-7 – but not vti1a containing – vesicles (Bal et al. 2013).

An open question is how dominant a role the noncanonical SNAREs play in asynchronous and spontaneous release under physiological conditions, given that the neuronal SNAREs – syntaxin-1, synaptobrevin-2/VAMP2, and SNAP-25 – are very abundant and also participate in those events, and knockout or expression of mutants of the neuronal SNAREs can eliminate most spontaneous events and asynchronous release in cultured neurons. Most likely, the relative expression levels and/or separation into different vesicle pools will determine the relative participation of the different SNAREs in fusion. In this scenario, the noncanonical SNAREs might play their largest role in minority neuron populations displaying stronger expression; for instance, VAMP-4 was found to be strongly expressed in CCK-interneurons (Raingo et al. 2012), which are known to display strongly asynchronous release (Hefft and Jonas 2005). Much more research is needed before the importance of the noncanonical SNAREs in synaptic transmission can be assessed.

4.6 Membrane Fusion and SNARE Complex: Beyond 2014

Most of the research work described in this review has been designed to explore the properties and mechanistic action of one or a few specific components of the membrane fusion machinery. These types of studies have been essential to understand how the SNARE machinery works and are leading the field towards a stage of maturity in which the inner workings of the fusion machinery in the living cell can be described concisely and reconstituted *in vitro*. A few key questions that will still have to be answered are:

- What is the minimal essential function of Munc18 in membrane fusion?
- Is the stoichiometry of SNARE complexes participating in fusion regulated, and is it predetermined or stochastic?
- What is the nature of the fusion pathway(s) downstream of SNARE-complex assembly?

While the basic discoveries in membrane fusion and SNAREs emerged from biochemical and electrophysiological studies, continuous progress in this research field will be driven by new technologies and methodologies. In recent years, new genomic, biophysical (computational and molecular modelling), and imaging (super-resolution microscopy) approaches have pointed to new properties of membrane fusion and SNARE-complex action. In the future, expression from

endogenous loci of SNARE proteins bearing genetically encoded fluorescent tags will likely provide new ways to study SNAREs and mechanisms of membrane fusion in space and time in living cells and organisms. Some of the key questions for this new phase of discovery are:

- How, and at which stage, is the SNARE mechanism regulated – and how are different levels of regulation (for instance, expression level, phosphorylation, Munc13-activation, and inhibition by tomosyn) integrated?
- How does regulation of the SNARE mechanism result in tissue- and cell-specific release properties?
- What role do the basal biophysical properties of the SNARE mechanism (and its regulation) play in the context of neuronal networks, and how does the SNARE mechanism influence the working of the brain in health and disease?

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