A molecular machine for neurotransmitter release: synaptotagmin and beyond

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Fifty years ago, Bernard Katz's seminal work revealed that calcium triggers neurotransmitter release by stimulating ultrafast synaptic vesicle fusion. But how a presynaptic terminal achieves the speed and precision of calcium-triggered fusion remained unknown. My colleagues and I set out to study this fundamental problem more than two decades ago.

How do the synaptic vesicle and the plasma membrane fuse during transmitter release? How does calcium trigger synaptic vesicle fusion? How is calcium influx localized to release sites in order to enable the fast coupling of an action potential to transmitter release? Together with contributions made by other scientists, most prominently James Rothman, Reinhard Jahn and Richard Scheller, and assisted by luck and good fortune, we have addressed these questions over the last decades.

As I describe below, we now know of a general mechanism of membrane fusion that operates by the interaction of SNAREs (for soluble N-ethylmaleimide–sensitive factor (NSF)-attachment protein receptors) and SM proteins (for Sec1/Munc18-like proteins). We also have now a general mechanism of calcium-triggered fusion that operates by calcium binding to synaptotagmins, plus a general mechanism of vesicle positioning adjacent to calcium channels, which involves the interaction of the so-called RIM proteins with these channels and synaptic vesicles. Thus, a molecular framework that accounts for the astounding speed and precision of neurotransmitter release has emerged. In describing this framework, I have been asked to describe primarily my own work. I apologize for the many omissions of citations to work of others; please consult a recent review for additional references[1].

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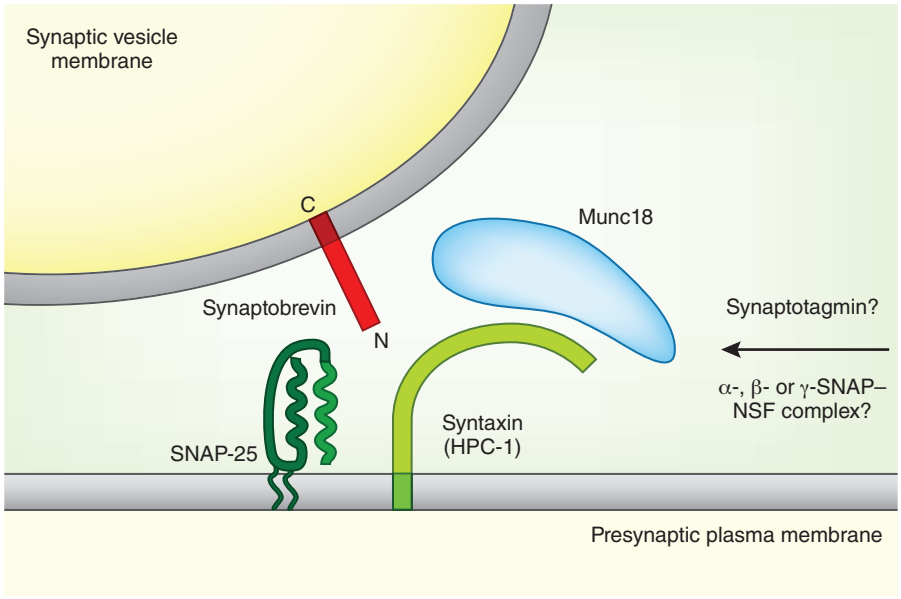

Figure 1 Description of the SNARE–SM protein complex that mediates synaptic vesicle fusion. The initial study[2] identified the SM protein Munc18-1 as a component of the fusion machinery that also contains the SNARE proteins synaptobrevin (also known as VAMP), SNAP-25 and syntaxin (also known as HPC-1), although the detailed protein-protein interactions involved were defined only later (reproduced from ref. 2).

Starting my lab

When I started my laboratory in 1986 at the University of Texas Southwestern in Dallas, exquisite electrophysiological studies had already characterized neurotransmitter release in detail. These studies showed that calcium triggers release within a few hundred microseconds, exhibits amazing plasticity and displays a nonlinear dependence on calcium. However, aside from calcium, not a single molecule important for release had been identified.

Genetic screens by Sidney Brenner, Randy Scheckman and their colleagues had isolated gene mutations that disrupt synaptic transmission in *Caenorhabditis elegans* or impair the secretory pathway in yeast, but the function of the corresponding proteins were unknown. In pioneering work, Rothman performed *in vitro* membrane fusion assays using non-neuronal cells, but the molecular mechanisms involved in these fusion reactions were unclear. The lack of knowledge about how synaptic vesicle fusion happens and how such fusion is controlled by calcium intrigued me and led me to search for molecular mechanisms.

We chose a simple approach: to isolate and clone all of the major proteins present in presynaptic terminals. Initially, in collaboration with Reinhard Jahn, we focused on synaptic vesicles because they could be isolated at high yield and purity. Later on, we expanded this approach to the presynaptic active zone and plasma membrane. The goal was to achieve a molecular catalog of protein components of the presynaptic terminal as a starting point for a functional dissection. For over a decade, we purified and cloned a series of major synaptic proteins—among others, synaptophysin, synaptobrevin,

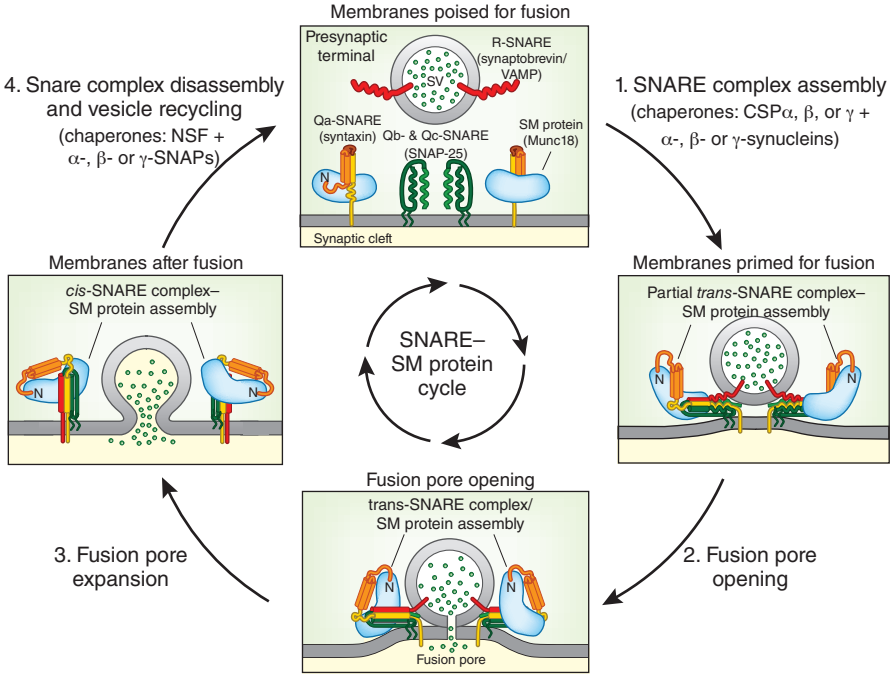

Figure 2 Model of the SNARE–SM protein cycle during synaptic vesicle fusion. During step 1, synaptic vesicles are primed for fusion; this step involves opening of the closed conformation of syntaxin, a switch of the Munc18-binding mode of syntaxin from the closed to the open conformation and partial assembly of *trans*-SNARE complexes. Step 1 is facilitated by recently discovered chaperones (cysteine string proteins (CSPs) and synucleins) that enhance SNARE complex assembly and whose dysfunction is related to neurodegeneration[3]. During step 2, the fusion pore opens, with full *trans*-SNARE complex assembly. During step 3, the fusion pore expands, converting *trans*-SNARE into *cis*-SNARE complexes. In step 4, NSF and SNAPs mediate disassembly of the SNARE complex, leading to vesicle recycling. The cycle shown here for synaptic vesicle fusion is paradigmatic for most cytoplasmic fusion reactions, although the details differ. In knockout experiments, deletion of the SM protein Munc18-1 produces the most severe phenotype[22], possibly because loss of SNARE components of the fusion machinery is better compensated for than loss of SM proteins. SNAREs are generally classified into four types (R, Qa, Qb and Qc) that assemble into SNARE complexes in an obligatory R-Qa-Qb-Qc combination.

synapsins, synaptotagmins, Munc13s, Munc18s, complexins, RIMs, RIM-BPs, neurexins and neuroligins.

Elucidating the mechanism of fusion

As a molecular catalog of synaptic vesicle proteins emerged, the question of how synaptic vesicles may interact with the presynaptic plasma membrane became approachable. Other laboratories cloned SNAP-25 and syntaxin-1, presynaptic plasma membrane proteins now known as t-SNAREs. In a crucial study, Rothman showed that syntaxin and SNAP-25 form a complex (which he named the SNARE complex) with the synaptic vesicle protein that we had identified and named synaptobrevin (also cloned by Scheller, who named it VAMP). Immediately afterwards, we identified Munc18-1 as a syntaxin-associated protein and proposed that Munc18-1 and the SNARE proteins constitute the fusion machine of synaptic vesicles (**Fig. 1**)[2].

In parallel, the laboratories of Cesare Montecucco and Jahn—partly in collaboration with our lab—identified synaptobrevin, SNAP-25 and syntaxin-1 as substrates for the proteolytic activities of botulinum and tetanus toxins, which were known to block neurotransmitter release. These findings strongly supported the idea that the three SNARE proteins function in synaptic vesicle fusion. Thus, at the end of 1993, most components of the synaptic fusion machinery had been identified. But how do SNARE and SM proteins mediate fusion? It took two decades until a plausible model emerged[1].

During fusion, SNARE and SM proteins undergo a cycle of association and dissociation, which is maintained by chaperones that support SNARE complex assembly (cysteine string proteins and synucleins)[3] and disassembly (NSF and SNAPs; **Fig. 2**). The underlying principle of SNARE and SM protein function is simple: SNARE proteins are attached to both membranes destined to fuse and form a *trans* complex that involves a progressive zippering of the four-helical SNARE complex bundle in an N- to C-terminal direction (step 1 in **Fig. 2**). The zippering of *trans*-SNARE complexes forces the two fusing membranes into close proximity, destabilizing the membrane surfaces (step 2). Assembly of the full *trans*-SNARE complex (together with the action of SM proteins; see below) opens the fusion pore (step 3). Fusion-pore expansion transforms the initial *trans*-SNARE complexes into *cis*-SNARE complexes that are then dissociated by the ATPase NSF, which binds SNARE complexes via α-, β- or γ-SNAP adaptor proteins, thereby completing the cycle (step 4).

In a physiological context, SNARE complex assembly forces opposing membranes into close proximity but is insufficient to mediate fusion. Our initial proposal that SM proteins generally contribute to membrane fusion[2] met with skepticism because biochemical data seemed to argue against this hypothesis. However, studies over the past decade have revealed that Munc18-1 remains associated during fusion with SNARE proteins throughout their assembly-disassembly cycle and that this association is essential for fusion[4]. This finding resolved the questions surrounding Munc18-1 and strongly supported the idea that Munc18-1 and other SM proteins are obligatory components of fusion machines[1].

How calcium controls membrane fusion

The above discussion describes the major progress that was made in determining the mechanism of membrane fusion. At the same time, my laboratory was focusing on a question crucial for neuronal function: how is this process triggered in microseconds when calcium enters the presynaptic terminal?

While examining the fusion machinery, we wondered how it could possibly be controlled so tightly by calcium. Starting with the description of synaptotagmin-1 (Syt1)[5], we worked over two decades to show that calcium-dependent exocytosis is mediated by synaptotagmins as calcium sensors.

Synaptotagmins are evolutionarily conserved transmembrane proteins with two cytoplasmic C_2 domains (**Fig. 3a**)[5,6]. When we cloned Syt1, nothing was known about C_2 domains except that they represented the 'second constant sequence' in protein-kinase C isozymes. Because protein kinase C had been shown to interact with phospholipids by an unknown mechanism, we speculated that Syt1 C_2 domains may bind phospholipids, which we indeed found to be the case[5]. We also found that this interaction is calcium dependent[6,7] and that a single C_2 domain mediates calcium-dependent phospholipid binding (**Fig. 3b**)[8]. In addition, the Syt1 C_2 domains also bind syntaxin-1 and the SNARE complex[6,9]. All of these observations were first made for Syt1 C_2 domains, but they have since been generalized to other C_2 domains.

As calcium-binding modules, C_2 domains were unlike any other calcium-binding protein known at the time. Beginning in 1995, we obtained atomic structures of calcium-free and

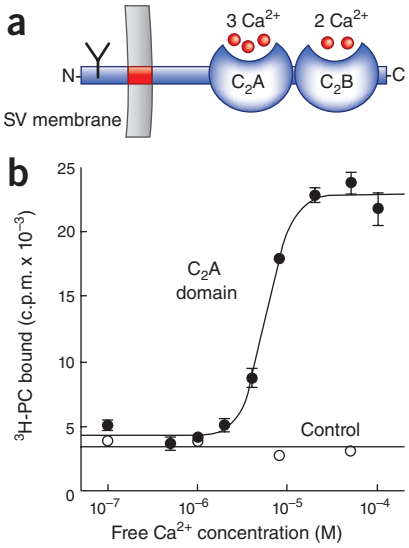

Figure 3 Discovery of synaptotagmins and of the calcium-binding properties of C_2 domains. (**a**) Syt1 cloning defined the canonical domain structure of synaptotagmins, which includes a single transmembrane region and two C_2 domains[4]. Only some synaptotagmins are glycosylated in their intravesicular sequence as shown; some other synaptotagmins bind calcium in a different stoichiometry or not at all. SV, synaptic vesicle. (**b**) Calcium-dependent phospholipid binding by the Syt1 C_2A domain. Note the steep calcium cooperativity and micromolar calcium affinity of binding (modified from ref. 7). Data shown are means ± s.e.m.; *y* axis depicts the amount of liposome binding to the immobilized Syt1 C2A domain measured as the amount of tritiated phosphotidylcholine (^{3}H-PC) bound. (**c**) Atomic structures of the Syt1 C_2A and C_2B domains with three and two bound calcium ions, respectively. Images show ribbon diagrams with cyan-colored β-strands, orange α-helices and orange balls for the calcium ions (courtesy of J. Rizo, University of Texas Southwestern).

calcium-bound Syt1 C2 domains[10] in collaboration with structural biologists, primarily Jose Rizo (**Fig. 3c**). These structures provided the first insights into how C_2 domains bind calcium and allowed us to test the role of Syt1 calcium binding in transmitter release[11].

The biochemical properties of Syt1 suggested that it constituted Katz's long-sought calcium sensor for neurotransmitter release. Initial experiments in *C. elegans* and *Drosophila*, however, disappointingly indicated otherwise. The 'synaptotagmin calcium-sensor hypothesis' seemed unlikely until our electrophysiological analyses of Syt1 knockout mice revealed that Syt1 is required for all fast synchronous synaptic fusion in forebrain neurons but is dispensable for other types of fusion (**Fig. 4**)[12]. These experiments established that Syt1 is essential for fast calcium-triggered release, but not for fusion as such.

Although the Syt1 knockout analysis supported the synaptotagmin calcium-sensor hypothesis, it did not exclude the possibility that Syt1 positions vesicles next to voltage-gated calcium channels (a function now known to be mediated by RIMs and RIM-BPs; see below), with calcium binding to Syt1 performing a role unrelated to calcium sensing and transmitter release. To directly test whether calcium binding to Syt1 triggers release, we introduced a point mutation into the endogenous mouse *Syt1* gene locus. This mutation decreased the Syt1 calcium-binding affinity by about twofold[11]. Electrophysiological recordings revealed that this mutation also decreased the calcium affinity of neurotransmitter release approximately twofold, formally proving that Syt1 is the calcium sensor for release (**Fig. 5**). In addition to mediating calcium triggering of release, Syt1 controls ('clamps') the rate of spontaneous release occurring in the absence of action potentials, thus serving as an essential mediator of the speed and precision of release by association with SNARE complexes and phospholipids (**Fig. 6a**,**b**).

It was initially surprising that the Syt1 knockout produced a marked phenotype because the brain expresses multiple synaptotagmins[6]. However, we found that only three synaptotagmins—Syt1, Syt2 and Syt9—mediate fast synaptic vesicle exocytosis[13]. Syt2 triggers release faster, and Syt9 slower, than Syt1. Most forebrain neurons express only Syt1, but not Syt2 or Syt9, accounting for the profound Syt1 knockout phenotype. Syt2 is the predominant calcium sensor of very fast synapses in the brainstem[14], whereas Syt9 is primarily present in the limbic system[13]. Thus, the kinetic properties of Syt1, Syt2 and Syt9 correspond to the functional needs of the synapses that contain them.

Parallel experiments in neuroendocrine cells revealed that, in addition to Syt1, Syt7 functions as a calcium sensor for hormone exocytosis. Moreover, experiments in olfactory neurons uncovered a role for Syt10 as a calcium sensor for insulin-like growth factor-1 exocytosis[15], showing that, even in a single neuron, different synaptotagmins act as calcium sensors for distinct fusion reactions. Viewed together with results by other groups, these observations indicated that calcium-triggered exocytosis generally depends on synaptotagmin calcium sensors and that different synaptotagmins confer specificity onto exocytosis pathways.

We had originally identified complexin as a small protein bound to SNARE complexes (**Fig. 6b**)[16]. Analysis of complexin-deficient neurons showed that complexin represents a cofactor for synaptotagmin that functions both as a clamp and as an activator of calcium-

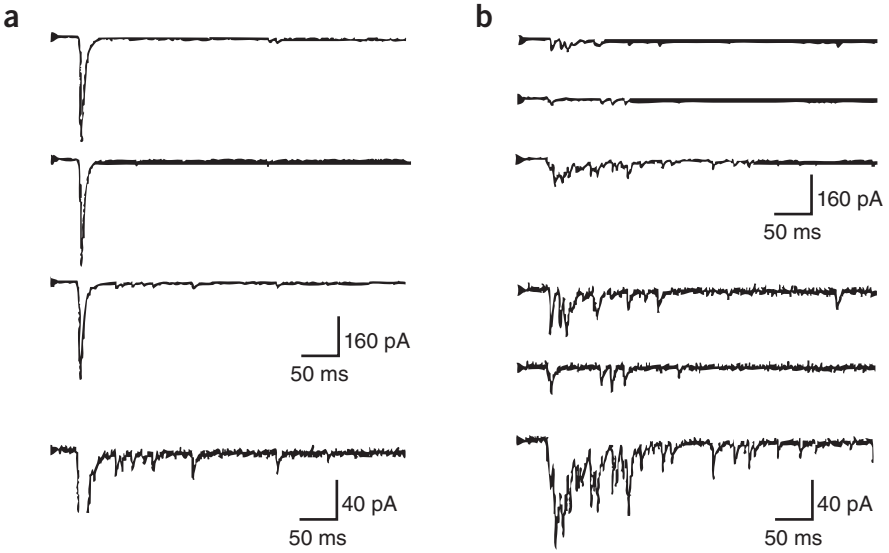

Figure 4 Syt1 knockout ablates fast synchronous transmitter release. (**a**,**b**) Traces showing recordings from neurons cultured from littermate wild-type (**a**) and Syt1-knockout (**b**) mice. Traces depict synaptic responses to isolated action potentials; standard (top) and expanded views (bottom) illustrate that the Syt1 knockout completely ablates fast synchronous release but not slow asynchronous responses (modified from ref. 12).

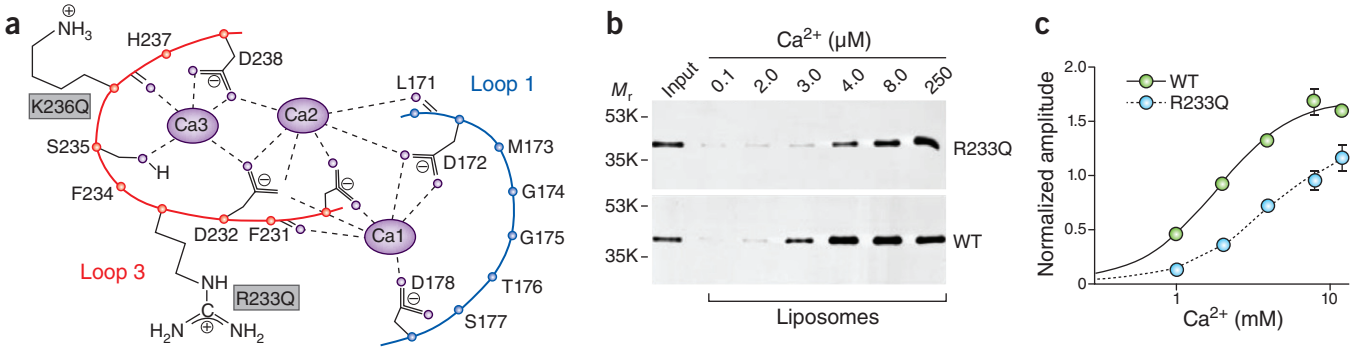

Figure 5 Introducing a mutation that changes the calcium affinity of Syt1 into endogenous Syt1 in mice establishes that Syt1 is a calcium sensor for neurotransmitter release. (**a**) Calcium-binding site architecture of the Syt1 C_2A domain and location of the amino acid substitution (R233Q) that we analyzed. A second innocuous mutation (K236Q) served as a control. (**b**) The R233Q substitution decreases the apparent calcium affinity of Syt1 during phospholipid binding approximately twofold. Data show binding of a native Syt1 fragment containing both C_2 domains to liposomes as a function of the free calcium concentration. M_r, apparent molecular mass. (**c**) The R233Q mutation decreases the apparent calcium affinity of synaptic exocytosis by about twofold. Figure depicts amplitudes of synaptic responses induced at different concentrations of extracellular calcium (modified from ref. 11). WT, wild type. Data show means ± s.e.m.; lines are fitted by a Hill function with an approximately twofold lower apparent calcium affinity for R233Q than for WT neurons.

triggered fusion[17]. Complexin-deficient neurons exhibit a phenotype milder than that of Syt1-deficient neurons, with a selective suppression of fast synchronous exocytosis and an increase in spontaneous exocytosis, which suggests that complexin and synaptotagmins are functionally interdependent.

How does a small molecule like complexin, composed of only ~130 amino acid residues, act to activate and clamp synaptic vesicles for synaptotagmin action? Atomic structures revealed that, when bound to assembled SNARE complexes, complexin contains two short α-helices flanked by flexible sequences (**Fig. 6c**). One of the α-helices is bound to the SNARE complex and is essential for all complexin function[18]. The second α-helix is required only for the clamping, and not for the activating function of complexin[17]. The flexible N-terminal sequence of complexin, conversely, mediates only the activating, but not the clamping, function of the protein. Our current model is that complexin binding to SNAREs activates the SNARE–SM protein complex and that at least part of complexin competes with synaptotagmin for SNARE complex binding. Calcium-activated synaptotagmin displaces this part of complexin, thereby triggering fusion-pore opening (**Fig. 6a**)[1,18].

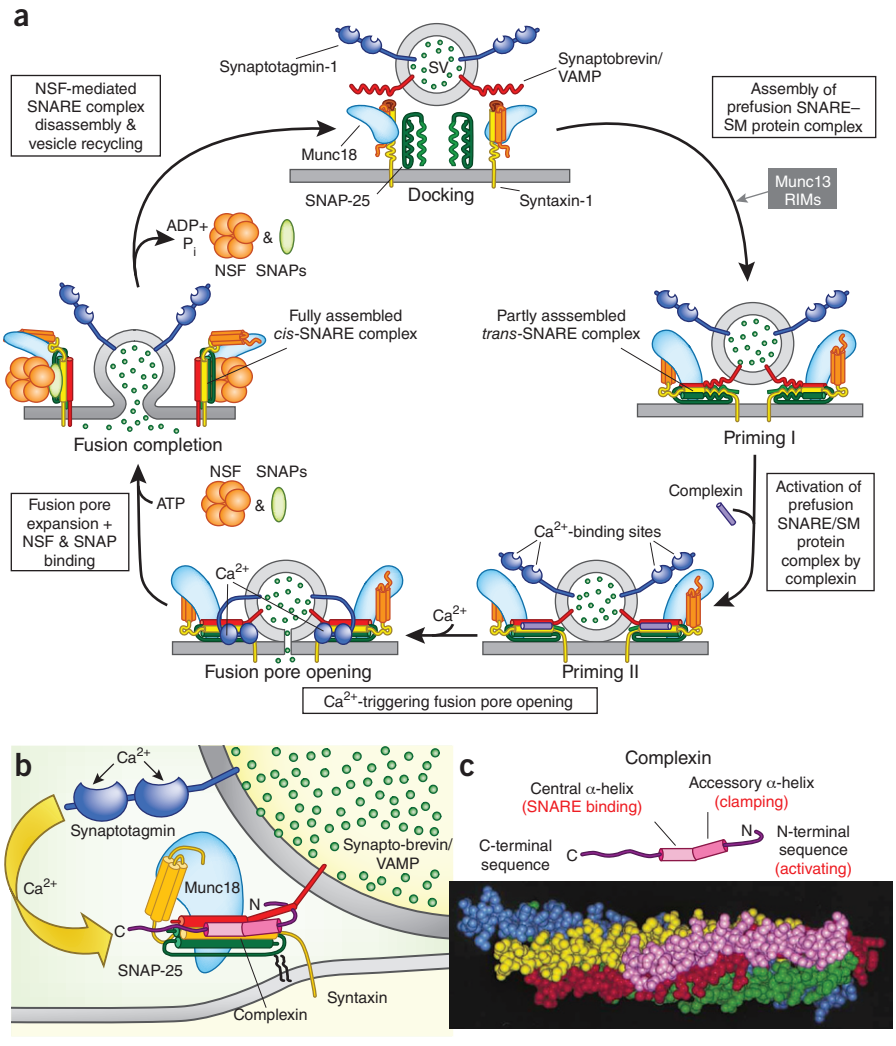

Figure 6 Model of the action of synaptotagmin and complexin in the SNARE–SM protein cycle. (**a**) Overview of the SNARE–SM protein cycle and the points of action of complexin and synaptotagmin. Superimposed on the SNARE–SM protein cycle (**Fig. 2**) is the association of complexin with partially assembled *trans*-SNARE complexes, which enhances vesicle priming for fusion, and the step of calcium triggering of fusion pore opening by synaptotagmin. (**b**) Expanded view of the primed synaptic vesicle fusion complex (an assembly of SNARE proteins, Munc18-1 and complexin) with synaptotagmin poised to trigger fusion pore opening upon calcium binding. (**c**) Modular domain structure of complexin with functional assignments of the complexin sequences, and a space-filling model of the atomic structure of the SNARE complex containing bound complexin (modified from ref. 17).

Calcium channel recruitment, vesicle docking and priming

Up to this point, I have focused on two of Katz's central questions—how membranes fuse and how such fusion is controlled by calcium. But

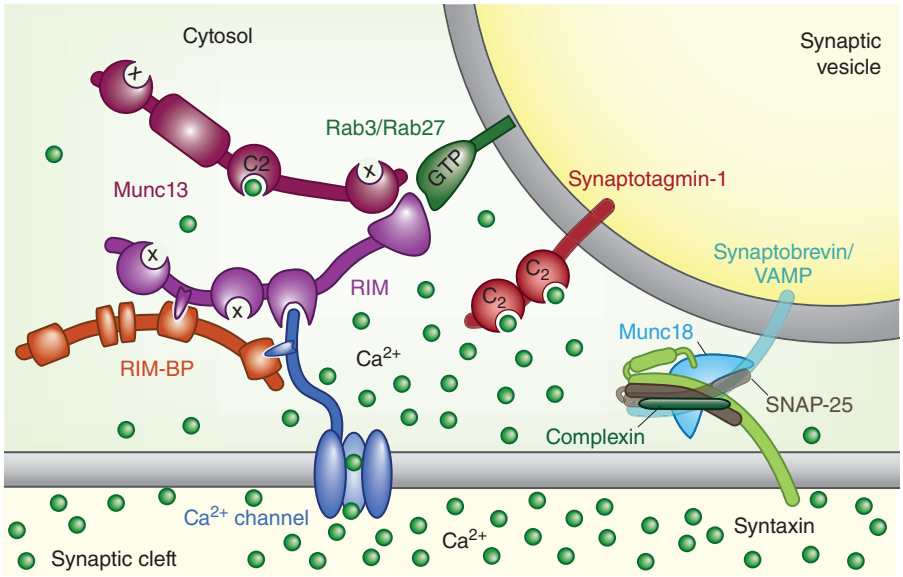

Figure 7 Diagram of the protein complex that mediates the recruitment of calcium channels and the docking of vesicles at release sites. RIM, RIM-BP and Munc13 are multidomain proteins that form a tight complex that mediates three essential functions of active zones: recruitment of Ca^{2+} channels to enable tight coupling of action potentials to release by localizing calcium influx next to the calcium sensor synaptotagmin, docking of vesicles at the release site and Munc13-dependent priming of the fusion machinery. Spheres denote calcium ions; of the domains shown, only calcium-binding C_2 domains are specifically labeled (modified from ref. 20).

I have not addressed the third question that is crucial to understand the speed of synaptic transmission: how is calcium influx localized to the active zone for rapid coupling of an action potential to neurotransmitter release? Only recently we identified a molecular mechanism that addresses this question and ties everything together.

A molecular mechanism that explains how calcium channels and synaptic vesicles are localized to the active zone emerged with the demonstration that two families of active-zone proteins, RIMs and RIM-BPs, collaborate to recruit calcium channels to release sites[19,20]. The same proteins also dock synaptic vesicles at release sites and bind Munc13 proteins, which catalyze the priming of SNARE–SM protein complexes for fusion[19–21]. Thus, in a parsimonious design, a single protein complex localizes synaptic vesicles and calcium channels next to release sites at the active zone and recruits the priming factor Munc13 to the vesicles (**Fig. 7**).

Our initial studies identified RIMs, RIM-BPs and Munc13 proteins as multidomain scaffolding proteins of the presynaptic active zone that form a complex with each other, prime vesicles for fusion and mediate short-term synaptic plasticity[19–21]. Identification of the role of these proteins in recruiting calcium channels to the active zone, however, was only possible when we deleted all RIM isoforms from presynaptic terminals[19]. We found that RIMs selectively bind calcium channels expressed in presynaptic active zones and that deletion of RIMs causes a decrease of presynaptic calcium influx, a loss of presynaptic calcium channels and a loss of the tight coupling of a presynaptic action potential to release.

RIMs perform their functions in a direct complex with the calcium channels, with other active zone proteins such as RIM-BPs (which, in turn, also bind calcium channels) and with Rab3/Rab27 GTPases on synaptic vesicles (**Fig. 3**). At the same time, we and others found that RIMs are essential for normal vesicle docking at the active zone, thus tying docking and calcium channel recruitment together (**Fig. 7**). The role of RIMs and RIM-BPs in recruiting calcium channels and docking vesicles to active zones is evolutionarily conserved and represents a fundamental mechanism underlying synaptic transmission.

Outlook

Our work, together with that of other researchers, uncovered a plausible mechanism explaining how membranes undergo rapid fusion during transmitter release, how such fusion is regulated by calcium and how the calcium-controlled fusion of synaptic vesicles is spatially organized in the presynaptic terminal. Nevertheless, many new questions now arise that are not just details but of great importance. For example, what are the precise physicochemical mechanisms underlying fusion, and what is the role of the fusion mechanism we outlined in brain diseases? Much remains to be done in this field.

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COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

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