Mechanisms of SNARE proteins in membrane fusion

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Abstract

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are a family of small conserved eukaryotic proteins that mediate membrane fusion between organelles and with the plasma membrane. SNAREs are directly or indirectly anchored to membranes. Prior to fusion, complementary SNAREs assemble between membranes with the aid of accessory proteins that provide a scaffold to initiate SNARE zippering, pulling the membranes together and mediating fusion. Recent advances have enabled the construction of detailed models describing bilayer transitions and energy barriers along the fusion pathway and have elucidated the structures of SNAREs complexed in various states with regulatory proteins. In this Review, we discuss how these advances are yielding an increasingly detailed picture of the SNARE-mediated fusion pathway, leading from first contact between the membranes via metastable non-bilayer intermediates towards the opening and expansion of a fusion pore. We describe how SNARE proteins assemble into complexes, how this assembly is regulated by accessory proteins and how SNARE complexes overcome the free energy barriers that prevent spontaneous membrane fusion.

Introduction

Biological membranes are bilayers formed by membrane lipids, with a hydrophobic core and hydrophilic surfaces. Membranes are generally stable in an aqueous environment and form diffusion barriers that segregate aqueous reaction spaces from the surroundings. However, it is essential that membrane-enclosed spaces such as cells and intracellular organelles can both merge (termed 'fusion') and split (known as 'fission') without becoming leaky in the process. Such transitions do not occur spontaneously because they are prevented by free energy barriers. To overcome these barriers, bilayers need to be perturbed, which frequently involves dedicated protein complexes undergoing exergonic conformational changes.

Membrane fusion and fission describe the forward and backward direction of a reversible reaction, but the two biological processes underlying these changes are fundamentally different. Although some steps of each process are reversible, both pathways employ different proteins that ensure that the reaction is unidirectional. Fusion proteins operate at the proximal contact site between the membranes, bringing them together and establishing hydrophobic connections. In contrast, fission proteins form either constricting helices and rings around a tubular neck of a budding vesicle (dynamins, discussed in ref. 1) or spirals on a flat membrane surface (ESCRT-III polymers, reviewed in ref. 2), extruding vesicles away from the assembly site. Consequently, the membrane geometry and, thus, the membrane curvatures of the intermediate steps are different between fusion and fission. Note, however, that members of the dynamin superfamily also mediate fusion, most notably in mitochondria[3], suggesting that in certain cases fusion and fission may share structurally related steps in the reaction pathway.

In the last decades we have witnessed enormous progress in the understanding of protein-mediated membrane fusion. The first fusion proteins to be characterized were surface glycoproteins of enveloped viruses such as haemagglutinin of influenza virus or Env of the human immunodeficiency virus (for review see refs. 4,5). In contrast, the protein machineries responsible for both extracellular and intracellular membrane fusions in eukaryotic cells remained enigmatic for a long time. This changed with the discovery of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins 30 years ago[6,7]. SNAREs are a protein superfamily whose members are responsible for almost all fusion events of both the exocytotic and endocytic branches of the secretory pathway. These include reactions that are highly diverse in structure and timing, such as exocytosis of synaptic vesicles that occurs at a millisecond timescale[8], or exocytosis of giant vesicles containing viscous material such as endothelial secretory granules termed Weibel–Palade bodies that require minutes to be released[9]. Moreover, SNAREs are conserved across the eukaryotic kingdom and are found in plants and animals inhabiting the entire biosphere[10]. Since the first description of SNAREs, additional and structurally unrelated eukaryotic fusion proteins have been discovered that function in fusion events not covered by SNARE proteins. These include, for instance, the fusion between mitochondria or the fusion between entire cells as it occurs during fertilization or during formation of syncytia (reviewed elsewhere[11–13]). In many cases, it is not yet understood by which mechanisms these proteins operate, and thus, in addition to viral membrane fusion, our understanding of SNARE-mediated fusion is most advanced.

Advances were also made in our understanding of the physical principles governing membrane fusion. Experimentally, it is still difficult to access the transition states of fusion reactions, particularly at molecular resolution, and thus simplified physical models are required in which all structural parameters are defined and which can be treated in accordance with the laws of physics. The structures of all intermediates are determined by the hydrophobic effect that creates forces aimed at minimizing the exposure of non-polar surfaces to the aqueous surroundings. Physical models describing the fusion of protein-free bilayers have advanced significantly and are increasingly supported by experiments as described in more detail in the next section. Thus, we presently have a much better understanding of the reaction pathway facilitated by fusion proteins such as SNAREs.

In this Review, we discuss the function of SNAREs in the context of modern physical concepts of lipid bilayer fusion. We will first describe the transitions that purely lipidic membranes undergo along the fusion pathway. Fusion proceeds through an ordered sequence of molecular rearrangements, termed the reaction coordinate, in which the positions of the participating molecules in the three-dimensional space are changed in a defined manner, beginning with the membranes approaching and contacting each other and ending with the opening of an aqueous fusion pore. Only if the reaction coordinate of the fusion pathway with its intermediate steps and the associated free energy barriers and valleys are known can the precise function of the SNARE machinery be determined, which we discuss in the following sections. For more comprehensive recent reviews on other aspects of SNARE structure and function, in particular the structural details of SNARE assembly and disassembly and the role of accessory proteins, see refs. 14–17.

Energy landscape of lipid bilayer fusion

Protein-free lipid membranes do not fuse spontaneously in aqueous media under physiological conditions. However, fusion can be induced if the energy barriers are overcome. This is investigated using both various types of models and experiments. We will first discuss fusion pathways of lipid bilayers (that is, a protein-free environment) as these models allowed researchers to define the tasks performed by fusion proteins.

Despite the structural and mechanistic diversity of fusion events, the reaction pathway of membrane fusion involves a series of common and sequential steps (Fig. 1). As mentioned above, the mechanisms by which fusion proteins such as SNAREs operate cannot be understood without detailed knowledge of the free energy profile of protein-free membranes moving along the fusion pathway, which is governed by the hydrophobic effect, in combination with electrostatic, solvation and steric forces. Once the reaction pathway(s) can be precisely described in all geometric and molecular detail, the free energy profile can be calculated using only the laws of physics, that is, independent of model-dependent assumptions. However, due to the complexity and fluid disorder of the transition states it is far from trivial to define a reaction coordinate, as the precise position of each atom is not experimentally accessible and is still difficult to predict by simulation models. Even variations that appear 'intuitively' minor with respect to geometry or molecular composition, such as local rearrangements of a few lipid molecules, may have drastic effects on the energy profile (see refs. 18,19 for a more detailed discussion). Thus, although frequently referred to, a 'general' fixed value (kT) for the overall free energy barrier for membrane fusion, which fusion proteins must overcome, cannot be given. This requires a fully defined reaction coordinate, which may vary widely between different fusion reactions.

Originally, the free energy profile of the fusion pathway was calculated using elastic continuum models in which the properties of membranes were approximated by modelling them as elastic and bendable sheets characterized by parameters such as membrane curvature, membrane stiffness or electrostatic repulsion (see for example refs. 20–22). Although these models were instrumental for describing fusion pathways in accordance with physical principles, it became clear early on that the free energy barriers along the reaction coordinate cannot be accurately described without incorporating molecular details[21,23–26].

To account for these parameters, simplified molecular models in which groups of atoms are consolidated into particles for simulation (termed 'coarse-grained') were developed that are becoming increasingly sophisticated and that are beginning to bridge between atomistic simulation and elastic continuum models[19,27–30]. Such coarse-grained models proved instrumental for our understanding of the molecular structure and the free energy profiles of the intermediate steps.

Figure 1 shows a hypothetical free energy profile along the fusion reaction coordinate, with molecular models of possible fusion intermediates (see for example refs. 31,32), that is discussed below in more detail[32]. As mentioned above, both free energies and numbers of the peaks and valleys depend on the specific reaction coordinate of a given fusion reaction. A barrier along the pathway means kinetic retardation of the reaction, which may result in the accumulation of metastable intermediates that can be trapped, thus becoming accessible to experimental characterization.

Step I: approach

At a distance of 3–10 nm the repulsive forces between bilayers are low and are easily overcome by thermal fluctuations. Thus, liposomes in a test tube spontaneously collide with each other. The transient contact distance can be very short and is governed by factors such as surface charge (electrostatic repulsion), hydrophilicity, charge shielding by counter ions, temperature and size of the contact area[33,34]. In biological membranes, close bilayer contact by diffusive collisions is sterically hindered by surface proteins or protein domains (Fig. 1, energy barrier 1), which can be overcome by proteins connecting the membranes, such as tethering factors.

Step II: tight docking

In order to proceed to the next stage, which involves intermediates where the bilayer structure is disrupted (Step III, see below), the membranes need to approach each other very closely, below a distance of 1 nm. To achieve this, water must be removed from between the surfaces of the membranes and electrostatic repulsive forces need to be overcome[35]. For most fusion reactions, the free energy difference between the preceding minimum and this second barrier is likely to be the highest along the entire pathway.

How can this energy barrier be reduced? Aside from electrostatic shielding of fixed negative surface charges by monovalent and divalent cations[36], one option is to minimize the contact area, which implies highly curved regions at the contact site. Moreover, positive curvature at the contact site increases hydrophobicity as the hydrophilic headgroups become spaced farther apart, resulting in hydrophobic 'defects' in the hydrated membrane surface. This facilitates merging of the monolayers, which results in the formation of an hourglass-shaped intermediate termed the fusion stalk (Step III; see Fig. 1 and below). Indeed, the fusion-promoting effect of membrane lipids with small headgroups (cone-shaped lipids such as phosphatidylethanolamine) is usually explained by stabilizing negative curvature in the distal monolayer (that is, opposite to the positively curved contact site) during transition to the stalk (reviewed elsewhere[23]). However, the fusion-promoting effect of positive curvature may also be explained by increased interfacial hydrophobicity, which due to

increased spacing of the charges reduces repulsion by electrostatic and dehydration forces and increases the probability of lipid tail fluctuations and, thus, stalk formation (Step III) at the surface of the contacting proximal monolayers[19]. Moreover, polypeptide chains that have a propensity to form amphipathic structures partition into membrane interfaces where they may perturb the bilayer structure and thereby lower the energy barrier, even up to the point that fusion occurs spontaneously. This is the case with certain amphiphilic viral fusion peptides[37,38] or peptides corresponding to transmembrane domains (TMDs) capped with hydrophilic residues[39].

Despite early experimental evidence to the contrary (see for example ref. 34), it was thought for many years that membranes cannot form tight connections due to the strong repulsive electrostatic forces, unless the contact sites are limited to very small areas involving local protrusions[23]. Thus, tight contacts were considered to represent an energy maximum along the pathway. Recently, however, tightly connected membranes were captured by cryo-electron microscopy (cryo-EM) as metastable intermediates after reconstituting several protein-mediated fusion reactions in vitro, including membrane fusion facilitated by SNAREs[40–42]. Such contacts persist after SNARE disassembly and may expand due to adhesive forces, contain negatively charged headgroups, are free of proteins and do not depend on (albeit being stabilized by) divalent cations. Simulations showed that at the contact site, the phospholipids are packed more tightly because of headgroup tilting, resulting in measurable thickening of the proximal monolayers[43].

Step III: lipid tail splaying and fusion stalk formation

The emerging consensus in the field is that the first hydrophobic connection between the two apposed membranes is mediated by the 'splaying' of phospholipid side chains (Step IIIa) (see for example refs. 44–46). As discussed above, the experimental capture of tightly docked intermediates suggests that, at least under certain circumstances, transition to this stage requires overcoming a free energy barrier (Fig. 1, energy barrier 3). However, it is also possible that there are fusion pathways in which a tightly docked metastable intermediate is bypassed, that is, that the transition from two separate membranes to the stalk involves only a single free energy barrier. In any case, perturbation of the hydrophilic–hydrophobic boundary in either of the monolayers that reduces the energetic penalty of hydrophobic lipid side chain exposure will lower this barrier. Once the first hydrophobic connection is established, other hydrophobic side chains reorient along the template provided by the splayed side chains, moving downhill on the free energy scale towards a fusion stalk in which the proximal leaflets are forming a highly curved continuous monolayer (Fig. 1, Step IIIb). Although stalks were originally predicted by elastic continuum models to be transient[20,23], they can be stabilized by mild dehydration of stacked membranes, representing a separate (termed rhombohedral) phase that is accessible to experimental characterization[47–50].

The free energies of the transition towards the stalk (energy barrier 4) and of the stalk itself can be reduced by lowering negative curvature stress. This can be achieved, for example, by introducing lipids with small headgroups (cone-shaped lipids, see above) with increased surface hydrophobicity[23], by decreasing membrane rigidity[51] and/or by perturbations of the

hydrophobic–hydrophilic boundary of either membrane interface (see above). This transition is the critical step for the final 'triggering' of fusion reactions as from here onwards the reaction generally proceeds downhill on the free energy scale, except for fusion pore opening that may require additional input of energy (see below).

Although stalks are metastable, and thus can be experimentally trapped, they can relax into extended hemifusion diaphragms in which the two distal monolayers connect by forming a bilayer (Fig. 1, Step IIIb). Such diaphragms may progress to fusion, but they may also extend, getting trapped in an energy minimum (Fig. 1, Step IIIb, dotted line in the energy profile) and likely represent dead-end off-pathways[52]. Intriguingly, although hemifusion diaphragms can be induced by experimental manipulation of fusion proteins resulting in the abortion of fusion[52], it is debated whether they occur during biological fusions in intact cells (see ref. 53 for a more comprehensive discussion). It needs to be taken into consideration that the formation of extended hemifusion diaphragms creates significant imbalances between the surface areas of the two leaflets that need to be compensated, for example by unidirectional flipping or selective removal of membrane lipids from the proximal monolayers of the contacting membranes.

Step IV: fusion pore opening

Fusion pores can only open at sites where the two distal leaflets are in contact, for example, by forming a stalk or a (possibly very small) patch of a bilayer diaphragm as described above. The development of a fusion pore requires the formation of a pathway for water across the bilayer, which depends on reorientation of the lipid headgroups until the headgroups of the proximal and distal membranes meet to form a hydrophilic channel, thus overcoming energy barrier 5.

Fusion pores are probably initiated by local defects in side chain packing at the centre of the stalk or at the rim of a hemifusion diaphragm. Even though single water molecules may penetrate through hydrophobic side chains, reorientation of lipid headgroups must precede any flux of water molecules and, thus, the initial formation of a fusion pore[18,54] (Fig. 1). It remains to be established whether the so-called 'flickering' of fusion pores, describing the repetitive opening and closing of an aqueous permeation pathway for ions carrying electric charge (which, although experimentally observed only in fusion of protein-free bilayers[55], appears to be a hallmark of protein-mediated fusion as well, see below), represents a reversal equilibrium of the pore opening or is governed by more complex mechanisms. To our knowledge, physical models that accurately describe the features and the parameters governing reversible fusion pores between bilayers are still lacking.

Step V: fusion pore expansion

Once the reorientation of the lipid headgroups is completed and an aqueous channel lined by headgroups is formed, the bilayer becomes stable again, and fusion pore opening is governed by minimizing curvature stress and lateral membrane tension. It is unclear whether, for expansion, an additional energy barrier needs to be overcome after initial fusion pore opening (energy barrier 6) or whether such barriers are dependent on proteins (see below).

The assembly–disassembly cycle of SNARE proteins

SNAREs represent a unique class of fusion proteins. Single SNARE molecules are not active and cannot fuse membranes even if present at high concentrations. For fusion, SNARE proteins residing in the two membranes destined to fuse need to interact in a highly regulated assembly reaction that connects the membranes and guides them along the fusion pathway. After fusion, these SNARE complexes are disassembled by a dedicated ATPase termed NEM-sensitive factor (NSF; also known as NEM-sensitive fusion protein), thus regenerating the SNARE proteins for another round of membrane fusion.

Domain structure of SNAREs

SNARE proteins represent a superfamily of small and mostly membrane-anchored proteins with a common domain structure (Fig. 2). The most characteristic feature is the SNARE motif, a stretch of 60–70 amino acids arranged in heptad repeats present in all SNAREs[56]. There are four different variants of SNARE motifs termed Qa, Qb, Qc and R, respectively, that were first distinguished by their conserved position in the structure of the assembled SNARE complex and later shown to be conserved in all eukaryotes using in-depth bioinformatic sequence analysis[56–59]. The other domains of SNAREs exhibit more diversity. For instance, although many SNAREs possess a carboxy-terminal hydrophobic TMD, connected to the SNARE motif by a short linker, there are some in which a classical TMD is lacking. These SNAREs may contain post-translational hydrophobic modifications or phospholipid binding domains, or lack membrane anchors altogether (reviewed elsewhere[10]). Examples include the members of the synaptosomal-associated protein 25 (SNAP25) subfamily, which contain a Qb motif and a Qc motif (see below)[60], synaptobrevin homologue Ykt6 (containing an R-type motif)[61] or the yeast SNARE vacuolar morphogenesis protein 7 (Vam7; with a Qc motif)[62–64].

In addition, many — but not all — SNAREs possess independently folded amino-terminal domains. Most commonly, the N terminus consists of antiparallel three-helix bundles connected to the SNARE motif by a short linker. Three-helix bundles are typical for Qa-SNAREs but are also observed in some Qb-SNAREs and Qc-SNAREs. Other structures include, for instance, a profilin-like domain called the long in domain that is present in some R-SNAREs (reviewed elsewhere[10]) (Fig. 2). Some SNAREs lacking a TMD contain two SNARE motifs (usually Qb or Qc motifs) that are separated by a linker of variable length and that may contain palmitoyl residues acting as a membrane anchor (Qbc-SNAREs, reviewed elsewhere[60]). Another exception includes the Qa-SNARE syntaxin 17 that contains two adjacent TMDs forming a hairpin, targeting the protein to autophagosomes[65]. Note that complete or partial SNARE motifs are also found in a few soluble proteins which may regulate SNARE function by interacting with complementary SNAREs. Examples include the neuronal proteins tomosyn (also known as syntaxin-binding protein 5)[66,67], amisyn (also known as syntaxin-binding protein 6)[68] and, although with lower similarity, lethal(2) giant larvae protein homologue 1 (LgL) and its homologues Sro7 and Sro77 (ref. 69).

SNARE assembly

Assembly of SNAREs between membranes is the key step in SNARE-mediated membrane fusion. In the following sections, we first give an overview over the general rules governing SNARE assembly in different fusion reactions. Next, we discuss structural details of the various interactions between free SNAREs, largely based on in vitro studies with purified proteins. We then outline how conserved accessory proteins that are recruited to the fusion sites are thought to control this conformational landscape for initiating assembly. The section is concluded by a discussion of the specialist proteins controlling the final steps of SNARE zippering in regulated exocytosis of neurons.

Common properties of SNARE complexes.—SNARE assembly requires the participation of four different SNARE motifs, one from each subfamily (Fig. 2). Starting from dissociated, largely unstructured monomers they assemble into a coiled-coil bundle of extraordinary stability, which consists of four α-helices aligned in parallel, one of each SNARE subfamily (the QabcR complex, reviewed elsewhere[10,16,17]). The number of SNARE complexes required for fusion is thought to range between one and three (at least in neuronal exocytosis), but whether this is generally the case is still debated[70–72]. Assembly is initiated at the N-terminal ends and progresses towards the C termini of the α-helices (termed zippering, see for example refs. 73–75). Although assembly does occur spontaneously when purified SNAREs are mixed, its initiation is controlled by accessory proteins that are required for SNARE function in vivo (see below). For fusion, SNAREs that are anchored to opposing membranes assemble into a complex ('*trans*-complex'), with the progression of zippering pulling the membranes together until the membranes merge. In the fully assembled complex ('*cis*-complex') the linker and the TMDs are aligned in parallel and also form α-helices, thus extending the helices of the SNARE motifs into the membrane[76] (Fig. 2).

The evolutionary success of the SNARE fusion engine is probably due not only to its structural simplicity but also to an enormous flexibility and robustness, which allows for adjustments to the specific needs of individual fusion reactions. For instance, only two SNAREs need to possess TMDs for fusion, and even replacement of one of these TMDs with lipid anchors maintains fusion, at least to some extent[77]. Accordingly, the SNAREs providing the other SNARE motifs can be structurally more variable. Thus, SNARE complexes contain not only 'canonical' SNAREs with TMDs but also SNAREs lacking them, with their function being limited to contributing additional helices required for the formation of the stable QabcR four-helix bundle (reviewed elsewhere[78]). Similarly, there is flexibility in the distribution of the SNARE subfamilies between the membranes before fusion: whereas in neuronal exocytosis the vesicle contains the R-SNARE and the plasma membrane contributes the Qa-SNARE, Qb-SNARE and Qc-SNARE motifs to the complex[6], a different topology is observed during the fusion of a transport vesicle generated at the endoplasmic reticulum (characterized by a COPII coat) with the *cis*-Golgi membrane — here, the vesicle contains Qb-SNAREs, Qc-SNAREs and R-SNAREs, whereas the target membrane contains the Qa-SNARE (reviewed elsewhere[79]). Although in these cases one membrane contributes three SNAREs and the other one SNARE, it is unclear whether this is always the case: at least in vitro, fusion is also observed if each membrane contributes

two SNAREs[80]. Moreover, one of the essential yeast R-SNAREs (Ykt6) lacks a TMD[61], requiring *trans*-assembly between membrane-anchored Q-SNAREs for fusion[81]. Thus, multiple permutations are possible. For instance, liposomes containing only Qc-SNAREs (carrying a TMD) fuse with their endogenous counterparts when injected into cells[82]. Moreover, SNAREs belonging to the same subfamily can substitute for each other, albeit only to a certain extent (for review see ref. 10), which provides additional flexibility due to partial redundancy and explains why deletion of some SNAREs in yeast results in surprisingly mild phenotypes[79,81].

Assembly is highly exergonic, with the resulting QabcR-SNARE complex being of extraordinary stability[83], and is considered to represent the main energy source ('power stroke') driving fusion. However, it has been far from trivial to determine the free energy of the assembly reaction. This is due to strong hysteresis that is observed when SNARE complexes are first dissociated by heat and then cooled for reassembly, suggesting that assembly and disassembly are not in equilibrium but, rather, follow different pathways[83]. Therefore, indirect approaches are needed such as single-molecule force experiments[84]; however, these are limited by spatial constraints of the force trajectories (when pulling SNAREs apart and allowing for their reassembly), which may not represent those of membrane-anchored SNAREs. Binding energies determined by such methods for the neuronal SNARE complex range from 30 to 85 kT (for discussion see refs. 15,85).

Assembly of free SNAREs.—Assembly of SNAREs is a complex molecular reaction that requires four different SNARE motifs on three or four separate proteins to interact and align in the correct sequence and spatial orientation. In vitro studies using purified SNAREs either in solution or after reconstitution in artificial membranes yielded structural details of intermediate complexes and associated conformational transitions, thus providing a framework for understanding how the accessory proteins (discussed in the next section) channel the reaction. Most in vitro studies of SNARE assembly were carried out using the SNAREs mediating neuronal exocytosis. These include syntaxin 1 (Qa-SNARE) and SNAP25 (Qbc-SNARE), both localized at the plasma membrane, and vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin 2) (R-SNARE), localized at the synaptic vesicle. Although these SNAREs spontaneously assemble into QabcR-SNARE complexes[86], assembly is very slow, requiring hours for completion, regardless of whether assembly proceeds in solution[87] or whether it proceeds between complementary SNAREs reconstituted in liposomes[74]. This may be caused by the tendency of the SNARE motifs to reversibly form homo-oligomeric and hetero-oligomeric complexes of varying stoichiometry and composition, resulting in a panoply of metastable helical assemblies that are facilitated by the propensity of many SNARE motifs for helix formation[88–92]. For instance, neuronal syntaxin 1 forms homo-oligomers of varying stoichiometry, both in solution and in membranes[93–95]. In addition, syntaxin 1 and SNAP25 form stable binary complexes with a 2:1 stoichiometry[86,96,97]. Moreover, individual SNARE motifs of SNAP25 (Qb, Qc) can associate with syntaxin alone or in combination with synaptobrevin[98]. Several of these complexes represent four-helix bundles that are reminiscent of the QabcR complex although less stable. Binary complexes between synaptobrevin and SNAP25, and also between synaptobrevin and syntaxin 1, have been reported, but they are of very low affinity (see

ref. 99 and references therein). Finally, some Qa-SNAREs can flip between a 'closed' and an 'open' conformation[100]. In the closed conformation, the N-terminal domain is folded back onto the N-terminal portion of the SNARE motif, preventing binding to other SNARE motifs[101].

Although the formation of SNARE complexes is likely to proceed in sequential steps, the diversity of such oligomeric assemblies and conformational transitions renders it difficult to distinguish which of these complexes are intermediates of the assembly pathway and which are kinetically trapped side reactions[102]. Intriguingly, it appears that trapping in such side reactions may not be a general feature of all SNAREs as it is not, or at least not to this extent, observed in other (non-neuronal) SNAREs. For instance, endosomal SNARE complexes spontaneously assemble and fuse much faster in vitro than the neuronal SNAREs, with the reaction completed within minutes[80,103] and with no evidence for helical off-pathway interactions[104].

Why are endosomal SNAREs more reactive than neuronal SNAREs, particularly when considering that neuronal SNAREs catalyse exocytosis of synaptic vesicles, the arguably fastest fusion reaction in our body? Although one can only speculate about the reasons, it needs to be considered that any accidental contact between SNARE-containing membranes may lead to spontaneous SNARE 'firing' and fusion unless prevented by kinetic barriers. The membrane concentration of neuronal SNAREs is extremely high, more than two orders of magnitude higher than that of endosomal SNAREs[105,106]. The low reactivity of the neuronal SNAREs may thus be a safeguard mechanism preventing uncontrolled exocytosis at contact sites crowded with SNAREs. In contrast, endosomes contain only very few copies of SNAREs[106], rendering it highly unlikely that four different SNAREs are accidentally present at such contact sites.

In conclusion, although free SNARE proteins are able to assemble spontaneously and mediate fusion in the absence of other proteins, their reactivity is low. Rather, regulatory proteins are required for activation, making sure that SNAREs only mediate fusion at predefined sites.

Proteins regulating initiation of assembly.—In the secretory pathway where the SNAREs operate, membranes destined to fuse first need to recognize and bind to each other. Initial connection between the membranes is mediated by diverse sets of so-called tethering factors that recognize molecular markers in both membranes. Such markers include specific Rab GTPases and phosphorylated variants of the membrane lipid phosphatidylinositol (reviewed elsewhere[107]). Efficient tethering only takes place if both membranes contain the appropriate combination of molecular markers[107,108]. Many (but not all) tethering factors form multiprotein particles, referred to as multisubunit tethering complexes (MTCs), and they are usually specific for a single intracellular trafficking step (for review see ref. 108). However, tethering is not sufficient for initiating SNARE assembly and fusion — it requires, in addition, specific regulatory proteins.

Two structurally conserved protein families are primarily responsible for controlling SNARE assembly. These are the Sec1/Munc18-like (SM) proteins[109,110] and proteins characterized

by bundles of helical rods, referred to as complexes associated with tethering containing helical rods (CATCHR) proteins[111,112]. These proteins act either as monomers that are separately recruited to SNAREs, such as the well-characterized neuronal proteins Munc18 (SM) or Munc13 (CATCHR), or they are integral components (subunits) of MTCs. Examples for the latter include the SM protein vacuolar protein sorting-associated protein 33 (Vps33) that is part of the homotypic fusion and protein sorting (HOPS) as well as the class C core vacuole/endosome tethering (CORVET) complex. Another example is provided by the CATCHR protein Tip20 that is part of the Dsl1 complex (also known as the syntaxin 18 complex).

Although SM and CATCHR proteins interact primarily with Qa-SNAREs, their main role probably consists of providing a scaffold for the ordered and sequential alignment of the other SNARE motifs. Accordingly, assembly proceeds through structurally defined intermediate states that not only prevent the SNAREs from entering off-pathway complexes but also result in a metastable acceptor site for the final SNARE motif completing the four-helix bundle, thus triggering efficient SNARE zippering and fusion. Indeed, it is possible to artificially create such highly reactive acceptor complexes using R-SNARE fragments without the need for SM or CATCHR proteins. For instance, if a Qabc-SNARE acceptor complex is stabilized by binding of a C-terminal R-SNARE fragment, yielding a free N-terminal acceptor site, binding of an intact R-SNARE is greatly accelerated, with rapid fusion occurring while the stabilizing C-terminal fragment is displaced[113]. Similarly, rapid zippering and fusion is observed if the R-SNARE is split into two fragments that each possess their own membrane anchor[114].

It is not known whether SNAREs need to be monomeric for the initial binding of SM and/or CATCHR proteins. In that case, kinetically trapped oligomers (see above) or inactive *cis*-complexes that are known to spontaneously form in membranes would first need to be dissociated by the ATPase NSF (see ref. 106) (Fig. 3) that disassembles all homo-oligomeric and hetero-oligomeric SNARE complexes (described in more detail below). In addition, it is still unclear exactly how assembly is initiated. This is at least partially due to the vexing properties of SM proteins that exhibit two distinct SNARE binding modes. In the first mode, the SNARE is kept in the closed conformation (with the N-terminal domain folded back on the SNARE motif, see above), being stabilized by binding to a cleft of the SM protein, essentially rendering the SNARE inactive. In the second mode, a short stretch at the N-terminal end of the Qa-SNARE binds to the outer surface of the SM protein, with no major changes in the structure of the SM protein. The relative distributions between the two binding modes vary between different Qa-SNAREs, and not all Qa-SNAREs may be able to adopt a closed conformation (for review see refs. 15,109,110).

How can these features be integrated into a working model of SNARE assembly? One scenario implies that the two binding modes occur consecutively, with the switch from closed to open possibly being mediated by simultaneous binding of a CATCHR protein (see Fig. 3, top panel), creating a metastable SM–CATCHR–Qa-SNARE acceptor complex[15,16,115]. The next steps are still unclear and may not be the same for all known SNARE complexes. Three possible scenarios, each supported by experimental evidence, are discussed (Fig. 3). According to the first model, the R-SNARE binds to a metastable

SM–CATCHR–Qa-SNARE acceptor complex (Fig. 3, top panel), thereby connecting the membranes and initiating *trans*-SNARE assembly. This view is supported by reports showing structures of SM proteins bound to either Qa-SNAREs or R-SNAREs (reviewed elsewhere[15]), or to both simultaneously[116,117]. Such a complex would then form a template for subsequent binding of Qb-SNAREs and Qc-SNAREs[15,117]. In a second model, SM proteins stabilize reactive Qabc-SNARE instead of QaR-SNARE complexes (Fig. 3, middle panel), providing an N-terminal and transient three-helix template for the rapid binding of the R-SNARE, possibly without directly contacting the SM protein[102,118,119]. According to the third model, CATCHR proteins, as part of MTCs, first bind to Qbc-SNAREs in one of the membranes before connecting with the target membrane, which is then followed by *trans*-SNARE assembly (Fig. 3, bottom panel). For instance, in the fusion of Golgi-derived vesicles with the endoplasmic reticulum, the CATCHR protein Tip20, as part of the Dsl1 tethering complex, binds to Qb-SNAREs and Qc-SNAREs[120,121], thus holding a SNARE acceptor complex in place. This assembly then interacts with the Qa-SNARE and its SM protein and recruits the incoming vesicle containing the R-SNARE. This process may involve some rearrangement of the SNAREs, possibly involving a SM protein-mediated QaR intermediate as in the first model, before SNARE zippering is initiated (for a more thorough discussion see refs. 15,16).

Why is it so difficult to identify the intermediates of the SNARE assembly pathway? Most probably, the intermediates are of low stability in order to maximize the energy gain from SNARE zippering for fusion (see for example ref. 122), rendering it highly challenging to isolate such states and to capture their structure[123]. For instance, stabilized neuronal SNARE complexes were created for the binding of the accessory proteins in which the SNAREs were either fully zippered or partly zippered by the use of truncated SNAREs, resulting in shortened bundles[124–126] (see Box 1). Although insightful, it remains to be established to what extent such structures resemble conformational intermediates in the fusion pathway (discussed elsewhere[123,127]). Moreover, several consecutive steps may be involved in which bound SNARE motifs may need to be rearranged before final nucleation, for example by dissociation–association cycles of individual SNARE motifs (reviewed elsewhere[15,16]). Finally, the activity of SNAREs towards assembly appears to be regulated at additional levels including, for instance, phosphorylation by an array of kinases of side chains within or adjacent to the SNARE motifs (for review see refs. 128,129).

SNARE zippering and its regulation by late-acting proteins.—Once SNARE assembly is initiated at the N-terminal end of the SNARE motifs, zippering proceeds downhill on an energy gradient but may be controlled by late-acting regulatory proteins. This is particularly conspicuous in neurons where Ca^{2+}-mediated triggering of synaptic vesicle exocytosis proceeds with a delay time of less than a millisecond[130]. Vesicles are docked in a 'release-ready' state only 1–2 nm apart from the plasma membrane (corresponding to Step II; see Fig. 1) before fusion[131]. The receptor for the Ca^{2+} ions is synaptotagmin, an abundant transmembrane protein of synaptic vesicles. Synaptotagmin contains two adjacent Ca^{2+}-binding C2 domains, termed C2A and C2B, that each possess several Ca^{2+}-binding sites but are somewhat different in their relative affinities[132,133]. Ca^{2+} binding does not appear to induce major conformational changes of the stably folded C2

domains but, rather, triggers electrostatic binding to clusters of negative charges such as acidic phospholipids in the plasma membrane or the acidic surface of (Q-)SNAREs, or both (reviewed elsewhere[16,17,134]). Effective triggering also requires complexin, a small protein that binds to the surface of the partially or fully assembled SNARE complex (reviewed elsewhere[135,136]). Note that synaptotagmins and complexins represent special adaptations of neuronal exocytosis, but are not part of the universal SNARE fusion machine. Importantly, phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), a membrane lipid specifically associated with the plasma membrane and found to cluster in the proximity of syntaxin[137], strongly modulates the binding of synaptotagmin, and probably plays a critical role in the final assembly of the SNAREs and progression to fusion[138,139].

How does Ca^{2+} binding to synaptotagmin trigger the final step of SNARE zippering and fusion? Whereas binding of synaptotagmin 1 and complexin to both SNAREs and lipids seems to be required, the underlying mechanisms and the assembly status of the SNAREs before the final triggering are still controversially discussed (see Box 1 and ref. 134 for more details). In particular, the interaction of one of the two calcium-binding C2 domains (C2B) with the surface of the SNARE complex appears to be essential for this step. Although C2 domains were shown to bind at several sites of the assembled SNARE complex, one of them (referred to as the primary binding site) appears to be critical as any interference with this interaction, for example by mutagenesis or small-molecule inhibitors, impairs calcium-dependent triggering of SNARE assembly (reviewed elsewhere[127]). However, it remains to be established how this interaction, or the lack thereof, is connected to the fusion reaction (Box 1).

SNARE disassembly

As discussed above, assembly of individual SNAREs into helical bundles is a highly exergonic reaction that allows for overcoming energy barriers along the fusion pathway. After fusion, the SNAREs within the complex are all aligned in the same membrane (*cis*-complexes) and need to be reactivated by dissociation. This reaction requires metabolic energy in the form of ATP. It is mediated by NSF[140], a member of the AAA+ superfamily of proteins, whereby AAA refers to their designation as ATPases associated with diverse cellular activities. These proteins are nanomechanical devices that are capable of driving major conformational changes in highly diverse client proteins and nucleic acids. Unlike some other AAA+ proteins, NSF does not directly interact with its substrate but requires soluble NSF attachment proteins (SNAPs) as adaptors (see below).

AAA+ proteins share a structurally conserved ATPase domain (also referred to as the AAA+ core domain) that forms ring-like or spiral-like oligomers[141,142]. NSF belongs to the type II ATPases that are hexameric. Each of the monomers contains three domains, two of which are represented by homologous ATPase core domains, referred to as D1 and D2, that are fused together and assembled into two stacked hexameric rings (Fig. 2). The third domain, referred to as the N domain, is independently folded and mediates contact with the client proteins, that is, SNARE complexes bound to SNAP proteins. The D2 ring binds ATP with very high affinity, which is not cleaved during the regular catalytic cycle and is responsible for stabilizing the hexameric assembly of the monomers. The catalytically active

D1 domain undergoes conformational changes during ATP cleavage, which are transferred to the N domain. As a result, the N domain undergoes large movements that are propagated through the SNAP intermediates to the SNARE complex, driving its disassembly (reviewed elsewhere[142]).

Both NSF and SNAPs are evolutionarily highly conserved and operate on all SNARE complexes studied so far (reviewed elsewhere[143]). Unlike NSF, of which only a single variant is expressed in most species, SNAPs are represented by three isoforms in mammals, termed α-SNAP, β-SNAP and γ-SNAP[144]. SNAPs are composed of stiff, twisted sheets of stacked α-helices connected to a C-terminal helix bundle that is negatively charged and, as shown in an elegant cryo-EM study, connects to a positively charged groove in the N domain of NSF[145]. Binding of SNAP to the grooves of the SNARE complex is dependent on a pattern of positive and negative surface charges that appears to be conserved in all SNARE complexes[146]. Moreover, SNAPs possess an extended loop containing exposed phenylalanine residues that mediate membrane binding, thus directing the disassembly machinery to membrane-anchored SNARE complexes[147]. As in many other AAA+ proteins, ATPase activity of NSF is stimulated by substrate binding[148].

It is still unclear how exactly the concerted action of NSF and SNAP leads to unwinding and dissociation of the SNARE helical bundle. Whereas in several other AAA+ proteins the substrates are threaded through the central hole of the hexameric ATPase ring, this does not appear to be the case in the NSF–SNAP–SNARE interaction, even though conserved pore-lining residues are likely involved in the catalytic cycle[145]. Rather, there is a conspicuous gap between the first and last protomers of the D1 ring, which may allow for sideways escape of dissociated SNARE monomers during disassembly[145,149]. Moreover, the number of SNAP molecules required for disassembly appears to be variable, with estimates ranging between two and four molecules (see ref. 142 for review). Finally, single-molecule experiments have revealed that disassembly occurs within 10 ms in a single and concerted round of ATP hydrolysis rather than by sequential disassembly of individual subunits[150]. The reaction involves only a single unfolding intermediate in which the membrane-adjacent C-terminal part of the SNARE complex is dissociated whereas the N-terminal parts of the SNAREs are still joined.

The NSF–SNAP disassembly machinery dissociates not only fully assembled SNARE complexes but also various off-pathway hetero-oligomeric and even homo-oligomeric SNARE complexes (see above) in which SNAREs otherwise may remain trapped. Moreover, in vitro experiments suggest that NSF can attack SNARE *trans*-complexes that are not yet fully zippered, that is, before fusion is completed[151]. Such complexes may be shielded by the scaffold and accessory proteins that compete with SNAP proteins for binding to the SNARE complex, preventing premature abortion of fusion[152,153]. Intriguingly, an opposite role of α-SNAP was also suggested: the protein was reported to bind after nucleation of SNARE assembly to recruit SM proteins[154] and promote full zippering in the absence of NSF[155]. Presently, it is unclear how these two views can be reconciled and to what extent assembling SNARE complexes are protected from attack by NSF before fusion is completed.

The function of SNAREs along the fusion pathway

As detailed above (Fig. 1), the pathway of membrane fusion involves sequential steps separated by energy barriers. Fusion proteins such as SNAREs transfer conformational energy onto the membranes and change the reaction coordinate in such a way that the fusion reaction follows a downhill energy gradient, giving directionality to the pathway. Moreover, they lower the energy barriers of transition states, allowing fusion to be completed instead of becoming trapped in one of the intermediate states. Experimental manipulations of SNAREs, by studying fusion both in vitro and in intact cells and organisms, suggest that SNARE assembly is mechanistically involved in all of the steps depicted in Fig. 1. However, despite a multitude of approaches for observing SNARE-mediated membrane fusion (see Table 1), it is experimentally far from trivial to differentiate the steps between the first transition towards close apposition (Fig. 1, Step II) and the opening of an aqueous fusion pore (Step IV). Thus, it is still controversially discussed how exactly SNARE zippering and its control by the regulatory proteins is associated with the progression towards fusion, that is, a unified model for the function of SNAREs along the fusion pathway is not yet available. SNAREs are conserved across the entire eukaryotic kingdom and function in a wide range of highly diverse fusion reactions. It is conceivable that the built-in flexibility of the SNARE machine and the fluid dynamic nature of lipid bilayers allow for multiple different reaction pathways in a complex free energy landscape. This landscape may resemble energy funnels with many valleys and obstructions along their slopes to eventually merge into a common energy minimum of the fully fused state, akin to a concept that is now frequently used to describe the energetics of protein folding[156]. Moreover, the free energy landscape is dependent on the overall lipid composition and local variations in lipid content. SNAREs and some of the accessory proteins may alter the local lipid composition, for example by de-mixing or binding lipids such as PtdIns species or phosphatidylserine[137], thus influencing free energy barriers along the reaction coordinate.

Despite this diversity, some common features are beginning to emerge, and increasingly sophisticated molecular models are being developed for SNARE action in individual steps of the pathway (see for example ref. 157).

Step I: SNARE action during membrane approach

As already discussed above, initial contact between membranes destined to fuse is mediated by tethering factors that interact with both polyphosphoinositides and Rab GTPases[108,158,159]. Although this step precedes activation and *trans*-interaction of SNAREs, it is becoming clear that SNAREs are instrumental in orchestrating tethering sites. For instance, at least in the plasma membrane, SNARE proteins are concentrated in nanodomains that may serve as beacons marking prospective fusion sites for incoming vesicles[160–164]. Moreover, most multisubunit tethering factors bind to SNAREs not only by means of their SM or CATCHR protein subunits but also by means of other subunits, thus recruiting them to the prospective fusion sites (reviewed elsewhere[165]). Linking the recruitment of SNAREs to tethering ensures that the SNAREs are in place when membrane contact is established, thus facilitating subsequent SNARE assembly.

Step II: SNARE zippering promotes tight membrane docking

Bringing the membranes sufficiently close to allow for lipid tail splaying is arguably the essential function of SNARE assembly. Indeed, SNAREs are perfectly designed as force-generating 'zippering' machines capable of overcoming large repulsive electrostatic and hydration forces.

Except in the case of regulated exocytosis in neurons and related cells in which SNARE assembly, and thus progress towards fusion, is arrested in a tightly docked state (Box 1), such intermediates appear to be transient and rapidly progress towards fusion. Although these intermediates can be stabilized in vitro, cryo-EM is required to distinguish this state from more loosely docked precursor states. So far, only few studies are available[131,166,167] and it is unclear how far SNARE zippering needs to proceed to reach this state, that is, how much of the energy of the SNARE power stroke is required. If the SNARE motifs fully assemble close to the TMDs, these may become tilted (see Box 1). Straightening out the TMDs in alignment with the SNARE complex bundle will buckle up the membranes and force them towards each other, as proposed in a recent model in which SNARE conformations respond to the action of Ca^{2+}-activated synaptotagmin and, thus, facilitate transition to Step III[168]. Polyunsaturated membrane lipids may facilitate the formation of such high curvature protrusions as they appear to reduce membrane rigidity and increase surface hydrophobicity[51].

Tight contact between the membranes at the prospective fusion site imposes steric constraints on the fusion proteins, which are excluded from the contact site[43]. Indeed, the small size of the SNAREs allows for their positioning directly adjacent to the protein-free contact area but this may be limited by bulky accessory proteins (such as SM and CATCHR proteins) which are thought to remain bound during zippering (see ref. 169 for review) (Box 1). Small contact areas are advantageous as they reduce the energy barrier, and thus may explain why, as mentioned above, neuronal exocytosis requires only one to three SNARE complexes for fusion[70–72,170]. It is likely that biological fusion reactions generally proceed through small contact sites even if the participating membranes are mostly flat (such as yeast vacuoles)[171]. Apparently, this is more difficult to reproduce in vitro where the contact area, and thus repulsion, scales inversely with curvature, explaining why larger vesicles need more SNARE complexes for fusion[40]. Once formed, however, tight contact zones expand in vitro, probably due to adhesive forces involving both electrostatic and hydrophobic components[43,166]. It is unclear whether such expanded tight contact areas exist in intact cells. Here, accessory proteins may prevent adhesive expansion and limit the contact area, which has also been suggested by a recent reconstitution study[167]. Note that in neurons, tight docking of synaptic vesicles is reversible[172]. This means that in that state SNARE zippering can still be undone, either by maintaining the SNAREs in an only loosely assembled state that is reversible or by dissociating zippered SNARE *trans*-complexes with the aid of NSF (see above).

Step III: SNARE-mediated perturbations promote fusion stalk formation

The closer the membrane surfaces are, the lower the energy barrier for the next step — the formation of a lipid stalk that establishes a hydrophobic connection between the core

of the two membranes. Even minor surface perturbations may suffice, such as transient dislocations of membrane lipids caused by tilting or outward pulling of the SNARE TMDs. Both the linkers connecting the SNARE motifs with the TMDs and the TMDs themselves appear to be critical for this transition: molecular dynamics simulations and various in vitro and in vivo experiments document that manipulations of SNARE TMDs, for example by mutations in the hydrophobic part leading to increased helicity or by adding charges to the C terminus, inhibit progression to fusion[19,173–175] (see also below). Similar impairments of fusion, albeit to different degrees, are observed when SNARE TMDs are truncated or replaced with lipid anchors (see for example refs. 77,173,176,177). Thus, it is possible that the motion associated with C-terminal zippering of the SNARE motif enhances TMD-mediated perturbations at the contact sites, causing lipid tail splaying and progression to hemifusion in a single continuous step.

Once tight contact is established by SNARE zippering, however, progress towards hemifusion may also be induced by other factors. For instance, it is well established that docked synaptic vesicles that are ready to be released can be driven towards exocytosis by increasing membrane tension (and thus surface hydrophobicity), for example by mechanical stretching[178] or osmotic pressure[179]. Mechanical stretching and osmotic pressure increase hydrophobicity at the surface of the bilayer, which, as discussed above, reduces the energy barrier for lipid tail splaying. This happens also when pure lipid bilayers are induced to fuse in the presence of polyethyleneglycol, which induces osmotic pressure by reducing the water activity at the membrane surface[180]. Moreover, the fusion-promoting effect of Ca^{2+}-activated synaptotagmin may be exerted by insertion of C2 domain(s) into the proximal monolayer of the plasma membrane, perhaps attracted to the contact site by $PtdIns(4,5)P_2$ clusters around the TMD of the SNARE syntaxin (see Box 1 and above). Also, the presence of bulky accessory proteins such as MTCs close to the contact area may cause membrane deformations that contribute to overcoming the final energy barrier[169,181]. Similarly, recruitment of the NSF adaptor SNAP to such states promotes fusion even if the SNAREs are not fully zippered, which appears to depend on membrane binding caused by the abovementioned hydrophobic loop region of SNAP[182] (Fig. 2).

Thus, the conserved and universal function of SNAREs in fusion is embedded in the zippering reaction. Its initiation is guided by protein machineries containing conserved components, followed by zippering that brings the membrane surfaces very close. Progression towards hemifusion requires less energy and little structural specificity. Although completion of SNARE zippering alone can be sufficient for driving the reaction towards full fusion, there may be fusion reactions where this is not the case, requiring other proteins to control the final step(s).

Steps IV and V: SNAREs shape fusion pore opening and expansion

Opening and subsequent expansion of an aqueous fusion pore are the final steps in the fusion trajectory. Originally, based on electrophysiological signatures reminiscent of ion channels, fusion pores were considered to be mainly lined by proteins[183]. However, fusion pore-like signatures such as rapid sequences of pore opening and closing (referred to as pore flickering) were also observed when fusion was forced to occur between protein-free

membranes[55] (see also above). Presently, the emerging consensus is that fusion pores are primarily lipidic but may contain TMDs of proteins[184].

Whereas in many systems fusion is rapidly completed after the initial opening of the first aqueous connection, a more complex pattern emerged from measuring exocytosis of secretory vesicles at high resolution using various electrophysiological and imaging approaches[185–187]. Accordingly, initial pores are small and may linger for a while before they either expand or close again. Of these, re-closure is the least understood, and it is still unclear whether it involves mechanistic reversal of fusion, that is, a reversal of SNARE zippering, particularly in cases where closure occurs only milliseconds after opening (fusion pore flickering)[188]. For a long time, it was debated whether synaptic vesicles recycle by direct recapture after such brief opening of a fusion pore (termed 'kiss-and-run'[189]) or by endocytosis at sites away from the fusion site (for review see ref. 190). Using an elegant combination of optogenetics and time-resolved cryo-EM, the latter hypothesis was proved to be correct[191]. Recapture of only partially fused vesicles by the endocytic machinery at the site of exocytosis was reported[192,193], but in these cases the pores remained open for much longer (seconds to minutes) and lipid mixing occurred before recapture[194].

Numerous studies have shown that SNAREs are instrumental in shaping the dynamic properties of fusion pores such as the size of the initial pore or the delay and kinetics of its expansion. For example, changes in SNARE expression levels or mutations affecting SNARE zippering, post-translational modifications or minor changes in the TMDs such as conservative substitutions of single amino acids may have profound effects on these parameters (for review see refs. 169,184,195–199). In addition, fusion pores are governed by membrane tension or stresses exerted by curvature, or by other means such as osmotic pressure caused by osmotic swelling of vesicle contents[174,200]. Evidently, the local composition of membrane lipids, particularly the cholesterol content[201,202], has a profound influence on these factors. For instance, addition of cholesterol promotes fusion pore opening while preventing stalling of fusion in a hemifusion state[203]. The lifetime and expansion of fusion pores can be readily determined from a shape analysis of single-vesicle fusion profiles measured by total internal reflection fluorescence microscopy, which also showed that addition of negative curvature-inducing phosphatidylethanolamine lipids in the *cis* leaflet, but not in the *trans* leaflet, increased the lifetime of fusion pores[204].

In intact cells the fusion apparatus is embedded in the physiological regulatory context, with a multitude of interacting proteins in the vicinity of the fusion site, complicating the interpretation of molecular perturbations. Molecular mechanisms governing fusion pores cannot be deduced from even the most sophisticated kinetic measurements — their interpretation is limited to supporting plausible models. Thus, in such experiments it is difficult to discern whether SNAREs or other proteins govern fusion pore dynamics by directly interacting with membrane lipids within the pore structure, for instance with the SNARE TMDs lining the pore rim, or whether their effects are exerted indirectly by affecting membrane tension from a distance, with the pore itself being lipidic. In recent years, in vitro assays for monitoring SNARE-mediated fusion involving ab initio reconstitution have become highly sophisticated (see Table 1), allowing for monitoring fusion pore opening using imaging or electrophysiology with high temporal resolution.

For instance, SNAREs reconstituted in nanodiscs can be fused with liposomes, planar membranes or even intact cells (using artificially 'flipped' SNAREs)[71,205–208] (for review see refs. 196,209). Although complete fusion cannot be achieved due to the spatial constraints of lateral lipid movement in nanodiscs, initial opening states can be captured with this approach, thus allowing for dissecting parameters affecting pore features such as lipid composition, membrane tension, structure of TMDs or protein stoichiometries.

Concluding remarks

Since the discovery of the SNARE fusion machinery about 30 years ago, major progress was made not only in the detailed understanding of the SNARE machinery with its complex conformational association–dissociation cycle and the multitude of proteins controlling this cycle but also in understanding the physical principles of fusion itself. Indeed, due to the structural simplicity of the SNARE 'core engine', SNAREs were instrumental in the development of refined models of fusion trajectories that are compatible with physical principles. There are still major unresolved questions concerning the structure and dynamics of the transition states governing the non-bilayer intermediates of the SNARE-mediated fusion pathway. For example, the structure of the neuronal pre-fusion complex that is activated by Ca^{2+} ions is still unclear, despite recent advances described above. Similarly, it is unclear to what extent the SNARE TMDs and the short linkers connecting them to the SNARE motifs are part of transition states such as hemifusion stalks and initial fusion pores. Moreover, in vitro reconstitutions of SNARE-mediated fusion have considerably improved in recent years and exhibit features approximating those of biological fusion reactions including Ca^{2+}-regulated exocytosis. Although these reconstitutions were crucial in achieving the insights discussed in this Review, there are still major uncertainties regarding to what extent findings obtained in such systems can be transferred to in vivo reactions. However, we expect that due to rapid technical advances, particularly in obtaining time-resolved high-resolution structures by cryo-EM and cryo-electron tomography and in the parallel development of increasingly sophisticated physical models (see for example refs. 117,127,168,210,211), a complete molecular description of the fascinating SNARE nanomachines appears to be within reach in the foreseeable future.

Acknowledgements

Glossary

Black lipid membranes

An experimental model in which single bilayers span a hole 10–100 μm wide created in a Teflon sheet immersed in aqueous media. They appear black because light reflected off the back of the membrane interferes with light reflected from the front

Content mixing

Mixing of the aqueous interior of two vesicles undergoing fusion. Only occurs after opening of a fusion pore

Content release

Release of the enclosed content of a vesicle into the environment upon fusion of a vesicle with a planar membrane

Hysteresis

The state of a system depends on the history (or pathway) by which the state has been reached. Hysteretic systems therefore cannot be modelled using equilibrium thermodynamics where a system can only assume a single state under a defined set of conditions, independent of how it got there

kT

The product of the Boltzmann constant k with the absolute temperature T, a measure of the thermal energy per molecule. Can be converted into other energy units such as calories, joules or electronvolts

Lipid mixing

Mixing of membrane lipids during fusion, often describing the stage when the contacting lipid leaflets have mixed and the two distal leaflets remain separated

Secretory pathway

A system that connects most intracellular membranes, including the plasma membrane of eukaryotic cells, by vesicles that dissociate from the 'donor' membrane and then fuse with the target membrane, including the plasma membrane, resulting in secretion of the vesicle content

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Box 1

Pre-fusion arrest in neuronal exocytosis: how far are the SNAREs zippered?

Although Ca^{2+}-triggered fusion of neuronal soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) has been reconstituted in artificial membranes using neuronal SNAREs, synaptotagmin and various combinations of the Sec1/Munc18-like (SM) protein Munc18, the complexes associated with tethering containing helical rods (CATCHR) protein Munc13 and complexin (see for example refs. 168,217,221,225,234), it has been difficult to determine the status of SNARE assembly before fusion and the mechanism by which synaptotagmin triggers progression to fusion. Three scenarios are discussed for the release-ready state that are not mutually exclusive:

1. According to the first model, SNAREs are not zippered. The Q-SNAREs syntaxin 1 and synaptosomal-associated protein 25 (SNAP25) are pre-assembled in an acceptor complex exposing a binding site for the R-SNARE vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin 2) to 'snap' in, for which the assembly proteins provide a scaffold. Alternatively, syntaxin and synaptobrevin may be bound to Munc18, with SNAP25 zippering not yet initiated (see Fig. 3). Synaptotagmin triggers zippering indirectly by pulling the vesicle closer to the target membrane[235]. Here, the full SNARE power stroke is available for the final fusion step. Complexin only binds after SNARE zippering is triggered in this model, which then cannot explain its clamping function (below). Also, docking of release-ready vesicles at a distance of 1–2nm requires SNAREs, arguing for SNARE *trans*-contact in this state[131].

2. According to a second model, SNARE zippering is halted halfway. Arrest may be caused by complexin that can bind to partially assembled Qabc complexes[236] or may interfere with the carboxy-terminal zippering of synaptobrevin[237], and/or by binding of synaptotagmin[238]. In this state, Munc18 and Munc13 are still thought to be bound. According to one model, the block is released by Ca^{2+}-dependent dissociation of the C2B domain from the surface of the SNARE complex, probably switching instead to the plasma membrane ('release-of-inhibition model'[238]). This model is supported by high-resolution structures showing SNARE complexes with bound C2 domains, Munc18, Munc13 and complexin in various combinations, sometimes in symmetrical arrangements[117,124,126,239]. Alternatively, Ca^{2+}-triggered binding of synaptotagmin to ionic lipids may change the lipid order and thus, indirectly, the conformation of the Q-SNARE acceptor complex by lifting the Qa-SNARE, thus pulling it towards the vesicle membrane[168]. Other models are also discussed, such as oligomerization of synaptotagmin between the vesicle and the plasma membrane which is then dissociated upon calcium binding[240]. Arrest in the partially zippered state is an attractive model as it preserves part of the SNARE power stroke for the last step but does not require major rearrangements of the SNAREs after Ca^{2+}-induced triggering.

Moreover, distinct steps in the force profile are observed when SNARE complexes are pulled apart with optical tweezers and allowed to reassemble, supporting an energy barrier upon zippering halfway[237,241]. However, it has been difficult to show Ca^{2+}-dependent binding or dissociation of C2 domains to or from SNAREs, or any direct effect of C2 domains on zippering, and synaptotagmin–SNARE interactions are abolished at physiological salt and metabolite concentrations[242]. Moreover, the arrested complex with bound synaptotagmin, complexin and assembly proteins is very large and difficult to fit close to the fusion site.

3. A third model postulates that SNAREs are fully zippered throughout the SNARE motifs and, possibly, into the linker regions, resulting in a tightly docked intermediate to which complexin is bound, requiring only minor energy input for fusion completion (reviewed elsewhere[134]) (Fig. 1, Step II). In this scenario, triggering by the C2 domains is mediated by perturbing the hydrophilic–hydrophobic boundary at the membrane contact site, for example by creating locally highly curved protrusions, perhaps assisted by the C-terminal final zippering steps into the membrane and/or rotational twisting of the SNARE complex, which may be carried out by synaptotagmin bound to the surface of the SNARE complex (alternative model, not shown). It is not clear whether, at this stage, accessory proteins such as Munc18 and Munc13 are still bound.
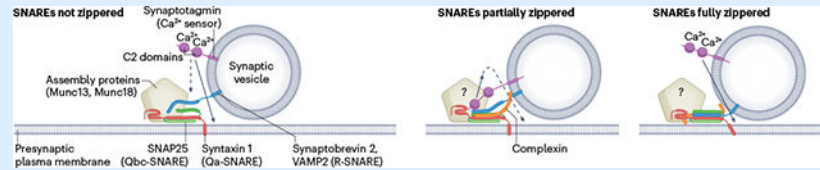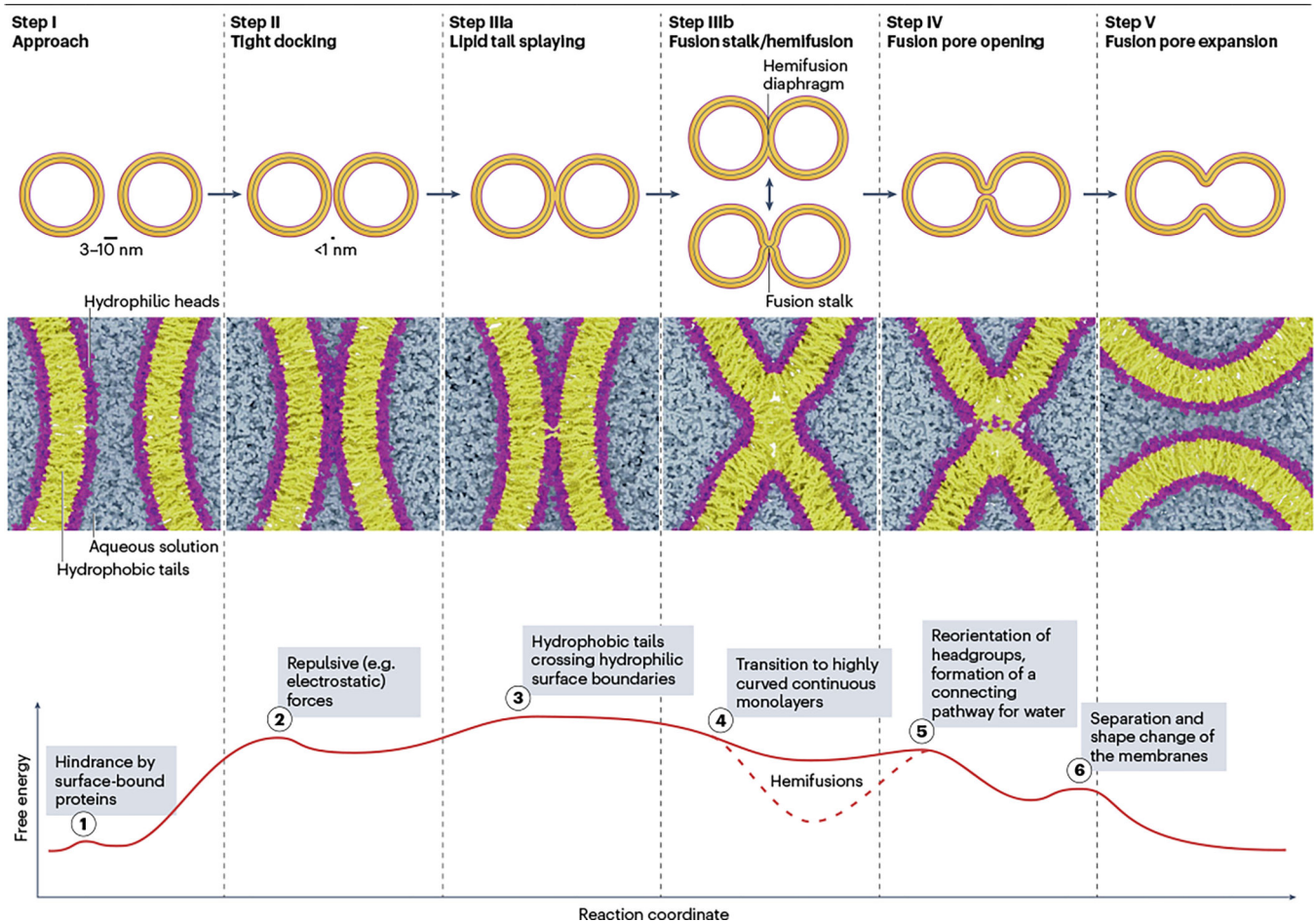
Fig. 1 |. Reaction pathway of fusion between two lipid vesicles.
Steps involved in the fusion of two lipid vesicles, with the two phospholipid monolayers
of the membrane indicated (black line in the middle) (top panel). Molecular details of the
intermediate steps, modelled by integrating information derived from various coarse-grained
simulation studies (see text). Coarse-grained models of the phospholipids of the membrane,
and the surrounding water molecules, are shown (middle panel). Hypothetical free energy
profile of the fusion pathway in the absence of proteins, with the energy barriers indicated
(encircled Arabic numbers) (bottom panel). Vesicle fusion is divided into steps, beginning
with the approach of two membranes, whereby bilayers destined to fuse become close
to each other (3–10 nm, Step I). Energy barrier 1 indicates that surface-bound proteins
may hinder a close approach. Once the membranes are in close proximity, a tight contact
is formed between the two hydrophilic bilayer surfaces, with headgroups being less than
1 nm apart (Step II). Major repulsive (mainly electrostatic) forces need to be overcome,
constituting the major energy barrier 2, but once this is achieved, tight contact is stabilized
by adhesive forces. The subsequent step involves lipid tail splaying, where aliphatic tails
of individual membrane lipids cross the hydrophilic gap between the connected proximal
monolayers, forming a hydrophobic link between the bilayer cores (Step IIIa). This state
is thought to be unstable (energy barrier 3) and rapidly proceeds towards the fusion stalk
or hemifusion stage (Step IIIb), but it is also possible that an additional small barrier

(energy barrier 4) needs to be overcome. In hemifusion, the proximal lipid monolayers are merged whereas the distal monolayers are still separated. Stalks may develop into extended hemifusion diaphragms with increased stability (dotted line in the energy profile) but may also overcome the next energy barrier 5 towards fusion pore opening (Step IV), which allows formation of an aqueous connection, which can only commence in areas where the two distal bilayers are in direct contact. Fusion pores are still not well understood. For instance, it is unclear which structural changes are involved in rapid opening–closing events referred to as fusion pore flickering. Finally, pores expand and re-establish stable bilayers (step V). Energy barrier 6 indicates that full opening may be hindered by transitions from a highly curved to a flatter state, which may involve major lateral rearrangements of membrane lipids.
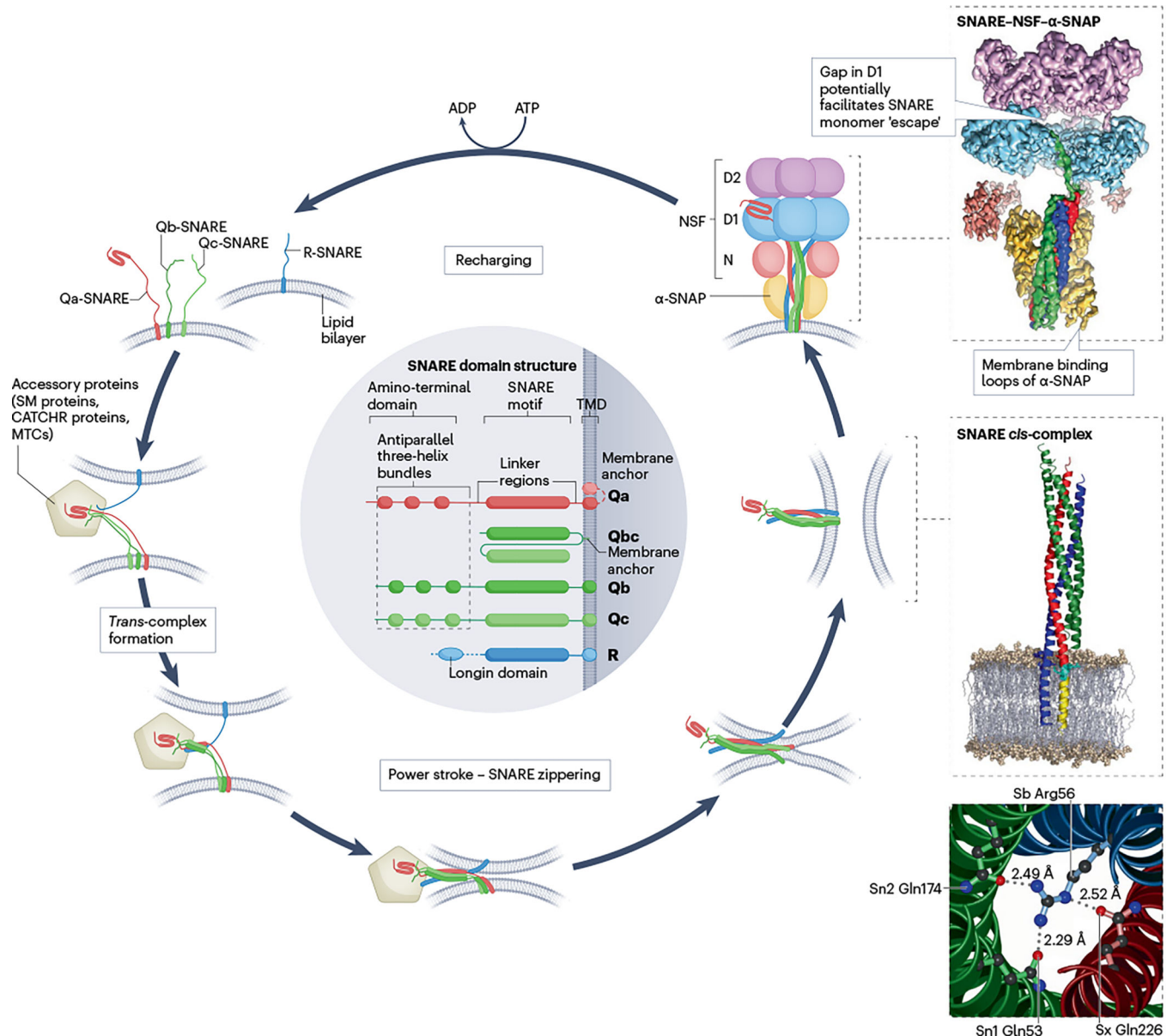
Fig. 2 |. SNARE assembly–disassembly cycle.
The cycle is depicted using an example in which three Q-SNAREs (Qa, Qb and Qc), each
with its own transmembrane domain (TMD), form a *trans*-complex with an R-SNARE
in the opposing membrane. This process is supported by accessory proteins such as Sec1/
Munc18-like (SM) proteins and complexes associated with tethering containing helical
rods (CATCHR) proteins, which may be part of multisubunit tethering complexes (MTCs)
containing additional proteins. Once *trans*-assembly is initiated at the amino-terminal ends
of the SNARE motifs, zippering of SNARE proteins progresses spontaneously towards
the carboxy-terminal membrane anchors ('power stroke', with the gradual formation of
the α-helical bundle indicated by progressively thicker lines), pulling the two membranes
together and allowing fusion. After fusion, the SNAREs are all aligned in parallel (SNARE
cis-complex). Disassembly of SNARE *cis*-complexes requires the vesicle-fusing ATPase

NEM-sensitive factor (NSF), which is composed of D2, D1 and N domains, and several copies of α-soluble NSF attachment protein (α-SNAP) that link NSF to SNARE complexes. Structures on the right show, from top to bottom, an assembled SNARE complex with bound α-SNAP and NSF [PDB:6MDM] (ref. 149), a model of a membrane-anchored neuronal SNARE *cis*-complex including linkers and TMDs of syntaxin 1 and synaptobrevin 2 (based on [PDB:3HD7] (ref. 76)), and an ionic layer of interacting side chains in the middle of the neuronal SNARE complex (based on [PDB:1SFC] (ref. 233)). This layer is highly conserved across all SNAREs, contains three glutamines (Q) and one arginine (R) and defines the four SNARE subfamilies Qa-SNAREs, Qb-SNAREs, Qc-SNAREs and R-SNAREs, respectively[57,233]. SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.
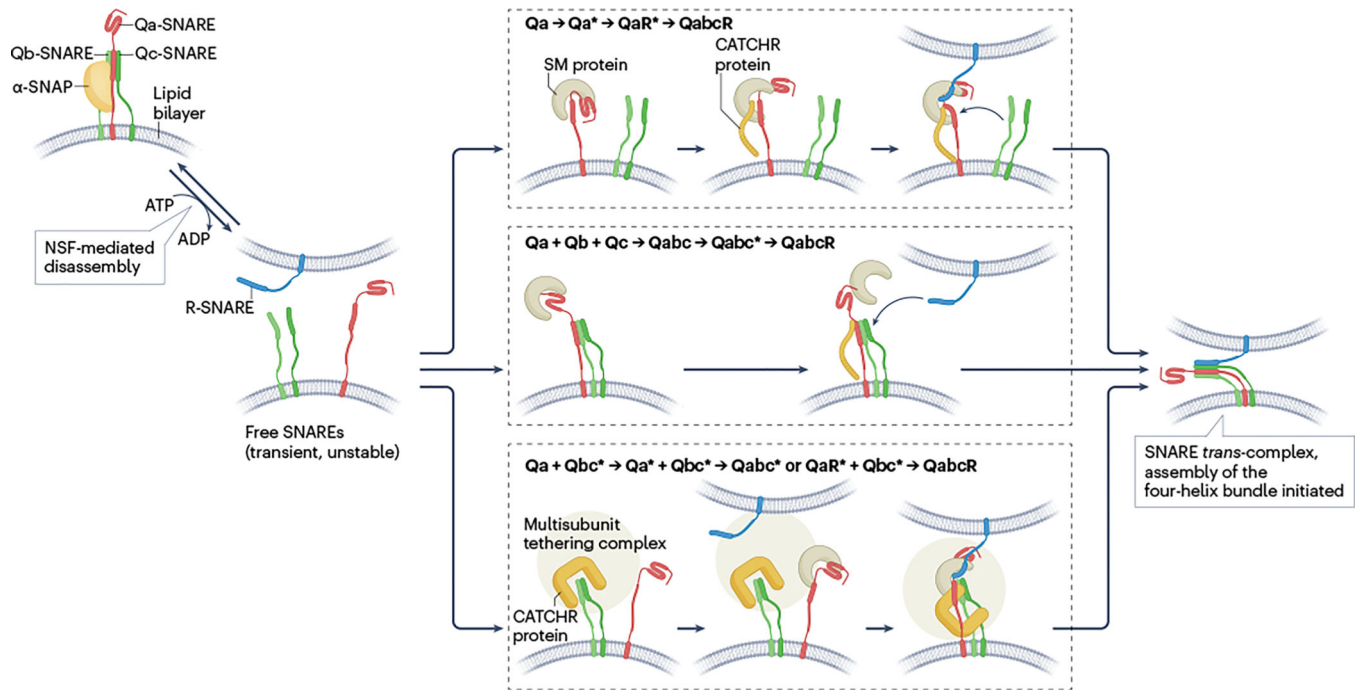
Fig. 3 |. Pathways for *trans*-SNARE assembly prior to fusion.
Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) zippering
requires the coordinated assembly of four conformationally flexible SNARE motifs between
membranes facing each other ('*trans*' configuration). Before assembly, NEM-sensitive factor
(NSF)-driven disassembly may be required to free SNAREs that are trapped in various
homomeric and heteromeric complexes. *trans*-assembly is guided by Sec1/Munc18-like
(SM) proteins and complexes associated with tethering containing helical rods (CATCHR)
proteins. Their sequential or simultaneous recruitment is thought to allow for an ordered
SNARE *trans*-assembly by stabilizing reactive intermediate complexes (indicated by
asterisks). Three possible assembly pathways are shown that are not necessarily exclusive.
According to one model (top panel), the SM protein first binds to the Qa-SNARE, yielding
a closed conformation that is then opened by the subsequent binding of the CATCHR
protein. The resulting SM–CATCHR–Qa-SNARE protein complex serves as a binding site
for the R-SNARE, followed by binding of the Qb-SNARE and Qc-SNARE motifs that
initiates zippering[15,116,117]. According to a second model (middle panel), SM proteins
form a metastable complex with Qa-SNARE, Qb-SNARE and Qc-SNARE motifs, with
the CATCHR protein possibly providing further stabilization, which allows for binding of
the R-SNARE as the final step in initiating zippering[102,118,119]. The third model (bottom
panel) accounts for variations that may occur if either the SM or the CATCHR protein are
integrated into multisubunit tethering complexes (MTCs). Shown is the example of the Dsl1
complex that contains a CATCHR protein and operates in the fusion of retrograde Golgi
vesicles with the endoplasmic reticulum. The complex first binds to the Qb-SNAREs and
Qc-SNAREs, and then associates with the Qa-SNARE that binds the SM protein separately.
The final step again involves recruitment of the R-SNARE to initiate zippering[120,121]. It
is still unclear at which point during (or after) SNARE zippering and in which sequence

the accessory proteins dissociate or whether they remain bound until fusion is completed. α-SNAP, α-soluble NSF attachment protein.
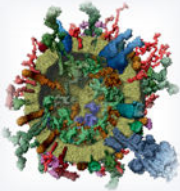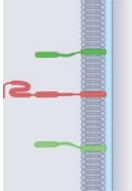
Table 1 |

Overview of techniques commonly used to study SNARE-mediated membrane fusion by in vitro reconstitution

Membrane type	Fusion partner	Fusion assays	Refs. (selection)
'Native' vesicles (purified from cells or tissues) For example, synaptic vesicles, neuroendocrine granules, endosomes	Native vesicles, SUVs, LUVs, GUVs, supported bilayers	Lipid mixing – measured using fluorescence dequenching, microscopic spreading of labelled lipids at the fusion site; Formation of SNARE complexes	212–215
Nanodiscs	Cells Liposomes	Fusion pore opening and closing – measured using electrophysiological methods, fluorescence microscopy	71,205,207, 209,216
Liposomes In solution or immobilized on solid support SUVs (30–60 nm) LUVs (70–300 nm) GUVs (>1 μm)	Native vesicles SUVs, LUVs, GUVs	Lipid mixing – shown using FRET, FCCS, fluorescence dequenching, microscopic spreading of labelled lipids or proteins; Content mixing – measured using various fluorescence assays; Formation of SNARE complexes– shown using FRET, biochemical assays	74,166, 217–226
Supported bilayers Immobilized on solid support	Native vesicles SUVs, LUVs	Lipid mixing – measured using FRET, FCCS, fluorescence dequenching, microscopic spreading of labelled lipids or proteins; Content release – measured using fluorescence spreading/dequenching Formation of SNARE complexes	227–231
Suspended bilayers	Native vesicles SUVs, LUVs	Lipid mixing – measured using FRET, FCCS, fluorescence dequenching, microscopic spreading of labelled lipids or proteins, or by monitoring current flow with electrophysiological methods using vesicles containing pores; Content release – measured by fluorescence spreading or dequenching	207,232

Membrane type	Fusion partner	Fusion assays	Refs. (selection)
Black lipid membranes			
Pore-spanning membranes			

FCCS, fluorescence cross-correlation spectroscopy; FRET, fluorescence resonance energy transfer; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SUV, small unilamellar vesicle.