Chapter 3
Roles of SNARE Proteins in Synaptic Vesicle Fusion

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Contents

Abstract Neurotransmitters are stored in small membrane-bound vesicles at synapses. Neurotransmitter release is initiated by depolarization of the neuron, which in turn activates voltage-gated calcium channels. Calcium influx then triggers the fusion of the synaptic vesicles with the plasma membrane. Fusion of the vesicular and plasma membranes is mediated by SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment receptor) proteins. The SNAREs are now known to be used in all trafficking steps of the secretory pathway, including neurotransmission. This chapter describes the discovery of the SNAREs, their relevant structural features, models for their function, the specificity of interactions, and their interactions with the calcium-sensing machinery.

Keywords SNARE, syntaxin, SNAP-25, synaptobrevin, membrane fusion

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SNARE Discovery: A Convergence of Genetics and Biochemistry

To understand the mechanisms of synaptic vesicle fusion, it is useful to think about the evolution of neurotransmission. Eukaryotic cells separate cellular functions into membrane-bound organelles. The content of these organelles are moved between compartments and the extracellular environment by transport vesicles. Cellular compartments must be kept distinct, but membrane-impermeable cargo must be transferred to the target organelle. To transfer cargo the lipid bilayers of the vesicle and the target must merge so that their luminal contents can intermingle. In some cases, cargo must be secreted into the extracellular space via exocytosis. It was perhaps a small step for the cell to develop a mechanism for calcium-dependent regulation of exocytosis, but it was a giant leap for evolution. The nervous system is arguably the universe's greatest invention.

A convergence of independent tracks led to the identification of SNAREs as the central players in membrane fusion. In the late 1980s SNARE proteins were identified in the brain as components of the synapse. Specifically, synaptobrevin (also called vesicle-associated membrane protein [VAMP]) was purified from synaptic vesicles (1). Subsequently, two additional SNAREs, syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), were found localized to the plasma membrane of neurons (2–4). The identification of homologues among the yeast *sec* genes linked the mechanisms of synaptic function to vesicular trafficking (5,6) and hinted at the universality of membrane fusion. Although the SNARE proteins were well placed to mediate synaptic vesicle fusion and were related to proteins required for trafficking, there was at this point no evidence that these proteins functioned in calcium-dependent exocytosis of synaptic vesicles.

The groups of Heiner Niemann, Reinhard Jahn, and Cesare Montecucco were looking for the targets of the clostridial toxins. The clostridial toxins from the anaerobic bacteria *Clostridium botulinum* and *Clostridium tetani* can potently inhibit neurotransmission (7). Thus, it was reasoned that their targets would identify essential proteins in synaptic transmission. Botulinum and tetanus toxins cleave the SNARE proteins, demonstrating the central role of the SNAREs in synaptic vesicle release (8–11). These were the first functional data that the SNAREs were involved in neurotransmission (12,13). The central role of the SNAREs in neurotransmission would later be confirmed from electrophysiologic studies on null mutants in the SNARE proteins in *Drosophila*, mice, and *Caenorhabditis elegans* (14–19). Thus, the functional data identified the SNAREs as perpetrators but their association had not been described.

The discovery that these proteins formed a complex was demonstrated soon after. Jim Rothman's group was taking a biochemical approach to understand trafficking in the Golgi apparatus. The toxin *N*-ethylmaleimide (NEM) potently inhibits Golgi trafficking (20). Wilson et al (21) found that the target of NEM was the mammalian homologue of a previously cloned yeast gene *SEC18* (22). Rothman's group named this new protein the NEM-sensitive factor (NSF) (23), and NSF was

found to bind, via the action of the soluble NSF adaptors (SNAPs) (24), to a set of proteins from brain detergent extracts that came to be collectively known as the soluble *N*-ethylmaleimide–sensitive factor attachment receptor proteins (SNAREs). The evidence for SNARE involvement in synaptic vesicle exocytosis was now overwhelming, but a list of names in a complex did not constitute a model.

The first coherent model, called the SNARE hypothesis, would arise from the melding of the genetic and biochemical observations described above. Although wrong in detail, it would catalyze a number of hypothesis-driven experiments that would lead to more accurate models. Based on the finding that unique SNAREs are found at each of the trafficking steps (25,26), Thomas Söllner and Jim Rothman proposed that SNARE interactions provided the specificity for vesicular trafficking by tethering the vesicle to its target membrane (27,28). The SNAREs would then be acted on by the adenosine triphosphatase (ATPase) NSF which, by disassembling the SNAREs, would drive fusion (27,29).

Further experiments from Bill Wickner's lab, using a purified vacuole fusion assay, demonstrated that NSF acted not at the final step of fusion, but rather to recover monomeric SNAREs for use in further rounds of fusion (28,29,30,31). NSF was acting as a chaperone to separate the embracing SNAREs on the plasma membrane to reactivate the system for further fusion (32,33). Thus assembly of the SNAREs, not disassembly, catalyzes fusion.

Finally, Rothman's group demonstrated that the SNAREs alone could fuse membranes. The SNAREs were incorporated into vesicles composed of artificial lipid bilayers. Donor vesicles containing synaptobrevin were capable of fusion with acceptor vesicles containing syntaxin and SNAP-25 (34). This experiment was extended to native membranes by engineering SNAREs to face out of the cell; in this configuration the SNAREs could induce fusion of whole cells (35). Thus, the current thinking is that the SNAREs function in the final steps in fusion and represent the minimal fusion machinery.

In the following sections we briefly define the steps leading to fusion, introduce the structure of the SNARE proteins, present a model for fusion, discuss SNARE specificity, and finally touch on the regulation of the SNARE complex by other proteins.

Definitions: The World Turned Upside Down and Given a Good Shake

In the past, synaptic vesicles were thought to dock with the plasma membrane, and then undergo a maturation step in which they became release ready. Depolarization activated a calcium sensor that then allowed the vesicle to fuse with the plasma membrane. Only a subset of docked vesicles were considered to be in the readily releasable pool (36). Thus, the life of a vesicle could be divided into four steps: docking, maturing to release-ready, calcium sensing, and fusing. The definition of these stages in vesicle fusion relied on morphologic and electrophysiologic criteria.

Current studies have sought to associate these pools with particular molecular interactions and thereby more precisely define these states.

Paradoxically, recent studies have tended to confuse rather than clarify the states of a vesicle. Although some have argued that very few docked vesicles are in the readily releasable pool (36,37), others suggest that docked vesicles are equivalent to the readily releasable pool (38–41). Studies of SNARE proteins have also muddied our previously clean definitions of these pools. The assembly of SNARE proteins between synaptic vesicle and plasma membrane is defined as vesicle "priming." Initial studies suggested that priming occurred after docking (15). However, recent studies suggest that the primed state may correspond to "docked" vesicles as observed in electron micrographs (14). Thus, the morphologic, electrophysiologic, and molecular definitions have seemingly converged on a single state. It is hoped that as the actions of various proteins are more precisely understood, we will once again refine synaptic vesicle fusion into discrete steps.

There is one last sorry note concerning our attempts to define steps in vesicle fusion: the terminology used for synaptic vesicle fusion is at odds with the terminology used in yeast. In yeast, *priming*" refers to the generation of free SNAREs rather than the formation of the SNARE complex, *tethering* rather than *docking* describes the initial membrane association, and *docking* includes SNARE engagement. Only the word *fusion* seems to mean the same thing in these different languages.

Molecular Characteristics of the SNAREs

The SNARE proteins are characterized by a conserved 60- to 70-amino-acid SNARE motif. Phylogenetic analysis indicates that SNARE proteins can be divided into four families (25,42,43). The individual SNARE motifs are largely unstructured in solution, but when all four family members are mixed, the SNARE motifs come together to form a four-helix parallel bundle known as the core complex (Fig. 3.1A,B) (44). The SNARE complex is remarkably stable and can only be separated by boiling in the presence of sodium dedocyl sulfonate (SDS) (45,46). The hydrophobic residues of the alpha-helical SNARE motifs are oriented inward to form layers like those in the coiled coil domains of classical leucine zippers. However, the layer in the middle of the complex, called the "0" layer, is formed by ionic interactions between an arginine (R-SNARE) and three glutamines (Qa, Qb, and Qc SNAREs) (Fig. 3.1B,C). The role for these conserved residues buried in the hydrophobic core is briefly discussed in the next section. At each fusion site a unique SNARE complex consisting of all four flavors is formed. While other complexes have been observed in vitro, the only complexes that have been shown to efficiently support fusion are QabcR complexes (47–52).

The SNAREs that are used for synaptic vesicle exocytosis are synaptobrevin (R-SNARE, also called VAMP2), syntaxin 1a (Qa SNARE), and SNAP-25 (contains both the Qb and Qc SNARE motifs) (Fig. 3.1) (1–4,53).

In addition to the SNARE motifs, all three SNAREs contain sequences that anchor them to the membrane (Fig. 3.1A). Syntaxin and synaptobrevin are anchored

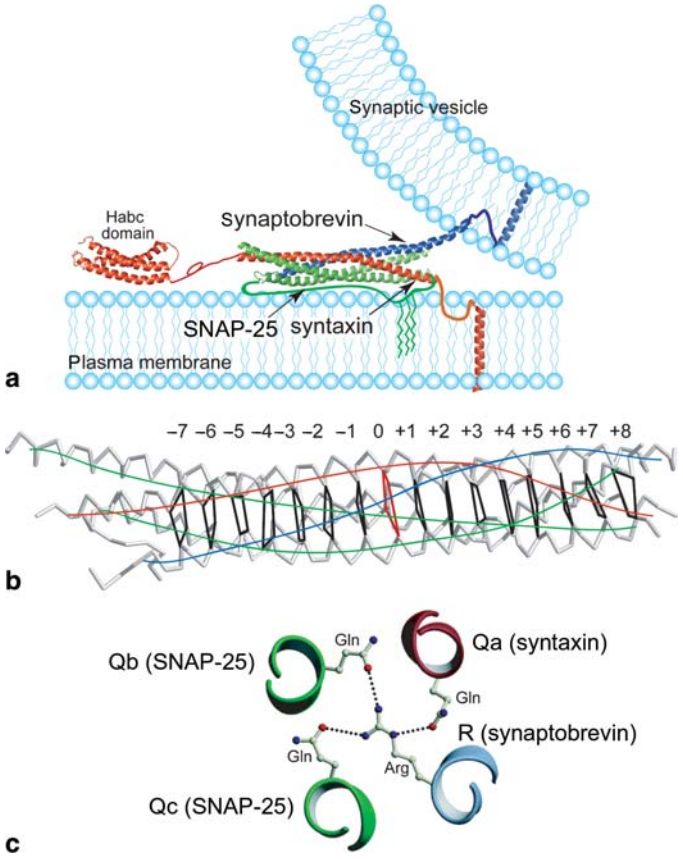

Fig. 3.1 Molecular description of the SNAREs. By assembling into a four-helix parallel bundle, the SNAREs bridge the gap between the two membranes destined to fuse. **(a)** In the case of the neuronal SNAREs, syntaxin (red) and SNAP-25 (green) are found on the plasma membrane and synaptobrevin (blue) is associated with the synaptic vesicle. The 60- to 70-amino-acid SNARE motifs form a four-helix bundle. Syntaxin and synaptobrevin contribute one SNARE motif and SNAP-25 contributes two. Syntaxin contains an additional regulatory domain composed of three alpha-helices called the Habc domain. Syntaxin and synaptobrevin are transmembrane proteins, while SNAP-25 is attached to the membrane via palmitoylation of the linker region. **(b)** The wire frame model shows the backbone of the SNARE motifs. The N-termini are at the left and the C-termini are at the right, matching the illustration in (A). The amino acids facing toward the center of this helix (denoted as layers −7 to +8) are largely hydrophobic in nature with the notable exception of the zero layer. **(c)** In the zero layer charged residues are oriented toward the center of the helix. Syntaxin contributes one glutamine (Qa), SNAP-25 contributes two glutamines (Qb and Qc), and synaptobrevin contributes one arginine (R). (A: Courtesy of Enfu Hui and Edwin R. Chapman. B: Adapted from Fasshauer et al [40]. C: Adapted from Bracher et al [193].)

via transmembrane domains. SNAP-25 is anchored via the palmitoylation of cysteines in the linker region connecting the two SNARE motifs. In all SNARE-based fusion reactions, each of the two membranes destined to fuse must contain a SNARE with a transmembrane domain; otherwise fusion will not occur (54).

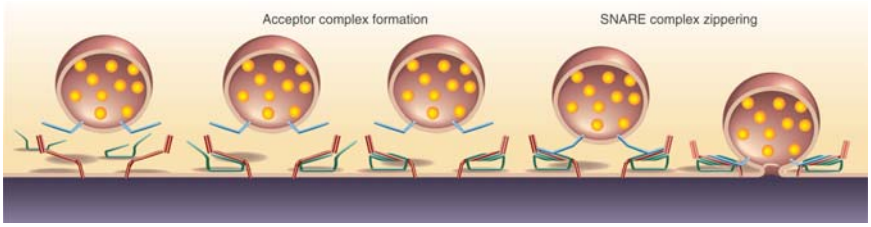

Fig. 3.2 Acceptor complex and zipper model for SNARE assembly. The Q SNAREs syntaxin and SNAP-25 assemble on the plasma membrane. This Qabc acceptor complex then contacts the distal N-terminus of synaptobrevin on the synaptic vesicle. This conformation is known as a "loose" SNARE complex. The "zippering" of the rest of the SNAREs into the complex serves two potential functions. First, full assembly of the SNAREs leads to close proximity between the membranes destined to fuse. Second, the zippering might provide torque that is transferred to the transmembrane domain leading to full fusion

Synaptobrevin is located on synaptic vesicles, while syntaxin and SNAP-25 are on the plasma membrane. The assembly of synaptobrevin, syntaxin, and SNAP-25 into the SNARE complex would thus bridge the vesicle and plasma membrane, forming what is known as a *trans* SNARE complex (Fig. 3.1A).

Assembly and Disassembly Cycles in SNARE Function

The steps in the assembly of the *trans* SNARE complex are still in dispute. Based on biochemical experiments using the yeast SNAREs, it was proposed that homologues of syntaxin (Sso1p) and SNAP-25 (Sec9p) might form an "acceptor complex" (55) (Fig. 3.2). A syntaxin–SNAP-25 complex was also subsequently proposed for the neuronal SNAREs (56). This acceptor complex greatly speeds up the assembly of the core complex (57). However, it is not known whether this complex exists *in vivo*. It has been shown that SNAP-25 and syntaxin can stably associate in cells (58). Specifically, a fluorescently tagged SNAP-25 generated an intramolecular fluorescence resonance energy transfer (FRET) signal upon assembly with syntaxin in PC12 cells.

This acceptor complex comprising one SNAP-25 molecule and one syntaxin molecule is highly reactive and will rapidly incorporate a second syntaxin molecule to form a dead-end Qaabc complex (55–58). This dead-end complex might be prevented in vivo by the action of tomosyn, a molecule with an R-SNARE domain (59,60). By occupying the synaptobrevin position in the complex, tomosyn might prevent the accumulation of the nonproductive Qaabc complexes and thus promote SNARE complex formation (60,61). However, this model is not consistent with the largely inhibitory role for tomosyn; genetic knockouts yield large increases in synaptic vesicle release (62,63). Tomosyn is thus more likely to bind to the acceptor complexes and, just like the Qaabc complexes, might form inactive complexes (62–64).

A second protein family that might serve to stabilize the acceptor complex is the SM (Sec1/Munc-18) family. At the synapse these proteins are called Unc18 proteins (UNC-18 in *C. elegans*, Munc18 in mammals, and ROP in *Drosophila*). It was originally thought that Unc18 exclusively bound to syntaxin monomers (65–69). However, more recent experiments have suggested alternative modes of binding (70–73). When reconstituted into lawns of plasma membrane, Unc18 was displaced from syntaxin by synaptobrevin but only when SNAP-25 was also present (70). Unc18 might therefore stabilize a syntaxin/SNAP-25 acceptor complex awaiting synaptobrevin (70). Nonetheless, it is still at present unclear how acceptor complexes are maintained or even whether they are true intermediates in core complex assembly. Indeed, synaptobrevin and syntaxin have been shown to assemble in vitro in the absence of SNAP-25 (74–76), suggesting that SNAP-25 might join the complex last. It has even been suggested that syntaxin might be the last molecule to enter the core complex in vivo (77). Until SNARE assembly can be monitored in vivo, we are forced to rely on these studies of in vitro SNARE interactions.

Once synaptobrevin enters the complex it is proposed to make contact at the N-terminal portion of the SNARE domain distal from the membrane. This conformation of the SNAREs is termed a loose configuration and is then thought to zipper down to a tight conformation (Fig. 3.2). Synaptic vesicles are held in a release-ready state in which the *trans* SNARE complex is likely to be arrested in a partially zippered state. Calcium binding to synaptotagmin would release arrest so that the SNARE complex could fully zipper to the tight conformation. This transition to the tight conformation would pull the transmembrane domains of the SNAREs, and hence the membranes, into close proximity and induce fusion (78,79). Models for the action of SNAREs in membrane fusion are described below.

Once the two membranes have merged, the core complex is now located in a single membrane and is referred to as a *cis* SNARE complex. To undergo further rounds of fusion, this *cis* complex must be disassembled and the SNAREs repartitioned to their appropriate compartments. Disassembly is mediated by the action of NSF and the SNAPs. Together NSF and the SNAPs are able to disassemble all SNARE complexes thus far tested (80). The ATPase NSF itself does not directly bind SNAREs; instead, it binds SNAREs through the action of the SNAPs. The SNAPs bind to the surface of the *cis* SNAREs around the central zero layer, which contains the conserved Q and R residues (81). The disassembly of the mammalian core complexes in PC12 cells is inhibited by mutation in these conserved residues (82). However, the disassembly of the *C. elegans* core complex is not affected by the same mutations (83). An alternative model for the function of these conserved residues is that they have a role before fusion in getting the four helixes to align in register to ensure that their transmembrane domains are directly opposed at their C-terminal ends (42,78). It has also been proposed that they might function in the prevention of full SNARE zippering (77). The next section explores how the formation of these SNARE complexes might catalyze fusion.

A Model for Membrane Fusion

Membranes do not spontaneously fuse, because of the high repulsive forces between two phospholipid bilayers 1 to 2 nm apart. How might the SNAREs fuse membranes? Three characteristics of the SNAREs are central to the current models for their function in fusing membranes. First, the assembled SNARE complex is remarkably stable. The formation of the SNARE complex is therefore an energy source that can be used to overcome barriers to fusion. Second, the SNARE complex must consist of at least two SNARE molecules with transmembrane domains (84). The transmembrane domains must be inserted into both of the membranes destined to fuse (54). Third, the SNAREs assemble in a parallel orientation (44,78,79,85). Due to the parallel orientation of the SNARE motifs, SNARE assembly leads to the close apposition of the transmembrane domains and hence the membranes themselves. This section describes how the assembly of the SNARE complexes might lead the membranes through the sequential intermediates of a lipid stalk, a hemifusion diaphragm, an initial fusion pore, and finally full fusion (Fig. 3.3).

The stability of the SNARE complexes combined with their parallel orientation led to the idea that their formation might provide the driving force for fusion. By first assembling at their N-terminals and subsequently "zippering" down to their membrane proximal C-terminals, the assembly of the SNAREs would bring the transmembrane domains of synaptobrevin and syntaxin into close proximity (77–79, 86–88) (Fig. 3.2). Evidence for zippering comes from two complementary experiments. First, biochemical and structural studies have shown that the membrane proximal domain of syntaxin becomes sequentially more ordered upon binding synaptobrevin in a directed N- to C-terminal fashion (55,57,87,89). The temperatures for assembly and disassembly of SNARE complex differ by as much as 10°C. Thus, assembly and dissociation follow different reaction pathways. This hysteresis suggests a kinetic barrier between folded and unfolded states (45). Mutations in the N-terminal hydrophobic core of the SNARE complex selectively slowed SNARE assembly, while those in the C-terminal did not slow assembly (56,87), suggesting that the N-terminal nucleates SNARE assembly. The kinetic barrier to assembly also suggests that loose SNARE complexes could be an intermediate.

The second line of evidence for zippering comes from in vivo disruption studies using clostridial toxins, antibodies directed toward the SNARE motifs, and mutations in the hydrophobic core of the SNARE complex (77,86–88,90). The toxin and antibody disruption studies demonstrated that the N-termini of SNAREs become resistant to cleavage or antibody block at early stages, while C-termini are only resistant to disruptions at late stages. As a specific example, Hua et al injected either botulinum toxin D, which cleaves free synaptobrevin at the N-terminal side of the SNARE motif, or botulinum toxin B, which cleaves synaptobrevin toward the C-terminal side of the SNARE motif (88). SNAREs cannot be cleaved once they have assembled into the four helix SNARE complex (46). Exocytosis from the crayfish neuromuscular junction was not sensitive to cleavage at the N-terminus of the SNARE motif, suggesting that this region was protected, presumably by the SNARE complex. By contrast, neurotransmitter release was blocked by cleavage at the C-terminus of the

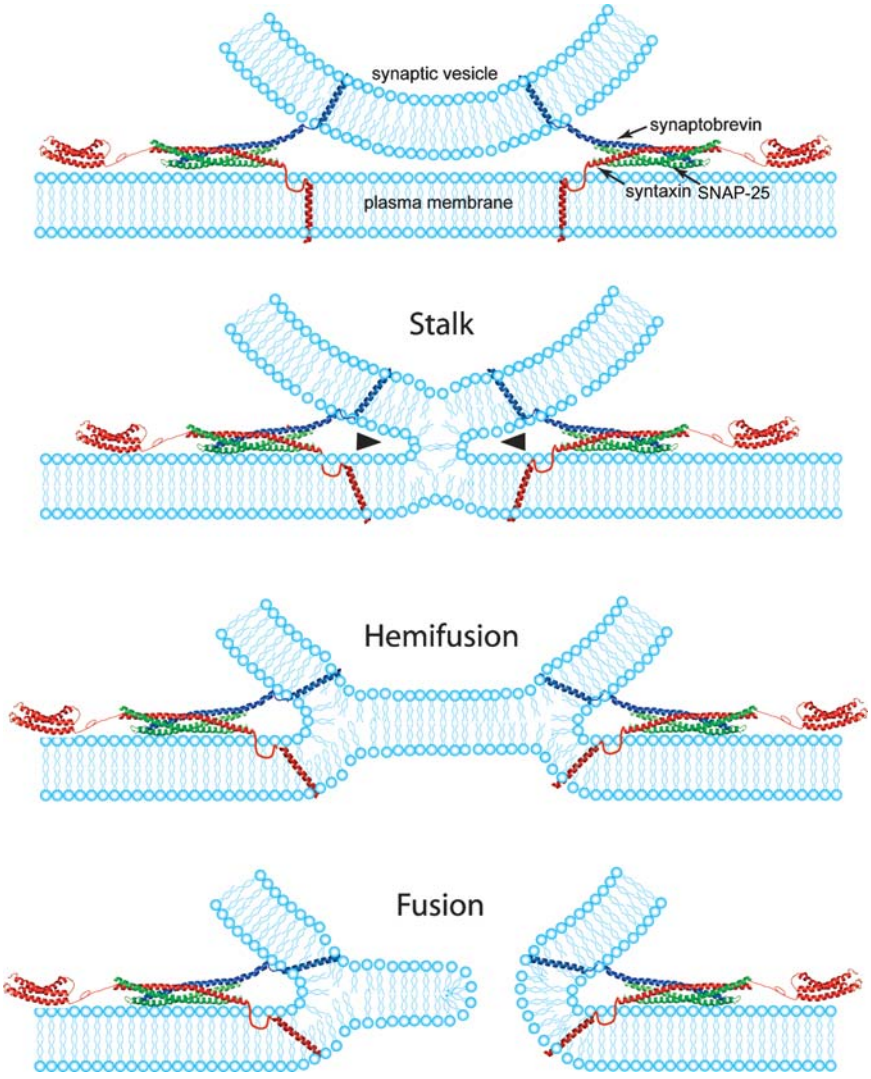

Fig. 3.3 A model for SNARE-mediated membrane fusion. The high repulsive forces between lipid membranes prevent them from fusing. The SNAREs are thought to provide the energy that enables the lipid rearrangements required for fusion. Pairing of the SNAREs brings the membranes into close proximity and leads to the merger of the proximal leaflet of the membranes to form a lipid stalk. The lipid stalk can then expand into a hemifusion diaphragm. Fusion is likely to require the transfer of energy from the SNARE motif to the transmembrane domains. It is thought that the weakest points lie at the edge of the hemifusion diaphragm. A rupture in the membrane at one of these points leads to fusion of the distal leaflet of the membranes and completes the fusion process. Regions of negative lipid curvature are indicated by arrowheads in the stalk. (Courtesy of Enfu Hui and Edwin R. Chapman.)

SNARE motif (86). Importantly, once the neuromuscular junction was electrically stimulated, botulinum toxin B was able to block exocytosis, demonstrating that the crayfish synaptobrevin monomers were indeed targets for the toxin. Thus, these data suggested that the N-terminus, but not the C-terminus, of synaptobrevin, is zippered into a SNARE complex in primed vesicles; presumably, calcium influx stimulates full zippering and membrane fusion.

As a second example, the mutations in the hydrophobic core of the SNARE complex have been expressed in neurosecretory chromaffin cells (87,90). Mutations in the C-terminal hydrophobic core incrementally reduced the kinetics of the rapid component of secretion, while those in the N-terminal reduced the sustained component of release, which is thought to correspond to engagement of new SNARE complexes (87). Importantly, the N-terminal mutants did not change the kinetics of the fast or slow components of release, only the amplitude of the response. Thus, it was interpreted that the C-terminal mutations were slowing "zippering" while those in the N-terminal were disrupting nucleation of the SNARE complexes (87). By contrast, when SNAREs bearing mutations in the hydrophobic core were introduced into the neurosecretory PC12 cells, there was no gradient in the efficacy of mutations in the kinetics of exocytosis (90).

The zippering of the membrane proximal portion of the SNARE complex likely serves two functions. First, the SNAREs are thought to catalyze the formation of a "hemifusion" transition state in which the proximal membrane leaflets have merged. This state can be achieved with comparatively low-energy requirements (91–94) and might simply need the SNAREs to bring the membranes into close proximity (95). Second, the SNAREs have been proposed to open up a fusion pore. This step requires the transmembrane domains of the SNAREs and likely involves the transfer of energy from the zippering of the SNARE cytoplasmic domains being passed to the transmembrane domain in order to locally disrupt lipid membranes (96).

Inspired by experiments in viral fusion and modeling of lipid bilayers, it is proposed that the initial steps of membrane merger result in a lipid stalk (97,98). The stalk corresponds to an hourglass-like structure that may contain as few as a dozen lipid molecules (98–100). The expansion of the stalk then results in a hemifusion diaphragm (91,101). These steps are not as highly energetically unfavorable as later steps and can be experimentally observed by dehydration of planar lipid bilayers, even in the absence of SNAREs (92,93,100,102). Direct evidence for lipid stalks has come from x-ray–scattering experiments that have given us a structure of this intermediate (100). The hemifusion state has been shown to be a metastable intermediate in vivo and can be observed for extensive periods of time in certain fusion reactions (103). Importantly, in vitro liposome fusion experiments have shown that hemifusion is an intermediate in the fusion pathway mediated by the synaptic vesicle SNAREs (104–106). Hemifusion intermediates have also been seen at central synapses using conical electron tomography; hemifused vesicles corresponded to those vesicles that were docked at the active zone (107).

Aside from the tomography and x-ray–scattering experiments, the evidence for stalk intermediates and hemifusion diaphragms comes from two observations:

the sensitivity of the fusion reaction to lipids of different intrinsic curvature (108), and the exchange of lipid membrane without luminal content mixing (103,109–111). The intrinsic curvature of lipids is determined by the ratio of the size of the lipid head group to their acyl chain tails. For example, a lipid with a single acyl chain would promote a positive intrinsic curvature (convex). At stalk structures and hemifusion diaphragms the outer, nonfused monolayer must adopt a negative curvature (concave) (arrowheads in Fig. 3.3) compared to the fused proximal monolayer, which adopts a net positive curvature (Fig. 3.3). This model predicts that, when added at the final steps of fusion, lipids with negative curvature would stimulate fusion while those with positive curvature would hinder fusion. Indeed, for all fusion reactions thus far tested, this prediction has been borne out (112). The application of lipids with altered curvature has been particularly useful in determining at which step fusion is arrested in various experimental manipulations (91,95,112).

When the SNARE transmembrane domain is replaced by artificial lipid anchors or when the transmembrane domain is truncated, fusion no longer proceeds (95,96,113). However, these perturbations do lead to a state in which lipids can exchange—a hallmark of hemifusion (91). Interestingly, replacement of the membrane anchor of the influenza hemagglutinin with an artificial membrane anchor, a glycosylphosphatidylinositol (GPI) tail, traps influenza viral fusion at a hemifusion stage (111). This observation demonstrates that membrane fusion events as varied as synaptic vesicle exocytosis and viral fusion might use a common mechanism to catalyze fusion. Importantly, the fusion arrest that results from the replacement of the transmembrane domain in both SNARE-based fusion and viral fusion can be bypassed by the addition of lipids with intrinsic negative curvature to the outer membrane or lipids that induce positive curvature to the inner membrane (95,114,115). This demonstrates that the proximity resulting from the SNARE pairing might be enough to achieve a hemifusion state, but that full fusion requires the transmembrane domains of the respective fusion proteins (95,111,114).

The dependence on the transmembrane domains for full fusion also suggests that the zippering of the SNAREs might result in the transduction of force to the transmembrane domain. The domain linking the SNARE motif to the membrane may be rather rigid; when synaptobrevin and syntaxin are placed in planar bilayers, they stand straight up from the membrane (116,117). Disrupting this rigidity by the addition of flexible linkers of incremental lengths, between the SNARE motif and the transmembrane domain, incrementally reduces fusion to complete elimination (50,96,113,118). In addition, mutations in the linker domain do not disrupt liposome fusion, while those in the SNARE motif have dramatic effects (119). This experiment favors the model of the linker as largely a force transducer (119). By contrast, mutations in the linker domain of yeast syntaxin (Sso1p) do cause dramatic decreases in fusion (120). Nonetheless, these results suggest that the winding of the SNARE proteins during core complex assembly transduces force to the transmembrane domains (96,116). Torque on the transmembrane domains might force dimples in the lipid bilayer at regions of *trans* SNARE complex formation (84) (Fig. 3.3).

It is likely that more than one core complex is required to catalyze fusion. Like viral fusion proteins, the SNAREs used in exocytosis also seem to work as higher order multimers (121). Thus, a ring of SNAREs could induce a controlled local disruption of lipids. One possibility is that the hemifusion diaphragm would be delineated by a ring of SNARE transmembrane domains (84,121). Alternatively, it has been suggested that the transmembrane domains of the SNAREs might serve as a proteinaceous pore (122). Though the interactions are quite weak (123), it has been shown that both syntaxin and synaptobrevin form higher order multimers via conserved regions located in their transmembrane domains (124–127). Electron microscopy has provided images of these multimers and shows that they form star-shaped structures with the transmembrane domains at the vertex (128). In vivo evidence for the existence of such multimers comes from the cooperative action of the SNAREs and dose dependency of inhibition by botulinum neurotoxins and SNARE peptide blockers (121,129–132). Together, the evidence has suggested multimers containing from between three and 15 complexes (121). Nonetheless, working models for multimerization are currently quite preliminary; it will remain to be seen how these multimers might aid in catalyzing fusion.

The Reliable Opposition: Protein Models for the Fusion Pore

Despite the appeal and considerable evidence for a lipidic fusion pore, there remain data suggesting that the fusion pore could be proteinaceous (133). First, it has been proposed that the SNAREs are the fusogen but that the pore is lined by the transmembrane of the five to eight syntaxin molecules rather than by lipids (122). This model derived from the observation that the replacement of residues in the transmembrane domain of syntaxin with bulky amino acids slowed the conductance of the initial fusion pore. Second, some data indicate that SNAREs were not involved in the fusion step. NSF disassembles SNARE complexes, yet in yeast overexpression of NSF (Sec18p) did not block vacuole fusion (134). Third, techniques that can detect early stages of pore formation, amperometry, and capacitance measurements indicate that the fusion pore in chromaffin cells might be formed by a protein. In these experiments the initial fusion pore was found to have a pore size equivalent to a large ion channel (approximately 1 to 2 nm in diameter) (135). In addition, these initial fusion pores "flickered" like ion channel fusion pores (132,135,136). Fourth, it has been proposed that the V_o sector of the vacuolar ATPase could act as a proteinaceous fusion pore (137,138). In yeast, calcium and calmodulin might be required in a step after SNARE complex formation in the process of fusion (139). The target of calcium-calmodulin in this late step in fusion was identified as the V_o sector of the vacuolar ATPase (137). Furthermore, analysis of *Drosophila* mutants indicated that the vacuolar ATPase was important for fusion of synaptic vesicles (140).

Nonetheless, several points are difficult to reconcile with a protein pore–based model for fusion. First, *trans* SNARE complexes are resistant to the action of NSF, suggesting that functional SNAREs were still present in yeast experiments (141).

Second, fusion pore sizes have been found to vary considerably in different fusion reactions, an observation more consistent with a lipid-based pore (142). Third, null mutants in many of the SNAREs proteins have been shown to have a stronger phenotype—often they are completely inviable—than respective mutants in the vacuolar ATPase (143). Fourth, lysophosphatidyl choline, a lipid that induces positive membrane curvature, is able to block all fusion reactions so far tested (112). Finally, the observed fusion pore flickering has been seen in pure lipid bilayers induced to fuse by polyethylene glycol (PEG) (144). PEG dehydrates the spaces between lipid bilayers and drives lipid mixing. Flickering is therefore not a hallmark solely of proteinaceous fusion pores.

Other observations that are apparently inconsistent with the lipid-based model for fusion have arisen from liposome fusion assays. For example, NSF and other proteins can catalyze the fusion of liposomes (145). However, the liposome fusion assay can be problematic (146). First, the lipid composition is critical in these assays and can produce misleading results; NSF could no longer fuse membranes when more physiologic lipid mixes were used (147). Second, many liposome fusion assays have used excessive and nonphysiologic concentrations of the SNARE molecules. Third, in most instances the speed of neurotransmitter release has not been replicated in this assay. Thus, results from liposome fusion assays must be interpreted cautiously and be supported by in vivo or genetic experiments.

SNAREs Encode Specificity

The original SNARE hypothesis proposed that compartmental specificity of fusion was encoded by SNARE proteins. Each intracellular fusion would be mediated by a specific set of SNARE proteins and thereby provide an addressing system for vesicle trafficking (27,28). This model makes several predictions. First, SNAREs should only bind their cognate SNARE partners. Second, SNAREs should only catalyze fusion when mixed with their SNARE partners. Third, SNAREs should be required for docking of vesicles to the correct target membrane. Fourth, the removal of a SNARE should selectively and completely eliminate fusion in one and only one fusion reaction. All of these hypotheses have been tested.

In vitro, the binding between cytoplasmic SNARE motifs is surprisingly promiscuous (148–150). However, these same SNAREs exhibited specificity in catalyzing fusion reactions when inserted into artificial lipid bilayers (151–152). Specifically, only cognate SNARE complexes could catalyze fusion reaction. To date, out of the 275 pairwise combinations of yeast SNAREs tried, only nine are functional in the liposome fusion assay. Eight of these nine SNARE combinations represented interactions that occur in vivo, thus the specificity of fusion is greater than 99% (274/275) accurate (151). This specificity is preserved among the neuronal SNAREs; after cleavage of SNAP-25 in PC12 cells, secretion could only be rescued by SNAP-25 itself and not other SNAP-25 homologues (152). Thus, the SNAREs can encode the specificity of fusion.

Morphologic docking of synaptic vesicles long appeared to be independent of SNAREs. Genetic or pharmacologic disruption of SNAREs did not perturb synaptic vesicle docking (12,13,15,154). However, more recent experiments indicate that docking of synaptic vesicles (14) and dense core vesicles requires syntaxin (155–157). Importantly, if syntaxin is required for docking, experiments claiming roles for syntaxin in fusion must be interpreted with caution since fusion is downstream of docking. Docking defects will lead by necessity to defects in fusion. The discrepancy for syntaxin's role in docking could be due to different morphologic definitions of docking, which has been defined as everything from direct contact with the plasma membrane to vesicles 50 nm from the plasma membrane. Alternatively, additional docking factors might be present in some cell types to ensure the specificity of fusion (155). For example, syntaxin is required for docking in neurosecretory cells but not neurons in mice (155,157). Perhaps tethering factors also contribute to docking of synaptic vesicles at the active zone (158–162). Overlapping roles for SNAREs and docking factors have been observed in yeast (163,164). Specifically, *sec35* encodes a tethering protein for Golgi trafficking in yeast; *sec35* mutants can be partially bypassed by overexpression of the relevant SNARE proteins (165). Similarly, overexpression of SNAREs can bypass mutations in the tethering complex for plasma membrane fusion (166,167). It is likely that these overlapping redundant functions are necessary to achieve the high level of fidelity seen in membrane trafficking.

Thus far in vivo perturbations of the SNAREs have mostly been shown to selectively eliminate single trafficking steps. However, in all cases fusion was not completely eliminated. There are two possible explanations. First, it is possible that the SNAREs are not executing fusion—an unlikely interpretation given the wealth of data described above. Second, the SNAREs might be partially redundant. Evidence so far points to the latter interpretation. Knockout mice in synaptobrevin II were found to retain some synaptic activity in hippocampal neurons (16). In chromaffin cells, this remnant activity could be attributed to the synaptobrevin paralog cellubrevin (168). Redundancy can also explain the remaining fusion events in synaptobrevin null *Drosophila* mutants. Syb, the *Drosophila* equivalent of cellubrevin, can functionally substitute for n-Syb, the *Drosophila* equivalent of synaptobrevin, when overexpressed in neurons (169). Redundancy is also seen in the Q SNAREs. SNAP-23, SNAP-47, and SNAP-24 can provide partial function when SNAP-25 is absent (19,170,171). Finally, redundancy might also explain the almost complete lack of phenotype in syntaxin 1a knockout mice (172), where it is likely that syntaxin 1b is sufficient to almost entirely replace syntaxin 1a action. These observations are supported by experiments in yeast where redundancy between SNAREs has also been conclusively demonstrated in numerous trafficking reactions (173–175). By contrast, loss of syntaxin (*unc-64*) in *C. elegans* neurons results in a 500-fold reduction in neurotransmitter release with no apparent developmental defects (14); UNC-64 is committed to synaptic vesicle fusion and is unlikely to have a redundant syntaxin, like in mice; nor is it involved in other cellular functions, like in flies (176). In summary, the SNAREs do encode specificity; nonetheless, in some instances it is likely that other factors can provide overlapping functions to ensure that fusion happens with the appropriate target membrane.

SNARE Regulation

We will only touch on SNARE regulation briefly in this chapter, since other chapters will cover this topic in greater depth. SNARE regulation can roughly be divided into two forms: before and after initiation of complex formation. Before core complex formation, regulation involves occlusion of the SNARE motif of syntaxin to prevent the assembly of SNARE core complexes. After the initiation of SNARE assembly regulation likely takes place at the level of complex zippering. The calcium-sensing machinery works at these later steps.

Syntaxin itself has its own regulatory domain; the N-terminal Habc domain can fold over and occlude the SNARE motif (Fig. 3.1). Syntaxin can adopt two conformations: a closed form, in which the SNARE motif is occluded, and an open form, in which the SNARE motif is available to interact with SNAP-25 and synaptobrevin. At least two synaptic proteins, Unc13 and Unc18 proteins, have been proposed to act directly on this N-terminal extension of syntaxin (65,177). In *C. elegans, unc-13* mutants can be partially bypassed by an open form of syntaxin, demonstrating a direct or indirect role of UNC-13 in the conversion of syntaxin from a closed to an open form (14,62,178). Several additional proteins may regulate SNARE complex assembly by directly occluding the SNARE motif of syntaxin. These molecules include tomosyn, amisyn, and syntaphilin (59,62–64,179–181).

At steps after core complex assembly, regulation might take place at the level of preventing full zippering of the SNARE proteins. Three proteins—Unc18, complexin, and synaptotagmin—may act at this late stage. The precise function of the SM superfamily of proteins, which include the Unc18 synaptic proteins, is not yet known (see Chapter 7), but Unc18 proteins might function in these later stages (70–73,182–184). Sec1p, the yeast SM homologue that acts at the plasma membrane, binds to the SNARE complex rather than syntaxin monomers (185). Recent data suggest that Unc18 also uses this mode of interaction (70–73).

Complexin and synaptotagmin serve as part of the calcium-sensing machinery. The coupling of fusion to calcium influx is the key evolutionary modifications of SNARE function to adapt it for neurotransmission. At synapses, the time delay between the elevation in calcium concentration and the postsynaptic response can be as little as 60 to 200 μs (186). Though calcium is needed for fusion in other membrane trafficking steps, it usually serves as a facilitator of fusion rather than directly functioning as a signal in triggering fusion (187,188). The addition of complexin and synaptotagmin appear to impart the calcium trigger to SNARE-mediated fusion (189, 190). Complexin appears to act as a fusion clamp—a brake preventing constitutive fusion from occurring (191–194).

Interestingly, recent experiments have shown that the complexin clamp holds the SNAREs in a state where the membranes are hemifused (193). This observation demonstrates that the transition from hemifusion to full fusion can be regulated at the cytoplasmic SNARE motifs. Complexin sits in a groove between syntaxin and synaptobrevin, potentially preventing the full zippering of the core SNARE complex (195,196). The calcium sensor is synaptotagmin (197–201). Synaptotagmin binds to lipids and to syntaxin and SNAP-25 in a

calcium-dependent manner (200–204). Importantly, synaptotagmin appears to compete with complexin for SNARE complex binding and relieves the clamp when calcium is present (reviewed in ref. 194). One possibility is that calcium binding allows synaptotagmin to actively displace complexin from the SNARE complex, which is then free to fully wind and to break the membrane of the hemifused intermediate. In this model the SNAREs could function like a wheel, with complexin the stick in the spokes preventing the wheel from turning. Calcium binding to synaptotagmin would pull the stick from the spokes and allow the wheel to turn and drive fusion. This model, however, remains speculative, and several pieces of data are currently incompatible with the above model. First, complexin knockout in mice do not have elevated synaptic vesicle fusion, as would be predicted (205). In addition, synaptotagmin when reconstituted with the neuronal SNAREs in the liposome fusion assay, can act alone as both a fusion clamp in the absence of calcium as well as an accelerator of fusion in the presence of calcium (206). However, a second group did not observe calcium sensitivity in SNARE-mediated liposome fusion assays by the addition of synaptotagmin; instead, synaptotagmin simply accelerated the rate of liposome fusion independent of calcium (207). Since subsequent chapters will delve further into the murky depths of calcium regulation, here we will suffice to stay in the shallow end of the pool.

Conclusion

Rounds of SNARE assembly and disassembly lie at the center of all vesicular trafficking. Assembly of the SNAREs into a four-helix bundle drives fusion of synaptic vesicles with the plasma membrane and thereby mediates the release of neurotransmitter. The entwined SNAREs are then pulled apart by the ATPase NSF, which reenergizes the system for further rounds of fusion. This model is widely accepted, yet its details are in considerable dispute. So far, reconstitution experiments have examined interactions between only a very few of the proteins involved in what is undoubtedly a complex and highly regulated fusion machine. As such, they have given us largely static images of the complex. Thus, the overarching challenge in the coming years will be to understand the regulation of the SNAREs and how the assembly of SNAREs catalyzes fusion.

Several questions must be resolved. First, is a preassembled Q-SNARE acceptor complex present on the plasma membrane in vivo, and if so how is it stabilized? Second, how is assembly of the SNAREs regulated? SNARE regulators, including MUN domain proteins such as Unc13, SM proteins, and Tomosyn, have been identified, yet their mechanism of action is unclear. Third, are SNAREs fully zippered prior to or during fusion? Fourth, is SNARE complex zippering arrested in the readily releasable pool of synaptic vesicles? Fifth, does formation of the SNARE complex generate a hemifusion intermediate? And finally, what rearrangements occur in the SNARE complex when synaptotagmin binds calcium and phospholipids?

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