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Neurotransmitter Release: The Last Millisecond in the Life of a Synaptic Vesicle

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Abstract

During an action potential, Ca^{2+} entering a presynaptic terminal triggers synaptic vesicle exocytosis and neurotransmitter release in less than a millisecond. How does Ca^{2+} stimulate release so rapidly and precisely? Work over the last decades revealed that Ca^{2+}-binding to synaptotagmin triggers release by stimulating synaptotagmin-binding to a core machinery composed of SNARE and SM proteins that mediates membrane fusion during exocytosis. Complexin adaptor proteins assist synaptotagmin by activating and clamping this core fusion machinery. Synaptic vesicles containing synaptotagmin are positioned at the active zone, the site of vesicle fusion, by a protein complex containing RIM proteins. RIM proteins simultaneously activate docking and priming of synaptic vesicles and recruit Ca^{2+}-channels to active zones, thereby connecting in a single complex primed synaptic vesicles to Ca^{2+}-channels. This architecture allows direct flow of Ca^{2+}-ions from Ca^{2+}-channels to synaptotagmin, which then triggers fusion, thus mediating tight millisecond coupling of an action potential to neurotransmitter release.

INTRODUCTION

Arguably, Emil du Bois-Raymond (1818-1896) initiated modern neuroscience with the discovery of the action potential and of synaptic transmission at the neuromuscular junction. 100 years later, Bernard Katz (1911-2003), working also on the neuromuscular junction, established the fundamental pathway of synaptic transmission whereby an action potential arrives at a presynaptic nerve terminal and gates Ca^{2+}-channels; the inflowing Ca^{2+} then triggers the exocytotic fusion of synaptic vesicles containing neurotransmitters, and the released neurotransmitters subsequently produce a postsynaptic signal (Katz, 1969). Katz's brilliant work built on George Palade's (1912-2008) studies on vesicular trafficking (Palay and Palade, 1955), and initiated a series of elegant electrophysiological experiments that characterized the process of synaptic transmission in exquisite detail. Among others, these studies revealed that Ca^{2+} triggers release in a highly cooperative manner (Dodge and Rahamimoff, 1967) within a few hundred microseconds (Sabatini and Regehr, 1996), which is not much slower than the opening of a voltage-gated ion channel. What molecular mechanisms enable fast vesicle fusion at a synapse, however, remained a mystery until molecular biology allowed mechanistic dissection of vesicle fusion and its control by Ca^{2+} (reviewed in Südhof and Rothman, 2009).

Katz's work posed three basic questions:

- How do vesicles fuse? This general question transcends neurobiology, and is important for all areas of vesicle traffic and cell biology since membrane fusion is a universal process in eukaryotic cells.
- How does Ca^{2+} induce rapid membrane fusion? This question is crucial for understanding synaptic transmission, and also relevant for other types of Ca^{2+}-regulated exocytosis, such as hormone secretion, mast cell exocytosis, and oocyte fertilization. Moreover, synapses exhibit diverse properties, and synaptic transmission is not a digital yes-or-no event but plastic. Thus, understanding how Ca^{2+} controls release additionally pertains to how neural circuits operate.
- How are Ca^{2+}-influx and vesicle docking co-localized to presynaptic release sites, and how are they coordinated with priming of vesicles for Ca^{2+}-triggered release? The last millisecond in the life of a synaptic vesicle depends on tight co-localization of primed vesicles with Ca^{2+}-channels – only their close proximity at release sites enables precise coupling of an action potential to release.

These three questions lie at the heart of a molecular understanding of synaptic transmission. As described below, we now have a plausible framework of answers to these questions, although much remains to be done.

In the following, I will first provide a brief broad outline of the general release machinery (Fig. 1), and then discuss in greater detail selected questions that in my personal view are particularly interesting. Due to space constraints, I do not aim to provide a comprehensive discussion of the field, and I apologize for the many omissions I am bound to commit. Moreover, owing to the same space constraints I will focus on physiological studies. In particular, I am unable to give appropriate consideration to the many elegant liposome fusion studies that have recently been performed; for a more complete treatment of this subject, please see Brunger et al. (2009) and Marsden et al. (2011).

A MOLECULAR FRAMEWORK FOR RELEASE

Work over the lifetime of *Neuron* – two and a half decades! – has produced a general framework for understanding neurotransmitter release that will be briefly summarized below (Fig. 1; see also reviews by Rizo and Rosenmund, 2008; Kochubey et al., 2011; Mohrmann and Sørensen, 2012).

Fusion

Intracellular membrane fusion is generally mediated by SNARE proteins (for 'soluble NSF attachment receptor proteins') and SM proteins (for 'Sec1/Munc18-like proteins') that undergo a cycle of association and dissociation during the fusion reaction (Fig. 2). At the synapse, the vesicular SNARE protein **synaptobrevin** (a.k.a. **VAMP**) forms a complex with the plasma membrane SNARE proteins **syntaxin-1** and **SNAP-25** (Söllner et al., 1993a). Prior to SNARE-complex formation, syntaxin-1 is present in a closed conformation that cannot engage in SNARE-complex formation; syntaxin-1 has to open for SNARE-complex assembly to proceed (Dulubova et al., 1999; Misura et al., 2000). The SM protein **Munc18-1** initially binds to the closed conformation of syntaxin-1 (Hata et al., 1993; Dulubova et al., 1999). When the closed conformation of syntaxin-1 'opens' in preparation to fusion and SNARE complexes form, Munc18-1 remains attached to syntaxin-1 in the assembling SNARE complex, but switches its binding mode to an interaction with the SNARE complex (Dulubova et al., 2007). Assembly of these SNARE/SM complexes mediates fusion, whereas disassembly of these complexes recycles SNARE and SM proteins for further use (Fig. 2; reviewed in Südhof and Rothman, 2009). The continued association of Munc18-1 to

SNARE complexes throughout their assembly/disassembly cycle is essential for fusion (Khvotchev et al., 2007; Zhou et al., 2013a). SNARE/SM-complex assembly is maintained by chaperones whose dysfunction causes neurodegeneration (**CSPs** and **synucleins**; Burre et al., 2010; Sharma et al., 2011), whereas disassembly is mediated by an evolutionarily conserved specialized ATPase (**NSF**) and its adaptors (**SNAPs**; Söllner et al., 1993b).

The underlying principle of SNARE and SM protein function is simple (Fig. 2): SNARE proteins embedded in the two fusing membranes form a trans-complex that involves a progressive zippering of the four-helical SNARE complex in an N- to C-terminal direction (Hanson et al., 1997). Zippering of trans-SNARE complexes forces the fusing membranes into close proximity, destabilizing their hydrophilic surfaces. Assembly of the full trans-SNARE complex together with the action of the SM protein opens the fusion pore. Fusion-pore expansion transforms the initial 'trans'-SNARE complexes into 'cis'-SNARE complexes that are then dissociated by NSF (that binds to SNARE complexes via SNAP adapter proteins), completing the cycle.

In a physiological context, SNARE-complex assembly alone does not mediate fusion. We initially proposed that the SM protein Munc18-1 is an essential component of the core fusion machinery (Hata et al., 1993). This proposal was supported by pioneering work from the Novick laboratory demonstrating that the yeast SM protein Sec1p (the Munc18-1 homolog in yeast exocytosis) binds to assembled SNARE complexes (Carr et al., 1999). Moreover Novick and colleagues showed that Sec1p mutations completely block fusion but do not impair SNARE-complex assembly (Grote et al., 2000). However, the proposal that Munc18-1 activates fusion met resistance because Munc18-1 binding to closed syntaxin-1 appeared to suggest that Munc18-1 is an inhibitor of fusion (Yang et al., 2000), despite the fact that Munc18-1 is absolutely required for synaptic fusion (Verhage et al., 2000). This issue was only resolved over the last decade when studies revealed that Munc18-1 remains associated during fusion with SNARE proteins throughout their assembly/disassembly cycle, thereby removing the notion of an inhibitor (Dulubova et al., 2007; Shen et al., 2007). Subsequent data showed that Munc18-1 binding to assembling SNARE complexes is essential for synaptic vesicle fusion, whereas Munc18-1 binding to closed syntaxin-1 is not (Khvotchev et al., 2007; Gerber et al., 2008; Deák et al., 2009; Zhou et al., 2013a).

In every SNARE-dependent fusion reaction studied, an SM protein participates and is essential for that fusion reaction. Although SM proteins always appear to bind to SNARE complexes, the molecular basis of their interactions varies. For example, several SM proteins (Munc18-1, Vps45, and Sly1) bind to the N-terminal 'N-peptide' of their cognate syntaxins (Yamaguchi et al., 2003; Dulubova et al., 2003), but the yeast SM protein Sec1p binds to assembled SNARE complexes independent of the syntaxin-1 N-peptide (Carr et al., 1999).

The role of NSF and SNAPs in dissociating and activating SNARE proteins prior to fusion was recognized early on (Söllner et al., 1993b; Mayer et al., 1996). More recently, two chaperones that maintain SNARE-protein function during multiple association/dissociation cycles were identified. CSPs are evolutionarily conserved co-chaperones containing a DNA-J domain that form a catalytically active complex with Hsc70 and SGT (for 'small glutamate-rich tetratricopeptide repeat protein'). The CSPα/Hsc70/SGT complex prevents misfolding of monomeric SNAP-25, thereby enabling SNAP-25 to engage in SNARE complexes (Sharma et al., 2011a). Synucleins are small soluble vertebrate proteins that increase SNARE-complex assembly by a non-classical chaperone activity (Burre et al., 2010). Loss of CSPα in mice causes fulminant neurodegeneration in neurons because of SNAP-25 misfolding and impaired SNARE-complex formation (Sharma et al., 2011b). This neurodegeneration can be fully rescued by modest increases in α-synuclein levels which

indirectly enhance SNARE-complex assembly and thereby rescue the CSPα knockout phenotype (Chandra et al., 2005).

Ca^{2+}-triggering of fusion-pore opening

Synaptotagmins are evolutionarily conserved transmembrane proteins with two cytoplasmic C_2-domains (Perin et al., 1990 and 1992; Fig. 2) that bind Ca^{2+} (Brose et al., 1992). C_2-domains were initially defined in protein-kinase C isozymes as a conserved sequence of unknown function. Studies on synaptotagmin-1 (Syt1) showed that C2-domains constitute autonomously folding Ca^{2+}/phospholipid-binding domains (Perin et al., 1990; Davletov and Südhof, 1993; Sutton et al., 1995). In addition, C2-domains constitute protein-interaction domains, and in the case of Syt1 bind to syntaxin-1 and to SNARE-complexes (Bennett et al., 1992; Söllner et al., 1993b; Li et al., 1995).

Although Syt1 was proposed to constitute Katz's long-sought Ca^{2+}-sensor for fast neurotransmitter release when it was cloned (Perin et al., 1990), initial experiments in C. elegans and Drosophila disappointingly indicated otherwise (Nonet et al., 1993; Di Antonio et al., 1993; Littleton et al., 1993). Electrophysiological analyses of Syt1 knockout mice, however, revealed that Syt1 is selectively essential for fast Ca^{2+}-triggered release in forebrain neurons, but is not required for fusion as such (Geppert et al., 1994).

The Syt1 KO analysis 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, see below), with Ca^{2+}-binding to Syt1 performing an unrelated role (Neher and Penner, 1994). Experiments with knock-in mice, however, proved that Ca^{2+}-binding to Syt1 triggers neurotransmitter release (Fernandez-Chacon et al., 2001; Sørensen et al., 2003; Pang et al., 2006). Introduction into the endogenous mouse Syt1 gene of a point mutation that decreased the Syt1 Ca^{2+}-binding affinity ~2-fold also decreased the Ca^{2+}-affinity of neurotransmitter release ~2-fold. In addition to mediating Ca^{2+}-triggering of release, Syt1 clamps mini release (Littleton et al., 1993; Xu et al., 2009), thus serving as an essential mediator of the speed and precision of release by association with SNARE complexes.

Sixteen synaptotagmins are expressed in brain, 8 or which bind Ca^{2+}. All initial functional studies were carried out with Syt1, but further analyses revealed that Syt2 and Syt9 also act as Ca^{2+}-sensors for synchronous synaptic vesicle exocytosis, albeit with different kinetics that correspond to the synapses in which these synaptotagmins are expressed (Xu et al., 2007). For example, Syt2 as the fastest synaptotagmin is expressed in the neurons mediating sound localization which requires extremely fast synaptic responses (Sun et al., 2007), whereas Syt9 is the slowest synaptotagmin that is primarily expressed in the limbic system mediating slower emotional responses (Xu et al., 2007).

Synaptotagmins do not act alone in fusion, but require **complexin** as a co-factor. Complexin was discovered by virtue of its tight binding to SNARE complexes (McMahon et al., 1995). Complexin-deficient neurons exhibit a milder phenocopy of Syt1-deficient neurons, with a selective suppression of fast synchronous exocytosis and an increase in spontaneous exocytosis (Reim et al., 2001). Complexin functions as a priming factor for SNARE complexes, as an activator of these SNARE complexes for subsequent synaptotagmin action, and as a clamp of spontaneous release (Giraudo et al., 2006; Tang et al., 2006; Xue et al., 2007; Maximov et al., 2009; Martin et al., 2011; Hobson et al., 2011; Kaeser-Woo et al., 2012; Jorquera et al., 2012).

Synaptotagmins also act as Ca^{2+}-sensors for other Ca^{2+}-dependent fusion reactions. For example, Syt1 and Syt7 are Ca^{2+}-sensors for catecholamine and peptide hormone secretion

(Schonn et al., 2008; Gustavsson et al., 2008 and 2009), and Syt2 is a Ca^{2+}-sensor for mast cell exocytosis (Melicoff et al., 2009). Moreover, experiments in olfactory neurons uncovered a role for Syt10 as a Ca^{2+}-sensor for IGF-1 exocytosis that differs from the Ca^{2+}-sensor function of Syt1 in synaptic vesicle and neuropeptide vesicle exocytosis (Cao et al., 2011). Thus, even in a single neuron, different synaptotagmins can act as Ca^{2+}-sensors for distinct Ca^{2+}-triggered fusion reactions. All synaptotagmin-controlled fusion reactions appear to require complexin as a co-factor (e.g., see Reim et al., 2001; Cai et al., 2008; Jorquera et al., 2012; Cao et al., 2013). It seems likely that all Ca^{2+}-triggered exocytosis depends on synaptotagmin Ca^{2+}-sensors and complexins, and that different synaptotagmins contribute to the specificity of exocytosis pathways.

Spatial organization of the release machinery

Ultrafast neurotransmitter release in response to an action potential can only be achieved by tethering Ca^{2+}-channels to docked and primed synaptic vesicles at the active zone. A large protein complex whose central components are three multidomain proteins called **RIM**, **RIM-BP**, and **Munc13** mediate the docking and priming of synaptic vesicles at the active zone and recruit Ca^{2+}-channels to the docked and primed vesicles (Kaeser et al., 2011). Thus, a single protein complex organizes release sites (Fig. 1; reviewed in Südhof, 2012).

RIM (for Rab3-interacting molecule; Wang et al., 1997) binds to small Rab3 and Rab27 GTP-binding proteins which are localized on synaptic vesicles, thereby docking the vesicles (Gracheva et al., 2008; Kaeser et al., 2011; Han et al., 2011; Fernández-Busnadiego et al., 2013). RIM also binds to Munc13 (no relation to Munc18; Brose et al., 1995; Betz et al., 2001), thereby activating Munc13 (Deng et al., 2011). Munc13 is a priming factor (Augustin et al., 1999) that catalyzes the conformational switch of syntaxin-1 from closed to open, promoting SNARE-complex assembly (Richmond et al., 2001; Ma et al., 2013). RIM-binding to both Munc13 and Rab3/27 is mediated by a composite N-terminal domain that contains a Munc13-binding zinc-finger surrounded by Rab3-binding α-helices (Dulubova et al., 2005; Lu et al., 2006).

Ca^{2+}-channels need to be localized adjacent to docked and primed vesicles for fast coupling of an action potential to Ca^{2+}-triggered exocytosis. Ca^{2+}-channels are generally positioned less than 100 nm away from docked vesicles (Eggermann et al., 2011). RIM and the RIM-interacting molecule RIM-BP (Wang et al., 2000) both bind to Ca^{2+}-channels in addition to binding to each other (Kaeser et al., 2011). Deletion of RIM in mice (Kaeser et al., 2011 and 2012; Han et al., 2011) and of RIM-BP in flies (Liu et al., 2011) causes a loss of Ca^{2+}-channels from presynaptic active zones, and a decrease in Ca^{2+}-influx. These data show that RIM and RIM-BP collaborate to recruit Ca^{2+}-channels to release sites. Thus, in a parsimonious design a single protein complex that contains RIM as a central element mediates the co-localization of all critical proteins to the active zone. This protein complex localizes synaptic vesicles, Ca^{2+}-channels, and vesicle priming factors next to release sites, thereby allowing fast coupling of an action potential to neurotransmitter release (Fig. 1C).

As sketched out above, we can now broadly account for the mechanism, speed, and regulation of neurotransmitter release. However, our understanding resembles an unfinished house with no plumbing and holes for windows, and raises major new questions. Below, I will briefly discuss those questions about fusion and Ca^{2+}-triggering that seem most important to me personally, and apologize for the rather incomplete treatment of the issues. Although fascinating advances were recently made in understanding the active zone, space constraints prevent me from discussing these findings and the new questions that now arise in this subject.

SETTING UP MEMBRANE FUSION

To set the stage for fast Ca^{2+}-triggering of release, the synaptic vesicle fusion machinery is primed into an energized, metastable state (Fig. 3A). Ca^{2+}-binding to synaptotagmin then triggers fusion-pore opening by acting on the metastable primed fusion machinery. The nature of priming, however, and the mechanism of fusion remain debated.

Partial SNARE-complex assembly may precede Ca^{2+}-triggering of exocytosis

Elegant studies in neurons, chromaffin cells, and liposomes showed that the energy released by assembly of only 1-3 SNARE complexes is sufficient to drive fusion (Hua and Scheller, 2001; van den Bogaart et al., 2010; Mohrmann et al., 2010; Sinha et al., 2011; Shi et al., 2012). However, careful quantifications by Jahn and colleagues showed that SNARE proteins are very abundant, with approximately 70 synaptobrevin molecules per vesicle (Takamori et al., 2006), indicating that physiological fusion is effected by assembly of many SNARE complexes.

A plausible model for priming posits that SNARE complexes are partially assembled to elevate a synaptic vesicle into an energized pre-fusion state (Fig. 3). This model is supported by significant evidence, but not proven (Jahn and Fasshauer, 2012). Complexin only binds to partly or fully assembled SNARE complexes (McMahon et al., 1995), and complexin binding to SNARE complexes is essential for priming and activating synaptic vesicle fusion (Cai et al., 2008; Maximov et al., 2009; Yang et al., 2010; Hobson et al., 2011). Thus, at least partly assembled SNARE complexes must be present prior to fusion to which complexin can bind. Moreover, Munc13 converts syntaxin-1 from a closed to an open conformation and is selectively required for synaptic vesicle priming upstream of fusion and of Ca^{2+}-triggering of release (Augustin et al., 1999; Richmond et al., 2001; Varoqueaux et al., 2002; Ma et al., 2013), again suggesting that SNARE complexes are at least partly assembled prior to fusion. Furthermore, t-SNARE complexes composed of ‘open’ syntaxin-1 and SNAP-25 can be visualized in native axonal membranes, and thus exist before Ca^{2+} triggers neurotransmitter release (Pertsinidis et al., 2013). Finally, Ca^{2+} can trigger synaptic vesicle fusion in less than 100 microseconds (Sabatini and Regehr, 1996), a time period that appears insufficient to accommodate opening of syntaxin-1, formation of SNARE complexes, and Ca^{2+}-triggering of fusion by synaptotagmin.

Of these four lines of evidence supporting patial assembly of SNARE complexes during priming, the time argument is the least compelling. Conceivably, Ca^{2+}-binding to synaptotagmin and formation of SNARE complexes could occur from an undefined intermediate and may be very fast (Jahn and Fasshauer, 2012). However, the required functions of complexin and Munc13 in priming upstream of Ca^{2+}-triggering are not easily explained by a model that postulates an action of Ca^{2+} upstream of SNARE-complex assembly, suggesting that SNARE complexes are at least partly pre-assembled prior to fusion.

How do SNAREs mediate fusion?

How precisely full SNARE-complex assembly induces fusion-pore opening is unclear, as is the role of SM proteins in fusion. Although only a few SNARE complexes are needed for fusion (Hua and Scheller, 2001; van den Bogaart et al., 2010; Mohrmann et al., 2010; Sinha et al., 2011; Shi et al., 2012), physiological synaptic vesicle fusion may involve tens of SNARE complexes. It seems likely that the number of SNARE complexes per vesicle has an effect on the speed and Ca^{2+}-dependence of neurotransmitter release because synaptotagmin acts on assembling SNARE complexes, and mass action law predicts that this interaction depends on the concentration of the substrate. Thus, it would be interesting to probe the

effect of changes in the number of SNARE complexes per vesicle on the properties of release. Indeed, we recently found that increasing SNARE-complex assembly by constitutively opening syntaxin-1 dramatically increases the speed of fusion-pore opening and decreases the amount of Ca^{2+} required to trigger fusion-pore opening (Acuna et al., submitted).

How does SNARE-complex assembly act on the membranes in which the SNAREs reside? Do SNARE proteins primarily pull membranes together, or is the force generated by SNARE-complex assembly transferred onto the SNARE transmembrane regions, such that the transmembrane regions mediate lipid mixing during fusion and/or form the fusion pore? Physiologically, increasing the distance between the SNARE motif and the transmembrane region within synaptobrevin impairs neurotransmitter release (Deak et al., 2006; Kesavan et al., 2007; Guzman et al., 2010). Similarly, adding only 3 residues to the linker separating the transmembrane region from the SNARE motif in syntaxin-1 severely impairs Ca^{2+}-triggered fusion (Zhou et al., 2013b). Thus, transferring of the force generated by SNARE complex assembly onto the membrane is essential.

In a test of the role of the SNARE transmembrane regions in fusion at a synapse, we recently found that SNAREs lacking a transmembrane region on both the plasma membrane (syntaxin-1) and the synaptic vesicle (synaptobrevin) are still competent for fusion (Zhou et al., 2013b). Lipid-anchored SNAREs fully substituted for regular SNAREs containing a transmembrane region in spontaneous vesicle fusion, but were less efficient in mediating Ca^{2+}-triggered fusion. Interestingly, although the transmembrane region was dispensable, the distance of the SNARE motif from the membrane anchor continued to be crucial in lipid-anchored syntaxin-1. A 3-residue insertion into lipid-anchored syntaxin-1 severely impaired Ca^{2+}-triggered fusion, suggesting that the mechanism of fusion is the same for syntaxin-1 independent of whether it contains a lipid anchor or a transmembrane region (Zhou et al., 2013b).

Viewed together, these data suggest that SNARE transmembrane regions may not directly form a fusion pore, but serve as membrane anchors. These data simplify our view of how SNAREs work by reducing their activity to that of a force generator that pulls membranes together in a vertical but not horizontal direction with respect to the plane of the membranes.

What do SM proteins do in fusion?

Deletion of Munc18-1 completely blocks synaptic vesicle fusion during exocytosis, and neurons subsequently degenerate (Verhage et al., 2000). No other protein's deletion (including deletion of any SNARE protein) produces a comparably severe block of fusion. Moreover, in yeast deletion of the SM protein that mediates exocytosis – Sec1p – also completely blocks fusion (Julius et al., 1984; Grote et al., 2000).

Several hypotheses have been advanced for SM protein function in fusion, which may be the most important unsolved question in understanding fusion. Here, I would like to propose a simple parsimonious hypothesis that arguably accounts for all available data and is consistent with the essential function of SM proteins in fusion (Fig. 3B). This hypothesis is suggested by the pioneering work of the Novick laboratory on yeast Sec1p (Carr et al., 1999; Grote et al., 2000), and proposes that SNARE proteins force fusing membranes into close proximity while SM proteins, riding on top of assembling SNARE complexes, enable lipid mixing between the fusing membranes (Fig. 3B).

The hypothesis that SM proteins mediate lipid mixing during fusion provides a parsimonious explanation for how fusion may work physiologically. It is consistent with the finding that fusion requires continuous association of Munc18-1 and Sec1p with SNAREs

after SNARE-complex assembly has started (Khvotchev et al., 2007; Zhou et al., 2013a; Grote et al., 2000a), and agrees with the observation that SNARE transmembrane regions are not essential for fusion (Zhou et al., 2013b). An apparent contradiction to this hypothesis is the fact that Munc18-1 is not required for SNARE-mediated liposome fusion (Weber et al., 1997). However, the lack of a requirement for Munc18-1 in liposome fusion contradicts the universal necessity for SM proteins in physiological SNARE-dependent fusion reactions, and may be due to differences between biological membranes and liposomes. Biological membranes contain high concentrations of both intrinsic and peripheral membrane proteins, and may require an activator of lipid mixing for fusion beyond the proximity of the phospholipid membrane surfaces provided by SNARE-complex assembly. SM proteins may enable lipid mixing by organizing lipid patches adjacent to SNARE membrane anchors, such that the action of the SNAREs on the membrane allows exposed lipids to become destabilized for fusion, or may actually promote lipid mixing. Indeed, recent experiments uncovered a strong fusion-promoting role of SM proteins even for liposome fusion (Shen et al., 2007; Diao et al., 2010; Rathore et al., 2010; Schollmeier et al., 2011; Yu et al., 2013).

The proposal that SM proteins act in fusion by enabling phospholipid mixing, riding on the assembling SNARE complexes, is at present only that – a hypothesis. Alternative, not necessarily mutually exclusive hypotheses are also plausible. Early on, the notion that SM proteins primarily inhibit fusion obtained significant support (Yang et al., 2000; Wu et al., 2001), but more recent experiments with in vivo and in vitro fusion reactions have argued against this notion (e.g., see Schollmeier et al., 2011; Rathore et al., 2010). More recently, the idea that Munc18-1 and other SM proteins catalyze SNARE-complex assembly, possibly by nucleating it, has received significant attention. This idea accounts for the binding of SM proteins to SNARE complexes. However, a sole function of SM proteins in promoting SNARE-complex assembly is difficult to reconcile with the essential role of SM proteins in fusion. A decreased rate of SNARE-complex assembly in the absence of an SM protein should decrease the rate of fusion, but not block fusion. Moreover, deletion of Sec1p in yeast blocks fusion but not SNARE complex assembly (Grote et al., 2000). Finally, other proteins are known to promote SNARE-complex assembly (e.g., Munc13 [Ma et al., 2013] and synucleins [Burre et al., 2010]). Although these data argue against a primary function of SM proteins as SNARE-complex assembly catalysts, it is quite possible that SM proteins also act to promote SNARE-complex assembly.

Besides the notion that SM proteins mediate fusion and/or catalyze SNARE complex assembly, a third plausible hypothesis for how SM proteins activate fusion is that they spatially organize assembled SNARE complexes around the fusion site (Rizo et al., 2006). This esthetically pleasing hypothesis also accounts for the binding of SM proteins to assembled SNARE complexes, and could be related to a lipid-mixing activity of SM proteins. However, fusion mediated by only 1-3 SNARE complexes (van den Bogaart et al., 2010; Mohrmann et al., 2010) presumably still requires Munc18-1, and thus according to this hypothesis an SM protein would organize isolated SNARE complexes for fusion.

Differentiating between these hypotheses and testing them will require novel assays that monitor lipid mixing with a high temporal resolution in an SM protein-dependent manner. Thus, answering the question of SM protein function in fusion remains a major challenge whose resolution requires not only a reductionist approach using liposome fusion, but also physiological tests of normal fusion reactions in a living cell.

HOW DOES Ca^{2+} TRIGGER FUSION IN MICROSECONDS?

As outlined above, the Ca^{2+}-sensor synaptotagmin and its assistant complexin transduce the presynaptic Ca^{2+}-signal for release. These proteins likely act on a primed fusion machinery

that is ready to go. However, the precise interplay of these two key players is incompletely understood.

Asynchronous neurotransmitter release

Although deletion of Syt1, Syt2, and Syt9 in vertebrate neurons, and of Syt1 in Drosophila neurons, blocks synchronous release, not all Ca^{2+}-dependent release is ablated (Geppert et al., 1994; Yoshihara and Littleton, 2002; Maximov and Südhof, 2005; Sun et al., 2007). In most synapses, the remaining Ca^{2+}-stimulated release is dramatically facilitated during action-potential bursts *in vitro* and *in vivo* (Xu et al., 2012). This remaining release is often referred to as 'asynchronous' because it lags after synchronous release and is not tightly coupled to an action potential.

Asynchronous release exhibits distinct properties in different types of neurons, and likely comprises multiple processes. Hippocampal Syt1 knockout neurons exhibit significant asynchronous release that is amplified by facilitation during action-potential trains (Maximov and Südhof, 2005), so much so that the total amount of asynchronous release in Syt1 knockout neurons becomes identical to that observed in wild-type neurons (Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004; Maximov and Südhof, 2005; Xu et al., 2012)! In contrast, Syt2 knockout synapses in the calyx of Held display relatively little asynchronous release that exhibits only modest facilitation during high-frequency stimulus trains (Sun et al., 2007). In yet another example for a difference between synapses, some neurons such as cholecystokinin-containing interneurons in the hippocampus use a facilitating type of asynchronous release as the dominant form of release even in wild-type conditions (Hefft and Jonas, 2005; Daw et al., 2009; Karson et al., 2009). These observations prompted the question, what is asynchronous release, and what Ca^{2+}-sensor mediates asynchronous release?

Studies in chromaffin cells provided the first clue to answering these questions. Earlier experiments had shown that deletion of Syt1 in chromaffin cells produced a small but significant decrease in Ca^{2+}-stimulated exocytosis and a delay in the rate of exocytosis (Sørensen et al., 2003). In a pivotal study, Schonn et al. (2008) then demonstrated that deletion of only Syt7, a Ca^{2+}-binding synaptotagmin that had previously been implicated as a Ca^{2+}-sensor in exocytosis in PC12 cells (Sugita et al., 2001; Fukuda et al., 2004) also produced a small decrease in Ca^{2+}-stimulated exocytosis in chromaffin cells. However, the double deletion of both Syt1 and Syt7 caused a dramatic ablation of nearly all Ca^{2+}-induced exocytosis (Schonn et al., 2008). This finding suggested that at least in chromaffin cells, Syt1 and Syt7 are redundant Ca^{2+}-sensors for exocytosis with distinct response kinetics.

Syt7 is also expressed at high levels in brain – even higher than Syt1 – and is localized to synapses (Sugita et al., 2001). However, initial studies to uncover a role for Syt7 in synaptic exocytosis using constitutive Syt1 and Syt7 knockout mice were disappointingly unsuccessful (Maximov et al., 2008). This situation changed with acute ablation of Syt1 or Syt7 on the background of constitutive deletions of Syt7 or Syt1, respectively (Bacaj et al., 2013). Such acute manipulations severely impaired not only synchronous but also asynchronous release in hippocampal neurons (Fig. 4). Consistent with the studies in PC12 and primary chromaffin cells, this result suggests that effectively all Ca^{2+}-triggered neurotransmitter release is mediated by a synaptotagmin. Moreover, this result agrees with studies indicating that Syt7 functions as a Ca^{2+}-sensor in neuroendocrine secretion and in lysosome exocytosis (Shin et al., 2002; Chakrabarti et al., 2003; Fukuda et al., 2004; Tsuboi and Fukuda, 2007; Schonn et al., 2008; Gustavsson et al., 2008 and 2009; Li et al., 2009; Segovia et al., 2010). Finally, a role for Syt7 as a Ca^{2+}-sensor in synaptic exocytosis agrees well with the similar Ca^{2+}-binding properties and Ca^{2+}-dependent phospholipid- and syntaxin-binding properties of Syt1 and Syt7 (Li et al., 1995; Sugita et al., 2002).

However, Syt7 exhibits two puzzling properties. First, in neurons Syt7 is not dectable in synaptic vesicles, but at least partly localized to the plasma membrane (Sugita et al., 2001; Takamori et al., 2006). This is puzzling given the localization of Syt7 to secretory vesicles in non-neuronal cells (Chakrabarti et al., 2003; Fukuda et al., 2004; Schonn et al., 2008; Gustavsson et al., 2008 and 2009). Second, whereas in all vesicular synaptotagmins tested up to date, the C2B-domain Ca^{2+}-binding sites are essential for Ca^{2+}-stimulation of exocytosis and the C2A-domain Ca^{2+}-binding sites only assist in Ca^{2+}-triggering of exocytosis (e.g., see Mackler and Reist, 2001; Nishiki and Augustin, 2004; Shin et al., 2009; Cao et al., 2011; Lee et al., 2013), in Syt7 the C2A-domain Ca^{2+}-binding sites were essential for asynchronous release and the C2B-domain Ca^{2+}-binding sites were dispensable (Bacaj et al., 2013). The differences in the localization and the relative C2-domain functions between Syt1 and Syt7 may be related to each other, and the plasma membrane localization of Syt7 may also explain, at least in part, why Syt7 is generally less effective than Syt1 in triggering exocytosis. Alternatively, it is possible that a small amount of Syt7 is present on synaptic vesicles, and its relatively low Ca^{2+}-triggering efficiency is due to its poor synaptic vesicle sorting efficiency. The recent Syt7 results suggest that different synaptotagmins collaborate and compete with each other as Ca^{2+}-sensors for release, and expand the finding that Syt1 is also co-expressed with Syt2 or Syt9 in some synapses where these synaptotagmins also complement each other physiologically (Xu et al., 2007; Pang et al., 2007).

In the non-physiological situation of a Syt1 or Syt2 knockout synapse, the observed remaining Ca^{2+}-dependent release may be more complex than simply allowing Syt7 function to become manifest (Fig. 5). This possibility is suggested by two observations. First, in Syt1 or Syt2 KO synapses, an approximately 10-fold increase of spontaneous miniature release is observed. The increased ‘minis’ in the Syt1 KO neurons are still largely Ca^{2+}-dependent (>90%), just like normal minis, but exhibit a different Ca^{2+}-cooperativity than normal minis (Xu et al., 2009; see below for a further discussion of ‘clamping’ of minis by synaptotagmin and complexin). These minis are thus driven by an unknown Ca^{2+}-sensor that is not Syt7 because ablation of Syt7 expression does not affect these minis (Bacaj et al., 2013). Second, in Syt2 KO calyx synapses which do not exhibit the facilitating asynchronous release observed for hippocampal neurons, biophysical studies revealed that the remaining ‘asynchronous’ release has an apparent Ca^{2+}-cooperativity of 1-2, whereas synaptotagmin-dependent release generally exhibits an apparent Ca^{2+}-cooperativity of 4-5 (Sun et al., 2007; Kochubey and Schneggenburger, 2011). This finding suggests that non-facilitating asynchronous release observed in the Syt2 KO calyx, similar to the increased mini release in Syt1 KO hippocampus, is due to a non-synaptotagmin dependent mechanism. The relationship between physiological synaptotagmin-induced release and non-physiological Ca^{2+}-induced release mediated by an as yet unknown Ca^{2+}-sensor is illustrated in Fig. 5.

What synaptotagmin-independent Ca^{2+}-sensor may mediate the increased mini release in Syt1 KO hippocampal neurons and the remaining release in Syt2 KO calyx synapses? Proteins like Doc2 and calmodulin were ruled out in loss-of-function experiments (Groffen et al., 2010; Pang et al., 2010 and 2011). It is striking that the priming factor Munc13 is activated by Ca^{2+}. Munc13 contains at least three regulatory domains that are directly (the central C2-domain) or indirectly (the central C1-domain and the calmodulin-binding sequence) controlled by Ca^{2+} (Rhee et al., 2002; Junge et al., 2004; Shin et al., 2010). In the absence of the synaptotagmin/complexin clamp, Ca^{2+}-stimulation of Munc13 may induce increased mini release in Syt1 and Syt2 KO neurons. However, this hypothesis implies that priming is rate-limiting in such neurons, i.e. that no reservoir of primed vesicles should be present, whereas the readily-releasable pool (RRP) of vesicles is not altered in Syt1 or Syt2 KO neurons (Geppert et al., 1994; Sun et al., 2007; Xu et al., 2007). These considerations

suggest that the Ca^{2+}-dependent pathway mediating the increased mini release in Syt1 KO neurons is downstream of priming and Munc13 (Fig. 5).

Activating vs. clamping functions of synaptotagmin

Deletion of Syt1 or Syt2 enhances the rate of spontaneous vesicle exocytosis approximately 10-fold to cause increased mini release (Littleton et al., 1993; Broadie et al., 1994; Maximov and Südhof, 2005; Sun et al., 2007; Xu et al., 2009). This is referred to as 'unclamping', with the notion that Syt1 and Syt2 normally clamp spontaneous mini release. The 'clamping' function of synaptotagmin is probably highly significant, but reflects an independent activity that is less efficacious than the Ca^{2+}-triggering function of synaptotagmin, as suggested by the following considerations.

First, the enhancement in release induced by unclamping is relatively minor compared to the increase in the rate of synaptic vesicle exocytosis produced by Ca^{2+}-binding to Syt1 or Syt2 (a ~10-fold increase in mini release vs. a ~1,000,000-fold increase produced by Ca^{2+}; Sun et al., 2007). Per excitatory synapse, normal spontaneous fusion translates to an average rate of ~0.004 Hz, and even in the unclamped state an excitatory synapse will fire only once every 4 minutes or so, a very low rate that is not sufficient to deplete the readily-releasable pool of vesicles (Xu et al., 2009).

Second, unclamping is not observed in all synapses; for example, the Syt1 knockout does not produce an increase in mini release in autapses (Geppert et al., 1994).

Third, mutations that block Ca^{2+}-binding to the Syt1 C2A-domain decrease Ca^{2+}-triggered synchronous release in hippocampal neurons by approximately 50%, but do not ablate it, whereas mutations that block Ca^{2+}-binding to the C2B-domain ablate fast synchronous release (Mackler and Reist, 2001; Nishiki and Augustin, 2004; Shin et al., 2009). In contrast, mutations of the Syt1 C2A- or C2B-domain equally abolish its clamping activity (Shin et al., 2009; Lee et al., 2013). Thus, activating and clamping functions of synaptotagmin are not obligatorily coupled. Interestingly, no Syt1 mutation is known that allows clamping but blocks Ca^{2+}-triggering of release, although such mutations are described for complexin (see below).

Fourth, as mentioned above, the majority of spontaneous 'mini' release events both in wild-type and in Syt1-deficient synapses are Ca^{2+}-dependent (Xu et al., 2009). However, their Ca^{2+}-dependence exhibits a dramatically different Ca^{2+}-cooperativity (~4 for normal minis vs. ~2 for minis in Syt1 knockout neurons; Xu et al., 2009), suggesting that the minis are carried by distinct Ca^{2+}-sensors.

Viewed together, these results suggest that the activating and clamping functions of synaptotagmin are independent. Thus Ca^{2+}-triggering of release by Ca^{2+}-binding to synaptotagmin does not involve the removal of a clamp that prevents a primed, partially assembled SNARE complex from fully assembling and completing fusion. Moreover, these results suggest that the clamping functions of synaptotagmin are relatively minor, and may primarily ensure the precision of synaptic transmission, of fine-tuning the release process.

However, the fact that the synaptotagmin clamping function is relatively minor does not imply that it is not significant – clearly it is of utmost importance for synapses to be silent when not activated. Large numbers of whispering synapses would make a lot of noise. This computational noise would be detrimental for the ability of the brain to process real signals in neural circuits, and tightly regulating the amount of action-potential independent spontaneous release is likely an important mechanism of information processing.

The available clamping data also raise additional questions. It is striking that the clamping function of Syt1 requires the wild-type sequences of both its C2A- and its C2B-domain Ca^{2+}-binding sites (Shin et al., 2009; Lee et al., 2013). Clamping itself cannot depend on Ca^{2+}-binding to these domains because such Ca^{2+}-binding triggers exocytosis (Fernandez-Chacon et al., 2001; Rhee et al., 2005; Pang et al., 2006; Xu et al., 2009). It is unlikely that the Ca^{2+}-binding sites of the C2-domains are partially occupied in nerve terminals at rest given the high cooperativity of Ca^{2+}-binding to C2-domains (Davletov and Südhof, 1993; Kohout et al., 2002). The most parsimonious interpretation is that prior to Ca^{2+}-binding, the sequences of the C2-domain Ca^{2+}-binding sites interact with an unidentified target in a Ca^{2+}-independent manner, such that this interaction clamps mini release but is severed by Ca^{2+}-binding (Fig. 2). Apart from prompting the question of the nature of this target, this interpretation implies that contrary to current models, Syt1 acts on the release process upstream of Ca^{2+}-triggering, before the last millisecond in the lifetime of a vesicle, during the stage during which the fusion machinery is set up to prepare for the demise of the vesicle and the popping of its fusion pore (Fig. 2). Future experiments will have to explore the nature of this activity.

The complexin enigma

Complexin is a universal co-factor for synaptotagmin in all Ca^{2+}-triggered fusion reactions that have been examined (e.g., see Reim et al., 2001; Tang et al., 2006; Cai et al., 2008; Jorquera et al., 2012; Cao et al., 2013). Three distinct changes caused by the loss-of-function of complexin have been defined: a decrease in Ca^{2+}-triggering of release, an increase in spontaneous mini release, and a decrease in the size of the RRP. In two of these activities – the Ca^{2+}-triggering of release and the clamping of mini release – complexin performs analogous roles to Syt1 and Syt2, but with considerably smaller effect sizes.

How does a small molecule like complexin, composed of only ~130 residues, act to activate and clamp synaptic vesicles for synaptotagmin action? Atomic structures revealed that complexin, when bound to assembled SNARE complexes, contains two short α-helices flanked by flexible sequences (Chen et al., 2001). The central, more C-terminal α-helix is bound to the SNARE complex, and is essential for all complexin function (Maximov et al., 2009). The accessory, more N-terminal α-helix is required only for the clamping but not for the activating 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). The equally flexible C-terminal sequence, in turn, is required only for the clamping and priming activities of complexin, but not for its Ca^{2+}-triggering activity (Kaeser-Woo et al., 2012). Thus, all three activities of complexin – clamping, priming, and activation of Ca^{2+}-triggering – require distinct complexin sequences.

For complexin's activity, its binding in the middle of the SNARE complex, close to the central 'zero layer', is crucial, as it implies that complexin can bind to partially assembled SNARE complexes prior to fusion-pore opening, consistent with its role in priming. Our current model is that complexin binding to SNAREs activates the SNARE/SM protein complex, and that at least part of complexin competes with synaptotagmin for SNARE-complex binding (Tang et al., 2006; Xu et al., 2013). Ca^{2+}-activated synaptotagmin displaces this part of complexin (although not necessarily the entire complexin molecule), thereby triggering fusion-pore opening.

The conclusions made above for synaptotagmin function in clamping similarly apply to complexin: Complexin also does not primarily act as a clamp that prevents SNARE-complex assembly, and does not activate fast Ca^{2+}-triggered release by being displaced. Apart from the fact that complexin clamping activity is variably observed in different

contexts (e.g., see Reim et al., 2001, and Xue et al., 2008, vs. Huntwork and Littleton, 2007, and Maximov et al., 2009), complexin 'poorclamp' mutants with an inactive accessory α-helix fully support Ca^{2+}-triggered fusion (Yang et al., 2010). As for synaptotagmin, the activation and clamping functions of complexin are not linked, and the cumulative evidence supports the notion that it is really the activation function of complexin that is most important, especially since that is also the only function observed in non-synaptic exocytosis (Cai et al., 2008; Cao et al., 2013).

How does complexin function? The clamping function is easier to address because it depends on the complexin accessory α-helix, suggesting that this accessory α-helix may insert into the partially assembled trans-SNARE complex to prevent full zippering (Giraudo et al., 2009). This hypothesis is supported by structural data showing that complexin can cross-link trans-SNARE complexes into a zigzag array (Kümmel et al., 2011). However, the relation of these observations to the activation functions of complexin is not clear. Moreover, these observations do not explain why the complexin C-terminus is required for clamping, even though it is not essential for Ca^{2+}-triggering, and thus the loss of the accessory α-helix does not interfere with the localization or expression of complexin (Kaeser-Woo et al., 2012).

At present, no plausible hypothesis is available for how complexin activates Ca^{2+}-triggering of release by synaptotagmin – possibly one of the most important questions in the field. Strikingly, such activation requires the N-terminal complexin sequences (Xue et al., 2007; Maximov et al., 2009), suggesting an as yet uncharacterized interaction, possibly with membrane phospholipids.

A contemporary model for synaptotagmin and complexin function

Based on the advances discussed above, we propose that fast (Syt1, Syt2, and Syt9) and slow (Syt7) synaptotagmin Ca^{2+}-sensors normally compete with each other during Ca^{2+}-stimulated neurotransmitter release (Fig. 5). Syt7 function is not readily apparent in a generic synapse in a cultured neuron because Syt1, Syt2, and Syt9 generally win the competition, but Syt7 function may be physiologically activated by extended stimulus trains, by alternative splicing of Syt7, and/or by phosphorylation which may inhibit Syt1, Syt2, or Syt9 or activate Syt7. Such physiological activation could account for the preponderance of asynchronous release in some synapses (Hefft and Jonas, 2005; Daw et al., 2009; Karson et al., 2009).

Mutagenesis experiments indicated that synaptotagmins induce fusion pore opening via Ca^{2+}-stimulated binding to phospholipids and SNAREs (Fernandez-Chacon et al., 2001; Zhang et al., 2002; Pang et al., 2006), suggesting that synaptotagmins act on a primed fusion machinery via a simple Ca^{2+}-induced interaction that may cause a mechanical push-and-pull, thereby opening the fusion pore. Consistent with this model, mutation of a conserved tryptophan-tryptophan sequence in the linker separating the SNARE motif from the membrane anchor of synaptobrevin did not block fusion as such, but ablated synchronous neurotransmitter release (Maximov et al., 2009). When the tryptophan-tryptophan motif was replaced by a double alanine motif, evoked release became desynchronized and spontaneous release was unclamped, suggesting that the clamping and activating functions of synaptotagmin and complexin act on the linker separating the SNARE motif from the membrane anchor. This hypothesis is further supported by experiments demonstrating that cleavage of the C-terminal residues of the second SNARE motif of SNAP-25 by botulinum toxin A impairs Ca^{2+}-triggered fusion much more severely, in relative terms, than fusion as such (Xu et al., 1998; Sørensen et al., 2002; Zhang et al., 2002; Sakaba et al., 2005).

Clearly, Ca^{2+} does not trigger release by unclamping the SNARE complex, for example via a Ca^{2+}-dependent displacement of complexin from SNARE complexes via synaptotagmin, although Ca^{2+}-binding to Syt1 likely displaces at least part of complexin from SNARE complexes (Tang et al., 2006; Xu et al., 2013). If synaptotagmin is not the major clamp of fusion, what 'clamps' the SNARE complexes, i.e. what keeps partly zippered up complexes from completely zippering up and opening the fusion pore? This may be the wrong question – in a physiological system, a partly zippered-up complex may be perfectly stable, and simply require an additional push for completing the zippering process, a push that we propose is provided by synaptotagmin and complexin. Synaptotagmin and complexin may interact in the absence of Ca^{2+} with the partly zippered complex, thereby setting up the fast Ca^{2+}-triggered reaction. Deletion of synaptotagmin and complexin may lead to a modest increase in spontaneous fusion that is still Ca^{2+}-dependent by allowing a non-physiological Ca^{2+}-dependent process – possibly a change in electrostatic properties at the site of fusion – to partly destabilize partly zippered SNARE complexes.

We thus propose that Ca^{2+} triggers release in a two-stage reaction that involves a close collaboration between synaptotagmin and complexin. Prior to Ca^{2+}-influx, both synaptotagmin and complexin interact with the fusion machinery composed of a partly assembled SNARE/SM protein complex to activate the complex and enable a fast response to Ca^{2+}. Such interaction is indicated by the 'clamping' activity of synaptotagmin and complexin, and by the priming activity of complexin. When Ca^{2+}-levels rise during an action potential, Ca^{2+}-binding to synaptotagmin triggers a rearrangement of the overall fusion complex containing also complexin and synaptotagmin, such that part of complexin is displaced from the complex via the Ca^{2+}-dependent SNARE-complex interaction of synaptotagmin, and the SNARE complex is moved with respect to the membrane via the Ca^{2+}-dependent phospholipid interaction of synaptotagmin. This overall proposal is consistent with the available data, but far from proven – no direct evidence for an upstream activity of synaptotagmin apart from its clamping activity is available, and the atomic basis of the various interactions has not been elucidated.

ANOTHER QUARTER CENTURY IS COMING!

Given the detailed current understanding of how a presynaptic terminal converts a presynaptic action potential into a trans-synaptic neurotransmitter signal, and how the terminal not only translates an action potential into neurotransmitter release, but also computes the action potential signal dependent on the previous use of a synapse and on extrinsic inputs – given this detailed understanding, is there anything left to be done? This question is particularly pertinent because of current views that the molecular and computational mechanisms of synaptic transmission do not matter for an understanding of the brain, and that not only synapses, but even entire neurons can be dealt with as unitary entities in the large information processing machine that constitutes the brain.

At present, a widely shared opinion is that understanding the architecture of the brain will be sufficient for explaining how the brain works, maybe combined with a description about information flow, similar to the beautiful drawings of Cajal that have dominated neuroscientists' vision for a century. However, understanding the brain is not like understanding a house where features like air ducts, electrical connections, and window locks are just details that you don't really need to know in order to live in it. Instead of one house, a brain is rather like an assembly of billions of houses – the synapses – each of which has their own air ducts, electrical connections, and window locks. To carry this analogy further, understanding Shanghai will not be possible by looking at street maps, even if one knows how the traffic flows – it is necessary to know what is going on in individual buildings, and how such buildings work. Moreover, it is necessary to know how the houses

change, as there is continuous construction activity, demolition and rebuilding of houses, or just renovations – a never ending series of changes. Thus, we would like to argue that until we understand how synapses work, how synapses differ from each other, and how synapses change as a function of use over milliseconds to years, we will not be able to understand how the brain works, no matter how many connections have been mapped and how many stimulated neurons have been shown to elicit a certain behavior. Among the key questions about neurotransmitter release that have not been addressed are questions such as how vesicles are made, how short- and long-term plasticity is effected, and how precisely complexin works at the atomic level.

Much of contemporary neuroscience and cell biology seems to believe that everything concerning molecules or purified proteins is a detail. The general perspective often is that the only attractive type of scientist corresponds to an architect who designs beautiful buildings but pays no attention to air ducts, electrical wiring, and window locks. The idea is that what counts is the overall design, and that the details are negligible. I hope that at least some of my readers have been convinced by my arguments that the molecules which make up a biological system are actually more than trivial details, but are the system, and that studying and understanding them is not just an unfortunate necessity but the only avenue to building the building in the first place.

Finally, increasing evidence implicates synapse dysfunction in neurological and psychiatric disorders. This evidence includes the observation that α-synuclein, which is centrally involved in multiple neurodegenerative disorders including Parkinson's disease, is a SNARE-complex assembly chaperone (Burre et al., 2010), the finding that the SM protein Munc18-1 is frequently mutated in Ohtahara syndrome (Saitsu et al., 2008), and the discovery that many 'synaptic' genes are mutated in schizophrenia and autism (Südhof, 2008). However, we know very little about how the pathophysiological mechanisms underlying any of these diseases. Thus, unraveling not only the normal mechanisms of release but also the abnormal processes producing neurological disorders will be a major challenge for future work.

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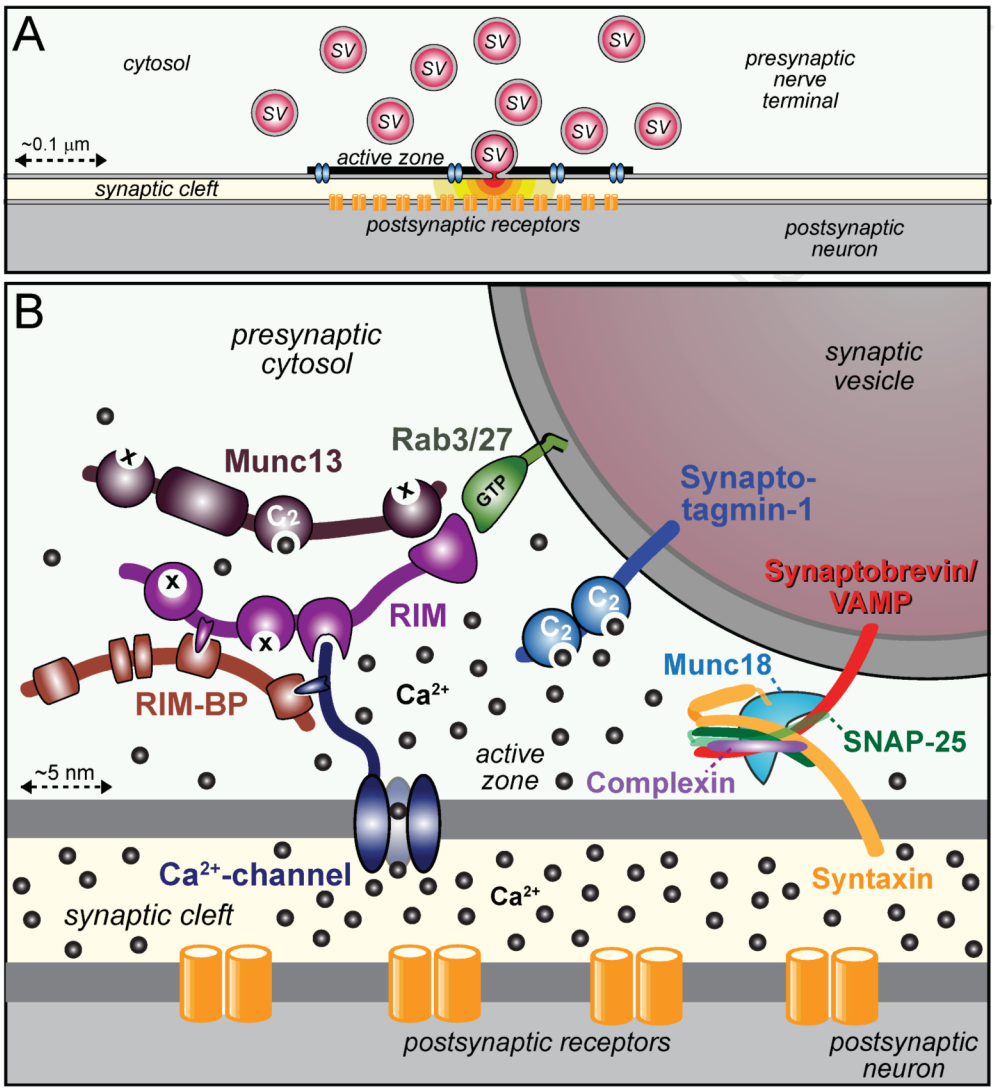

Figure 1. Organization of the presynaptic release machinery

A. Drawing of a synapse with synaptic vesicles (SV), an active zone containing Ca^{2+}-channels (blue), and a postsynaptic cluster of receptors (orange). One vesicle in the active zone is depicted in the process of fusing, with red neurotransmitters emitting from the fusion pore.

B. Schematic of the molecular machinery mediating Ca^{2+}-triggered vesicle fusion. The drawing depicts a segment of a docked synaptic vesicle on the top right, and the presynaptic active zone in the middle. The three functional elements of the neurotransmitter release machinery are depicted from right to left. On the right, the core fusion machine composed of the SNARE/SM protein complex is shown; this machine comprises the SNARE proteins synaptobrevin/VAMP, syntaxin-1, and SNAP-25 and the SM protein Munc18-1. The Ca^{2+}-sensor synaptotagmin-1 is depicted in the middle; it is composed of a short intravesicular sequence, a single transmembrane region, and two cytoplasmic C2-domains that bind Ca^{2+}, and it functions using complexin (bound to the SNARE complex) as an assistant. The active zone protein complex containing RIM, Munc13, and RIM-BP, and a Ca^{2+}-channel in the presynaptic plasma membrane is shown on the left. In this protein complex, RIM binding to specific target proteins coordinates all three functions of the active zone: RIM binding to

vesicular rab proteins (Rab3 and Rab27 isoforms) mediates vesicle docking; RIM binding to the central priming factor Munc13 activates vesicle priming; and RIM binding to the Ca^{2+}-channel, both directly and indirectly via RIM-BP, recruits the Ca^{2+}-channels within 100 nm of the docked vesicles for fast excitation-secretion coupling. The overall design of the neurotransmitter release machinery depicted here enables in a single nanodevice fast and efficient triggering of release in response to an action potential by combining a fusion machine with a Ca^{2+}-trigger and an active zone protein complex that positions all elements into appropriate proximity (modified from Kaeser et al., 2011).

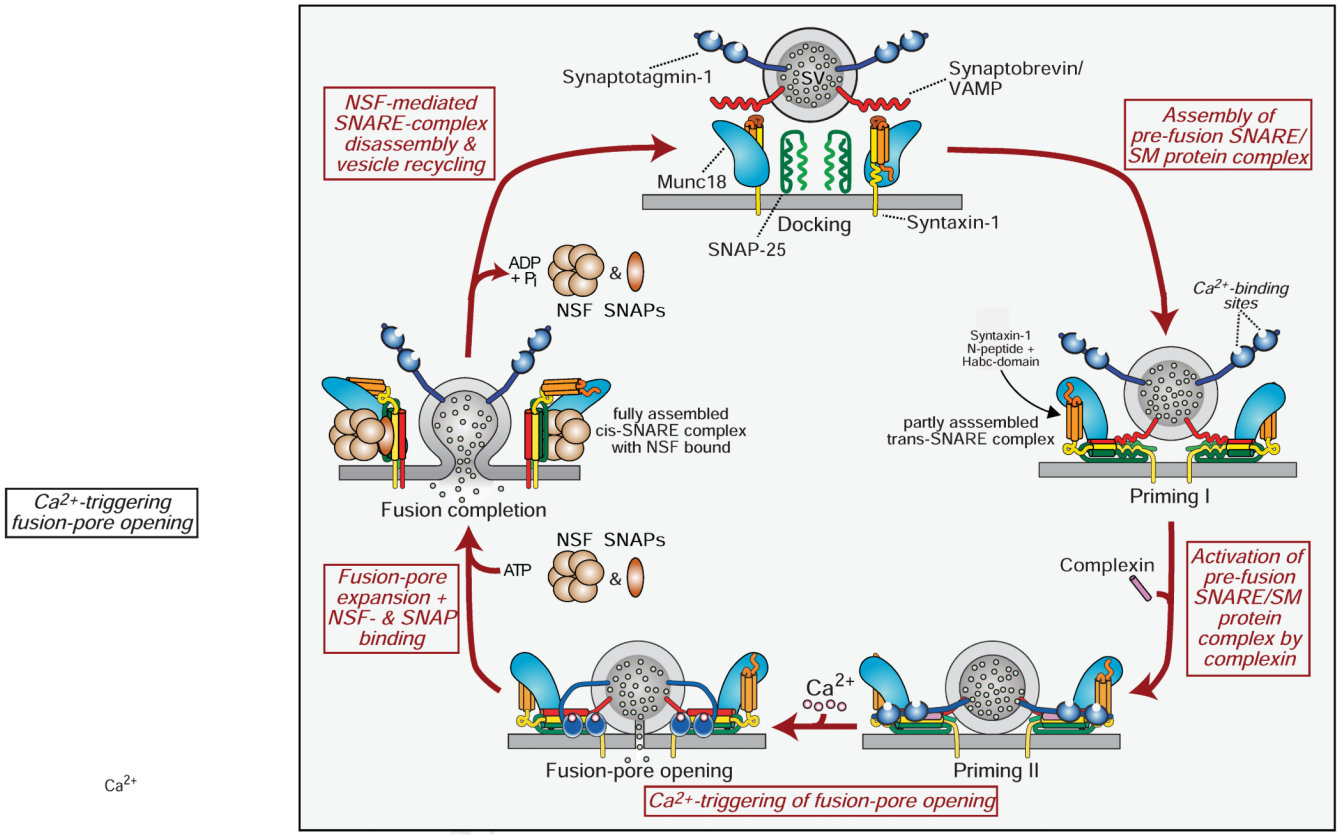

Figure 2. Schematic of the SNARE/SM protein cycle mediating fusion, and the role of synaptotagmin and complexin in Ca^{2+}-triggering of fusion

SNARE and SM proteins undergo a cycle of assembly and disassembly, such that the vesicular SNARE protein synaptobrevin assembles during priming into a trans-SNARE complex with the plasma membrane SNARE proteins syntaxin-1 and SNAP-25. Prior to SNARE-complex assembly, syntaxin-1 is present in a closed conformation in which its Habc-domain folds back onto its SNARE motif; this conformation precludes SNARE-complex assembly, and syntaxin-1 has to 'open' for SNARE-complex assembly to initiate. Prior to SNARE-complex assembly, Munc18-1 is associated with monomeric syntaxin-1 when syntaxin-1 is in a closed conformation; as syntaxin-1 opens during SNARE-complex assembly, Munc18-1 alters the mode of its binding to syntaxin-1 by binding to assembling trans-SNARE complexes via interacting with the syntaxin-1 N-peptide. Once SNARE complexes are partially assembled, complexin binds to further increase their priming. The 'superprimed' SNARE/SM protein complexes are then substrate for Ca^{2+}-triggered fusion pore opening by Ca^{2+}-binding to synaptotagmin, which causes an interaction of synaptotagmin with SNAREs and phospholipids. However, even before Ca^{2+}-triggering synaptotagmin likely at least partly interacts with the fusion machinery as evidenced by the unclamping of spontaneous mini release in Syt1 knockout neurons. After fusion-pore opening, the resulting cis-SNARE complexes are disassembled by the NSF/SNAP ATPases, and vesicles are recycled, refilled with neurotransmitters, and reused for release (modified from Südhof, 2013).

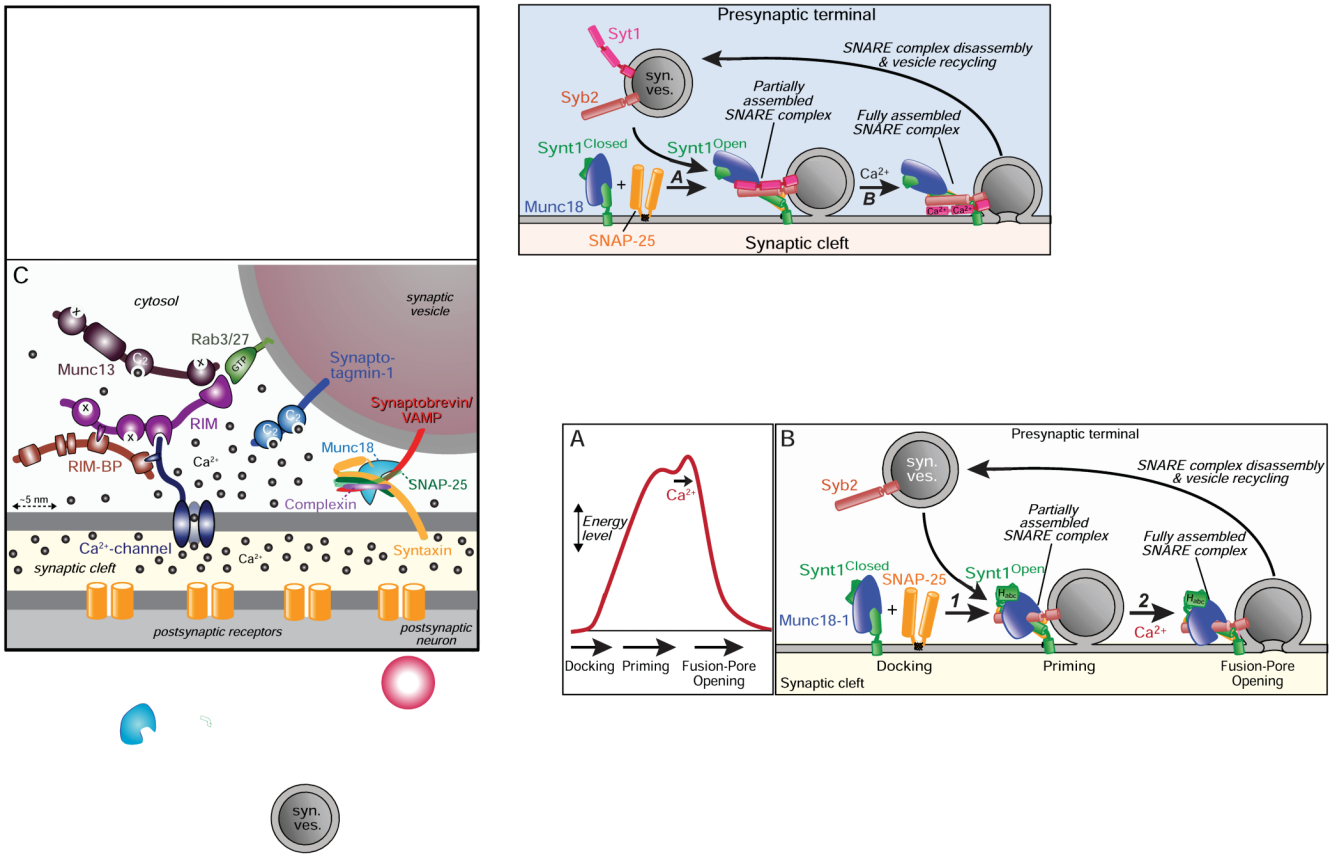

Figure 3. Energy landscape of fusion and proposed role of the SM protein Munc18-1 in promoting fusion-pore opening

A. Schematic diagram of the energy level of a vesicle that is docked, primed, and fused. The diagram illustrates that partial SNARE-complex assembly during priming is proposed to provide most of the energy required for fusion, such that Ca^{2+}-triggering only adds a small amount of additional energy to induce fusion-pore opening.

B. Model of Munc18-1 function in fusion. Prior to priming of docked vesicles, Munc18-1 is bound to the closed conformation of syntaxin-1; this interaction is primarily regulatory to maintain a defined rate of entry into the fusion reaction, and additionally serves for the mutual stabilization of Munc18-1 and syntaxin-1 for each other (Gerber et al., 2008; Zhou et al., 2013a). Partial SNARE-complex assembly during priming (middle) is associated with a dramatic conformational change in syntaxin-1 which has to open, and in Munc18-1 whose binding changes from that to a closed syntaxin-1 conformation to binding to the open syntaxin-1 conformation via the N-peptide of syntaxin-1 (Dulubova et al., 2007). Full SNARE-complex assembly (right) produces fusion-pore opening. Munc18-1 is proposed to ride on the assembly SNARE complexes throughout the fusion reaction, and to couple the approximation of the membranes produced by the energy released by SNARE-complex assembly to fusion, possibly by mediating phospholipid mixing.

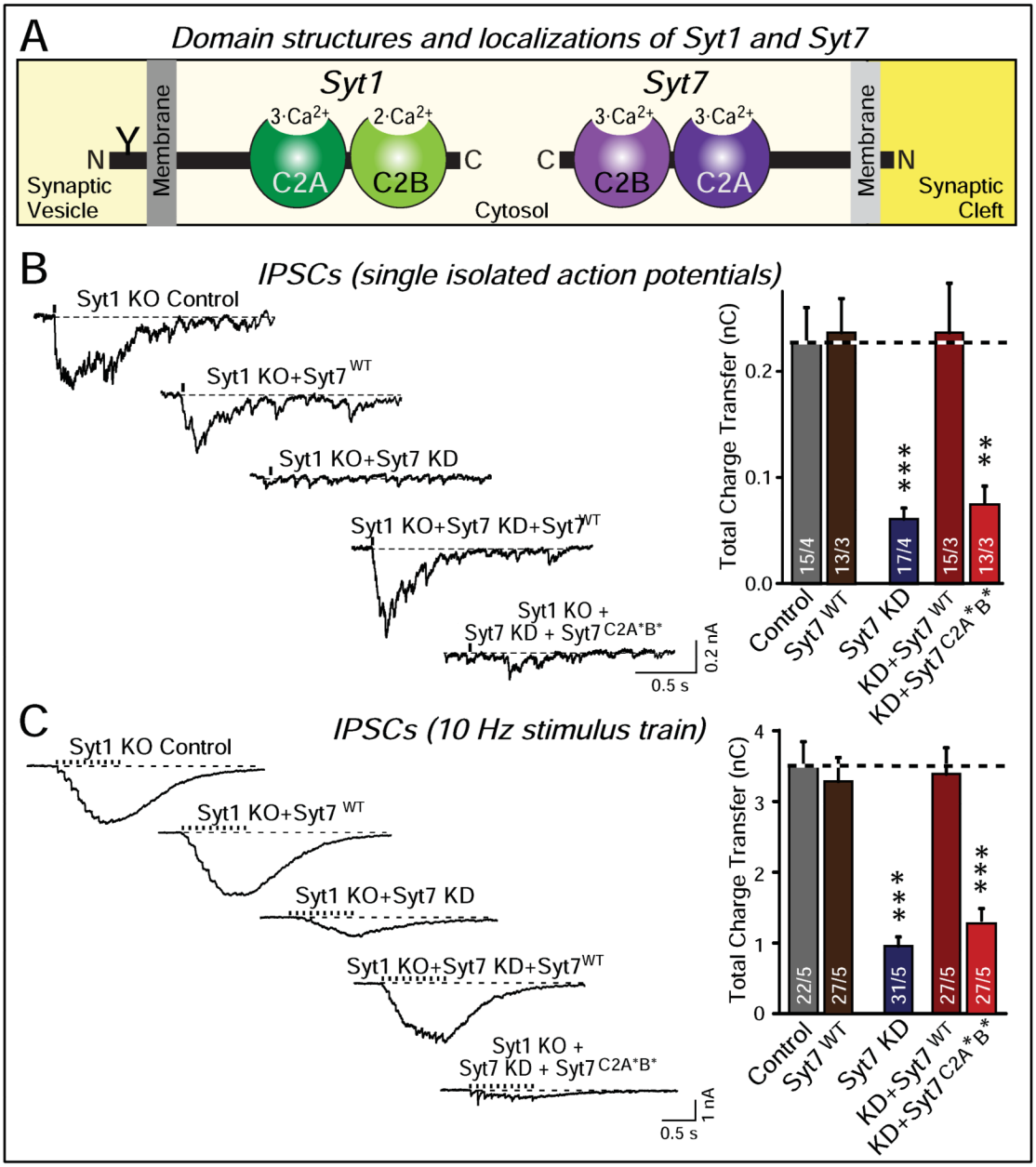

Figure 4. Complementary roles of synaptotagmin-1 (Syt1) and -7 (Syt7) Ca^{2+}-sensors in triggering synchronous and asynchronous neurotransmitter release

A. Domain structures and localizations of Syt1 as the paradigmatic fast synaptotagmin isoform, and of Syt7 as the slow synaptotagmin isoform. Syt1 and Syt7 have identical domain structures, except that Syt1 contains an N-terminal N-glycosylation site in the vesicle that is lacking from Syt7, and that Syt1 is primarily localized to synaptic vesicles where Syt7 is primarily absent from synaptic vesicles.

B. Inhibitory postsynaptic currents (IPSCs) measured in response to isolated action potentials in cultured hippocampal neurons from Syt1 knockout mice that were either infected with a control lentivirus, a lentivirus overexpressing wild-type Syt7, a lentivirus expressing a Syt7 shRNA that blocks most Syt7 expression, a lentivirus co-expressing the Syt7 shRNA together with wild-type Syt7, or a lentivirus co-expressing the Syt7 shRNA

with a mutant Syt7 in which the C2A- and C2B-domain Ca^{2+}-binding sites were mutated. Representative traces are shown on the left, and summary graphs on the right. Data shown are means ± SEM; numbers in the bars indicate the numbers of neurons/cultures analyzed. Statistical significance was assessed by one-way ANOVA comparing all test conditions to the control (**, $p<0.01$; ***, $p<0.001$).

C. Same as B, except that IPSCs were measured in response to a 10 Hz 1 sec stimulus train. Data and figure were adapted from Bacaj et al. (2013).

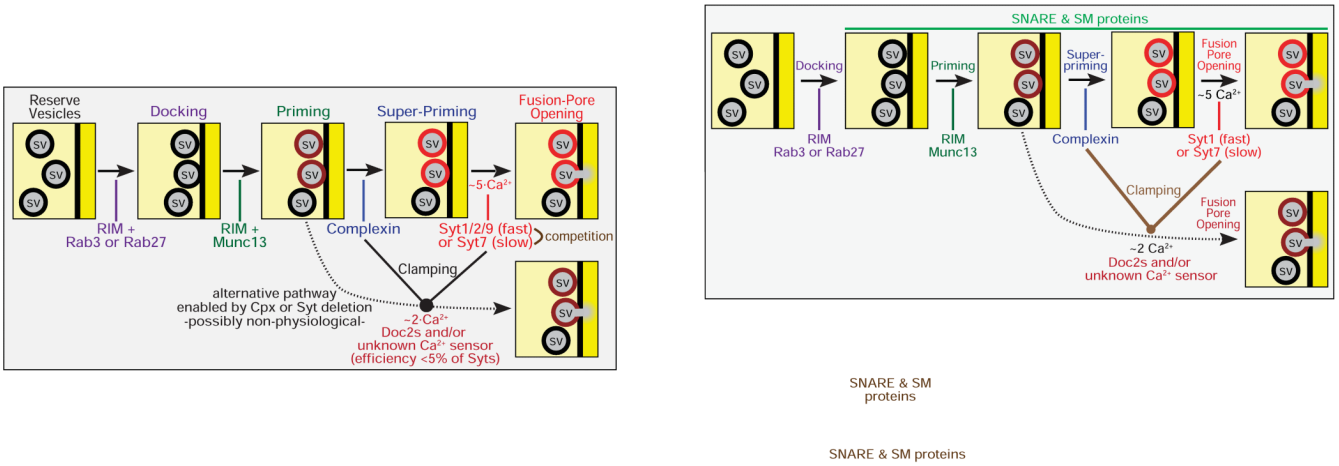

Figure 5. Schematic representation of different synaptic Ca^{2+}-controlled neurotransmitter release pathways

After docking and priming of synaptic vesicles mediated by the active zone protein complex containing RIM proteins as central elements, vesicles are further 'superprimed' by binding of complexin to the assembling SNARE complexes. The superprimed vesicles are the substrates of fast (Syt1/Syt2/Syt7) and slow (Syt7) synaptotagmins as synaptic Ca^{2+}-sensors. Fast and slow synaptotagmins are differentially expressed, compete with each other, and are further regulated by alternative splicing and phosphorylation, such that synapses differ in the amount and short-term plasticity of Ca^{2+}-induced release. In the absence of complexin or the fast synaptotagmin Ca^{2+}-sensors, an alternative nonphysiological pathway becomes enabled (dotted line) whereby an as yet unidentified Ca^{2+}-sensor with a Ca^{2+}-cooperativity that differs from that of synaptotagmins mediates spontaneous 'mini' release. Key proteins are listed in the drawing at their points of action; SNARE and SM proteins are not shown for clarity sake, but are obviously vital components for the entire fusion reaction.