The Molecular Machinery of Neurotransmitter Release

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1. THE NEUROTRANSMITTER RELEASE ENIGMA

Synapses have a long history in science. Synapses were first functionally demonstrated by Emil duBois-Reymond (1818–1896), were morphologically identified by classical neuroanatomists such as Rudolf von Kölliker (1817–1905) and Santiago Ramon y Cajal (1852–1934), and named in 1897 by Michael Foster (1836–1907). Although the chemical nature of synaptic transmission was already suggested by duBois-Reymond, it was long disputed because of its incredible speed. Over time, however, overwhelming evidence established that most synapses use chemical messengers called neurotransmitters, most notably with the pioneering contributions by Otto Loewi (1873–1961), Henry Dale (1875–1968), Ulf von Euler (1905–1983), and Julius Axelrod (1912–2004). In parallel, arguably the most important advance to understanding how synapses work was provided by Bernard Katz (1911–2003), who elucidated the principal mechanism of synaptic transmission (Katz, 1969). Most initial studies on synapses were carried out on the neuromuscular junction, and central synapses have only come to the fore in recent decades. Here, major contributions by many scientists, including George Palade, Rodolfo Llinas, Chuck Stevens, Bert Sakmann, Eric Kandel, and Victor Whittaker, to name just a few, not only confirmed the principal results obtained in the neuromuscular junction by Katz, but also revealed that synapses

exhibit an enormous diversity of properties as well as an unexpected capacity for plasticity.

Arguably, the most important property of synaptic transmission is its speed. At most synapses, synaptic transmission lasts for only a few milliseconds. This amazing speed is crucial for the overall workings of the brain—how else could a goalkeeper react to a shot in less than a second, or a ballerina pirouette without crashing to the floor? Synapses differ dramatically from each other in properties such as strength and plasticity, but always operate by the same canonical principle to achieve this speed, as first elucidated by Bernard Katz. When an action potential travels down an axon, it depolarizes the nerve terminals and opens presynaptic Ca^{2+}-channels. The in-flowing Ca^{2+} then triggers neurotransmitter release in less than a millisecond, with a delay of possibly less than 100 microseconds (Sabatini and Regehr, 1996). Amazingly given this speed, presynaptic neurotransmitter release is mediated by membrane traffic. Presynaptic terminals are chock-full with synaptic vesicles—uniformly small organelles with a 35 nm diameter—that contain high concentrations of neurotransmitters. Release is triggered when Ca^{2+} induces the rapid fusion of these vesicles with the presynaptic plasma membrane at a specialized region, the so-called active zone. The active zone is located exactly opposite the postsynaptic density containing the neurotransmitter receptors; as a result, neurotransmitters are released directly onto their receptors (Fig. 1).

The active zone is the organizing principle that ensures the speed and precision of synaptic transmission. The active zone recruits and docks synaptic vesicles at the release sites, transforms synaptic vesicles into a fusion-competent 'primed' state that is responsive to Ca^{2+}-triggering of release, and tethers Ca^{2+}-channels next to the docking sites (Südhof, 2012). By co-localizing Ca^{2+}-channels and primed vesicles at the synaptic cleft, the active zone enables the tight coupling of neurotransmitter release to an action potential and directs neurotransmitter release to the synaptic cleft. After exocytosis, synaptic vesicles recycle by different pathways, including fast endocytic mechanisms that are sometimes referred to as 'kiss-and-run' (Ceccarelli et al., 1973), as well as slower endocytic mechanisms involving clathrin-coated pits (Heuser and Reese, 1973; Fig. 1).

Compared to presynaptic neurotransmitter release, postsynaptic neurotransmitter reception is conceptually more straightforward since it is largely mediated by transmitter binding to ligand-gated ion channels. Postsynaptic ionotropic receptors are highly developed molecular machines that are clustered opposite to the presynaptic active zone, and quickly convert an extracellular neurotransmitter signal into an intracellular ionic signal (Fig. 1). The apparent simplicity of postsynaptic mechanisms, however, is deceptive because

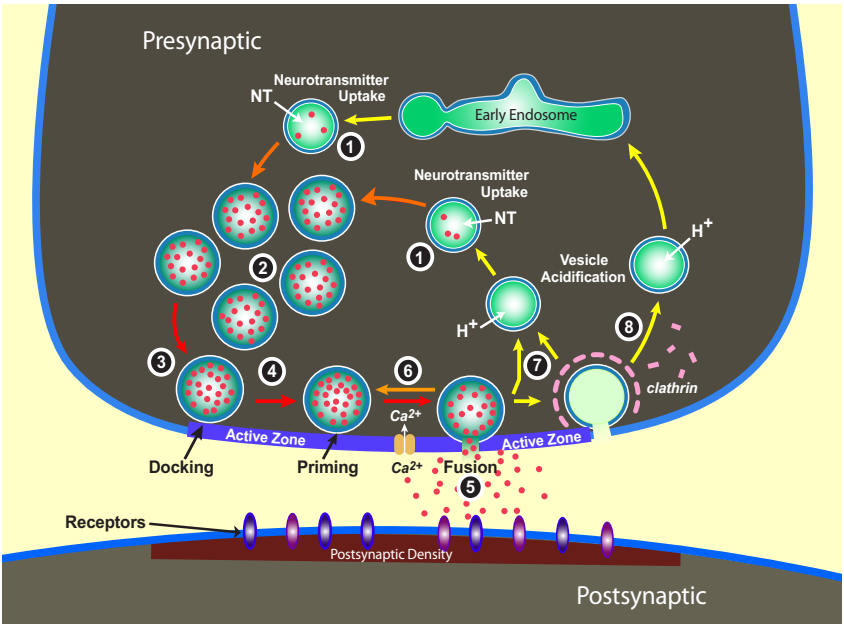


FIGURE 1. *The synaptic vesicle cycle.* Synaptic vesicles undergo a membrane trafficking cycle in presynaptic terminals that mediates neurotransmitter release. Step *1*, vesicles are replenished from endosomes or by recycling after exo- and endocytosis, and are filled with neurotransmitters (NT); Step *2*, vesicles are transported to the active zone of the presynaptic plasma membrane, where they reside in a cluster ready to be recruited for exocytosis; Step *3*, vesicles are tethered to the presynaptic active zone in a 'docking' reaction that depends on the synaptic vesicle proteins Rab3/27 and the active zone protein RIM (see Fig. 14); Step *4*, vesicles are 'primed' to render them competent for Ca^{2+}-triggered fusion; Step *5*, Ca^{2+} triggers fusion-pore opening, releasing the neurotransmitters; Steps *6–8*, vesicles recycle locally immediately after fusion-pore opening (6, 'kiss-and-stay'), by endocytosis via a rapid pathway that is likely clathrin-independent (7, 'kiss-and-run'), or by a clathrin-dependent pathway that involves an endosomal intermediate (8). Note that most of the recycling pathways were worked out in classical studies by Heuser and Reese (1973), Ceccarelli et al. (1973), and Zimmermann and Whittaker (1977). Drawing was adapted from Südhof and Jahn (1991) and Südhof (2004).

postsynaptic neurotransmitter receptors are subject to complex regulatory processes, including vesicular trafficking, that are incompletely understood. Moreover, postsynaptic signal-transduction pathways are organized in a sophisticated and compartmentalized manner that differs between various types of synapses. Considering the simple yet complex canonical design of a chemical synapse, one cannot but marvel at the ingenuity of this design that enables the requisite speed and plasticity of synaptic transmission using specialized pre- and postsynaptic machineries.

When I started my laboratory in 1986, neurotransmitter release had been described in exquisite physiological detail. However, there was no mechanistic understanding, not even a hypothesis, of how synaptic vesicles might fuse, how Ca^{2+} could possibly trigger such fusion so rapidly, and how the release machinery is organized by the presynaptic active zone. No molecular component of the release machinery had been characterized, and no conceptual framework was available to explain the extraordinary plasticity and precision of Ca^{2+}-triggered release. I focused on these questions, as opposed to studying postsynaptic neurotransmitter reception, because I was mesmerized by the apparent incomprehensibility of the speed of Ca^{2+}-triggered release, and intrigued by the general implications of understanding release for other membrane-trafficking reactions, such as hormone secretion.

In the following, I will provide a brief personal overview of what we found. I will present our work in the context of that of others which was indispensable for our progress, but given space constraints I will not be able to do justice to the many important contributions made by others. We performed our studies as part of a larger scientific community working on this problem, and I will try to provide as balanced an account of the field as I can within my space allowance.

2. MOLECULAR ANATOMY OF THE PRESYNAPTIC TERMINAL

When we started, we chose a simple approach to the understanding of neurotransmitter release: to isolate and clone all major proteins of presynaptic terminals. Largely in collaboration with Reinhard Jahn, we first focused on synaptic vesicles because they could be obtained at high yield and purity (Whittaker and Sheridan, 1965; Südhof and Jahn, 1991). Later on, we expanded this approach to the presynaptic active zone. With these initial experiments, we aimed to assemble a molecular catalogue of presynaptic proteins as a starting point for a functional dissection of release.

The first synaptic vesicle proteins we purified and cloned were synaptophysin (Südhof et al., 1987), cytochrome b561 (Perin et al., 1988), synapsins (Südhof et al., 1989a), synaptobrevins (Südhof et al., 1989b; also independently cloned by R.H. Scheller and named vesicle-associated membrane protein [VAMP]; Trimble et al., 1988), proton pump components (Südhof et al., 1989c; Perin et al., 1991), and synaptotagmins (Perin et al., 1990; Geppert et al., 1991; Li et al., 1995). In addition, we found that Rab3 proteins, the brain's most abundant GTP-binding proteins originally identified as ras-homologous sequences (Touchot et al., 1987), are associated with synaptic vesicles (von Mollard et al.,

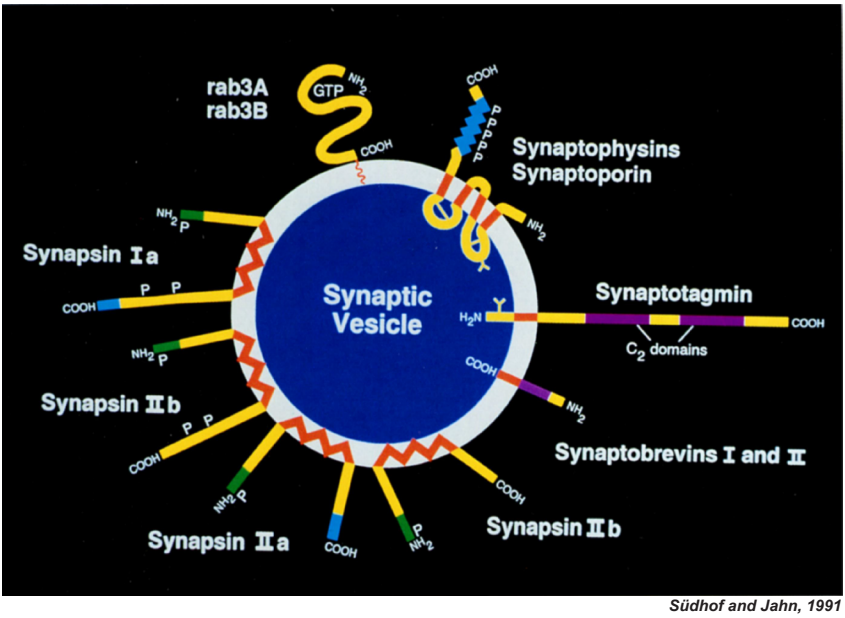


FIGURE 2. *Diagram of synaptic vesicle proteins involved in neurotransmitter release as seen in 1991.* At the beginning of the effort to map the molecular anatomy of synaptic vesicles, five major classes of synaptic vesicle proteins were identified: the synapsins that were at that time thought to be major candidates for regulating neurotransmitter release (Südhof et al., 1989a), Rab3 proteins (von Mollard et al., 1990), synaptophysins (Südhof et al., 1987), synaptotagmins (Perin et al., 1990), and synaptobrevins/VAMPs (Südhof et al., 1989b). Of these proteins, three classes (Rab3, synaptotagmins, and synaptobrevins) turned out to be crucial for release in subsequent studies (reproduced from Südhof and Jahn, 1991).

1990), and that Rab3 proteins cycle on and off synaptic vesicles during exocytosis (von Mollard et al., 1991).

Thus, in the beginning of the 1990s a fairly comprehensive characterization of the synaptic vesicle as an organelle had emerged (Südhof and Jahn, 1991; Fig. 2). Subsequently, we and others cloned a series of additional vesicle proteins, including SVOP (Janz et al., 1998) and SCAMPs (Fernandez-Chacon et al., 2000). Furthermore, we expanded our attempts to molecularly characterize the release machinery to the active zone, and identified Munc18s (Hata et al., 1993), complexins (McMahon et al., 1995), Munc13s (Brose et al., 1995), CASK (Hata et al., 1996), RIMs (Wang et al., 1997), RIM-BPs (Wang et al., 2000), and ELKS (Wang et al., 2002; independently described by Ohtsuka et al., 2002). These studies were complemented by those of others identifying as active-zone proteins α-liprins

(Zhen and Jin, 1999), bassoon (tom Diek et al., 1998) and piccolo (Wang et al., 1999; Fenster et al., 2000).

After having elucidated the primary structures of a growing number of synaptic proteins, we faced the challenge of determining their functions. We decided to examine these molecules broadly in an un-biased manner as systematically as possible, and used a combination of methods ranging from biochemistry and cell biology to structural biology, mouse genetics, and electrophysiology.

As I will describe in the following account, these studies enabled a new understanding of neurotransmitter release. However, not all efforts were productive, and not all abundant and conserved synaptic proteins were found to be important. For example, prominent proteins such as synapsins and synaptophysins turned out to have only ancillary roles in the synaptic vesicle cycle that may be important for the overall organism, but are not essential for the basic process of synaptic vesicle exo- and endocytosis (e.g., Rosahl et al., 1993 and 1995; Janz et al., 1999).

In the following description, I will divide neurotransmitter release into three processes, membrane fusion as the basic mechanism that mediates release by synaptic vesicle exocytosis, Ca^{2+}-triggering as the key event that enables fast synaptic transmission, and the spatial organization of the release machinery by the active zone that allows precise coupling of a presynaptic action potential to a postsynaptic response.

3. MECHANISM OF SYNAPTIC MEMBRANE FUSION

SNARE Proteins in Fusion

The first insights into how synaptic vesicles fuse with the presynaptic plasma membrane during neurotransmitter release came from studies of tetanus and botulinum toxins. These neurotoxins, which as disease agents cause tetanus and botulism but also have great therapeutic value, are among the most powerful neurotoxins known (Grumelli et al., 2005). Tetanus and botulinum toxins are metalloproteases that block neurotransmitter release at nanomolar concentrations by arresting the fusion of synaptic vesicles with the presynaptic plasma membrane.

In 1992, studies in Cesare Montecucco's, Heiner Niemann's, and Reinhard Jahn's laboratories—to which we contributed—showed that tetanus toxin and botulinum B toxin block synaptic vesicle fusion by proteolytic cleavage of Synaptobrevin-2/VAMPs (Link et al., 1992; Schiavo et al., 1992). In the following year, the same laboratories showed that other types of botulinum toxins cleave

two other presynaptic membrane proteins, SNAP-25 and Syntaxin-1 (Blasi et al., 1993a and 1993b; Schiavo et al., 1993). Moreover, we demonstrated that a ubiquitously distributed synaptobrevin isoform (Cellubrevin) is also a tetanus toxin substrate, suggesting that the inhibition of vesicle fusion by tetanus toxin-dependent cleavage of Synaptobrevin-2 reflects a general function of synaptobrevin-like molecules in membrane fusion (McMahon et al., 1993). Together, these findings provided the first, and arguably still most compelling evidence that Synaptobrevin-2, SNAP-25, and Syntaxin-1 are essential components of the presynaptic membrane fusion machinery. As we will see now, evidence about how these proteins, later named SNARE proteins (for 'soluble NSF-attachment protein receptors'), might work came from parallel studies in James Rothman's laboratory.

Rothman had been studying membrane fusion by biochemically reconstituting vesicular traffic between compartments of the Golgi apparatus (Balch et al., 1984). Using this assay, Rothman isolated an N-ethyl maleimide-sensitive factor (referred to as NSF) and NSF-adaptor proteins that attach NSF to membranes (referred to as SNAPs, an unfortunate coincidence of acronyms with SNAP-25). Both NSF and SNAPs were essential for *in vitro* fusion in Rothman's assay, and were found to be homologous to yeast genes involved in secretion, suggesting a fundamental function in membrane traffic (Wilson et al., 1989; Clary et al., 1990). In a crucial study, Rothman's laboratory then used immobilized NSF and SNAPs as an affinity matrix to purify SNAP 'receptors' (i.e., SNAREs) from brain because brain was the richest source of such receptors. He isolated Synaptobrevin-2, Syntaxin-1 and SNAP-25, just as these proteins were revealed to be tetanus and botulinum toxin substrates (Söllner et al., 1993a). Subsequently, Rothman went on to show in collaboration with Richard Scheller that Synaptobrevin-2, Syntaxin-1, and SNAP-25 formed a complex with each other, and that this complex is dissociated by NSF which acts as an ATPase (Söllner et al., 1993b). This brilliant experiment provided an explanation for how these proteins might work in fusion, although it took many more years to formulate a compelling mechanism for their fusion function. Collaborating with Heiner Niemann, we found that SNARE complexes are SDS-resistant and extremely tight, and that only the SNARE complex but not individual SNARE proteins binds to SNAPs and NSF, while only free SNARE proteins but not SNARE proteins in the complex are substrates for botulinum and tetanus toxins (Hayashi et al., 1994; McMahon et al., 1995a).

Viewed together, these studies suggested to us that formation of SNARE complexes between the synaptic vesicle and presynaptic plasma membranes may mediate fusion, but the mechanism of fusion was unclear. One hypothesis

was that NSF and SNAPs are the actual fusion proteins, and that SNARE proteins ensure the specificity of the fusion reaction mediated by NSF and SNAPs by acting as their receptors after SNARE complexes have assembled (Söllner et al., 1993a and 1993b). An alternative idea that we favored was motivated by the botulinum and tetanus toxin data, and stated that SNARE proteins, especially synaptobrevin, are actually directly involved in fusion, although we did not know by what mechanism (Südhof et al., 1993).

Two subsequent key experiments clarified the question whether NSF/SNAPs or SNAREs are the actual membrane-fusion proteins. First, Bill Wickner's laboratory elegantly showed in yeast vacuole fusion assays that yeast NSF does not function in fusion, but is only required to activate SNARE proteins for fusion and to recycle the SNARE machinery after fusion (Mayer et al., 1996). Second, in a seminal experiment Reinhard Jahn and John Heuser demonstrated that SNARE complexes assemble in a parallel manner, such that SNARE-complex assembly forces the C-terminal transmembrane regions of SNARE proteins into close proximity (Hanson et al., 1997). This key observation by Heuser and Jahn provided an immediate model for how SNARE proteins may mediate fusion, namely by zippering up in an N- to C-terminal direction, thereby forcing membranes that contain their C-terminal transmembrane regions into close proximity. This model was quickly confirmed using biophysical studies and crystallography (Lin and Scheller, 1997; Poirier et al., 1998, Sutton et al., 1998), and further elaborated by Rothman and others using *in vitro* reconstitution experiments with liposomes (Weber et al., 1998). It is now the standard model of the field.

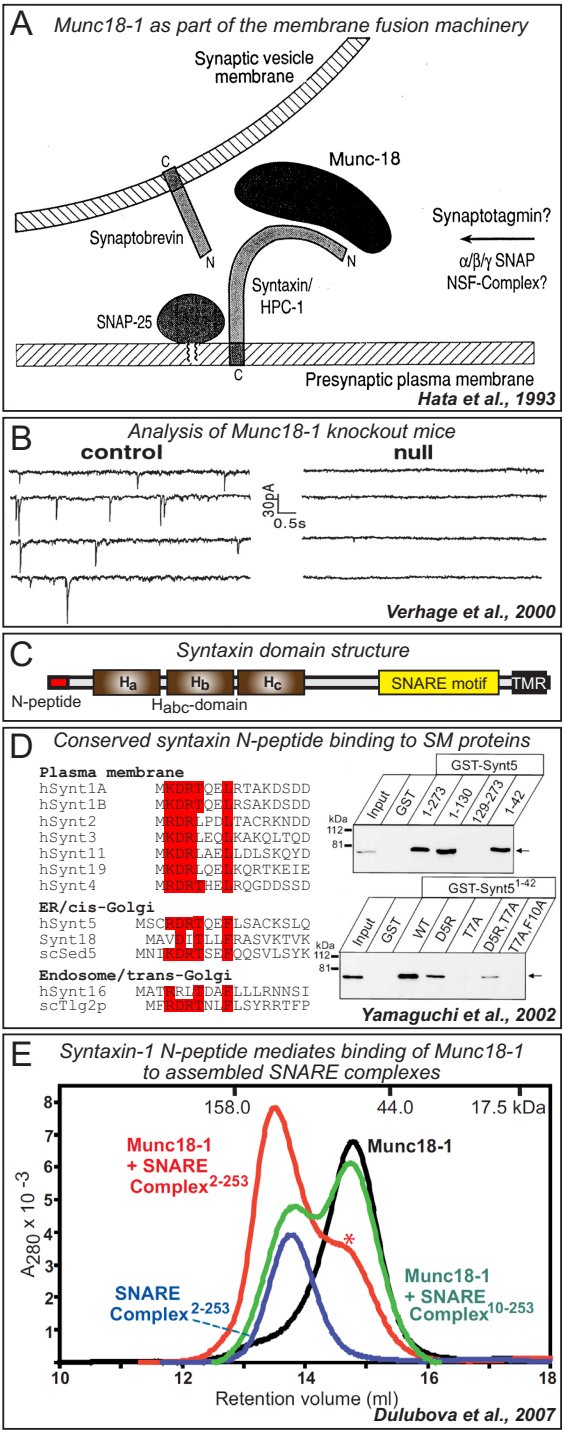
SM proteins are obligatory SNARE partners in membrane fusion

In 1993, just at the time at which SNARE proteins were being discovered as membrane fusion proteins, we searched for other components of the fusion machinery using affinity chromatography on immobilized Syntaxin-1 (Hata et al., 1993). We isolated a 65 kDa protein that we named Munc18-1 because of its sequence homology to the C. elegans *unc18* gene (Fig. 3A). Sidney Brenner had isolated *unc18*-mutants because the mutant worms did not move properly (were '*unc*oordinated'), but the function of the *unc18* gene was unknown (Brenner, 1974). However, because Munc18 bound to the SNARE membrane-fusion machinery and because C. elegans *unc18* was essential for movement, we hypothesized that Munc18-1 was an intrinsic component of the fusion machinery, and co-operates with SNARE proteins in fusion (Fig. 3A).

Further analyses revealed that Munc18-1 was also homologous to *sec1*, which was the first gene isolated by Peter Novick and Randy Schekman in screens for secretory yeast mutants, but whose function, like that of the previously described *unc18*, was unknown (Novick and Schekman, 1979). In fact, this homology led some investigators to refer to Munc18-1 as n-sec1 or rb-sec1 (Garcia et al., 1994, Pevsner et al., 1994). Multiple additional homologs of Sec1p and Munc18 were subsequently described, and the whole gene family is now referred to as *S*ec1/*M*unc18-like proteins (*SM* proteins; Rizo and Südhof, 2012).

After the discovery of Munc18-1, considerable confusion reigned about its function, fueled by paradoxical observations. On the one hand, in yeast *sec1* mutations blocked fusion (Novick and Schekman, 1979), in Drosophila deletion of the Munc18-1 gene (*rop*) abolished synaptic transmission (Harrison et al., 1994), and in mice knockout of Munc18-1 ablated neurotransmitter release (Verhage et al., 2000; Fig. 3B). These results suggested an essential role for Munc18-1 in fusion itself, a hypothesis that was further supported by Novick's elegant studies demonstrating that yeast Sec1p binds to assembled SNARE complexes (Carr et al., 1999), and acts downstream of SNARE-complex assembly (Grote et al., 2000). On the other hand, we found that outside of the SNARE complex, Syntaxin-1 assumes a 'closed' conformation in which its N-terminal Habc-domain folds back on its SNARE-motif, and that Munc18-1 specifically binds to this closed conformation of Syntaxin-1 (Dulubova et al., 1999). Habc-domains are a conserved feature of syntaxins, and account for half of their sequences, while the SNARE motifs of synaxins form SNARE complexes by assembling with similar SNARE motifs in synaptobrevins and SNAP-25 or their homologs into a four-helical bundle (Fig. 3C; Fernandez et al., 1998; Sutton et al., 1998). As a result, the intramolecular interaction in the closed Syntaxin-1 conformation of the N-terminal Habc-domain with the SNARE motif prevents Syntaxin-1 from assembling into SNARE complexes, suggesting that Munc18-1 may be a negative regulator of SNARE-complex assembly. Thus, paradoxically at this junction Munc18-1 seemed to be at the same time essential for fusion itself and preventing fusion by blocking SNARE-complex assembly.

We found a resolution to this apparent contradiction when we observed in collaboration with Josep Rizo that both in vertebrates and in yeast, the SM protein involved in vesicular transport from the endoplasmic reticulum to the Golgi apparatus (Sly1) binds to its cognate syntaxins (Syntaxin-5 and -18 in vertebrates, and Sed5p and Ufe1p in yeast) via a short, conserved N-terminal peptide (the 'N-peptide'; Yamaguchi et al., 2002; Figs. 3C and 3D). We also found that the same mechanism applies to another SM protein—Vps45—that


A
Munc18-1 as part of the membrane fusion machinery
Synaptic vesicle membrane
C
Munc-18
Synaptotagmin?
Synaptobrevin
N
N
α/β/γ SNAP
NSF-Complex?
Syntaxin/
HPC-1
SNAP-25
C
Presynaptic plasma membrane
Hata et al., 1993
B
Analysis of Munc18-1 knockout mice
control
null
30pA
0.5s
Verhage et al., 2000
C
Syntaxin domain structure
Ha
Hb
Hc
SNAP motif
TMR
N-peptide
Habc-domain
D
Conserved syntaxin N-peptide binding to SM proteins
Plasma membrane
hSynt1A MKDRTQELRTAKDSDD
hSynt1B MKDRTQELRSAKDSDD
hSynt2 MRDRLPDLTACRKNDD
hSynt3 MKDRLEQLKAKQLTQD
hSynt11 MKDRLAELLDLSKQYD
hSynt19 MKDRLQELKQRTKEIE
hSynt4 MRDRTHDLRQGDDSSD
ER/cis-Golgi
hSynt5 MSGRDSTQELLSACKSLQ
Synt18 MAVRITLLERASVKTVK
scSed5 MNIRDRISSLQQSVLSYK
Endosome/trans-Golgi
hSynt16 MATRRLADALLLRNNSI
scTlg2p MFRDRTNLLLSYRRTFP
GST-Synt5
Input
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1-273
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129-273
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kDa
112
81
GST-Synt5 1-42
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GST
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D5R
T7A
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T7A,F10A
kDa
112
81
Yamaguchi et al., 2002
E
Syntaxin-1 N-peptide mediates binding of Munc18-1 to assembled SNARE complexes
158.0
44.0
17.5 kDa
Munc18-1
+ SNARE
Complex 2-253
Munc18-1
SNARE
Complex 2-253
Munc18-1
+ SNARE
Complex 10-253
A280 x 10 -3
Retention volume (ml)
Dulubova et al., 2007

is involved in endosome and trans-Golgi fusion, and that binds to its cognate Syntaxin-16 (Tlg2p in yeast) again via a very similar N-peptide sequence (Dulubova et al., 2002). Owing to this binding mechanism, these SM proteins could remain associated with their cognate syntaxins throughout SNARE-complex assembly, consistent with Novick's studies on Sec1p (Grote et al., 2000; note, however, that the details of Sec1p binding in yeast to the SNARE complex may differ). We observed that the vertebrate plasma membrane syntaxins contain an extremely similar conserved N-terminal sequence (Fig. 3D), prompting us to search for a similar binding mode of Munc18-1 to Syntaxin-1.

FIGURE 3. *(opposite) Definition of the interactions of Sec1/Munc18-like ('SM') proteins with syntaxins and the SNARE complex during synaptic vesicle fusion.*

A. Diagram of the Munc18-1/SNARE interactions proposed in the description of Munc18-1 (originally referred to as 'Munc-18') as a Syntaxin-1 binding protein that contributes to the fusion machinery (reproduced from Hata et al., 1993).

B. Demonstration that Munc18-1 is essential for vesicle fusion and does not primarily function as a negative regulator of fusion. Images show synaptic activity recorded from the cortex of newborn littermate wild-type (control) and Munc18-1 knockout mice (null), demonstrating complete electrical silence in the absence of Munc18-1 (reproduced from Verhage et al., 2000).

C. Domain structure of syntaxins composed of a conserved N-terminal sequence (N-peptide), an autonomously folded Habc-domain comprising three α-helices (Fernandez et al., 1998), the SNARE motif that associates into a SNARE complex with the homologous sequences present in synaptobrevins and SNAP-25 or their homologs, and a C-terminal transmembrane region (TMR). Outside of the SNARE complex, syntaxins spontaneously form a 'closed' conformation in which the N-terminal Habc-domain folds back onto the SNARE motif, thereby occluding this motif and hindering SNARE-complex assembly (Dulubova et al., 1999).

D. Discovery of a conserved N-terminal sequence motif of syntaxins that mediates binding of most SM proteins to their cognate syntaxins. An alignment of the N-terminal syntaxin sequences is shown on the left (red, conserved residues involved in SM-protein binding), and immunoblots of the initial binding experiments demonstrating that the N-terminus of the ER/Golgi syntaxin-5 binds to the SM protein Sly1 in a manner dependent on the conserved N-terminal Syntaxin-5 sequence motif are shown on the right (reproduced from Yamaguchi et al., 2002).

E. Demonstration by gel-filtration of a stable complex containing Munc18-1 bound to fully assembled SNARE complexes. Munc18-1 or synaptic SNARE complexes containing the full N-terminal sequence of Syntaxin-1 were analyzed alone (black and blue traces, respectively), or Munc18-1 was analyzed together with SNARE complexes containing either the full N-terminal Syntaxin-1 sequence (red trace) or N-terminally truncated Syntaxin-1 lacking 8 residues (green trace). Note that in the presence of SNARE complexes containing full-length Syntaxin-1, most Munc18-1 co-elutes with SNARE complexes, whereas in the presence of SNARE complexes containing N-terminally truncated Syntaxin-1, no Munc18-1 co-elutes with the SNARE complexes (reproduced from Dulubova et al., 2007).

Indeed, we found that Munc18-1 bound tightly to assembled SNARE complexes in a manner that depended on the Syntaxin-1 N-peptide (Fig. 3E; Dulubova et al., 2007). The Munc18-1/SNARE-complex assembly was stable during size-exclusion chromatography, but disrupted by deletion of the N-peptide from Syntaxin-1 (Fig. 3E; note that James Rothman's laboratory simultaneously made similar observations [Shen et al., 2007]). Fusing as little as a Myc-epitope to the N-peptide of Syntaxin-1 impaired this binding mode, whereas binding of Munc18-1 to the monomeric closed conformation of Syntaxin-1 did not require the Syntaxin-1 N-peptide.

Viewed together, these results showed that Munc18-1 binds to Syntaxin-1 in two sequential modes that involve different Syntaxin-1 conformations (Fig. 4): an exocytosis-specific binding mode in which Munc18-1 binds to 'closed' Syntaxin-1 independent of the N-peptide (Dulubova et al., 1999), and a general

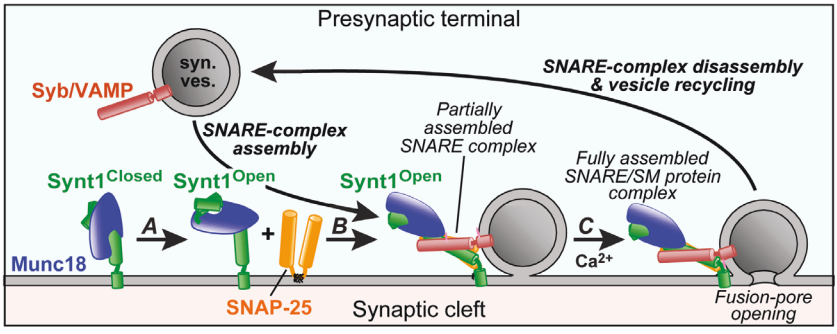


FIGURE 4. *Conformational changes of SNARE and SM proteins mediating synaptic vesicle fusion.* Prior to fusion, Syntaxin-1 assumes a default 'closed' conformation that binds Munc18-1 via an interaction which does not require the Syntaxin-1 N-peptide (Dulubova et al., 1999). In Reaction *A*, Syntaxin-1 is 'opened' (probably by Munc13-mediated catalysis; Ma et al., 2011) to initiate synaptic vesicle priming. In Reaction *B*, SNARE complexes partially assemble via N- to C-terminal zippering (Hanson et al., 1997), while Munc18-1 stays associated with Syntaxin-1 during SNARE-complex assembly via its binding to the Syntaxin-1 N-peptide (Khvotchev et al., 2007). In Reaction *C*, Ca^{2+}-triggers fusion-pore opening by stimulating the completion of SNARE-complex assembly; Munc18-1 contributes to this process and is required for fusion-pore opening during this step because the continuing association of Munc18-1 with SNARE-complexes is essential for fusion-pore opening (Zhou et al., 2013a). After fusion, vesicles are endocytosed (see Fig. 1), and SNARE complexes are disassembled by the NSF ATPase and its SNAP protein adaptor (no relation to the SNARE protein SNAP-25). Munc18-1 remains associated with Syntaxin-1, and reverts to the heterodimeric interaction with 'closed' Syntaxin-1. Thus, there are two major conformational transitions during exocytotic membrane fusion: opening of Syntaxin-1 with rearrangement of the mode of Munc18-1 binding, and folding of SNARE proteins into SNARE complexes.

binding mode shared with some other SM protein/SNARE complex interactions in which Munc18-1 binds to 'open' Syntaxin-1 assembled into SNARE complexes via the Syntaxin-1 N-peptide, and additionally interacts with other parts of the SNARE complex (Dulubova et al., 2007).

What are the functions of the two Munc18-1 binding modes to SNARE proteins, and which of the two modes is more important for fusion? Initial peptide competition experiments in the calyx-of-Held synapse showed that displacing the Syntaxin-1 N-terminus from Munc18-1 impairs synaptic vesicle fusion (Khvotchev et al., 2007). Further studies described that mutations in Munc18-1 which decrease Munc18-1 binding to the Syntaxin-1 N-terminal sequences also decrease fusion (Deak et al., 2009). It should be noted that in a later study in which this result was disputed using similar but weaker mutations (Meijer et al., 2012), the Munc18-1 mutations caused only a partial decrease in binding to the Syntaxin-1 N-peptide. In these studies, all physiology was performed with a high degree of overexpression, which could have easily compensated for the decrease in binding affinity. Furthermore, elegant experiments in C. elegans revealed that the Syntaxin-1 N-peptide was essential for fusion, but that it did not actually need to be on Syntaxin-1 in order to function, as long as it was positioned close to SNARE complexes (Rathore et al., 2010). Finally, we showed that in mammalian synapses, the Syntaxin-1 N-peptide was also required for fusion under physiological conditions (Zhou et al., 2013b).

These experiments show that binding of Munc18-1 to 'open' Syntaxin-1 within the SNARE complex is essential for fusion, and validate the function of Munc18-1—analogous to that of Sec1p—as an intrinsic component of the fusion machine. What then is the role of Munc18-1 binding to 'closed' Syntaxin-1? To test this role, we created knock-in mice in which Syntaxin-1 was rendered constitutively 'open' (Syntaxin-1^{Open}), and thus binding of Munc18-1 to 'closed' Syntaxin-1 was suppressed (Gerber et al., 2008). In these mice, both Munc18-1 and Syntaxin-1 were destabilized and decreased in levels, consistent with other evidence suggesting that the complex of Munc18-1 with the closed conformation of Syntaxin-1 stabilizes both proteins (Verhage et al., 2000). The decreased levels of Syntaxin-1 and Munc18-1 in Syntaxin-1^{Open} synapses resulted in decreased vesicle priming, presumably because fewer slots for vesicle fusion were available (Gerber et al., 2008; Acuna et al., 2014).

Nevertheless, the probability of Ca^{2+}-triggered neurotransmitter release was dramatically enhanced in Syntaxin-1^{Open} synapses, and fusion was accelerated. Even the fusion of individual vesicles, as judged by the kinetics of single miniature release ('mini') events, was faster in Syntaxin-1^{Open} than in wild-type synapses (Acuna et al., 2014). These data, together with the finding that the

Habc-domain of Syntaxin-1, different from its N-peptide, is not essential for fusion (Zhou et al., 2013a) demonstrate that Munc18-1 binding to the closed conformation of Syntaxin-1 is not required for fusion, whereas binding to 'open' Syntaxin-1 in the SNARE complex is essential for fusion. Binding of Munc18-1 to closed Syntaxin-1 appears to serve two other functions that are not directly part of fusion itself: to stabilize both proteins in the complex, and to 'gate' SNARE-complex assembly mediating fusion, i.e., to regulate the rate of fusion.

How do SNARE and SM proteins mediate fusion?

In principle, SNARE proteins act in fusion via a simple mechanism: 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, forcing the fusing membranes into close proximity and destabilizing their surfaces. This opens a fusion pore, whose expansion then converts the initial 'trans'-SNARE complexes into 'cis'-SNARE complexes which are subsequently dissociated by the NSF and SNAP adapter proteins, thereby allowing a recycling of the vesicles and the SNARE proteins for another round of fusion (Fig. 4).

However, at least two major questions arise at this point. First, do SNARE proteins primarily act as force-generators to pull membranes together (which may be sufficient for inducing *in vitro* fusion), or do SNARE proteins actually open the fusion pore? Second, what is the precise function of SM proteins in fusion—why are they required?

In vitro, the transmembrane regions of synaptobrevin and Syntaxin-1 interact with each other in the plane of the membrane. The SNARE motifs of these proteins form a continuous, rigid a-helix with their transmembrane regions, suggesting that the SNARE protein transmembrane regions may actively contribute to the fusion pore (Stein et al., 2009). However, in recent experiments we found that Synaptobrevin-2 and Syntaxin-1 still mediate fusion when both are attached to their resident membranes via lipid anchors, not transmembrane regions, demonstrating that SNARE transmembrane regions are not essential components of the fusion machine (Zhou et al., 2013b). These results support the notion that SNARE proteins act as force generators, and that their transmembrane regions do not act as fusion catalysts.

What then do SM proteins do in fusion? The fact that SM proteins are required continuously during SNARE-complex assembly argues for a role either in organizing proper SNARE-complex assembly and in preventing dead-end

inappropriate SNARE complexes, or in catalyzing lipid mixing during fusion. At present, no conclusive data argue one way or the other, and this question will clearly keep many of us busy for years to come.

SNARE chaperones are essential for maintaining the integrity of the presynaptic terminal

Neurons fire action potentials often in bursts or trains, with high frequencies, sometimes exceeding 100 Hz. Each neurotransmitter release event involves the folding and unfolding of reactive SNARE proteins, exposing the presynaptic cytosol to potentially deleterious misfolding of SNARE proteins and formation of inappropriate complexes by reactive SNARE motifs. It is thus not surprising that neurons express specialized chaperones which help proper folding of SNARE proteins, and that deletion of these chaperones leads to neurodegeneration.

We identified two classes of such chaperones, CSPα (for cysteine-string protein-α, so named because it contains an eponymous string of cysteine residues that are palmitoylated to attach CSPα to the synaptic vesicle membrane; Gundersen et al., 1994), and synucleins (so named because it was initially thought that these presynaptic proteins may also be in the nucleus; Maroteaux et al., 1988).

Our discovery that these proteins function as SNARE chaperones was pure serendipity. We found that deletion of CSPα in mice leads to massive neurodegeneration that kills affected mice in 3-4 months and is caused by an impairment in SNARE-complex formation (Fernandez-Chacon et al., 2004). Surprisingly, this neurodegeneration was suppressed by modest overexpression of α-synuclein (Chandra et al., 2005). Following up on these observations, we showed that CSPα—which contains a DNA-J domain and forms a catalytically active, ATP-dependent chaperone complex with Hsc70 and the tetratricopeptide-repeat protein SGT (Tobaben et al., 2000)—catalyzes the proper folding of SNAP-25, rendering SNAP-25 competent for SNARE-complex assembly (Sharma et al., 2011a, 2011b, and 2012). In CSPα KO mice, misfolding of SNAP-25 impaired SNARE-complex assembly which then caused neurodegeneration. α-Synuclein rescued this neurodegeneration by independently promoting SNARE-complex assembly via a non-classical, ATP-independent chaperone activity (Burre et al., 2010).

Although these observations uncovered a potentially interesting facet of SNARE protein biology, we do not yet understand how the physiological activities of α-synuclein relate to its neurotoxic role in Parkinson's disease. One

attractive hypothesis is that α-synuclein aggregation in Parkinson's disease may deplete neurons of all available functional α-synuclein, and thus cause SNARE protein misfolding that is then deleterious, but alternative hypotheses, such as a direct neurotoxic non-physiological activity of α-synuclein oligomers, are equally plausible.

4. Ca^{2+}-TRIGGERING OF FUSION: SYNAPTOTAGMINS AND MORE

At the same time as our work on synaptic membrane fusion was progressing, we were studying a related question: how is neurotransmitter release by synaptic membrane fusion triggered by Ca^{2+}? Ever since I was a graduate student in Victor Whittaker's laboratory in Göttingen, I had been fascinated by this question. The central importance of Ca^{2+}-triggered neurotransmitter release for brain function intrigued me, its improbable speed and plasticity puzzled me, and the similarity of Ca^{2+}-induced synaptic vesicle exocytosis to other types of Ca^{2+}-induced exocytosis, such as those underlying hormone secretion, mast cell degranulation, or fertilization, suggested to me that understanding Ca^{2+}-triggered neurotransmitter release may be generally relevant for cellular signaling processes. Although some key discoveries about synaptotagmins were made at the same time as those about SNARE and SM proteins, the work on synaptotagmins extended over a longer time period to satisfy even the most stringent critics, and some of the most important observations are quite recent.

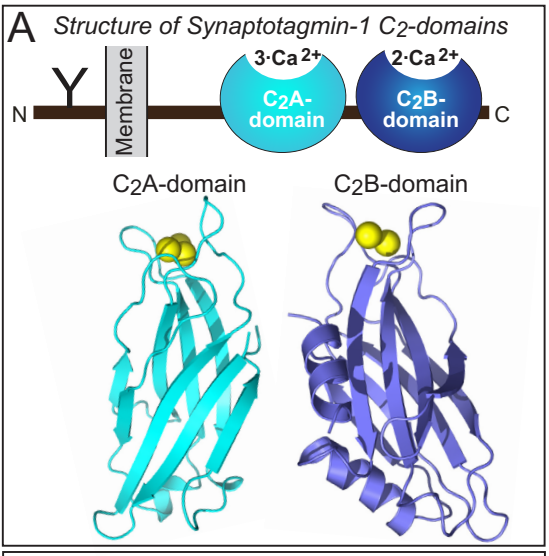
Discovery of Synaptotagmin-1: identification of C2-domains as versatile Ca^{2+}-binding domains

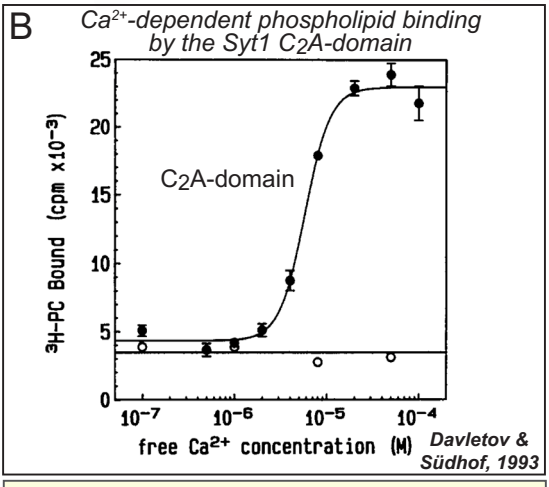
During our studies of the molecular anatomy of synaptic vesicles, we searched for a candidate Ca^{2+}-sensor that might mediate Ca^{2+}-triggering of synaptic vesicle exocytosis. When we purified and cloned Synaptotagmin-1 (Syt1)—which had been described earlier as a synaptic vesicle protein using a monoclonal antibody raised against synaptosomes (Matthew et al., 1981)—we were intrigued by its primary structure because Syt1 included two C_2-domains that were anchored on the vesicle membrane by a transmembrane region (Perin et al., 1990; Figs. 2 and 5A). At that time, nothing was known about C_2-domains except that they represented the "2nd constant sequence" in classical protein-kinase C (PKC) isozymes (Coussens et al., 1986). Since classical PKC isozymes are Ca^{2+}-regulated and interact with phospholipids, we speculated that the synaptotagmin C2-domains may represent Ca^{2+}-binding modules that interact with phospholipids, and that Syt1 may be a Ca^{2+}-sensor for neurotransmitter release (Perin et

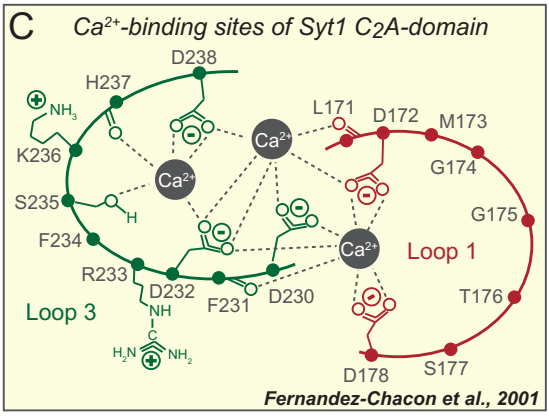
al., 1990). In pursuing this hypothesis over two decades, we showed that Ca^{2+}-triggering of neurotransmitter release is mediated by Ca^{2+}-binding to Syt1 and other synaptotagmins, and that different synaptotagmin isoforms additionally perform similar Ca^{2+}-sensor functions in other types of Ca^{2+}-dependent exocytosis in neuronal and non-neuronal cells.

The first challenge after describing Syt1 was to test whether the Syt1 C2-domains were indeed a novel type of Ca^{2+}/phospholipid-binding domain. We found that the Syt1 C2-domains bound to phospholipids (Perin et al., 1990), that such binding was mediated by purified brain Syt1 in a Ca^{2+}-dependent manner (Brose et al., 1992), and that a single C_2-domain of Syt1—the first 'C2A-domain—constituted an autonomously folded domain that bound Ca^{2+} and phospholipids in a ternary complex (Davletov and Südhof, 1993 and 1994; Fig. 5B). In addition, we and others observed that the Syt1 C_2-domains also bind to Syntaxin-1 and to SNARE-complexes as a function of Ca^{2+} (Li et al., 1995a and 1995b; Chapman et al., 1995). In collaboration with Steven Sprang and Josep Rizo, we obtained atomic structures of the C2-domains of Syt1, and defined the architecture of their Ca^{2+}-binding sites (Sutton et al., 1995; Shao et al., 1996 and 1997; Ubach et 1998 and 2001; Fernandez et al., 2001; Fig. 5A). Our structural studies demonstrated that the Syt1 C2-domains are composed of stable β-sandwiches with flexible loops emerging from the top and bottom, and that Ca^{2+} exclusively binds to the top loops of the C2-domains with incomplete coordination spheres (Figs. 5A and 5C). As a result, intrinsic Ca^{2+}-binding to Syt1 C2-domains exhibited low affinity, but was dramatically enhanced by binding of phospholipids which complete the Ca^{2+}-coordination spheres (Davletov and Südhof, 1993 and 1994; Ubach et al., 1998 ; Fernandez et al., 2001).

The biochemical and structural definition of the Syt1 C2A-domain as an autonomously folded Ca^{2+}-binding module – the first for any C2-domain—proved paradigmatic for all C2-domains, which are now known to represent a common Ca^{2+}-binding motif found in many proteins (Rizo and Südhof, 1998; Corbalan-Garcia and Gómez-Fernández, 2014). However, not all C2-domains bind Ca^{2+}. Some C2-domains are Ca^{2+}-independent phospholipid-binding modules (e.g., the PTEN C2-domain; Lee et al., 1999), while others are Ca^{2+}-independent protein interaction domains (e.g., the N-terminal C2-domain of Munc13 that binds to RIMs as discussed below; Dulubova et al., 2005; Lu et al., 2006). Even C2-domains that bind Ca^{2+} are functionally diverse. For example, different from Syt1 C2-domains, some C2-domains exhibit a high intrinsic Ca^{2+}-affinity also in the absence of phospholipids (e.g., the central C2-domain of Munc13-2; Shin et al., 2010). Thus, C2-domains are versatile protein modules that most often are Ca^{2+}/phospholipid-binding domains but can adopt multifarious other functions.


A
Structure of Synaptotagmin-1 C2-domains
N
Y
Membrane
3·Ca 2+
2·Ca 2+
C2A-domain
C2B-domain
C
C2A-domain
C2B-domain


B
Ca2+-dependent phospholipid binding by the Syt1 C2A-domain
3H-PC Bound (cpm x10−3)
25
20
15
10
5
0
C2A-domain
10−7
10−6
10−5
10−4
free Ca2+ concentration (M)
Davletov & Südhof, 1993


C
Ca2+-binding sites of Syt1 C2A-domain
D238
H237
NH3
K236
S235
H
F234
R233
D232
NH
H2N
NH2
Loop 3
F231
D230
Ca2+
Ca2+
Ca2+
L171
D172
M173
G174
G175
Loop 1
T176
S177
D178
Fernandez-Chacon et al., 2001

Demonstration that Syt1 is a Ca^{2+}-sensor for exocytosis

After the biochemical studies had established that Syt1 binds Ca^{2+}, the next challenge was to show whether Syt1 constitutes Katz's long-sought Ca^{2+}-sensor for neurotransmitter release. Initial experiments in C. elegans and Drosophila disappointingly indicated that at least some neurotransmitter release remained after deletion of Syt1, even though release was significantly reduced (Littleton et al., 1993; DiAntonio et al., 1993; Nonet et al., 1993). Our electrophysiological analyses of Syt1 knockout mice in which higher resolution measurements of release were possible then revealed that Syt1 is selectively and absolutely required for fast synchronous synaptic fusion in forebrain neurons, whereas it is dispensable for other, slower forms of Ca^{2+}-induced release (Fig. 6; Geppert et al., 1994; Maximov and Südhof, 2005). These experiments, carried out in collaboration with Chuck Stevens at the Salk Institute, accounted for the Drosophila and C. elegans phenotypes, and established that Syt1 is essential for fast Ca^{2+}-triggered release, but is not required for fusion as such—is not even necessary for all Ca^{2+}-triggered fusion. Moreover, deletion of Syt1 increased spontaneous 'mini' release in some synapses, suggesting that Syt1 normally contributes to clamping spontaneous synaptic vesicle exocytosis (Maximov and Südhof, 2005; Xu et al., 2009).

The Syt1 knockout analyses thus supported the 'synaptotagmin Ca^{2+}-sensor hypothesis', but did not exclude the possibility that Syt1 positions vesicles next to voltage-gated Ca^{2+}-channels (a function now known to be mediated by RIMs and RIM-BPs [Kaeser et al., 2011]). Such a 'positioning function' would enable another 'real' Ca^{2+}-sensor to do the actual Ca^{2+}-triggering, consistent with the remaining Ca^{2+}-induced release in Syt1 knockout synapses—an alternative hypothesis that was widely discussed (Penner and Neher, 1994), but could not account for why Syt1 itself binds Ca^{2+}.

FIGURE 5. *(opposite) Domain structure and Ca^{2+}-binding of Synaptotagmin-1*

A. Domain structure of Synaptotagmin-1 (Syt1) and structure of the Syt1 C2-domains (courtesy of J. Rizo; Shao et al., 1998; Fernandez et al., 2001).

B. Demonstration that the C2A-domain of Syt1, and by extension other C2-domains, are autonomously folding Ca^{2+}-binding domains. The data illustrate high-affinity and highly cooperative Ca^{2+}-regulation of phospholipid binding by the purified recombinant Syt1 C2A-domain (reproduced from Davletov and Südhof, 1993).

C. Architecture of the Syt1 C2A-domain Ca^{2+}-binding sites as determined by NMR-spectroscopy (modified from Fernandez-Chacon et al., 2001). Note that multiple Ca^{2+}-ions are ligated in incomplete coordination spheres by multiple overlapping aspartate residues.

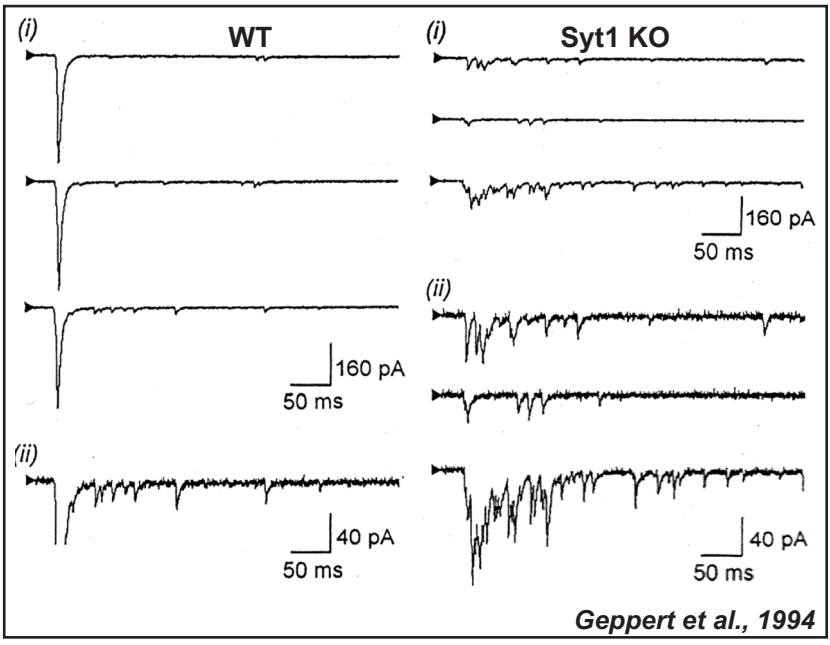


FIGURE 6. *Synaptotagmin-1 (Syt1) knockout selectively ablates fast synchronous neurotransmitter release.* Traces of evoked synaptic responses recorded from hippocampal neurons cultured from newborn littermate wild-type (WT, left) and Syt1 knockout mice (right). Synaptic responses were induced by isolated action potentials; two different scales are shown under (i) and (ii) as indicated by the calibration bars. Note that the Syt1 knockout completely ablates fast synchronous response, but not slow asynchronous responses (reproduced from Geppert et al., 1994a).

To directly test whether Ca^{2+}-binding to Syt1 actually triggers neurotransmitter release, we introduced into the endogenous mouse Syt1 gene a point mutation (R233Q) that decreased the Syt1 Ca^{2+}-binding affinity during phospholipid binding ~2-fold, but had no detectable effect on Ca^{2+}-dependent Syntaxin-1 binding (Figs. 7A and 7B; Fernandez-Chacon et al., 2001). Electrophysiological recordings, carried out in collaboration with Christian Rosenmund, revealed that the R233Q mutation converted synaptic depression during stimulus trains into synaptic facilitation, consistent with a decrease in release probability (Fig. 7C). Importantly, this decrease in release probability was revealed to be caused by a ~2-fold decrease in the apparent Ca^{2+}-affinity of neurotransmitter release, formally proving that Syt1 is the Ca^{2+}-sensor for release (Fig. 7D).

In subsequent studies, we extended this analysis, and introduced into knock-in mice other point mutations, including a mutation (D232N) that

increased the Ca^{2+}-dependent interaction of Syt1 with SNARE proteins (Fig. 7E; Pang et al., 2006a). We found that this mutation increased neurotransmitter release accordingly. We showed in a detailed comparison of the R233Q and D232N point mutations, which decrease or increase the apparent Ca^{2+}-affinity of Syt1, respectively, that they have corresponding opposite effects on the apparent Ca^{2+}-affinity of release (Figs. 7F and 7G). Moreover, in parallel experiments in chromaffin cells performed in collaboration with Erwin Neher, we found that Syt1 also functions as a Ca^{2+}-sensor for endocrine granule exocytosis (Voets et al., 2001; Sorensen et al., 2002), although here the Syt1 deletion causes only a very small impairment in Ca^{2+}-triggered exocytosis because Syt1 function is largely redundant with that of Syt7 in chromaffin cells (Schonn et al., 2008; see discussion below).

Together, these studies proved that Syt1 functions as a Ca^{2+}-sensor in synaptic vesicle exocytosis. We next wondered whether Ca^{2+}-binding to both of the C2-domains of Syt1 contributes to triggering release. Initial studies in Drosophila demonstrated that the C2B-domain Ca^{2+}-binding sites of Syt1 are essential for release (Mackler and Reist, 2001). A similar study suggested that the C2A-domain Ca^{2+}-binding sites are dispensable (Robinson et al., 2002), but the signal-to-noise ratio of this study was too low to rule out a significant contribution of the C2A-domain. Using systematic rescue experiments to perform a direct quantitative comparison of the Ca^{2+}-triggering activities of Syt1 mutants lacking either C2A- or C2B-domain Ca^{2+}-binding sites, we found that in addition to the C2B-domain Ca^{2+}-binding sites, the C2A-domain Ca^{2+}-binding sites significantly contribute to release (Shin et al., 2009). Moreover, we observed that in the absence of the C2A-domain Ca^{2+}-binding sites, Ca^{2+}-triggered release exhibited a significantly decreased apparent Ca^{2+}-cooperativity, documenting that Ca^{2+}-binding to the C2A-domain of Synaptotagmin-1 directly participates in the Ca^{2+}-triggering of fast release.

Diversity of synaptotagmins in fast Ca^{2+}-triggered neurotransmitter release

Mammalian genomes encode 16 synaptotagmins (defined as double C2-domain proteins with an N-terminal transmembrane region). The C2-domains of 8 synaptotagmins (Syt1-Syt3, Syt5-Syt7, Syt9, and Syt10) bind Ca^{2+}, whereas those of the other 8 synaptotagmins do not. The 8 Ca^{2+}-binding synaptotagmins comprise two classes which lack (Syt1, Syt2, Syt7, and Syt9) or contain N-terminal disulfide bond that covalently dimerizes the respective synaptotagmins (Syt3, Syt5, Syt6, and Syt10).

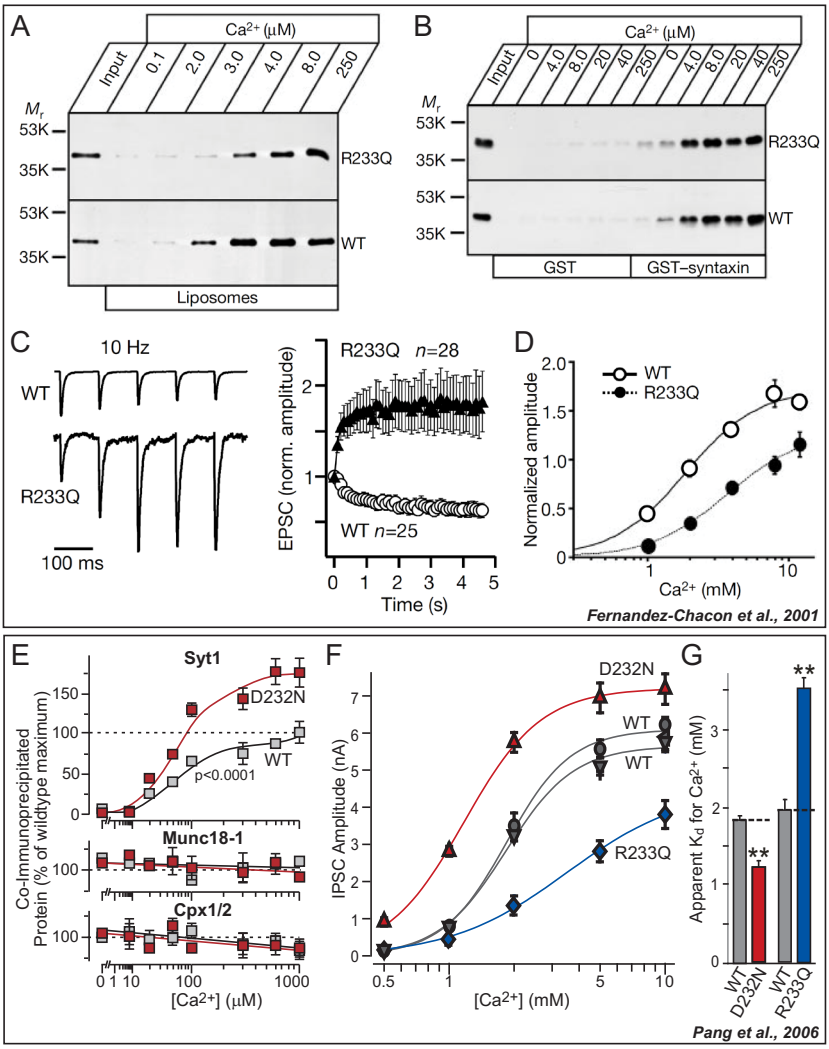


FIGURE 7. *Demonstration that Ca^{2+}-binding to Synaptotagmin-1 (Syt1) triggers neurotransmitter release using knock-in mice containing mutant Syt1 with altered Ca^{2+}-affinities*

A & **B.** A single amino-acid substitution in the Syt1 C2A-domain (R233Q) decreases the apparent Ca^{2+}-affinity of Syt1 during phospholipid but not during Syntaxin-1 binding. Data show measurements of Ca^{2+}-dependent binding of the entire cytoplasmic fragment of endogenous wild-type and R233Q-mutant mutant Syt1 obtained from littermate knock-in mice to liposomes (A) or immobilized GST-Syntaxin-1 (B).

C. The R233Q amino-acid substitution decreases the probability of neurotransmitter release as evidenced by a conversion of synaptic depression in wild-type synapses into synaptic facilitation in R233Q-mutant synapses. Synaptic responses during a 10 Hz stimulus train are measured (left, representative traces; right, normalized responses).

(continues)

When the diversity of synaptotagmins emerged (e.g., see Geppert et al., 1991; Li et al., 1995), it was surprising that the Syt1 knockout produced a dramatic phenotype because at least some of these other synaptotagmins are co-expressed with Syt1. However, using systematic rescue experiments we found that only three of the eight Ca^{2+}-binding synaptotagmins—Syt1, Syt2 and Syt9—mediate fast synaptic vesicle exocytosis (Xu et al., 2007). These synaptotagmins exhibit distinct kinetics, with Syt2 triggering release faster, and Syt9 slower than Syt1. Most forebrain neurons express only Syt1, accounting for the dramatic Syt1 knockout phenotype. Syt2 is the Ca^{2+}-sensor of fast synapses in the brainstem and the neuromuscular junction (Pang et al., 2006b; Sun et al., 2007; Figs. 8A and 8B), while Syt9 is primarily present in the limbic system (Xu et al., 2007). Thus, the kinetic properties of Syt1, Syt2, and Syt9 correspond to the functional needs of the synapses containing them.

In the initial Syt1 KO studies (Geppert et al., 1994), we observed that although fast release was ablated in Syt1-deficient synapses, a slower form of Ca^{2+}-triggered release remained (Fig. 6). We thus sought to biophysically define the

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D. The R233Q-mutation decreases the apparent Ca^{2+}-affinity of neurotransmitter release approximately 2-fold similar to its effect on the apparent Ca^{2+}-affinity of phospholipid binding (see A), which accounts for the decrease in release probability in C. Data show normalized amplitudes of synaptic responses as a function of extracellular Ca^{2+}-concentration.

E. Another single amino-acid substitution in the Syt1 C2A-domain (D232N) has a distinct effect on the Ca^{2+}-binding properties of Syt1: it increases Ca^{2+}-dependent binding of Syt1 to SNARE complexes. Data show measurements of Ca^{2+}-dependent binding of wild-type and D232N-mutant endogenous Syt1 to SNARE complexes in brain homogenates from knock-in mice solubilized with Triton X-100. SNARE complexes were immunoprecipated at the indicated concentrations of free Ca^{2+}, and immunoprecipiates were analyzed by quantitative immunoblotting for Syt1 (top graph), Munc18-1, and and complexins (bottom graphs). Note that Munc18-1 and complexin constitutively co-immunoprecipitate with SNARE complexes whereas the co-IP of Syt1 is dramatically enhanced at increasing Ca^{2+}-concentrations.

F & **G.** Direct comparisons of the effects of D232N- and R233Q-knock-in mutations in Syt1 demonstrate that these two mutations that have opposite effects on the Ca^{2+}-binding properties of Syt1 produce opposite shifts in the apparent Ca^{2+}-affinity of release. F. Measurements of the absolute amplitude of evoked inhibitory postsynaptic currents as a function of extracellular Ca^{2+} in neurons cultured from littermate D232N- or R233Q-mutant knock-in mice and their wild-type (WT) littermates; each mutant has its own wild-type control. Synaptic amplitudes are fit to a Hill function. G. Apparent Ca^{2+}-affinity for release calculated by Hill function fits of the data in F comparing wild-type controls to D232N- or R233Q-mutant synapses.

Panels A–D were reproduced from Fernandez-Chacon et al. (2001); and panels E–G from Pang et al. (2006) and Xu et al. (2009).

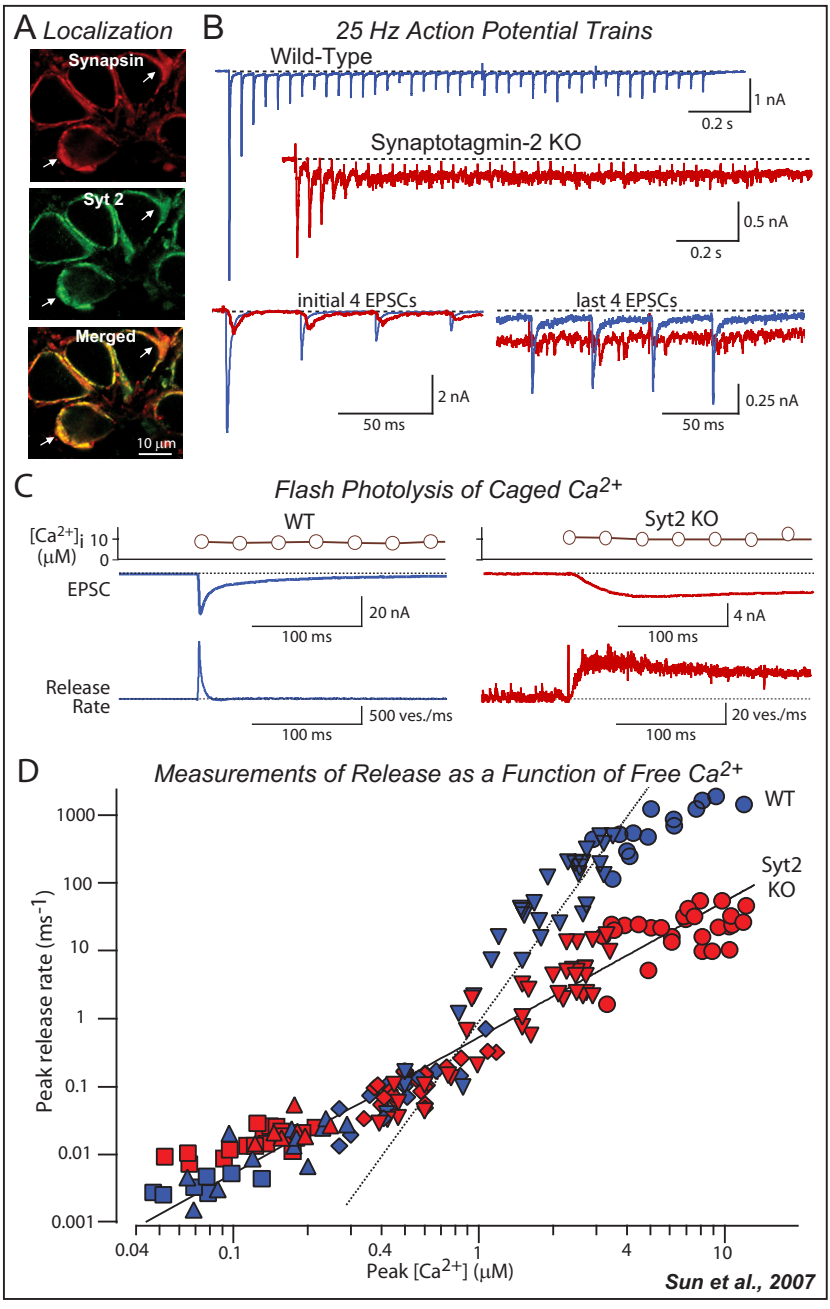

FIGURE 8. *Deletion of Synaptotagmin-2 (Syt2) the fast Ca^{2+}-sensor for release in the Calyx of Held synapse, uncovers a slower form of Ca^{2+}-triggered release that is controlled by a secondary Ca^{2+}-sensor with a much lower Ca^{2+}-cooperativity than Syt2.*

(continues)

contribution of the 'fast' synaptotagmin-dependent form of release, and to describe the properties of the slower remaining form. To do so, we used the calyx-of-Held synapse as a model system because it allows simultaneous patching of pre- and postsynaptic compartments, providing an unparalleled resolution of electrophysiological measurements (Forsythe, 1994; Borst and Sakmann, 1996). The calyx-of-Held synapse expresses only Syt2 among the 'fast' synaptotagmins (Fig. 8A; Sun et al., 2007). Knockout of Syt2 ablated all fast Ca^{2+}-triggered neurotransmitter release; only a slower form of release remained (Fig. 8B). In the Syt2 KO calyx synapse, this remaining Ca^{2+}-triggered release did not facilitate during high-frequency stimulus trains, different from what we observed in Syt1 KO synapses in hippocampal and cortical neurons (see Maximov and Südhof, 2005, and Fig. 9 below). As a result, the Syt2 KO blocked the vast majority of

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A. Localization of Syt2 by immunocytochemistry of Calyx synapses demonstrates abundant expression in presynaptic terminals.

B. Knockout (KO) of Syt2 in calyx synapses ablates most fast synchronous neurotransmitter release induced by a high-frequency action potential train (40 stimuli at 25 Hz). Representative traces of synaptic responses (EPSCs) recorded during the overall train are shown on top (note that wild-type and mutant traces are shown with different scales), and expansions of the initial and the final 4 EPSCs at the bottom (note that here wild-type and mutant traces have the same scales, but scales differ for the first and last 4 EPSCs). The baseline shift in the Syt2 KO traces reflects unclamping of unsynchronous release that is not observed in wild-type synapses.

C. KO of Syt2 severely impairs neurotransmitter release triggered by high concentrations of Ca^{2+} in the calyx of Held synapse. Presynaptic terminals were filled via a patch pipette with caged Ca^{2+} and a Ca^{2+}-indicator dye, and release was triggered by Ca^{2+} released by flash photolysis. The amount of release was measured postsynaptically by monitoring the EPSC and then calculating the number of vesicles released at a given time (release rate). Simultaneously, the presynaptic Ca^{2+}-concentration was measured by microfluorometry.

D. Ca^{2+}-triggered neurotransmitter release exhibits a biphasic Ca^{2+}-concentration dependence in wild-type (WT) calyx synapses with a low apparent Ca^{2+}-cooperativity of release (~2 Ca^{2+}-ions) at low Ca^{2+}-concentrations, and a high apparent Ca^{2+}-cooperativity at high Ca^{2+}-concentrations (~5 Ca^{2+}-ions). KO of Syt2 selectively ablates the high Ca^{2+}-cooperativity release phase, decreasing the release rates at physiological Ca^{2+}-concentrations nearly 100-fold without significantly affecting Ca^{2+}-triggered release at low Ca^{2+}-concentrations. Data show summary graph of EPSC peak release rates as a function of different free Ca^{2+}-concentrations in the presynaptic terminal. The dashed line represents a fit of a 5th power function to the data from wild-type terminals at >1 μM free Ca^{2+}; the solid line a 2nd power function to the data from mutant terminals at all Ca^{2+}-concentrations. Note that the solid line also fits the wild-type responses at low Ca^{2+}-concentrations.

All data were adapted from Sun et al. (2007).

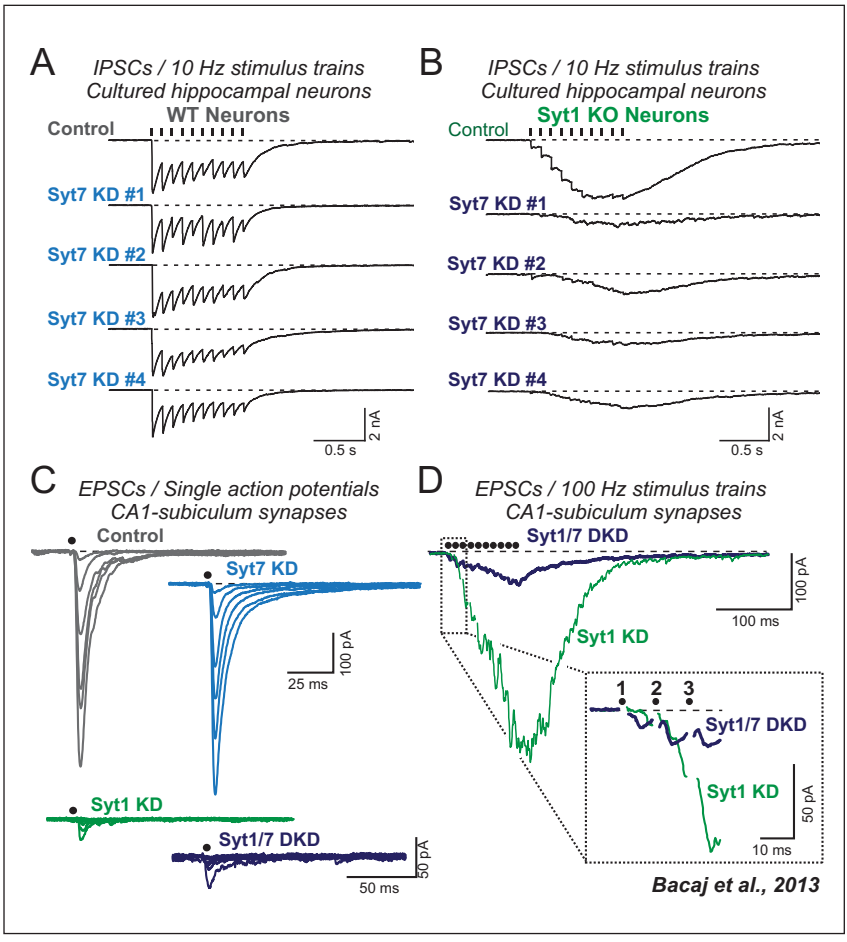

FIGURE 9. *Synaptotagmin-7 (Syt7) knockdown impairs the slow release remaining in Synaptotagmin-1 (Syt1) knockout neurons*

A & **B.** In cultured hippocampal neurons, suppression of Syt7 expression by knockdown (KD) has no major effect on neurotransmitter release evoked by a high-frequency stimulus train in wild-type synapses (A). However, suppressing Syt7 expression in Syt1-deficient neurons (Syt1 KO) impairs most of the slow and facilitating Ca^{2+}-triggered release that remains after the Syt1 KO (B). Data show representative traces of IPSCs evoked by a 10 Hz stimulus train obtained in control neurons and neurons expressing four different Syt7 shRNAs to assure reproducibility. Note that in hippocampal neurons, the high-frequency stimulus train induces in Syt1 KO neurons a strongly facilitating form of asynchronous release, such that the amount of total release during the train is similar in Syt1 KO and wild-type neurons. By contrast, in Syt2 KO calyx synapses no such facilitation of the residual release is observed (see Fig. 8B).

C & **D.** In acute slices, suppression of Syt7 expression by itself also has no significant effect on release, but here again suppression of Syt1 expression ablates only the initial fast phase of release but retains a strongly facilitating asynchronous form of release that is severely

(continues)

Ca^{2+}-triggered release in this synapse independent of the stimulation frequency (Sun et al., 2007).

We then analyzed the Ca^{2+}-dependence of neurotransmitter release in calyx synapses from wild-type and littermate Syt2 KO mice using flash-photolysis of caged-Ca^{2+}. We performed simultaneous measurements of the postsynaptic response (which allows precise calculations of synaptic vesicle exocytosis) and of presynaptic Ca^{2+}-levels by microfluorometry, an approach that had been pioneered by the Sackmann, Schneggenburger, and Neher laboratories (Bollmann et al., 2000; Schneggenburger and Neher, 2000). We found that as described previously (Bollmann and Sakmann, 2000), release triggered by physiological Ca^{2+}-concentrations exhibited an apparent Ca^{2+}-cooperativity of 5, similar to the number of Ca^{2+}-ions bound to synaptotagmins (Figs. 8C and 8D). However, the small amount of remaining Ca^{2+}-triggered release in Syt2 KO calyx synapses exhibited an apparent Ca^{2+}-cooperativity of only 2, suggesting that this release was mediated by a different Ca^{2+}-sensor that at least in the calyx of Held synapse has properties distinct from those of Syt1, Syt2, and Syt9 (Sun et al., 2007).

Testing the function of Synaptotagmin-7 in slow Ca^{2+}-triggered release

Which Ca^{2+}-sensor induces the remaining release in Syt1 and Syt2 KO synapses, and could this release be mediated by one of the other 5 Ca^{2+}-binding synaptotagmins? The remaining release in Syt1 KO neurons exhibits distinct, synapse-dependent properties. Whereas in Syt2-deficient calyx-of-Held synapses the remaining release remains small and constant even at high stimulation frequencies (Fig. 8B), in Syt1-deficient hippocampal and cortical synapses the remaining 'asynchronous' release is massively facilitating at high stimulation frequencies (Fig. 9A). As a result, in the latter synapses the total amount of Ca^{2+}-triggered release induced by high-frequency stimulus trains is similar in wild-type and Syt1-deficient synapses, even though the initial rate of fast release differs more than 10-fold (Maximov and Südhof, 2005; Xu et al., 2012).

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impaired by additional suppression of Syt7 expression. Data show measurements of EPSCs elicited by isolated stimuli applied with increasing strength (C) or by a 100 Hz, 0.1 sec stimulus train (D; representative traces with an expansion of the initial response below). Measurements were performed in acute hippocampal slices from mice whose CA1 region had been injected with viruses encoding shRNAs for knockdown of the indicated synaptotagmins two weeks prior to the experiments. EPSCs were measured in postsynaptic subiculum neurons after presynaptic stimulation of axons emanating from CA1 region neurons.

All data were adapted from Bacaj et al. (2013).

To define the Ca^{2+}-sensor for the remaining release in Syt1-deficient hippocampal neurons, we focused on Syt7. We had found earlier that Syt7, similarly to Syt1, functions as a Ca^{2+}-sensor for exocytosis in chromaffin and other neuroendocrine and endocrine cells (Sugita et al., 2001; Gustavsson et al., 2008 and 2009; Schonn et al., 2009), and Paul Brehm had observed a role for Syt7 in release at the neuromuscular junction (Wen et al., 2010). We found that although Syt7 loss-of-function did not produce a major change in neurotransmitter release in Syt1-containing wild-type neurons (Maximov et al., 2008), it impaired most of the remaining slow Ca^{2+}-triggered release in Syt1 knockout neurons (Bacaj et al., 2013; Fig. 9). The Syt7 loss-of-function phenotype in Syt1-deficient neurons could be rescued only by Syt7 containing functional Ca^{2+}-binding sites, suggesting that Syt7 functions as a Ca^{2+}-sensor. Different from Syt1 in which the C2B-domain Ca^{2+}-binding sites were more important than the C2A-domain Ca^{2+}-binding sites, blocking the Syt7 C2B-domain Ca^{2+}-binding sites of Syt7 had no effect on rescue. However, blocking the Syt7 C2A-domain Ca^{2+}-binding sites abolished its rescue activity (Bacaj et al., 2013). This result indicates that the mechanisms of action of Syt1 and Syt7 partly differ from each other.

Viewed together, these observations suggest that Syt7—like the other synaptotagmins of its class (Syt1, Syt2, and Syt9)—functions as a Ca^{2+}-sensor for exocytosis, but exhibits a slower kinetics than Syt1, Syt2, and Syt9. The relatively slow action of Syt7 normally occludes its function in many wild-type synapses in which the faster Syt1 or Syt2 probably outcompetes the slower Syt7. Although the function of Syt7 was not immediately apparent at most normal synapses (Fig. 9), paired recordings showed that Syt7 does contribute physiologically to release during stimulus trains even in the presence of Syt1 (Bacaj et al., 2013). Therefore four synaptotagmins (Syt1, Syt2, Syt7, and Syt9) together account for nearly all neurotransmitter release at a synapse. The different speed of action of Syt1 and Syt7 may be related to their localizations because Syt7 has been consistently found to be absent from synaptic vesicles (Sugita et al., 2002; Maximov et al., 2008), even though it is present on endocrine granules, suggesting that it is slow because it is not as close to the site of Ca^{2+}-triggered fusion as Syt1.

Complexins support synaptotagmin-dependent Ca^{2+}-triggering of fusion

We identified complexins as small proteins bound to SNARE complexes but not to individual SNARE proteins (McMahon et al., 1995; also later independently identified by Ishizuka et al., 1995). The crystal structure of complexin bound to the SNARE complex, obtained in collaboration with Josep Rizo, revealed that complexin contains a central α-helix that nestles in an antiparallel orientation

into the groove formed by the Syntaxin-1 and Synaptobrevin-2 SNARE motifs (Chen et al., 2002). The central α-helix of complexin is N-terminally preceded by an accessory α-helix and a short unstructured sequence, and C-terminally followed by a longer unstructured sequence. Analysis of complexin-deficient neurons showed that complexin represents a co-factor for synaptotagmin that functions physiologically both as a clamp and as an activator of Ca^{2+}-triggered fusion (Reim et al., 2001; Tang et al., 2006; Huntwork and Littleton, 2007; Maximov et al., 2009; Yang et al., 2010). Complexin-deficient neurons exhibited a milder phenocopy of Syt1-deficient neurons, with a partial suppression of fast synchronous exocytosis and an increase in spontaneous exocytosis, suggesting that complexins and synaptotagmins are functionally interdependent.

Some confusion developed regarding complexin function because *in vitro* fusion assays suggested that complexins act only as a clamp of fusion (Giraudo et al., 2006), whereas in analyses of synaptic transmission in autapses (in which isolated neurons form synapses with themselves for want of a better partner), complexins acted only as an activator of Ca^{2+}-triggered fusion (Reim et al., 2001). Subsequent studies in cultures of dissociated neurons readily uncovered both complexin activities in that the loss-of-function of complexin produced a large increase in spontaneous 'mini' release (interpreted as unclamping) and a major impairment in evoked release (interpreted as a lack of activation; Fig. 10 [Maximov et al., 2009]).

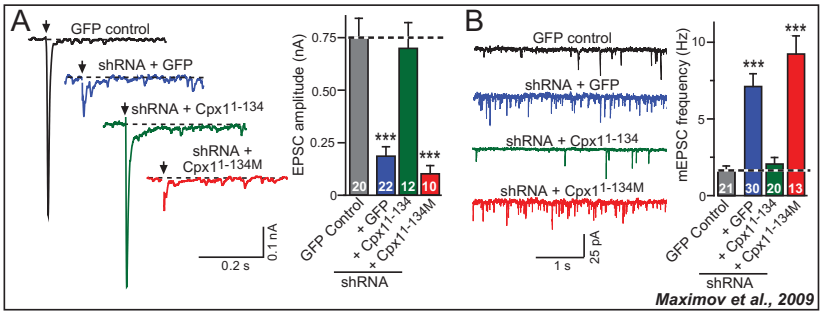

FIGURE 10. *Complexin functions both as an activator and as a clamp of synaptic vesicle fusion*

A & **B.** Excitatory postsynaptic currents (EPSCs) elicited by isolated action potentials (A) and spontaneous miniature EPSCs (mESPCs; B) monitored in control neurons and complexin knockdown neurons without or with expression of complexin rescue constructs (wild-type complexin-1 [$Cpx^{1\text{-}134}$] and mutant complexin-1 unable to bind to SNARE complexes [$Cpx^{1\text{-}134M}$]). Representative traces are shown on the left, and summary graphs on the right to illustrate the dual nature of complexin action as an activator of Ca^{2+}-triggered exocytosis (A) and as a clamp of spontaneous mini release (B). Data are adapted from Maximov et al. (2009).

How does a small molecule like complexin, composed of only ~130 residues, act to activate and clamp synaptic vesicles for synaptotagmin action? The central complexin α-helix that is bound to the SNARE complex is essential for all complexin function (Fig. 10; Maximov et al., 2009). The accessory α-helix is required only for the clamping but not the activating function of complexin, demonstrating that clamping is not a prerequisite for the activation function of complexin (Yang et al., 2010). The flexible N-terminal sequence of complexin, conversely, mediates only the activating but not the clamping function of complexin (Xue et al., 2007; Maximov et al., 2009). Recent results indicate that the activating function of complexin is unexpectedly complex (no pun intended) in that complexin also contributes to the priming of synaptic vesicles, but that for this facet of its activating function the C-terminal sequence is required (Yang et al., 2010; Kaeser-Woo et al., 2012).

Based on these studies, our current model posits 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 and clamps the complex to prevent its complete assembly (Tang et al., 2006). Ca^{2+}-activated synaptotagmin displaces this part of complexin, thereby enabling fusion-pore opening (Fig. 11). However, it is likely that the clamping function of complexin is relatively less important than its activation function. Even though a 10-fold increase in the rate of spontaneous mini release induced by loss of complexin function is significant, it is very small on a per synapse basis. If one considers that each neuron receives thousands of synaptic inputs, the increased mini rate still translates into only one release event or less per synapse and per minute (Yang et al., 2013). Moreover, some complexin isoforms that are generally expressed at low levels (complexin-3 and -4) do not exhibit a clamping function (Kaeser-Woo et al., 2012), and the function of complexin in Ca^{2+}-triggered exocytosis of IGF-1 containing vesicles (see below) does not involve clamping (Cao et al., 2013). Thus, it is likely that complexin primarily functions as an activator of exocytosis, and that its clamping function is either an epiphenomenon, or a more minor fine-tuning activity in synaptic transmission.

An approximation of how SNARE and SM proteins collaborate with synaptotagmins and complexins in Ca^{2+}-triggered fusion

The convergence of biochemical and biophysical studies on the neurotransmitter release machinery lead us to a preliminary model of how Ca^{2+}-triggered neurotransmitter release proceeds (Fig. 11).

Sketching the atomic structures of SNARE proteins, complexin and synaptotagmin into the context of a docked and primed synaptic vesicle in an in-scale drawing reveals a crowded space in which all partners are placed into close proximity, allowing for rapid interactions (Fig. 11A). When we consider how sequential interactions of SNARE and SM proteins with complexin and synaptotagmin may mediate Ca^{2+}-triggered fusion, the most plausible model is that complexin and synaptotagmin act on top of the two sequential major conformational changes involved in SNARE/SM protein complex assembly (Fig. 11B; see also Fig. 4). Specifically, after docked and tethered vesicles are primed for fusion by opening up the closed conformation of Syntaxin-1 and by partial trans-SNARE-complex assembly (Priming I, Fig. 11B), complexin binds to the partially assembled trans-SNARE complex to 'superprime' it and to energize the vesicles for Ca^{2+}-triggered fusion (Priming II). Synaptotagmins probably also constitutively bind to assembling SNARE complexes independent of Ca^{2+}, and the complexin- and synaptotagmin-binding may contribute to 'freeze' the partly assembled SNARE complex and thus 'clamp' it. Ca^{2+} then triggers fusion-pore opening by binding to synaptotagmin, which in turn binds to phospholipids and changes its interaction with the trans-SNARE complex to partly displace complexin. It is likely that synaptotagmin and complexin constitutively interact with the SNARE/SM protein complex in a Ca^{2+}-independent manner to form a single prefusion complex, and that Ca^{2+} does not cause an all-or-none binding of synaptotagmin to the SNARE complex as it does for binding of synaptotagmin to phospholipids, but instead causes a rearrangement of the prefusion complex (e.g., see Shin et al., 2003).

The simplest mechanism by which Ca^{2+}-binding to synaptotagmin could open the fusion pore would be by pulling on the SNARE/SM protein complex, a pulling action that could be induced by Ca^{2+}-triggered binding of synaptotagmin to phospholipids. After fusion-pore opening, the pore expands, and NSF and SNAPs are recruited to the assembled cis-SNARE complex. NSF then dissociates the cis-SNARE complex, the Munc18-1/SNARE complex assembly is transformed into the heteromeric Munc18-1/Syntaxin-1 complex, and synaptic vesicles recycle via one of several forms of endocytosis (see Fig. 1).

Parallel synaptotagmin-mediated pathways of Ca^{2+}-triggered exocytosis

The 4 synaptotagmins that lack N-terminal disulfide bonds (Syt1, Syt2, Syt7, and Syt9) function in synaptic vesicle and neuroendocrine exocytosis, but what about the other 4 Ca^{2+}-binding synaptotagmins that are disulfide-bonded

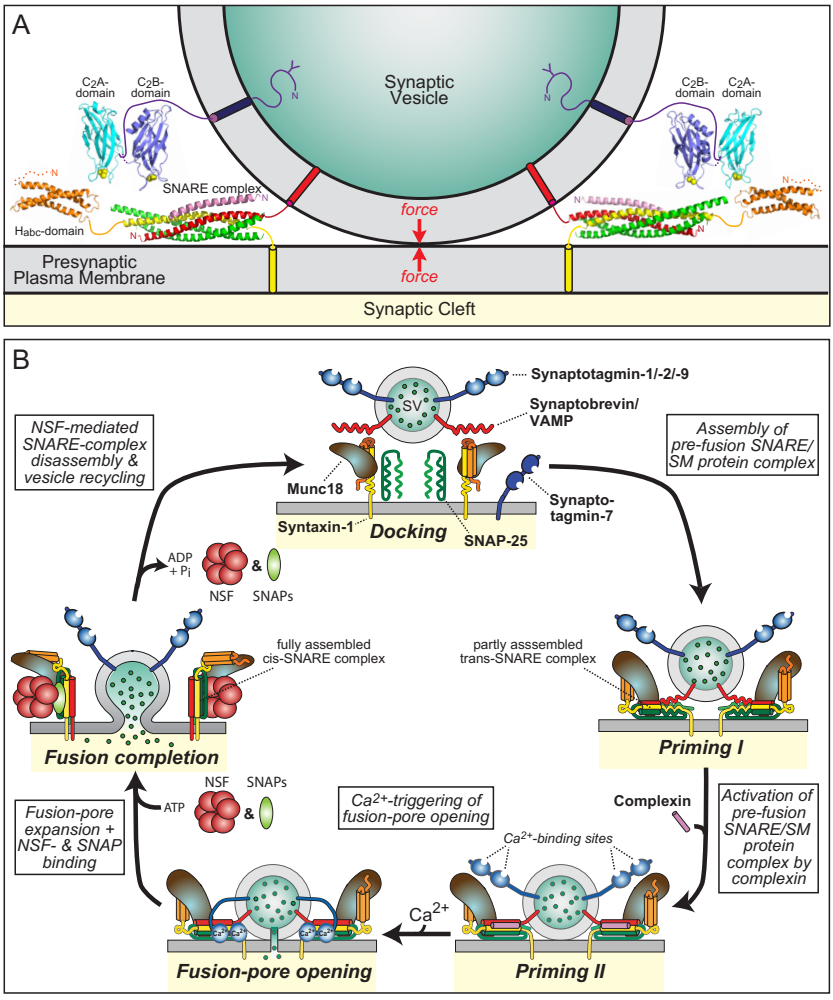

FIGURE 11. *Modeling how SNARE and SM proteins collaborate with synaptotagmins and complexins in Ca^{2+}-triggered neurotransmitter release*

A. Atomic structures of SNARE proteins, complexin, and Syt1 during synaptic vesicle fusion. The illustration summarizes atomic structures obtained in collaboration with Josep Rizo (UT Southwestern) of the Syt1 C2-domains (Shao et al., 1998; Fernandez et al., 2001), the Syntaxin-1 Habc-domain (Ferndandez et al., 1997), and the assembled SNARE complex containing bound complexin (pink; Chen et al., 2002). Transmembrane regions are depicted as cylinders, and linker sequences as lines. All structures are in scale relative to the synaptic vesicle, illustrating the space constraints of the collaboration between Syt1 and the SNARE complex/complexin assembly. Munc18-1 is also bound to the SNARE complex at the same time (see Figs. 3D and 4) but is not shown since no structure of Munc18-1 bound to the SNARE complex is available. The direction of the force produced by SNARE complex assembly that destabilizes the phospholipid membrane surfaces is indicated.

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dimers? Recent studies revealed that one of these synaptotagmins, Syt10, also acts as a Ca^{2+}-sensor in exocytosis, but in a form of exocytosis that differs from synaptic vesicle and neuroendocrine granule exocytosis. Specifically, we found that Syt10 functions in olfactory neurons as a Ca^{2+}-sensor for specialized vesicles containing IGF-1 (Cao et al., 2011). These vesicles differ from neuropeptide-containing vesicles present in the same neurons (which are more like neuroendocrine granules and contain Syt1; Cao et al., 2013). Among others, these experiments demonstrated that even in a single neuron, different synaptotagmins act as Ca^{2+}-sensors for distinct Ca^{2+}-triggered fusion reactions (Fig. 12). Moreover, these observations indicated that Ca^{2+}-triggered exocytosis generally depends on synaptotagmin Ca^{2+}-sensors, and that different synaptotagmins contribute to the specificity and differential properties of distinct exocytosis pathways.

Interestingly, complexin not only supports synaptotagmins acting in neurotransmitter release, but also Syt10-dependent IGF1 secretion, despite the different covalent structures of Syt1 and Syt10 (Cao et al., 2013). Thus, complexin likely is a general co-factor for all synaptotagmins in regulated exocytosis. This hypothesis is supported by the fact that complexin is ubiquitously present in all cells (McMahon et al., 1995), and is also central for the postsynaptic insertion of AMPA-type glutamate receptors during LTP (Ahmad et al., 2012), suggesting that complexins are general cofactors for regulated exocytosis.

B. Schematic diagram of the action of synaptotagmins and complexins in the SNARE/SM protein cycle. The SNARE/SM protein cycle is composed of the assembly of the SNARE proteins Synaptobrevin/VAMP, SNAP-25, and Syntaxin-1 into complexes whose full formation forces fusion-pore opening; the SM protein Munc18-1 remains associated with Syntaxin-1 throughout the cycle and is essential for fusion-pore opening. After fusion, the chaperone ATPase NSF and its SNAP adaptors catalyze SNARE-complex dissociation. Complexin binds to partially assemble SNARE complexes during priming, and serves as an essential adaptor that enables synaptotagmin to act as a Ca^{2+}-sensor in triggering fusion-pore opening (bottom limb of the cycle). Note that synaptotagmin likely constitutively interacts with the SNARE/SM protein complex in a Ca^{2+}-independent manner to form a single prefusion complex prior to Ca^{2+}-triggering of exocytosis, and that Ca^{2+} does not cause an all-or-none binding of synaptotagmin to the SNARE complex as it does for binding of synaptotagmin to phospholipids, but instead causes a rearrangement of the prefusion complex. However, this is not shown in the diagram due to difficulties of representing these multifarious three-dimensional interactions in a two-dimensional format. Both synaptotagmins and complexins additionally clamp spontaneous release, probably via their Ca^{2+}-independent constitutive binding to partly assembled SNARE complexes. Three vesicular synaptotagmins act as Ca^{2+}-sensors for fast exocytosis (Syt1, Syt2, and Syt9); in addition, Syt7 that is not present on synaptic vesicles but probably localizes to the presynaptic plasma membrane (Sugita et al., 2001) mediates slower forms of Ca^{2+}-triggered exocytosis (Syt7 is only shown in the top overview for simplicity). Drawing was modified from Südhof (2013).

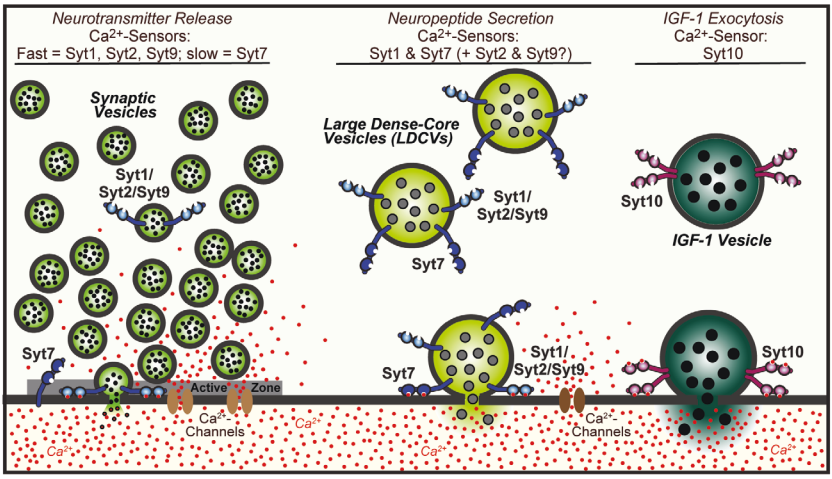

FIGURE 12. *Overlapping and non-overlapping functions of different synaptotagmins in Ca^{2+}-triggering of various types of exocytosis in a single neuron.* Three types of Ca^{2+}-triggered exocytosis are illustrated: Left, synaptic vesicle exocytosis mediating neurotransmitter release that uses Syt1, Syt2, and/or Syt9 as fast Ca^{2+}-sensors (Xu et al., 2007), and Syt7 as a slow Ca^{2+}-sensor (Bacaj et al., 2013). Center, large dense-core vesicle (LDCV) exocytosis that uses the same Ca^{2+}-sensors as synaptic vesicle exocytosis (Syt1 and Syt7 based on work in chromaffin cells [Sugita et al., 2001; Schonn et al., 2007], and probably also Syt2 and Syt9). Right, exocytosis of a separate class of peptidergic vesicles that are larger than LDCVs and utilize Syt10 as a Ca^{2+}-sensor (Cao et al., 2011 and 2013). Note that although Syt7 has been shown to operate in both synaptic vesicle and LDCV exocytosis, it is absent from synaptic vesicles but present on LDCVs, and is thought that act more slowly in neurotransmitter release because of its different localization. Diagram was modified from Cao et al. (2011).

5. ORGANIZING THE RELEASE MACHINERY AT THE ACTIVE ZONE

In a presynaptic terminal, synaptic vesicles dock and fuse at the active zone of the presynaptic plasma membrane. The active zone is a specialized area that appears dense in electron microscopy pictures of fixed tissue and is localized precisely opposite postsynaptic receptor clusters (Fig. 1). The first specific active zone protein we identified was Munc13-1 (Brose et al., 1995). Munc 13-1 was named like Munc18-1 (no relation!) after a homologous gene in C. elegans (*unc13*) that is essential for coordinated worm movements but whose function was unknown (Brenner, 1974). Based on this homology, the 'uncoordinated' worm phenotype, and its localization to the active zone, we speculated that Munc13-1 may be a component of the neurotransmitter release machinery (Brose et al., 1995).

This supposition was confirmed when we analyzed knockout synapses lacking Munc13-1, which exhibited a dramatic loss of synaptic vesicle priming (Augustin et al., 1999).

Quickly after Munc13-1, we identified a series of additional active zone proteins such as CASK (Hata et al., 1996), RIMs (for Rab3-interacting molecules; Wang et al., 1997), RIM-BPs (Wang et al., 2000), and ELKS (Wang et al., 2002; see also Ohtsuka et al., 2002), while others identified additional active zone proteins such as α-liprins (Zhen and Jin, 1999), bassoon (tom Dieck et al., 1998), and piccolo (Wang et al., 1999; Fenster et al., 2000). Interestingly, most of these proteins directly or indirectly bind to each other, forming a protein network at the active zone (Südhof, 2012). Specifically, RIMs bind to Munc13-1, to RIM-BPs, to ELKS (although it is not clear whether this binding is physiologically important) and to α-liprins, suggesting that RIMs are the central hub of this network, while additional interactions connect some of the other proteins with each other (Wang et al., 2000 and 2003; Betz et al., 2001; Schoch et al., 2002). Clearly, many questions about the active zone are still unanswered, most importantly what mechanisms position the active zone precisely opposite a postsynaptic specialization. Nevertheless, we now have a plausible view of how the active zone performs its three main functions, namely the tethering ('docking') of synaptic vesicles at the plasma membrane, the priming of such vesicles for fusion, and the recruitment of Ca^{2+}-channels next to docked and primed vesicles.

Tethering ('docking') of synaptic vesicles to the active zone

As in other membrane-trafficking processes, synaptic vesicle tethering involves Rab proteins, small GTPases that are distantly related to ras proteins. The central role of Rab proteins in membrane traffic was discovered in Novick's studies on Sec4p (Salminen and Novick, 1987). Following up on Novick's work, we observed in 1992 in collaboration with Reinhard Jahn that Rab3, the most abundant Rab protein in brain, is highly enriched on synaptic vesicles at rest but dissociates from the vesicles during exocytosis, suggesting a role in neurotransmitter release (von Mollard et al., 1990 and 1991). Subsequent mouse genetic analyses of the four different Rab3 isoforms (Rab3A, 3B, 3C, and 3D) confirmed that Rab3 plays a central role in neurotransmitter release (Geppert et al., 1994b; Schlüter et al., 2004 and 2006). Moreover, single Rab3 isoforms did perform essential functions on their own in that deletions of Rab3A or Rab3B caused major but distinct changes in short- and long-term forms of presynaptic plasticity (Geppert et al., 1994b; Schlüter et al., 2004 and 2006; Tsetsenis et al., 2011).

In our search for a mechanism of action for Rab3s, we initially tested the functional role of rabphilin, the first putative Rab3-effector identified by Yoshimi Takai (Shirataki et al., 1993). However, rabphilin deletions produced only minor changes in release, suggesting that is it not a major player (Schlüter et al., 1999; Deak et al., 2006).

We then searched for additional Rab3-effector proteins, defined by the GTP-dependent binding to Rab3 but not other major Rabs. We identified RIMs (for 'Rab3-interacting molecules'), a family of large multi-domain active zone proteins that are evolutionarily conserved (Wang et al., 1997 and 2000). In mammals, four RIM-related genes are expressed, of which only two (*RIMS1* and *RIMS2*) produce proteins that contain the Rab3-binding domain (Wang et al., 2000 and 2002). The RIMS1 and RIMS2 genes, however, include multiple independent promoters, resulting in five principal forms (RIM1α, RIM1β, RIM2α, RIM2β, and RIM2γ) that are further diversified by extensive alternative splicing.

Subsequent studies extending over 15 years revealed that RIMs perform multiple functions in the active zone which extend far beyond their role as Rab3-effectors. As we will see below, RIMs are critical not only for tethering/docking synaptic vesicles, but also for recruiting Ca^{2+}-channels to the active zone, for mediating short- and long-term presynaptic plasticity, and for activating the priming function of Munc13 proteins. As regards the tethering/docking function of RIMs that was suggested by their active zone localization and Rab3-binding, this function was first validated in C. elegans, which contains only a single RIM gene (referred to as *unc10*; Koushika et al., 2001; Gracheva et al., 2008). However, in mice deletions of single RIM isoforms, including that of the predominant RIM1α, did not detectably alter vesicle docking as analyzed by conventional electron microscopy (Schoch et al., 2002), but double conditional knockouts that deleted all isoforms produced by the *RIMS1* and *RIMS2* genes exhibited a dramatic decrease in vesicle docking (Kaeser et al., 2011; Han et al., 2011). Based on these studies, it is plausible that synaptic vesicles are tethered ('docked') to active zones via a GTP-dependent binding of active zone RIM proteins to synaptic vesicle Rab3/27 proteins.

It should be noted that no other proteins besides RIMs were found to be essential for synaptic vesicle docking when such docking was analyzed in electron micrographs of chemically fixed and traditionally stained sections. However, a completely different picture emerges when electron microscopy is performed on unfixed, rapidly frozen tissue—now, a large number of additional genes were found to be essential for 'docking'. In such preparations, even the single RIM1α

knockout exhibits a docking phenotype. However, it is implausible that so many proteins tether vesicles without redundancy, and these phenotypes may more closely reflect priming than docking. Thus, although multiple molecules can contribute to the stable attachment of synaptic vesicles to the active zone, only RIMs appear to be truly required for docking. It should also be noted that 'docking' of secretory granules in chromaffin cells behaves differently from docking of synaptic vesicles at the active zone. For example, the Syt1 KO blocks secretory granule docking (de Wit et al., 2009) but not synaptic vesicle docking (Geppert et al., 1994). However, the Syt1 KO has only a small effect on Ca^{2+}-triggered exocytosis in chromaffin cells in contrast to its large effect on synaptic exocytosis, probably because Syt1 is fully redundant with Syt7 in chromaffin exocytosis but not in synaptic exocytosis (Xu et al., 2007; Schonn et al., 2008). This discrepancy between docking and exocytosis suggests that different from synapses, docking may not even be essential for exocytosis in chromaffin cells.

Priming vesicles for fusion

Priming is thought to transfer vesicles into a readily-releasable pool (RRP) of vesicles that are then competent for Ca^{2+}-triggered fusion. A large number of proteins have been implicated in priming. In addition to those proteins that are involved in fusion itself (e.g., SNARE and SM proteins) and to complexin, the most important priming factors are probably Munc13 and RIMs that bind to each other.

Analyses largely carried out in Nils Brose's and Josep Rizo's laboratories revealed that Munc13 is essential for vesicle priming, probably because it catalyzes SNARE-complex assembly via its MUN domain (Augustin et al., 1999; Varoqueaux et al., 2002). The purified MUN domain can facilitate the opening of 'closed' Syntaxin-1 for subsequent SNARE-complex assembly, providing a mechanism for the phenotypes observed in mutant mice (Ma et al., 2011). A striking observation is that Munc13 function is tightly regulated by multiple signaling pathways. Among others, neuronal Munc13 isoforms contain a C1-domain N-terminal to the central Ca^{2+}-binding C2-domain. The Munc13 C1-domain binds diacylglycerol physiologically, and is activated pharmacologically by phorbol esters (Betz et al., 1998). Diacylglycerol binding to the Munc13 C1-domain regulates synaptic function since mouse mutants lacking phorbol ester binding to Munc13-1 exhibit a dramatic impairment in priming and short-term plasticity (Rhee et al., 2002). The Ca^{2+}-binding C2-domain of Munc13s is equally important since it also significantly contributes to short-term plasticity

of synapses (Shin et al., 2010). Finally, Munc13s bind to calmodulin which additionally modulates its function (Lipstein et al., 2013).

Deletions of RIMs also cause a major impairment in priming (Schoch et al., 2002; Koushika et al., 2001). The mechanism of this impairment, however, seems to be indirect because RIMs bind to Munc13s and activate Munc13 function (Deng et al., 2011). Specifically, the N-terminal sequence of RIMs includes a zinc-finger motif that avidly binds to the N-terminal Ca^{2+}-independent C2-domain of Munc13 (Dulubova et al., 2005; Lu et al., 2006). Without such binding, the Munc13-1 C2-domain forms a constitutive homodimer; upon RIM zinc-finger binding, the homodimer is converted into a RIM-Munc13 heterodimer. Strikingly, we found that the priming impairment in RIM-deficient synapses can be at least partly suppressed by overexpression of an N-terminally truncated Munc13-1 mutant that lacks the N-terminal C2-domain and no longer homodimerizes, whereas overexpression of wild-type Munc13-1 has no effect (Deng et al., 2011). These observations portray at least one mechanism by which RIMs regulate the priming function of Munc13, consistent with an overall central function of RIMs in all active zone activities.

Recruiting Ca^{2+}-channels to the active zone

In order to achieve fast synchronous neurotransmitter release that is precisely coupled to an action potential, the most important requirement is that Ca^{2+}-channels are localized at the active zone adjacent to docked and primed synaptic vesicles. Only such an arrangement produces the short Ca^{2+}-diffusion pathways required for the requisite speed of a synapse, and only a short Ca^{2+}-diffusion path can explain how the extremely brief presynaptic Ca^{2+}-transient triggers release—after all, the Ca^{2+}-sensors for neurotransmitter release and neuroendocrine exocytosis are the same, even though the latter are much slower than the former.

A molecular mechanism that explains how synapses achieve the required arrangement of Ca^{2+}-channels and synaptic vesicles emerged with the demonstration that RIMs collaborate with their binding partners RIM-BPs to recruit Ca^{2+}-channels to release sites (Kaeser et al., 2011; Fig. 13). Since RIMs are also the tethering agents for synaptic vesicles and contribute crucially to vesicle priming, RIMs are thus the central elements in the organization of the active zone that enable the amazing properties of neurotransmitter release. This simple architecture of the active zone, whereby a single protein is the central agent in assembling all components at one location, is at the same time parsimonious and effective (Fig. 14).

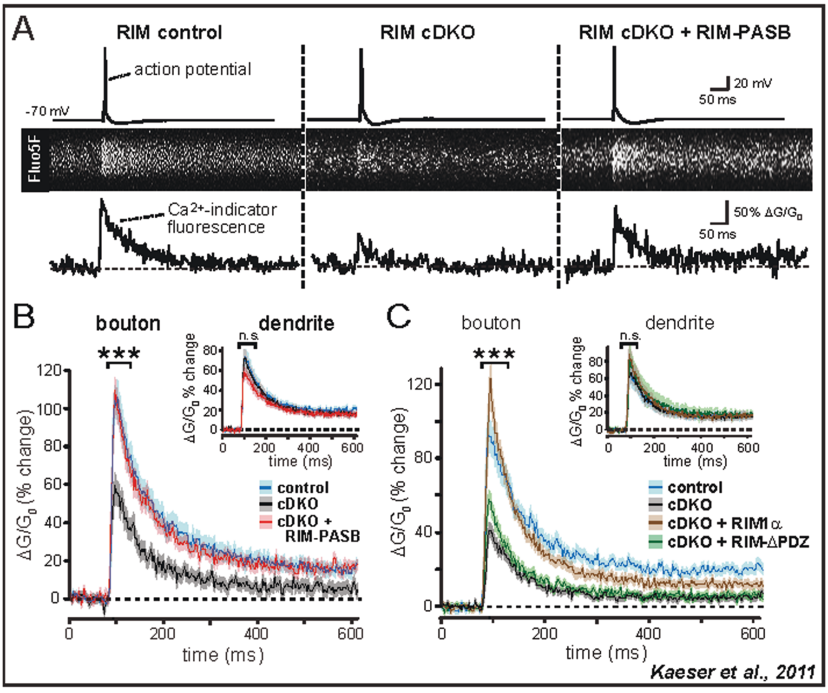

FIGURE 13. *RIM deletion decreases presynaptic Ca^{2+}-transients*

A. Isolated action potentials cause a rise in presynaptic Ca^{2+}-concentrations that is impaired by deletion of RIM proteins (RIM cDKO) but can be rescued by expression of a RIM1 fragment which binds to Ca^{2+}-channels (RIM-PASB). Data show representative traces of action potentials (top); line scans of Ca^{2+}-transients in presynaptic boutons induced by these action potentials, and monitored by fluorescence of the Ca^{2+}-indicator Fluo5F (middle); and quantitations of Ca^{2+}-transients (bottom).

B. Summary plots of the time course of the intracellular Ca^{2+}-concentration in presynaptic terminals and in dendrites (inset) during an action potential. Data show average Ca^{2+}-concentrations monitored as shown in A in multiple independent experiments in control neurons, neurons lacking RIM proteins (cDKO), and RIM-deficient neurons that express a RIM fragment binding to Ca^{2+}-channels (cDKO + RIM-PASB).

C. Same as B, except that rescue of impaired Ca^{2+}-transients in RIM-deficient synapses was tested for full-length wild-type RIM1α or for full-length RIM1α lacking the PDZ-domain.

All images are from Kaeser et al. (2011).

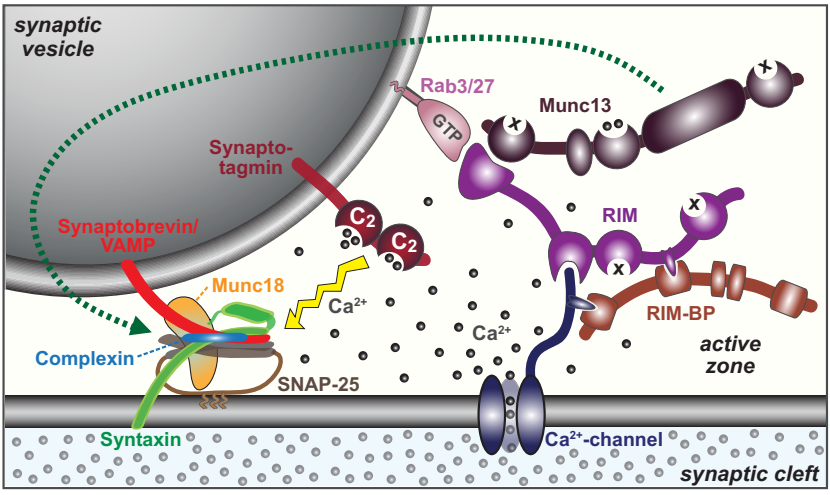

FIGURE 14. *Schematic diagram of the RIM, RIM-BP, and Munc13 protein complex that binds simultaneously to Rab3/27 on the synaptic vesicle and to Ca^{2+}-channels on the plasma membrane, thereby mediating the tethering ('docking') of vesicles at release sites, the priming of vesicles for release (arrow with dotted line), and the recruitment of Ca^{2+}-channels adjacent to tethered vesicles.* RIM, RIM-BP, and Munc13 are multidomain proteins that form a tight complex which mediates three essential functions of active zones: recruitment of Ca^{2+}-channels to enable tight coupling of action potentials to release by localizing Ca^{2+}-influx next to the Ca^{2+}-sensor synaptotagmin; docking of vesicles at the release site; and Munc13-dependent priming of the fusion machinery composed of the SNARE syntaxin, SNAP-25, synaptobrevin/VAMP, and Munc18-1. Spheres denote Ca^{2+}-ions; of the domains shown, only C2-domains are specifically labeled. Other active zone proteins bind to the RIM/Munc13/RIM-BP complex such as α-liprins and ELKS, and contribute to release but are not shown. Modified from Kaeser et al. (2011) and Südhof (2012).

We found that RIMs directly and selectively bind to Ca^{2+}-channels expressed in presynaptic active zones. Similar to the identification of the role of RIMs in vesicle tethering/docking, however, identification of the role of RIMs in recruiting Ca^{2+}-channels to the active zone only became possible when we deleted all RIM isoforms from presynaptic terminals (Kaeser et al., 2011). We found that deletion of RIMs causes a decrease of presynaptic Ca^{2+}-influx, a loss of presynaptic Ca^{2+}-channels, and a loss of the tight coupling of a presynaptic action potential to release (Fig. 13; Han et al., 2011; Kaeser et al., 2011 and 2012). RIMs perform their functions by forming a large complex with the Ca^{2+}-channels, with other active zone proteins such as RIM-BPs (which in turn also bind to Ca^{2+}-channels) and Munc13-1, and with synaptic vesicles. The role of RIMs and RIM-BPs in recruiting Ca^{2+}-channels and docking vesicles to active zones is

evolutionarily conserved (Liu et al., 2011; Graf et al., 2012), and represents a fundamental mechanism underlying synaptic transmission.

6. PUTTING IT ALL TOGETHER

The three levels of release that we have been studying—membrane fusion, Ca^{2+}-triggering of fusion, and the organization of the Ca^{2+}-controlled fusion machinery at the active zone—form a hierarchy of interdependent processes. Like a Russian doll, these three levels are nestled into each other, with membrane fusion as the inner core, and the scaffolding organizing the various components into a single machine as the outer layer. Our work, together with that of others, uncovered a plausible mechanism explaining how the synaptic vesicle membrane and the plasma membrane undergo rapid fusion during neurotransmitter release, how such fusion is triggered by Ca^{2+}, and how those processes are spatially organized in the presynaptic terminal, such that opening of Ca^{2+}-channels by an action potential allows rapid translation of the entering Ca^{2+} signal into a fusion event.

Together, the neurotransmitter release machinery that we uncovered accounts for the astounding speed and precision of Ca^{2+}-triggered release. Moreover, the overall design of this machinery and the identification of regulatory domains in it suggest mechanisms to explain the dramatic short- and long-term plasticity of release that plays a central role in determining circuit properties. Nevertheless, many crucial questions remain. For example, what are the physicochemical mechanisms underlying membrane fusion, how precisely do SNARE and SM proteins work, what is the role of the fusion machine as outlined here in disorders like Parkinson's disease, how do presynaptic terminals undergo long-term structural changes during plasticity, and what is the role of plasticity in long-term memory? Moreover, what mechanisms render various types of synapses different from each other—why do inhibitory synapses for example often exhibit a higher release probability than excitatory synapses, and what mechanisms confer distinct forms of plasticity onto different types of synapses? How is the presynaptic active zone precisely aligned with the postsynaptic density, and how is the size of a synapse regulated? Much remains to be done, and I hope to see at least some of these intriguing questions addressed in my lifetime!

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