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Review

Revisiting the role of SNAREs in exocytosis and membrane fusion

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Abstract

For over a decade SNARE hypotheses have been proposed to explain the mechanism of membrane fusion, yet the field still lacks sufficient evidence to conclusively identify the minimal components of native fusion. Consequently, debate concerning the postulated role(s) of SNAREs in membrane fusion continues. The focus of this review is to revisit original literature with a current perspective. Our analysis begins with the earliest studies of clostridial toxins, leading to various cellular and molecular approaches that have been used to test for the roles of SNAREs in exocytosis. We place much emphasis on distinguishing between specific effects on membrane fusion and effects on other critical steps in exocytosis. Although many systems can be used to study exocytosis, few permit selective access to specific steps in the pathway, such as membrane fusion. Thus, while SNARE proteins are essential to the physiology of exocytosis, assay limitations often prevent definitive conclusions concerning the molecular mechanism of membrane fusion. In all, the SNAREs are more likely to function upstream as modulators or priming factors of fusion.

Keywords: Secretion; Exocytosis; Clostridial toxin; Lipid; Fusion pore; Molecular mechanism

1. Introduction

> "*It is suggested that in BoTx poisoning the mechanism for transmitter release has a reduced sensitivity to Ca, and the level for activation by intracellular Ca is elevated...the release mechanism is in principle intact...*"
> Cull-Candy, Lundh and Thesleff (1976)

Although among the most fundamental and essential of biological mechanisms, the molecular process of membrane fusion, in both constitutive and triggered (regulated) release events, still eludes our extensive attempts to dissect and understand its underlying progression that results in the merger of two separate and distinct biological membranes and the compartments they previously delimited. This efficient, targeted process constitutes the defining step in mechanisms as seemingly disparate as neurotransmission, wound repair, hormone release, fertilization and blood coagulation. However, might these release events perhaps be more similar than they initially appear?

Compartmentalization and the necessity of (regulated) fusion has been a conserved theme in biology. Considering the extreme energy barriers inherent to the merger of two distinct membranes, and the molecular rearrangements necessary to accomplish leak-free coalescence of the apposed membranes into a single continuous bilayer structure [1–5], it is likely that fusion is a fundamentally conserved cellular mechanism, and there is evidence to support this concept [6–11]. It might be supposed that there had even been some evolutionary processing in this regard, with different molecular steps being optimized by selection until an energetically efficient series of reactions was available. Subsequent cell specialization would account for current differences in the Ca^{2+} sensitivity and speed of various systems; testing and selecting alternate Ca^{2+} sensors (different proteins and/or isoforms of a given protein) and assorted accessory proteins resulted in optimized functions in different cell types. The resulting 'variations on a theme' likely consist of the same, fundamentally conserved fusion mechanism [6–11] elaborated upon with different accessory and modulatory components to enhance efficiency, sensitivity to specific triggers and localization to optimized membrane domains.

Although we take a reductionist approach in our ongoing research directed toward dissecting the mechanisms under-

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lying the Ca^{2+}-triggered membrane fusion step(s) of exocytosis, we consider this from an inclusionist point of view; work from a range of model systems (from yeast-to-invertebrates-to-the-mammalian-CNS) contributes to the understanding of fundamental molecular processes. For example, let us consider the role of ATP. Across several cell types, the fusion step(s) of exocytosis has been shown to be independent of ATP. In 1975, the ATP independence of Ca^{2+}-triggered membrane fusion (of fully release-ready vesicles) was first demonstrated in membrane preparations from sea urchin eggs [12]. Subsequently, Baker and Whitaker [13] extended this finding in experiments designed to characterize the release mechanism. Elegant work from others [14], studying regulated exocytosis in paramecium, established that ATP was necessary for maintenance of a primed release state. Holz et al. [15] confirmed these findings in neuroendocrine cells, and identified an upstream ATP-dependent priming stage; a neuroendocrine cell line was used to identify priming factors [16,17]. The neuroendocrine cell studies also identified the rapid depriming of vesicles (within 2–4 min) that occurs in such cell types upon the depletion of ATP [15]. Time-resolved assays have built on these findings to show that only a subpopulation of morphologically docked vesicles are fully primed and release-ready [18–20]. The ATP independence of synaptic vesicle fusion has since been confirmed by Heidelberger et al. [21]. Thus, before the originally proposed SNARE hypothesis (suggesting that NSF-induced ATP hydrolysis disrupts an inter-membrane complex and thereby induces fusion [22,23]), it was known that ATP was required for exocytosis, but not for the membrane fusion step(s).

As every system and every research approach has its own inherent strengths and weaknesses as a 'tool,' we have tried to refine our functional assays, indeed our interpretations, to focus on the Ca^{2+}-triggered steps of membrane fusion. This is particularly relevant to the current review in that much of exocytosis research deals with 'readouts' of fusion rather than direct measurements of the fusion step(s) itself. Caveats to consider include (i) that we do not know all molecular components, steps or events in the pathways of exocytosis or fusion; and (ii) that most assays assess the pathway as a whole or at best multiple molecular steps underlying a part of the pathway. Thus, in our own work, we have found it necessary to use a specific terminology in reference to different pre-fusion, triggering and fusion events. We employ this terminology as an additional tool, to avoid semantic and thus interpretational ambiguities; similar definitions are broadly accepted in the field [24]. Thus, we define membrane fusion as the full, non-leaky merger of two previously distinct bilayer membranes into one continuous bilayer via a 'fusion pore;' hemifusion refers to the merger of only the proximal monolayers of two apposed bilayer membranes. By extension, we consider the 'fusogen,' or the 'fusion machine,' to be that molecular entity directly responsible for the membrane merger event itself. By definition, all other molecular components and steps are supportive or modulatory relative to the function of this molecular entity. We also consider 'pore expansion' to be a separate stage based on the existence of transient ('kiss-and-run') fusion events [25–27] and evidence for specific modulation of the expansion process [28–30]. Accordingly, vesicle content extrusion after fusion may be yet another mechanistically separable stage [31] or a simple physical consequence of pore expansion and full (irreversible) merger of the two fusing membranes.

Endocytosis, a multi-step fission process that recycles membrane from the plasma membrane (PM), is the next stage in the cyclic exocytotic pathway, but beyond the scope of this review. In very general terms, endocytotic vesicles pass either through the lysosomal system or rapidly re-enter the small pool of actively recycling vesicles ($\sim$ 10% of the total vesicle population in a presynaptic bouton) [32,33]. Vesicles in the cytosol appear to exist in one or more 'reserve' populations that can be recruited to PM release sites as required. Such vesicles must be appropriately targeted and transported to sites on the PM. Although possibly inter-related, we currently consider transport and targeting to be effected by distinct, separable mechanisms. Indeed, there appears to be much overlap of the subsequent (or parallel) pre-fusion mechanisms that are broadly defined as tethering, priming and docking. For the purposes of working definitions, we describe tethering as an initial stabilized contact between the vesicle and a targeted site on the PM; this is a developing research focus [34,35]. Although likely to be multi-step processes with substantial molecular and temporal overlap, we define priming and docking as a potentially separable series of events. Here, priming refers to any molecular reaction(s) that contributes to optimization of the fusogenic potential of the vesicle, and to its fully docked state; effective docking is required for the most efficient translation of subsequent signals ('triggers') for the fusion of the apposed vesicle and PM. As will be described, SNARE interactions may thus be key (perhaps culminating) events in priming. We consider 'docked' to be the most fully optimized stage of inter-membrane contact that can exist prior to actual fusion, and such vesicles (forming only a very small proportion of vesicles that appear morphologically attached to the PM) are often said to be immediately 'release-ready,' awaiting only the necessary trigger to initiate fusion (an appropriate increase in the local $[Ca^{2+}]_{free}$ in many instances) [18,20,36]; we do not, at the moment, subscribe to the idea that this represents a hemi-fused state per se [37], but recognize that rapidly passing through such a localized conformation is likely a requisite molecular rearrangement on the molecular pathway to fusion [38–40].

We stress that the actual molecular components essential (necessary and sufficient) to the stages described above are as yet largely unknown, or at least unverified in terms of definitive (but perhaps overlapping) roles in specific steps of the overall exocytotic pathway. Indeed, at the molecular level, many of these stages, particularly priming,

docking, triggering and fusion, may well be rapid, transient, multi-step processes that are not easily dissected experimentally. This emphasizes the fact that many established assays actually assess multiple stages of the exocytotic pathway, and it is thus often impractical to claim direct measure of fusion when either (i) changes in the release of vesicular content are detected from (semi)intact preparations; (ii) other stages in the pathway cannot be directly and independently assessed; or (iii) the membranes undergoing fusion cannot be directly verified. If intervention does not change the assay outcome, fusion is unlikely to have been affected, but if exocytosis is altered, this is not evidence for a direct effect on the fusion step(s) themselves.

2. An early tool: clostridial toxins

The clostridial neurotoxins have been among the most important tools in understanding the exocytotic pathway. Originally thought only to block neuronal function, this selectivity of binding and uptake was eventually shown to reside with the heavy chains of these toxins, while the catalytic light chains (Zn^{2+}-dependent metalloproteinases) are the actual active components that effect blockade of neurotransmitter exocytosis [41]. This distinction led to identification of the putatively selective substrates of these toxins, the SNARE proteins [42,43]. Many VAMP/synaptobrevin isoforms are cleaved by tetanus toxin (TeTx) and botulinum toxin (BoTx) B, D, F and G, SNAP-25 isoforms by BoTx A, C and E, and Syntaxins by BoTx C (reviewed in Refs. [41,44]). Notably, mutations have resulted in at least one cleavage resistant native isoform of each of the three SNARE proteins (reviewed in Ref. [44]).

For well over a century, medical science has sought to understand the actions of the clostridial neurotoxins. Many of these now classical works highlight initial studies into synaptic mechanisms and the vesicular hypothesis of neurotransmission, thus charting the course of modern neurophysiology. Considering that the molecular targets and even the proteolytic nature of the clostridial neurotoxins were unknown at the time, these studies were elegantly insightful. As early as 1939, Harvey [45] noted that spontaneous release (muscle activity) still occurred following poisoning with TeTx, and that repetitive stimulation (even by only two effectively spaced stimuli) resulted in increased activity; increased intra-terminal $[Ca^{2+}]_{free}$ overcame the block by TeTx. These findings were confirmed for preparations poisoned with BoTxA [46], in which depolarization with KCl was also shown to produce a transient recovery. The work of Burgen et al. [46] was also important for extending substantially earlier original observations that decreased activity (of the test subject/preparation and thus of synaptic activity) would slow the rate and extent of poisoning. These observations suggest that synaptic activity (turnover in the cyclic exocytotic pathway) enhances the effect of the toxins and that the block must lie upstream of the fusion reaction since enhanced stimulation can elicit some release (fusion), albeit from a now rapidly exhaustible pool [47] (see also Ref. [48]). Brooks [49,50] soon after concluded that BoTxA did not directly inactivate the release mechanism itself. Thesleff [51] also noted that doubling the external (bath) $[Ca^{2+}]$, adding TEA or inducing mechanical injury to the motor-endplate overcame the block of release by BoTx A. Spontaneous release events in denervated nerves were also much less sensitive to BoTxA, again suggesting that synaptic activity (vesicular turnover) promoted the ability of the toxin to affect a block of neurotransmission (see also Ref. [47]). Tetanic stimulation and reduced temperatures were also shown to overcome blocks by both BoTx A and TeTx [52].

Extensive studies by Parsons et al. [53], and by Harris and Miledi [54], confirmed many of the findings described above, illustrating $[Ca^{2+}]$-dependent recovery from TeTx and BoTxD intoxication, respectively, by increased external $[Ca^{2+}]$, KCl-depolarization and paired stimuli. Both studies reached conclusions of substantial long-term importance to understanding synaptic function: toxin (i) decreases the probability of transmitter release [53]; and (ii) interferes with stimulus-secretion coupling [54,55]. More recent work on factors affecting SNARE complexes has now reached similar conclusions regarding effects on release probability [56,57], but the underlying mechanism remains unknown. Notably, repetitive stimulation was also shown to transiently relieve a complete block of neurotransmission produced by TeTx [58]. The most extensive single analysis (even including a second dose of toxin) may well be that by Cull-Candy et al. [59], who concluded that the block by BoTxA affected the Ca^{2+} sensitivity of the release mechanisms while clearly leaving the actual fusion machinery intact. Thus, several decades of work indicated that the target(s) of the clostridial neurotoxins (then unknown) are not minimal essential components of the native fusion machine, but rather that they are important upstream modulators of synaptic efficacy. Subsequent elegant studies also demonstrated distinct pre-fusion sites of action of the different clostridial toxins, including effects on priming [52,60–64]. However, caveats to be considered include (i) the effectiveness and purity of some of the early toxin isolates; (ii) the possibility that some nerve fibres escape poisoning in the tissue preparations used; and (iii) the potential presence of toxin-insensitive SNARE isoforms that might still function in a manner consistent with the SNARE hypothesis. The first and second issues are well addressed by the general consistency of findings, using a variety of toxin and tissue preparations over several decades, and by the range of toxin doses and poisoning periods studied over this time. In addition, Cull-Candy et al. [59] used second applications of toxin to ensure full extent of poisoning. Gansel et al. [60] also used toxin combinations, all without additional effect. However, the potential presence of low levels of compensatory, toxin-

insensitive SNAREs cannot be easily ruled out in these or other studies, yet recovery after extensive, long-term blockade of exocytosis only occurs via the sprouting of new terminals.

3. Comparative studies of toxin action

Despite the availability of the clostridial toxin light chains, several factors have hampered the usefulness of these toxins in terms of clearly defining the role(s) of the SNARE proteins. These problems include the (i) existence of toxin-insensitive SNARE isoforms, and (ii) lack of routine, highly sensitive quantitative assays (to confirm the extent of SNARE cleavage). These issues are further complicated by the lack of selectivity of most assay systems for specific steps in the exocytotic pathway; an observed effect of these toxins in a given cell type clearly indicates a role for the SNAREs in the dynamics of the cyclic exocytotic pathway, but tends to provide little additional evidence as to the actual molecular contribution(s) of these proteins to membrane fusion. While such experiments have been carried out in a wide range of different constitutive and regulated secretory cell types, blockade of release is rarely consistent or complete [52,59,60,63–71]. While these studies clearly indicate strong evolutionary conservation of molecular mechanisms involving SNAREs at one or more stages of the exocytotic pathway, more definitive conclusions are difficult. However, the ability to bypass clostridial blockade clearly demonstrated that fusion mechanisms remained intact and functional. Ca^{2+}- but not GTPγS-triggered insulin release was blocked by TeTx and BoTx B [72]. The BoTx A and C block of neurotransmission in cultured hippocampal neurons could be partially overcome by increased intra-terminal $[Ca^{2+}]_{free}$, addition of cAMP or by substituting Sr^{2+} for Ca^{2+} [68]. Fassio et al. [73] showed that increased $[Ca^{2+}]_{free}$ (via ionophore) could bypass inhibition of exocytosis by TeTx and BoTx F. Similar to the early work on the neuromuscular junction, these studies indicate that the final membrane fusion steps of excocytosis are still functional after selective SNARE cleavage by the different clostridial toxins, but that upstream regulation by Ca^{2+} is perturbed. This conclusion is substantiated by work in three very different systems.

First, despite extensive treatment with clostridial toxins (singly or in combination) effecting the removal of the bulk of the resident SNARE proteins from fully Ca^{2+} sensitive, fusion-ready cortical vesicles (CV) isolated from unfertilized sea urchin eggs [36,74,75], there was no effect on Ca^{2+}-triggered homotypic fusion [76,77]. These findings (1997–1998) were confirmed and extended using a variety of coupled functional and biochemical assays, leading to the conclusion that the SNARE complex might promote the Ca^{2+} sensitivity of late triggered steps of exocytosis, but was not an essential component of the minimal native fusion machine [36,75–77]. Notably, fusion in the intact urchin egg can be disrupted by clostridial toxins if the eggs are first treated so as to un-dock CV from the PM, implying a more likely role for the SNAREs in targeting and docking [78].

Second, studies focussing on the homotypic fusion of isolated yeast vacuoles also indicated that SNAREs functioned upstream of fusion; *trans* SNARE complexes appeared to 'activate' a pathway to fusion, but the presence of these complexes was not required for subsequent progress through the fusion pathway [79–82].

Third, detailed time-resolved analyses of Ca^{2+}-triggered exocytosis from bovine chromaffin cells treated with different clostridial toxins indicated inhibition of both the fast and slow phases of exocytosis; BoTx A had the weakest effect [70]. Inhibition of the slower phases of exocytosis implies blockade of one or more steps upstream of fusion. Attempts to use higher $[Ca^{2+}]_{free}$ to bypass the clostridial block were confounded by a large release response that did not correlate to dense core vesicles; elevated $[Ca^{2+}]_{free}$ are known to trigger exocytosis of lysosomes in many cell types [83,84]. Nevertheless, there are several points to be considered in interpreting these experiments. An ultra-fast phase of vesicle release appeared still to be present in the toxin-treated cells; the change in membrane capacitance upon triggering was still equivalent to that measured for a small pool ($\sim$ 10–35) of fully release-ready vesicles [85–89]. A corresponding amperometric signal appeared in some experiments but not all [70]. The presence of this pool is somewhat surprising. This small pool is most easily interpreted to represent fully docked and release-ready vesicles, presumably with SNARE proteins already complexed and thus inaccessible to the clostridial toxins. However, observations of vesicle dynamics near the PM indicate that even attached ('docked') vesicles turn over within $\sim$ 100 s [90]; pre-stimulus incubations were for $\sim$ 10 min in the experiments with clostridial toxins [70]. If these vesicles were fully docked prior to toxin delivery through the patch pipette, why did they not dissociate from the membrane and become toxin sensitive during the pre-stimulus incubation? If they did, how did other vesicles, previously exposed to toxin, replace them? One possible explanation is that many intact SNAREs might still be operational in these experiments. There are clear differences in the cleavage susceptibilities of recombinant SNAREs in vitro and SNAREs in a native membrane. As current measures of SNARE density on secretory vesicles are $\sim$ 10 times higher than estimated for the in vitro tests used to assess toxin efficiency [70], substantial amounts of functional SNARE proteins likely remained [91]. Then why was exocytosis affected in the manner detected? Again, the results are most simply interpreted to indicate that the SNAREs function upstream of fusion, promoting the 'normal' physiological response because extensive secretion was inhibited, but the actual fusion event was not (ultra-fast phase). Combined with the findings described above, from the experiments using CV and yeast vacuoles, the results might be interpreted to indicate that once a group of vesicles have gone through a *trans* SNARE 'priming' step,

they, for some period of time, remain prepared to engage in rapid fusion, and therefore represent a highly release-ready pool. In some systems such as neuroendocrine cells, this dynamic primed and immediate fusion-ready state might last for ~2–4 min in the presence of cytosol, for up to 90 min in yeast vacuoles, and for tens of hours (or longer; in the absence of cytosol) in isolated CV [36,76]. This long-lived fully primed and fusion competent (stage-specific) state of docked CV, together with the Ca^{2+}-triggered disassembly of SNARE complexes prior to fusion, has led to one possible interpretation being that the SNAREs have not only carried out their essential function(s) in exocytosis by this point, but that their regulated removal from the site of inter-membrane contact represents a critical step to ensuring fast, efficient fusion [75].

A second study by Xu et al. [92] used an antibody (Fab fragment) to SNAP-25, known to block SNARE complex formation in vitro, to test the role of the complex in the late Ca^{2+}-triggered steps of exocytosis. Again, both the fast and slow phases of release were perturbed, but the fusion machinery was clearly intact. Slowing the kinetics of triggered release suggests a modulatory role for the SNARE complex, consistent with an influence on the probability of fusion in the physiological range of $[Ca^{2+}]_{free}$. The interpretation was that 'loose' inter-membrane SNARE complexes were sufficient to support fusion but that fully 'zippered' complexes were necessary for fast, triggered fusion; these different states of the SNARE complex may correlate with previously described differences in sensitivities to clostridial toxins [48]. However, as SNARE complex formation in vitro has been suggested to correlate with *cis* rather than *trans* complexes, and native complexes were not assayed in this study [92], more definitive statements concerning sites and effects of the Fab fragment binding cannot be made. As with the earlier experiments involving clostridial toxins, there is a question as to why an incomplete block of the burst phase of release occurs if fully docked vesicles turn over during the 10-min intracellular exposure to the blocking antibody. Alternatively, one might also postulate that binding of the Fab fragment prevented effective clearance of SNARE complexes thereby blocking fast, efficient fusion. However, considering as a whole all the studies described above, and noting that (i) the extent of inhibition of exocytosis by a given clostridial toxin varies widely among different cell types; and (ii) standard Western blotting or immunocytochemical analyses are inherently insensitive, potentially 'missing' thousands or more copies of a given protein (increased risk of 'false-negative' results) [91], firm conclusions as to the actual function(s) of the SNARE proteins remained difficult. There was no way of confirming whether the actual amounts of intact SNARE proteins remaining after clostridial toxin treatments were sufficient to account for the remaining function, whether SNARE fragments acted to promote function [93,94], whether residual function was due to the presence of toxin-insensitive SNARE isoforms, or some combination of the above. Additional clostridial toxin targets also could not be ruled out [95–100].

As an alternative, molecular genetic approaches have also been applied to the question of SNARE function. While these studies have taught us a great deal concerning details of protein–protein interactions, providing tools that will undoubtedly prove invaluable to future mechanistic studies, many of the same issues outlined above plague a definitive interpretation of SNARE functions in membrane fusion. While various SNARE mutations block or modify exocytosis to varying extents, the assay formats used do not permit detailed analyses of the Ca^{2+}-triggered fusion steps themselves. Early SNARE mutations/knockouts in *C. elegans* and *Drosophila* yielded results consistent with the early clostridial toxin work in neuromuscular junction preparations; fusion still occurred (spontaneous release), but regulation of the release process was disrupted [101–105]. Up-regulation of syntaxin 1A suggests a distinct role in defining vesicles for the regulated secretory pathway [106–108], and VAMP mutations suggest disruptions of vesicle targeting and docking [109]. Washbourne et al. [110] showed that SNAP-25 mutants incapable of binding VAMP still support exocytosis. In addition, SNAP-25 mutations that should disrupt SNARE complex formation and stability still support normal exocytotic release from neuroendocrine cells [111]. Most recently, knockouts of neural SNAP-25 [112] and VAMP2 [113] in mice (also showing perturbed regulation of release) have been suggested to indicate that SNAREs may not be essential to fusion. However, additional treatments with clostridial toxins were not performed [114], and the presence of compensatory SNARE isoforms cannot be ruled out. The evidence for such potential 'rescue' in vivo is clear [115–117]. Therefore, unless all the known isoforms of a given SNARE in a specific organism can be simultaneously knocked out, SNARE hypotheses remain difficult to test even with the power of molecular genetics. Given the critical role(s) of SNAREs in the exocytotic pathway, even this approach would not provide a definitive assessment as it would likely be fatal quite early in development. Inducible knockouts are an obvious alternative, but still cannot address the issue of multiple SNARE isoforms.

4. Fusion in vitro

One possible approach to testing SNARE hypotheses requires the use of a stage-specific preparation such as the urchin CV; by all available criteria [36,75,118], Ca^{2+}-triggered CV–CV fusion in vitro, in the absence of cytoplasmic factors, occurs through the same molecular pathway as exocytotic release. Since cytoplasmic components, including SNARE recruitment and assembly factors [119,120], are not required for triggered CV fusion, an upstream modulatory role (e.g. pre-fusion) for the SNAREs is suggested. Further-

more, despite extensive analysis by a number of laboratories, evidence suggests CV membranes contain only one isoform of each of the SNARE proteins [76,121]. Considering the sensitivity of both triggered fusion and the SNARE proteins to proteases such as trypsin, it was anticipated that treating isolated CV (thus exposing the entire complement of vesicular membrane proteins) with broad spectrum proteases might produce a 'biochemical knockout' of one or more of the SNARE proteins [36]. If fusion persisted after such treatments, a direct role for the SNAREs as essential components of the native fusion machine would be ruled out. This approach, utilizing stage-specific native membranes, makes no assumptions as to which proteins are important, and would theoretically result in complete SNARE removal as all known SNARE isoforms (including those insensitive to the clostridial toxins) have an extensively conserved number of potential cleavage sites for proteases such as trypsin. Indeed, the extensive cleavage of SNAREs in vitro and in vivo has been demonstrated. However, the effective analysis of a molecular mechanism requires the accurate identification and quantification of specific proteins. A quantitative, ultra-sensitive immunoblotting protocol has been developed and optimized [91].

Coupling sensitive detection with broad spectrum protease treatments has now permitted direct testing of SNARE hypotheses. Quantitative removal of all three types of SNARE proteins, in some cases including the complete removal of syntaxin from CV, does not block Ca^{2+}-triggered fusion [122,123]. Furthermore, despite substantial ablation of the resident CV SNAREs ($\geq \sim 90\%$), clostripain treatments had no effect on the Ca^{2+} sensitivity or extent of fusion or, perhaps most importantly, on the kinetics of fusion. This is perhaps the most direct evidence that SNAREs are unlikely to be essential components of the minimal native fusion machine; if essential, such substantial removal should most certainly have affected the kinetics of Ca^{2+}-triggered fusion. Furthermore, the estimated energy contributed by SNAREs and associated proteins ($\sim$ 2 kT) [123] is lower than that thought to be required to overcome the hydration energy barrier at the membrane surface [124] or for bilayer merger [3,5,125]. Differential protease effects suggest the existence of a native fusion machine with an inherently low Ca^{2+} sensitivity. One hypothesis is that SNAREs and their immediate binding partners may act to modulate the Ca^{2+} sensitivity of this native machine into the physiological range of $[Ca^{2+}]_{free}$ [123].

The simplest interpretation of the above study is that SNAREs are not essential components of the minimal native fusion machine; there is little possibility of compensatory proteins preserving function in the Ca^{2+}-triggered fusion pathway of this particular stage-specific system. These findings are consistent with work in the yeast vacuolar system suggesting the existence of a fusion mechanism functioning downstream of the SNAREs [81,82]. In addition, mutations in genes related to fatty acid elongation and sphingolipid synthesis bypass the need for vesicular SNAREs, suggesting that SNAREs contribute to docking and the efficiency of fusion, but not to the fusion mechanism per se [126]. Although not yet verified in other systems, these studies on CV and yeast vacuoles support the concept of an upstream 'priming' role for the SNAREs, with alternate factors underlying the actual membrane merger steps. In contrast, other work on constitutive exocytosis in yeast has led to an alternate view of SNARE function, suggestive of a role in fusion. In these studies, SNARE transmembrane regions were replaced with covalently attached lipid moieties (geranylgeranylated) and the slow constitutive pathway was blocked; these results have been interpreted to suggest that the transmembrane domain of SNAREs can function late in fusion [127], an idea supported by studies demonstrating that peptides corresponding to these transmembrane regions can induce fusion of artificial membranes [128]. For reasons that are not immediately apparent, these results are inconsistent with the work on yeast vacuole fusion that indicates SNAREs are not required at the fusion step [81,82]. Overall, both sets of results in the yeast system appear inconsistent with the fact that isolated CV and other native vesicles can fuse with pure lipid target membranes [129–132]. While consistent with a lipidic fusion pore, these results are more difficult to reconcile with a proteinaceous, 'channel-like' fusion-pore, although triggered conformational changes of a proteolipid could perhaps contribute. Furthermore, recognized viral fusogenic proteins, to which SNAREs have been compared, will also catalyse merger with protein-free target membranes [133–135]. This is most interesting when considering the saddle model of hemagglutinin-induced fusion, where the viral peptide inserts into its own membrane rather than the target membrane [136]. SNAREs appear incapable of triggering comparable events [137].

Thus, although hypotheses concerning transient intermembrane "SNAREpins" as fusion complexes are questionable in light of the results of studies in a number of model systems, alternate hypotheses concerning the involvement of SNARE transmembrane regions in hemifusion intermediates require further investigation. However, it remains unclear how these transmembrane regions would converge in cases of cytosolic domain disruptions (that obviate inter-membrane complex formation and function), or in the absence of additional cytosolic factors that are suggested to promote complex formation [119,120,138]. If SNARE clearance from the fusion site is required for fast, efficient fusion, convergence of these transmembrane regions seems unlikely unless it contributes to localized membrane destabilization well before the actual fusion event, which might be promoted by specific membrane domains [139].

5. Reconstitution

The strongest case for the SNAREs as fusogens has come from the work of Weber et al. [137] who have used recom-

binant SNAREs reconstituted into artificial lipid membrane systems to study the effects of SNARE interactions in vitro. This technically complicated experimental approach has become an elegantly routine assay system in this group [137,140–146]. The type of lipid vesicle preparation used (small unilamellar liposomes) was originally developed to retain small soluble content markers, the mixing of which could be used to assess actual fusion events. Simply assessing intermixing of membrane lipids was unsatisfactory as it did not represent complete fusion, and could even occur by transbilayer exchange under conditions of close inter-membrane apposition. One of the principal concerns with interpretation of the reconstituted SNARE assays is that this system has never demonstrated the capacity to retain low molecular weight solutes; even large (~ 5 kDa) oligonucleotide markers showed a high level of 'leakage' [141]. But SNAREs are clearly capable of interacting to yield complexes that could promote closer apposition of membranes [147]. Although SNAREs may contribute, in part, to overcoming the energy barrier imposed by the ubiquitous hydration layer [1], and thereby perhaps promoting nonspecific lipid exchange between the apposed membranes, this state would be unlikely to spontaneously yield a fusion site unless the bilayers were already destabilized. Strong, nonselective increases in inter-membrane attractive forces in vitro have indeed been shown to sufficiently reduce inter-bilayer distances such that local dehydration induces hemifusion [148,149]. It is unclear from the published reconstitution assays the extent to which lipid exchange and bilayer mixing each contribute to the measured signals, and to what extent the signals being assessed are actually comparable to a native fusion event. Lipid bilayer contact is required for bilayer mixing but not for nonspecific lipid exchange. The structure of the SNARE complex [147] suggests that an intervening hydrated interface of ~ 2nm must remain between the SNARE-apposed membranes, thus favouring nonspecific lipid exchange over true bilayer mixing. This concern is reinforced when SNARE densities are considered. Recent assessments reveal a striking similarity between the SNARE densities measured on CV and synaptic vesicle membranes [91]. These native SNARE densities are 50-fold lower than required in the reconstituted SNARE preparations [137]. Although a recent report indicates that the in vitro assays will work with lower VAMP densities, the VAMP was replaced with recombinant synaptotagmin, which does not allay concerns regarding the bilayer status (stability) of these preparations [142]. Clearly, such a substantial increase in the local energy contributed by SNAREs, relative to that in stable native membranes, could well promote a nonspecific membrane merger event, but this is unlikely to be representative of a biological fusion site. If this is indeed what is occurring, it might, in part, explain the rather slow fusion kinetics in these reconstituted systems relative to triggered fusion in vivo. Although an increase in $[Ca^{2+}]_{free}$ triggers fast fusion events in most secretory cell types [18,20,150,151], reconstitution of synaptotagmin, the putative essential Ca^{2+} sensor for triggered release does not enhance fusion in the reconstituted preparations [142]. Similarly, lipid mixing between isolated synaptic vesicles (containing synaptotagmin) and SNARE-containing liposomes can be triggered by aggregation alone and is relatively slow even at high $[Ca^{2+}]_{free}$ [152].

Over the last several decades, a substantial number of proteins have been shown to promote aggregation, adhesion, lipid mixing and even fusion in model membrane systems [149,153–163]. In many cases, this fusion also involves content mixing, is reasonably fast, and can be triggered in some fashion that is at least reminiscent of biological fusion events. Thus, there are clearly differences between what can be demonstrated in vitro and actual protein functions in vivo [164]. Although the native cellular localization of the SNAREs may make these more likely candidates than some of the other proteins shown to promote fusion in vitro, this does not constitute evidence that the results of assays in the reconstituted SNARE preparations actually describe a minimal biological fusion machine. Thus, although it has been argued that additional factors may be present in native systems and that these promote SNARE function, this is one possible interpretation, and not evidence supporting a role for the SNARE complex as the minimal fusogenic entity. Indeed, the SNARE interacting protein *N*-ethylmaleimide (NEM)-sensitive factor (NSF), which is thought to mediate post-fusion uncoupling of SNARE complexes and thus promote their functions in vivo, has also been shown to independently promote the fusion of liposomes [161,162,165]. It has been argued that this effect of NSF is limited to liposomes of specific lipid composition, and that since the effect of the SNAREs extends to other lipid mixtures, the SNARE complex represents a minimal fusogen [163]. However, liposomes are rarely used in an effort to mimic global lipid compositions of entire cellular systems; rather, they are most often used to mimic localized sites on membranes. If a proteinaceous fusion machine (SNARE or otherwise) has been evolutionarily conserved and optimized, it seems unlikely that the same rationale does not apply to the lipid components that form the bulk of the membrane, and are the local substrates on/with which the proteins must function. To postulate that one lipid mixture is more likely or appropriate than another implies knowledge of the actual lipidic species functioning at the native fusion site. To the best of our knowledge, such specifics remain unknown, although much elegant work has suggested that certain lipid species are more likely than others [129,131,132,159,165,166]. If optimization of the focal lipid mixture at the fusion site is not critical, then SNARE complexes at native densities [91] should also drive the full fusion of membranes composed primarily of saturated, long chain species of phosphatidylcholine, sphingomyelin or ceramide; we are unaware of such a study. However, as isolated CV are at a stage of fusion readiness that no longer requires cytosolic factors, we suggest that NSF and other

identified promoters of SNARE function [119,120,167] are more likely to act upstream of the triggered native fusion event, and possibly also in later modulatory roles [168]. Thus, the reconstituted systems have demonstrated that the SNAREs may code for some level of selectivity in regulating the interactions of specific intracellular compartments [169,170], and that the resulting inter-membrane complexes may contribute to defining potential fusion sites. Therefore, roles in targeting, docking and priming steps of exocytosis are possible (e.g. pre-fusion), and disruptions at one or more of these stages could explain the altered functions observed when the cellular complement of SNAREs is targeted by toxins, peptides, and so forth [59,70,92,171,172].

6. Biophysics of membrane fusion

Bilayer membrane fusion has been described in terms of a progression of intermediate structures of lower free-energy, which involve rearrangements of the lipid matrix. Detailed mathematical modeling has been used to explore a number of potential molecular-structural models; the sequence of structural rearrangements corresponding to the lowest-free energy states has been identified as that involving a 'stalk-pore' transition [3,40,125,173,174]. The initial step in this sequential rearrangement and merger of focally apposed bilayer domains requires an input of energy to overcome the hydration and molecular repulsive barriers [1,2,175], thus bringing the apposed membranes into molecular contact. The subsequent rearrangements of the lipid matrix reduce the net free-energy of the lipid assemblies, mainly through the reduction of interstitial and curvature energies. Interstitial energy can be reduced by hydrophobic molecules occupying the interstitial volumes (within the hydrophobic domain of the membrane) [176–179], or by alternate packing and tilt of the acyl chains [5]. Curvature energy is described in terms of spontaneous curvature, where the curvature of a monolayer minimizes the bending elastic free-energy [176]. Various lipid species influence the spontaneous curvature of a membrane, either by adding zero curvature energy, positive curvature energy (increased propensity to forming curved assemblies with the polar groups on the convex side) or negative curvature energy (increased propensity to forming curved structures with the polar groups on the concave side) [180]. Therefore, the curvature properties of the lipids that compose fusogenic membranes influence the transition of intermediate structures [39,181].

Briefly, the proximal (contacting) monolayers of the apposed bilayer membranes merge, forming a contiguous, highly curved 'hourglass' structure with a net negative curvature (polar groups on the concave side), sandwiched between the still intact distal (non-contacting) monolayers of the apposed membranes (see Fig. 1 of Ref. [3], or Fig. 4 of Ref. [181]); the result is an inter-membrane 'stalk,' which defines a hemifusion state. Once formed, the stalk expands, thinning until the distal monolayers merge, forming a lipidic fusion pore which has a net positive curvature (polar groups on the convex side). Recently, it was demonstrated that model lipid assemblies undergo fusion through a process that is consistent with stalk formation [182], and this has also been seen in molecular dynamic simulations [183]. Existence of a stalk-like intermediate in native membrane fusion is supported by the reversible block of a range of biological fusion reactions by lysophospholipids [39,184,185]. This block is downstream of SNARE function, and occurs irrespective of SNARE protein complexes functioning upstream [76]. A lysophosphatidylcholine block was used to show that SNARE interactions were not strict determinants of a docked vesicle. SNAREs may function early in tethering/docking, but their interactions are not essential to maintenance of the fully docked and release-ready state [75]. Similar conclusions have been reached using total internal reflection fluorescence microscopy of TeTx and BoTx A expressing chromaffin cells [186].

7. The Ca^{2+}-triggered fusion steps of exocytosis

A decade of testing SNARE hypotheses has thus far not provided the definitive experiment(s) to fully assess SNARE contributions to exocytosis, although work on protease-treated CV does indicate that SNAREs likely function upstream of the (regulated) fusion steps [123]. Identification of the SNAREs and a large number of interacting proteins has provided a substantial catalogue of components necessary for effective functioning of the exocytotic pathway. Considering tests of function since the earliest neurophysiological analyses of clostridial toxin effects, we have critically evaluated the most probable role(s) of the SNAREs in the exocytotic pathway. Despite a range of hypotheses, we currently view the SNAREs as modulatory (promoting), working upstream of fusion and not representing minimal essential components of the native mechanism directly responsible for the membrane merger events of exocytotic fusion. Clearly, there are important implications for current research directions, for mechanistic models of exocytosis and for a molecular-level understanding of the (regulated) fusion pathway.

While SNARE modulatory functions are clearly essential to the physiology of exocytosis, perhaps in part defining potential native fusion sites, there is clearly a difference between contributions to the establishment and efficiency of a mechanism, and the mechanism itself. We interpret the SNAREs and associated proteins to function in targeting, docking and priming, perhaps in the last priming step that ensures full Ca^{2+} sensitivity, and thus a rapid, triggered fusion response. Changes induced by SNARE complexation result in an activated, fusion-ready state, and subsequent mechanistic steps leading to fusion may well be SNARE-independent, possibly even requiring disassembly (clearance) of SNARE complexes in order to most fully facilitate

the membrane merger steps. In this respect, the earliest cartoons proposing a role for the SNAREs in exocytosis may still be the most relevant [187]; a 'black box' still exists between SNARE complex formation and the actual membrane merger steps defining native fusion. Although a mechanistic pathway to the fusion step has now been proposed [81,82], this model requires testing in other systems. A recent groundbreaking study clearly supports the concept of a lipidic fusion pore, perhaps consistent with the involvement of a proteolipid [188].

Strong evidence also exists for alternate SNARE functions, in particular the regulation of Ca^{2+} and other ion channels [189–197]; however, recent evidence suggests that this represents an adapted modulatory function in mammalian systems [198]. How does this, if at all, relate to the proposed roles of SNARE complexation in fusion? One possible explanation is that this is the actual function of the SNAREs, to target and attach vesicles to appropriate 'functional' sites, in part also regulating the channels and thereby signalling pathways. It may be that accessory proteins associated with the SNAREs (or the channel; [199,200]) mediate the actual fusion steps of exocytosis. Or, SNAREs may simply have two (or more) separate but overlapping roles in the exocytotic pathway. Alternatively, perhaps SNARE complexes are artefacts of the techniques we currently have at our disposal [201]. This is an intriguing suggestion, implying that most SNARE complexes assayed in vitro form as a result of sample aging and are therefore not representative of functional native complexes. If this is the case, it means that after tens of hours of aging in vitro, CV SNAREs should be relegated to nonfunctional complexes. Furthermore, as the same group also reports the extremely high stability of SNARE complexes [202], CV aged in vitro should be non-fusogenic. This is simply not the case [36,76] [unpublished observations], again suggesting that SNAREs do not function as the minimal fusogens of native membranes.

Considering the CV studies, it is now reasonably certain that the SNAREs (either alone or as complexes), having carried out their critical upstream function(s), are not required during the Ca^{2+}-triggered steps of membrane fusion. In a sense, the unfertilized urchin egg provides us with a stage-specific 'snapshot' of these particular steps in the exocytotic pathway. This would account for the speed of the first fusion events within the population of $\sim$ 15,000 fully docked and release-ready CV in an egg; fusion is at least as fast as that measured in neuroendocrine cells, with the lag-time after a rapid rise in $[Ca^{2+}]_{free}$ being <10 ms [151]. However, while eggs only undergo one such triggered round of release, neurons and neuroendocrine cells are capable of multiple high-rate rounds with appropriate stimuli. Cytoplasmic factors (e.g. NSF, α-SNAP and ATP) causing disruption of *cis* SNARE complexes promote such recycling by ensuring the availability of free SNAREs to form inter-membrane complexes (e.g. priming in *trans*) [145]. Thus, although we know that Ca^{2+}-triggered fusion is extremely fast and seemingly optimized in neurons [150], the implication is that the Ca^{2+}-triggered step(s) occurs after SNARE action; SNAREs can promote the mechanism without having an actual role in fusion itself. As protein folding and conformational changes occur in the nanosecond–microsecond time scale [203–205], numerous molecular steps that are simply unresolved by current electrophysiological or imaging methods could clearly occur during the lag phase between Ca^{2+} entry and membrane merger. Indeed, the time for membrane rearrangements and merger via the stalk-pore pathway is estimated to be $\sim$ 10 ns [183,206]. If Ca^{2+} has a role in clearing inter-membrane SNARE complexes to promote the fusion mechanism [36,76], this elevated $[Ca^{2+}]_{free}$ during strong stimulus conditions would tend to promote complex disassembly. Such a triggered loss of SNARE complexes appears to be balanced upstream by Ca^{2+}-dependent SNARE complex formation [94,207,208]. Late, fast Ca^{2+}-triggered disruption of rapidly 'zippered' inter-membrane complexes would not be detected in the majority of currently available assays due to limited temporal resolution and the sheer magnitude of concurrent reactions in (semi)intact preparations. To summarize, the essential role of the SNAREs during the process of exocytosis is undeniable, however, the evidence suggests that they function at a pre-fusion rather than the fusion stage.

In closing, we are reminded of Dr. G. Palade's Nobel Prize acceptance speech [209]:

> "*A distinction should be made between agents directly affecting fusion–fission and agents affecting the superimposed regulatory systems that activate and inactivate the coupling between stimulation and secretion.*"

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References

[1] R.P. Rand, V.A. Parsegian, Hydration forces between phospholipid bilayers, Biochim. Biophys. Acta 988 (1989) 351–376.

[2] R.P. Rand, The lipid–water interface: revelations by osmotic stress, Int. Rev. Cyt. 215 (2002) 33–48.
[3] P.I. Kuzmin, J. Zimmerberg, Y.A. Chizmadzhev, F.S. Cohen, A quantitative model for membrane fusion based on low-energy intermediates, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7235–7240.
[4] V.S. Markin, J.P. Albanes, Membrane fusion: stalk model revisited, Biophys. J. 82 (2002) 693–712.
[5] Y. Kozlovsky, M.M. Kozlov, Stalk model of membrane fusion: solution of energy crisis, Biophys. J. 82 (2002) 882–895.
[6] R.A. Chavez, S.G. Miller, H.P. Moore, A biosynthetic secretory pathway in constitutive secretory cells, J. Cell Biol. 133 (1996) 1177–1191.
[7] J.R. Coorssen, H. Schmidt, W. Almers, Ca^{2+} triggers massive exocytosis in Chinese hamster ovary cells, EMBO J. 15 (1996) 3787–3791.
[8] Y. Ninomiya, T. Kishimoto, Y. Miyashita, H. Kasai, Ca^{2+}-dependent exocytotic pathways in Chinese hamster ovary fibroblasts revealed by a caged-Ca^{2+} compound, J. Biol. Chem. 271 (1996) 17751–17754.
[9] Y. Dan, M.M. Poo, Quantal transmitter secretion from myocytes loaded with acetylcholine, Nature 359 (1992) 733–736.
[10] E. Bugnard, N. Taulier, A. Bloc, P. Correges, J. Falk-Vairant, P. Sors, F. Loctin, Y. Dunant, Quantal transmitter release by glioma cells: quantification of intramembrane particle changes, Neuroscience 113 (2002) 125–135.
[11] D.E. Knight, H. von Grafenstein, C.M. Athayde, Calcium-dependent and calcium-independent exocytosis, Trends Neurosci. 12 (1989) 451–458.
[12] V.D. Vacquier, The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge, Dev. Biol. 43 (1975) 62–74.
[13] P.F. Baker, M.J. Whitaker, Influence of ATP and calcium on the cortical reaction in sea urchin eggs, Nature 276 (1978) 513–515.
[14] J. Vilmart-Seuwen, H. Kersken, R. Stürzl, H. Plattner, ATP keeps exocytosis sites in a primed state but is not required for membrane fusion: an analysis with *Paramecium* cells in vivo and in vitro, J. Cell Biol. 103 (1986) 1279–1288.
[15] R.W. Holz, M.A. Bittner, S.C. Peppers, R.A. Senter, D.A. Eberhard, MgATP-independent and MgATP-dependent exocytosis, J. Biol. Chem. 264 (1989) 5412–5419.
[16] J.C. Hay, T.F. Martin, Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins, J. Cell Biol. 119 (1992) 139–151.
[17] J.C. Hay, P.L. Fisette, G.H. Jenkins, K. Fukami, T. Takenawa, R.A. Anderson, T.F.J. Martin, ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion, Nature 374 (1995) 173–177.
[18] T. Parsons, J.R. Coorssen, H. Horstmann, W. Almers, Docked granules, the exocytotic burst, and the need for ATP hydrolysis in endocrine cells, Neuron 15 (1995) 1085–1096.
[19] H. Plattner, A.R. Artalejo, E. Neher, Ultrastructural organization of bovine chromaffin cell cortex—analysis by cryofixation and morphometry of aspects pertinent to exocytosis, J. Cell Biol. 139 (1997) 1709–1717.
[20] S. Barg, L. Eliasson, E. Renström, P. Rorsman, A subset of 50 secretory granules in close contact with L-type Ca^{2+} channels accounts for first-phase insulin secretion in mouse β-cells, Diabetes 51 (2002) S74–S82.
[21] R. Heidelberger, P. Sterling, G. Matthews, Roles of ATP in depletion and replenishment of the releasable pool of synaptic vesicles, J. Neurophysiol. 88 (2002) 98–106.
[22] T.H. Söllner, M.K. Bennett, S.W. Whitheart, R.H. Scheller, J.E. Rothman, A protein assembly–disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion, Cell 75 (1993) 409–418.
[23] J.E. Rothman, Mechanisms of intracellular protein transport, Nature 372 (1994) 55–63.
[24] S.H. Gerber, T.C. Südhof, Molecular determinants of regulated exocytosis, Diabetes 51 (2002) S3–S11.
[25] B. Storrie, M. Desjardins, The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? BioEssays 18 (1996) 895–903.
[26] E. Alés, L. Tabares, J.M. Poyato, V. Valero, M. Lindau, G. Alvarez de Toledo, High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism, Nat. Cell Biol. 1 (1999) 40–44.
[27] A.W. Henkel, G. Kang, J. Kornhuber, A common molecular machinery for exocytosis and the 'kiss-and-run' mechanism in chromaffin cells is controlled by phosphorylation, J. Cell Sci. 114 (2001) 4613–4620.
[28] J. Hartmann, M. Lindau, A novel Ca^{2+}-dependent step in exocytosis subsequent to vesicle fusion, FEBS Lett. 363 (1995) 217–220.
[29] R. Fernández-Chacón, G. Alvarez de Toledo, Cytosolic calcium facilitates release of secretory products after exocytotic vesicle fusion, FEBS Lett. 363 (1995) 221–225.
[30] S. Scepek, J.R. Coorssen, M. Lindau, Fusion pore expansion in horse eosinophils is modulated by Ca^{2+} and protein kinase C via distinct mechanisms, EMBO J. 17 (1998) 4340–4345.
[31] R. Rahamimoff, J.M. Fernandez, Pre- and postfusion regulation of transmitter release, Neuron 18 (1997) 17–27.
[32] V.N. Murthy, T.J. Sejnowski, C.F. Stevens, Heterogeneous release properties of visualized individual hippocampal synapses, Neuron 18 (1997) 599–612.
[33] V.N. Murthy, C.F. Stevens, Reversal of synaptic vesicle docking at central synapses, Nat. Neurosci. 2 (1999) 503–507.
[34] J. Shorter, M.B. Beard, J. Seemann, A.B. Dirac-Svejstrup, G. Warren, Sequential tethering of golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115, J. Cell Biol. 157 (2002) 45–62.
[35] J.R.C. Whyte, S. Munro, Vesicle tethering complexes in membrane traffic, J. Cell Sci. 115 (2002) 2627–2637.
[36] J. Zimmerberg, P. Blank, I. Kolosova, M.S. Cho, M. Tahara, J.R. Coorssen, A stage-specific preparation to study the Ca^{2+}-triggered fusion steps of exocytosis: rationale and perspectives, Biochimie 82 (2000) 303–314.
[37] D. Bruns, R. Jahn, Molecular determinants of exocytosis, Pflugers Arch. 443 (2002) 333–338.
[38] S.L. Leikin, M.M. Kozlov, L.V. Chernomordik, V.S. Markin, Y.A. Chizmadzhev, Membrane fusion: overcoming of the hydration barrier and local restructuring, J. Theor. Biol. 129 (1987) 411–425.
[39] L. Chernomordik, Non-bilayer lipids and biological fusion intermediates, Chem. Phys. Lipids 81 (1996) 203–213.
[40] Y. Kozlovsky, L.V. Chernomordik, M.M. Kozlov, Lipid intermediates in membrane fusion: formation, structure, and decay of hemifusion diaphragm, Biophys. J. 83 (2002) 2634–2651.
[41] G. Schiavo, M. Matteoli, C. Montecucco, Neurotoxins affecting neuroexocytosis, Physiol. Rev. 80 (2000) 717–766.
[42] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, C. Montecucco, Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, Nature 359 (1992) 832–835.
[43] G. Schiavo, C.C. Shone, O. Rossetto, F.C.G. Alexander, C. Montecucco, Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin, J. Biol. Chem. 268 (1993) 11516–11519.
[44] Y. Humeau, F. Doussau, N.J. Grant, B. Poulain, How botulinum and tetanus neurotoxins block neurotransmitter release, Biochimie 82 (2000) 427–446.
[45] A.M. Harvey, The peripheral action of tetanus toxin, J. Physiol. 96 (1939) 348–365.
[46] A.S.V. Burgen, F. Dickens, L.J. Zatman, The action of botulinum toxin on the neuro-muscular junction, J. Physiol. 109 (1949) 10–24.
[47] D.A. Boroff, J. DelCastillo, W.H. Evoy, R.A. Steinhardt, Observa-

tions on the actions of type A botulinum toxin on frog neuromuscular junctions, J. Physiol. 240 (1974) 227–253.
[48] S.Y. Hua, M.P. Charlton, Activity-dependent changes in partial VAMP complexes during neurotransmitter release, Nat. Neurosci. 2 (1999) 1078–1083.
[49] V.B. Brooks, The action of botulinum toxin on motor-nerve filaments, J. Physiol. 123 (1954) 501–515.
[50] V.B. Brooks, An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials, J. Physiol. 134 (1956) 264–277.
[51] S. Thesleff, Supersensitivity of skeletal muscle produced by botulinum toxin, J. Physiol. 151 (1960) 598–607.
[52] F. Dreyer, A. Schmitt, Transmitter release in tetanus and botulinum A toxin-poisoned mammalian motor endplates and its dependence on nerve stimulation and temperature, Pflugers Arch. 399 (1983) 228–234.
[53] R.L. Parsons, W.W. Hofmann, G.A. Feigen, Mode of action of tetanus toxin on the neuromuscular junction, Am. J. Physiol. 210 (1966) 84–90.
[54] A.J. Harris, R. Miledi, The effect of type D botulinum toxin on frog neuromuscular junctions, J. Physiol. 217 (1971) 497–515.
[55] L.W. Duchen, D.A. Tonge, The effects of tetanus toxin on neuromuscular transmission and on the morphology of motor end-plates in slow and fast skeletal muscle of the mouse, J. Physiol. 228 (1973) 157–172.
[56] B.A. Stewart, M. Mohtashami, W.S. Trimble, G.L. Boulianne, SNARE proteins contribute to calcium cooperativity of synaptic transmission, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13955–13960.
[57] M.F. Finley, S.M. Patel, D.V. Madison, R.H. Scheller, The core membrane fusion complex governs the probability of synaptic vesicle fusion but not transmitter release kinetics, J. Neurosci. 22 (2002) 1266–1272.
[58] J. Mellanby, P.A. Thompson, The effect of tetanus toxin at the neuromuscular junction in the goldfish, J. Physiol. 224 (1972) 407–419.
[59] S.G. Cull-Candy, H. Lundh, S. Thesleff, Effects of botulinum toxin on neuromuscular transmission in the rat, J. Physiol. 260 (1976) 177–203.
[60] M. Gansel, R. Penner, F. Dreyer, Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor nerve terminals, Pflugers Arch. 409 (1987) 533–539.
[61] F. Dreyer, F. Rosenberg, C. Becker, H. Bigalke, R. Penner, Differential effects of various secretagogues on quantal transmitter release from mouse motor nerve terminals treated with botulinum A and tetanus toxin, Naunyn-Schmiedeberg's Arch. Pharmacol. 335 (1987) 1–7.
[62] A.C. Ashton, J.O. Dolly, Microtubule-dissociating drugs and A23187 reveal differences in the inhibition of synaptosomal transmitter release by botulinum neurotoxins types A and B, J. Neurochem. 56 (1991) 827–835.
[63] G.W. Lawrence, U. Weller, J.O. Dolly, Botulinum A and the light chain of tetanus toxin inhibit distinct stages of MgATP-dependent catecholamine exocytosis from permeabilised chromaffin cells, Eur. J. Biochem. 222 (1994) 325–333.
[64] G.W. Lawrence, P. Foran, J.O. Dolly, Distinct exocytotic responses of intact and permeabilized chromaffin cells after cleavage of the 25-kDa synaptosomal-associated protein (SNAP-25) or synaptobrevin by botulinum toxin A or B, Eur. J. Biochem. 236 (1996) 877–886.
[65] M.A. Bittner, R.W. Holz, Protein kinase C and clostridial neurotoxins affect discrete and related steps in the secretory pathway, Cell. Mol. Neurobiol. 13 (1993) 649–664.
[66] G. Ahnert-Hilger, U. Weller, Comparison of the intracellular effects of clostridial neurotoxins on exocytosis from streptolysin O-permeabilized rat pheochromocytoma (PC12) and bovine adrenal chromaffin cells, Neuroscience 53 (1993) 547–552.
[67] D.E. Glenn, R.D. Burgoyne, Botulinum neurotoxin light chains inhibit both Ca^{2+}-induced and GTP analogue-induced catecholamine release from permeabilised adrenal chromaffin cells, FEBS Lett. 386 (1996) 137–140.
[68] M. Capogna, R.A. McKinney, V. O'Connor, B.H. Gähwiler, S.M. Thompson, Ca^{2+} or Sr^{2+} partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin, J. Neurosci. 17 (1997) 7190–7202.
[69] J. Land, H. Zhang, V.V. Vaidyanathan, K. Sadoul, H. Niemann, C.B. Wollheim, Transient expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose induced insulin secretion in clonal beta-cells, FEBS Lett. 419 (1997) 13–17.
[70] T. Xu, T. Binz, H. Niemann, E. Neher, Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity, Nat. Neurosci. 1 (1998) 192–200.
[71] S.Y. Hua, D.A. Raciborska, W.S. Trimble, M.P. Charlton, Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction, J. Neurophysiol. 80 (1998) 3233–3246.
[72] R. Regazzi, C.B. Wollheim, J. Lang, J.M. Theler, O. Rossetto, C. Montecucco, K. Sadoul, U. Weller, M. Palmer, B. Thorens, VAMP-2 and cellubrevin are expressed in pancreatic b-cells and are essential for Ca^{2+}- but not for GTPgS-induced insulin secretion, EMBO J. 15 (1995) 2723–2730.
[73] A. Fassio, R. Sala, G. Bonanno, M. Marchi, M. Raiteri, Evidence for calcium-dependent vesicular transmitter release insensitive to tetanus and botulinum toxin type F, Neuroscience 90 (1999) 893–902.
[74] S.S. Vogel, J. Zimmerberg, Proteins on exocytotic vesicles mediate calcium-triggered fusion, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4749–4753.
[75] J.R. Coorssen, P.S. Blank, M. Tahara, J. Zimmerberg, Biochemical and functional studies of cortical vesicle fusion: the SNARE complex and Ca^{2+} sensitivity, J. Cell Biol. 143 (1998) 1845–1857.
[76] M. Tahara, J.R. Coorssen, K. Timmers, P.S. Blank, T. Whalley, R.H. Scheller, J. Zimmerberg, Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion, J. Biol. Chem. 273 (1998) 33667–33673.
[77] J.R. Coorssen, M. Tahara, P. Blank, J. Zimmerberg, Role of SNARE proteins in membrane fusion, Mol. Biol. Cell 8 (1997) 296a.
[78] G.Q. Bi, J.M. Alderton, R.A. Steinhardt, Calcium-regulated exocytosis is required for cell membrane resealing, J. Cell Biol. 131 (1995) 1747–1758.
[79] C. Ungermann, B.J. Nichols, H.R.B. Pelham, W. Wickner, A vacuolar v–t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion, J. Cell Biol. 140 (1998) 61–69.
[80] C. Ungermann, K. Sato, W. Wickner, Defining the functions of trans-SNARE pairs, Nature 396 (1998) 543–548.
[81] C. Peters, M.J. Bayer, S. Buhler, J.S. Andersen, M. Mann, A. Mayer, Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion, Nature 409 (2001) 567–568.
[82] O. Müller, M.J. Bayer, C. Peters, J.S. Andersen, M. Mann, A. Mayer, The Vtc proteins in vacuolar fusion: coupling NSF activity to V(0) trans-complex formation, EMBO J. 21 (2002) 259–269.
[83] J.E. Smolen, S.J. Stoehr, L.A. Boxer, Human neutrophils permeabilized with digitonin respond with lysosomal enzyme release when exposed to micromolar levels of free calcium, Biochim. Biophys. Acta 886 (1986) 1–17.
[84] A. Rodriguez, P. Webster, J. Ortego, N.W. Andrews, Lysosomes behave as Ca^{2+}-regulated exocytotic vesicles in fibroblasts and epithelial cells, J. Cell Biol. 137 (1997) 93–104.
[85] F.T. Horrigan, R.J. Bookman, Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells, Neuron 13 (1994) 1119–1129.
[86] S. Kirischuk, R. Grantyn, A readily releasable pool of single inhibitory boutons in culture, NeuroReport 11 (2000) 3709–3713.
[87] T. Moser, E. Neher, Rapid exocytosis in single chromaffin cells

recorded from mouse adrenal slices, J. Neurosci. 17 (1997) 2314–2323.
[88] J.Y. Sun, L.G. Wu, Fast kinetics of exocytosis revealed by simultaneous measurements of presynaptic capacitance and postsynaptic currents at a central synapse, Neuron 30 (2001) 171–182.
[89] Y. Yang, S. Udayasankar, J. Dunning, P. Chen, K.D. Gillis, A highly Ca^{2+}-sensitive pool of vesicles is regulated by protein kinase C in adrenal chromaffin cells, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 17060–17065.
[90] J.A. Steyer, H. Horstmann, W. Almers, Transport, docking and exocytosis of single secretory granules in live chromaffin cells, Nature 388 (1997) 474–478.
[91] J.R. Coorssen, P.S. Blank, F. Albertorio, L. Bezrukov, I. Kolosova, P.S. Backlund, J. Zimmerberg, Quantitative femto- to attomole immunodetection of regulated secretory vesicle proteins critical to exocytosis, Anal. Biochem. 307 (2002) 54–62.
[92] T. Xu, B. Rammner, M. Margittai, A.R. Artalejo, E. Neher, R. Jahn, Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis, Cell 99 (1999) 713–722.
[93] Y.A. Chen, S.J. Scales, J.R. Jagath, R.H. Scheller, A discontinuous SNAP-25 C-terminal coil supports exocytosis, J. Biol. Chem. 276 (2001) 28503–28508.
[94] Y.A. Chen, S.J. Scales, S.M. Patel, Y.C. Doung, R.H. Scheller, SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion, Cell 97 (1999) 165–174.
[95] P. Ray, J.D. Berman, W. Middleton, J. Brendle, Botulinum toxin inhibits arachadonic acid release associated with acetylcholine release from PC12 cells, J. Biol. Chem. 268 (1993) 11057–11064.
[96] P. Ray, C.B. Millaed, J.P. Petrali, J.D. Berman, R. Ray, Acetylcholine exocytosis in PC12 cells deficient in SNAP-25, NeuroReport 8 (1997) 2271–2274.
[97] P. Presek, S. Jessen, F. Dreyer, P.E. Jarvie, D. Findik, P.R. Dunkley, Tetanus toxin inhibits depolarization-stimulated protein phosphorylation in rat cortical synaptosomes: effect on synapsin I phosphorylation and translocation, J. Neurochem. 59 (1992) 1336–1343.
[98] R.V. Considine, C.M. Handler, L.L. Simpson, J.R. Sherwin, Tetanus toxin inhibits neurotensin-induced mobilization of cytosolic protein kinase C activity in NG-108 cells, Toxicon 29 (1991) 1351–1357.
[99] A. Najib, P. Pelliccioni, C. Gil, J. Aguilera, Clostridium neurotoxins influence serotonin uptake and release differently in rat brain synaptosomes, J. Neurochem. 72 (1999) 1991–1998.
[100] H. Caohuy, H.B. Pollard, Annexin 7: a non-SNARE proteolytic substrate for botulinum toxin type C in secreting chromaffin cells, Ann. N.Y. Acad. Sci. 971 (2002) 287–290.
[101] K.L. Schulze, K. Broadie, M.S. Perin, H.J. Bellen, Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in non-neuronal secretion and neurotransmission, Cell 80 (1995) 311–320.
[102] M.L. Nonet, K. Grundahl, B.J. Meyer, J.B. Rand, Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin, Cell 73 (1993) 1291–1305.
[103] M.L. Nonet, O. Saifee, H. Zhao, J.B. Rand, L. Wei, Synaptic transmission deficits in *Caenorhabditis elegans* synaptobrevin mutants, J. Neurosci. 18 (1998) 70–80.
[104] D.L. Deitcher, A. Ueda, B.A. Stewart, R.W. Burgess, Y. Kidokoro, T.L. Schwarz, Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the *Drosophila* gene neuronal-synaptobrevin, J. Neurosci. 18 (1998) 2028–2039.
[105] S.S. Rao, B.A. Stewart, P.K. Rivlin, I. Vilinsky, B.O. Watson, C. Lang, G. Boulianne, M.M. Salpeter, D.L. Deitcher, Two distinct effects on neurotransmission in a temperature-sensitive SNAP-25 mutant, EMBO J. 20 (2001) 6761–6771.
[106] M.A. Bittner, M.K. Bennett, R.W. Holz, Evidence that syntaxin 1A is involved in storage in the secretory pathway, J. Biol. Chem. 271 (1996) 11214–11221.
[107] S. Nagamatsu, T. Fujiwara, Y. Nakamichi, T. Watanabe, H. Katahira, H. Sawa, K. Akagawa, Expression and functional role of syntaxin 1/HPC-1 in pancreatic beta cells. Syntaxin 1A, but not 1 B, plays a negative role in regulatory insulin release pathway, J. Biol. Chem. 271 (1996) 1160–1165.
[108] S. Nagamatsu, Y. Nakamichi, C. Yamamura, S. Matsushima, T. Watanabe, S. Ozawa, H. Furukawa, H. Ishida, Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion, Diabetes 48 (1999) 2367–2373.
[109] R. Regazzi, K. Sadoul, P. Meda, R.B. Kelly, P.A. Halban, C.B. Wollheim, Mutational analysis of VAMP domains implicated in Ca^{2+}-induced insulin exocytosis, EMBO J. 15 (1996) 6951–6959.
[110] P. Washbourne, N. Bortoletto, M.E. Graham, M.C. Wilson, R.D. Burgoyne, C. Montecucco, Botulinum neurotoxin E-insensitive mutants of SNAP-25 fail to bind VAMP but support exocytosis, J. Neurochem. 73 (1999) 2424–2433.
[111] M.E. Graham, P. Washbourne, M.C. Wilson, R.D. Burgoyne, SNAP-25 with mutations in the zero layer supports normal membrane fusion kinetics, J. Cell Sci. 114 (2001) 4397–4405.
[112] P. Washbourne, P.M. Thompson, M. Carta, E.T. Costa, J.R. Mathews, G. Lopez-Bendito, Z. Molnar, M.W. Becher, C.F. Valenzuela, L.D. Partridge, M.C. Wilson, Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis, Nat. Neurosci. 5 (2001) 19–26.
[113] S. Schoch, F. Deak, A. Konigstorfer, M. Mozhayeva, Y. Sara, T.C. Südhof, E.T. Kavalali, SNARE function analyzed in synaptobrevin/VAMP knockout mice, Science 294 (2001) 1117–1122.
[114] S.J. Scales, M.F.A. Finley, R.H. Scheller, Fusion without SNAREs? Science 294 (2001) 1015–1016.
[115] I. Vilinsky, B.A. Stewart, J. Drummond, I. Robinson, D.L. Deitcher, A drosophila SNAP-25 null mutant reveals context-dependent redundancy with SNAP-24 in neurotransmission, Genetics 162 (2002) 259–271.
[116] T.T. Liu, C. Barlow, Analysis of Sec22p in endoplasmic reticulum/golgi transport reveals cellular redundancy in SNARE protein function, Mol. Biol. Cell 13 (2002) 3314–3324.
[117] K. Sadoul, A. Berger, H. Niemann, U. Weller, P.A. Roche, A. Klip, W.S. Trimble, R. Regazzi, S. Catsicas, P.A. Halban, SNAP-23 is not cleaved by botulinum neurotoxin E and can replace SNAP-25 in the process of insulin secretion, J. Biol. Chem. 272 (1997) 33023–33027.
[118] J. Zimmerberg, J.R. Coorssen, S.S. Vogel, P.S. Blank, Topical review: sea urchin egg preparations as systems for the study of calcium-triggered exocytosis, J. Physiol. 520 (1999) 15–21.
[119] K. Hu, J. Carroll, C. Rickman, B. Davletov, Action of complexin on SNARE complex, J. Biol. Chem. 277 (2002) 41652–41656.
[120] S.J. Scales, B.A. Hesser, E.S. Masuda, R.H. Scheller, Amisyn, a novel syntaxin-binding protein that may regulate SNARE complex assembly, J. Biol. Chem. 277 (2002) 28271–28279.
[121] S. Conner, D. Leaf, G. Wessel, Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg, Mol. Reprod. Dev. 48 (1997) 106–118.
[122] J.R. Coorssen, P.S. Blank, I. Kolosova, P. Backlund, J. Zimmerberg, Are SNARE proteins necessary and sufficient for membrane fusion? Mol. Biol. Cell 10 (1999) 219a.
[123] J.R. Coorssen, P.S. Blank, F. Albertorio, L. Bezrukov, I. Kolosova, X. Chen, P. Backlund, J. Zimmerberg, Regulated secretion: SNARE density, vesicle fusion, and calcium dependence, J. Cell Sci. 116 (2003) 2087–2097 (in press).
[124] S. Leikin, V.A. Parsegian, D.C. Rau, R.P. Rand, Hydration forces, Annu. Rev. Phys. Chem. 44 (1993) 369–395.
[125] D.P. Siegel, Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms, Biophys. J. 65 (1993) 2124–2140.
[126] D. David, S. Sundarababu, J.E. Gerst, Involvement of long chain

fatty acid elongation in the trafficking of secretory vesicles in yeast, J. Cell Biol. 143 (1998) 1167–1182.
[127] E. Grote, M. Baba, Y. Ohsumi, P.J. Novick, Geranylgeranylated SNAREs are dominant inhibitors of membrane fusion, J. Cell Biol. 151 (2000) 453–465.
[128] D. Langosh, J.M. Crane, B. Brosig, A. Hellwig, L.K. Tamm, J. Reed, Peptide mimics of SNARE transmembrane segments drive membrane fusion depending on their conformational plasticity, J. Mol. Biol. 311 (2001) 709–721.
[129] S.S. Vogel, L.V. Chernomordik, J. Zimmerberg, Calcium-triggered fusion of exocytotic granules requires proteins in only one membrane, J. Biol. Chem. 267 (1992) 25640–25643.
[130] A. Chanturiya, M. Whitaker, J. Zimmerberg, Calcium-induced fusion of sea urchin egg secretory vesicles with planar phospholipid bilayer membranes, Mol. Membr. Biol. 16 (1999) 89–94.
[131] S. Kagiwada, M. Murata, R. Hishida, M. Tagaya, S. Yamashina, S. Ohnishi, In vitro fusion of rabbit liver golgi membranes with liposomes, J. Biol. Chem. 268 (1993) 1430–1435.
[132] M. Vidal, D. Hoekstra, In vitro fusion of reticulocyte endocytic vesicles with liposomes, J. Biol. Chem. 270 (1995) 17823–17829.
[133] J.M. Smit, G. Li, P. Schoen, J. Corver, R. Bittman, K.C. Lin, J. Wilschut, Fusion of alphavirus with liposomes is a non-leaky process, FEBS Lett. 521 (2002) 62–66.
[134] H. Mizuguchi, T. Nakanishi, M. Kondoh, T. Nakagawa, M. Nakanishi, T. Matsuyama, Y. Tsutsumi, S. Nakagawa, T. Mayumi, Fusion of sendai virus with liposome depends on only F protein, but not HN protein, Virus Res. 59 (1999) 191–201.
[135] E.I. Hernandez-Jimenez, V.I. Razinkov, I.I. Mikhalyov, A.V. Kozminykh, F.S. Cohen, J.G. Molotkovsky, Selective labeling of the inner liposome leaflet by fluorescent lipid probes, and studies of liposome fusion with influenza virus, Membr. Cell Biol. 11 (1997) 515–527.
[136] M.M. Kozlov, L.V. Chernomordik, A mechanism of protein-mediated fusion: coupling between refolding of the influenza hemagglutinin and lipid rearrangements, Biophys. J. 75 (1998) 1384–1396.
[137] T. Weber, B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Söllner, J.E. Rothman, SNAREpins: minimal machinery for membrane fusion, Cell 92 (1998) 759–772.
[138] S. Pabst, M. Margittai, D. Vainius, R. Langen, R. Jahn, D. Fasshauer, Rapid and selective binding to the synaptic SNARE complex suggests a modulatory role of complexins in neuroexicytosis, J. Biol. Chem. 277 (2002) 7838–7848.
[139] C. Lang, D. Bruns, D. Wenzel, D. Riedel, P. Holroyd, C. Thiele, R. Jahn, SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis, EMBO J. 20 (2001) 2202–2213.
[140] F. Parlati, T. Weber, J.A. McNew, B. Westermann, T.H. Söllner, J.E. Rothman, Rapid and efficient fusion of phospholipid vesicles by the a-core of a SNARE complex in the absence of an N-terminal regulatory domain, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12565–12570.
[141] W. Nickel, T. Weber, J.A. McNew, F. Parlati, T.H. Söllner, J.E. Rothman, Content mixing and membrane integrity during membrane fusion driven by pairing of isolated v-SNAREs and t-SNAREs, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12571–12576.
[142] L.K. Mahal, S.M. Sequeira, J.M. Gureasko, T.H. Söllner, Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I, J. Cell Biol. 158 (2002) 273–282.
[143] T.J. Melia, T. Weber, J.A. McNew, L.E. Fisher, R.J. Johnston, F. Parlati, L.K. Mahal, T.H. Söllner, J.E. Rothman, Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE during zippering of SNAREpins, J. Cell Biol. 158 (2002) 929–940.
[144] R. Fukuda, J.A. McNew, T. Weber, F. Parlati, T. Engel, W. Nickel, J.E. Rothman, T.H. Söllner, Functional architecture of an intracellular membrane t-SNARE, Nature 407 (2000) 198–202.
[145] T. Weber, F. Parlati, J.A. McNew, R.J. Johnston, B. Westermann, T.H. Söllner, J.E. Rothman, SNAREpins are functionally resistant to disruption by NSF and αSNAP, J. Cell Biol. 149 (2000) 1063–1072.
[146] J.A. McNew, T. Weber, F. Parlati, R.J. Johnston, T.J. Melia, T.H. Söllner, J.E. Rothman, Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors, J. Cell Biol. 150 (2000) 105–117.
[147] R.B. Sutton, D. Fasshauer, R. Jahn, A.T. Brunger, Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution, Nature 395 (1998) 347–353.
[148] F. Pincet, L. Lebeau, S. Cribier, Short-range specific forces are able to induce hemifusion, Eur. Biophys. J. 30 (2001) 91–97.
[149] Y. Cajal, J.M. Boggs, M.K. Jain, Salt-triggered intermembrane exchange of phospholipids and hemifusion by myelin basic protein, Biochemistry 36 (1997) 2566–2576.
[150] B.L. Sabatini, W.G. Regehr, Timing of neurotransmission at fast synapses in the mammalian brain, Nature 384 (1996) 170–172.
[151] N.I. Shafi, S.S. Vogel, J. Zimmerberg, Using caged calcium to study sea urchin egg corticle granule exocytosis in vitro, Methods 6 (1994) 82–92.
[152] K. Hu, J. Carroll, S. Fedorovich, C. Rickman, A. Sukhodub, B. Davletov, Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion, Nature 415 (2002) 646–650.
[153] C.E. Creutz, Cis-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin, J. Cell Biol. 91 (1981) 247–256.
[154] S. Schenkman, P.S. De Araujo, A. Sesso, F.H. Quina, H. Chaimovich, A kinetic and structural study of two-step aggregation and fusion of neutral phospholipid vesicles promoted by serum albumin at low pH, Chem. Phys. Lipids 28 (1981) 165–180.
[155] R. Blumenthal, M. Henkart, C.J. Steer, Clathrin-induced pH-dependent fusion of phosphatidylcholine vesicles, J. Biol. Chem. 258 (1983) 3409–3415.
[156] R. Sundler, J. Wijkander, Protein-mediated intermembrane contact specifically enhances Ca^{2+}-induced fusion of phosphatidate-containing membranes, Biochim. Biophys. Acta 730 (1983) 391–394.
[157] T.M. Young, J.D. Young, Protein-mediated intermembrane contact facilitates fusion of lipid vesicles with planar bilayers, Biochim. Biophys. Acta 775 (1984) 441–445.
[158] G. Fujii, M.E. Selsted, D. Eisenberg, Defensins promote fusion and lysis of negatively charged membranes, Protein Sci. 2 (1993) 1301–1312.
[159] P.E. Glaser, R.W. Gross, Rapid plasmenylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceraldehyde-3-phosphate dehydrogenase: discrimination between glycolytic and fusogenic roles of individual isoforms, Biochemistry 34 (1995) 12193–12203.
[160] R.J. Hessler, R.A. Blackwood, T.G. Brock, J.W. Francis, D.M. Harsh, J.E. Smolen, Identification of glyceraldehyde-3-phosphate dehydrogenase as a Ca^{2+}-dependent fusogen in human neutrophil cytosol, J. Leukoc. Biol. 63 (1998) 331–336.
[161] M. Otter-Nilsson, R. Hendriks, E.I. Pecheur-Huet, D. Hoekstra, T. Nilsson, Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion, EMBO J. 18 (1999) 2074–2083.
[162] B. Brügger, W. Nickel, T. Weber, F. Parlati, J.A. McNew, J.E. Rothman, T.H. Söllner, Putative fusogenic activity of NSF is restricted to a lipid mixture whose coalescence is also triggered by other factors, EMBO J. 19 (2000) 1272–1278.
[163] A. Agirre, S. Nir, J.L. Nieva, J. Dijkstra, Induction of aggregation and fusion of cholesterol-containing membrane vesicles by an anti-cholesterol monoclonal antibody, J. Lipid Res. 41 (2000) 621–628.
[164] S.J. Cho, M. Kelly, K.T. Rognlien, J.A. Cho, J.K.H. Horber, B.P. Jena, SNAREs in opposing bilayers interact in a circular array to form conducting pores, Biophys. J. 83 (2002) 2522–2527.
[165] E.I. Pécheur, I. Martin, O. Maier, U. Bakowski, J.M. Ruysschaert, D. Hoekstra, Phospholipid species act as modulators in p97/p47-mediated fusion of golgi membranes, Biochemistry 41 (2002) 9813–9823.

[166] M.E. Haque, T.J. McIntosh, B.R. Lentz, Influence of lipid composition on physical properties and PEG-mediated fusion of curved and uncurved model membrane vesicles: "Nature's own" fusogenic lipid bilayer, Biochemistry 40 (2001) 4340–4348.
[167] A. Bracher, J. Kadlec, H. Betz, W. Weissenhorn, X-ray structure of a neuronal complexin-SNARE complex from squid, J. Biol. Chem. 277 (2002) 26517–26523.
[168] D.A. Archer, M.E. Graham, R.D. Burgoyne, Complexin regulates the closure of the fusion pore during regulated vesicle exocytosis, J. Biol. Chem. 277 (2002) 18249–18252.
[169] F. Parlati, J.A. McNew, R. Fukuda, R. Miller, T.H. Söllner, J.E. Rothman, Topological restriction of SNARE-dependent membrane fusion, Nature 407 (2000) 194–198.
[170] H.R.B. Pelham, SNAREs and the specificity of membrane fusion, Trends Cell Biol. 11 (2001) 99–101.
[171] L.M. Gutierrez, S. Viniegra, J. Rueda, A.V. Ferrer-Montiel, J.M. Canaves, M. Montal, A peptide that mimics the C-terminal sequence of SNAP-25 inhibits secretory vesicle docking in chromaffin cells, J. Biol. Chem. 272 (1997) 2634–2639.
[172] J.P. Apland, J.A. Biser, M. Adler, A.V. Ferrer-Montiel, M. Montal, M.G. Filbert, Peptides that mimic the carboxy-terminal domain of SNAP-25 block acetylcholine release at an aplysia synapse, J. Appl. Toxicol. 19 (1999) S23–S26.
[173] M.M. Kozlov, V.S. Markin, Possible mechanism of membrane fusion, Biofizika 28 (1983) 242–247.
[174] L. Chernomordik, A. Chanturiya, J. Green, J. Zimmerberg, The hemifusion intermediate and its conversion to complete fusion: regulation by membrane composition, Biophys. J. 69 (1995) 922–929.
[175] R.P. Rand, Interacting phospholipid bilayers: measured forces and induced structural changes, Annu. Rev. Biophys. Bioeng. 10 (1981) 277–314.
[176] S.M. Gruner, Intrinsic curvature hypothesis for biomembrane lipid composition: a role for non-bilayer lipids, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 3665–3669.
[177] K. Gawrisch, V.A. Parsegian, D.A. Hajduk, M.W. Tate, S.M. Gruner, Energetics of a hexagonal–lamellar–hexagonal-phase transition sequence in dioleoylphosphatidylethanolamine membranes, Biochemistry 31 (1992) 2856–2864.
[178] M.M. Kozlov, S. Leikin, R.P. Rand, Bending, hydration and interstitial energies quantitatively account for the hexagonal–lamellar–hexagonal reentrant phase transition in dioleoylphosphatidylethanolamine, Biophys. J. 67 (1994) 1603–1611.
[179] Z. Chen, R.P. Rand, Comparative study of the effects of several *n*-alkanes on phospholipid hexagonal phases, Biophys. J. 74 (1998) 944–952.
[180] V. Luzzati, F. Husson, The structure of the liquid-crystalline phases of lipid–water systems, J. Cell Biol. 12 (1962) 207–219.
[181] L. Chernomordik, M.M. Kozlov, J. Zimmerberg, Lipids in biological membrane fusion, J. Membr. Biol. 146 (1995) 1–14.
[182] L. Yang, H.W. Huang, Observation of a membrane fusion intermediate structure, Science 297 (2002) 1877–1879.
[183] S.J. Marrink, P. Tieleman, Molecular dynamics simulation of spontaneous membrane fusion during a cubic–hexagonal phase transition, Biophys. J. 83 (2002) 2386–2392.
[184] L.V. Chernomordik, S.S. Vogel, A. Sokoloff, H.O. Onaran, E.A. Leikina, J. Zimmerberg, Lysolipids reversibly inhibit Ca^{2+}-, GTP- and pH-dependent fusion of biological membranes, FEBS Lett. 318 (1993) 71–76.
[185] S.S. Vogel, E.A. Leikina, L.V. Chernomordik, Lysophosphatidylcholine reversibly arrests exocytosis and viral fusion at a stage between triggering and membrane merger, J. Biol. Chem. 268 (1993) 25764–25768.
[186] L.M. Johns, E.S. Levitan, E.A. Shelden, R.W. Holz, D. Axelrod, Restriction of secretory granule motion near the plasma membrane of chromaffin cells, J. Cell Biol. 153 (2001) 177–190.
[187] J. Pevsner, S.C. Hsu, J.E.A. Braun, N. Calakos, A.E. Ting, M.K. Bennett, R.H. Scheller, Specificity and regulation of a synaptic vesicle docking complex, Neuron 13 (1994) 353–361.
[188] N. Takahashi, T. Kishimoto, T. Nemoto, T. Kadowaki, H. Kasai, Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet, Science 297 (2002) 1349–1352.
[189] I. Bezprozvanny, R.H. Scheller, R.W. Tsien, Functional impact of syntaxin on gating of N-type and Q-type calcium channels, Nature 378 (1995) 623–626.
[190] S. Mochida, C.T. Yokoyama, D.K. Kim, K. Itoh, W.A. Catterall, Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14523–14528.
[191] W.A. Catterall, Interactions of presynaptic Ca^{2+} channels and SNARE proteins in neurotransmitter release, Ann. N.Y. Acad. Sci. 868 (1999) 144–159.
[192] S.E. Jarvis, G.W. Zamponi, Interactions between presynaptic Ca^{2+} channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex, Trends Pharmacol. Sci. 22 (2001) 519–522.
[193] S.E. Jarvis, G.W. Zamponi, Distinct molecular determinants govern syntaxin1A-mediated inactivation and G-protein inhibition of N-type calcium channels, J. Neurosci. 21 (2001) 2939–2948.
[194] E.F. Stanley, R.R. Mirotznik, Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels, Nature 385 (1997) 340–343.
[195] J. Ji, S. Tsuk, A.M.F. Salapatek, X. Huang, D. Chikvashvili, E.A. Pasyk, Y. Kang, L. Sheu, R. Tsushima, N. Diamant, W.S. Trimble, I. Lotan, H.Y. Gaisano, The 25-kDa synaptosome-associated protein (SNAP-25) binds and inhibits delayed rectifier potassium channels in secretory cells, J. Biol. Chem. 277 (2002) 20195–20204.
[196] I. Michaelevski, D. Chikvashvili, S. Tsuk, O. Fili, M.J. Lohse, D. Singer-Lahat, I. Lotan, Modulation of a brain voltage-gated K^+ channel by syntaxin 1A requires the physical interaction of $G\beta\gamma$ with the channel, J. Biol. Chem. 277 (2002) 34909–34917.
[197] E. Cormet-Boyaka, A. Di, S.Y. Chang, A.P. Naren, A. Tousson, D.J. Nelson, K.L. Kirk, CFTR chloride channels are regulated by a SNAP-23/syntaxin 1A complex, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12477–12482.
[198] J.D. Spafford, D.W. Munno, P. Van Nierop, Z.P. Feng, S.E. Jarvis, W.J. Gallin, A.B. Smit, G.W. Zamponi, N.I. Syed, Calcium channel structural determinants of synaptic transmission between identified invertebrate neurons, J. Biol. Chem. 278 (2003) 4258–4267.
[199] O. Wiser, M. Trus, A. Hernández, E. Renström, S. Barg, P. Rorsman, D. Atlas, The voltage sensitive Lc-type Ca^{2+} channel is functionally coupled to the exocytotic machinery, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 248–253.
[200] D. Atlas, O. Wiser, M. Trus, The voltage-gated Ca^{2+} channel is the Ca^{2+} sensor of fast neurotransmitter release, Cell. Mol. Neurobiol. 21 (2001) 717–731.
[201] T. Lang, M. Margittai, H. Holzler, R. Jahn, SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs, J. Cell Biol. 158 (2002) 751–760.
[202] D. Fasshauer, W. Antonin, V. Subramaniam, R. Jahn, SNARE assembly and disassembly exhibit a pronounced hysteresis, Nat. Struct. Biol. 9 (2002) 144–151.
[203] S.J. Hagan, J. Hofrichter, A. Szabo, W.A. Eaton, Diffusion-limited contact formation in unfolded cytochrome *c*: estimating the maximum rate of protein folding, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11615–11617.
[204] P. Wittung-Stafshede, J.C. Lee, J.R. Winkler, H.B. Gray, Cytochrome *b*562 folding triggered by electron transfer: approaching the speed limit for formation of a four-helix-bundle protein, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6587–6590.
[205] Y. Levy, E. Hanan, B. Solomon, O.M. Becker, Helix–coil transition of PrP106–126: molecular dynamic study, Proteins 45 (2001) 382–396.

[206] D.P. Siegel, W.J. Green, Y. Talmon, The mechanisms of lamellar-to-inverted hexagonal phase transitions: a study using temperature-jump cryo-electron microscopy, Biophys. J. 66 (1994) 402–412.

[207] G.W. Lawrence, J.O. Dolly, Multiple forms of SNARE complexes in exocytosis from chromaffin cells: effects of Ca^{2+}, MgATP and botulinum toxin type A, J. Cell Sci. 115 (2002) 667–673.

[208] S. Quetglas, C. Leveque, R. Miquelis, K. Sato, M. Seager, Ca^{2+}-dependent regulation of synaptic SNARE complex assembly via a calmodulin- and phospholipid-binding domain or synaptobrevin, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 9695–9700.

[209] G. Palade, Intracellular aspects of the process of protein synthesis, Science 189 (1975) 347–358.