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Review

Progress in understanding the neuronal SNARE function and its regulation

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Abstract. Vesicle budding and fusion underlies many essential biochemical deliveries in eukaryotic cells, and its core fusion machinery is thought to be built on one protein family named soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). Recent technical advances based on site-directed fluorescence labelling and nano-scale detection down to the single-molecule level rapidly unveiled the protein and the lipid intermediates along the fusion pathway as well as the molecular actions of fusion effectors. Here we summarize these new exciting findings in context with a new mechanistic model that reconciles two existing fusion models: the proteinaceous pore model and the hemifusion model. Further, we attempt to locate the points of action for the fusion effectors along the fusion pathway and to delineate the energetic interplay between the SNARE complexes and the fusion effectors.

Keywords. Membrane fusion, SNARE, neurotransmitter release, synaptotagmin 1, single molecule study, complexin.

Introduction

Considering innate high energy barriers for fusion of two membranes [1, 2], the fast sub-millisecond time scales characteristic of synaptic vesicle fusion suggest that Nature has developed an exquisitely regulated fusion machinery [3], which expedites the kinetics of every fusion step. Identification of the fusion proteins that release the required free energy has thus been one of the primary goals in studying the synaptic vesicle fusion. It has been thought that soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) provide the necessary force for membrane fusion, establishing the basic frame for the intracellular fusion machinery [4]. The most distinctive and ubiquitous molecular action of all SNARE families is formation of a parallel four helix bundle, referred to as the SNARE core complex [5–8]. Four SNARE motifs, each of which is basically heptad repeats of 60–70 amino acids, intertwine with each other into a four-stranded coiled coil that bridges two membranes. According to the 'zippering' hypothesis, formation of the SNARE complex initially starts at the N-terminal part, and propagates toward the membrane-proximal C-terminal part [9–11], where the transmembrane domains are expected to transduce tension from SNARE complex formation to two apposing membranes destined to fuse [12, 13]. In this review, we will primarily focus on a neuronal SNARE family in which synaptobrevin works as the

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vesicle (v-) SNARE offering one SNARE motif, while syntaxin and SNAP-25 are the heavy and the light chains of the target membrane (t-) SNAREs that offer one and two SNARE motifs, respectively.

There have been many excellent reviews that pedagogically summarize recent progresses in understanding the functions of SNAREs and the regulatory fusion effectors [3, 14–17]. In this short review, we intend to build on those other reviews and develop a new perspective which reflects recent technical breakthroughs. Major technical advances include SNARE proteins labelled with fluorescence resonance energy transfer (FRET) fluorophores at specific points, which enable to follow their gymnastics along the pathway of membrane fusion. Furthermore, the latest use of single molecule detection techniques reveals hidden biochemical reactions in real time with unprecedented accuracies, while forces at the pN to sub-nN levels are selectively applied to a handful of SNARE pairings. We will try to envision the conformational states of SNAREs in different fusion intermediates, and synchronize the conformational dynamics of the SNARE proteins with the dynamics of membrane remodelling. Finally, we will try to incorporate the molecular actions of the fusion effectors complexin, synaptotagmin 1, and Ca^{2+} along the developed fusion pathway.

Pre-cluster of target membrane SNAREs

It is thought that the SNARE complex is the fusion machine that provides the necessary energy to overcome the fusion energy barrier [18] (however, see also Su et al. [19]). Therefore, many scientific efforts have been devoted to the investigation of assembly and disassembly of individual SNARE complexes and the resultant energy release. It has however become increasingly clear that the success of a fusion process heavily hinges on higher-level ordering among the individual SNARE complexes [20–23]. Temporal and spatial coordination of multiple SNARE assembly events maximizes their impact on membrane structure, which would otherwise be fruitlessly dissipated with sporadic SNARE complex formation. Furthermore, because several intermediates are involved in sequence along the fusion pathway [24, 25], synchronized initiation of multiple SNARE complexes and keeping them in phase with each other must be a stringent requirement for building efficient fusion machinery made of SNAREs.

Forming clusters of SNARE proteins prior to SNARE core assembly offers a simple and effective solution for this requirement. Such SNARE clusters work as hot spots on a membrane where assembly of multiple SNARE complexes can take place simultaneously with a relatively high probability (Fig. 1A). For instance, syntaxin 1 has been proposed to exist in a clustered form. Using stimulation emission depletion (STED) microscopy that offers spatial resolution far beyond the diffraction limit, Sieber et al. imaged clusters of syntaxin 1 formed on the plasma membrane of PC12 cells, with each cluster having a diameter of 50–60 nm and harbouring 75 molecules on average [26]. The fluorescence recovery after photobleaching studies using various syntaxin 1 mutants show that homo-interaction via the SNARE motif is mainly responsible for this clustering [26]. Further, the electron paramagnetic resonance (EPR) investigation of Sso1p, a yeast analogue of syntaxin 1, reconstituted in synthetic vesicles, shows clustering of its transmembrane domain, strongly indicating the existence of multimers in the *in vitro* proteoliposome system [27]. The quantitative EPR spectral analysis revealed a stoichiometric number between 3 and 5, which is in contrast to the cluster of tens of proteins detected for syntaxin 1. Considering the need for lipid molecules to eventually be fed in between SNARE proteins for late fusion steps such as dilation of a lipidic fusion pore (see discussions in Section 3 and Fig. 1C and D), it seems somewhat difficult to envision a way to efficiently deliver lipid molecules into a 50–60 nm protein cluster. However, much bigger protein clusters of syntaxin 1 and SNAP-25, as large as 700 nm in diameter, have also been observed in chromaffin cells using confocal microscopy imaging [28]. Either way, the syntaxin 1 cluster has been proposed to be a very dynamic entity, with its constituent molecules being continuously exchanged with spatially scattered individual syntaxin 1 [26, 27].

The t-SNARE light chain SNAP-25 is thought to associate with syntaxin 1 without significantly altering the morphology of the cluster. The C-terminal SNARE motif of SNAP-25 may be largely dissociated from the binary complex of syntaxin 1 and SNAP-25 [29] or tightly bound to the binary t-SNARE complex as revealed by botulinum toxin E treatment [28]. Although such detailed molecular configuration of SNAP-25 in the t-SNARE complex is still elusive, the stoichiometry of the t-SNARE complex is found to be predominantly 1:1 *in vivo* [28, 29]. However, the structural analysis of the soluble SNAREs and the *in vitro* proteoliposome assay detect the existence of 2:1 association of syntaxin 1 and SNAP-25, in which the second syntaxin occupies the position of v-SNARE (synaptobrevin) making this 2:1 t-SNARE complex less receptive for synaptobrevin [30, 31]. Then, one fundamental question on the syntaxin 1 cluster is how cells maintain the t-SNARE complex in an active state. The syntaxin 1 cluster would provide a more crowded environment for the cluster-residing syntaxin

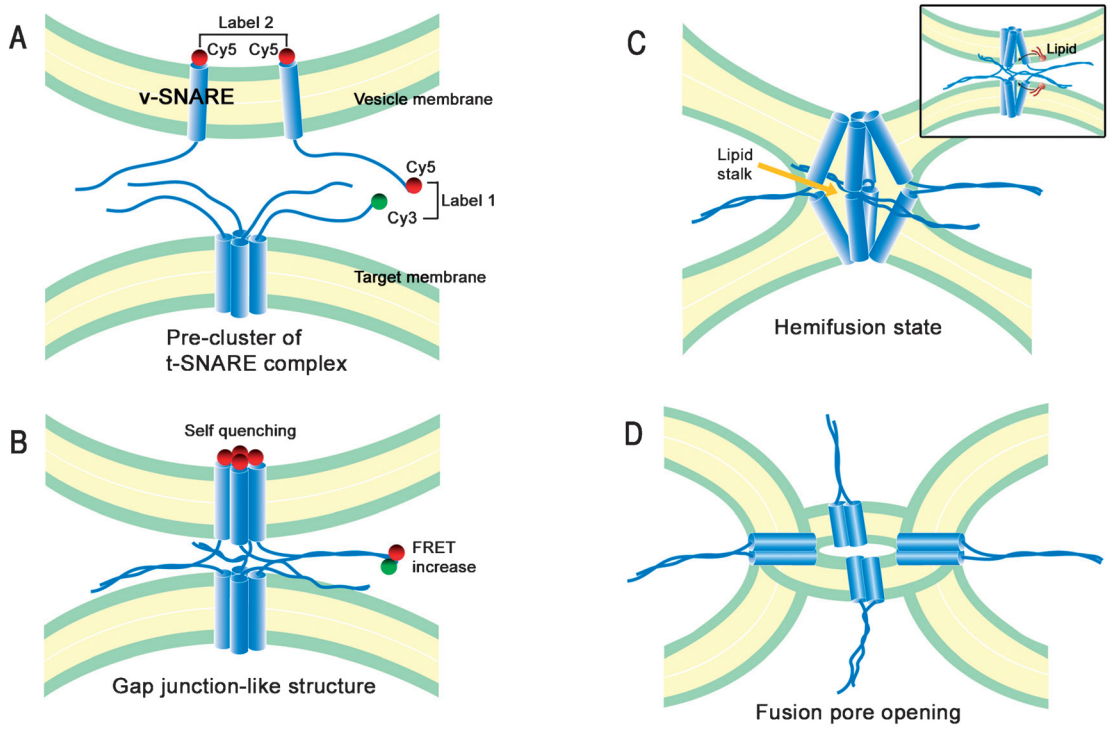

Figure 1. *(A)* Pre-docking stage: t-SNARE proteins in the target membrane exist in a clustered form. *(B)* Docking and gap junction-like structure: the t-SNARE cluster increases the probability of simultaneous formation of multiple SNARE complexes. The close proximity and alignment of the transmembrane domains of v- and t-SNAREs mimic the gap junction structure while the SNARE complexes bridge two membrane-spanning structures. *(C)* Hemifusion state: the transmembrane domain structures slightly expand to allow for a small number of lipid molecules inside. These lipid molecules may finally form membrane connectivity in the form of a lipid stalk. *(D)* Fusion pore opening: inner leaflets merge and the transmembrane domains completely dissociate from each other to open a fusion pore. Provided that zippering in the C-terminal part of the neuronal SNARE complex does not release enough energy for this step, fusion effectors such as complexin and synaptotagmin 1 should provide energy inputs to accomplish fusion pore opening on demand.

1 proteins, increasing the probability for formation of the 2:1 t-SNARE complex. Cells must have a way of preventing formation of the off-pathway 2:1 t-SNARE complex, and the recent single-molecule FRET study by Weninger et al. shows the possibility that various forms of fusion regulators, including the soluble domain of synaptotagmin 1, complexin, and Munc18, may have the effect of promoting a one-to-one combination of SNAP-25 and syntaxin 1 over a 2:1 complex [32].

Evolution of the fusion site from proteinaceous to lipidic structures

Once a fusion process commences, the t-SNARE complexes, gathered in a clustered form, rapidly start to recruit v-SNARE proteins to simultaneously initiate formation of multiple SNARE complexes (it seems that whether v-SNARE proteins also have a clustered form is yet a open question [33, 34]). The resultant higher-order protein complex containing multiple copies of the SNARE complex closely mimics the gap junction structurally (Fig. 1B), where two membrane-spanning structures are aligned in a stacked manner. In the plasma membrane, the transmembrane domains of syntaxin 1 should stay clustered and should be closely aligned to the counterpart composed of the transmembrane domains of synaptobrevin with SNARE complexes connecting both sides. This gap junction-like structure motivated several groups to propose that the transmembrane domain parts of syntaxin 1 form a pore that provides an aqueous passage to neurotransmitters [35, 36]. Considering the evidence that the syntaxin 1 cluster exists prior to SNARE complex formation, this 'proteinaceous pore' model indicates that the syntaxin 1 cluster must undergo structural changes in order to accommodate a pore structure subsequent to SNARE complex formation. This however needs to be experimentally verified.

It is highly likely that in the gap junction-like structure, the active fusion site is composed predominantly of the SNARE proteins. By contrast, there is compelling evidence that the active fusion site harbours predominantly lipidic intermediates such as the hemifusion state, in which the outer membranes are merged while the inner membranes remain intact [37]. The exis-

tence of hemifusion has been probed either by the observation of lipid mixing in the absence of content mixing [24, 38] or by the difference in kinetics between outer- and inner-leaflet mixing [39, 40]. In addition, single-vesicle fusion fluorescence imaging by Yoon et al. provided direct evidence for the hemifusion state [25]. The single-vesicle fusion assay developed by Yoon et al. uses a pair of v- and t-SNARE proteoliposomes as fusing objects that essentially constitute a closed system. As fusion progresses, donor and acceptor membrane fluorophores that are confined to respective vesicles mix together, and a fusion intermediate with a certain degree of lipid mixing is hallmarked by a defined value of the FRET efficiency. This assay enables to track the entire course of individual fusion events, from vesicle docking to full fusion, and to find that the FRET values of the first fusion intermediate are narrowly distributed around 0.35, which is exactly a half of the FRET value (0.7) for full fusion. Importantly, the same FRET range becomes more populated when the lipid composition of vesicles is enriched with 35 mol% phosphoethaloamine (PE) lipids that hinder progression to fusion pore opening after hemifusion. These observations are collectively consonant with the hypothesis that hemifusion is an on-pathway intermediate, and not simply a dead-end product of SNARE-mediated fusion [25].

These two hypothetical models, the gap junction-like structure and the hemifusion state, have been thought contradictory to each other, in the light of different molecule players involved: SNARE transmembrane domains in the former versus lipid molecules in the latter. Employing dye-labelled SNARE proteins in the *in vitro* fusion system, Lu et al. resolved sequential dynamic stages of SNARE assembly and placed these stages in relation to lipid mixing steps [27]. Technically, a FRET pair consisting of Cy3 and Cy5 was conjugated to the N-terminal parts of Sso1p and Snc2, respectively (yeast SNAREs analogous to syntaxin 1 and synaptobrevin; Fig. 1A, Label 1). In another preparation, Cy5 dye was attached to the C-terminal tip of Snc2 as a fluorescence reporter (Fig. 1A, Label 2). The FRET increase from the Label 1 sites probes the SNARE assembly at the N-termini, while the decrease in Cy5 fluorescence in the Label 2 experiment reports self quenching of the dyes perhaps due to jagged association of the transmembrane domains of the v-SNAREs. Of note, Lu et al. found that these two fluorescence changes, FRET increase from Label 1 and self quenching of Label 2 dyes, share the same kinetic constant [27]. Considering the pre-existing cluster of t-SNARE proteins, this observation of sharing kinetic constants strongly indicates formation of the gap junction-like structure (Fig. 1B). Moreover, Lu et al. showed that these fluorescence changes, indicative of formation of the gap junction-like structure, occur 'earlier' than lipid mixing between the outer leaflets that represents hemifusion.

This study not only finds the gap junction-like structure and the hemifusion state (or lipid stalk) in the course of SNARE-mediated fusion, two structures previously perceived at odds with each other, but it also provides interesting molecular insights into how the initially proteinaceous fusion active site might evolve to a predominantly lipidic structure. As discussed above, the clusters of transmembrane domains are not rigid ones, and lipid molecules continuously intrude on and get squeezed out of the space between transmembrane domains. The central region of the gap junction-like structure would be a 'melting pot' where two fusing membranes are brought into closest proximity. Therefore, one can envision that the transmembrane domains of the gap junction-like structure expand laterally to an extent that allows for a small number of lipid molecules between them (Fig. 1C, upper right inset), and the lipid molecules, newly fed into the fusion site, finally achieve a membrane connection which is in the form of a lipid stalk (Fig. 1C). Electrostatic repulsion between the highly basic membrane proximal regions of SNAREs might contribute to the lateral expansion of the transmembrane structures [41].

At the same time, we expect that the transmembrane domains would not be infinitely diluted by intercalating lipid molecules. Such hapless dilution into individual *trans* SNARE complexes is the result of dissociation of the transmembrane domain cluster, and such an individual *trans* SNARE complex would eventually lose its tension because the two transmembrane domains of v- and t-SNAREs soon migrate toward each other through the lipid stalk (i.e. fold into a *cis* SNARE complex). It has been repeatedly observed that there are two classes for the hemifusion state: productive and non-productive [42], and the non-productive hemifusion state may have suffered from severe dilution of *trans* SNARE complexes by lipid molecules. It is then conceivable that in the productive hemifusion state, the transmembrane domains of *trans* SNARE complexes remain largely associated with each other, perhaps fencing the lipid stalk that provides connectivity mainly between two outer leaflets. It is noteworthy that the protein-centric fusion model presented here differs from the traditional lipid-centric model. In the lipid-centric model, the SNARE proteins become laterally sequestered from the fusion site prior to hemifusion, in order to prepare the protein-free lipid patches for fusion. The lipid-centric model further predicts that a ring of sequestered SNARE complexes bend the membrane

into a hypothetical bilayer protrusion at the centre of the lipid patch, called 'dimple', that eases the merging of two bilayers into the lipid stalk.

Opening of the fusion pore

Ca^{2+}-triggered, synchronized release of neurotransmitters from synaptic vesicles constitutes the molecular mechanism underlying neuronal communication [3]. Fusion pore opening after the hemifusion state is believed to be the final energy barrier before successful release of neurotransmitters or other forms of biochemical delivery [1, 2]. Given the hemifusion model we depicted above, opening of a fusion pore would involve two processes: disruption and merging of the inner leaflet membranes and complete dissociation of the transmembrane domains to finally achieve a ring-like arrangement of *cis* SNARE complexes (Fig. 1D). The second process would be an exclusive feature of SNARE-mediated membrane fusion, which is absent in a purely lipidic fusion process.

Neuronal communication is a tightly regulated process, and the timing of fusion pore opening must be precisely controlled up to the millisecond scale [3]. As for the regulatory mechanism, there are two fundamentally different views depending on whether the SNAREpin assembly alone can drive all the fusion steps including fusion pore opening, fast enough to cope with neurotransmitter release. If SNARE assembly is efficient enough to foster fast fusion pore opening, the system would require a putative fusion clamp to gain control over fusion timing: the system would be spring-loaded by the fusion clamp. Upon Ca^{2+} influx, the break would be removed, and the remaining fusion steps would be completed. Recently, complexin, which is the major binding partner of neuronal SNARE complexes, was proposed as a fusion clamp because complexin was shown to have inhibitory effects in *in vitro* systems [43–46]. But, single-vesicle fusion experiments by Yoon et al. dissected individual fusion steps in each single fusion event, and found that the inhibitory effect is selective on the docking step that is prior to the SNARE complex formation (see discussions in the next section) [47], which is earlier than the docked or hemifused state that is an ideal fusion stage for clamping. In addition, a partial SNARE complex consisting of a c-terminal fragment of v-SNARE and the t-SNARE complex rapidly releases the v-SNARE fragment when the full length v-SNARE approaches to form a complete ternary SNARE complex (that is, the c-terminal fragment is replaced by the full-length v-SNARE) [48]. This result indicates that even the v-SNARE fragment that should have a very high affinity to the t-SNARE complex does not serve as a fusion clamp. Therefore, it appears that the identification of the fusion clamps of SNARE-mediated fusion is not conclusive yet.

More fundamentally, there is increasing evidence that the neuronal SNARE complex by itself is not an efficient fusogen, yielding very slow fusion kinetics [47, 49, 50]. The kinetics of such basal SNARE-mediated fusion may be accelerated by increasing the surface density of SNAREs on the membrane to some extent [50, 51]. However, experiments using the yeast vacuole system revealed that such excessively crowded SNAREs induce fusion through membrane lysis, bypassing the regular pathway that requires a much lower SNARE and fusion regulators such as the homotypic fusion and vacuole protein sorting (HOPS) complex [51]. Furthermore, detailed structural studies indicate that the C-terminal part of the neuronal SNARE complex (mostly spanning +4 to +8 layers) might have a lower binding energy than the N-terminal parts, suggesting that the SNARE complex is not an efficient force transducer. Consistently, the force measurement by Li et al. reported a binding energy as high as 35 k_BT for the N-terminal part, but the results show that the membrane proximal part still remained uncomplexed [52]. Further, the partial SNARE complex including the C-terminal part of the v-SNARE motif and the full-length t-SNARE motifs dissociates at a much lower temperature than another partial SNARE complex that has the N-terminal part of the v-SNARE motif [48]. Single-vesicle fusion imaging revealed that neuronal SNAREs induce an appreciable level of proteoliposome docking but very slow fusion kinetics on a time scale of tens of minutes, which is as much as six orders of magnitude slower than the time scale of the neurotransmitter release [47]. Based on these observations, one may conclude that the neuronal SNARE complex is effective in mediating docking, but that it is indeed a poor fusogen.

Presumably, yet unzipped SNARE motifs in the C-terminal parts are highly dynamic and flexible, and thermal uncoordinated motion of this membrane-proximal part dissipates much of the tension created through SNARE assembly at the N-termini. As discussed above, opening of a fusion pore requires an energy input to disrupt the membrane structure of inner leaflets plus to destabilize the association of transmembrane domains. This energy barrier was clearly signified in the single-vesicle fusion FRET data by an independent Gaussian distribution for the full fusion state and by the population valley at the border FRET value between hemifusion and fusion pore opening [25, 47].

Finding fusion stimulators

An immediate question is which fusion protein would provide an additional energy input to assist the SNARE complex to carry out the fusion reaction on much faster time scales. The *in vitro* proteoliposome fusion assay may be an ideal tool to tackle this task. Unlike the physiological assays, the well-defined setting does not suffer from complications arising from the existence of a myriad of other proteins. This reductionist approach provides a platform to tell if a fusion effector is a blue-collar worker for membrane fusion or just a white-collar signal mediator. Up to this point, three major fusion regulators have been reported to accelerate the SNARE-mediated fusion by directly interacting with SNAREs, namely Munc18-1 [53, 54], complexin [47], and C2AB which is the cytosolic part of synaptotagmin 1 [55, 56]. Munc 18-1, for example, was long hypothesized to be a fusion inhibitor based on the specific binding mode to the N-terminal domain of syntaxin 1. This binding is thought to stabilize the closed form of syntaxin 1, thus keeping individual syntaxin 1 proteins from taking part in SNARE complex formation [57–59]. However, using the *in vitro* proteoliposome system, Tareste et al. [53], Shen et al. [54] and Dulubova et al. [60] found that Munc18-1 stimulates SNARE-mediated fusion via direct interaction with the SNARE complex. This stimulatory effect is specific in a sense that only membrane fusion induced by the neuronal SNAREs (syntaxin 1, SNAP-25 and VAMP2) is selectively stimulated by Munc18-1, suggesting that Munc18-1 is a 'proofread' machine for neuronal exocytosis [54]. But we note that Munc18-1's inhibitory role of locking syntaxin 1 in the closed conformation also has a critical phenotype that elevates general seizures in a knockin/knockout mice study [61].

With the *in vitro* fusion system, it would be very useful if one could discern which particular step is accelerated by an effector protein. SNARE-mediated fusion passes through various stages: docking and gap junction-like structure, formation of a lipid stalk and hemifusion, and fusion pore opening. Single-vesicle fusion imaging would provide an unprecedented opportunity to dissect these different fusion steps. Recently, Yoon et al. determined the typical dwell times of the different stages using the single vesicle-vesicle FRET technique [25]. The experiments yielded crucial information that by and large, docking is the rate-limiting step for the proteoliposome fusion system. This observation implies that a bulk ensemble measurement may be sensitive to changes in the rate-determining docking kinetics, while being relatively insensitive to changes in the kinetics of the following fusion steps. This emphasizes the limited effectiveness of the bulk fusion assay in characterizing the precise functions of fusion effectors.

A recent study on the molecular effect of complexin on SNARE-dependent fusion illustrates an effective use of the single-vesicle fusion assay [47]. Single-vesicle fusion FRET imaging measured the kinetic rate of the docking as well as that of the subsequent steps by simultaneously investigating the number of docked vesicles and the fusion FRET values in a given reaction time. This new approach unexpectedly revealed the two-faceted effects of complexin on SNARE-mediated fusion. Complexin works as an inhibitor for vesicle docking, but once SNAREs are assembled, it significantly stimulates the rate of the late fusion steps (a larger FRET value). This observation finds the docking step to be critical for complexin's inhibitory effect. More importantly, the single vesicle technique identifies complexin as a blue-collar worker that interacts directly with the SNARE complex to accelerate SNARE-mediated membrane fusion. In contrast, a bulk ensemble measurement using the same SNARE-reconstituted vesicles showed a slowed FRET increase in time with the increased complexin concentration, thus predominantly reporting the inhibiting effect of complexin [45, 47].

Does complexin then help the C-terminal part of the SNARE complex make the SNARE complex a better energy transducer? Structural studies showed that the mid-region of complexin (residues 47–83) binds to the C-terminal part of the SNARE core in the anti-parallel manner (the N-terminus becomes the membrane-proximal part) [62, 63]. One can envision that complexin binding to the SNARE complex stabilizes the *trans* SNARE complex and thus delivers the tension from SNARE complexes more faithfully to the transmembrane domains [47, 63]. Further, it has been shown that the membrane-proximal N-terminal region of complexin is essential for its stimulatory role [64]. Although speculative, this N-terminal region might interact with the membrane, providing additional stability to SNARE-bound complexin (X. Lu and Y.-K. Shin, unpublished data). On the other hand, the inhibitory effect seems to arise from a much weaker interaction of complexin with the t-SNARE complex (dissociation constant ~50 μM) [47]. Therefore, complexin at unusually high concentrations competes with v-SNAREs for binding to t-SNARE complexes. In sum, complexin uses two divergent interaction modes with SNARE proteins, a weak interaction with the t-SNARE complex and a highly specific binding to the ternary SNARE complex, to attain the dual effects as well as switching between the two [47]. These observations provide a glimpse at how fusion regulators attain diverse functionalities for different fusion steps.

Outlook for synaptotagmin 1 function

In conclusion, on the basis of the above discussions, one may come up with a rather simple picture of the SNARE-mediated membrane fusion. Zippering at the N-terminal part of neuronal SNAREs creates force 1) to bring two vesicles into close proximity, 2) to form gap junction-like structure and 3) to induce a lipid stalk and hemifusion, but the lack of energy release from the C-terminal parts may keep the fusion process from proceeding to the opening of the fusion pore. The fusion energetics underlying this scenario are quite different from what is implied in the fusion clamp model. It is clear that SNARE complex formation essentially lowers the energy barrier against membrane fusion [65], which would otherwise show negligible fusion progression on practical time scales. However, at the same time, the SNARE complexes should be intimately aided by fusion stimulators and lipid molecules to accelerate fusion and to increase the Ca^{2+} sensitivity. A tangible idea may be obtained by extrapolating the time scales measured in the single vesicle study on complexin [47]. Firstly, with the neuronal SNAREs alone, a fusion event progresses on a time scale of tens of minutes. Through the stimulatory action discussed above, complexin lowers the fusion energy barrier, which shortens the average time required for full fusion to a few minutes. Finally, upon Ca^{2+} influx, fusion is further accelerated by almost two orders of magnitude to be on the scale of a few seconds. In addition, this final time scale of a few seconds, observed with SNARE, complexin and Ca^{2+}, is off the typical time scale of asynchronous release (hundreds of milliseconds) by only one order of magnitude [66–70]. Based on this analysis, we speculate that the supramolecular complex consisting of SNARE complexes, phospholipids and complexin serves as a substrate for Ca^{2+} for the asynchronous release.

For the synchronous exocytosis mode, many genetic and physiological data strongly evince that synaptotagmin 1 (or 2) should be the primary Ca^{2+} sensor, critical for fusion synchronization on (sub-) millisecond scales [3, 71–75]. Synaptotagmin 1 resides in synaptic vesicles with one transmembrane domain and catches five Ca^{2+} ions using tandem C2 domains (C2A and C2B domains) that are known to interact with both membranes and SNARE proteins [75, 76]. Most notably, genetic ablation of synaptotagmin 1 abolishes the synchronous exocytosis by more than 95%, leaving only the asynchronous release as the exocytosis output [69, 71, 77]. Capacitance measurements on PC12 cells reported that synaptotagmin 1 is one of the main players that determine the molecular structure and evolution of fusion pores [78]. In addition, although the C-terminal part of the neuronal SNARE may not drive the fusion process alone, this region must be intimately involved in late fusion steps, as evidenced by the impairment of exocytosis through the cleavage of the C-terminal part of SNAP-25 by the botulinum neurotoxins A, C1, and E [79, 80]. Therefore, it is highly likely that synaptotagmin 1 primarily functions to open a fusion pore in concert with the molecular action of the C-terminal part of neuronal SNAREs.

Despite the physiological evidence for synaptotagmin 1 as the primary Ca^{2+} sensor, *in vitro* reconstitution of synaptotagmin 1 function has proven very difficult. Tucker et al. [56, 81] and Matens et al. [55] showed that Ca^{2+} and the cytosolic domain of synaptotagmin 1 increase the speed of neuronal SNARE-mediated fusion by a factor of four to five. But the relevant time scale still stays at hundreds or thousands of seconds, making it difficult to be reconciled with the millisecond synchronous release. Furthermore, the *in vitro* experiments using the full-length synaptotagmin 1 failed to observe a Ca^{2+}-evoked acceleration of SNARE-mediated fusion [82–84]. These data report on fusion acceleration in the absence of $Ca^{2+,}$ but on fusion inhibition in the presence of Ca^{2+}, questioning the issues related to Ca^{2+}-evoked stimulation by synaptotagmin 1.

We note that several issues should be carefully scrutinized to ensure successful *in vitro* reconstitution of synaptotagmin 1 function. The C2AB domains of many synaptotagmin isoforms, when bound to Ca^{2+} ions, bind strongly to negatively charged membranes, inducing fast vesicle aggregation [85] and buckling of the membrane [55]. It is highly likely that a bulk ensemble measurement reports the acceleration in docking, but that the stimulatory effect on fusion pore opening must be assessed separately from that on docking. In fact, Stein et al. observed that the acceleration by Ca^{2+}-bound C2AB largely diminished when the docking stage was accelerated by using a v-SNARE fragment [82], indicating that the stimulatory effect of C2AB is mainly limited to docking and not translated well into boost of fusion pore opening. Furthermore, the C2AB domain is prone to the *cis* interaction, self-interaction with its resident v-SNARE (synaptic vesicle) membrane, which has adverse effects on fusion acceleration [82, 86]. Therefore, a new approach should be devised to direct the stimulatory effect of synaptotagmin 1 into the step of fusion pore opening. Also, the molecular action of the C2AB domain should be steered toward the target membrane in a vectorial way, for instance using disparate lipid compositions for fusing two membranes [82, 86]. We anticipate that the new arsenal of experimental techniques, especially the single-vesicle

fusion assay, will provide an avenue for recapitulating Ca^{2+}-triggered acceleration by synaptotagmin 1, shedding a new light on the regulation of the neurotransmitter release.

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